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14. ABSTRACT: We have demonstrated that the physical stress associated with confined cancer cell migration can result in transient nuclear envelope rupture in vitro and in vivo, and that the nuclear deformation and nuclear envelope rupture result in nuclear fragmentation and DNA damage. We were able to further show that ESCRT-III proteins play a critical role in the nuclear envelope repair, and that depleting or inhibiting ESCRT-III and associated proteins delays nuclear envelope repair. In addition, we showed that exposure of genomic DNA to the cytoplasm following nuclear envelope rupture activates the cGAS/STING pathway and promotes cancer metastasis. Inhibition of the cGAS/STING pathway or preventing nuclear envelope rupture by overexpression of lamin B2 significantly reduced metastasis in a mouse xenograft model. these findings suggest that targeting these pathways could be a potential therapeutic approach to prevent or reduce metastasis. During the current project period, our work on this project has so far resulted in 7 peer-reviewed publications (plus one currently in revision), as well as 13 seminar, conference or poster presentations by the PI or students/postdocs from the PI's laboratory. All tasks have been completed according to or ahead of the schedule listed in the original SOW.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	1
2. Keywords.....	1
3. Accomplishments.....	1
4. Impact.....	8
5. Changes/Problems.....	9
6. Products.....	9
7. Participants & Other Collaborating Organizations.....	12
8. Special Reporting Requirements.....	N/A
9. Appendices.....	13

1. INTRODUCTION

During invasion and metastasis, cancer cells move through narrow openings in dense extracellular matrix networks, tight interstitial spaces, and small capillaries. We found that the physical forces acting on the cell nucleus during passage through such confined spaces result in repetitive nuclear envelope rupture, as indicated by transient loss of nuclear envelope integrity, protrusion of chromatin from the nucleus, formation of micronuclei, and nuclear fragmentation. The major goal of this project is to investigate whether the nuclear deformation and nuclear envelope rupture during confined migration can promote genomic instability, i.e., an increased occurrence of gene deletions, amplifications, alterations, and rearrangements. At the same time, migration-induced nuclear envelope rupture and DNA damage could also offer an opportunity to specifically target invasive cancer cells, motivating us to study the mechanism of nuclear envelope repair, and the consequences of inhibiting nuclear envelope repair and DNA damage repair pathways in invasive cancer cells. Lastly, we are examining whether particularly invasive and metastatic cancer cells are particularly prone to nuclear envelope rupture, or have evolved adaptive molecular mechanisms to tolerate nuclear envelope rupture. Our experimental approach utilizes a broad panel of breast cancer cell lines and is carried out in vitro using novel microfluidic devices that mimic physiological microenvironments, collagen matrices, and in vivo breast cancer models. Insights from these studies could provide relevant information for new diagnostic, prognostic, and therapeutic approaches.

2. KEYWORDS

Breast cancer; metastasis; invasion; confined migration; nuclear envelope; lamins; DNA damage; genomic instability; mechanics; biophysics

3. ACCOMPLISHMENTS

Major goals and accomplishments

Aim 1 – Task 1: Generation of additional materials and reagents (month 1-36)

We have generated and analyzed a large panel of human and mouse breast cancer cell lines to measure the incidence and extent of nuclear envelope rupture during confined migration. To facilitate the generation of cells expressing NLS-GFP and histone H2B-tdTomato, which enables automated detection of nuclear envelope rupture, we created a novel retroviral vectors. We have generated more than 20 cell lines so far, including MDA-MB-231, MDA-MB-468, BT-549, HCC70, SKBR3, BT474, T47D, and MCF7 human breast cancer cell lines, the MCF10A human breast epithelial cell line tumor progression series, the 4T1 mouse breast cancer progression series, mouse PyMT breast cancer cells, and the highly metastatic Met1 cell line derived from the PyMT cells. In addition, we have generated MDA-MB-231 cells depleted for the mitotic centromere-associated kinesin (MCAK), which presents a model for increased micronuclei formation, associated with increased genomic instability and increased metastasis (Bakhoum et al. *Nature* 2018). To further accelerate and facilitate migration experiments with the microfluidic devices that mimic physiological environments while enabling imaging cells at high spatial and temporal resolution, we are continuing to improve specific features of the microfluidic device and its fabrication process. We recently published a detailed protocol (Keys et al., accepted for publication in *Methods in Molecular Biology*) describing the process. In addition, we developed an automated image analysis program that can track single cells moving through the migration devices and detect nuclear envelope rupture based on the ratio of NLS-GFP and H2B-tdTomato reporters, greatly speeding up the analysis of multiple cell lines. This work is currently in revision at PLoS one (Elacqua et al.).

Status: Stated goals completed; further improvements continuing.

Aim 1 – Task 2: Optimization of experimental parameters (months 1-9)

We have successfully established the experimental conditions for all of the current cell lines, including coating of microfluidic devices with suitable extracellular matrix proteins, fabrication of collagen matrices, cell seeding, etc. Since we experienced difficulties stably expressing the original full length 53BP1 reporter construct to detect double stranded DNA breaks used in our *Science* publication (Denais et al. 2016), we have obtained a new, truncated 53BP1 reporter construct that produces more consistent results. We have already validated this construct as suitable reagent to detect double stranded DNA breaks. We have generated a lentiviral vector to express an mCherry-tagged version of this reporter constructs and already modified several cell lines with this construct (See Task A1.1)

Status: Stated goals completed.

Aim 1 – Task 3: Development of nuclear rupture sensor (months 1-15)

The goal of this task is to develop a genetically encoded sensor/reporter to label cells that underwent nuclear envelope rupture. In addition to the NLS-GFP reporter, which helps to visualize the dynamics of nuclear envelope rupture, we have generated a nuclear rupture reporter based on the cytoplasmic DNA binding protein cGAS, which strongly binds to genomic DNA exposed to the cytoplasm upon nuclear rupture, and which is retained at the rupture site even after nuclear envelope integrity has been restored. We published the use of this reporter in our *Science* publication describing nuclear envelope rupture and repair in confined migration (Denais et al., *Science* 2016). We have modified a large panel of cell lines with this reporter. In addition, we are developing a new nuclear rupture sensor, which is based on the idea of having a Cre recombinase fused to a large, inert cytoplasmic protein, that can only enter the nucleus and act on a loxP-based fluorescent reporter when the nuclear envelope ruptures, with the goal to stably label cells after nuclear envelope rupture. We have already designed the full constructs, cloned it into a lentiviral expression vector, and are currently testing it in vitro.

Status: Stated goals completed; additional approaches for further improvements ongoing.

Aim 1 – Task 4: Nuclear rupture analysis (months 6-24)

The goal of this task was to quantitatively describe nuclear envelope rupture during confined and unconfined migration, and to assess the effect on cell viability. We have tested an extensive panel of breast cancer cell lines in the microfluidic migration devices and in collagen matrices. Additional experiments were performed in vivo, using intravital imaging. We showed that migration through tight spaces results in transient nuclear envelope rupture in vitro and in vivo, and that the incidence of nuclear envelope rupture substantially increases with confinement, i.e., decreasing pore size. Intriguingly, the vast majority of cells survived nuclear envelope rupture. These results were published in *Science* during the previous report period (Denais et al., 2016). Following up on these studies, we have now analyzed more than 20 cell lines expressing our nuclear rupture reporter. Surprisingly, we did not detect any correlation between the aggressiveness of the cancer cell lines (based on published in vivo studies) and the frequency or duration of nuclear envelope rupture. Instead, all cell lines showed similar frequency and duration of nuclear envelope rupture during confined migration. We did not detect any significant correlation between the aggressiveness of the cancer cell lines and their survival/death following nuclear envelope rupture, either, indicating that all tumor cells are capable of efficiently restoring nuclear envelope integrity. Consistent with this interpretation, we found that the various breast cancer cells expressed similar levels of ESCRT-III proteins, which we previously identified as crucial components for nuclear envelope repair (Denais et al. *Science* 2016).

Status: Stated goals completed. Follow-up experiments with additional cell lines in process.

Aim 1 – Task 5: Consequences of nuclear envelope rupture on genomic instability (months 8-24)

To assess the effect of nuclear envelope rupture on genomic integrity, we assessed cells during confined and confined migration for DNA damage and chromatin fragmentation. We found that increasing confinement resulted in a substantial fraction of cells displaying chromatin protrusions, and, in severe cases, complete chromatin fragmentation, i.e., chromatin fragments separating from the main nucleus. More than 60% of the chromatin fragments were positive for γ H2AX, indicating DNA damage, closely resembling previous findings in micronuclei. Our initial findings were published during the previous report period (Denais et al. *Science* 2016). During this report period, we analyzed the mechanism of DNA damage in more detail. Using live-cell imaging of cells co-expressing NLS-GFP and a 53BP1-mCherry reporter for DNA double strand breaks, we found that migration through confined spaces can result in DNA damage, even in the absence of nuclear envelope rupture, but that nuclear envelope rupture can further contribute to DNA damage. We are still in the process of determining the precise molecular mechanisms responsible for the DNA damage. In addition, we are currently in the process of assessing the long term consequences of nuclear protrusion and fragmentation. We have generated clonal populations of MDA-MB-231 cells and are currently in the process of collecting cells that have repeatedly migrated through tight spaces ($1 \times 5 \mu\text{m}^2$ constrictions) and cells that have migrated through larger control channels ($15 \times 5 \mu\text{m}^2$), which do not require nuclear deformation and which do not induce nuclear envelope rupture. These cells will be analyzed for chromosomal abnormalities (by spectral karyotyping) and copy number variations (based on single cell sequencing, in collaboration with Dr. Nicholas Navin at MD Anderson). Recent publications by the Discher group support our hypothesis that confined migration results in increased genomic instability (Irianto et al. *Curr Biol.* 2017; Pfeiffer et al. *Curr Opin Syst Biol.* 2018)

Working with Dr. Lew Cantley at Weill Cornell Medicine, we have further unraveled an intriguing connection between genomic instability and nuclear envelope rupture. Dr. Cantley's group had generated MDA-MB-231 breast cancer cells depleted for mitotic centromere-associated kinesin (MCAK), which leads to increased formation of micronuclei and genomic instability. Our collaboration revealed that nuclear envelope rupture of the micronuclei results in cytoplasmic genomic DNA, which activates the cGAS/STING pathway and promotes metastatic spreading of the cancer cells in a mouse xenograft model. Intriguingly, reducing nuclear envelope rupture by overexpression of lamin B2, or inhibiting the cGAS/STING pathway by depletion of STING, significantly reduced metastatic progression (Figure 1). These results were recently published in *Nature* (Bakhoum et al. *Nature* 2018).

Status: Chromatin herniation and DNA damage detection completed. Analysis of long-term changes in genomic stability ongoing, expected to be completed within the next 6 months.

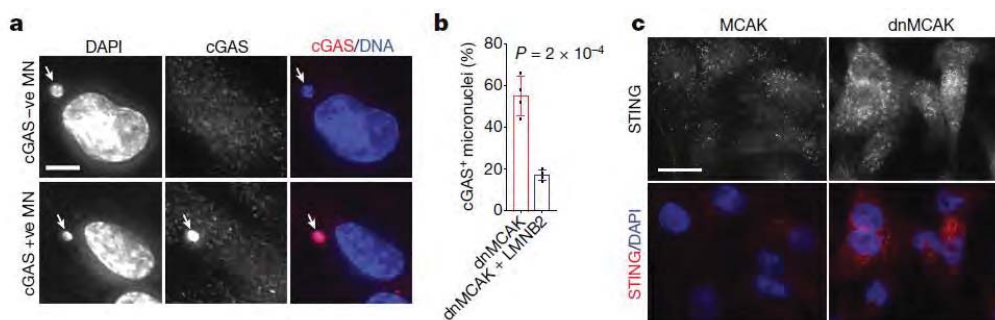


Figure 1. Nuclear envelope rupture of micronuclei activates cGAS/STING pathway. (a) MDA-MB-231 cells stained for DNA and cGAS, revealing accumulation of cGAS at ruptured micronucleus. (b) Percentage of micronuclei with cGAS localization (cGAS+), $n = 200$ cells. (c) Cells stained for DNA and STING. Figure reproduced from our recent *Nature* publication (Bakhoum et al. 2018).

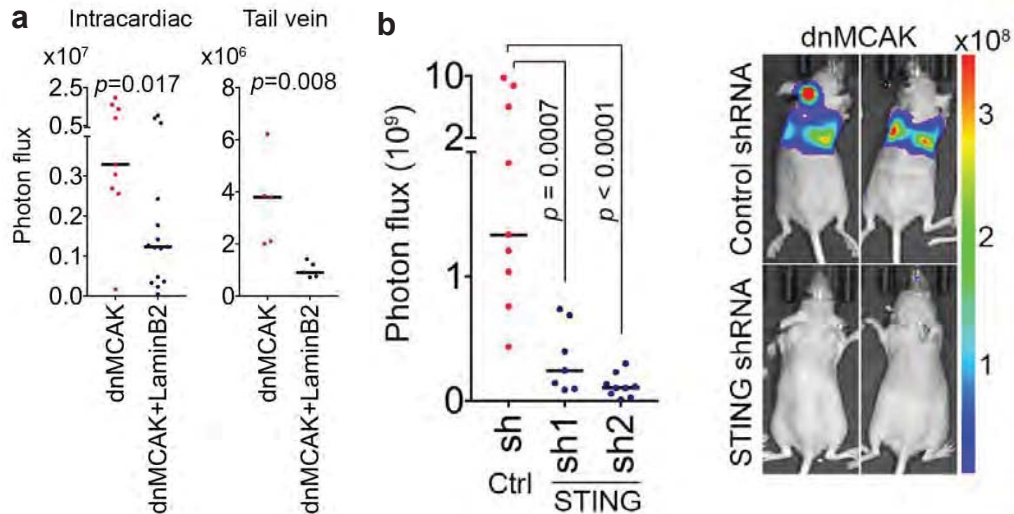


Figure 2. Stabilizing micronuclei with lamin B2 or depleting cGAS/STING pathway reduces metastatic progression. (A) Bioluminescence imaging results (photon flux) of mice after intracardiac or tail vein injection with MDA-MB-231 cells (depleted for MCAK) overexpressing lamin B2 or control construct, indicating that stabilization of the nuclear envelope prevents/reduces metastasis. (b) Bioluminescence imaging results (photon flux) of mice after intracardiac injection with MDA-MB-231 cells expressing control shRNA, or STING shRNA, indicating that depletion of cGAS/STING pathway components prevents/reduces metastasis. Figure panels reproduced from our recent *Nature* publication (Bakhoum et al. 2018).

Aim 2 – Task 1. Acquisition of additional cell lines and patient samples (months 8-36)

As described under Aim 1, Task 1, we have acquired additional breast cancer cell lines and modified these cells with the various fluorescent reporters. We have also obtained the MCF10A human breast cancer progression series, and the 4T1 mouse breast cancer progressions series, which will enable comparison of isogenic cell lines with different metastatic potential, and we have already used these progression series in our experiments. We have also obtained a large sets of human breast cancer patient samples, including tissue microarrays of 122 patient samples that includes 54 triple negative samples. Patients did not receive systemic adjuvant treatment and were followed up for at least 5 years. We have already used these samples to assess whether specific nuclear envelope proteins or indicators of nuclear envelope rupture correlate with clinical outcome in these patients (see Aim 2 – Task 2). We are already in the process of acquiring patient derived explants and organoids, which we plan to use later in the project (listed as month 24-36 in the SOW).

Status: Progress according to schedule described in the SOW.

Aim 2 – Task 2. Comparison of nuclear rupture incidence in cells/tissues (months 8-36)

In our analysis of more than 20 different breast cancer cell lines, we did not detect any significant correlation between the incidence of nuclear envelope rupture and metastatic potential/aggressiveness. Some of these findings were reported as part of our work with the Cantley group (Bakhoum et al. *Nature* 2018), while others are still in preparation for publication. Our comparison between the different breast cancer cell lines, including an isogenic cancer progression series, revealed one other striking finding though: breast cancer cell lines that are particularly invasive and metastatic have reduced levels of the nuclear envelope protein lamin A and increased nuclear deformability, which may increase their ability to migrate through tight spaces smaller than the nuclear diameter. To test whether this correlation could yield prognostic value, we assessed levels of lamin A (normalized by lamin B) in a large panel of tumor samples (tissue micro array) from breast cancer patients who did not received systemic adjuvant therapy

and who had been followed up for at least 5 years. Patients whose tumor cells had low levels of lamin A had significantly reduced disease free survival compared to patients whose tumors had high levels of lamin A (Figure 3). We are currently preparing a manuscript with these results for publication. Another manuscript describing lower levels of lamin A/C and enhanced migration in confined environments in an isogenic metastatic breast cancer model was published last year (Mekhdjian et al. *Mol Biol Cell*. 2017).

Status: Progress ahead of schedule described in the SOW.

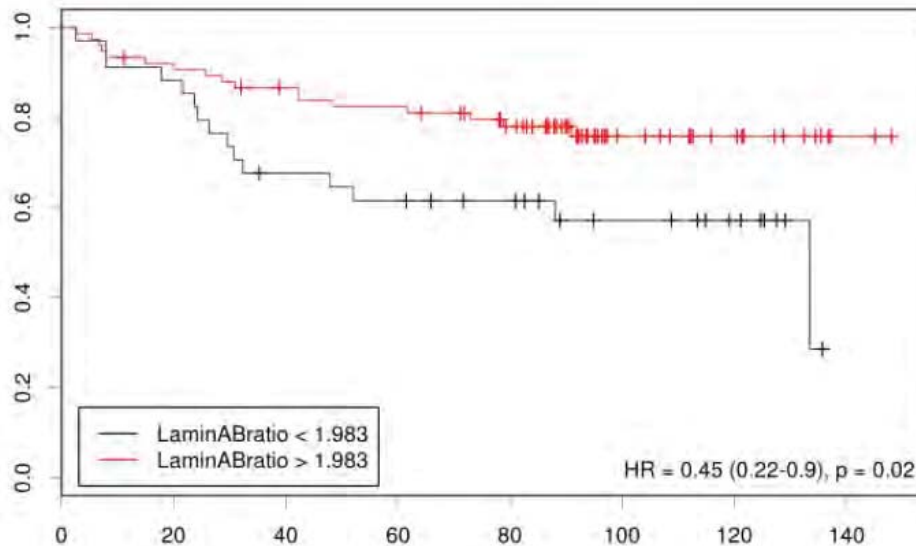


Figure 3. Low levels of lamin A correlate with reduced disease-free survival. Kaplan-Meier plot for disease free survival (months) for patients whose tumor cells had low levels of lamin A (normalized to lamin B) vs. those with tumors with high levels of lamin A. Patients with low levels of lamin A had significantly reduced disease-free survival ($p = 0.02$). Analysis based on tissue microarray of 122 breast cancer patient samples, including 54 triple-negative breast cancer samples. Patients did not receive systemic adjuvant treatment and were followed up for at least 5 years. The tissue microarray is described in Nagelkerke et al. *Radiother Oncol*, 2011.

Aim 2 – Task 3. Investigate the mechanism(s) of nuclear envelope rupture and survival (months 12-24)

By varying the degree of confinement (pore size) in our microfluidic migration devices and collagen matrices, we demonstrated that nuclear envelope rupture increases dramatically with increasing confinement. The formation of nuclear membrane blebs that collapse upon nuclear envelope rupture indicate that nuclear rupture is caused by intranuclear pressure. The intranuclear pressure results from compression of the nucleus by the cytoskeleton, particularly the actin-myosin network, and inhibition of myosin II with blebbistatin significantly reduces nuclear envelope rupture. Our findings were published in *Science* (Denais et al. 2016), together with an independent study by Matthieu Piel's group (Raab et al. *Science* 2016) that produced very similar results in dendritic cells. Subsequent work by Emily Hatch and Martin Hetzer (*JCB* 2016, published with a commentary by Jan Lammerding) confirmed these results. In collaboration with Vivek Shenoy (University of Pennsylvania), we carried out computational modeling to describe the biophysical aspects of nuclear deformation during confined migration in more detail, showing that the front of the nucleus, where most of the nuclear envelope ruptures occurs, is exposed to particularly large mechanical stresses (Cao et al. *Biophys J* 2016).

Status: Task completed ahead of schedule. Currently investigating further details of nuclear rupture, and how cells survive the nuclear envelope rupture process.

Aim 2 – Task 4. In vivo experiments with xenograft mouse model (months 1-33)

We have already obtained the regulatory approval for the proposed experiments by USAMRMC ACURO. During my sabbatical in Val Weaver's laboratory at UCSF (2015/16), I was trained in the procedures of mammary fat pad injection, observation of tumor growth, and harvest of primary tumors and other tissues for analysis of metastatic spreading. We are currently in the process of preparing cells for the in vivo experiments, by stably modifying MDA-MB-231 cells and cells from the 4T1 progression series to express luciferase (for bioluminescence imaging of metastatic progression), along with fluorescent reporters for nuclear envelope rupture, and variations in their lamin A/C levels. Due to some change in personnel and one postdoctoral fellow taking maternity leave, the start of the in vivo experiments has been slightly delayed, but is anticipated to start in March/April 2018, i.e. within the scheduled project period (months 18-33). In the meantime, our collaboration with the Cantley group at Weill Cornell Medicine revealed that cells from metastases had increased genomic instability compared to cells from the primary tumor, and that increasing genomic instability increases metastasis in a mouse model (Bakhom et al. *Nature* 2018). Status: Progress according to the schedule described in the SOW.

Aim 3 – Task 1. Identification of nuclear membrane repair mechanism (months 1-27)

Motivated by previous reports of a role of ESCRT-III proteins in mediating nuclear envelope re-sealing in late mitosis, we investigated the localization of the ESCRT-III proteins CHMP4B, CHMP7, and the ESCRT-III associated protein VPS4B during confined migration and nuclear envelope rupture induced by laser ablation or physical cell compression. Using time-lapse microscopy and superresolution imaging, we showed that these proteins rapidly accumulated at sites of nuclear envelope rupture, and that depletion of ESCRT-III proteins or expression of a dominant negative VPS4B mutant significantly delayed nuclear envelope repair after nuclear envelope rupture. Intriguingly, while inhibiting nuclear envelope repair alone (using the dominant negative VPS4B mutant) had no effect on cell viability after nuclear envelope rupture, inhibiting both nuclear envelope repair and DNA damage repair using the ATM inhibitor Ku-55933 resulted in significantly increased cell death after migrating, suggesting that this approach could potentially be used as a therapeutic strategy to target invasive cancer cells. These findings were described in detail in our *Science* publication (Denais et al. *Science*. 2016) and confirmed in an independent study published in parallel (Raab et al. *Science* 2016), as well as in a subsequent study by the De Vos group (Robijns et al. *Sci Reports* 2016). We recently published an extended description with additional details of the nuclear envelope repair (Isermann and Lammerding, *Nucleus*. 2017). We are currently generating cells with inducible shRNA against ESCRT-III proteins or non-target controls, which will enable us to study the effect of inhibiting nuclear envelope repair in more detail.

Status: All items completed ahead of schedule; currently performing additional studies to explore the best approaches to inhibit nuclear envelope repair.

Aim 3 – Task 2. Targeting DNA damage and stress response pathways in vitro (months 18-24)

We have already conducted experiments using the ATM inhibitor Ku-55933, showing that combined inhibition of nuclear envelope repair and DNA damage repair significantly increases cell death after nuclear envelope rupture (Denais et al. *Science* 2016). Experiments with the HSP90 inhibitor 17-AAG resulted in overall decreased cell viability and proliferation, independent of confined migration. We have already started experiments exploring inhibition of alternative DNA damage response pathways, including ATR (VE-821 inhibitor) and DNA-PK (NU-7741 inhibitor). These experiments are currently being analyzed. In addition, we are currently analyzing mouse embryo fibroblasts lacking specific DNA damage response genes (e.g. ATM, ATR) from Robert Weiss' lab at Cornell to complement pharmaceutical inhibition of DNA damage response pathways. Intriguingly, cells lacking ATM migrate significantly faster through tight constrictions than wild-type controls. Our biophysical assays revealed significantly more deformable

nuclei in the ATM-null cells, providing a possible explanation for this behavior. We are currently confirming these role of ATM in nuclear stability using rescue experiments.

Status: Progress according to schedule described in the SOW.

Aim 3 – Task 3. Targeting DNA damage and stress response pathways in vivo (months 12-36)

We have already obtained regulatory approval for the experiments by USAMRMC ACURO. We are currently in the process of preparing cells for the in vivo experiments, by stably modifying MDA-MB-231 cells and cells from the 4T1 progression series to express luciferase (for bioluminescence imaging of metastatic progression), along with fluorescent reporters for nuclear envelope rupture, and variations in their lamin A/C levels. In parallel, we are testing different combinations of nuclear envelope repair inhibition and DNA damage repair inhibition to determine the best strategy to specifically target metastatic cells. Due to some change in personnel and one postdoctoral fellow taking maternity leave, the start of the in vivo experiments has been slightly delayed, but is anticipated to start in March/April 2018, i.e. within the scheduled project period (months 18-36). In the meantime, working with the Cantley group at Weill Cornell Medicine, which has established protocols for in vivo models, we showed that preventing nuclear envelope rupture of micronuclei by overexpression of lamin B2, or inhibiting cGAS/STING signaling, which is activated by exposure of genomic DNA to the cytoplasm, significantly reduces metastasis in a mouse xenograft model (Fig. 3)

Status: Progress according to the schedule described in the SOW.

Opportunities for training/professional development

The project, coupled with additional funds from other sources (NSF graduate research fellowship, start-up funds from the Lammerding lab) has provided excellent opportunities to train undergraduate students, graduate students, and a postdoctoral fellow in multidisciplinary research. It also has provided opportunities for trainees to present their research at national conferences, interact with breast cancer patients through outreach efforts (coordinated with the Cancer Resource Center of the Finger Lakes), and thus contributed to the training and professional development of the next generation of researchers.

Dissemination of results to communities of interest

We have disseminated the research results from this project through peer-reviewed publications, conference presentations, and invited seminars (see Products). To reach the local patient community, Alexandra McGregor, a graduate student in the Lammerding lab working on this project, is organizing regular presentations by cancer researchers to breast cancer patients through a joint project with the Cancer Resource Center of the Finger Lakes.

Plans for the next reporting period

During the first 24 months of the project, we have generated a large array of assays, reagents, and tools to study the role of nuclear mechanics and nuclear envelope rupture in breast cancer progression, yielding already several new and important insights. In the coming project period, we will focus on the following tasks: 1) Assess the long-term effect of DNA damage and nuclear fragmentation during confined migration. We will use a complementary approach using both imaging based techniques, which enable us follow up individual cells for several generations after confined migration, as well as sequencing based approaches and spectral karyotyping, which will reveal copy number variations and chromosomal abnormalities induced by migration through tight spaces. For these experiments, we will compare clonal populations of breast cancer cells that have passed repeatedly through confined spaces or through wider control channels. 2) We will continue to determine the molecular mechanism underlying the observed DNA damage during confined migration, including whether it is caused by nuclear deformation (e.g.,

shearing) or by exposure of genomic DNA to normally cytoplasmic nucleases during nuclear envelope rupture. 3) We will determine the best strategies to target cells during confined migration, including combination therapies that target nuclear envelope repair and DNA damage response pathways. 4) We will assess the extent of nuclear envelope rupture during invasion and metastatic progression in vivo, using both human and mouse breast cancer cell lines (the latter to enable the use of immune-competent syngeneic models), and we will test whether interfering with nuclear envelope repair and/or DNA damage repair can reduce/prevent metastatic spreading.

4. IMPACT

Impact on the development of the principal discipline(s) of the project

Our group, together with a Matthieu Piel's group, were the first to demonstrate that the physical stress associated with confined migration can result in transient nuclear envelope rupture in vitro and in vivo, and that the nuclear deformation and nuclear envelope rupture result in nuclear fragmentation and DNA damage. These findings suggest that the physical process of invasion could further drive genomic instability in metastatic cancer cells. Subsequent work by the Discher group (Irianto et al. *Curr Biol* 2017; Irianto et al. *Mol Biol* 2017) further support these findings. We were able to show that ESCRT-III proteins play a critical role in the nuclear envelope repair, and that depleting or inhibiting ESCRT-III and associated proteins delays nuclear envelope repair. We also showed that combined inhibition of nuclear envelope repair and DNA damage repair results in significantly increased cell death after nuclear envelope rupture. These findings suggest that targeting these pathways could be a potential therapeutic approach to induce cell death in invasive cancer cells. Our finding that nuclear envelope rupture allows cytoplasmic cGAS to bind to genomic DNA (Denais et al. *Science* 2016) has stimulated tremendous interest in) the cytosolic DNA-sensing protein cyclic-GMP-AMP synthase (cGAS), which was first described as a key component of the innate immune system inflammatory response. Several recent reports demonstrate that loss of nuclear envelope integrity ('nuclear envelope rupture') exposes genomic DNA to the cytoplasm, enabling cytosolic cGAS to activate pro-inflammatory cytokine signaling and senescence (Mackenzie et al. *Nature* 2017; Dou et al *Nature* 2017; Gluck et al. *Nat Cell Bio.* 2017). In our work with the Cantley group, we showed that binding of cGAS to genomic DNA activates non-canonical NF- κ B signaling and pro-inflammatory cytokine signaling, which is required for metastatic progression. Inhibiting nuclear envelope rupture or cGAS/STING signaling was sufficient to significantly reduce metastatic progression in a mouse model with breast cancer cells with increased chromosomal instability (Bakhoum et al. *Nature* 2018).

Impact on other disciplines

Our work was also positively received in the membrane trafficking and ESCRT community, as our results showed an important role of ESCRT-III proteins, particularly the previously not well characterized CHMP7 protein, in nuclear envelope repair. Two other publications following our work have since then provided further insights into how CHMP7 can mediate nuclear envelope repair/reformation (Olmos et al. *Curr Biol* 2016; Gu et al. *PNAS* 2017). As discussed above, the finding that exposure of genomic DNA to cytoplasmic cGAS can activate the cGAS/STING pathway has resulted in a flurry of recent publications in a variety of field, ranging from immune cells to cell senescence research.

Impact on technology transfer

We are currently working with a team of engineering students to develop a stand-alone version of our microfluidic assay to perform high-throughput measurements of nuclear stiffness, which could have important applications in cancer prognosis. In addition, *The Scientist* published an article highlighting our

microfluidic cell migration device as a novel tool in mechanobiology: <http://www.the-scientist.com/?articles.view/articleNo/49860/title/The-Mechanobiology-Garage/>

Impact on society beyond science and technology

The You-Tube video produced by *Science* featuring the results (<https://www.youtube.com/watch?v=lg91eM8c7U>) has already had more than 14,000 views, raising broad public awareness for this research. We used our research on cancer cell invasion as a topic for outreach work “Squishing cells to stop cancer in its tracks” with female high school students interested in engineering through the Cornell CURIE Academy: https://www.bme.cornell.edu/news/index.cfm?news_id=96011. We collaborated with the Cancer Resource Center of the Finger Lakes to connect breast cancer patients with cancer researchers: <https://ejs349.wixsite.com/website>

5. CHANGES/PROBLEMS

We have not encountered any substantial problems or delays in the project. There have been some delays in the design of the Cre-based nuclear rupture sensor, which we are currently addressing. As an alternative approach, we have successfully developed another nuclear rupture sensor, based on the cGAS cytoplasmic DNA binding protein, which we described in our *Science* publication (Denais et al. 2016). While the cGAS reporter does not permanently label cells after nuclear envelope rupture, its binding to newly exposed genomic DNA is so strong that the signal is retained for 6-8 hours after nuclear envelope rupture, in contrast to the very transient nature of the NLS-GFP nuclear rupture sensor.

For the in vivo studies, we plan to include syngeneic mouse breast cancer cells in addition to or as an alternative to human breast cancer cells. The advantage of using syngeneic mouse breast cancer cells from the 4T1 progression series is that experiments can be performed in immune-competent mice and thus in a more physiologically relevant scenario. In contrast, experiments with human breast cancer cells in xenograft models requires the use of immune-compromised mice. We are in the process of modifying the cell lines with the various fluorescent reporter constructs. We have already submitted an amendment to our approved animal protocol to include the use of mouse breast cancer cells, with the approval expected for late March 2018. We will not start any experiments until the animal protocol has been approved, and we will provide you with documentation of the amended protocol and its approval.

6. PRODUCTS

The work performed for this project has already resulted in 7 journal publications (+ 1 in revision), and numerous seminar and conference presentations. Our recent *Science* publication describing our findings of nuclear envelope rupture, DNA damage, and nuclear envelope repair was broadly covered by numerous news outlets and websites.

Journal publications

1. Mekhdjian AH, Kai F, Rubashkin MG, Prahls LS, Przybyla LM, McGregor AL, Bell ES, Barnes JM, DuFort CC, Ou G, Chang AC, Cassereau L, Tan SJ, Pickup MW, Lakins JN, Ye X, Davidson MW, Lammerding J, Odde DJ, Dunn AR, Weaver VM. Integrin-mediated traction force enhances paxillin molecular associations and adhesion dynamics that increase the invasiveness of tumor cells into a three-dimensional extracellular matrix. *Mol Biol Cell*. 2017. 28(11): 1467-1488
2. Morelli FF, Verbeek DS, Bartacchini J, Vinet J, Mediani L, Marmiroli S, Cenacchi G, Nasi M, De Biasi S, Brunsting JF, Lammerding J, Pegoraro E, Angelini C, Tupler R, Alberti S, Carra S. Aberrant

- Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function. **Cell Reports**. 2017. 20(9): 2100-2115. Federal support acknowledged.
3. Bakhoum SF, Ngo B, Bakhoum AL, Cavallo JA, Murphy CJ, Ly P, Shah P, Sriram RK, Watkins TBK, Taunk NK, Duran M, Pauli C, Shaw C, Chadalavada K, Rajasekhar VK, Genovese G, Venkatesan S, Birkbak NJ, McGranahan N, Lundquist M, LaPlant Q, Healey JH, Elemento O, Chung CH, Lee NY, Imielenski M, Nanjangud G, Pe'er D, Cleveland D, Powell SN, Lammerding J, Swanton C, Cantley LC. Chromosomal instability promotes metastasis through a cytosolic DNA response. **Nature**. 2018. 553(7689): 467-472. Federal support acknowledged.
 4. Kirby TJ, Lammerding J. Emerging views of the nucleus as a cellular mechanosensor. **Nature Cell Biology**. Published online 2/23/2018. Federal support acknowledged.
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 7. Keys J, Windsor A, Lammerding J. Assembly and use of a microfluidic device to study cell migration in confined environments. **Meth Mol Biol**. Accepted for publication.
 8. Elacqua JJ, McGregor AL, Lammerding J. Automated analysis of cell migration and nuclear envelope rupture in confined environments. **PLoS one**. In revision. Federal support acknowledged.

Conferences/Seminars/Meetings

1. Squish and squeeze – Nuclear mechanics and mechanotransduction in physiology and disease. Invited seminar for the Department of Chemistry Colloquium. Binghamton University. Binghamton, NY (January 19, 2018)
2. Cell and nuclear mechanics during migration in 3-D environments. Invited presentation at the “Mechanics in Cancer Biology” meeting by the French Society of Cancer. Paris, France (Nov. 30, 2017)
3. Squish and squeeze – Nuclear mechanics and mechanotransduction in physiology and disease. Invited seminar for the Distinguished Mechanical Engineering Seminar series. Department of Mechanical Engineering, University of Houston. Houston, TX (Nov. 9, 2017)
4. Comparison of confined migration and nuclear envelope rupture in a panel of breast cancer cell lines. McGregor AL, Elacqua JJ, Bell ES, Lammerding J. Poster presentation selected from submitted abstract. Physical Science Oncology Network (PS-ON) Annual Meeting. Boston, MA (Oct. 17-18, 2017). AL McGregor was awarded top poster prize in the category “In Vivo & Organoid Models of Growth and Metastasis”
5. Nuclear Deformability and Expression of Lamin A/C as Predictors of Metastatic Potential in Breast Cancer Cells. Platform presentation selected from submitted abstract. Biomedical Engineering Society (BMES) Annual Meeting. Phoenix, AZ (October 12, 2017)
6. Squish and squeeze – the role of the nucleus and lamins in cancer metastasis. Invited presentation at the Physics of Cancer conference. Leipzig, Germany (October 5, 2017)
7. Squish and squeeze – Nuclear mechanics and mechanotransduction in physiology and disease. Invited seminar at the Department of Biochemistry and Cellular and Molecular Biology. University of Tennessee. Knoxville, TN (September 6, 2017)
8. Squish and squeeze – lamins and nuclear mechanics regulate confined migration of breast cancer cells. Invited talk. National Cancer Institute (NCI) Physical Science-Oncology Network (PS-

- ON) & Cancer Systems Biology Consortium (CSBC) 2017 Junior Investigator Meeting. Bethesda, MD (July 12, 2017)
9. Lamin mutations cause progressive nuclear envelope rupture in muscle fibers in vitro and in vivo. Invited talk at the COST School and The Batsheva de Rothschild Seminar: The Nuclear Lamina and Nuclear Organization. Ye'arim, Judean Hills, Israel (June 27, 2017)
 10. Squish and squeeze – Nuclear mechanics and mechanotransduction in physiology and disease. Student-invited seminar and tutorial at the Max Planck Institute of Molecular Cell Biology and Genetics. Dresden, Germany (June 8, 2017)
 11. Stressed out nuclei – the interplay between the nucleus and cytoskeleton during confined migration and muscle contraction. Invited presentation. European Cytoskeletal Forum. Helsinki, Finland (June 6, 2017)
 12. Squish and Squeeze – Nuclear Mechanics in Physiology and Disease. Invited seminar. Turku BioNet Seminars, University of Turku, Abo Akademi University. Turku, Finland (June 1, 2017).
 13. The role of nuclear deformation and rupture in breast cancer cell migration. McGregor AL, Elacqua JJ, Bell ES, Lammerding J. Poster presentation selected from submitted abstract. American Association for Cancer Research (AACR) Annual Meeting 2017. Washington, DC (April 4, 2017)

Websites and other media

- Coverage of our recent *Nature* article with the Cantley group on chromosomal instability in cancer and activation of the STING pathway upon nuclear envelope rupture of micronuclei (February 23, 2018):
<https://www.nature.com/articles/nrc.2018.16>
- Coverage of our research through the Cornell Research website:
<https://research.cornell.edu/news-features/cell-mechanics-cancer-and-muscle-disease>
- BME News Article covering our outreach work with female high school students interested in engineering through the Cornell CURIE Academy (July 27, 2017):
https://www.bme.cornell.edu/news/index.cfm?news_id=96011
- The Scientist article highlighting our microfluidic cell migration device as a novel tool in mechanobiology (July 17, 2017): <http://www.the-scientist.com/?articles.view/articleNo/49860/title/The-Mechanobiology-Garage/>
- Coverage of our cancer research featured by a new website/blog through a collaboration with the Cancer Resource Center of the Finger Lakes:
<https://ejs349.wixsite.com/website>

Technologies or techniques

We have already distributed several of the microfluidic migration devices developed in our laboratory to other laboratories, both as prepared devices and by providing design plans to recreate the devices. A manuscript with a detailed protocol describing the fabrication and use of the devices was just accepted for publication (Keys et al. *Meth Mol Bio*). We have also developed MATLAB programs to automate the image analysis of migration experiments and detection of nuclear envelope rupture. This manuscript is currently in revision as PLoS one.

In addition to the migration devices, we have developed a novel microfluidic device to rapidly measure nuclear deformability in a higher throughput approach, which greatly facilitates our analysis of multiple cancer cell lines. A manuscript to describe this technology will be submitted for publication in April 2018.

Other products

We have already generated more than 20 different breast cancer cell lines, including human and mouse, expressing various fluorescent reporters to monitor nuclear deformation and nuclear envelope rupture. We have modified these cells with a variety of fluorescent reporters to detect nuclear envelope rupture, nuclear fragmentation, and double strand DNA breaks by time-lapse microscopy. In addition, we have generated clonal populations of MDA-MB-231 cells that we will use to assess long-term DNA damage after confined migration through tight spaces, as the heterogeneity of standard MDA-MB-231 cell cultures would preclude drawing meaningful conclusions after karyotyping or other genomic analysis.

7. PARTICIPANTS

Individuals working on the project

Name:	Jan Lammerding
Project Role:	PI
Researcher Identifier:	ORCID ID: 0000-0003-4335-8611
Person Months:	1.7 (19%)
Contribution:	Planning and analysis of experiments; supervision of researchers; coordination with collaborators; writing/editing publications
Funding Support:	This award

Name:	Philipp Isermann
Project Role:	Visiting Scholar
Person Months:	1.2
Contribution:	Generation of fluorescent reporters and cell lines; analysis of nuclear envelope repair mechanism
Funding Support:	This award

Name:	Pragya Shah
Project Role:	Graduate student
Person Months:	4.4
Contribution:	Generation of cell lines; in vitro migration experiments; generation of new 53BP1-mCherry lentiviral vector; analysis of migration-induced DNA damage
Funding Support:	This award

Name:	Chieh-Ren (Jeremiah) Hsia
Project Role:	Graduate student
Person Months:	1.2
Contribution:	Generation of cell lines; in vitro migration experiments
Funding Support:	This award

Name:	Alexandra McGregor
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Project Role:	Graduate student
Person Months:	4.4
Contribution:	Generation of cell lines; in vitro migration experiments to assess nuclear envelope rupture and migration efficiency; assessment of DNA fragmentation in confined migration; dissemination of work to local breast cancer community
Funding Support:	This award; NSF Graduate Student Fellowship
Name:	Joshua Elacqua
Project Role:	Undergraduate student
Person Months:	1.9
Contribution:	Development of automated image analysis program to track cells and detect nuclear envelope rupture
Funding Support:	This award; research credit

Change in the support of the PI

Nothing to report

Other organizations involved as partners

Organization Name:	University of California San Francisco
Location:	San Francisco, CA
Contribution:	Hosted Jan Lammerding during his sabbatical from Sept. 2015 – July 2016; provided training in in vivo studies in mouse breast cancer models, including injection of mouse breast cancer cells into mammary fat pads, collection of primary tumor and metastasis, etc.
Organization Name:	MD Anderson Cancer Center
Location:	Houston, TX
Contribution:	Collaboration on nuclear envelope rupture studies in fibrosarcoma cells, performing intravital imaging studies.
Organization Name:	Radboud University Medical Center
Location:	Nijmegen, Netherlands
Contribution:	Collaboration on nuclear envelope rupture studies, performing migration assays in collagen matrix. De-identified tissue microarrays.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDIX

The appendix contains reprints of the following publications:

1. Mekhdjian AH, Kai F, Rubashkin MG, Prahls LS, Przybyla LM, McGregor AL, Bell ES, Barnes JM, DuFort CC, Ou G, Chang AC, Cassereau L, Tan SJ, Pickup MW, Lakins JN, Ye X, Davidson MW, Lammerding J, Odde DJ, Dunn AR, Weaver VM. Integrin-mediated traction force enhances

- paxillin molecular associations and adhesion dynamics that increase the invasiveness of tumor cells into a three-dimensional extracellular matrix. **Mol Biol Cell**. 2017. 28(11): 1467-1488
2. Morelli FF, Verbeek DS, Bartacchini J, Vinet J, Mediani L, Marmiroli S, Cenacchi G, Nasi M, De Biasi S, Brunsting JF, Lammerding J, Pegoraro E, Angelini C, Tupler R, Alberti S, Carra S. Aberrant Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function. **Cell Reports**. 2017. 20(9): 2100-2115. Federal support acknowledged.
 3. Bakhoum SF, Ngo B, Bakhoum AL, Cavallo JA, Murphy CJ, Ly P, Shah P, Sriram RK, Watkins TBK, Taunk NK, Duran M, Pauli C, Shaw C, Chadalavada K, Rajasekhar VK, Genovese G, Venkatesan S, Birkbak NJ, McGranahan N, Lundquist M, LaPlant Q, Healey JH, Elemento O, Chung CH, Lee NY, Imielenski M, Nanjangud G, Pe'er D, Cleveland D, Powell SN, Lammerding J, Swanton C, Cantley LC. Chromosomal instability promotes metastasis through a cytosolic DNA response. **Nature**. 2018. 553(7689): 467-472. Federal support acknowledged.
 4. Kirby TJ, Lammerding J. Emerging views of the nucleus as a cellular mechanosensor. **Nature Cell Biology**. Published online 2/23/2018. Federal support acknowledged.
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Integrin-mediated traction force enhances paxillin molecular associations and adhesion dynamics that increase the invasiveness of tumor cells into a three-dimensional extracellular matrix

Armen H. Mekhdjian^{a,†}, FuiBoon Kai^{b,†}, Matthew G. Rubashkin^{b,†}, Louis S. Prah^c, Laralynne M. Przybyla^b, Alexandra L. McGregor^d, Emily S. Bell^d, J. Matthew Barnes^b, Christopher C. DuFort^b, Guanqing Ou^b, Alice C. Chang^a, Luke Cassereau^b, Steven J. Tan^a, Michael W. Pickup^b, Jonathan N. Lakins^b, Xin Ye^e, Michael W. Davidson^f, Jan Lammerding^d, David J. Odde^c, Alexander R. Dunn^{a,*}, and Valerie M. Weaver^{b,g,*}

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ABSTRACT Metastasis requires tumor cells to navigate through a stiff stroma and squeeze through confined microenvironments. Whether tumors exploit unique biophysical properties to metastasize remains unclear. Data show that invading mammary tumor cells, when cultured in a stiffened three-dimensional extracellular matrix that recapitulates the primary tumor stroma, adopt a basal-like phenotype. Metastatic tumor cells and basal-like tumor cells exert higher integrin-mediated traction forces at the bulk and molecular levels, consistent with a motor-clutch model in which motors and clutches are both increased. Basal-like non-malignant mammary epithelial cells also display an altered integrin adhesion molecular organization at the nanoscale and recruit a suite of paxillin-associated proteins implicated in invasion and metastasis. Phosphorylation of paxillin by Src family kinases, which regulates adhesion turnover, is similarly enhanced in the metastatic and basal-like tumor cells, fostered by a stiff matrix, and critical for tumor cell invasion in our assays. Bioinformatics reveals an unappreciated relationship between Src kinases, paxillin, and survival of breast cancer patients. Thus adoption of the basal-like adhesion phenotype may favor the recruitment of molecules that facilitate tumor metastasis to integrin-based adhesions. Analysis of the physical properties of tumor cells and integrin adhesion composition in biopsies may be predictive of patient outcome.

Monitoring Editor

Alpha Yap
University of Queensland

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The authors declare no conflicts of interest.

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Abbreviations used: AFM, atomic force microscopy; 3B, Bayesian analysis of bleaching and blinking; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; FA, focal adhesion; FAK, focal adhesion kinase; FRET, Förster resonance energy transfer; ILK, integrin-linked kinase; MEC, mammary epithelial cell;

MTS, molecular tension sensor; PA, polyacrylamide; PINCH, particularly interesting new cysteine-histidine-rich protein; SAIM, scanning angle interference microscopy; TFM, traction force microscopy; TGF β , transforming growth factor β .

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INTRODUCTION

Metastasis is the main cause of death in patients with malignant tumors (Weigelt *et al.*, 2005; Johnson *et al.*, 2013; Lobbzoo *et al.*, 2015). Tumor metastasis proceeds through a series of critical steps that include invasion of malignant cells across the basement membrane and their directed migration into the parenchyma, where they form invasive carcinomas (Rowe and Weiss, 2008; Egeblad *et al.*, 2010). Thereafter, to metastasize, the malignant cells must migrate through the interstitial extracellular matrix (ECM) toward blood vessels and intravasate into the vasculature, where they can circulate as single cells, as cellular aggregates, or within fibrin-rich emboli (Matsugué and Obenauf, 2016). Eventually, the disseminated “circulating” tumor cells either die or adhere to the capillaries of distal tissues, where they can extravasate. Once at the secondary site, the tumor cells must proliferate and recruit a new vasculature to form metastases (Nguyen *et al.*, 2009; Telerman and Amson, 2009; Chaffer and Weinberg, 2011). Each of these steps favors tumor cells that migrate efficiently through a dense, collagen- and fibronectin-rich ECM, as well as cells that are able to retain their viability during intravasation and extravasation—processes that demand considerable cellular and nuclear squeezing and distortion (Friedl and Wolf, 2003; Egeblad *et al.*, 2010; Roussos *et al.*, 2011; Bravo-Cordero *et al.*, 2012; Nguyen-Ngoc *et al.*, 2012; Denais *et al.*, 2016). Despite the clinical importance of metastasis and an appreciation of the physical hurdles that a tumor cell must overcome in order to disseminate, it remains unclear whether tumor cells exploit specific biophysical states or acquire unique mechanical properties to participate in the metastatic journey.

Cancer cell invasion and metastasis are influenced by the mechanical properties of the primary tumor and in particular the stiffness of the stromal ECM (Paszek *et al.*, 2005; Levental *et al.*, 2009; Pickup *et al.*, 2013; Bae *et al.*, 2014; Mouw *et al.*, 2014; Rubashkin *et al.*, 2014a; Acerbi *et al.*, 2015; Kai *et al.*, 2016). A stiffened ECM fosters malignant transformation and metastasis of mammary tissue *in vivo* and stimulates the invasion of human and murine mammary epithelial cells (MECs) *in vitro* (Paszek *et al.*, 2005; Levental *et al.*, 2009; Schedin and Keely, 2011; Pickup *et al.*, 2013; Rubashkin *et al.*, 2014a). The invasive front of human breast tumors is also stiffer, and the level of stiffness correlates with human breast tumor aggression (Acerbi *et al.*, 2015). These findings are consistent with the general observation that cells tune their cytoskeletal tension to the stiffness of their underlying ECM (Engler *et al.*, 2004; Discher *et al.*, 2005; Paszek *et al.*, 2005; Yeung *et al.*, 2005; Saha *et al.*, 2008; Butcher *et al.*, 2009; Allen *et al.*, 2012; Cosgrove *et al.*, 2014; Chaudhuri *et al.*, 2015). Invading tumor cells engage the ECM through integrins, a class of heterodimeric, transmembrane proteins that link the cytoskeleton to the surrounding ECM. In response to a stiff ECM, integrin receptors assemble into focal adhesions (FAs), micrometer-sized assemblies consisting of hundreds of proteins that, in addition to their mechanical roles, mediate signaling through multiple pathways (Hoffman *et al.*, 2011). Mechanically activated signaling via integrin-based adhesions not only enhances tumor cell growth and survival, but it also drives tumor cell invasion and migration (Jalouk and Lammerding, 2009; DuFort *et al.*, 2011; Batra *et al.*, 2012; Carisey *et al.*, 2013; Ross *et al.*, 2013; Yao *et al.*, 2014; Rubashkin *et al.*, 2014b; Paluch *et al.*, 2015). Consistently, treatments that inhibit FA formation or that ablate FA signaling reduce cancer progression and metastasis (White *et al.*, 2004; Huveneers *et al.*, 2007; Provenzano *et al.*, 2008; Sulzmaier *et al.*, 2014; Winograd-Katz *et al.*, 2014). These observations support a picture in which a stiffened stroma, contractile cancer cell cytoskeleton, and integrin-dependent intracellular signaling combine to support cancer growth

and metastasis. However, the colonization of distant metastasis sites requires passage through a series of narrow constrictions. This would seem to pose a contradiction because a stiffened tumor cell should have a distinct disadvantage when attempting to navigate confined microenvironments during the process of metastasis.

One plausible explanation that could link matrix mechanics, adhesion dynamics, and metastatic potential may reside in the observation that metastatic tumor cells often exhibit traits consistent with an epithelial-to-mesenchymal transition (EMT)—a developmental process that is often hijacked by cancer cells (Egeblad *et al.*, 2010; Taube *et al.*, 2010; Craene and Berx, 2013; Jung *et al.*, 2015). An EMT allows formerly epithelial cells to access a mesenchymal form of cell migration that conceivably facilitates cancer cell invasion within dense ECMs, permitting their efficient dissemination to form distant metastatic colonies (Chaffer and Weinberg, 2011; Craene and Berx, 2013; Lamouille *et al.*, 2014). Canonical EMT is characterized by expression of key transcriptional regulators, including Twist, Snail, and Slug, leading to a loss in cadherin-dependent cell–cell adhesion and apical-basal polarity and increased cell–ECM adhesion and matrix metalloproteinase (MMP) expression (Yilmaz and Christofori, 2009; Przybyla *et al.*, 2016). Moreover, whereas an EMT-like transition can be triggered by exposure to chemokines such as transforming growth factor β (TGF β), it is also promoted by a stiff ECM that is located at the invasive front of tumors (Acerbi *et al.*, 2015; Wei *et al.*, 2015; Przybyla *et al.*, 2016).

Of importance, the idea that tumor cells require an EMT to metastasize and that this transformation is associated with reduced cortical tension has been questioned (Thoenkeling *et al.*, 2010; Buckley *et al.*, 2012; Schneider *et al.*, 2013; Fischer *et al.*, 2015; Zheng *et al.*, 2015). In some circumstances, metastasis is favored by tumor aggregates (Friedl *et al.*, 2012; Nguyen-Ngoc *et al.*, 2012; Cheung *et al.*, 2016). Furthermore, intravital imaging clearly illustrates that tumor cells can invade as cellular collectives (Gaggioli *et al.*, 2007; Hidalgo-Carcedo *et al.*, 2010; Manning *et al.*, 2015), a finding that accords well with the observation that metastatic cells often retain E-cadherin expression (Cheung *et al.*, 2013; Aceto *et al.*, 2014). A plausible alternative explanation for the apparently disparate theories that argue for either single EMT or collective tumor metastasis is that tumor cells may engage in a “partial” or “transient” EMT to disseminate (Chaffer and Weinberg, 2011; Jolly *et al.*, 2015, 2016). Consistent with this proposition, live-cell imaging of tumor aggregates with a high propensity to metastasize revealed that the leader “invading” cells exhibited a basal-epithelial phenotype (for convenience, “basal-like”), with features reminiscent of a partial EMT (Cheung *et al.*, 2013).

In summary, although evidence indicates that matrix mechanical properties can exert strong effects on cancer metastasis, the molecular mechanisms are poorly understood. In particular, whether a stiff ECM could foster tumor metastasis by inducing a “basal-like” or pseudo-EMT phenotype via mechanical, integrin-mediated signaling has yet to be determined. Moreover, whether this “basal-like switch” endows tumor cells with biophysical properties that potentiate the metastasis of the EMT-like cells and, if so, how, are not known. To investigate these possibilities, we interrogated the relationship between integrin adhesion dynamics, composition, and forces and the cellular biophysical properties that favor tumor cell invasion within a confined, stiffened three-dimensional (3D) ECM.

RESULTS

ECM stiffness stimulates tumor cell invasion and induces a basal-like phenotype

We first asked whether a stiffened tumor ECM could promote the metastatic behavior of mammary tumors by inducing a basal-like,

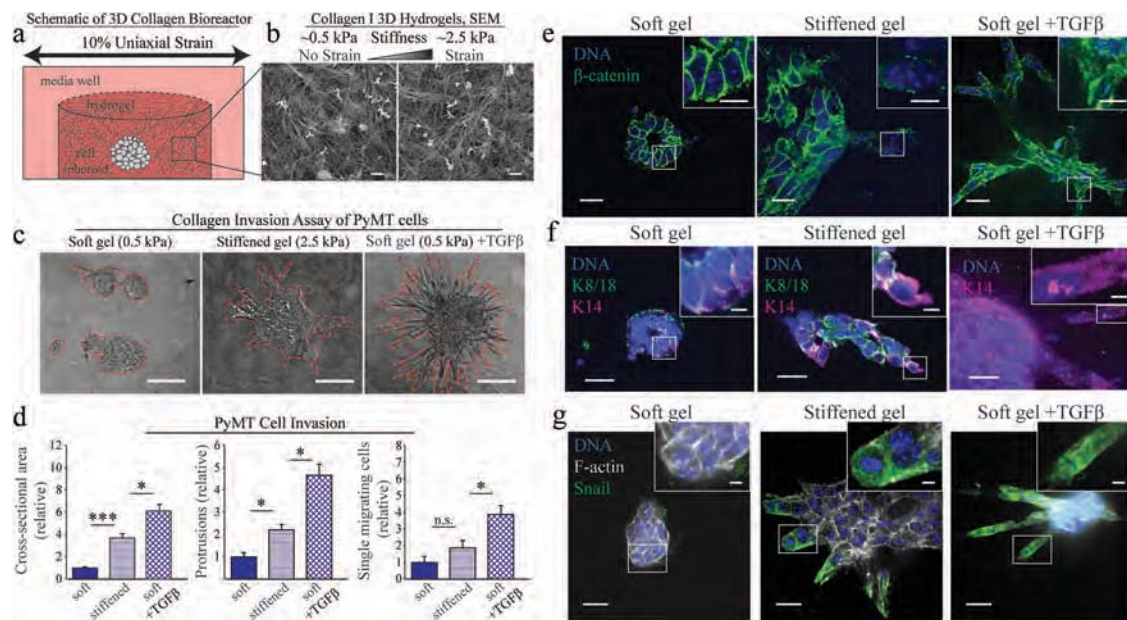


FIGURE 1: Tumorigenic cancer cells exhibit a basal-like phenotype that is fostered by ECM stiffness and EMT. (a) Schematic of the 3D collagen bioreactor. Collagen I hydrogels are made in a media reservoir as described previously (Cassereau *et al.*, 2015), and the application of 10% strain leads to an increase in effective gel stiffness from 0.5 to 2.5 kPa. (b) SEM images of representative 2.5 mg/ml collagen hydrogels in strained and unstrained conditions. Porosity and fiber orientation are unaltered at 10% strain; increased stiffness may reflect fiber bundling. Scale bar, 2 μ m. (c) A 3D collagen invasion assay of multicellular spheroids composed of tumorigenic PyMT cells in 2.5 mg/ml 3D collagen gels that are unstrained (soft, 0.5 kPa) or strained (stiffened, 2.5 kPa) for 72 h. Tumorigenic PyMT cells recapitulate invasive behavior when induced to undergo EMT via treatment with TGF β . Scale bar, 100 μ m. (d) Quantification of invasion parameters, total cross-sectional area of spheroid and protrusions, number of protrusions, and number of migrating cells normalized to the unstrained, untreated condition. Mean $\pm$ SEM (* p < 0.05; *** p < 0.001; >20 cells). (e) Immunofluorescence imaging of PyMT cell spheroids in 3D collagen gel stained for β -catenin (green) and DNA (DAPI, blue). β -Catenin strongly localizes to cell–cell junctions in cells cultured in soft 3D gels, whereas it is found more diffusely within the cytoplasm and nucleus in strain-stiffened gels and TGF β -treated cells. (f) PyMT cell spheroids in a 3D collagen bioreactor express basal epithelial marker K14 at the leader cells when cultured in strain-stiffened gels but maintain luminal epithelial K8/18 in the noninvasive core. Induction of EMT via TGF β treatment also leads to expression of K14 in leader cells. (g) Snail transcription visualized by Snail-YFP is up-regulated in leader cells in strain-stiffened gels and TGF β -treated cells. Scale bar, 50 μ m (e–g), 10 μ m (inset).

invasive phenotype (Pickup *et al.*, 2013). We examined the behavior of mouse tumor colonies (Borowsky *et al.*, 2005) generated by growing mammary epithelial cell (MEC) PyMT tumor cells within a reconstituted basement membrane (rBM; 3–5 d) to form colonies, followed by their embedment within a 3D collagen/fibronectin bioreactor (72 h), where they were either maintained without modification or subjected to uniaxial stretch (10%; 72 h). The uniaxial stretch induces local strain stiffening of the collagen gel, increasing its stiffness fivefold (from ~0.5 to ~2.5 kPa) without altering bulk ECM concentration or pore size (Cassereau *et al.*, 2015; Figure 1, a and b). Phase contrast images of colonies 72 h after their embedment and manipulation showed that the PyMT tumor colonies within the soft collagen/fibronectin gels exhibited little to no invasion. However, PyMT tumor colonies subjected to a stiffened ECM invaded significantly, although not to the same extent as those treated with TGF β to induce an EMT (Figure 1, c, and quantified in d). On further investigation, confocal images revealed that the PyMT colonies within the soft (~0.5 kPa) collagen/fibronectin hydrogels retained their epithelial phenotype, as indicated by β -catenin localization to cell–cell junctions, maintenance of cortical actin organization, and intense cytokeratin K8/18 expression (Figure 1, e and f, and Supplemental Figure S1). In contrast, the PyMT MECs invading into the stiffened ECM gels exhibited hallmarks of a basal-like phenotype.

This basal-like phenotype included loss of junctional β -catenin, reorganization of actin into linearized bundles parallel to the axis of invasion, and acquisition of cytokeratin K14 in the leader cells, reminiscent of, but not identical to, the PyMT MECs that had undergone an EMT in response to TGF β (Figure 1, e and f, and Supplemental Figure S1). Furthermore, expression of a knock-in IRES (internal ribosomal entry site) yellow fluorescent protein (YFP) Snail reporter (Ye *et al.*, 2015) indicated that only the invading PyMT leader cells within the stiff gels had abundant Snail transcripts that approximated the level uniformly expressed by all cells in PyMT colonies that had undergone a complete EMT (Figure 1g). These findings are consistent with the idea that a stiffened ECM could promote tumor metastasis by inducing a basal-like or partial EMT phenotype.

Metastatic tumor cells and tumor cells with a basal-like phenotype exhibit enhanced invasion within confined spaces

A prerequisite for tumor metastasis is the efficient migration of tumor cells through a dense and stiffened ECM, followed by their intravasation into the vasculature and extravasation into the metastatic site. These behaviors would plausibly be favored by more-compliant cells, as predicted to be exhibited by cells with either a basal or a mesenchymal phenotype after EMT (Sahai, 2007;

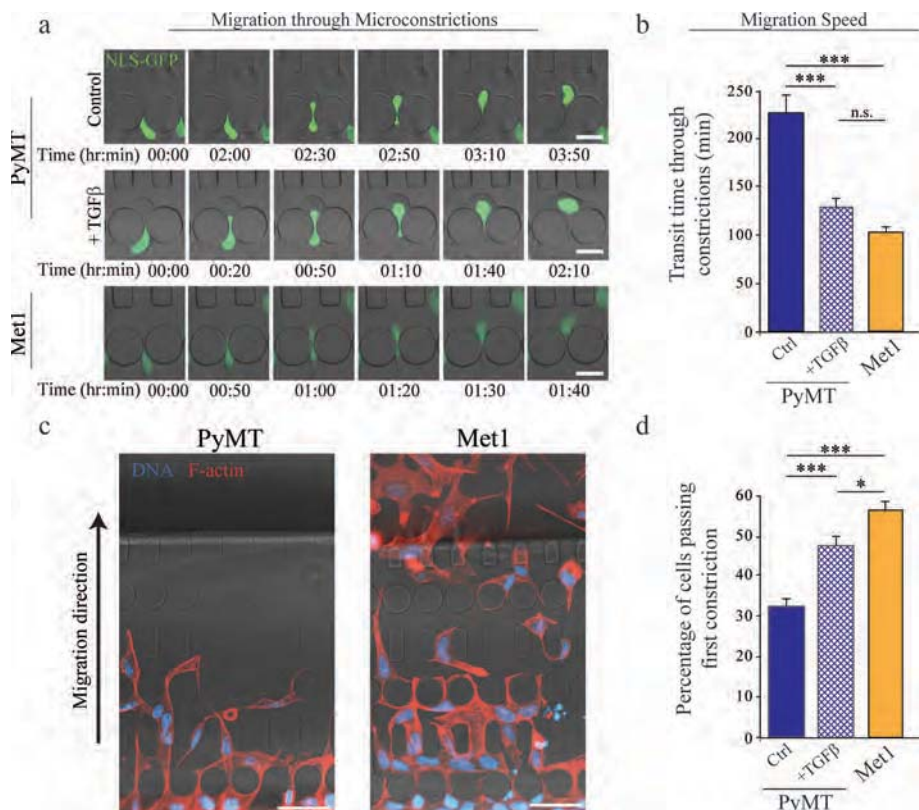


FIGURE 2: Cells with a basal phenotype display enhanced migration through microconstrictions. (a) Representative time-lapse image sequences of PyMT cells, PyMT cells treated with TGF β , and Met1 cells migrating through a $1 \times 5 \mu\text{m}^2$ constriction inside a microfluidic device. Cells express NLS-GFP to visualize the nucleus. Time displayed as hours:minutes. Scale bar, $20 \mu\text{m}$. (b) Transit times for cell passage through constrictions $\leq 10 \mu\text{m}^2$ in cross-sectional area, demonstrating that both Met1 cells and PyMT cells treated with TGF β migrate faster through the small constrictions. Mean $\pm$ SEM ($*p < 0.05$, $***p < 0.001$, by Kruskal-Wallis test with Dunn's multiple comparisons; >70 cells from three independent experiments). (c) Representative images of cells inside the microfluidic devices after 48 h of confined migration. Fixed cells were stained for F-actin (phalloidin) and DNA (Hoechst 33342). Scale bar, $50 \mu\text{m}$. (d) Incidence of cells successfully migrating through the first row of constrictions within 36 h relative to the total number of cells inside the microfluidic channels. Error bars represent SE of the binomial distribution ($*p < 0.05$; $***p < 0.001$; >500 cells by Fisher's exact test with Bonferroni correction).

Swaminathan *et al.*, 2011; McGrail *et al.*, 2015). To test this idea, we examined the migration of PyMT tumor MECs through constricted microchannels that mimic the confined, stiff microenvironment through which metastasizing tumor cells would likely navigate (Davidson *et al.*, 2015; Denais *et al.*, 2016; Figure 2). We compared cell behaviors after induction of EMT by treatment with TGF β to that of Met1 MECs, a PyMT variant that was previously shown to exhibit a propensity to metastasize and yet retain epithelial-like characteristics (Borowsky *et al.*, 2005; Qian *et al.*, 2011). TGF β -treated PyMT MECs migrated more efficiently than untreated MECs through the confined channels, as indicated by significantly shorter transit times through the constrictions (Figure 2, a and b) and higher percentages of cells migrating through constrictions with a cross-sectional area of $<10 \mu\text{m}^2$ (Figure 2, c and d). Of importance, Met1 tumor MECs migrated as efficiently as the TGF β -treated PyMT tumor MECs, implying that complete EMT is not a prerequisite for efficient migration through a confined space.

Because cells with increased mechanical deformability pass more easily through confined spaces, we concluded that it was possible that increased migration of TGF β -treated PyMT MECs and

Met1 MECs was due to altered cytoskeletal mechanical deformability. To test this idea, we used atomic force microscopy (AFM) to measure the average cortical elasticity of multiple tumor cells plated on 2.7-kPa gels, using a $5\text{-}\mu\text{m}$ bead-ligated cantilever with multiple indentations across the cellular surface (Figure 3a). The resulting force-indentation curves were fit to a Hertz model to estimate an effective elastic modulus (Figure 3b). Consistent with the notion that tumor cells with a higher propensity to metastasize are more compliant (Swaminathan *et al.*, 2011), our AFM measurements revealed that the Met1 MECs had a modest but significant reduction in effective stiffness compared with the PyMT MECs (Figure 3, c and e). Nevertheless, the compliance of the EMT-induced PyMT MECs was similar to that of the less invasive, untreated PyMT MECs (Figure 3, c and e).

Another component of cellular deformability that modulates cell migration through a confined microenvironment is nuclear stiffness (Davidson *et al.*, 2014; Harada *et al.*, 2014; Lautscham *et al.*, 2015; Denais *et al.*, 2016). Micropipette aspiration experiments indicated that there was a modest but statistically significant increase in nuclear deformability in the EMT-induced PyMT MECs (Figure 3, f and g). The findings are consistent with the argument that an EMT increases nuclear deformability and not cortical stiffness to facilitate cell migration through confined spaces and that this could potentiate tumor metastasis.

Tumor cells that migrate more efficiently within a confined space are more contractile

We next explored whether the enhanced invasive phenotype observed by the meta-

static and EMT-induced MECs in the confined channels was associated with differences in cell-ECM interactions, as measured by the degree of cell spreading and/or traction force generation. We used traction force microscopy (TFM; Munevar *et al.*, 2001; Paszek *et al.*, 2005) and compared traction forces exerted by the Met1 and the untreated versus EMT-induced PyMT tumor MECs plated on fibronectin-conjugated polyacrylamide (PA) gels with a stiffness calibrated to mimic that measured at the invasive front of the malignant human and murine mammary tissue stroma (~ 2.7 kPa; Munevar *et al.*, 2001; Paszek *et al.*, 2005; Laklai *et al.*, 2016). We observed that both the TGF β -treated PyMT MECs and the Met1 MECs generated higher maximal and total cellular traction forces (Figure 4, a, and quantified in b) and higher spread area (unpublished data). We also measured higher total and maximal adhesion traction forces and enhanced cell spreading in premalignant human MCF10AT MECs induced to undergo EMT by treatment with TGF β as compared with their nontreated controls (Figure 4, c, and quantified in d). As expected, TGF β -treated MCF10ATs also spread progressively more on hydrogel surfaces functionalized with increasing concentrations of fibronectin (Supplemental Figure S2).

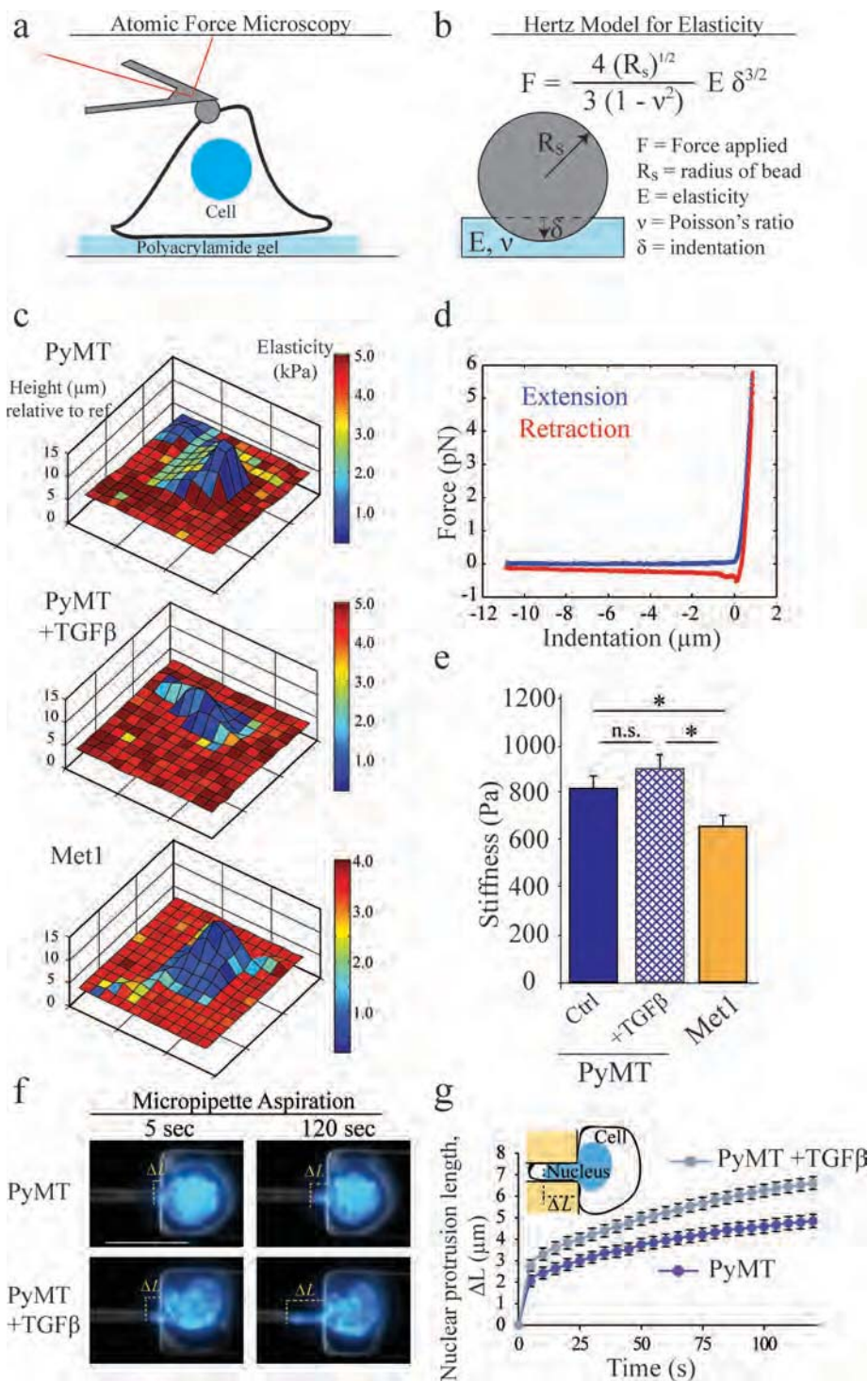


FIGURE 3: Cells with a basal phenotype are mechanically more compliant. (a) Schematic of AFM used to measure effective cell stiffness. Cells are seeded on a 2.7-kPa PA gel conjugated with fibronectin and indented using a 2.5-μm beaded tip on the AFM cantilever. (b) Hertz model and parameters used to estimate elasticity from a bead contacting a flat plane. (c) Representative example surface plots of elasticity for PyMT, TGFβ-treated PyMT, and Met1 cells, showing elasticity as function of cell topology. (d) Representative force-indentation curves for a given pixel in the surface map, showing the extension and retraction of the cantilever. The Hertz model is fit to these data to extract an effective cell elasticity. (e) Quantification of cell indentation stiffness for tumorigenic PyMT cells, with and without TGFβ treatment, and metastatic Met1 cells. Met1 cells are modestly softer on average than PyMT cells, with TGFβ treatment not eliciting a statistically significant response. Mean ± SEM (* p < 0.05; >15 cells). (f) Micropipette aspiration of PyMT and TGFβ-treated PyMT cells showing the deformation of the nucleus over time. Cell nuclei were visualized by staining DNA with Hoechst

Cells that underwent EMT frequently increase $\alpha_5\beta_1$ integrin expression, and fibronectin-ligated $\alpha_5\beta_1$ integrin can significantly enhance cell spread area and cell-ECM traction force (Mierke *et al.*, 2011; Lamouille *et al.*, 2014). Consistently, immunoblot and fluorescence-activated cell sorting (FACS) revealed more total and cell surface α_5 integrin expression in the TGFβ-treated MCF10A and PyMT MECs (unpublished data). To determine whether higher α_5 integrin expression could account for the observed gain in cell spreading and traction force generation observed in the TGFβ-treated MECs, we engineered MCF10A MECs to ectopically express higher total α_5 integrin. After confirming more than a two-fold increase in cell surface α_5 integrin expression by FACS, we assayed for cell spread area and traction force compared with paired control and TGFβ-treated MCF10A MECs. Whereas the MCF10A MECs expressing higher levels of α_5 integrin spread more and exerted higher traction force in response to a fibronectin gel, the magnitude of the increase was significantly lower than that after induction of EMT by TGFβ treatment (Supplemental Figure S3).

In summary, these findings suggest that in addition to an increase in nuclear deformability, there may also be an association between the ability of cells to exert higher cell-ECM traction forces and their ability to migrate more efficiently through a confined microenvironment. The data also argue that this phenotype cannot be solely explained by differences in integrin heterodimer expression.

Motor-clutch model of cellular traction predicts higher force and cell spread area for cells that have an enhanced ability to migrate within a confined space

We next used computational modeling to explore the interplay between cell adhesion and cell-generated traction forces exerted on compliant substrates. We first modified an existing computational motor-clutch model of cellular traction generation on compliant substrates (Chan and Odde, 2008). In this model, myosin motors slide an F-actin bundle and transmit forces to a compliant substrate through physical extension of molecular "clutch" molecules that

33342. (g) Compiled nuclear protrusion length (ΔL) as a function of time, indicating some nuclear softening in response to TGFβ treatment. Mean ± SEM (>120 cells from three independent experiments). Scale bar, 20 μm.

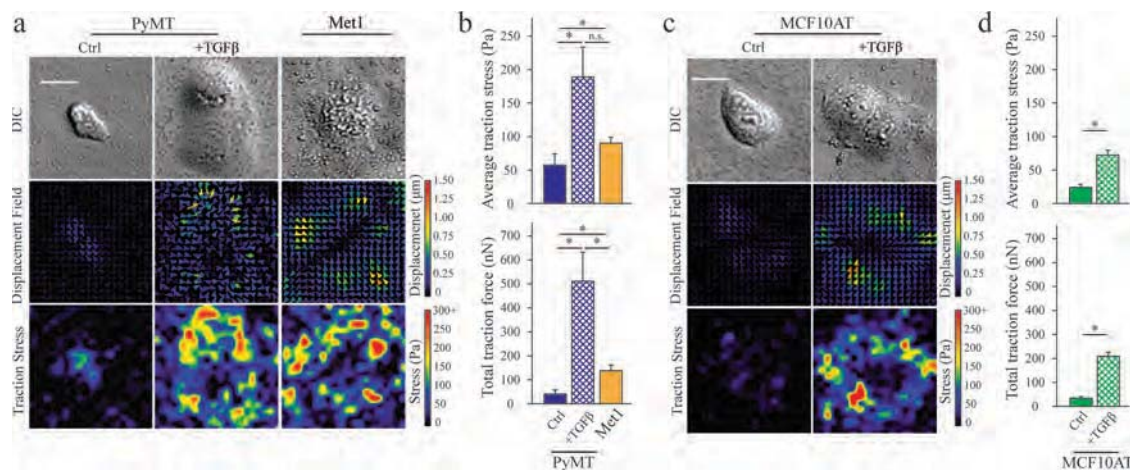


FIGURE 4: Cells with a basal phenotype exert higher traction forces. (a) PyMT and Met1 cells were seeded on 2.7-kPa PA gels conjugated with fibronectin and embedded with fluorescent beads for TFM. The displacement fields reflect the relative positions of beads before and after cell lysis. At a substrate stiffness comparable to that of tumor ECM (~2.7 kPa), Met1 cells are more spread and exert more traction forces on the substrate. Induction of EMT in PyMT cells by treatment with TGFβ recapitulates the increase in traction forces observed with Met1 cells, with total traction force even greater than for the Met1 cells. (b) Quantification of cell traction parameters. Metastatic cells have significantly increased total cellular and maximum traction forces. Mean ± SEM (* $p < 0.05$; >5 cells). (c) TFM of MCF10ATs. Cells that undergo TGFβ-induced EMT exert higher traction forces than untreated cells. (d) Quantification of cell traction parameters. TGFβ-treated MCF10ATs have significantly higher traction forces. Mean ± SEM (* $p < 0.05$; >5 cells). Scale bar, 20 μm (a, c).

transiently engage the actin filament (Figure 5a). The model revealed that efficient force transmission depends on the number and biophysical properties of the motors and clutches, clutch binding and force-dependent unbinding rates, and substrate mechanical properties (Figure 5b; Bangasser et al., 2013). Maximal force transmission occurs at an “optimum” substrate stiffness, whereas clutch unbinding increases on softer or stiffer substrates, leading to inefficient force transmission (Bangasser et al., 2013). Actin retrograde flow is biphasic and inversely correlated with traction force, and a leading-edge protrusion rate can be used as a proxy for spread area, defined by the difference between protrusion and retrograde flow (Chaudhuri et al., 2015).

Simulated cells with larger numbers of both myosin II motors and integrin-mediated adhesion clutches are better spread and generate larger tractions on surfaces with increasing stiffness but then go through a maximum so that at even higher stiffness, area and force are decreased (Figure 5, c and d). The prediction of increasing area and force is consistent with the larger adhesion sizes and increased traction generation observed for Met1 MECs and TGFβ-treated PyMT and MCF10AT MECs relative to untreated PyMT and MCF10AT cells (Paszek et al., 2005), at least below the optimal stiffness. Within the stiffness range of ~0.1–10 kPa, our results are consistent with nontransformed MECs employing a “weak clutch” regime. In this regime, an imbalance of contractile force and clutch number results in a failure to spread and low traction forces that modestly increase near disease-level stiffness. On addition of TGFβ and induction of an EMT phenotype, the simulated MECs express larger numbers of both motors and integrin-mediated adhesions and shift to a “balanced motor-clutch” regime in which the clutch strength matches that of the motors, resulting in slower actin flow, enhanced cell spreading, and higher traction forces (Figure 5, c and d).

As previously described (Chan and Odde, 2008; Bangasser et al., 2013), the motor-clutch model predicts a decrease in cell spread area and traction force at a substrate stiffness extending beyond an optimum stiffness (here ~10 kPa for TGFβ-treated

MCF10As). By contrast, many cell types remain well spread and generate large traction forces even on stiff substrates (Ghibaudo et al., 2008; Eloegui-Artola et al., 2014, 2016). We developed a modified motor-clutch model that includes force-dependent recruitment of adhesion components in response to substrate stiffness, similar to recently proposed models (Eloegui-Artola et al., 2014, 2016). Briefly, we incorporated a clutch recruitment feedback rate (k_{add}), which is proportional to the number of clutches experiencing force above a threshold ($F_{threshold}$), as well as a maximum number of clutches available to be recruited ($n_{clutch,max}$; see the Supplemental Material for model description). By varying key parameters within the feedback response (in particular, k_{add}), we recapitulate varying degrees of reinforcement on stiff substrates, with higher reinforcement values ($k_{add} = 0.1–1 \text{ s}^{-1}$), yielding a monotonic trend (Supplemental Figure S4). Hence our PA gel results alone did not provide enough information to distinguish between the two models—either TGFβ-treated cells have a higher optimum stiffness due to increased motor and clutch number, or our results are consistent with engaging adhesion reinforcement on stiff substrates.

Cells with a basal-like phenotype exert higher integrin-dependent force

To explore the model predictions further, we tested whether the increased overall cellular traction forces observed in Met1 and TGFβ-treated PyMT and MCF10A MECs translated into an increase in molecular forces at the level of individual adhesion complexes. To do so, we employed molecular tension sensors (MTSs), which report the forces exerted on integrin molecules in living cells (Morimatsu et al., 2013, 2015; Chang et al., 2016). Briefly, this technique incorporates an elastic protein spring flanked by two fluorophores that form a FRET pair (Figure 6a). The MTSs have an integrin-recognition domain containing the linear RGD tripeptide, which binds fibronectin-binding integrins. When cells adhere specifically via their integrins to an MTS-coated substrate, tension on individual sensors causes the elastic protein to stretch and the fluorophores to move

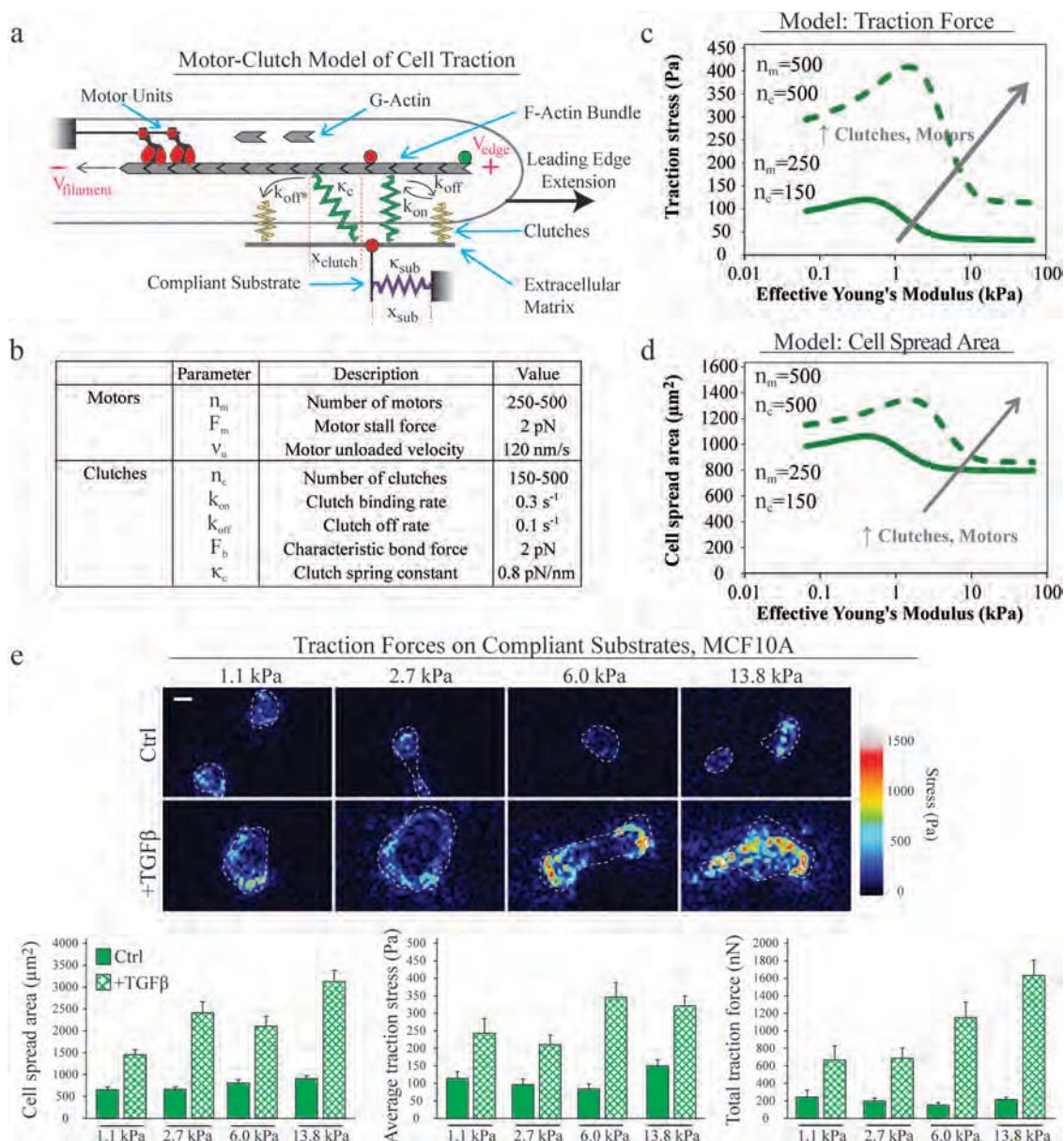


FIGURE 5: Motor-clutch model of cellular traction predicts higher force and spread area for cells with a basal phenotype. (a) Motor-clutch model of cell traction, including myosin contractility, actin assembly/treadmilling/disassembly, clutches (linear elastic springs) with constant k_{on} and variable k_{off} rates (dependent on clutch strain, x_{clutch}), and a linear elastic compliant substrate. (b) Parameter descriptions and values used in motor-clutch simulations. (c) Model predictions of traction force calculated from the results of the simulation. Increasing number of motors and clutches leads to an increase in traction force with substrate stiffness and shifts the peak traction to a higher stiffness. (d) Model predictions of cell adhesion area that is extrapolated from the length of assembled filaments, which in turn depends on the number of motors and clutches. (e) TFM of MCF10A cells with and without TGFβ treatment seeded on PA gels of varying stiffness. Cell spread area was quantified from bright-field images, average stress was calculated from the displacement fields, and total force was quantified by multiplying average traction stress by the spread area of the cell (>10 cells). The total force values represent the sum of individual pixel forces underneath the cell body as a whole (average stress times spread area). Scale bar, 20 μm.

farther apart, yielding a decreased fluorescence resonance energy transfer (FRET) signal. An average force exerted on an ensemble of sensors within a given pixel is then calculated using a previously determined calibration curve (Grashoff *et al.*, 2010) modified to incorporate the specific fluorophores used in these studies (Figure 6b). Using this approach, we found that both the TGFβ-treated PyMT MECs and the metastatic Met1 MECs generated higher total forces at their integrins than did the untreated PyMT MECs, consistent

with the TFM data (Figure 4). In addition, both the Met1 MECs and the TGFβ-treated PyMT tumor MECs exhibited a modest but statistically significant increase in the average force per MTS sensor (Figure 6, c, and quantified in d). This integrin-force phenotype appears to be conserved: MCF10As that had undergone a TGFβ-induced EMT also spread more, assembled larger adhesions, generated higher total tractions as measured using MTSs, and exhibited a modest but consistent increase in the force per MTS compared

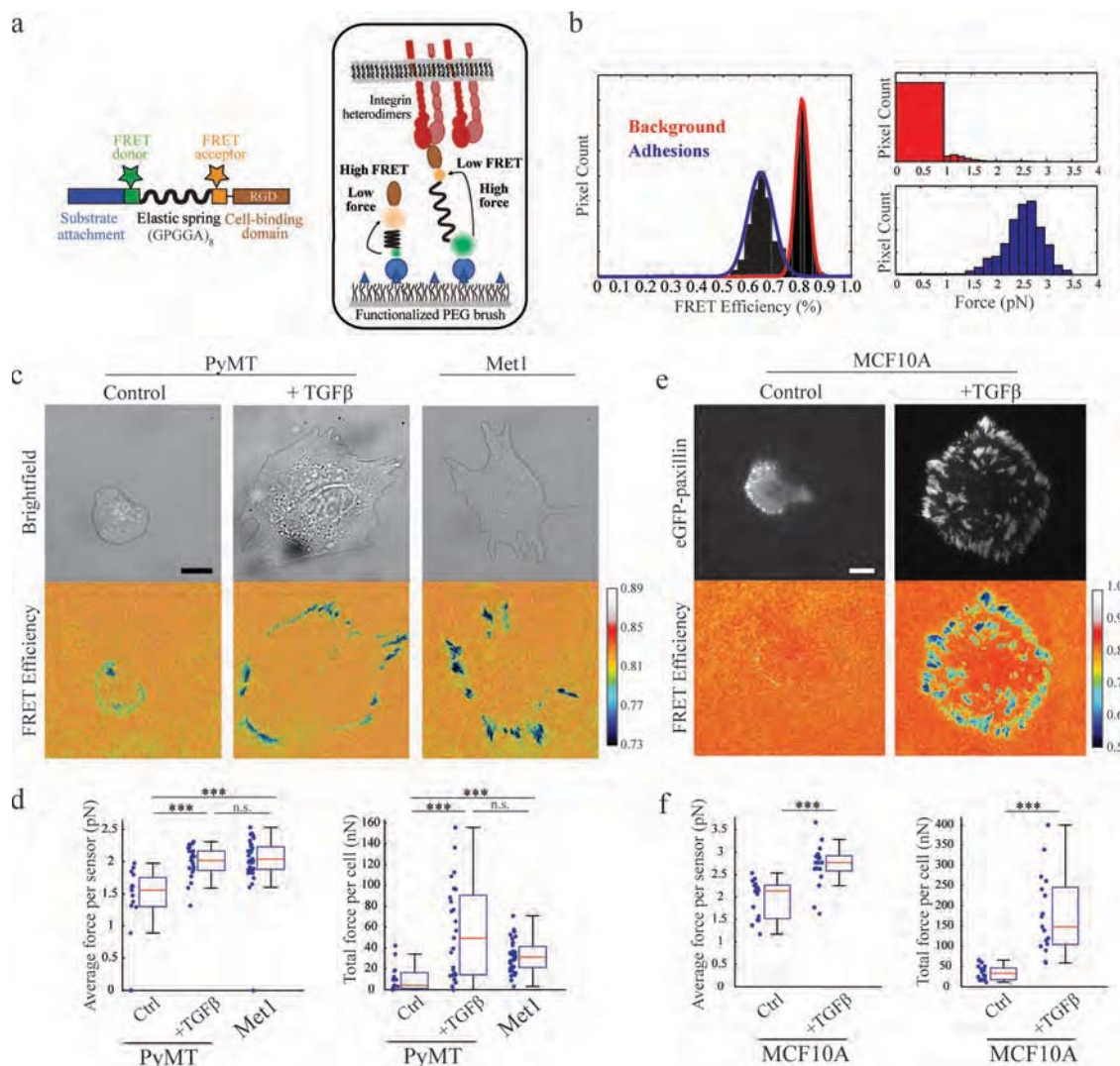


FIGURE 6: Cells with a basal phenotype exert higher integrin-dependent force. (a) Schematic of integrin MTSs. (b) FRET efficiency is converted into force using a calibration curve previously determined (Grashoff *et al.*, 2010) and modified for the fluorescent dyes used here. (c) When seeded on a coverslip densely coated with MTSs, Met1 cells and TGFβ-treated PyMT cells spread more readily and generate higher integrin-mediated forces than untreated PyMT cells. (d) Quantification of the total force and average force per integrin MTS, which are both higher for Met1 than with PyMT cells. TGFβ treatment of PyMT cells resulted in an increase in cell spread area (unpublished data), total integrin-mediated force per cell, and average force per MTS, similar to the values measured for Met1 cells ($***p < 0.001$; >15 cells by the Wilcoxon rank sum test). Box-and-whisker plots display the median (red), 25th and 75th percentiles (bottom and top edges of the box, respectively), and the most extreme data points not considered outliers (edge of whiskers). (e) Integrin MTS measurements for MCF10A cells treated with TGFβ. (f) There is an increase in total force and average force for the TGFβ-treated MCF10As compared with the untreated ones ($***p < 0.001$; >15 cells by the Wilcoxon rank sum test). In d and f, the total force values represent the sum of individual pixel forces specifically within segmented adhesions. Scale bar, 10 μm (c, e).

with untreated MCF10A MECs (Figure 6, e, and quantified in f). Note that the forces reported in Figure 6 are calculated specifically within adhesions; analyzing the MTS sensor data in a similar manner as the TFM data (namely, by including all pixels underneath the cell body) yields similar magnitudes of total force between the two techniques (Supplemental Figure S5; Chang *et al.*, 2016). Because the MTS substrates are effectively much stiffer than PA gels, we used this to test the reinforcement-model prediction that total traction forces and spread areas for TGFβ-treated cells remain uniformly high on stiff substrates. Of interest, contrary to this prediction, TGFβ-treated cells show lower traction forces and reduced spread area on the MTS substrates compared with 13.8-kPa gels (Supple-

mental Figure S5), consistent with cells adhering to surfaces that are stiffer than their optimal stiffness (Bangasser *et al.*, 2013). In addition, whereas the increased force per bond is statistically significant, it is relatively modest compared with the increase in overall traction force (Figure 6, c–f), implying that the number of motors and clutches both increased. Therefore we conclude that the results are consistent with a model in which EMT transition induces an increase in both motors and clutches while also shifting from a “weak clutch” regime to “balanced motor-clutch” regime (as in Figure 5, c and d) but are inconsistent with a model that relies entirely on a force-feedback reinforcement mechanism (as in Supplemental Figure S4, c and d).

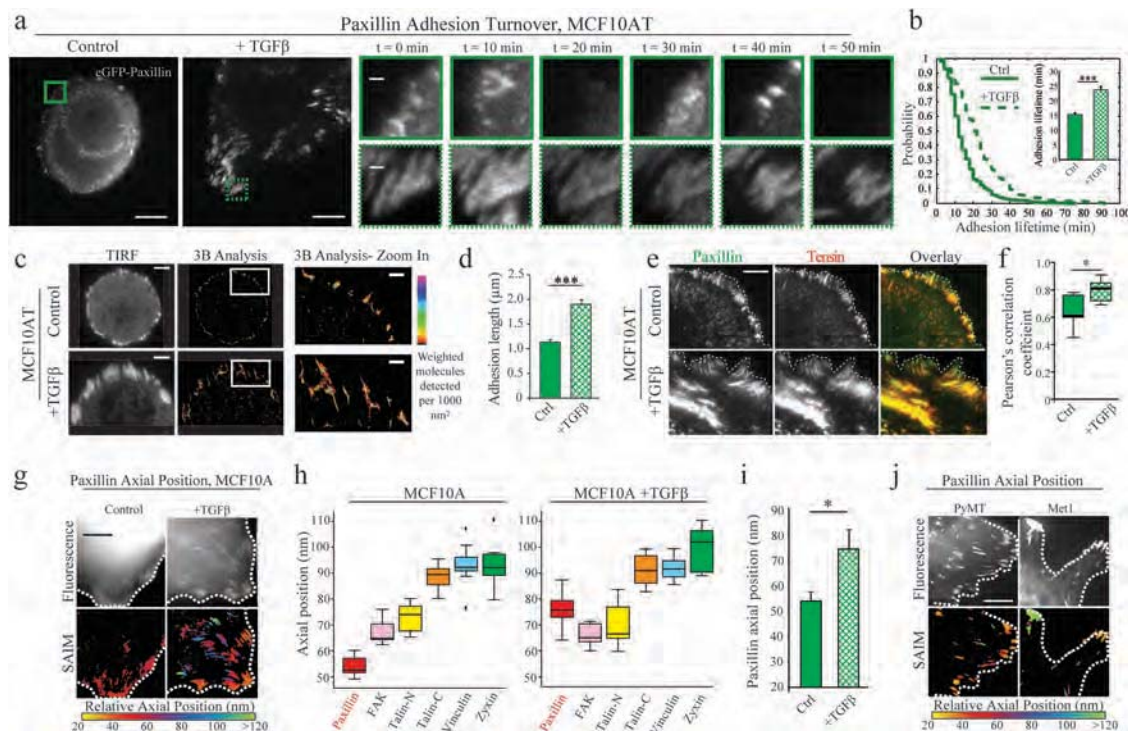


FIGURE 7: TGFβ-induced EMT alters adhesion organization and stability. (a) Live-cell imaging of paxillin-mEmerald and tensin-mCherry to visualize focal adhesion turnover in MCF10AT cells with and without TGFβ treatment. Top row: control, bottom row: TGFβ-treated. Scale bar, 10 μm (main image), 1 μm (inset). (b) Cumulative distributions of adhesion lifetimes. Inset, quantification of mean adhesion lifetime; mean ± SEM (****p* < 0.001; >150 adhesions from five cells total). (c) Focal adhesion microstructure of MCF10AT seeded on MTSs overexpressing paxillin-eGFP and imaged with TIRF and 3B superresolution microscopy. Scale bar, 3 μm (main image), 1 μm (zoom). (d) Quantification of mean adhesion length of MCF10AT cells. TGFβ-treated cells have significantly longer adhesions; mean ± SEM (****p* < 0.001; >100 adhesions from five cells). (e) Tensin-positive fibrillar adhesion formation in MCF10AT overexpressing paxillin-mEmerald and tensin-mCherry. Scale bar, 3 μm. (f) Quantification of the spatial colocalization between tensin and paxillin in adhesions. TGFβ-treated cells have significantly more paxillin-tensin colocalization (**p* < 0.05; >10 cells). Pearson's correlation was calculated based on pixels within the focal adhesion areas. (g) Paxillin-mEmerald (N-terminal) axial position in focal adhesions in MCF10As measured with SAIM. Paxillin position is plotted as the relative axial distance from the silicon wafer coated with fibronectin. Scale bar, 5 μm. (h) SAIM axial height measurements of adhesion proteins tagged with fluorescent proteins in MCF10As with and without TGFβ treatment. Paxillin is the only protein to significantly change axial position >10 nm, suggesting that the position of paxillin moves relative to that of the adhesion as a whole. (i) Quantification of paxillin-GFP axial position in MCF10As with and without TGFβ treatment. Paxillin in treated cells is located at a significantly higher axial nanoscale position (**p* < 0.05; >10 cells). (j) Representative images of the axial position of paxillin-mEmerald (N-terminal) in focal adhesions in PyMT and Met1 cells. Scale bar, 5 μm.

In sum, these data indicate that tumor MECs with an elevated propensity to metastasize (here, Met1) exert higher traction forces than less metastatic but malignant MECs (here, PyMT), due primarily to an increase in adhesion size, but also to an increase in the average force per integrin. This phenotype is shared by MECs that have undergone a TGFβ-induced EMT and should therefore also be recapitulated in the basal-like tumor MECs encountering a stiffened ECM (Figure 1, e–g). Indeed, when considered with the behavior of MECs invading into 3D, stiffened, confined ECMs or within microchannels (Figures 1 and 2), these findings suggest that tumor MECs that assemble larger adhesions and exert higher integrin-generated forces may be able to migrate more efficiently in confined microenvironments.

The integrin adhesions in cells with a basal-like phenotype are more stable and elongated

To clarify the molecular origins of the increased force per integrin within adhesions, we examined the organization and stability of the

adhesions in epithelial MCF10AT MECs and compared our findings to those measured in MECs that had been induced to undergo an EMT by TGFβ treatment. We conducted live-cell imaging of focal adhesion dynamics using paxillin-mEmerald as a proxy adhesion marker in live cells (Figure 7a). Consistent with our traction force data and the model predictions, we noted that the stability of the integrin adhesions was increased in the TGFβ-treated MCF10AT MECs, as evidenced by slower adhesion turnover (Figure 7b). To explore whether differences in adhesion lifetime reflected an altered adhesion microstructure, we performed superresolution microscopy on cellular paxillin-enhanced green fluorescent protein (eGFP) using Bayesian analysis of bleaching and blinking (3B), which uses the fluctuations in the fluorophore intensities to infer molecular localization at subpixel resolutions (Cox *et al.*, 2011). Whereas adhesions in MCF10AT MECs were roughly circular in aspect, the adhesions assembled in TGFβ-treated MCF10AT MECs were elongated and featured well-developed linear structures (possibly templated by actin bundles; Figure 7, c and d), similar to the adhesions

observed in fibroblasts and other mesenchymal cells (Morimatsu *et al.*, 2015).

Alterations in adhesion stability and structure plausibly reflect differences in molecular composition (Zamir *et al.*, 1999; Yoshigi *et al.*, 2005; Wolfenson *et al.*, 2013). To investigate this possibility, we assayed the colocalization of tensin, a marker for fibrillar adhesions, with paxillin in MCF10A MECs with and without TGF β treatment (Figure 7e). Pearson's correlation coefficient between paxillin and tensin was significantly higher for the TGF β -treated MECs, suggesting that the differences in integrin adhesion size and shape were mirrored by changes in molecular composition (Figure 7f). To further probe potential differences in molecular architecture, we performed scanning angle interference microscopy (SAIM; Paszek *et al.*, 2012; DuFort and Paszek, 2014) to measure the axial position of known adhesion proteins (Figure 7, g–i). We found that focal adhesion kinase (FAK), talin, vinculin, and zyxin retained the same relative axial positions in both MCF10A and TGF β -treated MCF10As (referenced to the coverslip; Figure 7 h). Intriguingly, the position of paxillin relative to the silicon wafer substrate increased from an average of ~50 nm in the epithelial MCF10As to ~65 nm in the TGF β -treated, EMT-induced MCF10As (Figure 7i). SAIM imaging revealed a similar qualitative shift in paxillin height between the epithelial PyMT tumor MECs and their TGF β -treated EMT-induced counterparts (Figure 7j). Of importance, paxillin phosphorylation alone could not account for the shift in molecular height observed after EMT induction, as revealed by studies in which a nonphosphorylatable paxillin (mutated at tyrosine residues 31 and 118 to phenylalanine; paxillin^{Y31/118F}) localized at the same height as a wild-type paxillin in paxillin-null mouse fibroblasts transfected with equal quantities of transgene (Supplemental Figure S6). These findings suggest that MECs that recapitulate a basal-like phenotype or that underwent a mesenchymal transition not only exert more cellular–ECM integrin traction forces, but also assemble integrin adhesions that may be more stable and acquire a distinct cellular and subcellular organization.

Proteomics identifies a protein complex implicated in tumor cell invasion and metastasis recruited to integrin adhesions in cells with a basal-like phenotype

We next sought to determine whether the differences in integrin adhesion force, stability, and cellular and subcellular organization observed in the cells with a basal-like phenotype translated into distinct differences in molecular composition. Given the significant height changes we observed in paxillin in the EMT-induced MECs, we used a mass spectroscopic proteomics approach termed Bio-ID (Roux *et al.*, 2012) to explore whether this was reflected by altered molecular interactions. We fused a promiscuous biotin ligase protein (BirA*) to the N-terminus of paxillin-eGFP and expressed this construct in MCF10A MECs treated with vehicle or TGF β to induce an EMT (Figure 8a; Dong *et al.*, 2016). Analogous measurements used BirA* fused to eGFP as a control for nonspecific biotinylation. Focusing on the consensus list of integrin adhesome proteins (Horton *et al.*, 2015; Figure 8b), we found that particularly interesting new cysteine-histidine-rich protein (PINCH; gene name LIMS1) was the most significantly enriched associated protein proximal to paxillin in the TGF β -treated MCF10A MECs (highly abundant in TGF β -treated cells and not present in untreated cells). Intensity correlation analysis of immunostained human MCF10A MECs showed that the amount of PINCH in paxillin-rich adhesions increased significantly in MCF10A MECs after TGF β treatment (Supplemental Figure S7). Indeed, even in the epithelial MCF10A MECs that assembled sizeable integrin adhesions, PINCH was largely absent or diffusely colocal-

ized. PINCH forms a complex with integrin-linked kinase (ILK) and α -parvin and interacts with paxillin at focal adhesions (Legate *et al.*, 2006), and knockout of PINCH suppresses migration and vinculin localization to adhesions (Gonzalez-Nieves *et al.*, 2013). Consistent with observations that ILK modulates breast tumor aggression and metastasis in vivo and has been linked to an EMT phenotype (White *et al.*, 2001; Persad and Dedhar, 2003; Hannigan *et al.*, 2005), we observed ILK enrichment at paxillin adhesions in MCF10A MECs that had been induced to undergo EMT by TGF β treatment (Figure 8d). Immunostaining also revealed ILK localized to the paxillin adhesions in the PyMT tumor MECs after TGF β treatment and in the PyMT tumor MECs interacting with a stiff ECM compared with those plated on a soft ECM (Figure 8, e and f). Of importance, immunoblot analysis showed that overall levels of total paxillin and phosphorylated paxillin, as well as of ILK, remained largely unchanged after TGF β treatment (Supplemental Figure S8a). Thus the enhanced association of ILK proximal to paxillin in adhesions in TGF β -treated cells was not the result of increased ILK expression. Further, abrogating myosin-related contractility reduced the association of ILK to paxillin-rich adhesions, indicating that the enhanced molecular interactions to paxillin were favored under conditions of elevated actomyosin-generated force (Supplemental Figure S8, b and c; Schiller *et al.*, 2013). These findings imply that the more prominent, longer-lasting integrin adhesions fostered by the elevated traction force in the metastatic and basal-like MECs facilitated the recruitment of a suite of paxillin-associated proteins that fosters tumor cell invasion within a dense and stiffened ECM.

Phosphorylation of paxillin by Src family kinases is elevated in the mammary tumor cells with a basal-like phenotype and enhances invasion within a stiff 3D ECM

Tumor cell invasion requires integrin adhesion turnover stimulated by the phosphorylation of key adhesion plaque proteins, including paxillin (Tumbarello *et al.*, 2005). Immunofluorescence analysis revealed that phosphorylation of paxillin (tyrosine 31) was elevated in the TGF β -treated MCF10A MECs (Figure 9a). Paxillin tyrosine 31 and 118 phosphorylation was also enhanced in the PyMT MECs induced to undergo EMT by TGF β treatment and in the invasive Met1 tumor MECs (Figure 9b and Supplemental Figure S9). We also detected strong phosphopaxillin staining at paxillin adhesions in basal-like leader PyMT tumor MECs invading into the 3D collagen/fibronectin gels in response to ECM stiffening (Figure 9c). Critically, preventing paxillin phosphorylation through ectopic expression of a nonphosphorylatable paxillin mutated at tyrosine residues 31 and 118 to phenylalanine (paxillin^{Y31/118F}) to hinder adhesion turnover (Webb *et al.*, 2004; Pasapera *et al.*, 2010; Plotnikov *et al.*, 2012) inhibited Met1 tumor cell invasion into a 3D stiffened collagen gel (Figure 9, d, and quantified in e). Paxillin phosphorylation is mediated predominantly by Src family kinases (Schaller, 2001), and our Bio-ID assay revealed that GIT2, a Src substrate, was enriched proximal to paxillin in TGF β -treated MCF10A MECs (enrichment factor, ~4; Yu *et al.*, 2009, 2010; Bianchi-Smiraglia *et al.*, 2013; Figure 8b). We therefore examined whether Src activity was critical for the elevated paxillin phosphorylation and enhanced invasive phenotype of the Met1 MECs in response to a stiffened 3D collagen/fibronectin in the bioreactor. Consistently, treatment of the metastatic Met1 and PyMT multicellular spheroids with the Abl/Src inhibitor dasatinib (20 nM) reduced paxillin phosphorylation and significantly decreased invasion of the tumor cell within the stiffened collagen/fibronectin hydrogel (Figure 9f).

The clinical relevance of these findings was investigated by analysis of the Cancer Genome Atlas (TCGA) data set and patient

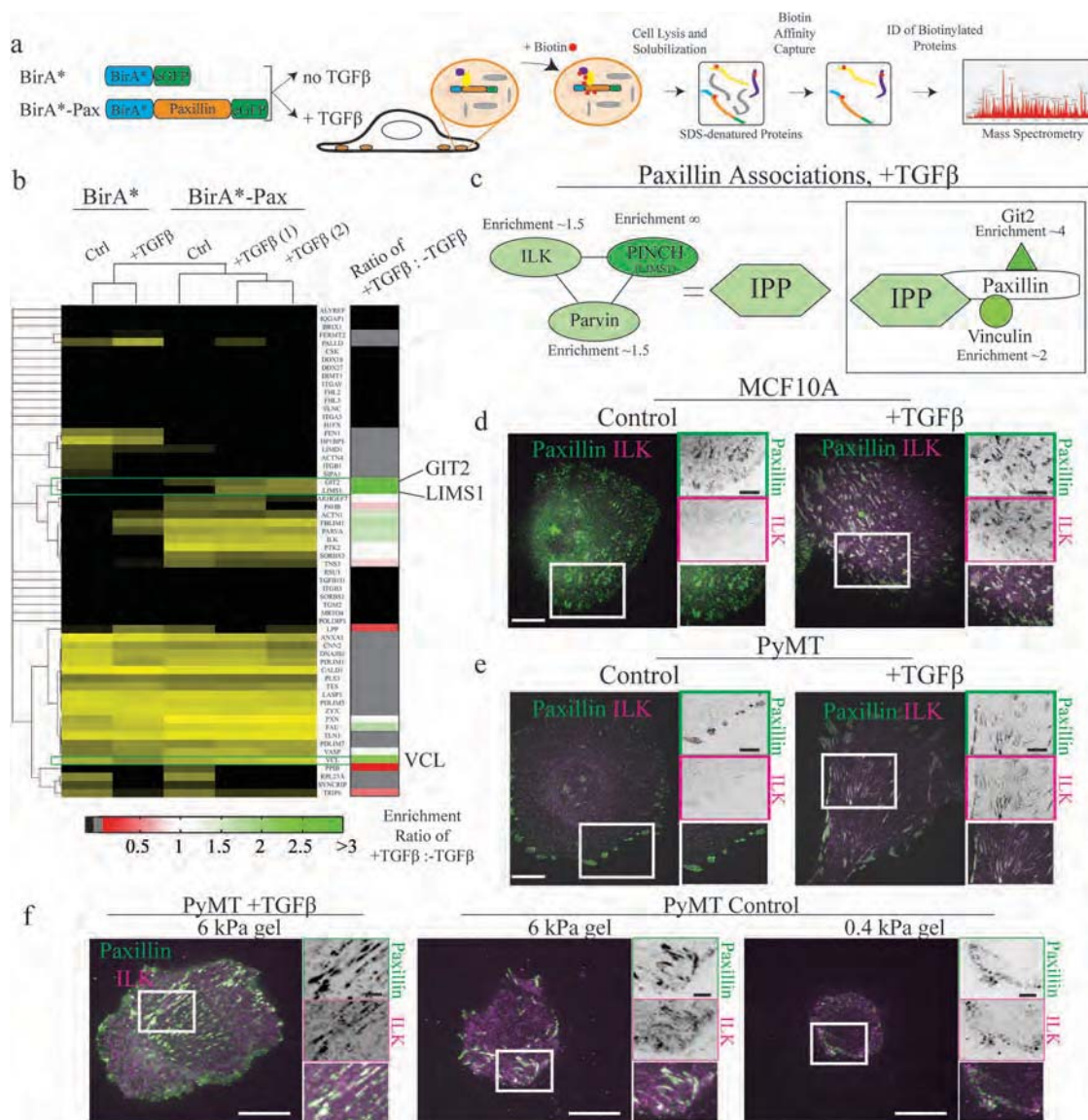


FIGURE 8: Proteomics identifies unique proteins recruited to paxillin in the integrin adhesions during EMT. (a) BioID technique. A control BirA*-eGFP construct and a BirA*-paxillin-eGFP construct are stably expressed in MCF10A cells, which are then treated with TGFβ to induce EMT. BirA*-paxillin-eGFP localizes to FAs and biotinylates proteins adjacent to paxillin when biotin is added to the medium. Biotinylated proteins are isolated and identified by mass spectrometry. (b) Normalized spectral counts for the consensus adhesome were clustered on the basis of an uncentered Pearson correlation using Cluster 3.0 and visualized using Java TreeView. From a subgroup of known paxillin interactors, we identify GIT2, LIMS1 (PINCH), and vinculin as enriched in TGFβ-treated cells. Black indicates no protein detected in the +TGFβ; -TGFβ scale bar, and gray indicates no enrichment relative to the control. (c) Model for potential paxillin associations altered in response to TGFβ treatment. PINCH, GIT2, and vinculin localize adjacent to paxillin, and these associations are enriched in TGFβ-treated cells. (d-f) Immunofluorescence images of paxillin (green) and ILK (magenta). ILK is enriched in paxillin-rich adhesions in (d) MCF10A cells treated with TGFβ and (e) PyMT cells treated with TGFβ. Scale bar, 10 μm (main image), 5 μm (inset). (f) Immunofluorescence images of paxillin and ILK in PyMT cells on stiff (6 kPa) and soft (0.4 kPa) PA gels coated with fibronectin, showing that stiffness and TGFβ treatment modulate the recruitment of ILK to paxillin-rich plaques. Scale bar, 10 μm (main image), 2 μm (inset).

survival (Györfy *et al.*, 2013). Gene expression data analysis revealed that patients with invasive breast cancer stratified into those with lymph node-positive cancers that had high levels of paxillin transcript also had a significantly worse chance of survival ($p < 0.001$; hazard ratio of 1.46 at 10 yr vs. patients with low paxillin levels; Figure 9g). Examination of the mortality hazard ratio for breast cancer patients with high gene expression of other integrin adhesion proteins also showed that patients with high FAK expres-

sion had a significantly higher risk of death ($p < 0.02$; hazard ratio, 1.34), whereas high expression of β1 integrin or talin did not associate with increased patient mortality (Supplemental Figure S10). Intriguingly, Src regulates FAK activity, FAK regulates integrin adhesion turnover, and FAK critically regulates cancer progression and metastasis (Deramaudt *et al.*, 2014; Sulzmaier *et al.*, 2014). Evaluation of reverse-phase protein array data derived from 892 human breast cancer patients further indicated that patients with

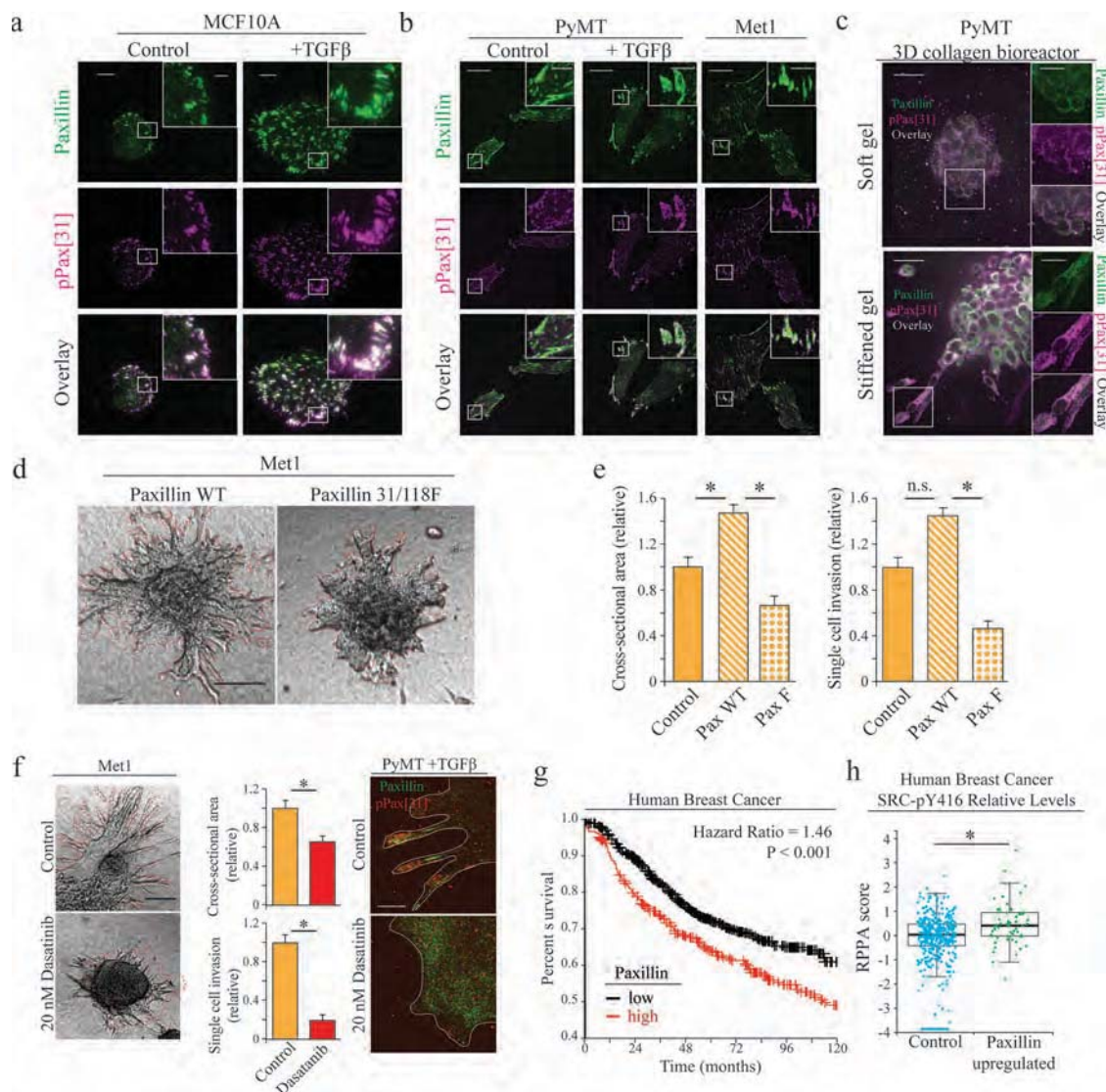


FIGURE 9: Enhanced paxillin phosphorylation permits invasion in cells with a basal phenotype. (a) Paxillin localization to the basal surface in MCF10A cells with and without TGFβ treatment visualized with immunofluorescence staining and TIRF imaging of paxillin and phosphorylated paxillin, pPax[31]. Scale bar, 10 μm (main image), 1 μm (inset). (b) Paxillin expression in PyMT, Met1, and PyMT + TGFβ visualized with immunofluorescence staining and confocal imaging of paxillin and phosphorylated paxillin pPax[31]. Scale bar, 10 μm (main image), 1 μm (inset). (c) Paxillin expression of PyMT cell spheroids visualized in soft and stiffened 3D collagen gel. Leader cells in stiffened gel have higher levels of phosphorylated paxillin indicated by pPax[31] immunostaining. Scale bar, 20 μm (main image), 10 μm (inset). (d) A 3D collagen invasion assay of multicellular spheroids composed of Met1 cells overexpressing either paxillin^{WT} or paxillin^{Y31/118F} in 2.5 mg/ml 3D collagen gels for 72 h. Scale bar, 100 μm. (e) Quantification of invasion. Paxillin^{WT} overexpression significantly increased invasion compared with control or paxillin^{Y31/118F} overexpression, as determined by cross-sectional area of spheroid and protrusions and single-cell invasion (*p < 0.05; >20 spheroids). (f) A 3D collagen invasion assay of multicellular spheroids composed of metastatic Met1 cells treated with dimethyl sulfoxide or 20 nM dasatinib, a Src/Abl inhibitor, in 2.5 mg/ml 3D collagen gels for 72 h. Dasatinib addition significantly decreased cell invasion, as determined by cross-sectional area and single-cell invasion (*p < 0.05; >15 spheroids). Scale bar, 100 μm. Dasatinib addition decreased levels of paxillin phosphorylation visualized by pPax[31] immunofluorescence. Scale bar, 5 μm. (g) Kaplan-Meier survival curve of invasive breast cancer patients expressing either high or low levels of paxillin. High paxillin levels are associated with a significantly worse chance of survival (p < 0.001; hazard ratio 1.46 at 10 yr). (h) Src phosphorylation levels via RPPA in TCGA breast cancer patients separated into control and paxillin-up-regulated groups. Paxillin up-regulation is correlated with a significant increase in Src phosphorylation at tyrosine 416 (*p < 0.05; >4200 patients).

elevated levels of paxillin also had significantly higher levels of phosphorylated Src-416, likely reflecting increased Src activity (Figure 9h). Thus the clinical data suggest that elevated total and Src family kinase-dependent phosphorylated paxillin may consti-

tute tractable prognostic markers for decreased survival of patients with breast cancer. The cell biology studies imply that Src family kinase-dependent phosphorylation of paxillin may detect tumor cells with an intrinsic or tension-induced basal-like phenotype.

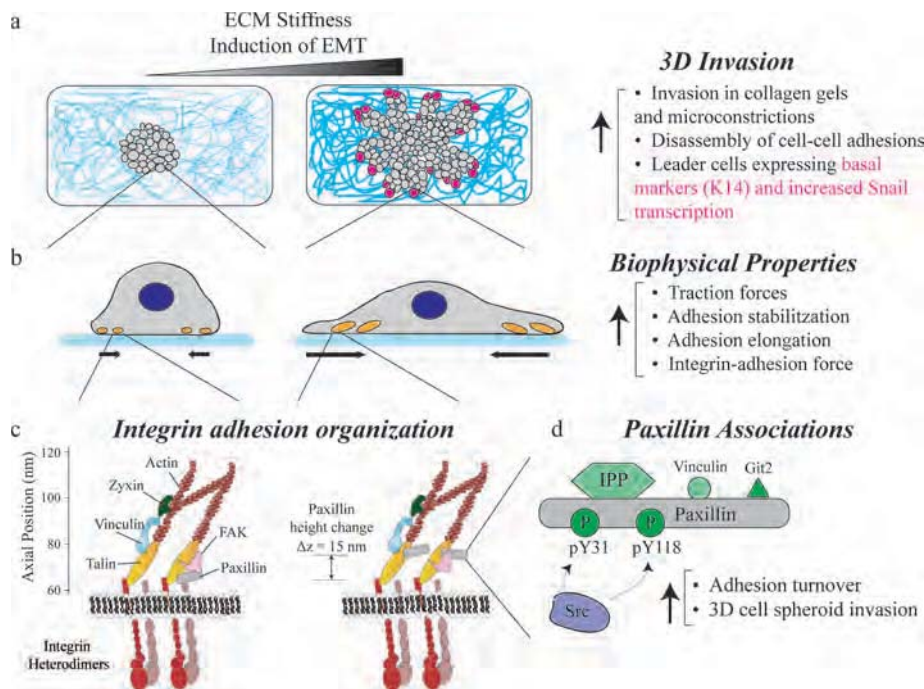


FIGURE 10: Mechanistic model. (a) ECM stiffness and induction of EMT enhance metastatic behavior, evidenced by increased invasion in 3D collagen matrices, expression of basal epithelial markers and Snail transcription in leader cells, and a higher propensity to migrate through small constrictions. (b) Adoption of a mesenchymal phenotype results in biophysical properties similar to those of basal-like cells, including higher integrin-mediated traction forces and more stable, elongated focal adhesions. (c) Axially scaled model of adhesion architecture changes in basal-like cells (TGF β treated), showing the average increase in paxillin height of 15 nm. (d) EMT promotes the association of the ILK-PINCH-parvin (IPP) complex, vinculin, and Git2 directly to or in the vicinity of paxillin. Paxillin phosphorylation, mediated through kinases such as Src, regulate adhesion turnover and cell invasion.

DISCUSSION

In this study, we sought to understand the key cellular biophysical properties that endow breast tumor cells with a propensity to metastasize and in particular the ability to invade and migrate through confined spaces. We present evidence of underlying similarities between metastatic breast tumor cells, EMT-induced breast tumor cells, and breast tumor cells interacting with a stiffened ECM that exhibit a basal-like phenotype. In each of these cases, we found that the breast tumor cells assembled large, elongated, “mesenchymal-like” focal adhesions that exerted markedly higher forces than those assembled in analogous cells lines that were predominantly epithelial and less metastatic in nature. Our findings revealed that these intrinsic or microenvironment-induced differences in adhesion size and force output reflected alterations in adhesion architecture, turnover, and molecular composition. Of note, in each of these conditions, the paxillin adhesions in the invasive basal-like tumor cells associated with the recruitment of a suite of adhesion plaque proteins, including PINCH and ILK—components of the PINCH/parvin/ILK complex previously implicated in tumor cell invasion and metastasis (Persad and Dedhar, 2003; Legate et al., 2006). In each of the invasive, basal-like cells, the paxillin that was recruited to focal adhesions was highly phosphorylated, a finding that is consistent with the notion that integrin adhesion turnover is essential for tumor cell invasion (Dermaudt et al., 2014). Consistently, when the invasive breast tumor cells were treated with a broad-spectrum Src family kinase inhibitor that is currently used in the clinic to treat cancer, paxillin phosphorylation was reduced at focal adhesions, and the invasiveness of the metastatic breast tumor cells into a 3D stiff ECM

was markedly decreased. Accordingly, our findings suggest that metastasis would be favored by tumor cells that either adopt or temporarily borrow a conserved, “EMT-like” or “basal-like” integrin adhesion phenotype with higher integrin-mediated traction force generation (Figure 10).

EMT has been proposed as a central, and even required, process in cancer metastasis (Chaffer and Weinberg, 2011; Tsai et al., 2012). Cells in epithelial tissues are polarized, nonmigratory, and linked by mechanically robust intercellular adhesion complexes (Przybyla et al., 2016). An EMT offers a conceptually attractive mechanism by which carcinoma cells could reverse these defining characteristics in order to migrate through surrounding tissues and establish distant metastases. Furthermore, invading malignant cells often exhibit at least some of the molecular hallmarks of EMT—for example, the expression and engagement of transcription factors such as Twist or Snail, which are considered defining features of EMT in a developmental context (Yilmaz and Christofori, 2009; Przybyla et al., 2016). However, recent work has challenged the centrality of EMT to malignancy. In mouse models, distant metastases preferentially formed from multicellular aggregates rather than single cells. Further, metastasizing cells remained linked by robust cell–cell adhesions and maintained expression of E-cadherin, whose loss is classically

considered a hallmark of EMT (Cheung et al., 2013, 2016). In addition, cells invading into a 3D ECM from murine mammary tumor colonies, as well as human tumor colonies isolated from women with aggressive breast tumors, did not undergo a canonical EMT; instead, the leader cells adopted key features of a “basal-like” response while maintaining their epithelial phenotype (Nguyen-Ngoc et al., 2012; Cheung and Ewald, 2014). More broadly, mesenchymal cells—for example, fibroblasts—are not intrinsically tumorigenic, and in humans are less likely to become cancerous than cells from epithelial tissues (Quail and Joyce, 2013). Our findings are consistent with these observations and imply that tumors cells may merely temporarily or permanently exploit basal-like features to facilitate their metastasis.

In the context of breast cancer, the perspective that a tumor need not undergo a complete EMT to metastasize has led to a revised model in which malignant epithelial cells adopt key features of basal-like/partial EMT cells and whose phenotypic characteristics may facilitate multicellular invasion into the surrounding tissue. Our data showed that an ECM with a stiffness matching that of the breast cancer stroma induced the leader PyMT epithelial cells within a tumor colony to adopt characteristics consistent with the “basal-like/partial EMT” phenotype, including cytokeratin 14 and Snail expression and partial disassembly of cell–cell adhesions, that was associated with their invasion into the surrounding matrix as loose cellular aggregates extending from the primary colony. The findings illustrate how the tissue microenvironment can promote the adoption of a presumably transient “basal-like” program that fosters tumor cell dissemination without compromising the overall epithelial

phenotype (Cheung *et al.*, 2013; Pickup *et al.*, 2013). Our studies thus provide a plausible explanation for the seemingly irreconcilable multicellular versus EMT phenotype as metastatic promoters. Critically, our observations suggest that malignant cells can either permanently or transiently borrow critical elements of a mesenchymal phenotype, notably the ability to exert large tractions on their surrounding ECM that would facilitate their dissemination through confined spaces. Our findings also predict that tumor cells should be able to adopt this metastatic adhesion phenotype without surrendering essential elements of their epithelial phenotype, such as cell-cell adhesion, which we and others showed can enhance survival in response to chemotherapy and during metastasis and would favor their ability to form macroscopic tumors (Weaver *et al.*, 2002; Loessner *et al.*, 2010; Longati *et al.*, 2013).

Metastatic cells that penetrate through confined spaces experience extensive cell deformation (Doyle *et al.*, 2013; Wolf *et al.*, 2013). In some studies, biophysical measurements revealed that metastatic tumor cells have a lower effective stiffness than their primary tumor counterparts (Darling *et al.*, 2007; Swaminathan *et al.*, 2011). A decrease in cell stiffness, believed to be linked to induction of an EMT, presumably provides the metastatic cell with a means to migrate through confined spaces (Osborne *et al.*, 2014; McGrail *et al.*, 2015). In our hands, the prometastatic Met1 and EMT-induced PyMT MECs exhibited an enhanced ability to pass through confined channels relative to the less metastatic parental PyMT MECs. However, AFM indentation revealed that this enhanced migratory ability did not consistently correlate with an increase in cortical deformability. However, we recognize that the AFM indentation measurements performed in the present studies report cell cortical stiffness, whereas the overall elasticity of the cytoskeleton may differ significantly. Another important proviso is that alterations in nuclear deformability, as we showed, also change after TGF β treatment-induced EMT, and this elevated compliance may itself significantly contribute to the cells' ability to navigate through constrictions. Nevertheless, our findings do suggest that an increase in cytoskeletal compliance is not an absolute prerequisite for cell migration through a confined microenvironment (Pathak and Kumar, 2012; Charras and Sahai, 2014; Lautscham *et al.*, 2015). Instead, our studies suggest that an obvious likely contributor to the ability of cells to pass through constrictions is the ability of the disseminating cells to generate substantial tractions at their ECM adhesions. In short, our observations are consistent with the idea that cells that have a higher propensity to metastasize are more contractile, and yet their nucleus is more deformable. Thus cell contractility and stiffness are not synonymous, particularly in a diseased state in which cells adopt different phenotypes and/or behaviors than during normal physiological function.

More broadly, generation of integrin-ECM traction by cancer cells is linked to matrix stiffening and matrix remodeling (Paszek *et al.*, 2005; Schedin and Keely, 2011; Jansen *et al.*, 2013; Rubashkin *et al.*, 2014a), both of which are believed to contribute to metastasis (Lu *et al.*, 2012; Pickup *et al.*, 2014). Consistently, prior studies correlated increased traction stresses with metastatic potential, albeit using a variety of cancer cell types with diverse origins that may or may not reflect a true acquisition of a metastatic phenotype (Kraning-Rush *et al.*, 2012). Here we found that the Met1 MECs and EMT-induced PyMT MECs increased their traction forces relative to the parental PyMT MECs, findings that are consistent with results reported here and elsewhere showing that a stiff ECM increases cellular traction force and fosters tumor cell metastasis (Paszek *et al.*, 2005; Kraning-Rush *et al.*, 2012; Pickup *et al.*, 2013). Of interest, an increase in cellular traction force is required for endoderm

specification and is proposed to guide osteogenesis and myogenesis (Lee *et al.*, 2015; Taylor-Weiner *et al.*, 2015). Our data are thus consistent with the general idea that increased traction forces exhibited by prometastatic cancer cells may reflect a selective hijacking of one critical aspect of the EMT phenotype.

We present a simple physical model, modified from previous iterations (Chan and Odde, 2008; Bangasser *et al.*, 2013; Elosegui-Artola *et al.*, 2016), that could explain the cell-level changes in adhesion and contractility that facilitate metastasis. A principal prediction arising from this model is that both enhanced cell ECM adhesion (an increased number of "clutches") and increased contractility (an increased number of "motors") are required for the increase in cellular traction that accompanies the malignant phenotype. Our experimental measurements of cellular ECM traction and adhesion size, architecture, and dynamics are consistent with this general prediction. In particular, our MTS measurements indicated large increases in cellular traction forces in all of the breast tumor cell lines with a prometastatic or an EMT-like phenotype and revealed that the forces were likely transmitted through larger force-producing integrin adhesions. Intriguingly, our data revealed that the average force per integrin complex was modestly increased, suggesting that, in addition to an increase in clutch number, individual bond properties were also altered by addition of TGF β . For example, the increased force per bond could be due to a modest increase in the characteristic bond force (F_{bond}) or modest decrease in basal clutch unbinding rate (k_{off}). Higher forces at the single-integrin level may plausibly reflect force-mediated "outside-in" signaling to drive clutch and motor addition (as in our biophysical model), which would then link to integrin-dependent intracellular signaling to drive actomyosin-dependent cytoskeletal tension. In the case of metastatic tumors, which exhibit increased integrin-linked forces and elevated "onco-gene-dependent" actomyosin contractility, this relationship is predicted to reset the state of cellular tensional homeostasis to a higher level (Paszek and Weaver, 2004; Laklai *et al.*, 2016).

Our data suggest that differences in adhesion properties reflect changes in adhesion molecular composition. We found that the large, force-producing adhesions assembled in the MECs that had undergone an EMT recruited more PINCH and ILK, which comprise part of the PINCH/Parvin/ILK complex, which has been strongly implicated in tumor cell invasion and metastasis (Persad and Dedhar, 2003; Legate *et al.*, 2006). Our findings are also consistent with cell studies implicating ILK in an EMT and transgenic mouse work showing that ILK overexpression promotes breast tumor progression, whereas its knockdown reduces tumor aggression (White *et al.*, 2001; Hannigan *et al.*, 2005; Serrano *et al.*, 2012; Pang *et al.*, 2016). Thus adoption of the EMT-like adhesion phenotype by tumor cells may favor the recruitment of key signaling molecules that facilitate tumor cell invasion. Consistently, we found that paxillin displayed a pronounced shift in z height relative to the membrane in cells that had gone an EMT, suggestive of a differential role in epithelial versus mesenchymal cells.

Adhesion turnover is required for cell migration and for the metastatic dissemination of malignant cells (Friedl and Alexander, 2011). Consistent with this concept, despite the fact that the mammary tumor cells that had undergone an EMT assembled large, distinct focal adhesions and recruited a large repertoire of adhesion plaque proteins, they were also highly dynamic. The integrin adhesions formed by the metastatic breast tumor cells, breast tumor cells induced to undergo an EMT, and the basal-like PyMT tumor colonies interacting with a stiffened ECM also had elevated levels of phosphorylated paxillin, an observation echoing prior reports that paxillin phosphorylation is higher in metastatic cells and

in cells treated with TGF β (Tumbarello *et al.*, 2005). Phosphorylation of paxillin by Src family kinases acts to induce both adhesion growth and disassembly and hence serves as a master regulator of adhesion stability (Turner, 2000; Webb *et al.*, 2004; Zaidel-Bar *et al.*, 2006; Wolfenson *et al.*, 2011). We found that tumor cells interacting with a stiffened 3D ECM also exhibited higher levels of phosphorylated paxillin at their integrin adhesions, which may reflect an adaptation of the cells' inherent sensing response to a soft substrate (Plotnikov *et al.*, 2012). Regardless, we showed that blocking paxillin phosphorylation either by point mutagenesis or by adding a small-molecule inhibitor of a Src family kinase used in the clinic blocked the 3D invasion of Met1 MECs. Moreover, epidemiological data indicate that high paxillin expression correlates with decreased patient survival, and phospho-array data show that elevated tumor paxillin associates with high Src kinase levels and activity. Thus alterations in adhesion structure and dynamics may be directly linked to patient outcome, and assessment of integrin adhesion composition may constitute an untapped prognostic indicator.

MATERIALS AND METHODS

Cell culture and reagents

MECs, including nonmalignant MCF10A (American Type Culture Collection; verified by epithelial cellular morphology in two dimensions, ability to form hollow, polarized acini in 3D rBM gels, and expression of epithelial markers, including E-cadherin and cytokeratins 8 and 14) and premalignant Ha-Ras-transformed MCF10AT (Karnanos Cancer Institute; verified by epithelial cell morphology in two dimensions) were cultured as described (Rubashkin *et al.*, 2014a). Tumorigenic MEC lines PyMT Flox3 and metastatic Met1 (kind gift of the Moses laboratory, Vanderbilt University) were cultured as described previously (Borowsky *et al.*, 2005).

The 3D multicellular spheroids were generated in rBM (Matrigel; Corning) suspension cultures as described (Rubashkin *et al.*, 2014a). MCF10A and Met1 MECs were flow sorted on a FACSARIA II cell sorter (BD Biosciences) via primary and secondary antibody staining for top ~20% or bottom ~20% expression of α_5 integrin, β_1 integrin, or $\alpha_v\beta_3$ integrin. The chemokine human TGF β at 10 nM (PeproTech or Sigma-Aldrich) was used for 3 d to induce mesenchymal behavior. The small-molecule inhibitor dasatinib was used at 20 nM (Cayman Chemical).

Expression constructs

Transient plasmid constructs included Clontech vectors containing N- and C-terminus-tagged vinculin-mEmerald, paxillin-mCherry, paxillin-mEmerald, farnesyl-mEmerald, farnesyl-mCherry, FAK-mEmerald, Talin-mEmerald, Talin-mCherry, Zyxin-mEmerald, Tensin-mEmerald, and Tensin-mCherry. Expression constructs were provided by the Davidson Lab. Piggybac transposable plasmids containing eGFP-paxillin and eGFP-paxillin^{Y31/118F} were synthesized and cloned (Epoch Life Science, Sugar Land, TX) into Bsal-digested pHULK piggyBac Mammalian Expression Vector–CometGFP (DNA 2.0).

Stable cell lines inducibly expressing BirA*-paxillin derivatives were prepared using a custom-built PiggyBac transposable vector, pPB puro Tet. Briefly, pPB puro Tet includes the hepatamerized tetracycline (tet) operator minimal CMV promoter, which drives expression of a transgene with a 5' chimeric intron and polyadenylation signal derived from bovine growth hormone. pPB puro Tet also includes an expression cassette for puromycin selection in reverse orientation. BirA* (Roux *et al.*, 2012) was modified with three tandem repeats of the myc 9E10 epitope at the N-terminus and a 22-amino acid, C-terminal glycine- and serine-rich flexible linker (N22).

The modified BirA* was then cloned in-frame with full-length paxillin-eGFP (Addgene plasmid 15233) at the N-terminus, such that the final construct had 3xmyc-BirA*-N22-paxillin-eGFP in order from N- to C-terminus. A fusion of mEmerald to the C-terminus of myc-tagged BirA* served as a control.

Transient transfection was performed using either Lipofectamine 2000 reagent or the Lonza Nucleofector Kit V, with program T024 for all epithelial cell lines and U024 for other cell lines. Recombinant lentivirus of the tet-promoter rtTA (neo) was prepared by transient transfection into HEK-293T cells, generating lentivirus titer used to transduce cells, as previously described (Rubashkin *et al.*, 2014a). Target cells constitutively expressing the synthetic reverse Tet transcriptional transactivator rTAs-M2 were prepared by transduction with a G418 selectable lentiviral vector. These cells were transiently transfected with pPB puro Tet constructs and an expression construct for a hyperactive piggyBac transposase pCMV-HAhyPBase (generous gift of Allan Bradley, The Wellcome Trust Sanger Institute; Yusa *et al.*, 2011).

Stable cell lines were selected in 200 ng/ml G418, 380 ng/ml hygromycin, or 1 μ g/ml puromycin and sorted on a FACSARIA II cell sorter (BD Biosciences) for fluorescent protein expression. Expression of inducible protein-encoding gene was induced with 200 ng/ml doxycycline in growth medium for 1 d before experimentation.

Hydrogel and substrate preparation

Collagen gels were fabricated by diluting 2.5 mg/ml acid-solubilized rat tail collagen (BD) in 1:1 DMEM-F12 (Invitrogen), neutralizing to pH 7.4 with 1 M NaOH. For 3D collagen gels, a base layer of ~250- μ m thickness was cast at 37°C for 30 min, followed by a second layer that included the cell suspension, which was also allowed to solidify over 30 min. Two-dimensional (2D) PA gels (height ~200 μ m) were fabricated by varying the acrylamide and cross-linker concentration and assessing stiffness using either ATM or a shear rheometer (Johnson *et al.*, 2007). Red fluorescent beads (100 nm; Invitrogen) were incorporated in the PA gels at 1:300 dilution relative to the manufacturer's concentration and centrifuged at 200 RCF to move the beads to the top surface of the gels for TFM. PA gels were functionalized with 10 μ g/ml human fibronectin (Millipore) as described (Lakins *et al.*, 2012).

N-type [100]-orientation silicon wafers with 1933-nm silicon oxide (1 cm x 1 cm; Addison Engineering) and borosilicate glass coverslips (~150 μ m thick) were cleaned by successive sonication in acetone and 1 M KOH (20 min), followed by chemical activation with silane (0.5% (3-aminopropyl)trimethoxysilane) and glutaraldehyde (0.5% in water). The substrates were then ultraviolet- and ethanol-sterilized and incubated overnight (4°C) in extracellular matrix (human fibronectin, 10 μ g/ml; Millipore). Before use, substrates were washed in phosphate-buffered saline (PBS; five times; pH 7.4), treated with sodium borohydride (20 mg/ml), and washed with PBS. SAIM calibration wafers were prepared by sonicating carboxylate-modified red fluorescent spheres (100 nm; Invitrogen), following by bead deposition (5×10^8 beads/ml) in NaCl (100 mM) PBS solution as described (Paszek *et al.*, 2012).

Immunofluorescence

Cells were cultured on various substrates overnight and then fixed with 4% paraformaldehyde (PFA; 2D: 20 min, room temperature; 3D: overnight, 4°C) and stained as described (Levental *et al.*, 2009). Primary antibodies against paxillin (5H11, Millipore; 610620, BD; P1093, Sigma-Aldrich; 32084, Abcam), phospho-paxillin-tyrosine 118 (44-722G, Life Technologies; 2541, Cell Signaling), phospho-paxillin-tyrosine 31 (44-720F, Life Technologies), vinculin (hVIN-1,

Sigma-Aldrich; 700062, Invitrogen; V284, Santa Cruz Biotechnology), phospho-FAK-tyrosine 397 (44-625G, Invitrogen), vimentin (D21H3, Cell Signaling), β -catenin (8480, Cell Signaling), K14 (PRB-115P, Covance), K8+18 (20R-CP004, Fitzgerald Industries), PINCH (EP1944, Abcam), ILK (611803, BD), and Alexa Fluor-phalloidin (Invitrogen) were used. Secondary antibodies used include Alexa Fluor goat anti-mouse, anti-rabbit, and anti-rat (488, 568, and 633 conjugates). For ILK immunofluorescence (IF), cells were fixed in 100% ice-cold methanol.

Microscopy and analysis

Unless otherwise noted, SAIM, TFM, confocal, and spheroid invasion assay imaging were performed on motorized total internal reflection fluorescence (TIRF) inverted microscopes (Ti-E Perfect Focus System; Nikon) controlled by MetaMorph (Molecular Devices) and Nikon Elements software and equipped with 488-, 561-, and 640-nm lasers, 350/50 epifluorescence, a CSU-X1 spinning-disk confocal unit (Yokogawa Electric Company), electronic shutters, motorized stage, a scientific complementary metal-oxide-semiconductor (sCMOS) camera (Zyla 5.5 megapixel, Andor; or Orca Flash, Hamamatsu), and an electron-multiplying charge-coupled device (EMCCD) camera (QuantEM 512; Photometrics). Temperature and CO₂ were controlled by an environmental chamber and proportional integral derivative-controlled heater (In-Vivo Scientific). Samples were imaged with 100 $\times$ /1.49 numerical aperture (NA) TIRF oil-immersion, 40 $\times$ /1.2 NA long-working-distance water-immersion, 20 $\times$ /0.75 NA air, and 10 $\times$ /0.45 NA air objectives. For live-cell imaging, phenol red-free growth medium with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4) was used. For 3D imaging, confocal image slices were taken at 0.2- or 1.0- μ m slices and the individual planes were maximum intensity z-projected for 2D visualization. Custom software packages for image analysis were written in Python, using ImageJ and the Eclipse Development Environment in Linux. Images were subject to a Gaussian blur of 1.0 pixel to smooth background noise. Intensity and colocalization measurements of single confocal planes were calculated on a pixel-by-pixel basis. Single-cell invasion, acini and cell protrusions, and cell adhesion length were quantified manually and verified qualitatively across three independent experiments. Acini cross-sectional area was quantified with the free-hand selection tool in ImageJ. Focal adhesion lifetimes were quantified manually and verified qualitatively across three independent experiments. SAIM was performed as described (Paszek et al., 2012; Rubashkin et al., 2014a), using data analysis and 3D visualization programs written in Matlab and Python using libraries distributed by Enthougt.

Collagen invasion assays in 3D bioreactor

PyMT organoids were cultured in rBM (Corning) for 3–5 d. The organoids were isolated by solubilizing the gels using PBS-EDTA. The isolated organoids were subsequently embedded in 2.5 mg/ml collagen I solution supplemented with fibronectin (1 μ g/ml) and rBM (1%). The collagen gels were stretched in a tension bioreactor with 0 or 10% strain for 72 h (Cassereau et al., 2015). Organoids were fixed with 1% PFA overnight for subsequent IF staining. Snail-YFP knock-in reporter tumor cells were kindly provided by Robert Weinberg (Ye et al., 2015). Tumor cells were cultured in rBM for 3–5 d and transferred to either untreated or ribose-stiffened collagen gels. After 48 h, organoids were fixed and stained for indicated proteins.

Microchannel migration assays

Migration experiments in microfluidic devices were carried out as described previously (Davidson et al., 2014, 2015; Denais et al.,

2016). Briefly, the migration devices contain 5- μ m-tall channels with constrictions ranging from 1 to 15 μ m in width. Migration channels are flanked on either side by two unconfined regions with a height of 250 μ m (Davidson et al., 2015). Molds of the migration devices were generated using two-layered SU-8 photolithography as described previously (Davidson et al., 2014). Replicas of the molds were made from a two-component Sylgard 184 polydimethylsiloxane (PDMS) kit (Dow Corning) using the (1:10) manufacturer's recipe and cured for 2 h at 65°C. After curing, devices were cut to size, and holes were punched for cell-seeding ports and media reservoirs. A plasma cleaner (Harrick Plasma) was used to covalently bind microfluidic devices to glass coverslips that had been soaked in 0.2 M HCl overnight, rinsed with water and isopropanol, and dried. To improve adhesion after plasma cleaning, the completed devices were heated on a hot plate at 95°C for 5 min before sterilization with 70% ethanol. Devices were rinsed and treated with 20 μ g/ml fibronectin (Millipore) overnight at 4°C. After incubation, the devices were rinsed with 1 $\times$ PBS and cell culture medium. For the experiments, 30,000 cells/device were loaded into each device and allowed to attach overnight. Experiments were conducted with a 0–10% fetal bovine serum (FBS) gradient across the constrictions to stimulate directed migration, with cell medium supplemented with 25 mM HEPES. Devices were sealed with a glass coverslip to minimize evaporation and placed on a temperature-controlled stage (37°C) of an inverted Zeiss Observer Z1 microscope equipped with a CCD camera (Photometrics CoolSNAP KINO) and a motorized stage. Images for time-lapse sequences were acquired every 10 min for at least 12 h through a 20 $\times$ air objective (0.8 NA) using ZEN software (Zeiss).

Images were analyzed using ZEN and ImageJ. Cell transit times through constrictions were measured for individual cells based on time-lapse sequences, with the starting point defined by the frame showing the first signs of nuclear deformation as a cell entered into a constriction, and the ending point defined by the frame at which the cell had migrated through the constriction and the nucleus was no longer "pinched." The fraction of cells that had passed through the first row of constrictions was determined using the final frame of each time-lapse series (i.e., 36-h after cell seeding). We quantified the number of cells that were located beyond the first constriction and the total number of cells that entered the 5- μ m-tall channels. All experimental results are based on at least three independent experiments. Statistical analysis of transit times was determined using a Kruskal–Wallis test with Dunn's multiple comparisons because the transit times did not follow a Gaussian distribution. Fisher's exact test was used to compare the fraction of cells that had passed the first constriction between different conditions. The SE of the cell fraction was calculated according to the equation describing the SE for a binomial distribution:

$$SE = \sqrt{\left(\frac{p(1-p)}{n}\right)}$$

where p is the fraction of cells that passed through the first row of constrictions and n is the total number of cells. Statistical tests were performed in GraphPad Prism.

Atomic force microscopy

Cells were seeded on polyacrylamide gels with 2.7-kPa stiffness for 18 h. Hydrogels were then placed on the center of a standard microscopy slide and anchored using two-sided tape. Cells were kept hydrated in a droplet of HEPES-buffered medium (at room temperature) while force measurements were performed on an Asylum Research (Santa Barbara, CA) MFP-3D atomic force microscope.

All samples were measured in liquid in contact mode using NovaScan 2.5- μm -radius beaded silica glass tip ($k = 0.06 \text{ N/m}$) cantilevers, which were calibrated by the thermal tune method. Force measurements were collected at multiple points across the surface of the gels and cells at a maximum trigger force of 2.0 nN. The resulting force data were converted into elastic modulus using the Hertz Model program in IgorPro v6.22A. Measurements were also repeated with pointed-tipped Asylum Research TR400PB silicon nitride ($k = 0.02 \text{ N/m}$) cantilevers, showing a similar trend. Surface plots were generated using the *surf()* function in Matlab. To determine cell elasticity, height cutoffs were chosen based on the height maps to assign pixel values associated with the cell versus the gel.

Micropipette aspiration

Micropipette aspiration through 3- μm -wide, 5- μm -high channels was performed using microfluidic devices fabricated as described in Denais *et al.* (2016). PDMS molds of the devices were filled with a 2% bovine serum albumin (BSA)/0.2% FBS solution. Cells were trypsinized and resuspended in the 2% BSA/0.2% FBS solution containing Hoechst 33342 for nuclear visualization. The cell suspension was perfused into the devices at constant pressure using a MCFS-EZ pressure controller (Fluigent). Channels were cleared by manual backflushing, and nuclear deformation of cells entering the channels was observed at 5 s intervals using a 20 $\times$ /0.4 NA phase contrast objective and Hamamatsu ORCA Flash 4.0 V2 camera. Images were analyzed using a custom-written MATLAB algorithm.

Traction force microscopy

Cells were seeded overnight on PA gels with 100-nm fluorescent red beads. Images of cells and beads were taken at 20 $\times$ before adding 0.01% SDS to lyse the cells from the surface. A second picture of the beads was taken afterward that was later aligned via the ImageJ (<http://rsb.info.nih.gov/ij/>) registration plug-in StackReg. To assess bead movement, a particle image velocimetry (PIV) program was implemented in ImageJ as described previously (Tseng *et al.*, 2012). The iterative scheme included a first pass at 32/64 (i.e., interrogation and search window size in pixels), followed by a second pass at 16/32, all at a correlation threshold of 0.60. Cross-correlation PIV was used for traction measurements on gels of different stiffness, with three iterative schemes of 128, 64, and 32 as the interrogation window sizes. For PIV postprocessing, a normalized mean test (NMT) was performed as described (Tseng *et al.*, 2012) with NMT noise of 0.2 and threshold of 2.0 as starting parameters. Parameters were altered to correct for erroneous vectors. The traction force was calculated by the Fourier transform traction cytometry (FTTC) method with a Poisson ratio of 0.5. For measurements of the PyMT/Met1 and MCF10AT system, no regulation factor was used. To quantify the traction forces of MCF10A MECs on gels of different stiffness, we performed a regularization sweep to determine an optimal value and chose a constant of 10^{-10} for all of the conditions. Total and maximum traction forces were calculated using a mask of cell area from the bright-field image overlaid onto a per-pixel magnitude plot of the traction vectors. The PIV and FTTC microscopy software for ImageJ was kindly made available by Qingzong Tseng (<https://sites.google.com/site/qingzongtseng/tfm>).

Motor-clutch model of cell traction

Stochastic simulations of a motor-clutch model of cellular traction were modified from previous models (Chan and Odde, 2008; Bangasser *et al.*, 2013). The base model contains eight parameters that describe numbers and physical properties of myosin motors and molecular clutches (e.g., integrin-mediated adhesion complexes),

as well as kinetic rates for binding and unbinding of molecular clutches. Monte Carlo simulations were executed using a Gillespie stochastic simulation algorithm (Gillespie, 1977) run on in-house simulation computers using Matlab (MathWorks, Natick, MA), version 2014b. For a detailed description of the simulation and relevant parameters, refer to the Supplemental Materials. The modified reinforcement model includes kinetic rates for addition of molecular clutches to the ensemble when a threshold force is reached by engaged clutches:

$$K_{\text{add}} = K_{\text{add,base}} \times n_{\text{clutch}} (F > F_{\text{threshold}}) \times \frac{n_{\text{clutch,max}} - n_{\text{clutch}}}{n_{\text{clutch,max}}}$$

where K_{add} is a basal first-order clutch addition rate constant, $F_{\text{threshold}}$ is a threshold force for clutch reinforcement signaling, and $n_{\text{clutch,max}}$ represents the total number of available clutches in the cell.

Cell areas in all simulations were approximated from simulation results by calculating the size of a disk-shaped cell from the length of an F-actin bundle. Actin filament assembly and cycling between G- and F-actin pools is calculated by an actin mass balance,

$$[\text{Actin}_{\text{total}}] = [\text{G-actin}] + [\text{F-actin}]$$

where [G-actin] and [F-actin] are relative lengths of actin assigned to a soluble pool and filaments, respectively. Leading-edge protrusion velocity ($v_{\text{protrusion}}$) scales with the amount of available actin ([G-actin]) and is opposed by actin retrograde flow (v_{filament}) to give the following relationship for cell spreading rate:

$$V_{\text{spread}} = V_{\text{protrusion}} - V_{\text{filament}}$$

Traction force outputs from simulations are in one-dimensional (1D) forces, whereas experimental measures of traction force result in 2D stresses on a compliant gel. To draw comparisons between simulation and experiment, we used a previously described approach to derive a relationship between 1D forces and 2D stresses on a continuous substrate (Ghibaudo *et al.*, 2008), as was previously described for stochastic simulations of cell traction (Elosegui-Artola *et al.*, 2014, 2016). Here P_{traction} is traction stress, and F_{traction} is traction force:

$$P_{\text{traction}} = \frac{F_{\text{traction}}}{\pi r_{\text{adhesion}}^2 (\approx 1 \mu\text{m}^2)} \rightarrow P_{\text{traction}} = F_{\text{traction}}$$

A compliant substrate is modeled as an elastic spring with stiffness constant $\kappa_{\text{substrate}}$ and effective Young's modulus (E_{eff}) given by

$$E_{\text{eff}} = \frac{9 \times \kappa_{\text{substrate}}}{4\pi r_{\text{adhesion}}} \rightarrow E_{\text{eff}} \sim 1.3 \kappa_{\text{substrate}}$$

Integrin force sensor measurements

The MTSs were adapted from sensors described previously (Morimatsu *et al.*, 2015), using cysteine-maleimide chemistry to attach the donor Alexa 546 dye and the acceptor Alexa 647 dye (Life Technologies). This modified MTS presents an RGD peptide derived from fibronectin TVYAVTGRGDSPASSAA. Perfusion chambers were attached to PEGylated coverslips as previously described (Morimatsu *et al.*, 2015). Briefly, labeled MTSs ($\sim 100 \text{ nM}$) were added to the flow cell and incubated for 30 min, followed by Pluronic F-127 (0.2% wt/vol) for ~ 1 min to prevent nonspecific cell attachment. All of these steps were preceded by a PBS wash to remove excess reagent from

the prior step. The cells were then added and incubated for at least 1 h at 37 °C before imaging.

Images were taken using TIRF microscopy on an inverted microscope (Nikon TiE) with an Apo TIRF 100x oil objective lens, NA 1.49 (Nikon). Data were acquired with a 200-ms exposure using either an EMCCD camera (Andor iXon) or an sCMOS camera (Hamamatsu Orca Flash). The no-load FRET efficiency and gamma factor (γ) were measured at the single-molecule level as described previously. To calculate FRET index (FRET_i), we divide the acceptor intensity, A (background subtracted), by the sum of the acceptor and donor (D) intensities (also background subtracted): $\text{FRET}_i = A/(A + D)$. Using the measured FRET index, we calculate the FRET efficiency (E), using the measured fraction of double-labeled sensor ($\alpha \approx 0.90$):

$$E = \frac{\text{FRET}_i}{\alpha(\gamma - \text{FRET}_i\gamma + \text{FRET}_i)}$$

We then convert from FRET efficiency, E , to an average force per sensor molecule at each pixel, using a previously measured calibration curve modified to incorporate the photophysical properties of the dyes used here (Morimatsu *et al.*, 2015).

A detailed explanation of calculations of the average force per sensor and total force per cell was given previously (Morimatsu *et al.*, 2015). Briefly, to measure the average force per sensor, we first segmented the images from the paxillin-eGFP signal or the FRET index signal. These images were normalized, boxcar averaged with a rotating reference frame, and then thresholded to create a binary mask. The binary mask was used for a watershed algorithm to isolate individual islands, which were then combined and indexed with custom-written MATLAB code with minimum adhesion size of 0.5 μm^2 . The average force per sensor was calculated by converting the FRET efficiency values to force values of individual pixels within the segmented regions. The total force per cell was calculated using FRET images of the entire cell taken on a Hamamatsu sCMOS camera. The FRET values in the segmented pixels were then converted to force and summed over all of the adhesions in the entire cell.

3B superresolution analysis

The 3B algorithm uses a Bayesian analysis technique to determine the positions of fluorophores based on patterns of blinking and bleaching events (Cox *et al.*, 2011). Data were recorded on an EMCCD camera (Andor iXon) for ~200–300 frames at 20 frames/s. The images were then processed with the 3B algorithm on virtual machines hosted on Amazon's EC2 cloud computing service (Hu *et al.*, 2013). Each calculation ran on the compute-optimized c3. large servers for ~40 iterations. The positions and intensities of the fluorophores as determined by the 3B analysis were plotted on a coordinate system scaled by a factor of three, representing a three-fold increase in effective resolution.

Proteomics experiment and analysis

The biotin ligase protocol was modified from the previously published method (Roux *et al.*, 2012), with some modifications outlined here. Four 10-cm dishes (~20 million cells) of MCF10A MECs stably expressing BirA* constructs were cultured on tissue culture plastic and treated with TGF β for 3 d. The cells were then incubated in medium supplemented with 50 μM biotin (B4639; Sigma-Aldrich) for 24 h before lysate collection. Cells were detached using trypsin-EDTA, washed three times with cold DPBS, and resuspended in 1.6 ml of ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) with protease inhibitor

cocktail (539134; Calbiochem). The lysates were incubated on ice for 1 h, followed by three sonication repeats using 10-s bursts at 30% amplitude and 30-s rest (Sonics VCX130). Supernatant was collected after 30-min centrifugation at 16,000 $\times g$ at 4 °C and incubated with 500 μl of DPBS-pretreated streptavidin Dynabeads (65001; ThermoFisher) overnight at 4 °C on an end-over-end mixer.

Dynabeads were collected the next day and washed for 5 min at room temperature twice with 1 ml of buffer 1 (2% SDS in H_2O), once with 1 ml of buffer 2 (0.1% sodium deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, 50 mM HEPES, pH 7.5), once with 1 ml of buffer 3 (250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.1), and three times with 1 ml of buffer 4 (50 mM NaCl, 50 mM Tris, pH 7.4). After the last wash, beads were resuspended in 35 ml of SDS sample buffer saturated with biotin (250 mM Tris, pH 6.8, 0.57 M β -mercaptoethanol, 8% SDS, 40% glycerol) and boiled at 95 °C for 10 min. Purified proteins were separated on SDS-PAGE gel (NP0321BOX; ThermoFisher), stained with Coomassie dye (24615; ThermoFisher), and excised for mass spectrometry analysis.

Gel fractions were submitted to the Proteomics Facility at the Fred Hutchinson Cancer Research Center for mass spectrometry analysis. Briefly, desalted peptide samples were analyzed by liquid chromatography-electrospray ionization tandem mass spectrometry with a Thermo Scientific Easy-nLC II nano HPLC system coupled to a hybrid Orbitrap Elite ETD mass spectrometer using an instrument configuration as described (Yi *et al.*, 2003). In-line desalting was accomplished by using a reversed-phase trap column packed with Magic C₁₈AQ (5- μm 200- $\text{\AA}$ resin; Michrom Bioresources, Auburn, CA), followed by peptide separations on a reversed-phase column packed with Magic resin and then directly mounted on the electrospray ion source.

Data analysis was performed using Proteome Discoverer 1.4 (Thermo Scientific, San Jose, CA). The data were searched against a Uniprot human database that included common contaminants. Sequest HT was used for database searching, and Percolator was used for scoring. Data acquired from the Orbitrap Elite were processed to generate normalized spectral count values for each protein. Similar to previously published techniques, each protein's spectral counts were divided by the total number of spectral counts for that sample and then divided by the protein's molecular weight (Horton *et al.*, 2015). The resulting value was multiplied by 10^6 and $\log_{10}$ -transformed to yield a score for each protein. Protein groups that had the same gene names (e.g., different isoforms or fragments of a larger protein) were combined to decrease redundancies in the analysis. Proteins or arrays were hierarchically clustered on the basis of an uncentered Pearson correlation using Cluster 3.0 and visualized using Java TreeView, as described previously (Horton *et al.*, 2015).

Human bioinformatics studies

To analyze survival as a function of transcriptomic data in human breast cancer patients, we used an online tool (www.kmplot.com; Györfy *et al.*, 2013). The Affymetrix ID for paxillin was 201087_at. Briefly, the KMplot tool employs gene expression data and relapse-free and overall survival information from the Gene Expression Omnibus, the European Genome-Phenome Archive, and The Cancer Genome Atlas (TCGA) from >4000 patients. To analyze the effect of specific genes in survival, patient samples were split into two groups by predefined metrics for calculating quantile expression. Affymetrix transcriptome data for a user-selected probe were analyzed in KMplot by autoselecting the best cutoff of gene expression, computing the median of expression over the entire database, and analyzing all

patient subtypes as long as they were lymph-node positive in the 2014 TCGA data set. All suggested quality controls were implemented (Györfy *et al.*, 2013).

Reverse-phase protein array (RPPA) data from TCGA patients were analyzed using an online tool (www.cbioportal.org; Gao *et al.*, 2013). Patients were separated into those with paxillin gene amplification, mRNA up-regulation, or protein up-regulation; and those without significant changes in paxillin at the gene, mRNA, or protein level. These two patient groups were then compared for differences in activated and phosphorylated signaling proteins (including Src phosphorylation at tyrosine 416) via previously conducted RPPA arrays.

Statistics

Statistical analysis for two groups was performed with an unpaired, two-tailed Student's *t* test or Wilcoxon rank sum test where appropriate. For multiple comparisons, an analysis of variance test and Holm-Bonferroni *t* test were performed. Calculations were implemented in MATLAB, Python, and Prism.

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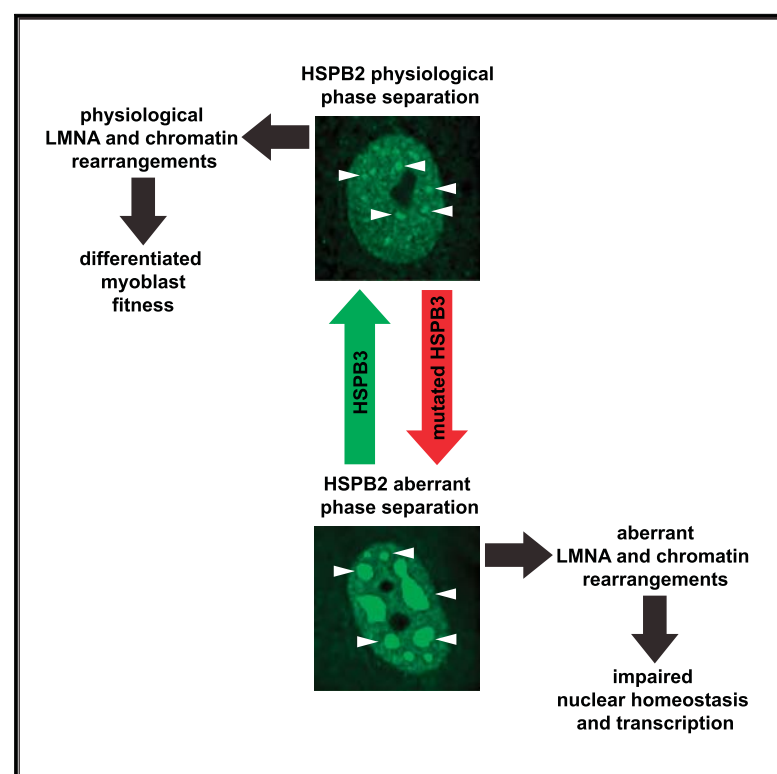
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Aberrant Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function

Graphical Abstract



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In Brief

Morelli et al. show that, in mammalian cells, HSPB2 forms liquid-like nuclear compartments that affect lamin A localization and mobility, with detrimental consequences for chromatin organization and nuclear integrity. Aberrant compartment formation by HSPB2 is regulated by HSPB3, but not by two identified HSPB3 mutants linked to myopathy.

Highlights

- HSPB2 undergoes concentration-dependent liquid-liquid phase separation in cells
- HSPB2 phase separation requires its intrinsically disordered C-terminal tail
- Aberrant HSPB2 phase separation mislocalizes lamin A
- HSPB3, but not two HSPB3 myopathy mutants, inhibits HSPB2 phase separation



Aberrant Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function

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SUMMARY

Small heat shock proteins (HSPBs) contain intrinsically disordered regions (IDRs), but the functions of these IDRs are still unknown. Here, we report that, in mammalian cells, HSPB2 phase separates to form nuclear compartments with liquid-like properties. We show that phase separation requires the disordered C-terminal domain of HSPB2. We further demonstrate that, in differentiating myoblasts, nuclear HSPB2 compartments sequester lamin A. Increasing the nuclear concentration of HSPB2 causes the formation of aberrant nuclear compartments that mislocalize lamin A and chromatin, with detrimental consequences for nuclear function and integrity. Importantly, phase separation of HSPB2 is regulated by HSPB3, but this ability is lost in two identified HSPB3 mutants that are associated with myopathy. Our results suggest that HSPB2 phase separation is involved in reorganizing the nucleoplasm during myoblast differentiation. Furthermore, these findings support the idea that aberrant HSPB2 phase separation, due to HSPB3 loss-of-function mutations, contributes to myopathy.

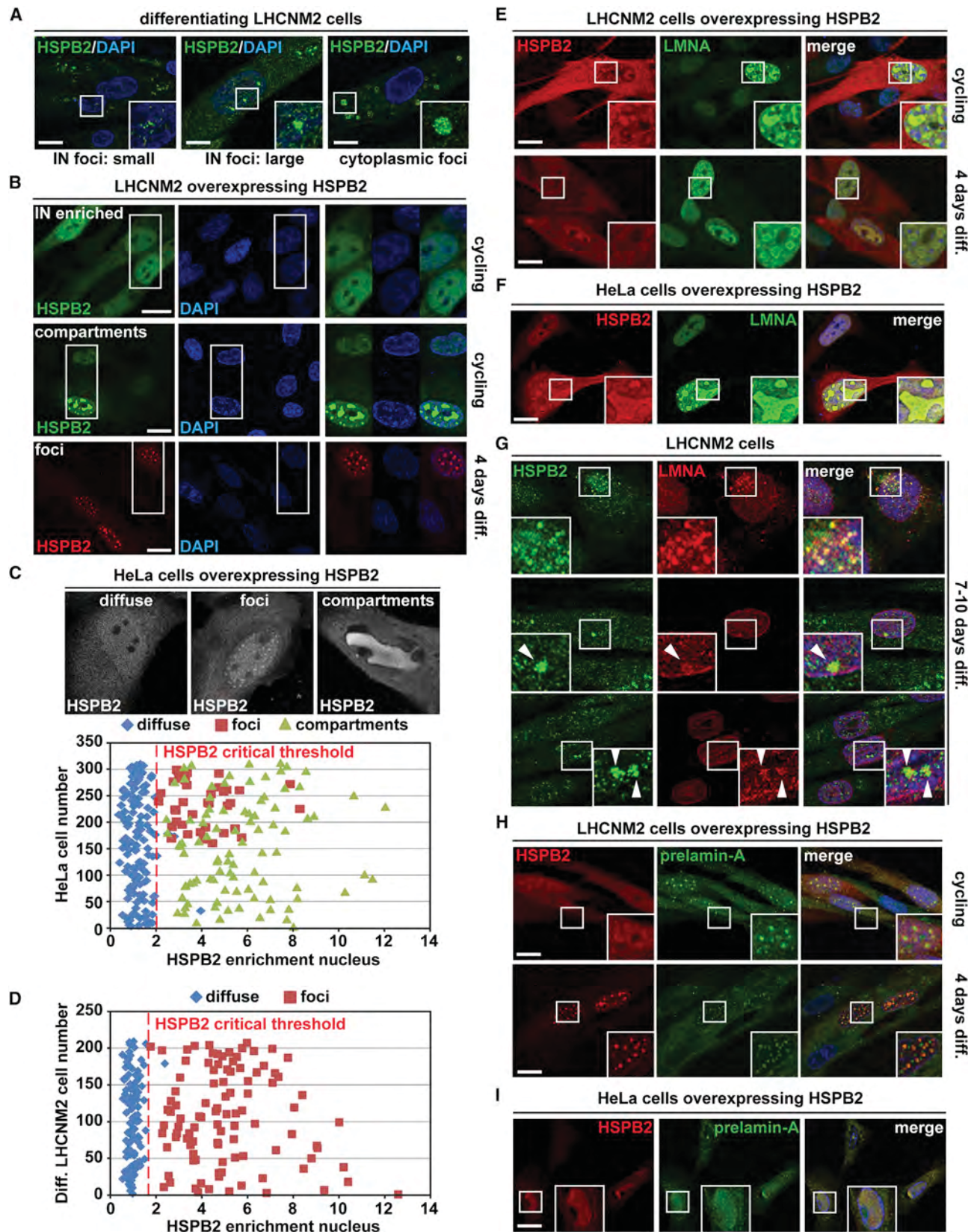
INTRODUCTION

Mammals have ten small heat shock proteins (HSPBs: HSPB1–HSPB10), which belong to the family of molecular chaperones. While some HSPBs are ubiquitously expressed and have been

widely studied, such as HSPB1 and HSPB5, others are expressed only in few tissues and are poorly characterized (Boncoraglio et al., 2012). One example of the latter category is HSPB2, which is expressed in differentiated skeletal and cardiac muscle cells, where it can form a complex with HSPB3 (den Engelsman et al., 2009; Sugiyama et al., 2000). Defects in HSPB2 deregulate the expression of metabolic and mitochondrial genes in response to pressure overload in the mammalian heart (Ishiwata et al., 2012). Moreover, myocardial overexpression of HSPB2 protects, with yet-unknown mechanisms, cardiomyocytes from ischemia (Grose et al., 2015), a pathological condition characterized by early transcriptional changes aimed at promoting damage repair (Wechsler et al., 1994). However, how changes in HSPB2 levels affect gene expression is still unknown.

From a structural point of view, HSPBs are composed of a well-conserved α -crystallin domain (ACD) and less conserved N- and C-terminal domains. HSPBs are disordered proteins, which contain domains of low complexity, referred to as intrinsically disordered regions (IDRs) (Sudnitsyna et al., 2012).

Over the past six years, proteins with low-complexity IDRs have been intensively studied, because they are able to drive the formation of liquid droplets in cells via a process known as liquid-liquid phase separation (Zhu and Brangwynne, 2015; Banani et al., 2017). These liquid droplets have been proposed to function as membrane-less organelles (MLOs) (Zhu and Brangwynne, 2015; Banani et al., 2017). Examples of MLOs are nuclear bodies such as nucleoli, promyelocytic leukemia protein (PML) bodies, Cajal bodies, speckles and paraspeckles, and cytoplasmic ribonucleoprotein particles, such as stress granules and P bodies (Banani et al., 2017). These MLOs differ in size, number, and composition, but they are all highly dynamic and show liquid-like behavior.



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Our understanding of the signals that drive self-assembly of MLOs in mammalian cells is still limited; however, it is known that specific stressors and an increase in the concentration of IDR-containing proteins can induce the formation of liquid droplets in the cytoplasm (Patel et al., 2015) and nucleus (Schmidt and Rohatgi, 2016). For example, overexpression of TDP-43 drives the assembly of nuclear droplets that segregate portions of the nucleoplasm and regulate specific nuclear functions (Schmidt and Rohatgi, 2016).

Recruitment of HSPBs into MLOs was documented previously. For example, HSPB1, HSPB5, and HSPB7 are recruited into nuclear speckles, which are splicing factor compartments (Bryantsev et al., 2007; van den IJssel et al., 2003; Vos et al., 2009), while HSPB8 and, to a lesser extent, HSPB1 are recruited inside cytoplasmic stress granules, ribonucleoprotein particles that store and protect mRNAs upon stress (Ganassi et al., 2016). What drives the recruitment of these HSPBs into MLOs and whether recruitment depends on the presence of IDRs in HSPBs are largely unknown. Moreover, direct evidence that members of the HSPB family can phase separate and form liquid droplets has not yet been provided.

Here, we demonstrate that, in differentiating myoblasts, HSPB2 forms cytoplasmic and nuclear spherical foci. While HSPB2 cytoplasmic foci partly colocalize with HSPB3, nuclear foci partly colocalize with the nuclear intermediate filament protein lamin-A/C (LMNA). LMNA exerts different nuclear functions, including the regulation of nuclear stability, genome organization, and transcription. Moreover, mutations in the *LMNA* gene cause skeletal and cardiac myopathy (Davidson and Lammerding, 2014). Overexpression of HSPB2 in several cell types, including human myoblasts, promotes HSPB2 assembly into cytoplasmic and nuclear compartments, which behave as liquid droplets. Aberrant phase separation of HSPB2 changes LMNA and chromatin distribution with detrimental consequences for nuclear function and integrity. Importantly, HSPB2 phase separation is negatively regulated by its binding partner HSPB3. Depletion of HSPB3 enhances HSPB2 compartmentalization, decreases myogenin expression, and leads to micronuclei formation. Finally, we identified two mutations in the *HSPB3* gene in myopathic patients. Both myopathy-linked mutations disrupt the binding of HSPB3 to HSPB2 and trigger phase separation of HSPB2 into aberrant compartments. Our data suggest that a developmentally regulated increase in HSPB2 concentration reorganizes nucleoplasmic LMNA distribution during myoblast differentiation. Deregulation

of HSPB2 assembly, due to HSPB3 mutations, may contribute to myopathy.

RESULTS

HSPB2 Forms Intranuclear Compartments in Mammalian Cells

To gain insights in HSPB2 properties, we studied its expression and subcellular distribution in human immortalized myoblasts (LHCNM2 cells) (Zhu et al., 2007). Differentiation of myoblasts follows an ordered sequence of events. The first step is commitment to differentiation, with upregulation of the transcription factor myogenin, followed by cell-cycle arrest, cell migration, adhesion, and phenotypic differentiation. This goes along with expression of genes, coding for contractile proteins, and fusion of mononucleated cells into multinucleated myotubes (Andrés and Walsh, 1996).

To characterize our LHCNM2 cells, we compared the expression levels of myogenin and desmin, markers of myoblast differentiation. Myogenin mRNA and desmin protein were absent from cycling (non-differentiating) LHCNM2 cells; they were both induced during differentiation (Figures S1A and S1B) (Kaufman and Foster, 1988). In agreement with published data (Sugiyama et al., 2000), HSPB2 and HSPB3 mRNA and protein were undetectable in cycling LHCNM2 cells but upregulated during differentiation (Figures S1A and S1B).

Next, we performed an immunohistochemical analysis of cycling and differentiating human myoblasts. We found a surprising heterogeneity in HSPB2 subcellular localization. Seven days post-differentiation, we found many multinucleated cells with homogeneous distribution of HSPB2 and HSPB3 both in the cytoplasm and nuclei (Figure S1C). However, some cells showed nuclear foci containing HSPB2, but not HSPB3; also, the number and size of these HSPB2-containing foci varied from dozens of small foci to one or a few large nuclear structures (Figures 1A and S1C). After 10 days of differentiation, we found mono- and multinucleated cells with undetectable nuclear HSPB3 staining and nuclear HSPB2 foci and cells with cytoplasmic HSPB2 spherical foci that partly colocalized with HSPB3 (Figures 1A and S1C). Thus, during the early steps of myoblast differentiation, HSPB2 forms two types of structures: nuclear foci that do not colocalize with HSPB3, in mono- and multinucleated cells, and cytoplasmic spherical foci that partly colocalize with HSPB3. The functional significance of these HSPB2 nuclear and cytoplasmic foci is currently unknown.

Figure 1. HSPB2 Forms Nuclear Compartments that Sequester LMNA in Cells

- (A) Immunofluorescence on differentiating (diff.) LHCNM2 cells showing HSPB2 and DAPI. IN, intranuclear.
 (B) Immunofluorescence on cycling and diff. LHCNM2 cells overexpressing HSPB2 showing HSPB2 and DAPI. IN, intranuclear.
 (C) Immunofluorescence on HeLa cells overexpressing HSPB2 for 48 hr. Based on HSPB2 intranuclear distribution, cells were grouped into three categories: diffuse, foci, and compartments. The representative graph reports HSPB2 fluorescence intensity in each compartment normalized for the fluorescence intensity in the cytoplasm. $n = 70\text{--}122$. Data indicate mean $\pm$ SEM; $p < 10^{-26}$ (compartments versus diffuse HSPB2) and $p < 10^{-34}$ (foci versus diffuse HSPB2).
 (D) Representative graphic showing HSPB2 critical threshold required for nuclear compartmentalization in diff. LHCNM2 cells. $n = 100\text{--}111$. Data indicate mean $\pm$ SEM; $p < 10^{-52}$ (foci versus diffuse HSPB2).
 (E and F) Immunofluorescence on cycling and diff. LHCNM2 cells (E) or HeLa cells (F) overexpressing HSPB2. Staining: HSPB2 and LMNA.
 (G) Immunofluorescence on diff. LHCNM2 cells showing nuclear foci containing endogenous HSPB2 and LMNA.
 (H and I) Immunofluorescence on cycling and diff. LHCNM2 cells (H) or HeLa cells (I) overexpressing HSPB2. Staining: HSPB2 and prelamin-A.
 In (A), (B), and (E)–(I), DAPI was used to stain nucleic acid. Scale bars, 10 μ m.
 See also Figures S1, S2, and S3.

To further investigate HSPB2 subcellular distribution, we overexpressed HSPB2 in cycling and differentiating LHCNM2 cells using lentiviral particles. By confocal microscopy, we confirmed that, similarly to endogenous HSPB2, overexpressed HSPB2 accumulated in the nucleus in cycling and differentiating LHCNM2 cells (Figure 1B). Again, we noticed heterogeneity in HSPB2 distribution. While some cells showed diffuse nuclear HSPB2 staining (Figure 1B, upper panel), others showed nuclear HSPB2 foci with variable size, ranging from 0.3 μm to 1.7 μm or more in diameter (Figure 1B, middle and lower panels; average size, 0.86 μm $\pm$ 0.02 μm ; $n = 167$). Because HSPB3 is absent in cycling LHCNM2 cells, these findings suggest that overexpressed HSPB2 forms nuclear compartments in a HSPB3-independent manner. Moreover, HSPB2 assembly is independent of the developmental status of the cell, because we observed HSPB2 compartments in cycling and differentiating human myoblasts.

We then asked whether the formation of nuclear compartments by HSPB2 is specific to human myoblasts or whether it could also occur in other cell types. HSPB2 overexpressed in HeLa cells also showed a heterogeneous distribution: it was diffusely localized in the cytoplasm and nucleus, or enriched in nuclear foci of size ranging from ca. 0.4 μm to 3.8 μm in diameter (average size, 1.17 μm $\pm$ 0.03 μm ; $n = 169$), or it accumulated in one large nuclear compartment (Figure 1C). Because MLO formation is dependent on protein concentration (Banani et al., 2017), we asked whether there is a critical concentration at which HSPB2 starts to form nuclear foci and larger compartments. Fluorescence density measurements allowed us to identify a critical threshold above which HSPB2 assembled into nuclear compartments (Figure 1C). We also identified a critical threshold at which endogenous HSPB2 formed nuclear compartments in differentiating human myoblasts (Figure 1D). The latter was similar to the one measured in HeLa cells overexpressing HSPB2. This result shows very clearly that compartmentalization by HSPB2 also occurs at endogenous expression levels and under physiological conditions.

Nuclear compartments of HSPB2 were also observed in immortalized motor neuronal (NSC34) and HEK293T cells (Figure S1D). Thus, HSPB2 compartmentalization is not cell type specific.

HSPB7 is also mainly expressed in myoblasts, similar to HSPB2 (Vos et al., 2009). In agreement with Vos et al. (2009), HSPB7 colocalized with the nuclear speckle marker ASF/SF2 in some HSPB7-overexpressing HeLa cells (data not shown). However, no nuclear compartments similar to the ones formed by HSPB2 were observed upon overexpression of HSPB7 in HeLa cells, even when a nuclear localization signal (NLS) was added to force HSPB7 nuclear accumulation (Figure S1E). HSPB1 and HSPB5, which translocate in the nucleus (Bryantsev et al., 2007; van den Ijssel et al., 2003), did not form nuclear foci upon overexpression in HeLa cells either (Figure S1F). We conclude that compartment formation is a specific property of HSPB2, which is independent of the cell type but dependent on HSPB2 concentration.

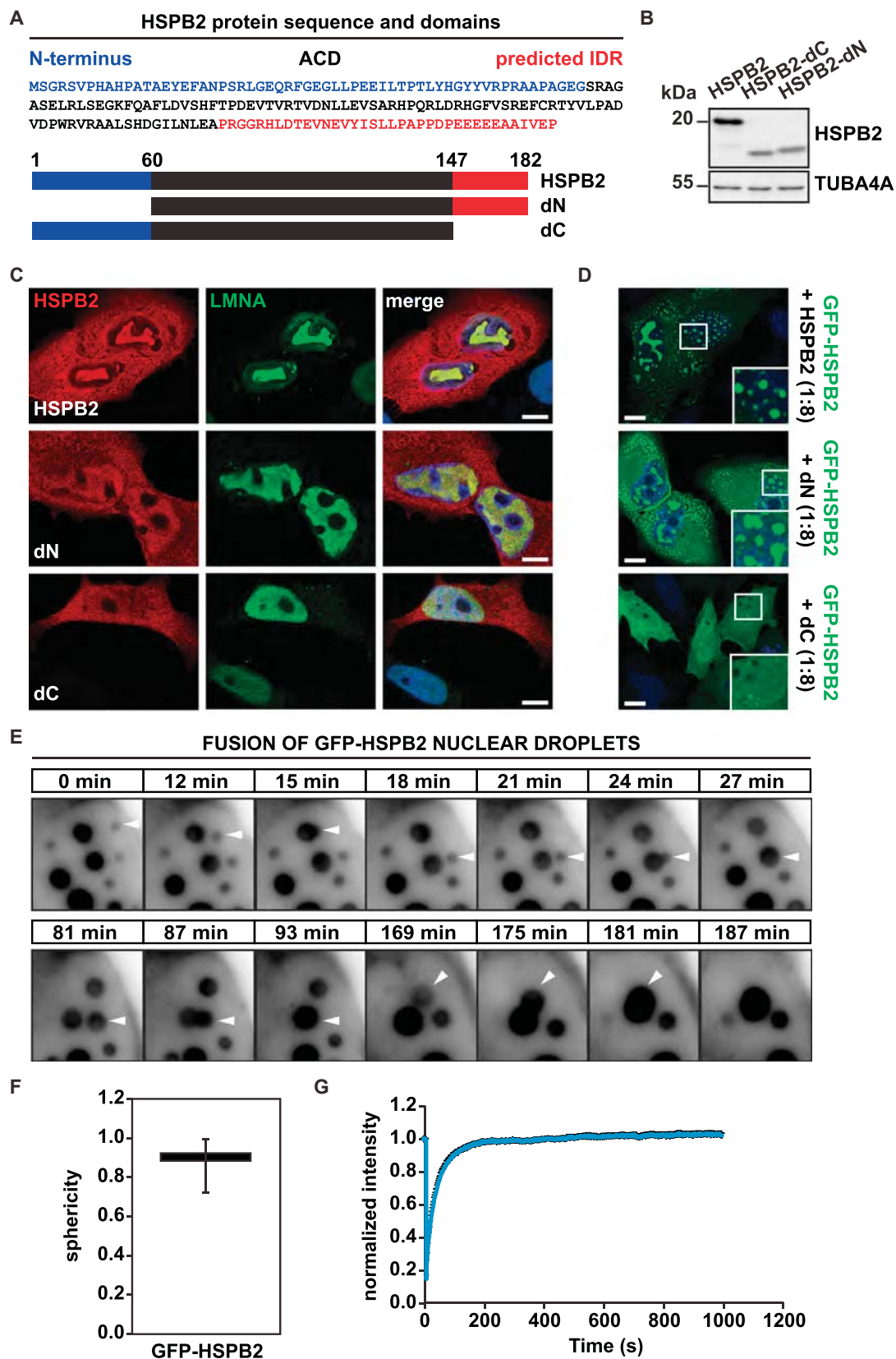
HSPB2 Nuclear Compartments Sequester LMNA in Mammalian Cells

In LHCNM2 and HeLa cells, the large nuclear HSPB2 compartments affected chromatin distribution (judged by DAPI staining)

and, occasionally, nuclear shape (Figures 1B and 1C). Nuclear shape and chromatin organization are regulated by nuclear lamins, which include LMNA, lamin-B1 (LMNB1), and B2 (LMNB2). These lamins form separate but interconnected meshwork underneath the nuclear envelope and throughout the nucleoplasm (Dechat et al., 2010). Thus, we asked whether HSPB2 nuclear compartments affected nuclear lamin distribution in myoblasts. Indeed, nuclear HSPB2 compartments colocalized with LMNA independent of their size; moreover, HSPB2 also changed LMNA distribution (Figure 1E). The changes in LMNA distribution induced by overexpressed HSPB2 also occurred in HeLa cells (Figure 1F).

We next verified whether the nuclear foci that HSPB2 forms during myoblast differentiation colocalize with LMNA. HSPB2 nuclear foci partly colocalized with LMNA (Figure 1G). These LMNA-positive HSPB2 foci were only observed in mononucleated cells that were in the process of differentiation. Thus, we conclude that HSPB2 forms intranuclear compartments in differentiating myoblasts, which sequester LMNA.

Mature LMNA originates from a precursor form, prelamin-A, which undergoes sequential post-translational modifications that include farnesylation and C terminus cleavage (Davies et al., 2011). Prelamin-A processing intermediates contribute to large-scale chromatin rearrangements, affecting the expression of specific genes. In particular, during myoblast differentiation, prelamin-A regulates the expression of key genes such as caveolin 3 and troponin T (Capanni et al., 2008). We asked whether HSPB2 nuclear compartments may also sequester immature forms of LMNA. Due to its rapid processing, prelamin-A is undetectable in mammalian cells unless its maturation is inhibited with, e.g., a specific farnesyltransferase inhibitor (FTI) (Verstraeten et al., 2008). In agreement, using an antibody specific for prelamin-A, we detected it only after treatment of cycling myoblasts with FTI (Figure S2A). Cycling and differentiating myoblasts overexpressing HSPB2 were characterized by the accumulation of prelamin-A in the form of nuclear foci that partly colocalized with HSPB2 compartments (Figure 1H). In contrast, prelamin-A was undetectable in cycling myoblasts overexpressing GFP (Figure S2B), excluding any artifact due to cell infection with lentiviral particles. Accumulation of prelamin-A inside HSPB2 nuclear compartments was also observed in HeLa cells (Figure 1I). The antibody used recognizes both non-farnesylated and farnesylated prelamin-A. To determine whether HSPB2 compartments equally recruit non-farnesylated and farnesylated prelamin-A, we co-expressed in HeLa cells HSPB2 with cDNAs expressing FLAG-prelamin-A (processed into mature lamin-A) and two mutants that cannot undergo complete maturation, FLAG-prelamin-A-C661M (non-farnesylatable) and FLAG-prelamin-A-L647R (uncleavable farnesylated) (Mattioli et al., 2011). HSPB2 compartments sequestered only FLAG-prelamin-A and non-farnesylated FLAG-prelamin-A-C661M, while leaving the distribution of farnesylated FLAG-prelamin-A-L647R unaffected (Figure S2C). Thus, HSPB2 sequesters immature non-farnesylated LMNA as well as mature LMNA in nuclear compartments and might interfere with LMNA maturation. Whether HSPB2-induced changes in prelamin-A and LMNA distribution have consequences on chromatin rearrangements and gene expression is unknown.



(legend on next page)

During differentiation, the solubility and distribution of LMNA change and these changes in the properties of LMNA are required for proper myoblast differentiation (Mariappan and Parnai, 2005). Our data demonstrated that HSPB2 and LMNA colocalize in nuclear compartments during early myoblast differentiation and that overexpression of HSPB2 in LHCNM2 cells promotes targeting of nucleoplasmic LMNA, as well as non-farnesylated prelamin-A, to intranuclear compartments. We propose two possible explanations for these observations. First, HSPB2 forms compartments that directly sequester LMNA. Alternatively, LMNA itself could be enriched in nuclear compartments and then recruit HSPB2. To differentiate between these possibilities, we overexpressed HSPB2 in mouse embryonic fibroblasts (MEFs) derived from LMNA wild-type mice (*Lmna*^{+/+}), LMNA-deficient mice (*Lmna*^{-/-}), or mice that lack lamin A and express lamin-C only (LCO) (Figures S2D and S2E) (Lammerding et al., 2006). The absence of lamin-A (in LCO) and LMNA (in *Lmna*^{-/-}) did not abrogate HSPB2 assembly into compartments (Figure S2E). We conclude that HSPB2 forms intranuclear compartments independently of LMNA and that LMNA is recruited into HSPB2 compartments and not vice versa. Our results suggest a working model in which a local increase in HSPB2 concentration promotes HSPB2 compartmentalization, which, in turn, affects the intranuclear distribution of non-farnesylated prelamin-A and LMNA.

HSPB2 Compartments Behave as Liquid Droplets

HSPBs are disordered proteins because they contain N- and C-terminal IDRs (Sudnitsyna et al., 2012). However, the functional role of these IDRs is still unclear. Recent studies suggest that many IDRs promote the assembly of proteins into MLOs by liquid-liquid phase separation. The intranuclear assemblies formed by HSPB2 are reminiscent of MLOs. The fact that compartment formation by HSPB2 only occurs above a critical concentration (Figures 1C and 1D) is also typical for liquid-liquid phase separation (Banani et al., 2017; Schmidt and Rohatgi, 2016). Thus, we next tested whether the IDRs promote HSPB2 compartmentalization by phase separation.

We generated two truncated forms of HSPB2, lacking either the N terminus (dN-HSPB2) or the predicted disordered C-terminal domain (dC-HSPB2; Figure 2A). Both deletion mutants were expressed at similar levels in HeLa cells (Figure 2B). While HSPB2 and dN-HSPB2 still formed intranuclear compartments that sequestered LMNA, dC-HSPB2 showed a diffuse staining

in the cytoplasm and nucleus and did not affect the distribution of LMNA (Figure 2C).

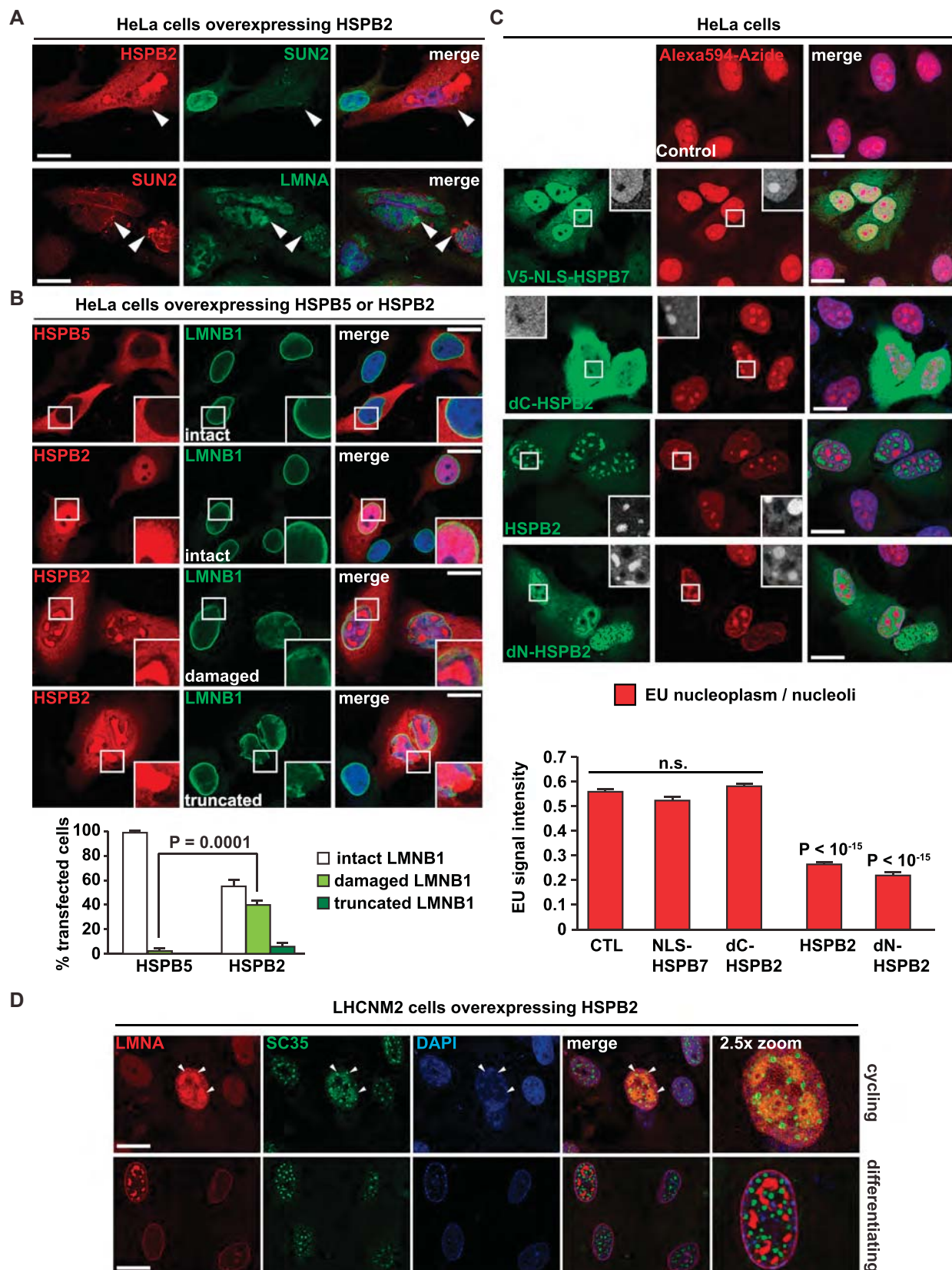
We next used live cell imaging with GFP-tagged HSPB2 to test whether HSPB2 compartments behave like liquid droplets. Because the GFP tag could influence the behavior of HSPB2, we overexpressed GFP-HSPB2 at a very low concentration, together with higher concentrations of untagged HSPB2, dN-HSPB2, or dC-HSPB2 (ratio of GFP-HSPB2:untagged HSPB2 constructs, 1:8). This strategy allowed us to recapitulate the previously observed subcellular distribution of untagged HSPB2 constructs (Figure 2; compare Figures 2C and 2D). Instead, GFP, per se, showed a homogeneous staining in HeLa cells (Movie S1). Upon co-transfection with full-length HSPB2 or dN-HSPB2, GFP-HSPB2 formed cytoplasmic and nuclear foci as well as large intranuclear assemblies (Figure 2D; Movies S2 and S3). However, GFP-HSPB2 displayed a diffuse distribution when co-expressed with dC-HSPB2 (Figure 2D; Movie S4).

We next characterized the behavior of GFP-HSPB2 compartments by live cell imaging. GFP-HSPB2 compartments have the typical properties of liquid droplets: they fuse after touching one another and then relax into one large droplet (Figure 2E; Movie S2), and they are roughly spherical, presumably due to the surface tension (Figure 2F). We next investigated the mobility of GFP-HSPB2 in the intranuclear compartments. Photobleaching analysis revealed that GFP-HSPB2 molecules rapidly redistribute within the nuclear assemblies, in agreement with a liquid material state (Figure 2G). Based on these data, we conclude that HSPB2 compartments have all the typical hallmarks of a liquid state, suggesting that HSPB2 compartments form via liquid-liquid phase separation.

We then verified whether HSPB2 compartments colocalize with known nuclear membrane-less organelles or recruit other IDR-containing proteins that undergo phase separation. The nuclear foci formed by overexpressed HSPB2 did not colocalize with nuclear speckles (SC35), Cajal bodies (SMN), nucleoli (fibrillarin), or nuclear stress bodies (Sam68) (Figure S3A). Adjacency and partial colocalization between some, but not all, HSPB2 foci and PML-nuclear bodies were observed. However, we also observed nuclear HSPB2 foci that were not adjacent to and did not colocalize with PML. Thus, HSPB2 nuclear foci can form independently of the presence of PML nuclear bodies (Figure S3A). Concerning RNA-binding proteins that undergo phase separation and are recruited to MLOs such as TIA-1, TDP-43 and FUS, these were not recruited at HSPB2 foci (Figure S3B).

Figure 2. The Intrinsically Disordered C-Terminal Domain of HSPB2 Is Required for Phase Separation in Cells

- (A) Schematic representation of HSPB2 protein and of dN- and dC-HSPB2 deletion constructs generated. Blue indicates the N terminus; black indicates alpha-crystallin domain (ACD); red indicates predicted IDR.
- (B) Immunoblot showing the expression levels of HSPB2, dC-HSPB2, and dN-HSPB2 in HeLa cells transfected for 48 hr. TUBA4A: loading control.
- (C) Immunofluorescence on HeLa cells overexpressing HSPB2, dN-HSPB2, and dC-HSPB2.
- (D) Microscopy on HeLa cells overexpressing GFP-HSPB2 with untagged HSPB2, dN-HSPB2, or dC-HSPB2 (1:8 ratio).
- (E) Inverted black-and-white images of GFP-HSPB2 in HeLa cells overexpressing GFP-HSPB2 with HSPB2 (1:8 ratio). Fusion events of GFP-HSPB2 nuclear droplets are indicated by arrowheads.
- (F) A boxplot showing the sphericity values of GFP-HSPB2 nuclear droplets (n = 72). Number 1 represents a sphere.
- (G) Quantification of the fluorescence intensity recovery after bleach of GFP-HSPB2 nuclear droplets in HeLa cells overexpressing GFP-HSPB2 with HSPB2 (1:8 ratio). The timescale of a bleach experiment is shown. n = 18. Data indicate mean ± SEM.
- Scale bars, 5 μm.
- See also Figures S3 and S4 and Movies S1, S2, S3, and S4.



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Combined, these results suggest that HSPB2 forms a type of MLO in mammalian cells.

HSPB2 Phase Separation Affects LMNA and Chromatin Distribution and Impairs Gene Transcription and Nuclear Integrity

To further study the functional consequences of HSPB2 phase separation, we performed live imaging and fluorescence recovery after photobleaching (FRAP) studies in cells co-expressing untagged HSPB2 and GFP-LMNA. Co-expression of untagged HSPB2, dN-HSPB2, or dC-HSPB2 with GFP-LMNA recapitulated the previously observed distribution of endogenous LMNA (compare Figure S4A with Figure 2C).

As expected, GFP-LMNA was almost completely immobile when expressed alone (Figure S4B; Movie S5), and the majority of GFP-LMNA was incorporated in the nuclear lamina (Gilchrist et al., 2004). In contrast, when co-expressed with HSPB2, a large fraction of GFP-LMNA formed dynamic droplets in the nucleoplasm (Movie S6). The pool of GFP-LMNA accumulating inside these nuclear droplets was highly mobile, as evidenced by FRAP measurements (Figure S4C) and droplet fusion events (Figure S4D). Instead, the pool of GFP-LMNA incorporated in the nuclear lamina was immobile, also upon co-expression of HSPB2 (data not shown). These results demonstrate that HSPB2 changes the intranuclear distribution and mobility of nucleoplasmic LMNA.

This result prompted us to investigate the localization of SUN2, an integral protein of the inner nuclear membrane (INM) whose anchoring to the nuclear envelope depends on LMNA (Liang et al., 2011). SUN2 was largely absent from the INM in cells with nuclear HSPB2 compartments (Figure 3A). This correlated with LMNA mislocalization and aggregation in the perinuclear region of the cell (Figure 3A, arrowheads). These results suggest that, by accumulating inside the nucleus, HSPB2 affects LMNA distribution and mobility, which, in turn, impairs SUN2 anchoring at the nuclear envelope, ultimately damaging its integrity. Consistently, HSPB2-overexpressing cells showed a significant increase in the percentage of cells with damaged or disrupted LMNB1 meshwork, compared to cells overexpressing HSPB5 (Figure 3B).

LMNA, together with nuclear-envelope-associated proteins, regulates DNA replication, chromatin organization, and gene transcription (Andrés and González, 2009). We studied whether HSPB2 compartments change chromatin distribution. We co-expressed GFP-HSPB2 in HeLa cells stably expressing histone H2B-mCherry and monitored the distribution of both proteins by time-lapse imaging. GFP-HSPB2 droplets displaced H2B-

mCherry, changing chromatin distribution. These H2B-mCherry rearrangements were very dynamic because of the fusion events of GFP-HSPB2 droplets (Figure S4E; Movie S7). Displacement of H2B-mCherry and chromatin (judged by DAPI staining) by GFP-HSPB2 droplets was further confirmed by immunostaining on fixed cells (Figure S4F).

We then asked whether the changes in chromatin reorganization caused by HSPB2 phase separation could have functional consequences for gene transcription, which we measured using the uridine analog 5-ethynyl uridine (EU) (Jao and Salic, 2008). EU incorporation into newly synthesized RNA could be blocked by co-treatment of HeLa cells with actinomycin D, validating the experimental setup (Figure S5A). Overexpression of NLS-HSPB7, used as a negative control, did not affect EU incorporation, as compared to untreated HeLa cells (Figure 3C). In contrast, HSPB2 significantly decreased RNA synthesis (Figure 3C). Similar results were obtained in cycling myoblasts overexpressing GFP, used as control, or HSPB2. EU incorporation was not observed in areas that contained HSPB2 droplets, regardless of their small or larger size, and global RNA transcription in the nucleoplasm was reduced by HSPB2 phase separation (Figure S5B).

Next, we studied whether HSPB2 compartments would also impair RNA transcription in LMNA knockout cells. Similarly to what is observed in HeLa and LHCNM2 cells, HSPB2 compartments locally inhibited EU incorporation in LMNA-proficient MEFs (Figure S5C). In contrast, in LMNA knockout MEFs, EU was still efficiently incorporated into HSPB2 compartments (Figure S5C). This result demonstrates that HSPB2 compartmentalization leads to a spatial rearrangement of lamin-A and locally impairs RNA synthesis.

Another indirect measure of RNA transcription is reflected by the change in the shape of nuclear speckles. Speckles are dynamic MLOs that store splicing factors (Lamond and Spector, 2003). While, in resting cells, speckles have an irregular shape, they reorganize into spherical foci when polymerase-II-mediated transcription is blocked with actinomycin D (Lamond and Spector, 2003). Moreover, changes in the levels and distribution of LMNA inhibit polymerase II-mediated transcription, with consequences on speckle shape (Shimi et al., 2008; Spann et al., 2002). These findings open the possibility that HSPB2 phase separation, by changing LMNA and chromatin distribution, may also indirectly lead to the reorganization of speckles into spherical foci. To verify this hypothesis, we overexpressed HSPB2 in myoblasts, and we studied the subcellular distribution of LMNA and SC35, a speckle marker. Speckles had an irregular shape in myoblasts with diffuse LMNA distribution

Figure 3. HSPB2 Aberrant Phase Separation Inhibits RNA Synthesis

(A) Immunofluorescence on HeLa cells overexpressing HSPB2 showing SUN2 recruitment at the nuclear envelope.

(B) Immunofluorescence on HeLa cells overexpressing HSPB5 or HSPB2. Damaged LMNB1: irregular and discontinuous nuclear rim; truncated LMNB1: nuclear rim missing one or more portions in the proximity of HSPB2 compartments. Data indicate mean $\pm$ SEM.

(C) 40 hr post-transfection, HeLa cells, control or overexpressing V5-NLS-HSPB7 or HSPB2, were incubated with 5-ethynyl uridine (EU; 200 μ M) for 6 hr. Staining: Alexa594-Azide, V5, HSPB2, and DAPI. EU intensity was quantified in nucleoli and nucleoplasm; nucleoplasm/nucleoli ratio was calculated. $n = 30$ –36. Data indicate mean $\pm$ SEM; $p < 10^{-15}$, n.s., not significant.

(D) Cycling and differentiating (diff.) LHCNM2 cells overexpressing HSPB2 were stained for LMNA, SC35, and DAPI.

Scale bars, 10 μ m.

See also Figures S4 and S5 and Movies S5, S6, and S7.

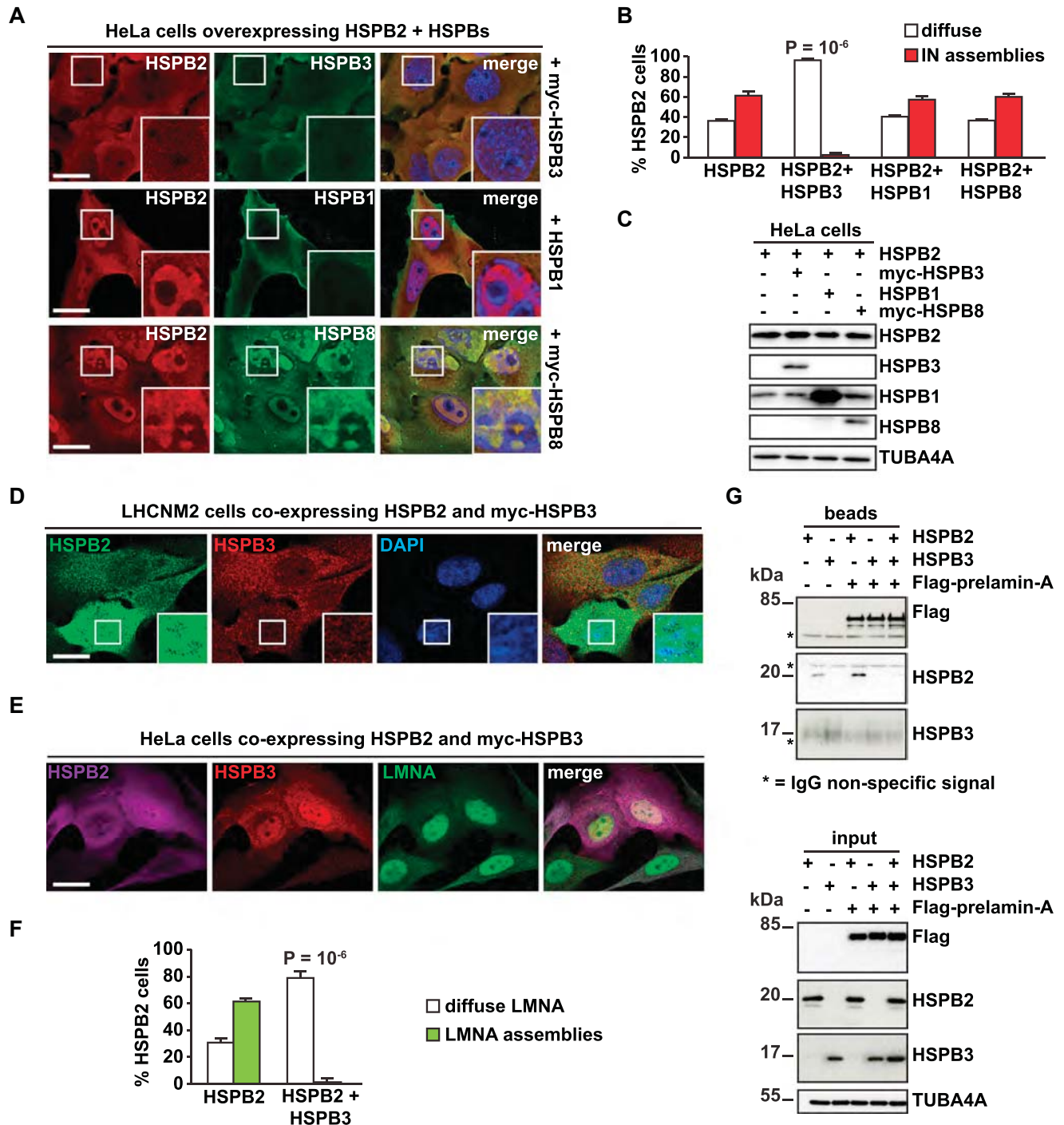


Figure 4. HSPB3 Inhibits HSPB2 Aberrant Phase Separation

(A) Immunofluorescence on HeLa cells overexpressing HSPB2 with myc-HSPB3, HSPB1, or myc-HSPB8.
 (B) Quantitation of the percentage of HSPB2-overexpressing cells with diffuse staining or HSPB2 nuclear assemblies. $n = 3$ experiments/conditions. Data indicate mean $\pm$ SEM; $p = 4.17 \times 10^{-6}$. 143–180 cells per experiment were analyzed.
 (C) Immunoblot showing levels of HSPB2, HSPB1, myc-HSPB3, and myc-HSPB8 in transfected HeLa cells. TUBA4A: loading control.
 (D) Immunofluorescence on cycling myoblasts overexpressing HSPB2 and HSPB3. One representative image of 478 cells analyzed, all showing diffuse HSPB2.
 (E) Immunofluorescence on HeLa cells overexpressing HSPB2 with myc-HSPB3 showing endogenous LMNA.
 (F) Microscopy on HeLa cells overexpressing HSPB2 alone or with myc-HSPB3. Quantitation of the percentage of cells with LMNA nuclear compartments is shown. $n = 3$ –4 experiments/conditions. Data indicate mean $\pm$ SEM; $p = 3.72 \times 10^{-6}$. 125–200 cells per experiment were analyzed.

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and a spherical shape in myoblasts where LMNA was sequestered into HSPB2 compartments (Figure 3D). These data further support the interpretation that HSPB2 phase separation, by altering LMNA and chromatin distribution, inhibits RNA transcription.

Based on these results, we define the nuclear droplets formed in differentiating human myoblasts as “physiological” HSPB2 compartments. In contrast, we refer to HSPB2 droplets that form upon transient overexpression as “aberrant” HSPB2 compartments because they mislocalize LMNA and chromatin, with detrimental consequences for nuclear integrity and function.

HSPB3 Inhibits Aberrant Compartment Formation by HSPB2 and Restores Nuclear LMNA Distribution

HSPB2 forms a stoichiometric complex with HSPB3 (3:1), and their interaction co-stabilizes both proteins (den Engelsman et al., 2009). Physiological intranuclear HSPB2 compartments that form in differentiating myoblasts did not colocalize with HSPB3 but partly colocalized with LMNA (Figure 1G). However, the cytoplasmic HSPB2 foci partly colocalized with HSPB3 (Figure S1C). We thus asked whether HSPB3 could influence HSPB2 phase separation. We co-transfected HSPB2 and HSPB3 in HeLa cells and compared the propensity of HSPB2 to form intranuclear assemblies in the presence and absence of HSPB3. HSPB3 prevented the formation of nuclear HSPB2 droplets, because both proteins were homogeneously distributed throughout the cells (Figures 4A and 4B). This effect was not a mere consequence of lower HSPB2 expression levels upon its co-transfection with HSPB3, as confirmed by immunoblotting (Figure 4C; see also Figure 5G). In contrast, co-expression with HSPB1 or HSPB8 did not prevent HSPB2 compartmentalization (Figures 4A and 4B). While HSPB8 was sequestered inside HSPB2 nuclear compartments, HSPB1 was not (Figure 4A). This result suggests some additional specificity in sequestering proteins in HSPB2 compartments. Also, in cycling LHCNM2 cells co-expressing HSPB3 and HSPB2, the latter showed a homogeneous distribution (Figure 4D), further confirming that HSPB3 negatively regulates HSPB2 compartmentalization.

Since LMNA distribution is rearranged due to HSPB2 aberrant phase separation, we tested whether co-expression of HSPB2 with HSPB3 could rescue LMNA distribution. Indeed, overexpression of HSPB3 inhibited HSPB2 aberrant phase separation and maintained proper LMNA distribution (Figures 4E and 4F). These results demonstrate that nuclear phase separation is a specific property of HSPB2 that is negatively regulated by HSPB3.

We next asked whether HSPB2 directly binds to LMNA and whether/how HSPB3 affects HSPB2-LMNA association. We overexpressed FLAG-tagged LMNA with HSPB2 and HSPB3, separately or combined. Co-immunoprecipitation studies revealed that HSPB2, but not HSPB3, weakly binds to LMNA (Figure 4G). When co-expressed with HSPB2, HSPB3 abolished the association between HSPB2 and LMNA (Figure 4G). These results support the interpretation that, by directly binding to

HSPB2, HSPB3 prevents both HSPB2 compartmentalization and association with LMNA.

Identification of Two Putative HSPB3 Mutations in Myopathy Patients

Mutations in the *HSPB1*, *HSPB5*, and *HSPB8* genes cause myopathy and/or neuropathy (Boncoraglio et al., 2012). Recently, the missense variant p.R7S (rs139382018) in *HSPB3* was linked to axonal motor neuropathy (HMN2C). However, the underlying mechanism is yet unknown (Kolb et al., 2010). Because of the high expression of HSPB2 and HSPB3 in skeletal muscles (Sugiyama et al., 2000), we hypothesized that mutations in this complex could cause (neuro)muscular diseases. Therefore, we sequenced the genomic DNA of 400 myopathy patients of unknown origin to identify potential mutations in the coding regions of *HSPB2* and *HSPB3*. We identified two variants in *HSPB3* in two independent cases that included the A33AfsX50-HSPB3 truncation variant and the R116P-HSPB3 rare missense variant (rs150931007), with a minor allele frequency of 0.0001730 in the ExAC browser (assessed March 2017) (Figures 5A and S6A–S6C). Unfortunately, we were unable to test the parents of case 1 (Figure S6A); the father of case 2 (Figure S6B), who also carried the R116P-HSPB3 variant, displayed only moderate symptoms at the time of testing. The R116P-HSPB3 variant affects a highly conserved, key amino acid in the α -crystallin domain of HSPB3 (Figure S6C), whereas the A33AfsX50-HSPB3 mutation disrupts the reading frame at alanine 33, leading to a premature stop codon 50 amino acids later. This event very likely leads to a non-functional protein.

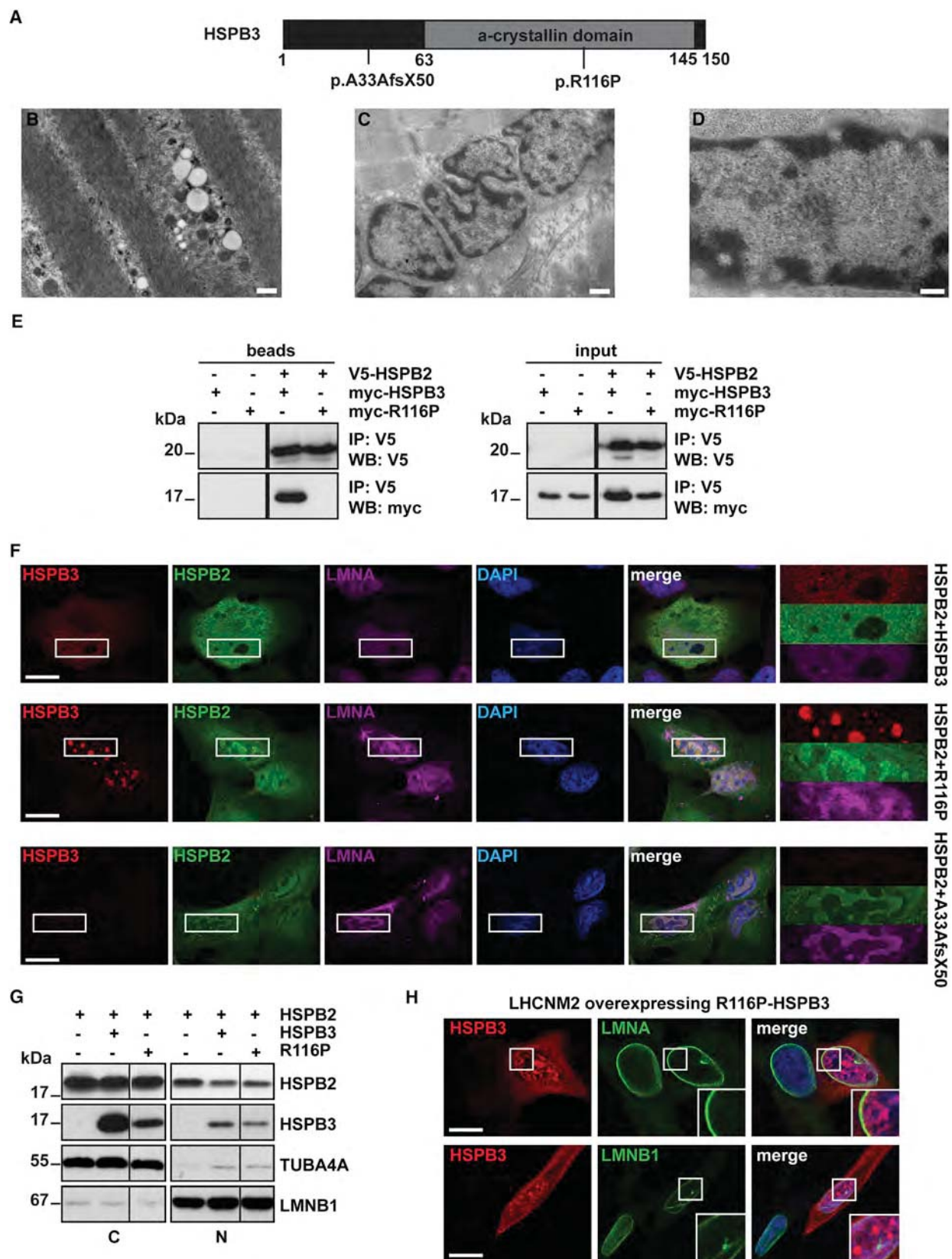
The A33AfsX50-HSPB3 mutation was found in a 70-year-old Italian man who presented shoulder-girdle muscle weakness and atrophy. The R116P-HSPB3 mutation was found in a 25-year-old woman of Italian origin. The father of the affected patient, carrier of the R116P-HSPB3 mutation, was pauci-symptomatic and presented sciatic irregular pains, similar to his daughter with intermittent myalgia. By the age of 32 years, the affected patient developed weakness of the upper and lower limbs, along with neurogenic changes in the lower limbs compatible with axonal neuropathy. Electron microscopy of her muscle biopsy showed severe myofibrillar disarray with loss of Z-disk, enlargements of sarcoplasmic reticulum cisternae, few lysosomes, and sub-sarcolemmal and intermyofibrillar glycogen accumulations in several fibers (Figure 5B). Importantly, the nuclei were plurisegmented with marginated chromatin (Figures 5C and 5D), a hallmark reminiscent of cells overexpressing HSPB2.

These two cases join the growing list of disease-causing mutations reported in HSPB genes.

R116P-HSPB3 and A33AfsX50-HSPB3 Disrupt HSPB2-HSPB3 Complex Formation and Cannot Regulate HSPB2 Aberrant Phase Separation

To study whether the two newly identified mutations affect HSPB3 expression and interaction with HSPB2, we overexpressed

(G) Immunoprecipitation with anti-FLAG of HEK293T cells overexpressing for 24 hr FLAG-prelamin-A and an empty vector or HSPB2 alone or with myc-HSPB3. Total cell lysates (input) and immunocomplexes (beads) were processed for western blot. Asterisk indicates immunoglobulin G (IgG) or non-specific signal. Scale bars, 10 μ m. See also Figure S6.



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myc-tagged HSPB3, R116P-HSPB3, and A33AfsX50-HSPB3 in HeLa cells and measured HSPB3 mRNA and protein levels. The mRNA levels of both mutants were higher compared to those of HSPB3 (Figure S6D). However, the R116P-HSPB3 protein was expressed at similar levels compared to HSPB3, whereas A33AfsX50-HSPB3 was undetectable. This suggested that A33AfsX50-HSPB3 is rapidly degraded after synthesis. This hypothesis was tested and confirmed using Bortezomib, a proteasome inhibitor (Figure S6E).

Next, we verified by co-immunoprecipitation whether the R116P mutation affects HSPB3 binding to HSPB2. While the wild-type proteins interacted (den Engelsman et al., 2009), no association was detected between HSPB2 and R116P-HSPB3 (Figure 5E). A33AfsX50-HSPB3 levels were barely detectable; therefore, its binding to HSPB2 was not tested. Thus, the two mutations identified in myopathy patients directly (R116P) or indirectly (A33AfsX50) disrupt the formation of the HSPB2-HSPB3 complex.

In light of these results, we then tested whether A33AfsX50-HSPB3 and R116P-HSPB3 can inhibit HSPB2 aberrant phase separation. Neither A33AfsX50-HSPB3 nor R116P-HSPB3 could inhibit the formation of HSPB2 nuclear droplets (Figure 5F). Intriguingly, R116P-HSPB3 formed intranuclear aggregates, which were often adjacent but excluded from HSPB2 droplets (Figure 5F). Importantly, in cells co-expressing R116P-HSPB3 or A33AfsX50-HSPB3 together with HSPB2, LMNA was sequestered into HSPB2 nuclear droplets. The nuclear amount of HSPB2 was similar in HeLa cells co-expressing HSPB2 with HSPB3 or R116P-HSPB3; however, HSPB2 formed compartments that sequestered LMNA only in cells co-expressing R116P-HSPB3 (Figure 5G). In agreement, LMNA could still co-immunoprecipitate HSPB2 in cells co-expressing R116P-HSPB3; as an additional control, the neuropathy-linked R7S mutant of HSPB3, which still binds to HSPB2 as efficiently as wild-type HSPB3, abrogated the HSPB2-LMNA interaction (Figures S6F and S6G). These results demonstrate that both HSPB3 mutants cannot negatively regulate HSPB2 aberrant phase separation.

Formation of nuclear aggregates by R116P-HSPB3 was confirmed using cycling myoblasts. Similarly to what was observed in HeLa cells, R116P-HSPB3 nuclear aggregates did not colocalize with LMNA and LMNB1 (Figure 5H). Since cycling myoblasts do not express endogenous HSPB2, these results

demonstrate that formation of nuclear aggregates is an intrinsic property of R116P-HSPB3.

Depletion of HSPB3 Enhances HSPB2 Compartmentalization and Leads to Nuclear Morphological Defects

We demonstrated that HSPB3 disease-linked mutants inhibit the formation of the HSPB2-HSPB3 complex. This result opens the possibility that the accumulation of a free pool of HSPB2, which can self-assemble, might affect gene expression and nuclei morphology, thereby contributing to HSPB3-linked disease. To address this hypothesis, we silenced HSPB3 in differentiating myoblasts. LHCNM2 cells were infected with lentiviral particles expressing a non-targeting short hairpin RNA (shRNA) control sequence or a specific HSPB3 shRNA sequence and GFP as reporter. Five days post-differentiation, cells were processed for qPCR, immunoblotting, or immunohistochemistry. HSPB3 depletion was efficient and did not affect the expression of HSPB2, compared to control cells (Figures 6A and 6B). HSPB3 depletion significantly decreased the expression of myogenin, one of the key genes required for myoblast differentiation (Figure 6A). Concerning the subcellular distribution of HSPB2, 5 days post-differentiation, it was mainly homogeneous in control differentiating cells, with few nuclear HSPB2-positive foci detectable in ca. 34% of the infected cells. In contrast, more than 67% of HSPB3-depleted cells were characterized by the presence of HSPB2-positive foci in the nucleus and cytoplasm (Figure 6C). Besides, compared to control cells, HSPB3-depleted cells were characterized by nuclear morphology abnormalities, with a significant increase of micronuclei (Figures 6D and 6E). These data support the idea that imbalances in the expression of HSPB2-HSPB3 and accumulation of a free pool of HSPB2 correlate with nuclear alterations and impaired myogenin expression.

DISCUSSION

Phase separation of IDR-containing proteins drives the assembly of intracellular components into MLOs that behave like liquid droplets and exert specific functions. The most extensively studied MLOs are cytoplasmic stress granules, P bodies, nuclear speckles, Cajal bodies, and nucleoli (Banani et al., 2017). Recently, phase separation has also been implicated in the

Figure 5. Two HSPB3 Myopathy Mutants Cannot Inhibit HSPB2 Aberrant Phase Separation

- (A) Schematic representation of HSPB3 protein structure and position of the two HSPB3 mutations identified in myopathy patients (p.A33AfsX50 and p.R116P).
(B–D) Electron microscopy on the muscle biopsy of the 25-year-old patient carrying the R116P mutation of HSPB3.
(B) Scale bar, 5 μ m.
(C and D) Scale bars, 0.1 μ m.
(C) Nuclear deformation and chromatin margination.
(D) Chromatin margination.
(E) Immunoprecipitation with anti-V5 on cells overexpressing for 24 hr V5-tagged HSPB2 and myc-tagged HSPB3 or R116P-HSPB3. Input and immunocomplexes (beads) were processed for immunoblotting. WB, western blot.
(F) Immunofluorescence on HeLa cells overexpressing HSPB2 with myc-tagged HSPB3, R116P-HSPB3, or A33AfsX50-HSPB3 showing HSPB2, HSPB3, LMNA, and nucleic acid (DAPI).
(G) Nucleus/cytoplasm fractionation of HeLa cells overexpressing HSPB2 alone or with myc-tagged HSPB3 or R116P-HSPB3.
(H) Immunofluorescence on cycling myoblasts overexpressing R116P-HSPB3 and stained for HSPB3 and LMNA or LMNB1.
Scale bars, 10 μ m.
See also Figure S6.

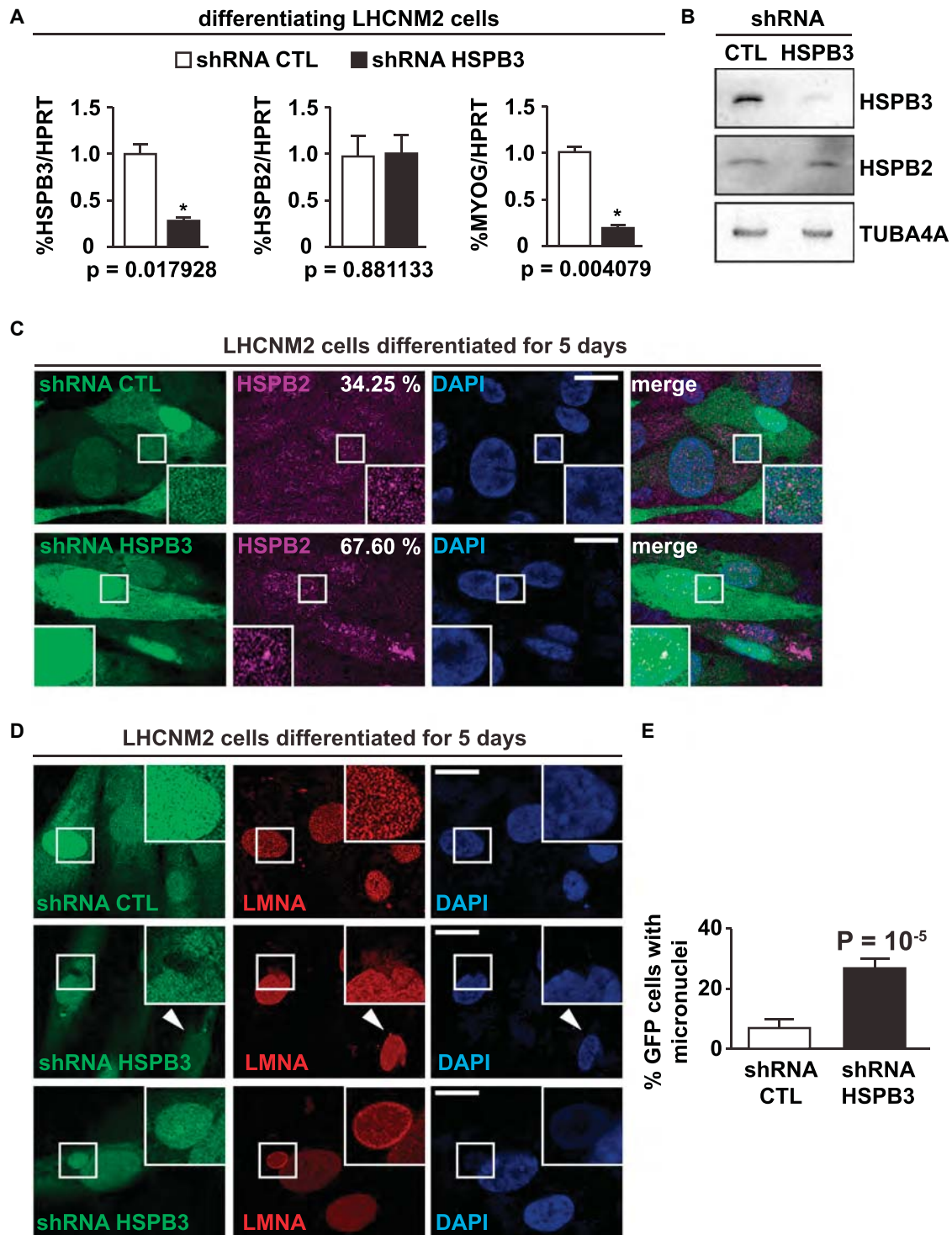


Figure 6. Depletion of HSPB3 Increases HSPB2 Compartmentalization, Decreases Myogenin Expression, and Affects Nuclear Morphology
(A) mRNA levels of HSPB3, HSPB2, and myogenin in LHCNM2 cells infected with non-targeting shRNA (CTL) or HSPB3 shRNA and differentiated for 5 days. HPRT was used for normalization.
(B) Protein levels of HSPB2, HSPB3, and TUBA4A (loading control) in samples treated as described in (A).
(C) Cells were treated as described in (A) and processed for immunofluorescence using the HSPB2 antibody. The percentage of cells with HSPB2-positive foci is shown (181 cells expressing shRNA CTL and 179 cells expressing shRNA HSPB3 were analyzed).
(D) Microscopy on LHCNM2 cells infected as described in (A), showing LMNA distribution and nuclear morphology (DAPI).

(legend continued on next page)

DNA damage response (Aguzzi and Altmeyer, 2016). Poly(ADP-ribose) (PAR) nucleates liquid demixing of IDR-containing proteins at sites of DNA damage. PAR-seeded liquid demixing enables the dynamic rearrangement of nuclear architecture, concentrating and organizing the players that orchestrate DNA repair (Aguzzi and Altmeyer, 2016). Phase separation is, thus, emerging as an efficient mechanism by which cells control the spatio-temporal localization and mobility of specific macromolecules, and regulate complex cellular functions.

Myoblast differentiation is characterized by changes in nuclear architecture, reorganization of nuclear LMNA, and a transient increase in targeted DNA strand breaks, which enhances muscle gene expression (Fernando et al., 2002; Larsen et al., 2010; Markiewicz et al., 2005). DNA damage is followed by caspase-triggered XRCC1 repair foci; the latter allows proceeding along muscle differentiation (Al-Khalaf et al., 2016). Interestingly, LMNA promotes DNA repair, stabilizing 53BP1 and preventing DNA damage and cell senescence (Gonzalez-Suarez et al., 2009; Lees-Miller, 2006). Moreover, LMNA remodeling is required for proper gene expression during myoblast differentiation (Markiewicz et al., 2005), and overexpression of LMNA upregulates muscle-specific genes (Lourim and Lin, 1992). Thus, changes in LMNA concentration and distribution directly affect chromatin and gene expression, and LMNA remodeling and DNA repair are interconnected processes, at least during myoblast differentiation.

Our study shows that HSPB2 forms liquid compartments that partly colocalize with LMNA in differentiating myoblasts. This suggests that HSPB2 compartments regulate nucleoplasmic LMNA rearrangements that take place during the early steps of muscle differentiation. Phase-separated HSPB2 may temporarily store discrete pools of LMNA, delaying their incorporation in the nuclear envelope (Markiewicz et al., 2005). Recruitment of LMNA into nuclear HSPB2 storage compartments could drive subtle changes in chromatin organization, influencing transcription of specific genes. In line, LMNA remodeling due to HSPB2 phase separation leads to a redistribution of chromatin, with direct consequences for nuclear transcription.

Muscles are subjected to oxidative and mechanical stress. LMNA remodeling activates the transcription of genes required in response to mechanical stress, a process known as mechanotransduction (Lammerding et al., 2006). HSPB2 may regulate specific LMNA rearrangements, indirectly regulating chromatin organization in mechanically stressed cells. Accordingly, upon exposure to oxidative stress, *HSPB2* knockout mice fail to properly modulate the transcription of metabolic and mitochondrial regulatory genes required for the stress response; this, in turn, has detrimental consequences for muscle viability (Ishiwata et al., 2012). Moreover, *HSPB2/HSPB5* double-knockout mice develop myopathy with aging, supporting the conclusion that HSPB2 (with HSPB5) is required for myoblast adaptation and response to chronic stress.

An increasing body of evidence shows that deregulated phase separation has strong implications in aging and disease, including, e.g., amyotrophic lateral sclerosis and inclusion body myopathy (Aguzzi and Altmeyer, 2016; Alberti and Hyman, 2016). Here, we provide further evidence that derailed phase separation can be a determinant of cellular dysfunction and human disease. First, deregulated HSPB2 phase separation mislocalizes LMNA, compromising nuclear architecture and transcription; however, aberrant compartmentalization by HSPB2 is counteracted by HSPB3, which is co-upregulated with HSPB2 during myoblast differentiation (Sugiyama et al., 2000). Intriguingly, HSPB3 depletion in differentiating myoblasts decreases myogenin expression and enhances HSPB2 compartmentalization and nuclear morphological defects such as micronucleus accumulation. These observations suggest that imbalances of HSPB2-HSPB3 expression and enhanced HSPB2 foci formation may have deleterious consequences on myoblast viability and differentiation. Second, we identified two HSPB3 mutations in myopathy patients. While A33AfsX50-HSPB3 is unstable and rapidly degraded, R116P-HSPB3 can no longer interact with HSPB2. Thus, both HSPB3 mutants cannot control HSPB2 aberrant phase separation. Intriguingly, a muscle biopsy from the patient with the R116P-HSPB3 mutation shows alterations of nuclear morphology with chromatin margination. Although we do not know whether aberrant HSPB2 compartmentalization occurred in patient cells, these changes are similar to the ones induced by HSPB2 overexpression. Concerning R116P-HSPB3-linked myopathy, we cannot exclude that R116P-HSPB3 is associated with a gain of toxic function, as suggested by its propensity to form nuclear aggregates. It is possible that both a gain of toxic function of HSPB3 and a loss of function resulting from deregulated HSPB2 phase separation and LMNA mobility contribute to disease.

In line, increased retention of LMNA in the nucleoplasm, increased LMNA mobility and solubility, and accumulation of micronuclei have been documented in mammalian and patient cells expressing mutated forms of LMNA associated with laminopathies (Gilchrist et al., 2004; Markiewicz et al., 2002, 2005). Laminopathies include a variety of human diseases, such as Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy, and Hutchinson-Gilford progeria syndrome, that are characterized by muscle atrophy, together with other symptoms (Davidson and Lammerding, 2014). Moreover, loss of LMNA disrupts nuclear envelope integrity and causes muscular dystrophy in mice (Sullivan et al., 1999). Altogether, these data highlight the importance of LMNA for muscle cell function and how aberrant changes in its distribution are detrimental for muscle viability.

In summary, we propose that HSPB2 phase separation regulates dynamic LMNA and chromatin remodeling in response to differentiation stimuli and upon stress, maintaining myoblast viability. In contrast, deregulation of HSPB2 compartmentalization, due to decreased HSPB3 expression or HSPB3 mutations

(E) Cells were treated as described in (A). Quantitation of the percentage of cells with micronuclei. $n = 8$. Data indicate mean $\pm$ SEM; $p = 1.23 \times 10^{-5}$ (423 cells expressing shRNA CTL and 390 cells expressing shRNA HSPB3 were analyzed).

Scale bars, 10 μ m.

See also Figure S6.

that disrupt the HSPB2-HSPB3 complex, may contribute to muscle aging and disease.

EXPERIMENTAL PROCEDURES

Cell Lines

In this study we used: LHCNM2 human myoblasts; HeLa, NSC34, and HEK293T cells; *Lmna*^{+/+}, *Lmna*^{-/-}, and LCO mouse embryonic fibroblasts; and HeLa-Kyoto cells expressing mCherry-tagged human H2B.

Collection of Human Samples

Procedures for collection of human blood and muscle biopsy were in accordance with the ethical standards of the regional committee (approval received on 09/10/2007). Informed consent was obtained from all subjects. At the time of muscle biopsy, the proband was a 25-year-old woman. When she was re-evaluated at 32 years of age, the proband showed a mild proximal and distal muscle weakness. Muscle biopsy was done at the left deltoid. Muscle was flash frozen in isopentane cooled in liquid nitrogen and stored at -80 degrees until analysis.

Statistical Analysis

Student's *t* test was used for comparisons between two groups. One-way ANOVA followed by a Bonferroni-Holm post hoc test was used for comparisons between three or more groups.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures and seven movies and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.08.018>.

AUTHOR CONTRIBUTIONS

Conceptualization, F.F.M. and S.C.; Methodology, F.F.M., J.B., and S.C.; Formal Analysis, F.F.M., J.B., J.V., and S.C.; Investigation, F.F.M., J.B., D.S.V., J.V., G.C., L.M., S.D.B., M.N., and S.C.; Resources, S.M., J.L., J.F.B., R.T., E.P., and C.A.; Writing – Original Draft, F.F.M. and S.C.; Writing – Review & Editing, S.A. and S.C.; Supervision, S.A. and S.C.; Funding Acquisition, S.C.

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Supplemental Information

Aberrant Compartment Formation by HSPB2

Mislocalizes Lamin A and Compromises

Nuclear Integrity and Function

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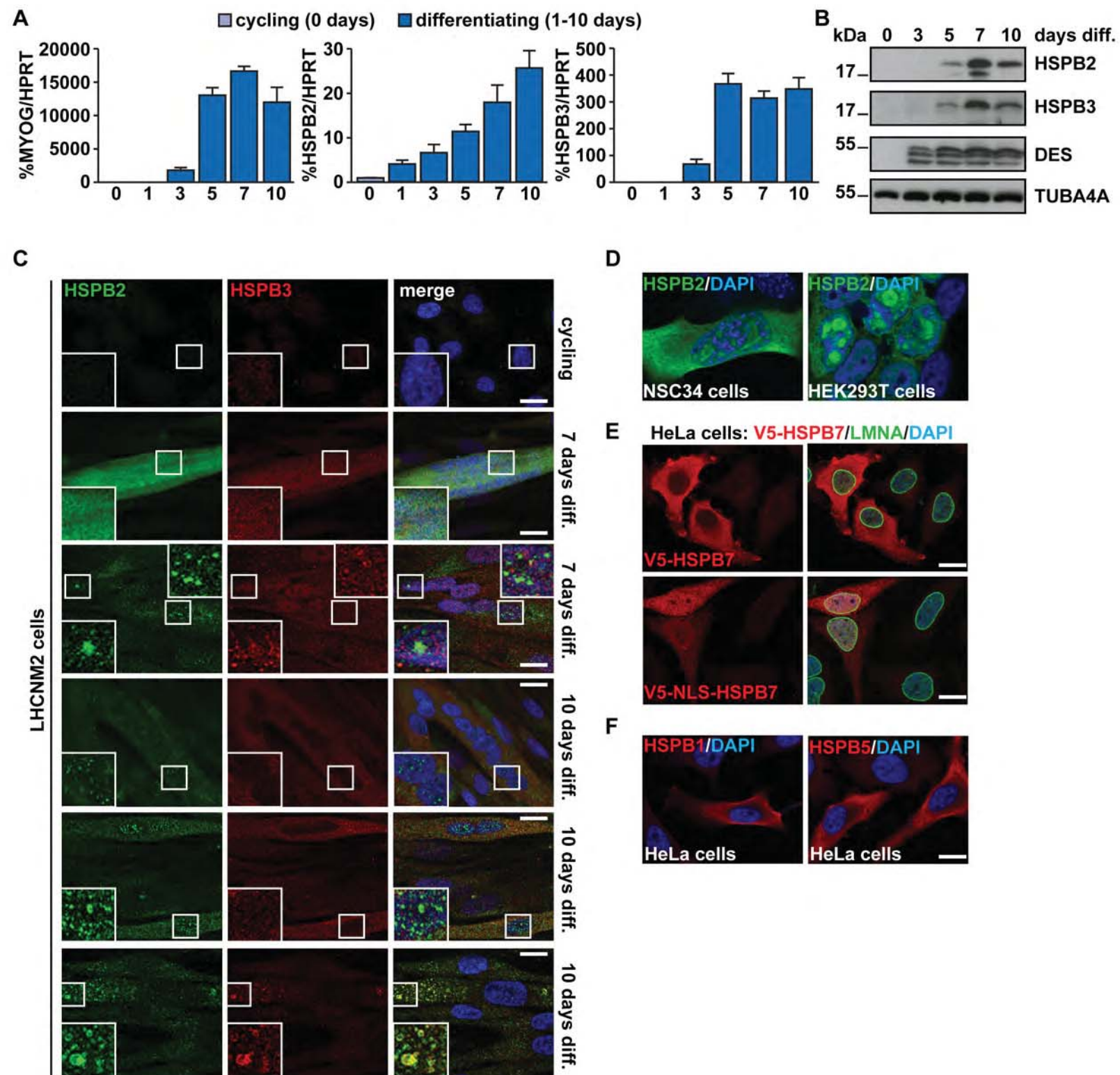


Figure S1: HSPB2 forms nuclear foci in differentiating myoblasts.

Related to Figure 1.

A: RT-qPCR showing the expression levels of myogenin, HSPB2 and HSPB3 in LHCNM2 cells differentiated for 0 days (cycling), 1, 3, 5, 7 or 10 days. HPRT was used as reference gene for qPCR normalisation. **B:** Immunoblot showing the expression levels of HSPB2, HSPB3 and desmin in LHCNM2 cells differentiated for the indicated days (0-10). TUBA4A was used as loading control. **C:** Immunofluorescence showing the subcellular distribution of HSPB2 and HSPB3 in LHCNM2 cells differentiated for 0 days (cycling), 7 or 10 days. **D:** Immunofluorescence showing the subcellular distribution of overexpressed HSPB2 in NSC34 and HEK293T cells. **E:** Immunofluorescence showing the subcellular distribution of overexpressed V5-tagged HSPB7 or NLS-HSPB7 in HeLa cells. Cells were stained for V5, LMNA and nucleic acid (DAPI). **F:** Immunofluorescence showing the subcellular distribution of overexpressed HSPB1 and HSPB5 in HeLa cells. Scale bars 15 μ m.

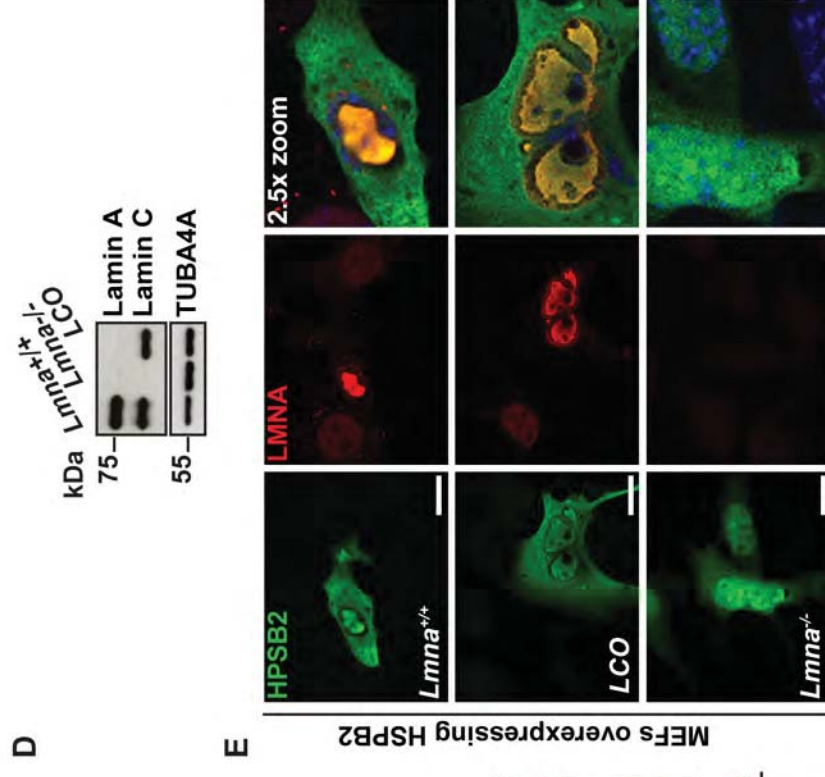
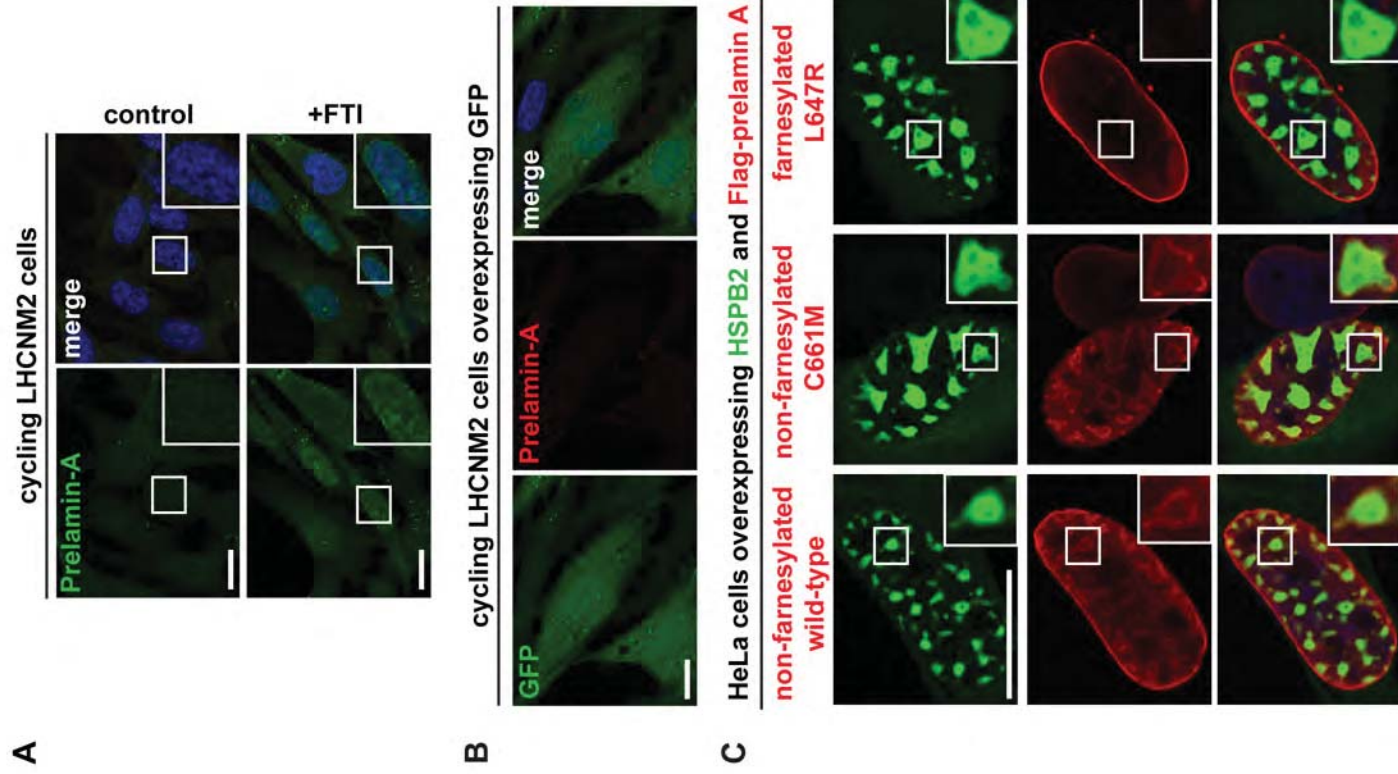


Figure S2: HSPB2 nuclear compartment sequester also immature non-farnesylated forms of prelamin-A. Related to Figure 1.

A: Microscopy on cycling LHCNM2 cells mock infected and untreated (control) or treated for 16 hrs with FTI (15 μ M). **B:** Microscopy on cycling LHCNM2 cells infected for 72 hrs with GFP lentiviral particles. **C:** Microscopy using anti-Flag on HeLa cells transfected for 48 hrs with Flag-prelamin-A, Flag-prelamin-A-C661M or Flag-prelamin-A-L647R together with GFP-HSPB2 and untagged HSPB2 (1 : 8). **D:** Immunoblot showing the expression levels of lamin A and C in MEFs from *Lma*^{+/+}, *LCO* (expressing only lamin-C) and *Lma*^{-/-} (lacking both lamin-A and lamin-C). TUBA4A was used as loading control. **E:** Immunofluorescence showing the subcellular distribution of HSPB2 overexpressed for 48 hrs in *Lma*^{+/+}, *LCO* and *Lma*^{-/-} MEFs. Cells were fixed and labeled with anti-HSPB2, anti-lamin-A/C and DAPI. Scale bars 10 μ m.

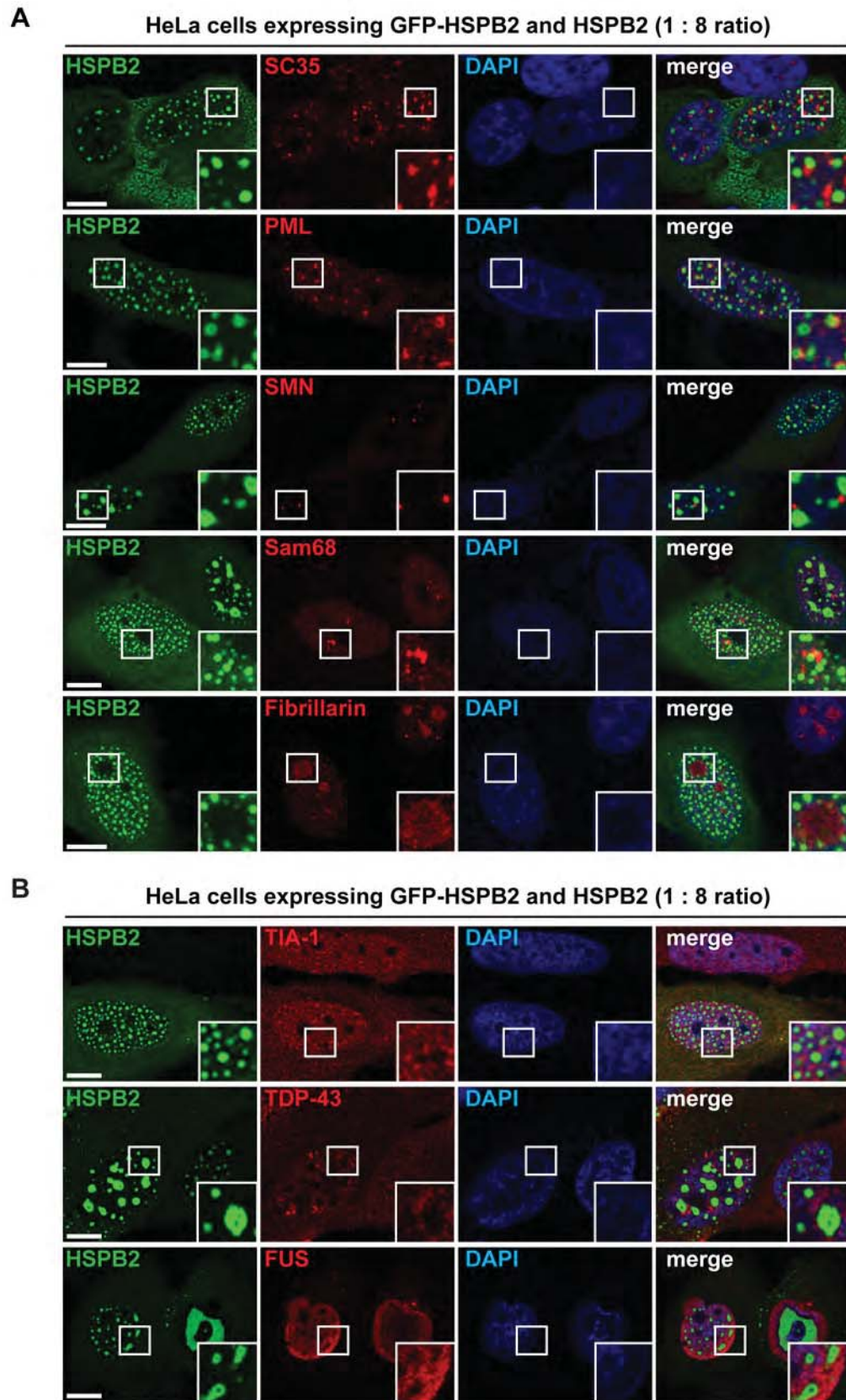


Figure S3: HSPB2 compartments do not colocalize with other known nuclear membrane-less organelles.

Related to Figures 1 and 2.

A: Microscopy on HeLa cells transfected for 48 hrs with GFP-HSPB2 and untagged HSPB2 (1 : 8) using antibodies specific for membrane-less nuclear body markers: speckles (anti-SC35), PML-nuclear bodies (anti-PML), Cajal bodies (anti-SMN), nucleoli (anti-fibrillarin) and Sam68 nuclear bodies (anti-Sam68). **B:** Microscopy on HeLa cells transfected for 48 hrs with GFP-HSPB2 and untagged HSPB2 (1 : 8) using antibodies specific for the RNA-binding proteins TIA-1, TDP-43 and FUS. Scale bars 5 μ m.

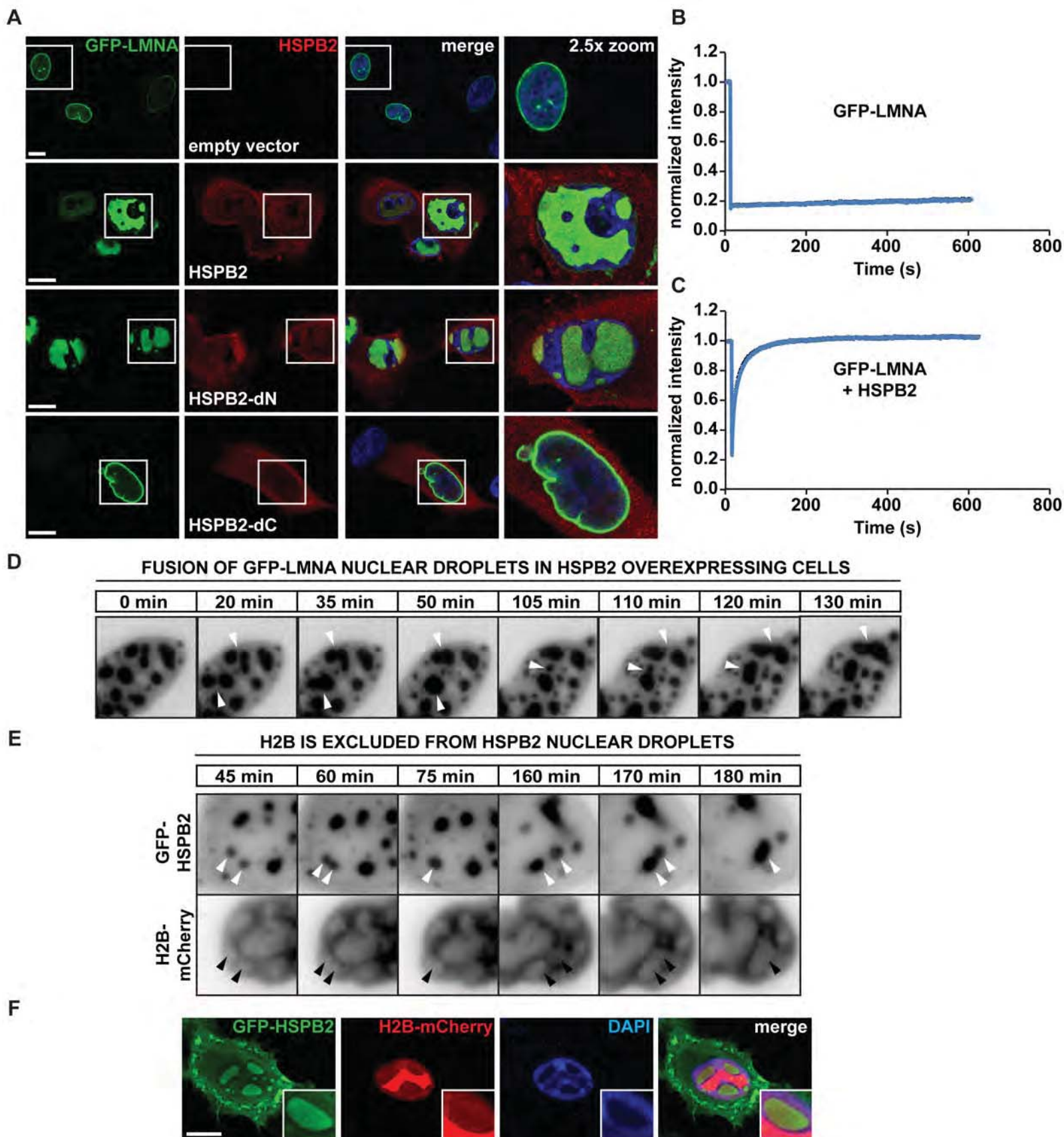


Figure S4: HSPB2 aberrant phase separation affects LMNA and H2B mobility and distribution.

Related to Figures 2, 3 and Movies S5-7.

A: Immunofluorescence on HeLa cells overexpressing for 48 hrs GFP-LMNA and an empty vector, HSPB2, HSPB2-dN or HSPB2-dC. **B:** Quantification of the fluorescence intensity recovery after bleach of GFP-LMNA expressed alone. The time scale of a bleach experiment is shown. $n = 39$, $\pm$ SEM. **C:** Quantification of the fluorescence intensity recovery after bleach of GFP-LMNA nuclear droplets in HeLa cells overexpressing GFP-LMNA with HSPB2. The time scale of a bleach experiment is shown. $n = 43$, $\pm$ SEM. **D:** Inverted black and white images of GFP-LMNA in HeLa cells overexpressing GFP-LMNA with HSPB2. Fusion events of GFP-LMNA nuclear droplets are indicated by arrowheads. **E:** Inverted black and white images of GFP-HSPB2 and H2B-mCherry in HeLa cells. Fusion events of GFP-HSPB2 nuclear droplets and exclusion of H2B-mCherry are indicated by arrowheads. **F:** Immunofluorescence on HeLa cells overexpressing for 48 hrs GFP-HSPB2 and H2B-mCherry and showing that GFP-HSPB2 displaces H2B-mCherry and DNA (DAPI). Scale bars 10 μ m.

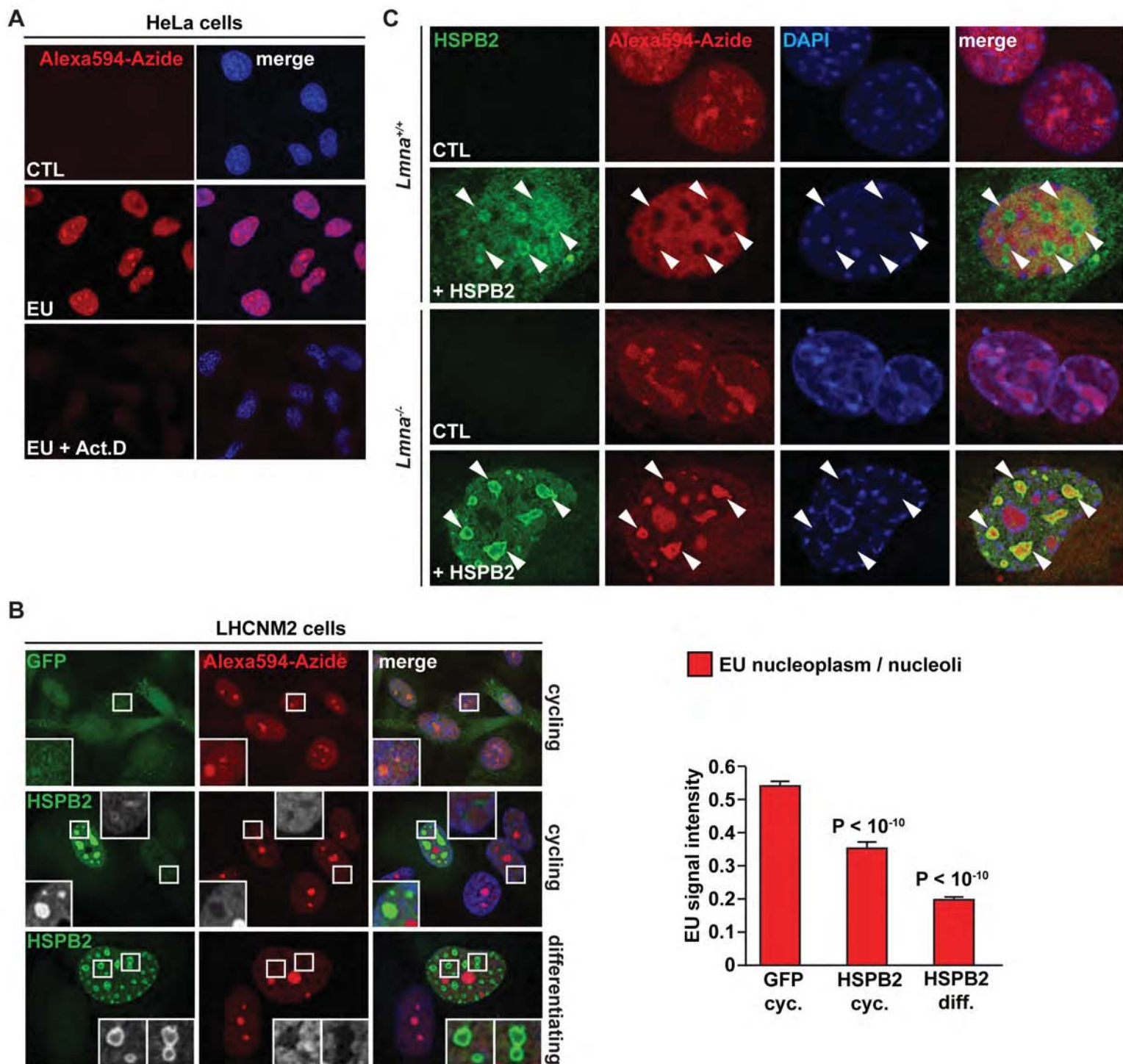


Figure S5: HSPB2 phase separation inhibits RNA transcription.
Related to Figure 3.

A: HeLa cells were left untreated (CTL) or incubated with 5-ethynyl uridine (EU; 200 μ M) alone or with actinomycin D (EU + Act.D; 2 μ M) for 6 hrs. Cells were then fixed and stained with Alexa594-Azide to visualize newly synthesized RNAs.

B: Cycling and differentiating LHCNM2 cells overexpressing GFP or HSPB2 were incubated with EU (200 μ M) for 6 hrs. Cells were fixed, stained with Alexa594-Azide and subsequently, where indicated, for HSPB2. The signal intensity of EU was quantified in the nucleoli and in the nucleoplasm and nucleoplasm/nucleoli ratio was calculated. Quantitation of EU signal intensity is reported. $n = 105-117$, $\pm$ SEM; $P < 10^{-10}$.

C: *Lmna*^{+/+} and *Lmna*^{-/-} (lacking both lamin-A and lamin-C) MEFs were transfected with an empty vector (CTL) or HSPB2. 48 hrs post-transfection, cells were incubated with 5-ethynyl uridine (EU; 200 μ M) for 6 hrs, fixed and stained with Alexa594-Azide, anti-HSPB2 and DAPI. Confocal microscopy shows that in *Lmna*^{+/+} MEFs HSPB2 nuclear compartments are all devoid of EU (257 HSPB2-overexpressing cells analyzed). Instead, in *Lmna*^{-/-} MEFs, HSPB2 nuclear compartments all colocalize with newly synthesized RNAs (196 HSPB2-overexpressing cells analyzed).

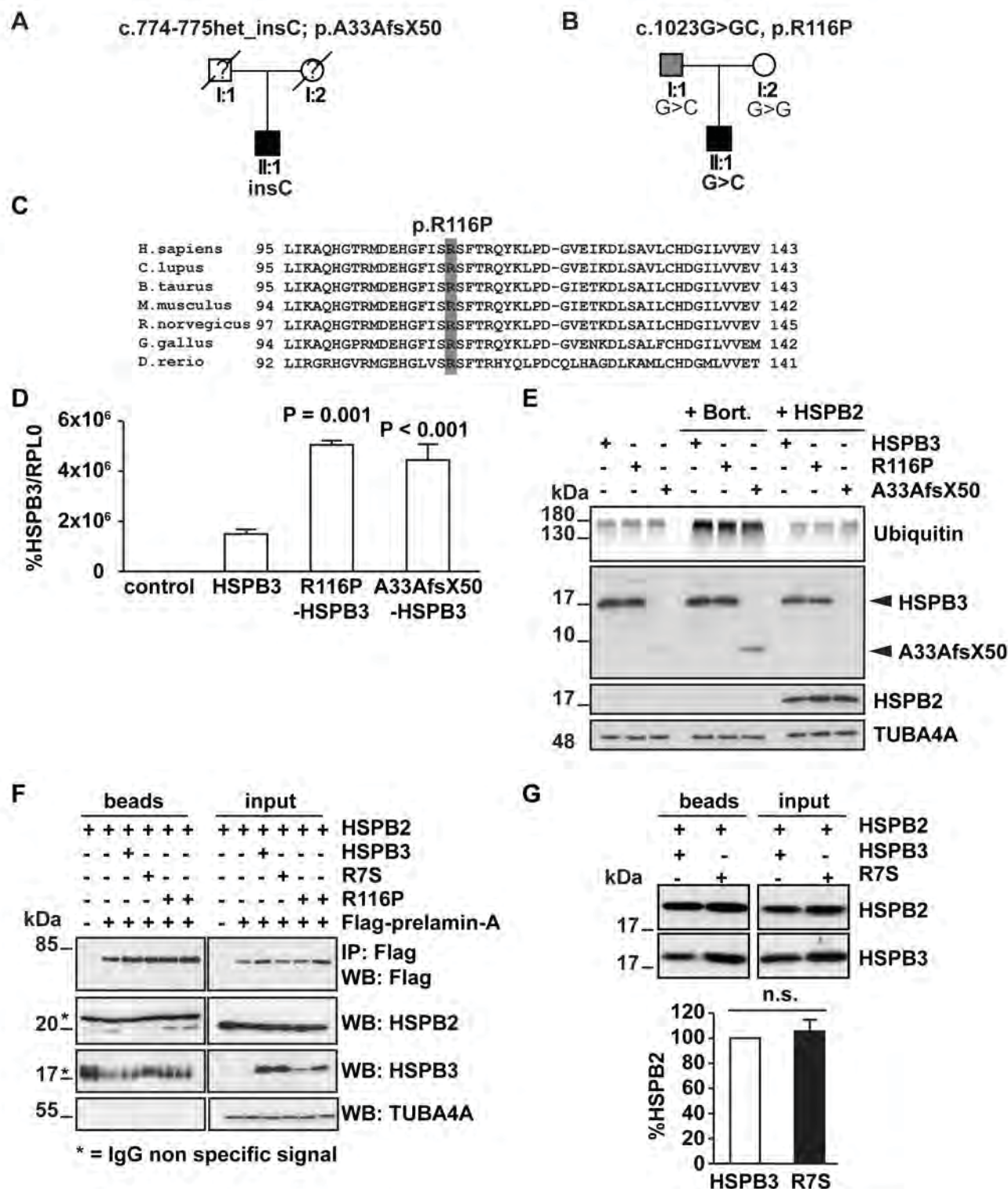


Figure S6: Sequencing analysis identified two putative mutations in HSPB3 in two patients affected by myopathy. Related to Figures 4, 5 and 6.

A, B: Black symbols = affected individuals; grey symbol = asymptomatic individual; white symbols = unaffected individuals, diagonal line = person diseased, and question marks = diagnosis unavailable. **C:** Comparison of the HSPB3 protein sequence in different species shows the conservation of the R116 residue. R116 is located in the α -crystallin domain of HSPB3. **D:** RT-qPCR analysis of myc-tagged HSPB3, HSPB3-R116P and HSPB3-A33AfsX50 mRNA expression levels after overexpression for 48 hrs in HeLa cells. $n = 3$, $\pm$ SEM. $P < 0.001$. **E:** Immunoblot showing the expression levels of myc-tagged HSPB3, HSPB3-R116P and HSPB3-A33AfsX50 in HeLa cells transfected for 48 hrs. Where indicated cells were co-transfected with HSPB2 (+ HSPB2) or treated with Bortezomib (+ Bort.; 100 nM) overnight prior to protein extraction. TUBA4A was used as loading control. **F:** HEK293T cells were transfected with cDNAs encoding for Flag-prelamin-A and an empty vector or HSPB2 alone or combined with myc-HSPB3, myc-R7S-HSPB3 or myc-R116P-HSPB3. Due to myc-R116P-HSPB3 high aggregation propensities and in order to reach in the supernatant fraction used for co-immunoprecipitation expression levels of myc-R116P-HSPB3 similar to the ones of myc-HSPB3 or myc-R7S-HSPB3, two increasing amounts of cDNAs encoding for myc-R116P-HSPB3 were transfected. 24 hrs post-transfection, cells were subjected to immunoprecipitation with a Flag antibody. * indicates IgG or nonspecific signal. **G:** HeLa cells were transfected with vectors encoding for his-HSPB2 and myc-HSPB3 or myc-R7S-HSPB3. 24 hrs post-transfection, cell lysates were subjected to NiNTA pull-down. Quantitation of data of five independent samples is shown. n.s.: non-significant. **F, G:** Total cell lysates (input) and immuno-

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture, Transfection and Treatments

HeLa, Lmna^{+/+}, Lmna^{-/-} (Sullivan et al., 1999), LCO (Fong et al., 2006) MEFs, NSC34 and HEK293T cells were cultured in DMEM (ECB7501L; EuroClone, Milan, Italy) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 10% Fetal Bovine Serum (F7524; Sigma-Aldrich, Milan, Italy) in a 37°C incubator with 5% CO₂. Lmna^{+/+}, Lmna^{-/-}, LCO MEFs were kindly provided by Dr. J. Lammerding (Cornell University). Cycling LHCNM2 cells were kindly provided by Prof. E. Pegoraro and were cultured in HAM's F12 (ECB7502L; EuroClone, Milan, Italy) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 20% FBS (Gibco 10106-169; Invitrogen, USA) and 25 ng/mL of rh FGF-b/FGF-2. Differentiated LHCNM2 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 2% horse serum (Gibco 26050-088; Life Technologies, USA) and 30 µg/mL of insulin. We thank Dr. E. Galletta, Dr. C. Stewart and Dr. S. Young for providing LHCNM2, Lmna^{-/-} and LCO cells, respectively. Stable HeLa-Kyoto cells expressing C-terminally mCherry-tagged human H2B were generated by random integration into the genome via BAC recombineering technology (Poser et al., 2008). H2B-mCherry cells were kept under selection using Puromycin (Sigma-Aldrich P8833, 0.5 µg/ml). Transfections were performed using the calcium phosphate method as previously described (Carra et al., 2005). Unless otherwise indicated, cells were transfected with cDNAs for 48 hrs.

Cells were treated with the following drugs at the concentrations indicated here: arsenite 0.5 mM; actinomycin D (ActD) 2 µM; bortezomib (Bort) 100 nM; 5-Ethynyl Uridine (EU) 200 µM.

Generation of cDNAs

The generation of the human HSPB plasmid library was described before (Vos et al., 2010). The sequence encoding for human non-tagged HSPB2, HSPB5 and HSPB3 wildtype, myc-HSPB1, myc-HSPB3, myc-R7S-HSPB3, myc-R116P-HSPB3, myc-R7S-HSPB3 and myc- A33AfsX50-HSPB3 were subcloned by PCR into the pCDNA5.1-FRT/TO.

FLAG-tagged plasmids containing wild-type prelamin A, the non-farnesylatable mutant LA-C661M and the farnesylated mutant LA-L647R were previously described (Mattioli et al., 2011).

Immunofluorescence on cultured cells

HeLa, Lmna^{+/+}, Lmna^{-/-}, LCO and HEK293T cells were grown on polylysine-coated glass coverslip, while NSC34 cells were grown on uncoated glass coverslip. Cycling LHCNM2 cells were grown on coverslips coated with gelatin (0.1%), while differentiating LHCNM2 cells were grown on plastic chamber slides. Cells were washed with cold PBS and fixed with 3.7% formaldehyde in PBS for 9 minutes at room temperature, followed by permeabilization with cold acetone for 5 minutes at -20°C. Alternatively, cells were fixed with ice-cold methanol for 10 minutes at -20°C. Blocking and incubation with primary and secondary antibodies were performed in PBS containing 3% BSA and 0.1% Triton X-100. The primary and secondary antibodies used are listed in the section of supplemental experimental procedures. Analyses of the cells were done by confocal imaging using a Leica SP2 AOBS system (Leica Microsystems) and a 63x oil-immersion lens.

Reagents and Antibodies

The reagents used in this study include: 5-Ethynyl Uridine (E10345, Life Technologies); actinomycin D (A1410, Sigma-Aldrich); bortezomib (S1013, Selleck Chemicals); DAPI (sc-3598, Santa Cruz Biotechnology); insulin (I1882, Sigma-Aldrich); polybrene (107689, Sigma-Aldrich); sodium arsenite (Carlo Erba Reagents, Cornaredo, Italy); rh FGF-b/FGF-2 (11343625, ImmunoToos); Farnesyltransferase inhibitor (BML-G242; Enzo Life Sciences).

The antibodies used in this study are: mouse monoclonal anti-HSPB2 (sc-136339, Santa Cruz Biotechnology); rabbit polyclonal anti-HSPB3 (SAB1100972, Sigma-Aldrich); rabbit polyclonal anti-ubiquitin (Z 0458, DakoCytomation); mouse monoclonal anti- α -tubulin (T6074, Sigma-Aldrich); goat polyclonal anti-lamin B (sc-6217, Santa Cruz Biotechnology); rabbit polyclonal anti-lamin A/C (sc-20681, Santa Cruz Biotechnology); goat polyclonal anti-lamin A (sc-6214, Santa Cruz Biotechnology); rabbit polyclonal anti-Desmin (sc-14026, Santa Cruz Biotechnology); rabbit polyclonal anti-Myogenin (sc-576, Santa Cruz Biotechnology); mouse monoclonal anti-V5 (R960-25, Life Technologies); mouse monoclonal anti-SC-35 (S4045, Sigma-Aldrich); mouse monoclonal anti-myc (9E10; sc-40, Santa Cruz Biotechnology; used for western blotting); mouse monoclonal anti-myc (9E10; kindly provided by Prof. R. Tanguay; used for western blotting and immunofluorescence); mouse monoclonal anti- α B Crystallin (SMC-159A, StressMarq Biosciences Inc); rabbit polyclonal anti-PML (sc-5621, Santa Cruz Biotechnology); mouse monoclonal anti-SMN (sc-365909, Santa Cruz Biotechnology); goat polyclonal anti-TIA-1 (sc-1751, Santa Cruz Biotechnology); mouse monoclonal anti-TDP-43 (60019-2-Ig, Proteintech); mouse monoclonal anti-FUS/TLS (sc-373698, Santa Cruz Biotechnology); mouse monoclonal

anti-Fibrillarin (sc-374022, Santa Cruz Biotechnology); rabbit polyclonal anti-Prelamin A (ANT0045, Diatheva); mouse monoclonal anti-SAM68 (sc-1238, Santa Cruz Biotechnology); mouse monoclonal anti-Flag (F1804, Sigma-Aldrich). Rabbit polyclonal anti-SUN2 was kindly provided by Prof. S. Marmioli; rabbit polyclonal anti-HSPB1 and anti-HSPB8 are homemade antibody kindly provided by Prof. J. Landry.

The Alexa-conjugated secondary antibodies used in this study were from Life Technologies and include: donkey-anti-goat-Alexa488, donkey-anti-goat-Alexa647, donkey-anti-mouse-Alexa594, donkey-anti-rabbit-Alexa594, donkey-anti-goat-Alexa594, donkey-anti-rabbit-Alexa488, donkey-anti-mouse-Alexa488, goat-anti-rabbit-Alexa488, goat-anti-rabbit-Alexa594, goat-anti-mouse-Alexa488, goat-anti-rabbit-Alexa594 or goat-anti-mouse-Alexa647. For click-chemistry on EU-treated cells we used Alexa fluor 594 Azide (A10270, Life Technologies). Mouse and rabbit HRP-conjugated secondary antibodies for western blot were from GE Healthcare Europe GmbH.

Preparation of samples for western blotting

For the preparation of total protein extracts, cells were lysed in Laemmli sample buffer and homogenized by sonication. Protein samples were boiled for 3 minutes at 100 °C, reduced with β -mercaptoethanol and separated by SDS-PAGE, followed by western blot.

5-Ethynyl Uridine (EU) Detection by Click Chemistry

The protocol for EU labelling was performed basically as described by Jao and Salic (2008). Briefly, 48 hrs post-transfection cells were incubated at 37°C with 5% CO₂ with 200 μ M of EU for 6 hrs. After EU labelling, cells were fixed (125 mM Pipes pH 6.8, 10 mM EGTA, 1 mM magnesium chloride, 0.2% Triton X-100, 3.7% formaldehyde) for 30 minutes at room temperature and processed for Alexa Fluor 594 Azide staining (100 mM Tris pH 8.5, 1 mM CuSO₄, 10 μ M Alexa Fluor 594 Azide, 100 mM ascorbic acid) for 30 minutes at room temperature and in dark condition. Cells were next processed for immunofluorescence microscopy as described above.

Viral vector production and lentiviral vectors

Lentiviral particles were generated by transient co-transfection using the calcium phosphate method of HEK293T cells with the 3rd generation packaging systems (pMDlg/pRRE #12251; pRSV-Rev #12253; pMD2.G #12259; Addgene) and lentiviral vectors encoding for Human HSPB2 (EX-Q0523-Lv105; Tebu-bio), Human myc-HSPB3 wt (EX-T1904-Lv107; Tebu-bio) or myc-HSPB3 R116P (CS-T1904-Lv107-01). For lentiviral particles expressing GFP the modified pLKO.1 GFP plasmid (Benatti et al., 2011) and the 2nd generation packaging systems (pCMV-dR8.74 #22036; pMD2.VSVG #12259; Addgene) were used. Shcontrol (RHS4346; Dharmacon) and shHSPB3 (VGH5518-200215240; Dharmacon) lentiviral particles were generated using GIPZTM Lentiviral shRNA (Dharmacon) according to the manufacturer's instructions. Cell medium was replaced 16 hrs after transfection. 32 hrs later, the medium containing the lentiviral particles was harvested, filtered by 0.45 μ m filter and stored at -80°C or ultracentrifuged at 19.000 rpm for 2 hrs and 30 minutes at 4°C on SW-28 rotor in a Beckman refrigerated centrifuge. Then the viral pellet was re-suspended with sterile DMEM and stored at -80°C.

In vitro transduction

Cycling LHCNM2 were transduced by adding viral supernatant to infection medium (HAM's F12, 2 mM L-glutamine, 10% heat-inactivated FBS, 25 ng/mL of rh FGF-b/FGF-2) supplemented with 8 μ g/mL polybrene. 24 hrs post-transduction, the medium was replaced with fresh cycling medium, and left for 48 hrs prior to fixation. For experiments under differentiation conditions, 24 hrs post-transduction, the medium was replaced with differentiation medium and left for ca. 120 hrs prior to fixation. For lentiviral particles expressing HSPB2 or HSPB3, the infection efficiency was calculated via immunofluorescence, while it was calculated both via immunofluorescence and FACS for GFP expressing lentiviral particles. For western blot analysis, 24 hrs post-transduction, LHCNM2 cells infected with lentiviral particles expressing HSPB2 or HSPB3 were selected with puromycin (4 μ g/mL). 48 hrs after, cells were lysed in Laemmli buffer.

Immunoprecipitation assay and Ni-NTA purification assay

24 hrs post-transfection cells were lysed in lysis buffer (150 mM NaCl, 0.5% NP40, 1.5 mM MgCl₂, 20 mM Tris-HCl pH 7.4, 3% glycerol, 1 mM DTT, complete EDTA-free, Roche). The cell lysates were centrifuged and cleared with A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C for 1 h. Mouse TrueBlot beads (88-7788-31; tebu-bio) at 4°C for 1 h. Mouse TrueBlot beads (88-7788-31; Tebu-bio) complexed with specific antibodies were added to the precleared lysates. After incubation for 1 h at 4 °C, the immune complexes were centrifuged. Beads were washed four times with the lysis buffer; both co-immunoprecipitated proteins and input fractions were resolved on SDS-PAGE.

For the purification of His-tagged HSPB2 with Ni-NTA, 24h post-transfection cells were lysed in lysis buffer (150 mM NaCl, 50 mM NaH₂PO₄, 10 mM imidazole, 0.5% NP40, 1.5 mM MgCl₂, 3% glycerol, 0.9 mM DTT and protease inhibitors). Lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C. An aliquot of the supernatant was kept as input fraction. Ni-NTA agarose beads (Qiagen) were added to the rest of the supernatant and incubated at 4°C for 1 h with slow agitation. The beads were washed once with lysis buffer and four times with washing buffer (300 mM NaCl, 50 mM NaH₂PO₄, 20 mM imidazole, 0.5% NP40, 1.5 mM MgCl₂, 3% glycerol).

Proteins bound to the beads were recovered by boiling in 2% SDS laemmli buffer supplemented with β -mercaptoethanol and processed for SDS-PAGE followed by western blot.

Fractionation of cytoplasmic and nuclear proteins

For the fractionation of cytoplasmic and nuclear proteins, cells were harvested and homogenized in a buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH 8, DTT 1 mM, NP40 0.15% and 1% complete EDTA-free (Roche). Cell lysates were centrifuged at 12,000 rpm for 30 seconds at 4°C, to separate the supernatant (cytosolic fraction) from the pellet. The pellet fraction was lysed in buffer containing 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 1 mM DTT, 0.5% NP40 and 1% complete EDTA-free (Roche), sonicated and centrifuged. The supernatant (nuclear fraction) was transferred into a new tube. Laemmli buffer (to a final 2% SDS concentration) was added to the cytosolic and nuclear fractions prior to SDS-PAGE followed by western blot.

RNA extraction, RT-PCR and Real-Time PCR

Total RNA was isolated using Trizol reagent (15596-026; Life Technologies) and subsequently treated with DNase I according to the manufacturer's instructions. First-strand cDNA was generated using High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems) according to the manufacturer's instructions. The relative changes in the levels of mRNAs for human HSPB2, HSPB3, myogenin and hypoxanthine guanine phosphoribosyltransferase (HPRT), used as housekeeping gene, were determined using CFX96 Touch Thermal cycler (Bio-Rad, Hercules, CA, USA) in combination with SYBR green master mix. The primers used were all purchased from Sigma-Aldrich and are listed below: HSPB2 For (CATGGTCCACAATGTATGGT); HSPB2 Rev (ATTTGGGTTTATTCAGCTCCAC); HSPB3 For (GACTAAGTGACATCGTATCGG); HSPB3 Rev (ACAAACATTCTCGTAGTACCAG); myogenin For (CACTCCCTCACCTCCATCGT); myogenin Rev (CATCTGGGAAGGCCACAGA); HPRT For (CGTCGTGATTAGCGATGATGA); HPRT Rev (TCCAAATCCTCGGCATAATGA). The real-time PCR was performed as follows: one cycle of denaturation (95°C for 3 minutes) and 40 cycles of amplification (95°C for 10 seconds, 60°C for 30 seconds). A triplicate of each sample was analyzed. Data were analyzed with Bio-rad CFX Manager 3.1 (Windows 7.0).

Electron Microscopy (EM)

Cell pellets were washed with PBS and fixed with 2.5 M glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, for 1 h at room temperature. After post-fixation with 1% osmium tetroxide (OsO₄) in cacodylate buffer 0.1 M for 1h at 4°C, the pellets were dehydrated in an ethanol series, infiltrated with propylene oxide and embedded in Epon resin. Ultrathin sections (60 nm thick) were stained with uranyl acetate and lead citrate and were observed with a JEOL JEM 1011 transmission electron microscope, operated at 100kV. At least 200 cells were examined for each sample.

Live imaging

For live-imaging, every 3 min for GFP-HSPB2 and every 5 min for GFP-LMNA and H2B-mCherry, 6 sections with 800-nm spacing were acquired and the maximum intensity projections were created in Fiji (www.fiji.sc).

Fluorescence recovery after photobleaching (FRAP) and fluorescence density analysis

Using a 100x oil immersion objective a region of approximately 2.09 x 2.09 μm^2 was bleached for 50 ms using a laser intensity of 20% at 405 nm (2.3 mW for GFP). Recovery was recorded for 510-600 time points after bleaching (600-1020 s). Analysis of the recovery curves were carried out with the FIJI/ImageJ.

The flow of the protein within the droplet was measured by quantifying the recovery of the bleached area at the cost of the unbleached region by a custom written FIJI/ImageJ routine. During image acquisition, the bleached region was corrected for general bleaching. We quantified the molecules that move from the unbleached region to the bleached region, leading to the recovery of the bleached region.

Prior to FRAP analysis the images were corrected for drift using the StackReg plug-in function of the FIJI software suite. FRAP analysis was carried out using the following equation: $((I_{\text{bleach}} - I_{\text{background}}) / (I_{\text{bleach}(t_0)} - I_{\text{background}(t_0)})) / ((I_{\text{total}} - I_{\text{background}}) / (I_{\text{total}(t_0)} - I_{\text{background}(t_0)}))$, where I_{bleach} is the fluorescence intensity in the bleach area, $I_{\text{background}}$ the background the camera offset and I_{total} is the fluorescence intensity of the entire cellular structure. Mean and standard deviation were obtained from averaging FRAP curves.

Analysis of fluorescent density was performed using FIJI/ImageJ and selecting specific ROI (region of interest). Briefly, for the analysis of HSPB2 critical threshold, HSPB2 fluorescence intensity in the ROI (nucleoplasm or nuclear droplets) was divided for the HSPB2 fluorescence density in the cytoplasm, selecting a region where no HSPB2 phase separation occurred. For the analysis of EU incorporation, Alexa594-Azide fluorescence intensity in the ROI (nucleoplasm) was divided for the Alexa594-Azide fluorescence intensity in the nucleoli.

Sequencing of *HSPB2* and *HSPB3*

The genomic DNA of all participating subjects in this study was used to amplify the *HSPB2* and *HSPB3* coding regions (GenBank accession numbers: NM 001541 and 006308) by PCR. The coding exons were amplified by a standard PCR using the following primers for *HSPB3*: HSPB3-For 3' GACTGAAGGCAGTGGAAGGT '5 and HSPB3-Rev 3'TGTGGGTAATACAAACATTCTCG and for *HSPB2*: HSPB2_FOR: GGCCTATTGTTGGGTGGTGT; HSPB2_REV: AGGGGATGAGGGGTAGTCTG; HSPB2_IFOR: CTGTGCCAGCCTCATCCT; HSPB2_1REV: GATGCTGCTACCTCTGGAGTG. The resulting amplicons, including the intron-exon boundaries, were screened for mutations via Sanger sequencing on an ABI 3700 (Applied Biosystems). The DNA sequences were analyzed using Mutation Surveyor software (Softgenetics).

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Chromosomal instability drives metastasis through a cytosolic DNA response

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Chromosomal instability is a hallmark of cancer that results from ongoing errors in chromosome segregation during mitosis. Although chromosomal instability is a major driver of tumour evolution, its role in metastasis has not been established. Here we show that chromosomal instability promotes metastasis by sustaining a tumour cell–autonomous response to cytosolic DNA. Errors in chromosome segregation create a preponderance of micronuclei whose rupture spills genomic DNA into the cytosol. This leads to the activation of the cGAS–STING (cyclic GMP–AMP synthase–stimulator of interferon genes) cytosolic DNA–sensing pathway and downstream noncanonical NF- κ B signalling. Genetic suppression of chromosomal instability markedly delays metastasis even in highly aneuploid tumour models, whereas continuous chromosome segregation errors promote cellular invasion and metastasis in a STING-dependent manner. By subverting lethal epithelial responses to cytosolic DNA, chromosomally unstable tumour cells co-opt chronic activation of innate immune pathways to spread to distant organs.

Chromosomal instability (CIN) correlates with tumour metastasis^{1,2}, but it remains unclear whether it is a mere bystander or a driver of metastatic progression. Chromosomally unstable cells show evidence of chromosome missegregation during anaphase^{3,4}, offering an attractive bottleneck in which to target CIN and probe its selective contribution to metastasis. Destabilization of microtubule attachments to chromosomes at the kinetochores, through overexpression of the non-motile microtubule-depolymerizing kinesin-13 family proteins KIF2B or KIF2C (also known as MCAK), directly suppresses CIN in otherwise chromosomally unstable cells^{5–7}. Cells overexpressing KIF2B or MCAK continue to propagate abnormal aneuploid karyotypes, albeit in a stable manner⁷. As such, this approach permits direct experimental interrogation of CIN, as defined by the rate of ongoing chromosome missegregation, independently of aneuploidy, which is defined as a state of abnormal chromosome numbers.

Increased CIN in human metastases

First, to determine whether CIN is associated with human metastases, we applied the weighted-genomic integrity index (wGII) as a proxy for CIN⁸ to 79 matched pairs of primary tumour and brain metastasis from a published cohort⁹. Metastases showed higher wGII than primary tumours (Fig. 1a and Extended Data Fig. 1a, b).

Next, karyotype analysis of primary breast tumours and metastases archived in the Mitelman Database of chromosomal translocations¹⁰ revealed a predilection for near-diploid ($2n$) karyotypes in primary tumours. Conversely, metastases were enriched for cells with near-triploid ($3n$) karyotypes and had twice as many structural or numerical

chromosomal aberrations per clone as primary tumours. The number of chromosomal aberrations was highest in tumour samples with karyotypes ranging between the diploid and tetraploid ($4n$) range (Fig. 1b, c and Extended Data Fig. 1c, d).

Finally, histological analysis of primary tumours from patients with locally advanced head and neck squamous cell carcinoma¹¹ revealed a significant association ($P < 0.05$) between anaphase chromosome missegregation and the incidence of lymph node metastasis (Fig. 1d and Extended Data Fig. 1e).

CIN is a driver of metastasis

To determine whether CIN is causally involved in metastasis, we used transplantable metastatic tumour models of human (MDA-MB-231) or mouse (4T1) triple-negative breast cancer and human lung adenocarcinoma (H2030), in which 47%, 55%, and 67% of anaphase cells, respectively, show evidence of chromosome missegregation. Overexpression of KIF2B or MCAK suppressed chromosome missegregation, whereas overexpression of a dominant-negative MCAK mutant¹² (dnMCAK) led to a modest increase in chromosome missegregation in MDA-MB-231 cells. Overexpression of KIF2B or MCAK did not alter cellular proliferation or the number of centrosomes per cell (Fig. 2a, b and Extended Data Fig. 1f–i). As a control, we overexpressed KIF2A, a third member of the kinesin-13 family that lacks kinetochore and centromere localization domains¹³; although KIF2A showed microtubule-depolymerizing activity on interphase microtubules, it had no observable effect on CIN (Fig. 2b and Extended Data Fig. 1i–k). We ruled out a direct role for kinesin-13-mediated microtubule depolymerization in

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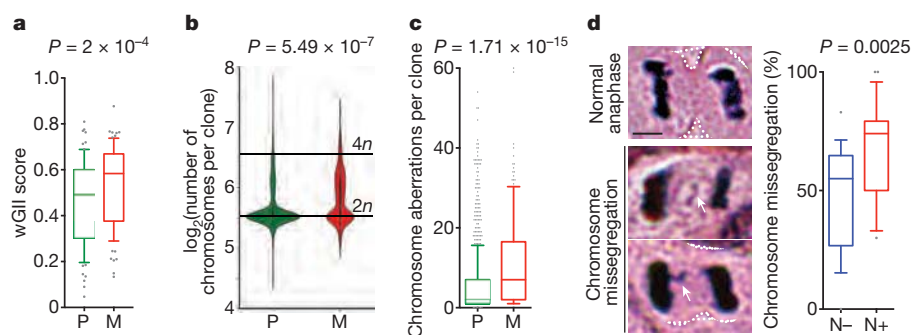


Figure 1 | Human metastases enrich for CIN. **a**, wGII of matched primary tumours (P) and brain metastases (M), $n = 79$ patients. **b**, **c**, Karyotype probability density (**b**) and chromosomal aberrations (**c**) in 983 primary tumour and 186 metastatic breast cancer clones. **d**, Left, images of head and neck squamous cell carcinoma cells undergoing anaphase. Arrows point to chromosome missegregation; scale bar, 5 μm .

Right, chromosome missegregation in tumours from patients with (N+, $n = 22$ patients) or without (N-, $n = 18$ patients) clinically detectable lymph node metastases. Boxes represent median $\pm$ interquartile range, confidence intervals (whiskers) denote 10th–90th percentiles (**a**, **c**, **d**), significance tested using two-sided Wilcoxon matched-pairs signed rank test (**a**) and two-sided Mann–Whitney test (**b**–**d**).

activating small GTPases¹⁴ by performing RhoA and Rac1 pull-down assays, which revealed low basal levels of activity and no correlation with overexpression of kinesin-13 family proteins (Extended Data Fig. 2a, b). Hereafter, we refer to cells expressing MCAK or KIF2B as CIN-low and to control cells or those expressing KIF2A or dnMCAK as CIN-high.

Karyotyping of the parental MDA-MB-231 cells revealed a widely aneuploid (approximately $3n$) chromosome content with widespread karyotypic heterogeneity (Extended Data Fig. 2c). Suppression of CIN reduced both numerical and structural karyotypic heterogeneity in single-cell-derived clones, as supported by the presence of fewer chromosomes exhibiting non-clonal structural abnormalities and decreased numerical chromosome heterogeneity in CIN-low cells (Extended Data Fig. 2c–h). Notably, CIN-low cells maintained highly aneuploid karyotypes, but faithfully propagated them in a stable manner. Thus, by comparing chromosomally stable aneuploid cells to their chromosomally unstable aneuploid counterparts, we can experimentally examine the role of CIN, independent of aneuploidy, in metastasis.

We injected MDA-MB-231 cells into the left cardiac ventricles of athymic mice to enable systemic dissemination while tracking metastatic colonization using a bioluminescence reporter. Differences in chromosome missegregation rates had a marked effect on colonization: mice harbouring CIN-high cells rapidly succumbed to metastatic disease, with a median survival of 70 days, whereas mice injected with CIN-low cells had a lower metastatic burden and a median survival of 207 days. Many metastases from CIN-low cells waxed and waned and, at times, spontaneously resolved, whereas metastases from CIN-high

cells involved multiple organs and progressed rapidly, leading to death. Similar results were obtained after injection of lung adenocarcinoma H2030 cells (Fig. 2c–e and Extended Data Fig. 3a–c). Overexpression of the spindle assembly checkpoint protein MAD2 in MCAK-expressing cells partially rescued chromosome missegregation¹⁵ and correspondingly augmented metastasis (Fig. 2c and Extended Data Fig. 3f).

We performed orthotopic injections of MDA-MB-231 or 4T1 cells into the mammary fat pads of athymic or immune-competent BALB/c mice, respectively, followed by surgical excision of the primary tumour. Suppression of CIN had no effect on the efficiency of primary tumour implantation, and even enhanced primary tumour growth in the 4T1 model. However, in both models, suppression of CIN significantly reduced spontaneous metastasis and prolonged survival (Extended Data Fig. 3d, e).

We then assessed chromosome missegregation in the injected cells and in cells derived from primary tumours and metastatic colonies (Fig. 3a). We performed this analysis using MDA-MB-231 cells and two metastasis-competent xenografts (PDX) derived from patients with oestrogen receptor-positive (ER+) and triple-negative breast cancer (TNBC). Regardless of the CIN status of the injected cells, the majority of metastases were enriched for higher rates of chromosome missegregation, whereas cells derived from most primary tumours had significantly lower rates of CIN (Fig. 3b–d). For instance, when CIN-high cells (Fig. 3d, dnMCAK, blue bars) were injected into the mammary fat pad, chromosome missegregation rates decreased in the primary tumours (green bars) before increasing once more in metastases spontaneously arising within the same animal (orange bars).

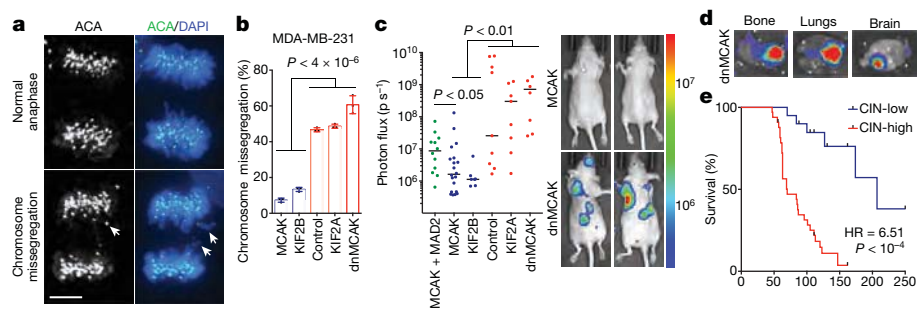


Figure 2 | CIN is a driver of metastasis. **a**, Anaphase cells stained for centromeres (ACA) and DNA (DAPI); scale bar, 5 μm . **b**, Chromosome missegregation in MDA-MB-231 cells expressing kinesin-13 proteins. Bars represent mean $\pm$ s.d., $n = 150$ cells. **c**, Whole animal bioluminescence (BLI) seven weeks after intracardiac injection of MDA-MB-231 cells. Left, bars represent the median and data points represent individual mice; $n = 12$ (MCAK + MAD2), 20 (MCAK), 7 (KIF2B), 9 (control), 9 (KIF2A), 8 (dnMCAK) mice. Right, representative images; colour scale shows

photon flux. **d**, Ex vivo BLI of organs with metastases from MDA-MB-231 cells expressing dnMCAK; colour scale as in **c**. **e**, Disease-specific survival of mice injected with CIN-high ($n = 33$) or CIN-low ($n = 20$) MDA-MB-231 cells. Significance tested using two-sided t -test (**b**), two-sided Mann–Whitney test (**c**), and two-sided log-rank test (**e**); $n = 3$ (**a**, **b**) and 5 (**d**) independent experiments. Throughout the paper, pairwise comparisons between individual CIN-low and CIN-high conditions are smaller than the stated P value. HR, hazard ratio.

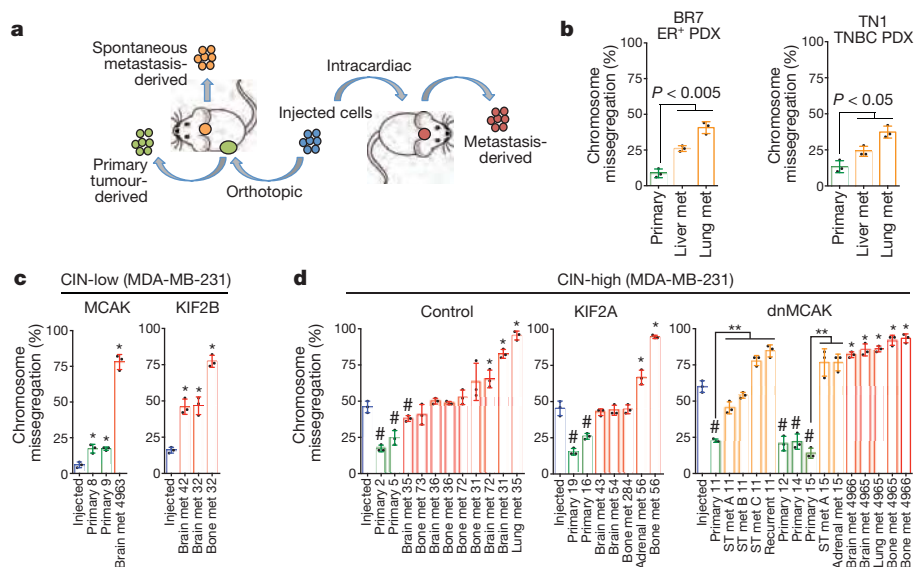


Figure 3 | Opposing roles for CIN in primary tumours and metastases. **a**, Experimental schema. **b–d**, Chromosome missegregation in injected cells (blue), cells derived from primary tumours (green), spontaneous metastases (met) (orange) or metastases arising after intracardiac injection (red). ST, soft tissue. Data shown as mean $\pm$ s.d., $n = 150$ cells, three independent experiments; * $P < 0.05$, # $P < 0.05$, ** $P < 0.05$ denote samples with higher or lower chromosome missegregation than the injected lines, and spontaneous metastases with higher missegregation than matched primary tumours, respectively. Significance tested using two-sided t -test (**b–d**).

CIN enriches for mesenchymal traits

Bulk RNA sequencing (RNA-seq) identified 1,584 genes that were differentially expressed between CIN-low and CIN-high MDA-MB-231 cells. Principal component analysis and unsupervised clustering accurately separated samples according to their CIN status. Metastasis-related and epithelial-to-mesenchymal transition (EMT) gene sets were relatively enriched in CIN-high cells. The top 23 differentially expressed genes in CIN-high cells (referred to as CIN signature) predicted distant metastasis-free survival (DMFS) in a meta-analysis¹⁶ as well as a validation cohort¹⁷ of patients with breast cancer, irrespective of tumour subtype, grade, or lymph node status (Extended Data Figs 4, 5).

RNA-seq of primary tumour-derived and metastasis-derived cells revealed pathways that were shared among metastases and CIN-high cells. However, metastases contained a large number of differentially upregulated EMT and inflammation-related genes that were disproportionately clustered on chromosome 1, signifying chromosome 1-specific selection. Karyotype analysis revealed that the injected cell lines and most metastases had three copies of chromosome 1, whereas primary tumours consistently had two copies. Thus chromosome 1 loss is a recurrent event during primary tumour growth in this model (Extended Data Figs 4c–f, 5a–e).

We then performed single-cell RNA-seq (scRNA-seq) on three MDA-MB-231 cell lines—two CIN-low (KIF2B and MCAK) and one CIN-high (dnMCAK)—comprising a total of 6,821 cells. Clustering of single cells using EMT genes successfully classified most cells according to their CIN-status and revealed a fraction of cells (primarily CIN-high)

that was highly enriched in mesenchymal markers (Fig. 4a). Unsupervised graph-based clustering, based on all expressed genes, identified 12 phenotypically distinct subpopulations. One subpopulation was defined by increased expression of genes involved in EMT and metastasis (referred to as subpopulation ‘M’) and was concomitantly enriched for CIN signature genes. This subpopulation comprised 45% of dnMCAK expressing cells compared to 6% of CIN-low cells (Fig. 4b and Extended Data Fig. 6a, b).

In agreement with the scRNA-seq data, CIN-high cells exhibited increased migratory and invasive behaviour *in vitro*, and displayed evidence of actin cytoskeletal reorganization, diffuse vimentin staining, and increased cytoplasmic and nuclear localization of β -catenin (Extended Data Figs 6c, d, 7a–d). As expected, MAD2 overexpression rescued invasion and migration of MCAK-expressing cells. Furthermore, the ability of KIF2B or MCAK overexpression to suppress invasion *in vitro* was dependent on the cell cycle, as the addition of thymidine after transient transfection of either protein abrogated this phenotype (Extended Data Figs 6f, 7e, f and Supplementary Fig. 2).

CIN generates cytosolic DNA

To better define CIN-responsive pathways, we performed a gene–gene Pearson correlation analysis using scRNA-seq data and identified two large gene modules: module 1 was characterized by proliferative and metabolic pathways, whereas module 2 comprised EMT and inflammation gene sets (Fig. 5a). There was a strong positive correlation between inflammation-related, CIN signature, and EMT genes in the scRNA-seq and bulk RNA-seq data (Figs 4b, 5b and Extended Data Fig. 4b, c).

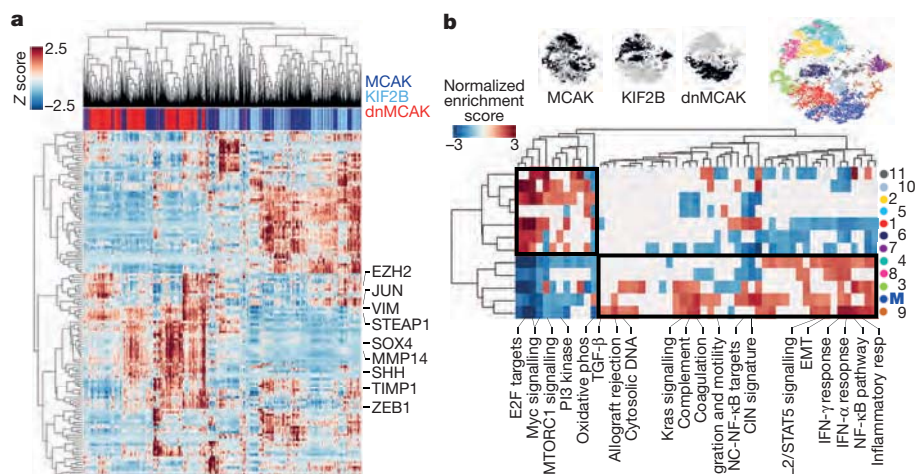


Figure 4 | CIN enriches for mesenchymal cell traits. **a**, Heat map showing expression of EMT genes in 6,821 MDA-MB-231 cells expressing MCAK, KIF2B, and dnMCAK. **b**, Top, t-stochastic neighbour embedding (tSNE) projection of all cells in **a**. Bottom, heat map of normalized enrichment score (NES) for pathways with an FDR $q < 0.05$ based on gene set enrichment analysis using one-sided weighted Smirnov–Kolmogorov test on subpopulations identified using Phenograph. Boxes outline populations with mutually exclusive transcriptional profiles.

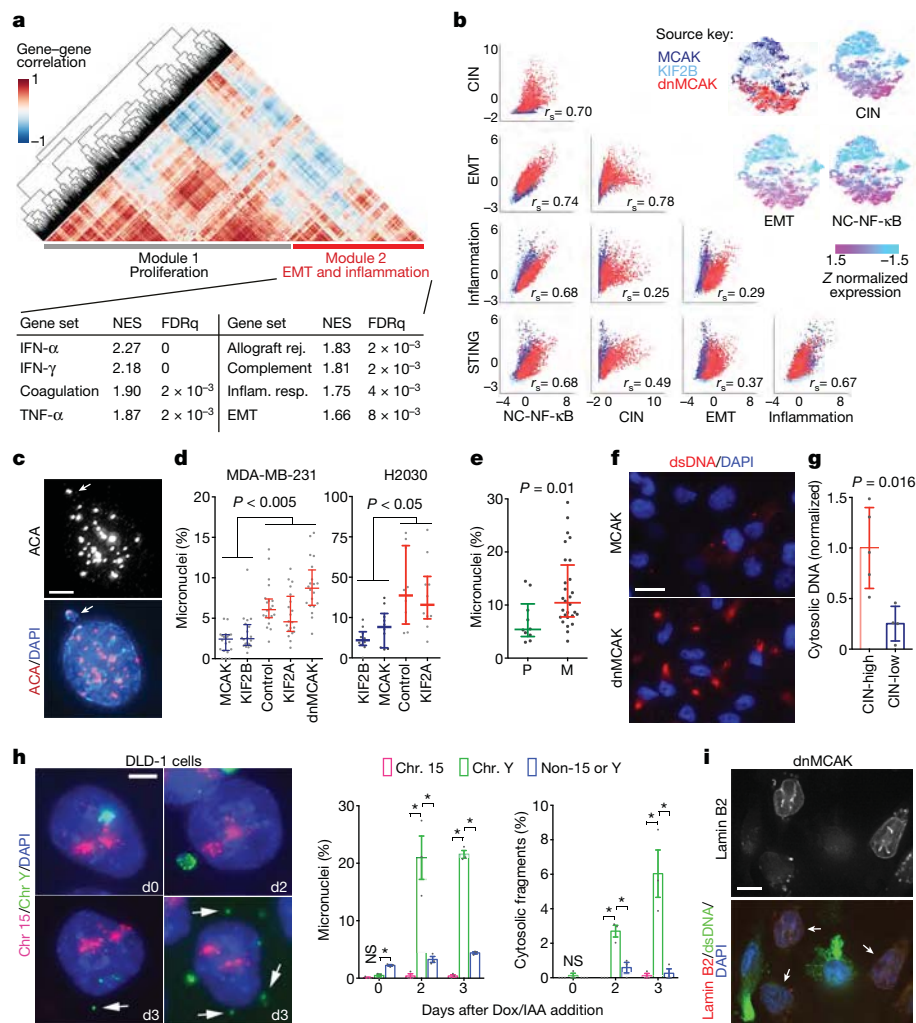


Figure 5 | CIN generates cytosolic DNA.

a, Heat map showing gene-gene correlations in 6,821 cells and HALLMARK gene sets significantly enriched in module 2, one-sided weighted Smirnov-Kolmogorov test. **b**, Top right, normalized expression of key gene signatures in 6,821 MDA-MB-231 cells expressing MCAK, KIF2B, and dnMCAK. Bottom left, correlation plots for key gene signatures. **c**, A primary nucleus and a micronucleus stained for centromeres and DNA; scale bar 5 μ m. **d, e**, Percentage of micronuclei cells expressing various kinesin-13 proteins (**d**) or in cells derived from 10 primary tumours and 28 metastases (**e**). $n = 20$ high-power fields per sample (**d**) or average values derived from 10 high-power fields per sample (**e**). **f**, MDA-MB-231 cells expressing MCAK and dnMCAK stained using DAPI and anti-dsDNA antibody; scale bar, 20 μ m. **g**, Cytosolic-to-nuclear DNA ratios in CIN-high ($n = 5$) and CIN-low ($n = 4$) MDA-MB-231 ($n = 5$) and H2030 ($n = 4$) cells. **h**, Left, DLD-1 cells stained for DNA and hybridized to chromosome-specific FISH probes; scale bar, 5 μ m. Right, percentage of cells containing micronuclei or small cytosolic DNA fragments ($n > 500$ cells per condition). **i**, MDA-MB-231 cells stained using DAPI, anti-dsDNA antibody, or mCherry-lamin B2 (arrow); scale bar, 10 μ m. Data shown as median $\pm$ interquartile range (**d, e**), mean $\pm$ s.d. (**g**), mean $\pm$ s.e.m. (**h**). $n = 6$ (**c, d**), 3 (**e, f, h**), 1 (**g**) and 2 (**i**) independent experiments. Significance tested using two-sided Mann-Whitney test (**d, e**) or two-sided t -test (**g, h**); $*P < 0.05$.

The induction of inflammatory pathways in response to CIN was unexpected and was reminiscent of a viral infection. We investigated whether CIN might introduce genomic DNA into the cytosol, thereby eliciting cellular responses normally reserved for anti-viral immunity^{18,19}. The exposure of genomic DNA to the cytosol can result from either primary nuclear or micronuclear envelope ruptures^{20–24}. We performed live-cell imaging using a GFP reporter with a nuclear localization signal (NLS-GFP)²⁵ and found no correlation between CIN and the frequency of NLS-GFP leakage into the cytosol in unconfined conditions. There was even a trend towards more efficient primary nucleus repair in CIN-high cells. CIN-high nuclei ruptured more frequently only during confined migration, and this was primarily attributed to their increased ability to go through a larger number of small constrictions (Extended Data Fig. 7g–j) that mimic confined migration during metastasis²⁵.

Instead, CIN-high cells and those derived from metastases exhibited a higher preponderance of micronuclei than did CIN-low or primary tumour-derived cells, respectively (Fig. 5c–e and Extended Data Fig. 8a–c). To test whether the presence of rupture-prone²⁰ micronuclei correlated with increased cytosolic DNA, we stained cells using two different anti-dsDNA antibodies after selective plasma membrane permeabilization and found increased cytosolic dsDNA and single-stranded DNA (ssDNA) in CIN-high cells. The dsDNA signal, which was distinct from mitochondrial staining, disappeared after treatment with double-strand-specific—but not single-strand-specific—nuclease and after overexpression of DNASE2, confirming the specificity of these antibodies (Fig. 5f and Extended Data Fig. 8d–h). Quantification of dsDNA levels after subcellular fractionation revealed a fourfold reduction in

cytosolic DNA in CIN-low cells compared to CIN-high cells (Fig. 5g). Whole-genome sequencing of subcellular fractions at 30 $\times$ coverage confirmed the genomic origin of cytosolic DNA (data not shown).

To determine whether missegregated chromosomes provide a source of cytosolic DNA, we used an inducible Y-chromosome-specific missegregation system established in chromosomally stable DLD-1 colorectal cancer cells²⁶. Whole-chromosome fluorescence *in situ* hybridization (FISH) probes targeting the Y chromosome or an independent autosome (chromosome 15) revealed selective incorporation of the Y chromosome into micronuclei two days after chromosome missegregation induced by doxycycline and auxin (Dox/IAA) treatment. Notably, Y-chromosome-specific fragments were found dispersed within the cytosol 2–3 days after Dox/IAA addition, whereas the control autosome remained confined to the nucleus (Fig. 5h), demonstrating that cytosolic DNA is generated from chromosomes undergoing high rates of missegregation.

Suppression of micronuclear envelope rupture by mCherry-lamin B2 overexpression²⁰ reduced cytosolic dsDNA staining without influencing chromosome segregation errors. Accordingly, such overexpression reduced metastasis after intracardiac or tail vein injection of MDA-MB-231 cells (Fig. 5i and Extended Data Fig. 3g, h).

Metastasis from cytosolic DNA response

In chromosomally stable cells, cytosolic dsDNA is scarce and is sensed by the cGAS–STING pathway¹⁹, leading to induction of type I interferon stimulated genes (ISGs)^{22,23,27}. Indeed, induced missegregation of the Y chromosome led to the upregulation of OAS2, an ISG, and increased interferon- β production by DLD-1 cells

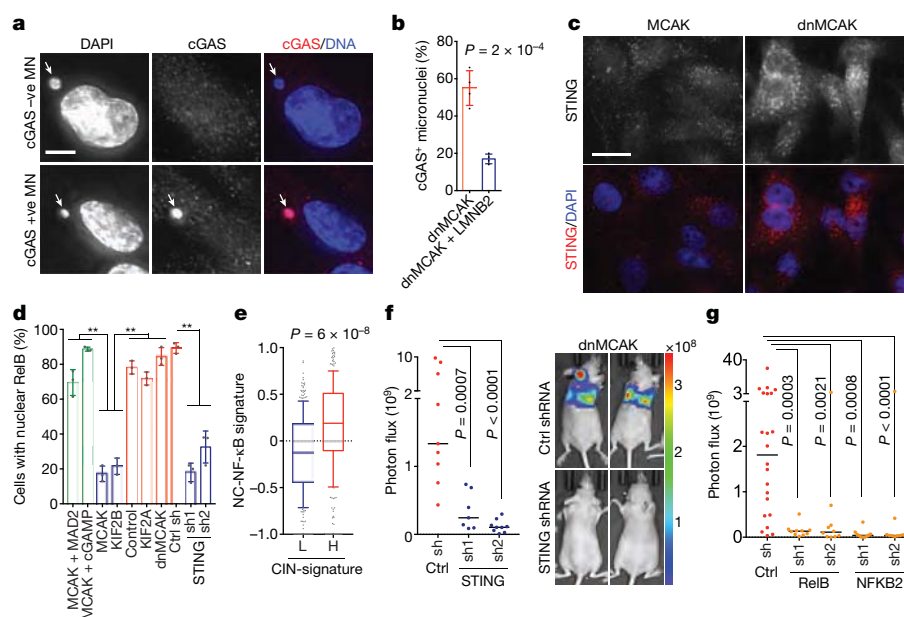


Figure 6 | Metastasis from a cytosolic DNA response. **a**, MDA-MB-231 cells stained for DNA and cGAS; scale bar, 5 μm. **b**, Percentage of micronuclei with cGAS localization (cGAS⁺), $n = 200$ cells. **c**, Cells stained for DNA and STING; scale bar, 20 μm. **d**, Percentage of MDA-MB-231 cells with nuclear RELB, $n = 150$ cells. **e**, Average z-normalized expression of CIN-responsive noncanonical NF-κB target genes in breast cancer patients with low (<30th percentile, $n = 330$) or high (>30th percentile, $n = 332$) CIN gene expression signature. **f**, **g**, Photon flux ($p \text{ s}^{-1}$) of whole animals after intracardiac injection with

MDA-MB-231 cells expressing control shRNA, STING shRNA (**f**), RELB shRNA (**g**) or NFKB2 shRNA (**g**); $n = 9$, 7, and 9 mice for the control, STING shRNA1, and STING shRNA2 groups, respectively (**f**); $n = 22$, 10, 10, 10, and 9 mice for the control, RELB shRNA1, RELB shRNA2, NFKB2 shRNA1, and NFKB2 shRNA2 groups, respectively (**g**). Data shown as mean $\pm$ s.d. (**b**, **d**), median $\pm$ interquartile range with bars spanning 10th–90th percentiles (**e**), median (**f**, **g**); significance tested using two-sided t -test (**b**, **d**), two-sided Mann–Whitney test (**e**–**g**); $n = 4$ (**a**, **b**) and 3 (**c**, **d**) independent experiments.

(Extended Data Fig. 9f, g). It is unclear how chromosomally unstable cancer cells cope with the constant presence of cytosolic DNA. We found notable localization of cGAS to approximately half of all micronuclei, as previously observed^{22,23,27}. Impeding micronuclear rupture through lamin B2 overexpression²⁰ significantly diminished the relative fraction of cGAS⁺ micronuclei (Fig. 6a, b). Furthermore, CIN-high cells exhibited increased levels and perinuclear localization of STING, congruent with pathway activation (Fig. 6c).

Notably, there was no evidence for robust activation of downstream canonical NF-κB or type I interferon signalling in CIN-high cells, as evidenced by the lack of a significant increase in p65 or IRF3 phosphorylation, absence of p65 or IRF3 nuclear translocation, undetectable levels of interferon-β, and failure to induce ISGs (Extended Data Figs 8i, j, 9), in line with previous observations^{28–30}.

Cytosolic DNA, however, can activate the noncanonical NF-κB pathway in a STING-dependent and TBK1-independent manner¹⁸. We found evidence for noncanonical NF-κB activation in CIN-high cells, as revealed by lower levels of the precursor protein p100, a trend towards higher ratios of p52 and phosphorylated p100 relative to total p100, and reduced levels of the noncanonical NF-κB pathway inhibitor TRAF2³¹ (Extended Data Fig. 8i, j). Given the subtle differences seen at the protein level, we assessed the nuclear localization of RELB, the binding partner of p52, and observed increased nuclear localization in CIN-high cells. This was often accompanied by cytosolic staining, indicative of chronic pathway activation. STING depletion reduced nuclear localization of RELB and led to downregulation of EMT and inflammatory pathways, whereas the addition of cGAMP or overexpression of MAD2 increased nuclear RELB in MCAK-expressing cells (Fig. 6d, Extended Data Figs 4e, 9c–e).

Bulk RNA-seq data identified a number of noncanonical NF-κB target genes that were upregulated in response to CIN (CIN-responsive NC-NF-κB genes). There was a robust correlation between the CIN signature, STING, and the CIN-responsive NC-NF-κB genes in scRNA-seq data, in contrast to a weaker correlation between CIN and

type I interferon targets (Fig. 5b and Extended Data Fig. 6e). Similarly, RNA-seq data from primary breast cancer in the TCGA database demonstrated increased expression of CIN-responsive NC-NF-κB genes in tumours with higher levels of CIN signature gene products (Fig. 6e), and higher expression of key regulators of the noncanonical NF-κB pathway or its CIN-responsive target genes was associated with shorter DMFS and disease-free survival in breast and lung cancers. Conversely, increased expression of canonical NF-κB or type I interferon regulatory factors was associated with an improved prognosis (Extended Data Fig. 10a).

cGAS activation by cancer cells has been invoked in brain metastasis through a tumour cell-non-autonomous mechanism³². We found that STING and downstream noncanonical NF-κB activity mediate metastasis in a tumour cell-autonomous fashion, as evidenced by reduction in metastatic dissemination, lifespan extension, and reduction in *in vitro* and *in vivo* invasion of CIN-high cells depleted of STING, RELB, or p100 (encoded by *NFKB2*). Conversely, the addition of cGAMP increased invasion and migration of CIN-low cells (Fig. 6f, g and Extended Data Figs 6f, 9j, 10b–d). These findings are in line with reported roles for the noncanonical NF-κB pathway in EMT, cellular invasion, and metastasis^{33–35}. The benefits of the noncanonical pathway may justify the scarcity of inactivating mutations in cGAS and STING among breast and lung cancers (Extended Data Fig. 10e).

Discussion

Our work reveals an unexpected link between CIN, chronic activation of cytosolic DNA sensing pathways, and metastasis. In addition to fuelling karyotypic heterogeneity that serves as a substrate for natural selection, ongoing chromosome missegregation is required to replenish cytosolic DNA pools and to maintain cells in a pro-metastatic state. Consequently, suppression of CIN reduces metastasis even in highly aneuploid cells. The repercussions of STING activation are context-dependent and range from senescence to tumorigenesis^{21–23,27,28,30}.

Given that chromosomally unstable cells are awash with cytosolic DNA, our results raise the possibility that by suppressing downstream type I interferon signalling³⁰ and instead upregulating the alternative NF- κ B pathway, such cells have substituted a lethal epithelial response to inflammation with that of myeloid-derived cells^{36,37}, thereby engaging in some form of immune mimicry. Restoration of normal responses to inflammation would constitute a viable therapeutic strategy to target chromosomally unstable cells.

The emergence, and subsequent tolerance, of CIN represents an important bottleneck during tumour evolution^{38–40}. We found that CIN induces a transcriptional shift from a proliferative and highly metabolic state, ideally suited for primary tumour growth, to a mesenchymal state associated with upregulation of inflammatory pathways (Figs 4b, 5a). These two mutually exclusive states, which were recently observed in a pan-cancer genomic analysis of metastatic tumours⁴¹, are likely to account for the reversibility in chromosome missegregation rates seen in primary tumours and metastases, and provide an explanation for the negative effect of aneuploidy during early tumorigenesis^{38,40,42}. These findings also lead us to suggest that CIN drives the subset of human metastases characterized by EMT and inflammation⁴¹.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions S.F.B. and L.C.C. conceived the project; S.F.B., B.N., J.-A.C. and R.K.S. performed animal experiments; V.K.R. derived PDX models; P.L. performed experiments using DLD-1 cells; S.F.B., B.N., J.A.C., R.K.S., M.D., S.V. and Q.L. performed immunostaining, immunoblotting, microscopy and qPCR; P.S. performed live-cell imaging; T.B.K.W., N.J.B. and N.M. analysed matched primary tumour–metastasis data; N.K.T., C.H.C. and S.F.B. analysed the HNSCC data; S.F.B. analysed breast cancer karyotype data; C.P. performed histological analysis; C.S., K.C. and G.N. performed cytogenetic analysis; A.M.L. analysed scRNAseq data; M.L. and S.F.B. analysed survival data; C.J.M. analysed bulk RNAseq data; M.I. analysed whole-genome sequence data; G.G., M.L., Q.L., J.H.H., O.E., C.H.C., N.Y.L., D.P., D.W.C., S.N.P., J.L., C.S. and L.C.C. assisted with data interpretation. All authors contributed to the writing and editing of the manuscript.

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METHODS

Genomic analysis of primary tumour–metastasis matched pairs. Whole-exome DNA sequence data from 79 brain metastases with matched primary tumour and normal tissue⁹ were downloaded from the database of Genotypes and Phenotypes (dbGAP) and processed as described⁴³ to derive allele-specific segmented DNA copy number data for each sample. The weighted genome instability index (wGII), which describes the proportion of the genome that was classified as aberrant relative to tumour ploidy, was determined as described⁸.

Mitelman database analysis. All available breast adenocarcinoma cases in the Mitelman database¹⁰ were analysed. The primary literature was reviewed to determine the source of the sample (primary tumour or metastasis). When clonal karyotype was reported as a range, the average value was used for that given clone. Karyotype aberrations included structural aberrations as well as numerical deviations from the overall karyotype of the clone.

Analysis of chromosome segregation in head and neck squamous cell carcinoma. We analysed primary tumour specimens from 60 patients with head and neck squamous cell carcinoma (HNSCC)¹¹. Haematoxylin and eosin-stained primary tumour samples of sufficient quality for high-resolution microscopy analysis were available for forty patients. Analysis was restricted to cells fixed while undergoing anaphase, as previously described^{44,45}. Chromosome missegregation was defined by the presence of haematoxylin staining between the remaining segregating chromosomes during anaphase and was reported as the percentage of cells undergoing anaphase with evidence of chromosome missegregation, as previously described⁴⁴. Clinical lymph node status was defined on the basis of clinical examination or radiographic evidence of lymph node tumour involvement¹¹.

Single-cell karyotyping. Cultures were treated with colcemid at a final concentration of $0.1 \mu\text{g ml}^{-1}$. Following 45 min incubation at 37°C , the cultures were trypsinized, resuspended in pre-warmed 0.075 M KCl , incubated for an additional 10 min at 37°C and fixed in methanol:acetic acid (3:1). The fixed cell suspension was then dropped onto slides, stained in $0.08 \mu\text{g ml}^{-1}$ DAPI in $2\times\text{SSC}$ for 5 min and mounted in antifade solution (Vectashield, Vector Labs). Metaphase spreads were captured using a Nikon Eclipse E800 epifluorescence microscope equipped with GenASI Cytogenetic suite (Applied Spectral Imaging). For each sample a minimum of 20 inverted DAPI-stained metaphases were fully karyotyped and analysed according to the International System of Human Cytogenetic Nomenclature (ISCN) 2013.

FISH analysis. FISH analysis was performed on fixed cells prepared for single-cell karyotyping. Based on karyotype data, four chromosomes were selected to further evaluate numerical instability. Probes specific for centromere 3 (red), centromere 4 (orange) and centromere 9 (green) were purchased from Abbott-Vysis. The chromosome 6 centromeric probe was home-brew (PAC clone P308; labelled with green dUTP, MSKCC Molecular Cytogenetics Core Facility). Probe labelling, hybridization, post-hybridization washing, and fluorescence detection were performed according to standard laboratory procedures²⁶. For each probe, three normal peripheral blood samples (GM07535, GM06875 and GM00558), obtained from Coriell Institute, were also analysed to derive cut-off values (false positive).

RHOA and RAC1 pull-down assays. The activity of RHOA and RAC1 was determined using bead-based pull-down assay kits (Cytoskeleton, RHOA: BK036S, RAC1: BK035S). Cells were lysed on the tissue culture dish and rapidly snap frozen for storage until further processing. cGAMP was added for 18 h before lysis. In addition to His-tagged RHOA and RAC1, the positive and negative controls were total cell lysates supplemented with non-hydrolysable GTP or GDP, respectively.

Cell culture. Cell lines were purchased from the American Type Culture Collection (ATCC). Tumour (MDA-MB-231, 4T1, HEK293, and H2030), cells were cultured in DMEM or RPMI (4T1) supplemented with 10% FBS and 2 mM L-glutamine in the presence of penicillin (50 U ml^{-1}) and streptomycin ($50 \mu\text{g ml}^{-1}$). All cells tested negative for mycoplasma. Cell confluence was measured using InCuCyte live-cell analysis system (Essen Bioscience).

Immunofluorescence microscopy. Cell fixation and antibody staining were performed as previously described⁷. In brief, cells were fixed with ice-cold (-30°C) methanol for 15 min (when staining for centromeres, centrosomes, cGAS, Vimentin, β -actin, IRF3, or α -tubulin) or 4% paraformaldehyde (when staining for RELB, p65, STING, ssDNA, dsDNA, COXIV, or β -catenin). Subsequently, cells were permeabilized using 1% triton for 4 min. See Supplementary Table 1 for antibody information. For selective plasma membrane permeabilization used for cytosolic dsDNA and ssDNA staining, cells were treated with 0.02% saponin for 5 min after fixation. For single-stranded (Thermo Fisher Scientific, FEREN0321) and double stranded (Life Technologies, EN0771)-specific nuclease treatment, cells were also permeabilized with 0.02% saponin for 2 min and treated with either nuclease for 10 min before fixation using 4% paraformaldehyde. TBS–BSA was used as a blocking agent during antibody staining. DAPI was added together with secondary

antibodies. Cells were mounted with Prolong Diamond Antifade Mountant (Life Technologies, P36961). cGAMP (Invivogen, tlr-nacga23) was transfected into cells at a concentration of $4 \mu\text{g ml}^{-1}$ using lipofectamine 2000 that was added for 3–4 h and then replaced with regular serum-containing medium.

Immunoblotting. Cells were pelleted and lysed using RIPA buffer. Protein concentration was determined using BCA protein assay and 20–30 μg total protein was loaded in each lane. Proteins were separated by gradient SDS–PAGE and transferred to PVDF or nitrocellulose membranes. Full blots are shown in Supplementary Fig. 1. See Supplementary Table 2 for antibody information. Band intensities on immunoblots were obtained using ImageJ (<https://imagej.nih.gov/ij/>) or the LI-COR Odyssey software, normalized to loading control, and background was subtracted. Ratios were normalized to control cells. Interferon- β levels from conditioned medium were measured using the Human IFN beta Array 1-plex (Eve Technologies, HIFNB-01-31).

Y chromosome missegregation and FISH. Flp-In T-REx DLD-1 cells were engineered to express the TIR1 auxin-dependent plant E3 ligase, an auxin-inducible degron (AID)-tagged CENP-A modified at the endogenous allele (CENP-A^{AID/-}), and a doxycycline-inducible CENP-A^{C-H3} rescue gene integrated into the Flp-In locus as previously described²⁶. Cells (4.0×10^4) were seeded into 4-well chamber slides and treated with doxycycline (DOX, Sigma-Aldrich) and indole-3-acetic acid (IAA, Sigma-Aldrich) for up to 3 days to induce Y chromosome missegregation and micronuclei. Slides were washed in PBS, fixed in 3:1 methanol:acetic acid for 15 min at room temperature, and dehydrated with 80% ethanol. Chromosome paint FISH probes targeting chromosomes Y and 15 (MetaSystems) were mixed at equal ratios, applied to cells, sealed with a coverslip, and co-denatured at 75°C for 2 min followed by overnight hybridization at 37°C in a humidified chamber. Slides were washed in $0.4\times$ saline–sodium citrate (SSC) buffer for 2 min at 72°C , followed by a 30 s wash in $2\times\text{SCC}$, 0.05% Tween-20 buffer at room temperature. Cells were counterstained with DAPI and captured on a DeltaVision Elite (GE Healthcare) microscope system at $60\times$ magnification ($25 \times 0.2 \mu\text{m z}$ -stacks) followed by image deconvolution and maximum intensity quick projection.

Knockdown and overexpression constructs. Luciferase expression was achieved using pLVX plasmid (expressing tdTomato) and cells stably expressing luciferase were selected using hygromycin and sorted for tdTomato expression. Kinesin-13 family protein expression was achieved using plasmid (pEGFP) transfection or lentiviral (pLenti-GIII-CMV-GFP-2A-Puro) expression where cells were selected using G418 (0.5 mg ml^{-1}) or puromycin ($5 \mu\text{g ml}^{-1}$), respectively. DNASE2 overexpression was achieved using a pLenti-GIII-CMV-RFP-2A-Puro plasmid with puromycin used for selection. Plasmids containing kinesin-13 proteins or lamin B2 (pQCXIB–mCherry–lmbn2) constructs were offered by the Compton and Hetzer Laboratories, respectively. Blasticidin was used to select for *lmbn2*-expressing cells at $10 \mu\text{g ml}^{-1}$. All other plasmids were purchased from Applied Biological Materials (<https://www.abmgood.com/>). Stable knockdown of STING, NFKB2, RELB, and cGAS were achieved using shRNAs in pRRL (SGEP or SGEN) plasmids obtained from the MSKCC RNA Interference Core. Two to four distinct shRNA hairpins were screened per target. Targeted shRNA sequences are listed in Supplementary Table 3. To visualize primary nuclear rupture, cells were stably modified with a retroviral construct expressing both NLS–GFP²⁵ and H2B–Tdtomato ($3\times\text{NLS}$ ScopGFP-P2A-H2BtdTomato-IRES-puromycin). Cells were cultured for 24 h after viral transduction before selection with $1 \mu\text{g ml}^{-1}$ puromycin and subsequently sorted to select for NLS–GFP and H2B–Tdtomato expression.

Cell migration in microfluidic devices. Microfluidic migration devices with precisely defined constrictions were prepared as described previously^{25,46}. Devices were coated with $50 \mu\text{g ml}^{-1}$ type-I rat tail collagen (BD Biosciences) in 0.02 M acetic acid overnight at 4°C . Approximately 80,000 cells were seeded (in DMEM supplemented with 10% FBS and 1% PenStrep) per migration chamber. Devices were placed in a tissue-culture incubator (37°C) for 5–6 h to allow the cells to adhere. Subsequently, the medium was changed to phenol-red free Leibovitz L15 medium supplemented with 10% FBS and 1% PenStrep before the device was mounted on an inverted microscope (Zeiss Observer Z1) equipped with a temperature-controlled stage (37°C) for live-cell imaging. The medium reservoirs of the device were covered with glass coverslips to minimize evaporation during live-cell imaging. Cells were imaged for 14–16 h at 10-min intervals with a CCD camera (Photometrics CoolSNAP KINO) using a Zeiss $20\times/\text{NA } 0.8$ air objective. Acquired image sequences were analysed for nuclear rupture frequency, duration, and transit time of cells through $1 \times 5\text{-}\mu\text{m}^2$, $2 \times 5\text{-}\mu\text{m}^2$, and $15 \times 5\text{-}\mu\text{m}^2$ constrictions using Zen software (Zeiss) and a custom-written MATLAB 2016a script for automated image analysis.

Animal studies. Animal experiments were performed in accordance with protocols approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee. For disease-specific survival in MDA-MB-231 experiments, power analysis indicated that ten mice per group would be sufficient to detect a difference

at relative hazard ratios of <0.2 or >5 with 80% power and 95% confidence, given a median disease-specific survival of 3 months in the control group and a total follow up period of 250 days. For the 4T1 experiments, power analysis indicated that ten mice per group would be sufficient to detect a difference at relative hazard ratios of <0.25 or >4.0 with 80% power and 95% confidence, given a median survival of 58 days in the control group and a total follow up period of 180 days. There was no need to randomize animals. Investigators were not blinded to group allocation. Intracardiac injection was performed as previously described³². In brief, cells were trypsinized and washed with PBS and 1×10^5 cells (in 100 μ l PBS) were injected into the left cardiac ventricle of female athymic 6–7-week-old athymic nude (*nu/nu*) mice (Jackson Laboratory strain 002019). Cells (2×10^5) were injected into the tail vein cohort of animals. Mice were then immediately injected with D-luciferin (150 mg kg⁻¹) and subjected to bioluminescence imaging (BLI) using an IVIS Spectrum Xenogen instrument (Caliper Life Sciences) to ensure systemic dissemination of tumour cells. Metastatic burden was measured 5–7 weeks after injection using BLI. BLI images were analysed using Living Image Software v2.50. Disease-specific survival endpoint was met when the mice died or met the criteria for euthanasia under the IACUC protocol and had radiographic evidence of metastatic disease. For orthotopic tumour implantation, 2.5×10^5 cells in 50 μ l PBS were mixed 1:1 with Matrigel (BD Biosciences) and injected into the fourth mammary fat pad. Only one tumour was implanted per animal. MDA-MB-231 primary tumours were surgically excised before they reached ~1.5 cm in the largest dimension (which was the maximum allowable under our IACUC protocol) and metastatic dissemination was assessed using BLI imaging at 1–3-week intervals for up to 30 weeks. The distant metastasis-free survival endpoint was met when BLI signal was seen outside the site of primary tumour transplantation. 4T1 tumours were excised 8–9 days after implantation. To derive short-term culture from primary tumours and metastases, anesthetized mice (isoflurane) were imaged then killed. *Ex vivo* BLI was subsequently performed on removed organs to define the precise location of the metastatic lesion. Primary tumours and metastases were subsequently mechanically dissociated and cultured in DMEM with selection medium (G418 or hygromycin) to select for tumour cells and exclude host cells. All subsequent assays (karyotyping, RNA-seq, immunofluorescence, and subcellular fractionation) were performed after a single passage from the primary sample. To assess chromosome missegregation from primary tumour-derived and metastasis-derived cells, we performed high-resolution immunofluorescence analysis on passage no. 1 cells, staining for DNA (DAPI) and centromeres (ACA). The presence of cells with DNA or centromere staining in the middle of the anaphase plate was taken as evidence of chromosome missegregation.

Patient-derived xenograft assays. Patient-derived xenograft (PDX) models of human metastatic breast cancers were generated by transplanting the freshly obtained surgically excised tumour specimens from patients who had given consent under the IRB approved protocol (MSKCC IRB #97-094) in female NOD/SCID/IL2R γ ^{null} (NSG) mice (Jackson Laboratories strain 005557). All relevant ethical regulations were followed. The oestrogen receptor-positive PDX was derived from breast cancer metastatic to the bone. The TNBC PDX was established from an axillary lymph node metastasis from a patient with inflammatory breast cancer. PDXs were maintained for a maximum of three serial passages. In brief, freshly obtained tumour tissue specimens were either directly transplanted in the mammary fat-pad of the mice or minced into 1–2-mm pieces in serum-free MEM medium with nonessential amino acids (Cat. No. 41500018, Thermo Fisher Scientific) transduced with lentiviral vectors expressing either GFP-luciferase or pUltra-Chili-Luc plasmid (Addgene plasmid: 48688) followed by transplantation into mice. Typically PDX tumour growth became evident during the first 1–3 weeks after engrafting and tumours continued to grow for an additional 4–8 weeks. Primary tumour growth and metastases were followed using BLI or spectrum CT imaging. At the time of removal of primary tumours and metastases, we derived primary cell cultures directly from primary tumours as well as lung and liver metastases. In brief, 500 mg of fresh bulk tumour tissue was chopped into 1–2-mm³ pieces and incubated in Accutase (AT104; Innovative Cell Technologies) for cell detachment and separation over 1–2 h. The dissociated tissues were sieved through 100- μ m cell strainers and the cells were pelleted by centrifugation at 1,200 r.p.m. The pellets were washed and resuspended in the above MEM buffer with 3% FBS. Cells were analysed for chromosome missegregation after one passage.

RNA sequencing and analysis. Bulk RNA was extracted from cells using the QIAshredder (Qiagen, 79654) and the RNA extraction kit (Qiagen, 74106) and sequenced using HiSeq2500 or HiSeq4000 (Illumina). The quality of the raw FASTQ files were checked with FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). For samples originating from mouse xenografts, FASTQ reads were classified as originating from either mouse (GRCm38) or human (GRCh38) genomes using xenome⁴⁷, and human-specific reads were used for mapping. Reads were mapped to human reference GRCh38 using STAR (v2.4.1d,

2-pass mode)⁴⁸. Gene expression was estimated using cufflinks (v2.2.1, default parameters) and HTSeq (v0.6.1)^{49,50}. Differential expression analyses were performed using DESeq2 (v1.14.1)⁵¹. Pre-ranked gene set enrichment analyses were performed on the DESeq2 log₂ fold changes⁵². Prior to any unsupervised analyses, expression counts were transformed using variance stabilizing transformation using the DESeq2 R package. Gene signatures used in the study are listed in Supplementary Table 5. Differentially expressed gene sets and their associated statistics are listed in Supplementary Table 6. To detect potential copy number changes, positional gene enrichment analysis (PGE) was performed on the upregulated and downregulated differentially expressed genes, separately ($\text{padj} \leq 0.1$)⁵³. Only significant regions with four or more genes and with $P \leq 0.01$ were kept for further analysis. Circos plots were made using the circize R package⁵⁴.

Reverse-transcriptase quantitative polymerase chain reaction. Cells were collected into trizol reagent (Thermo Fisher Scientific) and total RNA was extracted using ‘PureLink RNA mini kit’ (Thermo Fisher Scientific) according to the manufacturer’s instructions. Total RNA (5 μ g) was used for RT-PCR using the RNA to cDNA Ecodry premix (oligo dT) cDNA synthesis kit (Clontech) according to the manufacturer’s instructions. Resulting cDNA corresponding to 50 ng total RNA was used in each 20 μ l of quantitative real time PCR reaction. qRT-PCR was performed using SybrGreen master mix (Biorad) and the relative expression of each gene was calculated after normalizing to *ACTB* endogenous control and using the comparative ΔC_t method. A list of the primers used is in Supplementary Table 4.

Single-cell RNA sequencing. Cells were trypsinized and resuspended in PBS. Twenty-one microlitres of a cellular suspension at 400 cells per microlitre, $>95\%$ viability, were loaded onto the 10X Genomics Chromium platform to generate barcoded single-cell GEMs. scRNA-seq libraries were prepared according to 10X Genomics specifications (Single Cell 3’ Reagent Kits User Guide PN-120233, 10X Genomics). GEM-reverse transcription (RT) (55°C for 2 h, 85°C for 5 min; held at 4°C) was performed in a C1000 Touch Thermal cycler with 96-Deep Well Reaction Module (Bio-Rad). After RT, GEMs were broken and the single-strand cDNA was cleaned up with DynaBeads MyOne Silane Beads (Thermo Fisher Scientific) and SPRIselect Reagent Kit (0.6 $\times$ SPRI; Beckman Coulter). cDNA was amplified for 14 cycles using the C1000 Touch Thermal cycler with 96-Deep Well Reaction Module (98°C for 3 min; 98°C for 15 s, 67°C for 20 s, and 72°C for 1 min $\times$ 14 cycles; 72°C for 1 min; held at 4°C). The quality of the cDNA was analysed using an Agilent Bioanalyzer 2100. The resulting cDNA was sheared to ~200 bp using a Covaris S220 instrument (Covaris) and cleaned using 0.6 $\times$ SPRI beads. The products were end-repaired, ‘A’-tailed and ligated to adaptors provided in the kit. A unique sample index for each library was introduced through 10 cycles of PCR amplification using the indexes provided in the kit (98°C for 45 s; 98°C for 20 s, 60°C for 30 s, and 72°C for 20 s $\times$ 14 cycles; 72°C for 1 min; held at 4°C). After two SPRI cleanups, libraries were quantified using Qubit fluorometric quantification (Thermo Fisher Scientific) and the quality assessed on an Agilent Bioanalyzer 2100. Four libraries were pooled and clustered on a HiSeq2500 in rapid mode at 10 pM on a pair end read flow cell and sequenced for 98 cycles of R1, followed by 14 bp I7 Index (10X Barcode), 8 bp I5 Index (sample index) and 10 bp on R2 (UMI). Primary processing of sequencing images was done using Illumina’s Real Time Analysis software. Demultiplexing and post processing was done using the 10X Genomics Cell Ranger pipeline per the manufacturer’s recommendations. scRNA-seq data were processed from raw reads to a molecule count array using the Cell Ranger pipeline⁵⁵. Additionally, to minimize the effects of experimental artefacts on the analysis, data were pre-processed to filter out cells with low total molecule counts (library size), low complexity and high mitochondrial content, identified by a bimodal fit. The remaining cells were normalized by dividing the expression level of each gene in a cell by its total library size and then scaling by the median library size of all cells. After normalizing by library size, we performed principal component analysis to improve the robustness of the constructed Markov matrix generated when computing diffusion eigenvalues for imputation of dropout noise⁵⁶. We chose the number of principal components to retain approximately 80% of variance in the data and excluded the first principal component, which was highly correlated with library size. Imputation of both the normalized and unnormalized count matrix was performed using a Markov matrix raised to the power of 3 (power corresponds the approximate number of weighted nearest neighbours) and with a gene expression distribution computed according to 21 nearest neighbouring cells, as described⁵⁶. Our analysis was robust to imputation and we obtained similar results without imputed data (not shown). Subpopulations were identified using Phenograph⁵⁷ and genes differentially expressed in at least one subpopulation were identified by the Kruskal–Wallis rank statistic using a bootstrapping method for random downsampling of matched molecule and cell counts from each subpopulation. t-SNE was used to visualize subpopulation structure based on the first 20 principle components of the imputed count matrix, subsetted by the top

5,150 differentially expressed genes (FDR q of Kruskal–Wallis rank statistic <0.05). The mean expression of key gene signatures in population M versus other subpopulations was z -normalized and visualized using violin plots. All gene signatures are annotated in Supplementary Table 5. The correlation between gene signatures was computed using the Spearman rank correlation coefficient according to the mean expression of all genes per signature per cell. Ward's minimum variance method was applied to hierarchically cluster cells by their normalized expression of differentially expressed EMT genes.

Patient survival analysis. Genes used for survival analysis are listed in Supplementary Table 5. For the meta-analysis cohort, we used aggregate data from KMPlot^{16,58} (<http://www.kmplot.com>) using only JetSet best probe set and auto-selection for best cutoff between the 25th and 75th percentiles. For the validation cohort in which DMFS data were available¹⁷, we used the z -normalized expression data for a data set and the median value was used as a cutoff. DMFS curves were compared using the log-rank test.

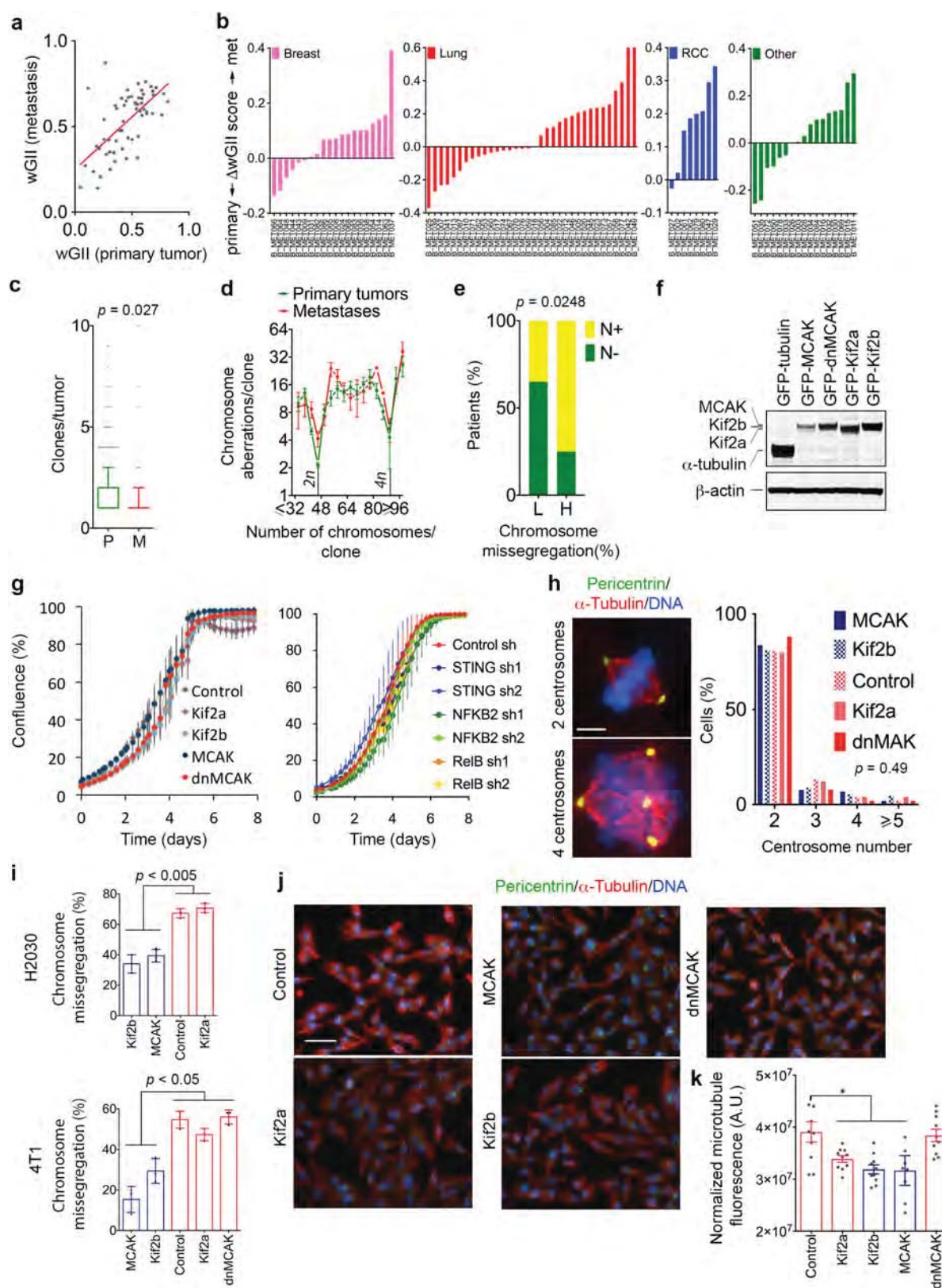
In vitro invasion and migration assays. For the invasion and migration/chemotaxis assays we used the CytoSelect cell invasion (CBA-110) and cell migration (CBA-100) kits, respectively. In brief, 3×10^5 cells were suspended in serum-free medium and placed on top of the membrane. Medium containing serum was placed at the bottom and cells that had invaded to the inferior surface of the collagen membrane were stained and counted 18–24 h later. For experiments involving transient transfection, cells were transfected, and thymidine (2 mM) was added 18 h later. Cells were plated on the membrane 3 days after transfection. For the chemotaxis assay, we used a colorimetric approach (OD 560 nm) for quantification. For the scratch assay, cells were treated with mitomycin C ($10 \mu\text{g ml}^{-1}$) for 1 h when they reached $>90\%$ confluence and then placed in DMEM containing 1% FBS. Wounds were applied using a p200 pipette tip and images of the wounds were taken immediately and at subsequent regular intervals. ImageJ was used for quantification of wound surface area.

Quantification of cytosolic DNA. Approximately 1×10^7 cells were lysed and the nuclear, cytosolic, and mitochondrial fractions were obtained using the mitochondrial isolation kit (Thermo Fisher Scientific, 89874). Protease inhibitors were not used to enable subsequent DNA purification. Mitochondria were purified by centrifugation at 12,000g to minimize their contamination in the cytosolic fraction. DNA was subsequently isolated from the nuclear, cytosolic, mitochondrial fractions using the Qiagen DNeasy blood and tissue kit (Qiagen, 69506) and dsDNA was quantified using Qubit 2.0 (Invitrogen) with Qubit dsDNA HS Reagent.

Code availability. All custom code, statistical analysis, and visualizations were performed in Python or R, and used Nextflow to manage some of the computational pipelines⁵⁹. Code for the RNA sequencing analysis is available online at: <https://github.com/murphycj/manuscripts/tree/master/BakhoumEtAl2017>. The live-cell tracking MATLAB 2016a code can be found at <https://github.com/Lammerding/MATLAB-CellTracking>.

Data availability. Source data for Figs 1–3, 5, 6, and Extended Data Figs 1–3, 5–10 are provided with the paper. Single-cell RNA sequencing data (shown in Figs 4, 5 and Extended Data Fig. 6) have been deposited in the Sequence Read Archive under accession number SRP104750. Bulk RNA-seq data (shown in Extended Data Figs 4, 5) have been deposited in the Gene Expression Omnibus under accession number GSE98183.

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Extended Data Figure 1 | See next page for caption.

Extended Data Figure 1 | Generation of isogenic tumour models of CIN.

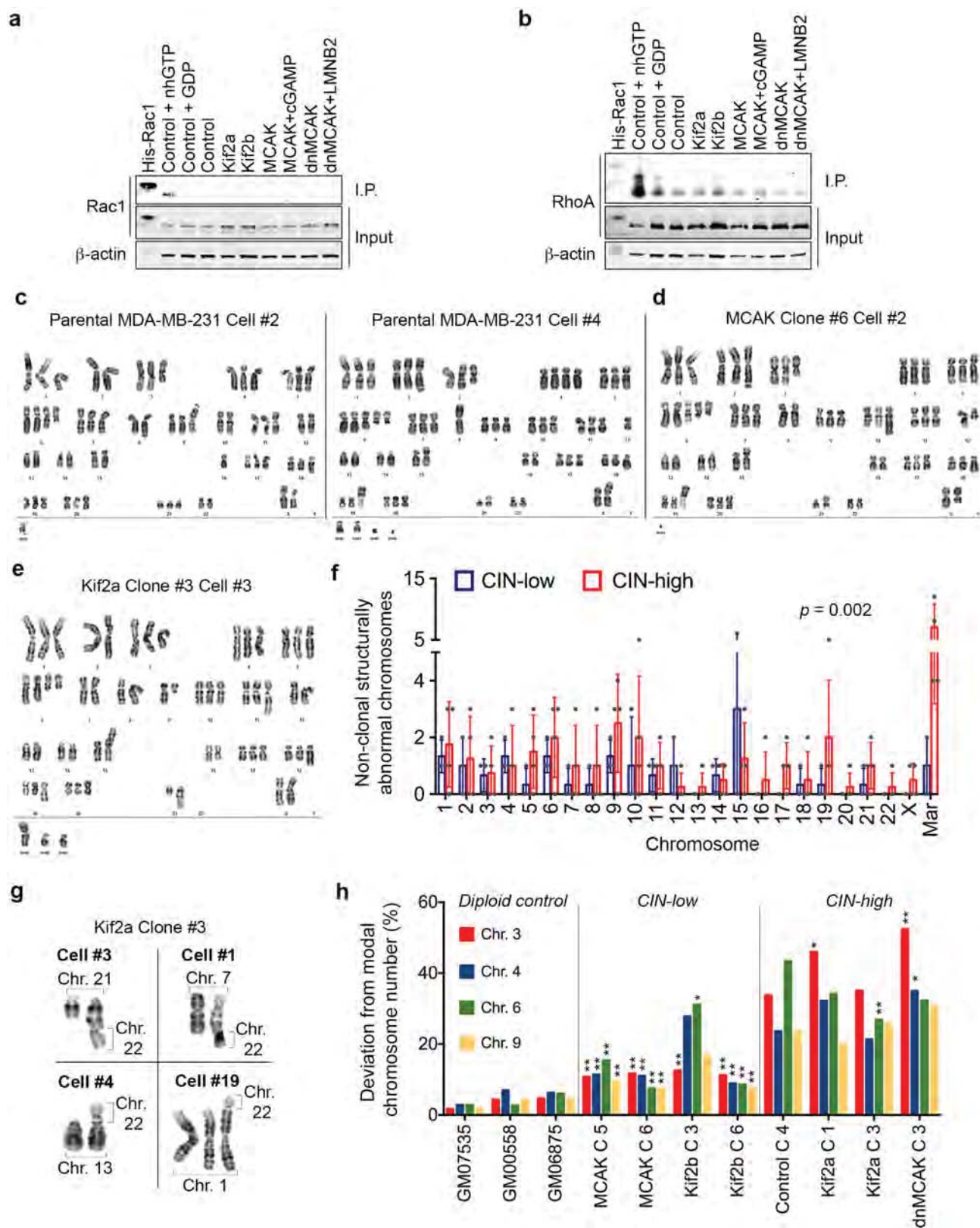
a, wGII of brain metastases as a function of the wGII of the matched primary tumour. Red line represents linear regression, $n = 79$ patients.

b, Differences in wGII between metastases and matched primary tumours. RCC, renal cell carcinoma; other includes melanoma, sarcoma, and ovarian, thyroid, and salivary gland cancers. **c**, Number of clones (based on single-cell karyotypes) in primary breast tumours (P; $n = 637$) or metastases (M; $n = 131$) found in the Mitelman database. Boxes represent median $\pm$ interquartile range and bars span the 10th and 90th percentiles; significance tested using two-sided Mann–Whitney test.

d, The number of chromosome aberrations per clone as a function of the total number of chromosomes in a given clone in samples derived from primary breast tumour clones ($n = 983$) and metastatic clones ($n = 186$); data shown as mean $\pm$ s.d. **e**, Percentage of N– or N+ patients as a function of chromosome missegregation frequency ($n = 20$ patients per condition); significance tested using two-sided Fisher's exact test.

f, Immunoblots of cells expressing various GFP-tagged kinesin-13 proteins stained using anti-GFP antibody; β -actin used as a loading control, two independent experiments performed. **g**, Cellular confluence as a function

of time in MDA-MB-231 cells expressing various kinesin-13 proteins or dnMCAK-expressing cells depleted of components of the cytosolic DNA-sensing machinery or the noncanonical NF- κ B pathway. Data shown as mean $\pm$ s.d., $n = 4$ independent experiments. **h**, Left, MCAK- and dnMCAK-expressing cells stained for microtubules (α -tubulin, DM1A), centrosomes (pericentrin) and DNA (DAPI). Scale bar, $5\mu\text{m}$; two independent experiments performed. Right, frequency distribution of the number of pericentrin foci per cell. Significance tested using ANOVA. $n = 100$ cells per condition, two independent experiments performed. **i**, Chromosome missegregation in H2030 and 4T1 cells expressing kinesin-13 proteins. Data shown as mean $\pm$ s.d., $n = 150$ cells, three independent experiments performed, significance tested using two-sided t -test. **j**, Cells expressing kinesin-13 proteins stained for microtubules (DM1A), centrosomes (pericentrin) and DNA (DAPI). Scale bar, $50\mu\text{m}$, two independent experiments performed. **k**, Fluorescence normalized to cell count of MDA-MB-231 cells expressing kinesin-13 proteins. Data shown as mean $\pm$ s.e.m., $*P < 0.05$, two-sided t -test, $n = 10$ high-power fields encompassing 477–612 cells, two independent experiments performed.

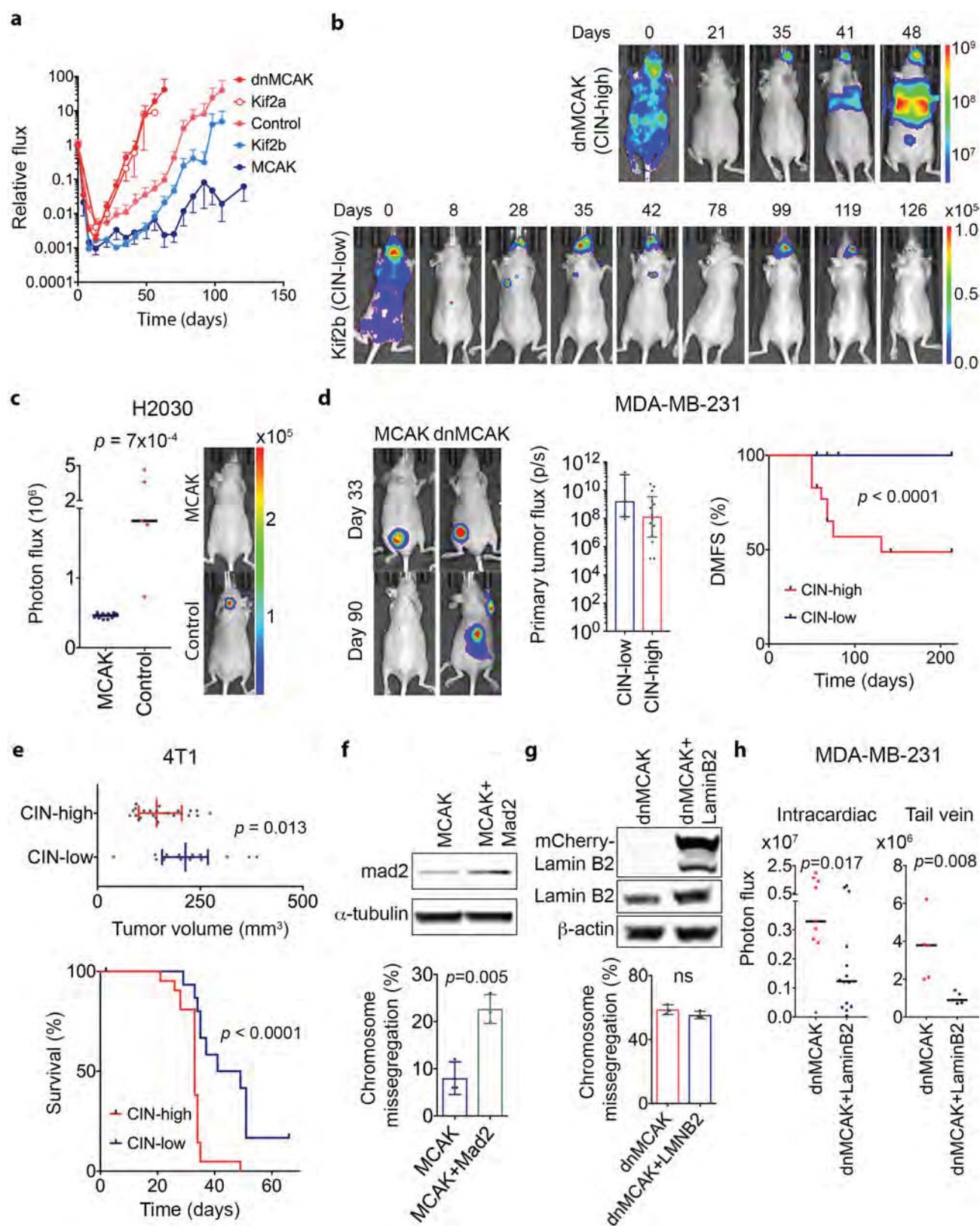


Extended Data Figure 2 | See next page for caption.

Extended Data Figure 2 | Karyotype analyses of human tumour cells.

a, b, Immunoblots showing total RAC1 (**a**) or RHOA (**b**) levels as well as RAC1 or RHOA that was pulled down using antibodies that were specific to the GTP-bound form of RAC1 (**a**) or RHOA (**b**). Positive and negative controls were total MDA-MB-231 cell lysates supplanted with non-hydrolysable GTP (nhGTP) and GDP, respectively. β -actin was used as a loading control, two independent experiments performed. **c–e**, Representative karyotypes (DAPI-banding) from parental MDA-MB-231 cells (**c**) or populations derived from single cells expressing MCAK (**d**) or KIF2A (**e**) that were allowed to divide for 30 days. **f**, The number of non-clonal (present in less than 25% of the cells in a single clone) structurally abnormal chromosomes in CIN-low or CIN-high MDA-MB-231 cells.

Mar, chromosomes so structurally abnormal that they could not be identified by conventional banding; data shown as mean $\pm$ s.d., $n = 140$ cells from 7 clonal populations, significance tested using two-way ANOVA test. **g**, Examples taken from four distinct cells belonging to the same clonal population (derived from a single KIF2A-expressing cell) showing convergent translocations involving chromosome 22 with four other chromosomes. **h**, Deviation from modal chromosome number in single-cell-derived clones grown for 30 days. Four chromosomes were assayed for each clone using centromere-specific probes. $*P < 0.05$, $**P < 0.005$ compared to control clone 4 by two-sided χ^2 -test, $n = 300$ cells per clone. Diploid controls were used to determine the false-positive rate of the centromeric probes.

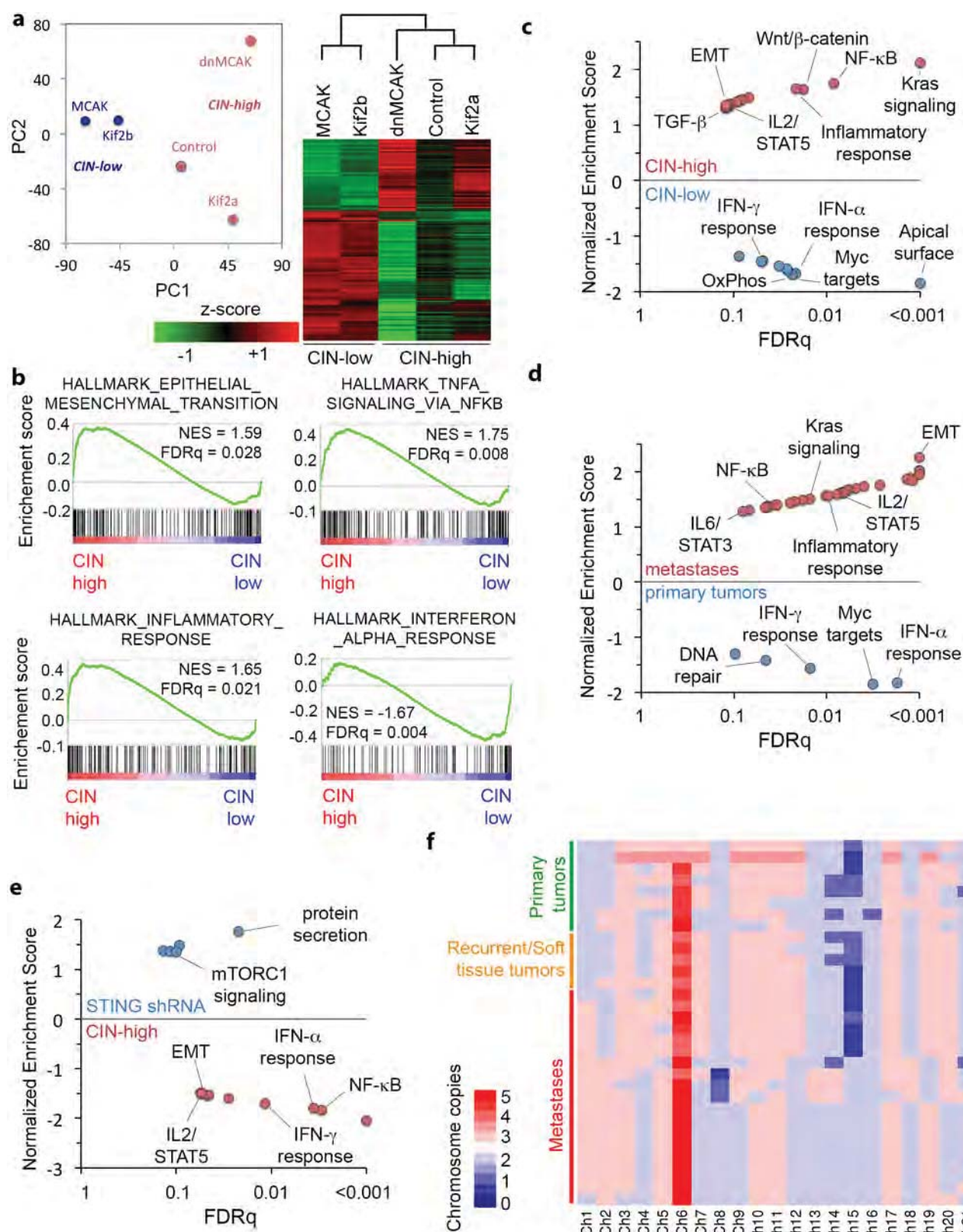


Extended Data Figure 3 | See next page for caption.

Extended Data Figure 3 | CIN promotes the formation and maintenance

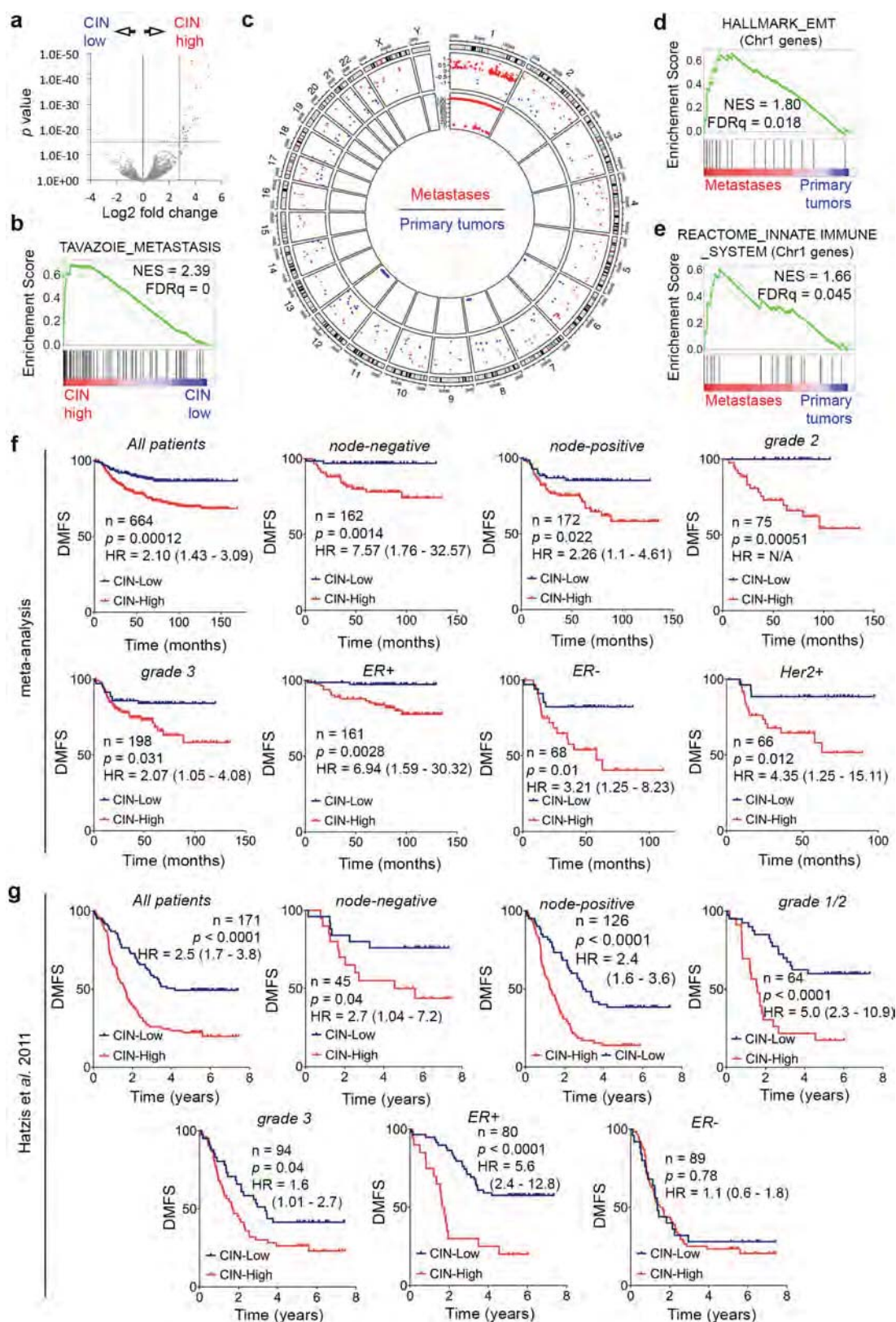
of metastasis. a, Normalized photon flux over time of whole animals injected with MDA-MB-231 cells expressing kinesin-13 proteins. Data shown as mean $\pm$ s.e.m. $n = 8$ (MCAK), 7 (KIF2B), 5 (control), 4 (KIF2A), and 9 (dnMCAK) mice per group; three independent experiments performed. **b**, Representative images of mice injected with MDA-MB-231 cells expressing dnMCAK (above) or KIF2B (below) with disease burden tracked using BLI; three independent experiments performed. **c**, Photon flux (p s^{-1}) of whole animals imaged 5 weeks after intracardiac injection with control or MCAK-expressing H2030 cells. Horizontal bars represent the mean, significance tested using two-sided Mann–Whitney test, $n = 10$ mice in the MCAK group and 5 mice in the control group. **d**, Left, representative BLI images (from two independent experiments) of mice orthotopically transplanted with MDA-MB-231 cells. Images taken before (day 33) and after (day 90) tumour excision. Metastasis can be detected in the mouse transplanted with dnMCAK-expressing cells at day 90. Middle, total flux (p s^{-1}) emitted from primary tumours 52 days after transplantation. Data shown as mean $\pm$ s.d., $n = 5$ (CIN-low) and 14 (CIN-high) mice, $P = 0.13$, two-sided Mann–Whitney test. Right, DMFS of mice orthotopically transplanted with MDA-MB-231 cells with various levels of CIN. $n = 15$ (CIN-low) and 29 (CIN-high) mice, pairwise significance tested with two-sided log-rank test. **e**, Tumour volume at 8 days (top) and survival (bottom) of mice transplanted with mouse

4T1 cells into the mammary fat pad. Bars represent median $\pm$ interquartile range, pairwise significance tested with two-sided t -test (top) and two-sided log-rank test (bottom). $n = 20$ (CIN-low) and 30 (CIN-high) mice. **f**, Top, immunoblots of MDA-MB-231 cells overexpressing MCAK or MCAK and MAD2 stained for MAD2 using anti-MAD2 antibody with α -tubulin used as a loading control; three independent experiments performed. Bottom, percentage of anaphase cells exhibiting evidence of chromosome missegregation in cells overexpressing MCAK or MCAK and MAD2. Data shown as mean $\pm$ s.d., $n = 150$ cells, three experiments performed, significance tested using two-sided t -test. **g**, Top, immunoblots of MDA-MB-231 cells overexpressing dnMCAK or dnMCAK and lamin B2 stained for lamin B2 using anti-lamin B2 antibody with β -actin used as a loading control. Two experiments performed. Bottom, percentage of anaphase cells exhibiting evidence of chromosome missegregation in cells overexpressing dnMCAK or dnMCAK and lamin B2. Data shown as mean $\pm$ s.d., $n = 150$ cells, three experiments performed, significance tested using two-sided t -test. **h**, Photon flux (p s^{-1}) of whole animals after intracardiac (left) or tail vein (right) injection with MDA-MB-231 cells expressing dnMCAK or dnMCAK and lamin B2. Bars represent the median, significance tested using two-sided Mann–Whitney test, $n = 9$ (dnMCAK), 15 (dnMCAK and Lamin B2) mice in the intracardiac injection cohort and 5 mice per group in the tail vein injection cohort.



Extended Data Figure 4 | Transcriptional consequences of CIN in cancer cells. a, b, Principal component analysis (left) and unsupervised clustering (right) of five MDA-MB-231 cell lines expressing different kinesin-13 proteins based on bulk RNA expression data. **b–e,** Gene set enrichment analysis results showing HALLMARK gene sets that are highly enriched in CIN-high (control, KIF2A, and dnMCAK) compared with CIN-low cells (MCAK and KIF2B) (b, c) or STING-depleted cells (e),

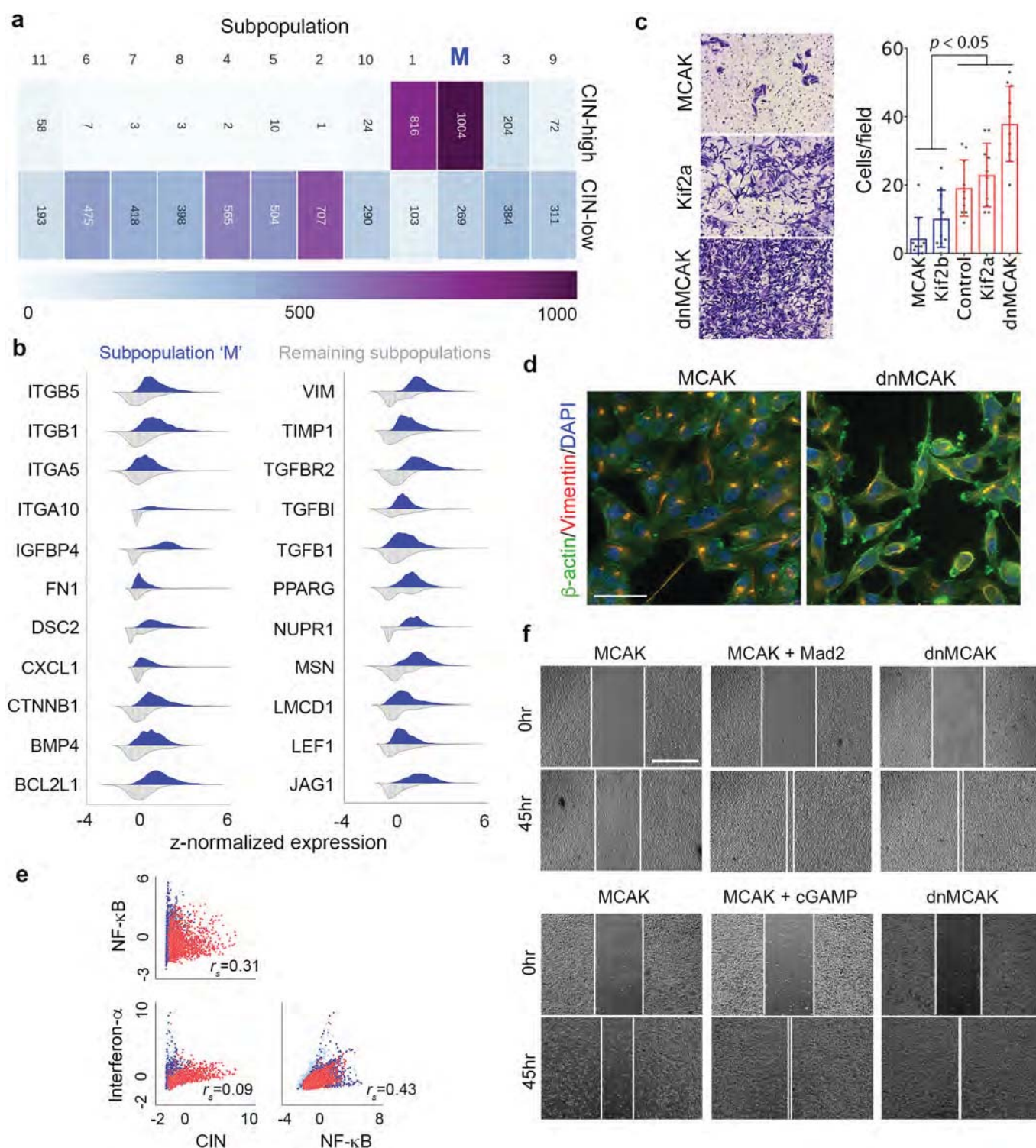
or after comparing metastases with primary tumours (d). Significance tested using one-sided weighted Smirnov–Kolmogorov test corrected for multiple tests. **f,** Heat map of consensus chromosomal karyotypes of cells derived from primary tumours and metastases showing selective increase in chromosome 1 copy number in metastases compared with primary tumours.



Extended Data Figure 5 | Prognostic impact of CIN signature.

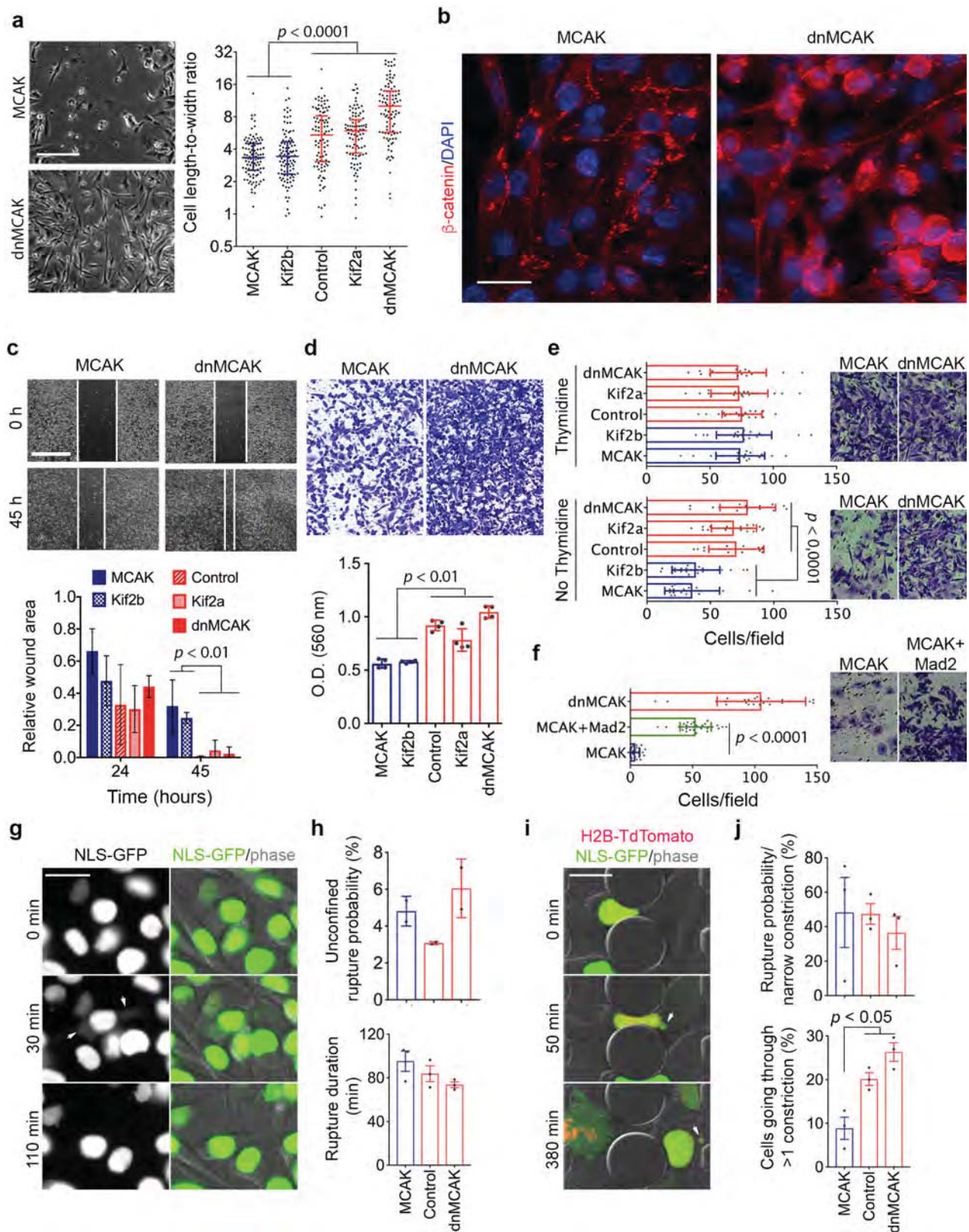
a, Volcano plot showing genes that were differentially expressed between CIN-high and CIN-low MDA-MB-231 cells. Red data points denote genes subsequently used for determining the CIN signature. **b–e**, Enrichment plots for all differentially expressed genes (**a**) or those on chromosome 1 (**d, e**). **c**, Circos plot shows genomic location (outer circle), log₂ fold expression of genes significantly differentially expressed in metastases compared to primary tumours (middle circles), and log₁₀ P (inner circle) for genomic amplifications (red) or deletions (blue) in metastases relative to primary

tumours. *n* = 2 (CIN-low), 3 (CIN-high), 11 (primary tumours), 28 (metastases). Significance tested using two-sided Wald test (**a**), one-sided weighted Smirnov–Kolmogorov test (**b, d, e**), and one-sided hypergeometric test (**c**), all corrected for multiple testing. **f, g**, DMFS of breast cancer patients stratified by lymph node status, grade, and receptor status, from a meta-analysis (**f**, *n* = 664 patients) or a validation cohort (**g**, *n* = 171 patients) divided on the basis of average expression of the CIN gene expression. Significance tested using two-sided log-rank test.



Extended Data Figure 6 | Single-cell sequencing and population detection. **a**, The cellular composition of every subpopulation presented Fig. 4b. **b**, Violin plots showing expression probability density of key metastasis and invasion genes in a subpopulation of cells ($n = 1,273$ cells) enriched for EMT and CIN genes (subpopulation M) compared with the remaining subpopulations ($n = 5,548$ cells) that were identified using graph-based unsupervised K -nearest neighbour embedding. **c**, Representative low-power field images (left) and numbers (right) of MDA-MB-231 cells that invaded through a collagen membrane within 18 h of culture. Data shown as mean $\pm$ s.d., significance tested using

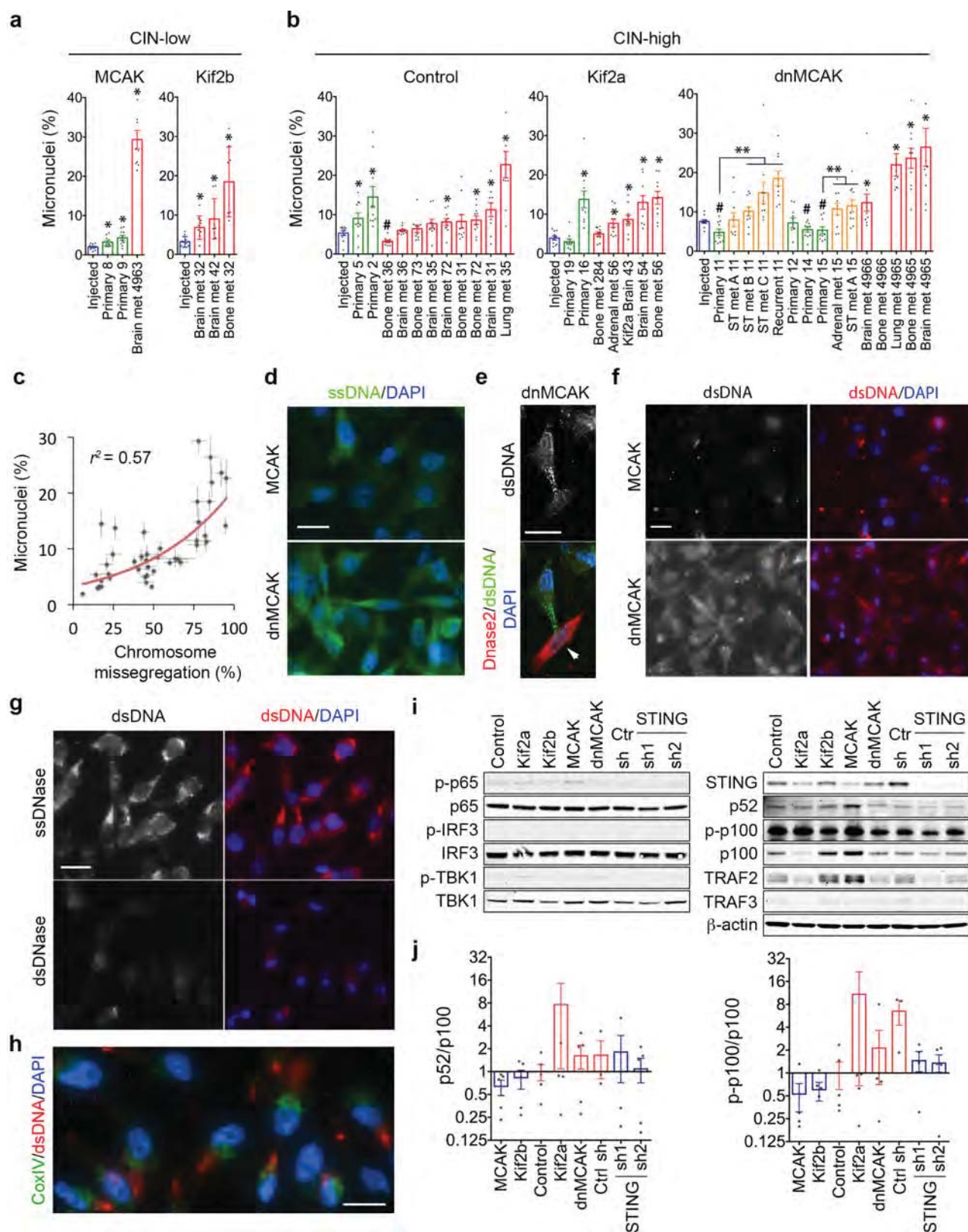
two-sided Mann–Whitney test, $n = 10$ high-power fields, two independent experiments performed. **d**, Representative images of MDA-MB-231 cells expressing MCAK or dnMCAK stained for β -actin, vimentin, and DNA. Scale bar, 50 μ m, $n = 2$ independent experiments. **e**, Single-cell correlation plots between CIN signature genes, canonical NF- κ B and type I interferon target genes, $n = 6,821$ cells. **f**, Representative phase-contrast images of a wound-healing assay of MDA-MB-231 cells expressing MCAK, MCAK and MAD2 or dnMCAK, and MCAK-expressing cells treated with cGAMP. Scale bar, 800 μ m, four experiments performed.



Extended Data Figure 7 | See next page for caption.

Extended Data Figure 7 | CIN promotes *in vitro* invasion and migration. **a**, Left, representative phase-contrast images of MDA-MB-231 cells in the wound area, 36 h after wound creation. Four experiments performed. Right, length-to-width ratio of cells expressing different kinesin-13 proteins. Bars span the interquartile range, $n = 100$ cells, two independent experiments performed, significance tested using two-sided Mann–Whitney test. **b**, Representative MDA-MB-231 cells stained for β -catenin (anti- β -catenin antibody) or DNA (DAPI). Changes in β -catenin are seen upon alteration of CIN; it is enriched at cell–cell junctions in MCAK-expressing cells but is found in the cytoplasm and nucleus in dnMCAK-expressing cells. Scale bar, $30\ \mu\text{m}$, two experiments performed. **c**, Top, phase-contrast images of a wound-healing assay of cells expressing kinesin-13 proteins. Scale bar, $800\ \mu\text{m}$, two experiments performed. Bottom, wound area (normalized to the 0 h time point) 24 h and 45 h after wound creation. Data shown as mean $\pm$ s.d., $n = 4$ experiments, significance tested using two-sided t -test. **d**, Top, low-power field images of MDA-MB-231 cells that have migrated through a polycarbonate membrane containing $8\text{-}\mu\text{m}$ pores within 18 h of culture. Bottom, normalized OD of cells scraped from the bottom of the membrane. Data shown as mean $\pm$ s.e.m., significance tested using two-sided t -test, $n = 3$ experiments. **e**, **f**, Left, number of MDA-MB-231 cells

that have successfully invaded through a collagen basement membrane 24 h after plating. Data shown as mean $\pm$ s.d., $n = 20$ high power fields from two independent experiments, significance tested using two-sided Mann–Whitney test. Right, representative images from high-power fields. Two independent experiments performed. **g**, **i**, Representative time-lapse fluorescence and phase-contrast image sequences of control cells expressing NLS–GFP undergoing unconfined migration (**g**) or going through $1 \times 5\text{-}\mu\text{m}^2$ constrictions (**i**). Scale bars, $20\ \mu\text{m}$. Arrows in **g** indicate cytoplasmic NLS–GFP. Arrows in **i** indicate formation of nuclear protrusion and subsequent fragments during confined migration. Three independent experiments performed. **h**, **j**, Top, the probability of primary nuclear rupture during unconfined conditions (**h**) or after migration through $1 \times 5\text{-}\mu\text{m}^2$ constrictions (**j**). Bottom, the number of cells migrating through more than one $1\text{-}\mu\text{m}$ -wide constrictions (**j**) and the duration of nuclear rupture (**h**), as measured by the length of time for which NLS–GFP signal is observed in the cytosol. Data shown as mean $\pm$ s.e.m., $n = 3$ independent experiments (except for unconfined rupture probability, 2 independent experiments) encompassing 390–665 (**h**) and 150–336 (**j**) cells observed during unconfined and confined migration, respectively. Significance tested using two-sided t -test.



Extended Data Figure 8 | See next page for caption.

Extended Data Figure 8 | CIN generates micronuclei and cytosolic DNA. **a, b**, Percentage of micronuclei in samples depicted in Fig. 3c, d: injected cells (blue), first-passage cells derived from primary tumours (green), or metastases (orange denotes spontaneous metastases arising from primary tumours, red denotes metastases obtained from direct intracardiac implantation). Data shown as mean $\pm$ s.e.m., $n = 10$ high-power fields encompassing 500–1,500 cells per sample, three independent experiments performed, $*P < 0.05$ (denotes samples with higher missegregation rates than the injected lines), $\#P < 0.05$ (denotes samples with lower missegregation rates than the injected lines), $**P < 0.05$ (denotes significant differences between metastases and matched primary tumours from the same animals), two-tailed t -test. **c**, Correlation between the percentage of cells exhibiting evidence of chromosome missegregation and the percentage of micronuclei in all injected cell lines as well as cells derived from primary tumours and metastases. Data shown as mean $\pm$ s.e.m., $n = 44$ samples.

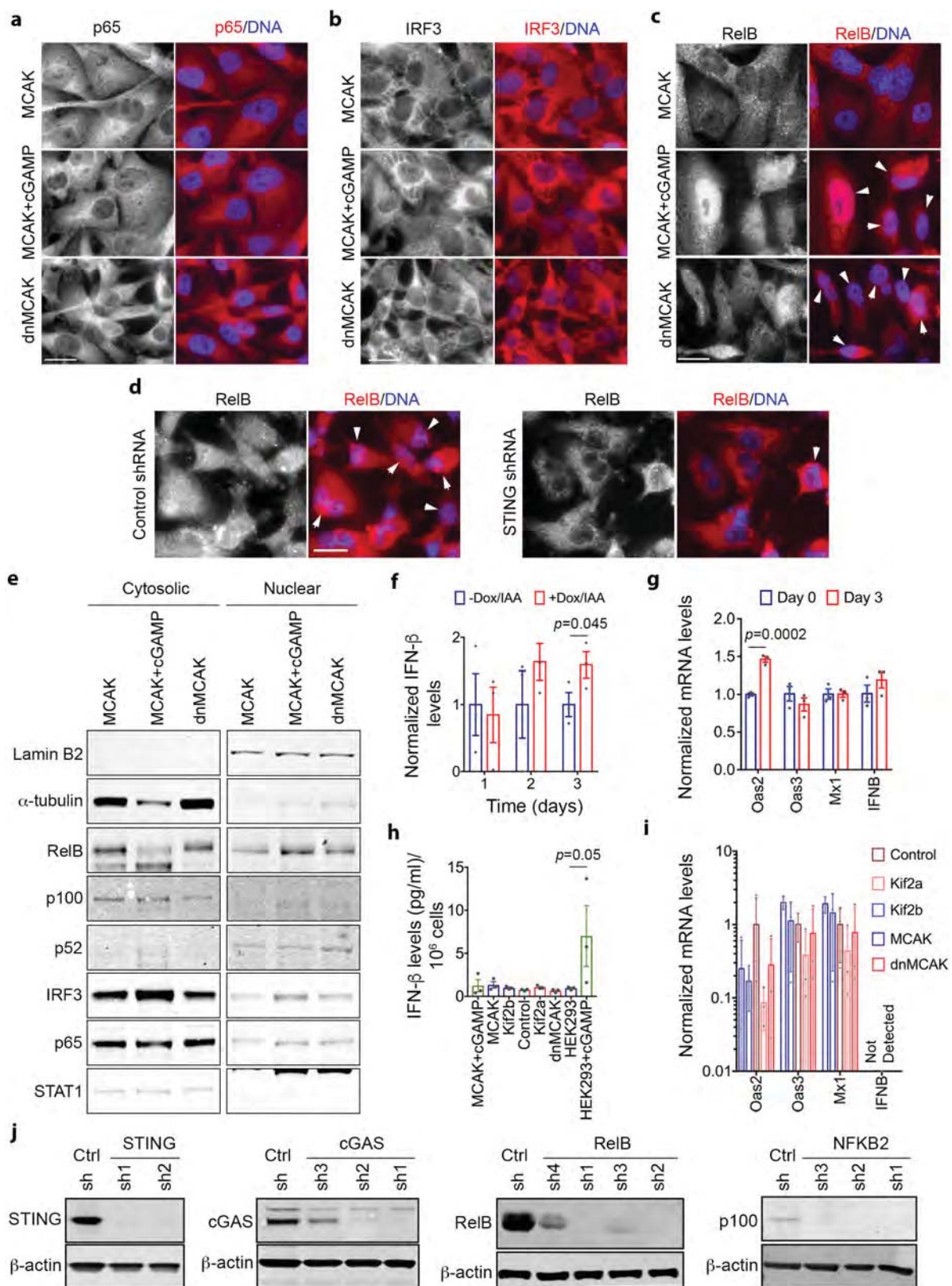
d–f, Representative images of cells stained for DNA (DAPI), cytosolic single-stranded DNA (ssDNA) (**d**), DNASE2 (RFP reporter) (**e**), or cytosolic dsDNA (**f**). Scale bar, 20 μm , arrows in **e** denote DNASE2-expressing cell, two independent experiments performed.

g, Representative images of dnMCAK-expressing cells treated with ssDNASE or dsDNASE for 10 min after selective plasma membrane permeabilization (using 0.02% saponin) and stained for DNA (DAPI) and cytosolic dsDNA. Scale bar, 20 μm , one experiment performed.

h, Representative images of dnMCAK-expressing cells stained for mitochondria (anti-CoxIV antibody), DNA (DAPI) or for cytosolic DNA (anti-dsDNA antibody). Scale bar, 20 μm , two independent experiments performed.

i, Immunoblots of lysates from cells expressing different kinesin-13 proteins, control or STING shRNA. β -actin used as a loading control.

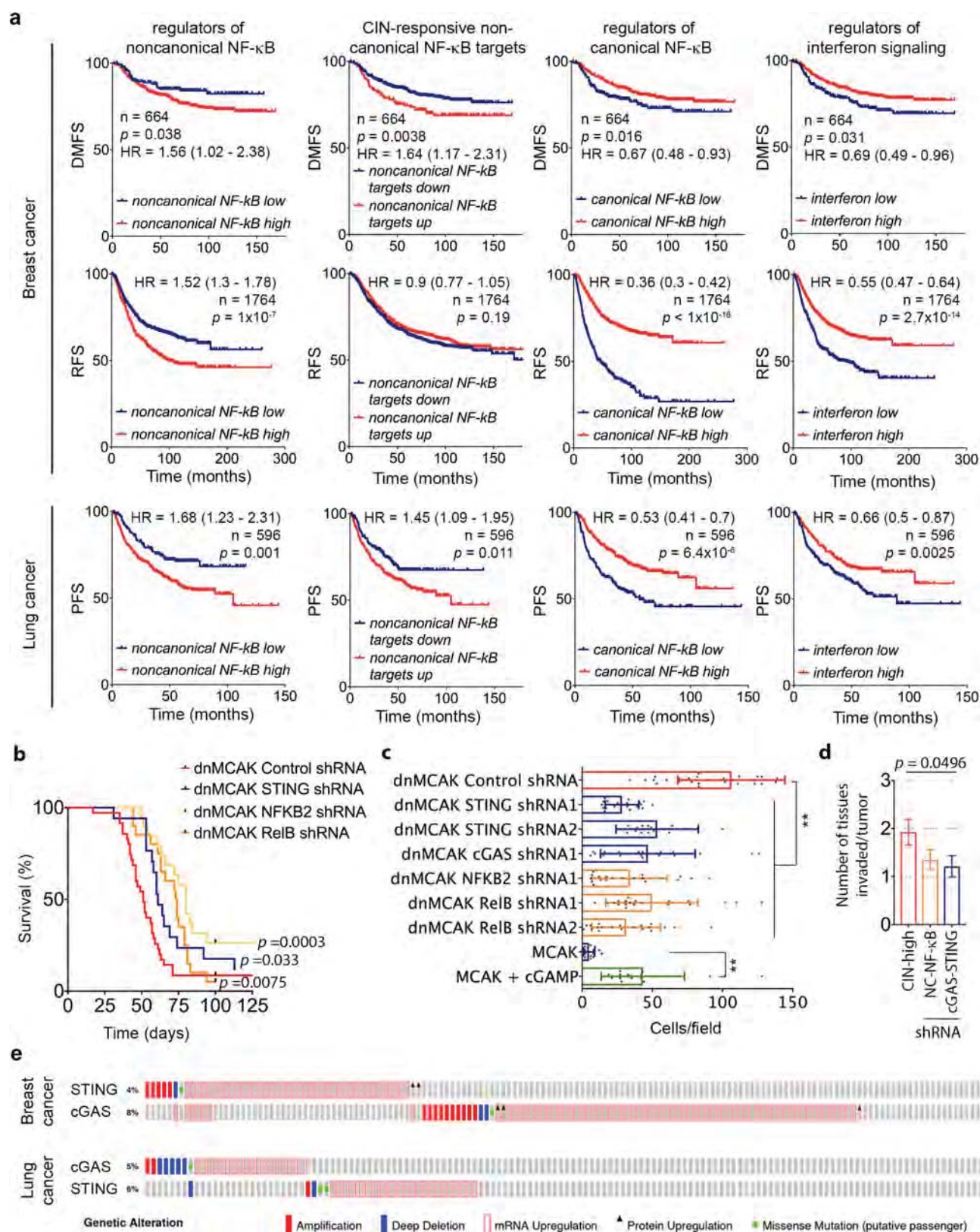
j, Normalized ratio of phosphorylated p52 to p100 (left) and p100 to total p100 (right) protein levels. Data shown as mean $\pm$ s.e.m., $n = 5$ independent experiments.



Extended Data Figure 9 | See next page for caption.

Extended Data Figure 9 | Alternative response to cytosolic DNA in cancer cells. **a–d**, Representative images of MDA-MB-231 cells stained for DNA (DAPI) and for p65 (**a**), IRF3 (**b**), or RELB (**c, d**). Images were individually contrast-enhanced to emphasize nuclear versus cytosolic localization of p65, IRF3, and RELB. For quantitative comparisons of identical images, see Supplementary Fig. 3. Arrows (**c, d**) point to RELB-positive nuclei. Scale bars, 20 μm , three independent experiments performed. **e**, Immunoblots of fractionated lysates. α -tubulin and lamin B2 were used as loading controls for the cytoplasmic and nuclear fractions, respectively; three independent experiments performed. **f, h**, Interferon- β levels in conditioned medium from DLD-1 cells (**f**), MDA-MB-231 or

HEK293 cells with and without cGAMP addition (**h**). Data shown as mean $\pm$ s.e.m. $n = 3$ experiments, significance tested using one-sided Mann–Whitney test. **g, i**, Relative levels of interferon-responsive genes obtained by RT–qPCR in DLD-1 cells (**g**) normalized to untreated conditions or MDA-MB-231 cells (**i**) normalized to control cells. Data shown as mean $\pm$ s.d. $n = 3$ experiments, significance tested using two-sided *t*-test. **j**, Immunoblots of lysates of dnMCAK-expressing cells that also co-expressed control shRNA or shRNAs targeting components of the cytosolic DNA-sensing or noncanonical NF- κ B pathways. shRNA hairpins are numbered in ascending order according to the efficiency of protein knockdown. Two independent experiments performed.



Extended Data Figure 10 | See next page for caption.

Extended Data Figure 10 | Effect of cytosolic DNA-sensing pathways on prognosis. **a**, Distant metastasis-free survival (DMFS), relapse-free survival (RFS) and progression-free survival (PFS) of patients with breast and lung, stratified according to their expression of NF- κ B and interferon pathways. Significance tested using two-sided log-rank test. **b**, Disease-specific survival of mice injected with dnMCAK-expressing MDA-MB-231 cells co-expressing control shRNA, STING shRNA, NFKB2 shRNA, or RELB shRNA. $n = 35, 16, 19$, and 20 mice in the control, STING shRNA, NFKB2 shRNA, and RELB shRNA groups, respectively; significance tested using two-sided log-rank test. **c**, Number of MDA-MB-231 cells expressing shRNA targeting genes belonging to the DNA-sensing or noncanonical

NF- κ B pathways that invaded through a collagen membrane within 24 h of culture. Data shown as mean $\pm$ s.d., ** $P < 0.0001$, two-sided Mann-Whitney test, $n = 20$ high-power fields, two independent experiments performed. **d**, Number of different normal tissues (vascular, neuronal, or soft tissue) invaded by orthotopically transplanted tumours. Data shown as mean $\pm$ s.e.m., * $P < 0.05$, two-tailed t -test, $n = 13$ tumours (CIN-high), 20 tumours (noncanonical NF- κ B depleted), 19 tumours (cGAS-STING depleted). **e**, Oncoprints showing genomic alterations in STING (TMEM173) and cGAS (MB21D1) in breast and lung cancers from the TCGA database.

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► Experimental design

1. Sample size

Describe how sample size was determined.

For disease-specific survival in MDA-MB-231 experiments, power analysis indicated that 10 mice per group will be sufficient to detect a difference at relative hazard ratios of <0.2 or >5 with 80% power and 95% confidence, given a median disease-specific survival of 3 months in the control group and a total follow up period of 250 days. For the 4T1 experiments, Power analysis indicates that 10 mice per group will be sufficient to detect a difference at relative hazard ratios of <0.25 or >4.0 with 80% power and 95% confidence, given a median survival of 58 days in the control group and a total follow up period of 180 days.

2. Data exclusions

Describe any data exclusions.

No data were excluded

3. Replication

Describe whether the experimental findings were reliably reproduced.

No attempts for replication failed. Immunofluorescence and immunoblot experiments were performed in three or more biological replicates (see figure legends). Key animal experiments were performed in up to 5 independent experiments

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

No method of randomization was used

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Investigators were not blinded to group allocation.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- ☐ ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ A statement indicating how many times each experiment was replicated
- ☐ ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- ☐ ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- ☐ ☒ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- ☐ ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- ☐ ☒ Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

Bulk RNA seq code is made publicly available (link in the methods section). Single cell RNAseq was analyzed using Python and already-published data and was appropriately referenced in the methods section. Wound healing assay (surface area) was analyzed using ImageJ. Single cell analysis was performed using Python 3.0. Cell migration using microfluidics devices was performed using MATLAB 2016a. RNAseq data was performed using R

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are no restrictions for distribution of research materials produced during this study.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Pre-validated antibodies were purchased from reputable sources (Cell signaling technology and abcam). Antibodies and their catalog numbers are listed in Supplementary information. Most antibodies were further validated when the target protein was depleted using shRNA.

Supplementary Table 1. Antibodies used in immunofluorescence.

Antibody against	Company	Catalog number
α -tubulin	Sigma Aldrich	T9026
β -actin	Abcam	ab8227
β -catenin	Abcam	ab16051
cGAS	Sigma Aldrich	HPA031700
Cox IV	Abcam	ab16056
dsDNA (Extended Fig. 8d)	Abcam	AB27156
dsDNA (Fig. 5f)	Thermo Fisher Scientific	MAB1293MI
Human centromere proteins	Antibodies Incorporated	15-234-0001
IRF3	Abcam	ab68481
p65	Abcam	ab16502
Pericentrin	Abcam	ab4448
RelB	Abcam	ab180127
ssDNA	Thermo Fisher Scientific	MAB3299MI
Vimentin	Abcam	ab201637
STING	Abcam	ab181125

Supplementary Table 2. Antibodies used in immunoblots.

Antibody against	Company	Catalog number
β -actin	Abcam	ab8227
cGAS	Sigma Aldrich	HPA031700
GFP	Life Technologies	A11122
IRF3	Abcam	ab68481
p100/p52	Cell Signaling Technology	4882
p65	Abcam	ab16502
phospho-IRF3	Cell Signaling Technology	4947
phospho-p100	Abcam	194919
phospho-p65	Cell Signaling Technology	3033
phospho-TBK1	Cell Signaling Technology	5483
RelB	Cell Signaling Technology	4922
STING	Cell Signaling Technology	13647
TBK1	Cell Signaling Technology	3013
TRAF2	Cell Signaling Technology	4712
TRAF3	Cell Signaling Technology	4729
STAT1	Abcam	ab47425

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

ATCC

b. Describe the method of cell line authentication used.

None as directly purchased from ATCC

c. Report whether the cell lines were tested for mycoplasma contamination.

Yes. Cells were tested each 4-6 months.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

None of the cell lines used are listed in the ICLAC database.

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Animal experiments were performed in accordance with protocols approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee. There was no need to randomize animals. Investigators were not blinded to group allocation. Intracardiac injection was performed as previously described³¹. Briefly, cells were trypsinized and washed with PBS and a 1×10^5 cells (in 100 μ l of PBS) were injected into the left cardiac ventricle of female athymic 6-7 week old athymic nude (nu/nu) mice (Jackson Laboratory strain 002019). 2×10^5 cells were injected into the tail vein cohort of animals. Mice were then immediately injected with D-luciferin (150 mg/kg) and subjected to bioluminescence imaging (BLI) using an IVIS Spectrum Xenogen instrument (Caliper Life Sciences) to ensure systemic dissemination of tumor cells. Metastatic burden was measured 5-7 weeks after injection using BLI. BLI images were analyzed using Living Image Software v.2.50. Disease-specific survival endpoint was met when the mice died or met the criteria for euthanasia under the IACUC protocol and had radiographic evidence of metastatic disease. For Orthotopic tumor implantation, 2.5×10^5 cells in 50 μ l of PBS were mixed 1:1 with Matrigel (BD Biosciences) and injected into the fourth mammary fat pad. Only one tumor was implanted per animal. MDA-MB-231 primary tumors were surgically excised before they reached ~1.5 cm in the largest dimension (which was the maximum allowable under our IACUC protocol) and metastatic dissemination was assessed using BLI imaging at 1-3 week intervals for up to 30 weeks. Distant metastasis-free survival endpoint was met when BLI signal was seen outside of site of primary tumor transplantation. 4T1 tumors were excised 9 days after implantation. To derive short-term culture from primary tumors and metastases, anesthetized animals (isofluorane) were imaged then sacrificed. Ex-vivo BLI was subsequently performed on harvested organs to define the precise location of the metastatic lesion. Primary tumors and metastases were subsequently mechanically dissociated and cultured in DMEM with selection media (G418 or hygromycin) to select for tumor cells and exclude host cells. All subsequent assays (karyotyping, RNAseq, immunofluorescence, and subcellular fractionation) were performed after a single passage from the primary sample. To assess chromosome missegregation from primary tumor-derived and metastases-derived cells, we performed high-resolution immunofluorescence analysis on passage #1 cells, staining for DNA (DAPI) and centromeres (ACA). Cells with DNA or centromere staining in the middle of the anaphase plate was taken as evidence of chromosome missegregation.

Policy information about [studies involving human research participants](#)

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

All human samples reported in the manuscript were from studies that were previously published and are adequately referenced in the manuscript. Details of patient characteristics - if available - is listed in the referenced manuscript.

Emerging views of the nucleus as a cellular mechanosensor

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The ability of cells to respond to mechanical forces is critical for numerous biological processes. Emerging evidence indicates that external mechanical forces trigger changes in nuclear envelope structure and composition, chromatin organization and gene expression. However, it remains unclear if these processes originate in the nucleus or are downstream of cytoplasmic signals. Here we discuss recent findings that support a direct role of the nucleus in cellular mechanosensing and highlight novel tools to study nuclear mechanotransduction.

Cells are constantly exposed to mechanical forces, such as shear forces on endothelial cells¹, compressive forces on chondrocytes² and tensile forces in myocytes³. The ability of cells to sense and respond to these mechanical cues is critical for numerous biological processes, including embryogenesis^{4,5}, development^{4,5} and tissue homeostasis^{6,7}. Although it has long been recognized that mechanical forces can influence cell morphology and behaviour^{8,9}, the understanding of the molecular pathways involved in mechanosensing, and how disruption of these pathways can give rise to various diseases, is still in progress^{10–13}. Stretch-activated ion channels, adhesion complexes, cell–cell junctions and cytoskeletal components have all been identified as mechanosensitive elements that can activate cellular signalling pathways, such as Rho-family GTPases or the mitogen-activated protein kinase–extracellular signal-regulated kinase (MAPK–ERK), induce nuclear translocation of the transcriptional regulators YAP/TAZ and MKL1, and ultimately result in expression of mechanoresponsive genes (see refs^{14–18} for review). Over the last two decades, the question whether the nucleus itself can sense mechanical stimuli has received increasing attention^{19,20}. Such ‘nuclear mechanotransduction’ could provide a more rapid and direct method to transduce forces into cellular events^{21,22} and act in concert with or independent of cytoplasmic mechanotransduction pathways. In this scenario, forces applied to the nucleus through the cytoskeleton may modulate the effect of cytoplasmic signals or even be sufficient to directly trigger changes in gene expression. Such multi-faceted mechanotransduction may enable cells to distinguish between small forces that only affect the cell surface, and larger forces that result in large-scale deformations of the cell and nucleus. Encouraged in part by advances in biophysical, biochemical and imaging assays, multiple mechanisms have been proposed to explain how forces acting on the nucleus could influence chromatin organization, transcription and other cellular processes^{19,22–24}. However, distinguishing between nuclear events that are downstream of cytoplasmic mechanosensitive signalling pathways, and those that reflect true nuclear mechanotransduction events, remains challenging.

One aspect, which is commonly accepted now, is that extracellular and cytoplasmic forces are transmitted across the nuclear envelope to the nuclear interior, where they can cause deformation of chromatin and nuclear bodies^{20,25–27}. Intriguingly, a recent study has demonstrated that force application to the nucleus can induce chromatin stretching and expression of a reporter transgene²⁸. These findings provide some of the most direct evidence to date

for the nucleus as a mechanoresponsive organelle. Below we discuss current findings that support nuclear mechanotransduction, explain potential molecular mechanisms and highlight emerging technologies to study nuclear mechanotransduction.

The nucleus and the nuclear lamina

The nucleus is the largest and stiffest organelle in the cell^{29,30}. It can broadly be separated into the nuclear interior (which contains chromatin), nuclear bodies and other intranuclear elements, and the surrounding nuclear envelope. The nuclear envelope consists of the outer and inner nuclear membranes (ONM and INM, respectively), which contain a large number of membrane-bound proteins^{31,32}, as well as nuclear pore complexes (NPCs) that control entry of large molecules into the nuclear interior³³. Underneath the INM lies the nuclear lamina, a filamentous protein network that consists of A-type and B-type lamins, and lamin-binding proteins^{34,35}. In mammalian somatic cells, the major A-type lamin isoforms are lamin A and C, encoded by the *LMNA* gene. One major reason to study the role of the nucleus in mechanotransduction came from the identification of *LMNA* mutations as the genetic cause for various forms of muscular dystrophy and cardiomyopathy^{36–38}. Diseases caused by lamin mutations (commonly referred to as laminopathies) remain both intriguing and perplexing. Although A-type lamins are nearly ubiquitously expressed, many of the *LMNA* mutations predominantly affect mechanically active tissue, such as skeletal muscle, cardiac muscle and tendons. These tissue-specific disease phenotypes suggest that defects in the nucleus can impair the ability of cells to respond appropriately to mechanical forces. It is now well-recognized that the nuclear lamina governs numerous biological functions, both biophysical and biochemical, including determining nuclear size and stiffness^{39–43}, regulating translocation and activity of transcription factors^{44–47}, interacting with chromatin and regulating its epigenetic state^{48,49}, and controlling cell polarization and migration^{50–52}. Consequently, cells lacking lamin A/C or expressing lamin A/C mutations linked to human diseases display severe defects in nuclear stability^{53–55}, cytoskeletal dynamics^{47,51} and nucleo-cytoskeletal force transmission^{55,56}. Furthermore, lamin A/C-deficient and -mutant cells fail to adequately activate mechanoresponsive genes when subjected to mechanical stimulation^{43,57,58}, suggesting an important role of the nucleus, and lamin A/C in particular, in cellular mechanotransduction. However, it remains incompletely understood to what extent lamins directly respond to mechanical stress in vivo,

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and whether changes in lamin levels and organization are downstream of other mechanotransduction pathways^{26,59–61}. The importance of the nuclear lamina in fundamental biological processes is highlighted by the early death of mice that lack functional lamin A/C. These mice are born without any overt defects, but develop severe muscular dystrophy and dilated cardiomyopathy and die at 2–8 weeks of age^{62,63}. Uncovering how lamins mediate nuclear processes and mechanosensitive gene expression will not only enhance our understanding of mechanotransduction per se, but may also provide insights into the pathophysiology of laminopathies, with the potential to inform therapeutic approaches for these currently incurable diseases.

Force transmission to the nucleus

Work by the Ingber group in the 1990s provided some of the first evidence that forces can be transmitted from the cell surface to the nucleus through the cytoskeleton²⁰. It is now recognized that these forces are transmitted across the nuclear envelope through the 'linker of nucleoskeleton and cytoskeleton' (LINC) complex^{64,65}. The LINC complex consists of nesprin proteins that reside within the ONM and contain a C-terminal KASH (Klarsicht, ANC-1, Syne homology) domain, which interacts with SUN (Sad1 Unc-84) domain-containing proteins located on the INM. The SUN proteins in turn bind to the nuclear lamina, nuclear pores and chromatin⁶⁶ (Fig. 1a). On the cytoplasmic side, nesprins can interact with each other and with all major cytoskeletal filaments. The composition of the LINC complex and LINC complex-associated proteins varies depending on the cell type. Furthermore, both nesprin-1 and nesprin-2 contain alternative start and stop sites that produce a number of isoforms, including the so-called 'giant' variants, which contain an N-terminal actin-binding domain⁶⁷. Nesprin-1 and nesprin-2 can bind to actin filaments⁶⁷ and the microtubule-associated motor proteins kinesin⁶⁸ and dynein⁶⁹, nesprin-3 binds to plectin⁷⁰, which connects to intermediate filaments, whereas nesprin-4 interacts with kinesin-1⁷¹ (Fig. 1a). Additional KASH-domain-containing proteins and LINC complex-associated proteins have recently been characterized and are often cell-type-specific. We refer the readers to recent reviews on the LINC complex for further details^{34,66,67,72}.

Whereas external forces can be applied to the nucleus independently of the LINC complex, for example, during compression of the nucleus⁷³ or cell migration through confined environments⁷⁴, cells in many cases require an intact LINC complex to effectively transmit forces between the cytoskeleton and the nucleus. Consequently, depletion or expression of dominant-negative nesprin or SUN proteins severely impairs nucleo-cytoskeletal force transmission²⁷ and mechanosensitive gene expression^{28,75}. Nonetheless, it remains to be tested whether impaired mechanotransduction is due to the role of components of the LINC complex in intracellular force transmission, or whether these proteins contribute through other functions, such as serving as signalling scaffolds or regulating other aspects of nuclear organization including chromatin mobility and nuclear envelope tethering^{49,76}. Force-induced nuclear deformation further requires an intact and adequately tensed cytoskeletal network^{77,78} to transmit forces from the cell surface to the nucleus²². If the actin cytoskeleton is disrupted through pharmacological or genetic approaches, force transmission to the nucleus is impaired^{78,79}, which is accompanied by changes in chromatin dynamics⁷⁷. Notably, mechanically induced changes in the nucleus, cytoskeleton and extracellular matrix appear to be interrelated. For example, the mechanical properties of the extracellular matrix affect both cytoskeletal organization⁸⁰ and expression of lamin A/C^{61,81}, resulting in cells that are finely tuned to their physical environment.

The intricate relationship between the cytoskeletal network, nuclear mechanics and the mechanical environment is particularly important for skeletal and cardiac muscle cells. These contractile cells have a highly organized cytoskeleton, including a

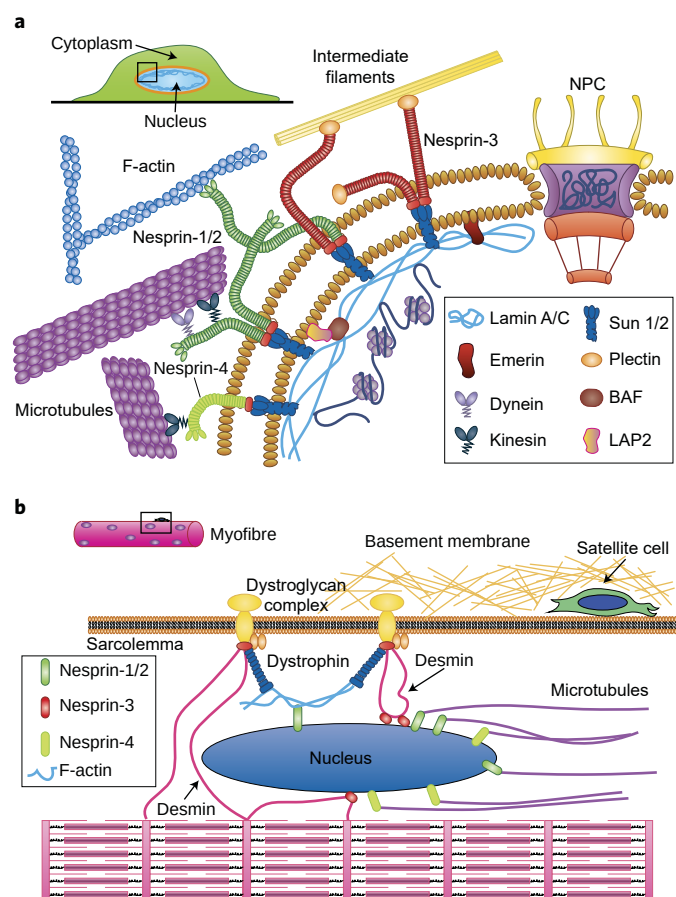


Fig. 1 | Schematic overview of nuclear envelope proteins involved in force transmission to the nucleus. **a**, Force transmission to the nucleus involves interaction of cytoskeletal elements (actin filaments, intermediate filaments, microtubules) with nesprin proteins on the ONM that transmit force through SUN domain proteins on the INM to the nuclear lamina and interior. **b**, Organization of the cytoskeletal network within muscle cells, including the highly ordered actin-myosin structures that form contractile sarcomeres and myofibrils. Nuclei are positioned at the periphery of the cell, where they interact with the muscle-specific proteins dystrophin (through actin filaments) and desmin. Additional proteins, such as LINC-complex proteins and lamins, may be involved in anchoring the myonuclei as well as generating and transmitting forces between the nucleus and cytoskeleton.

specialized perinuclear network that anchors the nucleus in place (Fig. 1b). Desmin is a muscle-specific cytoplasmic intermediate filament that interacts with the nuclear envelope through plectin⁸². This interaction is important for myofibre health⁸³ and functional loss of plectin releases tension on the nucleus resulting in altered expression of mechanoresponsive genes⁸². LINC-complex proteins have similarly important functions in muscle cells. The LINC complex is required for myonuclear movement^{84–87}, including the effective spacing of nuclei along the myofibre length. Loss of LINC-complex function causes muscular dystrophies^{88–90}, suggesting that adequately connecting the nucleus to the cytoskeleton is crucial for skeletal muscle health and maintenance. This idea is further supported by the finding that *LMNA* mutations that cause muscular dystrophy and dilated cardiomyopathy result in impaired nucleo-cytoskeletal coupling^{55,91,92} and loss of structural function, whereas *LMNA* mutations associated with lipodystrophy have little or no effect on nuclear mechanics and nucleo-cytoskeletal force transmission^{55,91}.

Although striated muscle tissues are affected the most by disruption in nuclear mechanics and nucleo-cytoskeletal coupling, many other cell types are also affected by impaired nucleo-cytoskeletal force transmission^{93,94}. For example, T cell activation requires lamin A/C and the LINC complex to function properly to regulate T cell receptor clustering and F-actin formation⁹³. In fibroblasts and endothelial cells, depletion of lamin A/C or disruption of the LINC complex reduces migration capabilities^{94–96}. Similarly, the LINC complex is important in outer hair cells for hearing⁹⁷, proper function of the ciliary rootlets in photoreceptors and ependymal cells⁹⁸, hair follicle structure⁹⁹ and radial neuronal migration during neurogenesis¹⁰⁰. These findings demonstrate the broad importance of nucleo-cytoskeletal force transmission on cellular function.

Potential mechanisms for nuclear mechanotransduction

The negative effects of lamin mutations and LINC complex disruption are well-documented, but the underlying molecular mechanisms remain incompletely understood. External forces are transmitted across the cytoskeleton to the nucleus, where they result in substantial deformation^{101–103}. These forces and deformations could modulate transcriptional activity and chromatin organization through a number of mechanisms.

One potential mechanism to transduce forces acting on the nucleus into altered transcriptional activity is by modulating the physical organization of chromatin. The spatial location of the DNA within the nucleus exists in a non-random organization. This '4D nucleome' (meaning the 3D chromatin architecture and its change over time) is important for transcriptional regulation and cellular functions^{104–107}. Heterochromatic DNA, which is tightly wrapped around histones and largely inaccessible for the transcriptional machinery, is often localized at the nuclear periphery⁴⁹. This peripheral localization promotes gene silencing, whereas repositioning of genes towards the interior of the nucleus generally facilitates gene activation¹⁰⁸, although additional regulatory networks exist. Thus, force-induced changes in gene positioning relative to the nuclear periphery could alter the transcriptional activity of specific genes and contribute to nuclear mechanotransduction. Supporting this idea, altering cytoskeletal organization and tension by culturing cells on micropatterned substrates alters nuclear shape and chromosome distribution, accompanied by changes in gene expression^{103,109}. It remains unclear to what extent these changes are the direct result of altered cytoskeletal forces acting on the nucleus versus upstream signalling pathways that may be sensitive to cytoskeletal organization. Extrinsic force application to cells can also induce repositioning of nuclear bodies and the associated chromatin^{110–112}, which could affect additional nuclear processes. Lastly, whereas changes in chromatin organization may be downstream of forces acting on the nucleus, the epigenetic state of chromatin also contributes to the mechanical properties of the nucleus: chromatin decondensation increases nuclear deformability and chromatin condensation decreases nuclear deformability^{110,113–116}, both of which may occur independently of changes in lamin levels¹¹⁷. Thus, changes in nuclear organization, even when downstream of other pathways, can have a direct effect on nuclear deformation and may thus modulate other nuclear mechanotransduction processes.

In addition to changes in gene or chromosome positioning, mechanical forces may directly alter chromatin organization and transcription. *In vitro* experiments indicate that 5 pN of force is sufficient to decondense single chromatin fibres¹¹⁸. Recent work from the Wang and Belmont laboratories demonstrated that applying force to the cell surface results in instantaneous stretching of chromatin inside the nucleus, associated with rapid induction of transcription of a transgene that is located within the stretched region of chromatin²⁸. Notably, the level of transcription correlated with the frequency and magnitude of the applied forces, and disruption of the LINC complex abolished the force-mediated transcription

response²⁸. The finding that force-induced transcription occurred extremely rapidly (<30 seconds) suggests that the stretching of chromatin alters the accessibility of the transcriptional machinery to the gene or its activity, rather than altering the epigenetic state of the locus. Although highly intriguing, the directly mediated modulation of gene expression has not yet been demonstrated for endogenous genes. Furthermore, it remains to be investigated whether this mechanism of modulating gene transcription only applies to genes that are already 'primed' for transcription, or if it could also activate silenced genes, such as those in heterochromatic regions. Notably, prolonged force application induces an increase in heterochromatin and transcriptional repression⁴, which could serve as a negative feedback mechanism. Lastly, it remains unclear how force-induced chromatin stretching would be able to confer specificity, as it is likely that multiple genomic loci are subjected to a similar level of mechanical force, and a direct association between mechanoreponsive genes and components of the LINC complex has not been demonstrated to date.

Force-induced molecular crowding could present another potential nuclear mechanotransduction mechanism. Nuclear deformation could also alter nuclear processes by local crowding and exclusion of soluble factors in areas where chromatin has been compacted. For example, exclusion of DNA-damage repair factors delays repair of DNA breaks^{119,120}. Similarly, exclusion of transcriptional regulators or chromatin remodelling proteins could alter transcriptional activity.

Recent studies revealed that mechanical stress can induce conformational and post-translational changes (for example, phosphorylation) in nuclear envelope proteins^{26,60,61,121} (Fig. 2). Force application on the nucleus results in apical-to-basal differences in the conformation of lamin A/C, as shown by the masking of certain C- and N-terminal epitopes under tension⁶⁰. Exposing isolated nuclei to shear stress exposes a cryptic cysteine residue (Cys552) in the Ig-domain of lamin A/C, which is normally inaccessible during periods of low mechanical stress⁶¹. It remains to be investigated whether this residue becomes exposed under physiological forces in intact cells, as the N-terminal portion of the Ig-domain appears to be mostly inaccessible during periods of high mechanical stress *in vivo*⁶⁰. Recent findings further indicate that reduced cytoskeletal tension, for example when cells are cultured on soft substrates, results in increased lamin A/C phosphorylation, which is associated with increased solubility and degradation^{61,121}. By contrast, increased cytoskeletal tension results in decreased lamin A/C phosphorylation and higher lamin A/C levels¹²¹. Similarly, force application to isolated nuclei through the LINC complex causes phosphorylation of the INM protein emerin²⁶, which binds to lamin A/C. It is unclear whether these phosphorylation events are triggered by increased residue accessibility after force-induced conformational changes, or whether force application modulates the activity of nuclear kinases such as Src¹²². Regardless of the specific mechanism, mutating the relevant Tyr74 and Tyr95 sites in emerin leads to decreased stress-fibre formation and decreased expression of SRF-dependent genes²⁶. In response to prolonged force application, emerin may also serve to reinforce the actin network at the ONM and facilitate chromatin remodelling⁴. Although additional work is needed to elucidate the specific pathways involved, including whether emerin and lamin are downstream of other mechanosensitive signalling events and which biochemical signals are activated by their phosphorylation, these findings demonstrate the relevance of nuclear envelope proteins for modulation of transcriptional activity as well as nuclear and cytoskeletal organization.

Force-induced stretching of nuclear membranes could present an additional mechanism for nuclear mechanotransduction. Hypotonic swelling of the nuclear membranes results in translocation of nucleoplasmic phospholipase A2 (cPLA2) to the INM, which is inhibited when the nucleus is stabilized by either F-actin or lamin A/C¹²³. This translocation directly activates cPLA2 and 5-LOX¹²³,

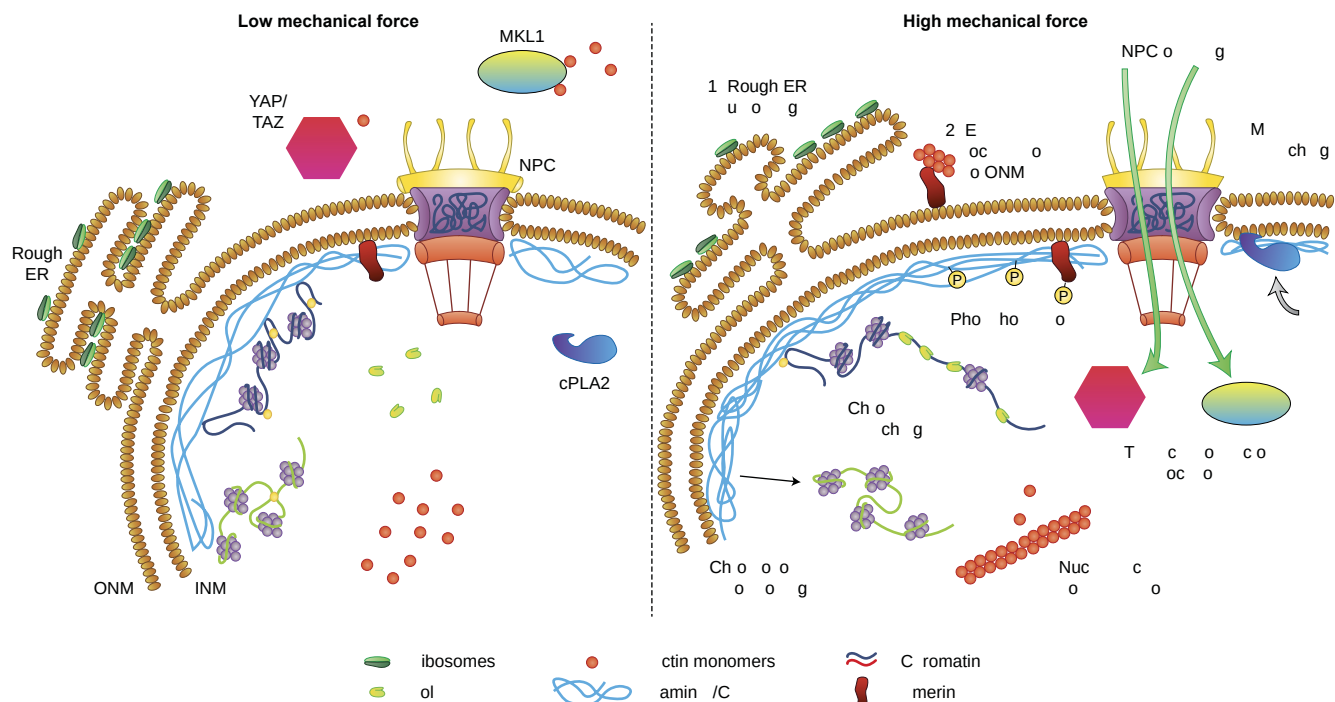


Fig. 2 | Proposed mechanisms for how the cell nucleus could respond directly to mechanical forces. (1) Stretching of the nuclear membrane could alter the conformation of the rough endoplasmic reticulum (ER), exposing more ribosomes to the cytoplasm. (2) Force application promotes translocation of emerin from the INM to the ONM, modulating chromatin organization and facilitating actin polymerization at the ONM. (3) Increased membrane tension could open NPCs and modulate NPC permeability. (4) Stretching of the nuclear membrane recruits cPLA2 to the INM. (5) Force transmission to the nucleus results in post-translational modification and altered dynamics of lamin A/C and INM proteins, such as emerin (see also (2)), which can modulate the mechanical properties of the nucleus and induce downstream signalling. (6) External forces can induce chromatin stretching, altering polymerase and transcription factor accessibility and activity. (7) Nuclear-pore opening and sequestration at the nuclear envelope can modulate localization and activity of transcription factors. (8) Forces acting on the nucleus may reposition chromatin domains, altering their transcriptional activity. (9) Mechanically induced polymerization of nuclear actin can modulate the export and activity of the transcriptional regulator MKL1, and affect other nuclear processes that require monomeric actin.

which are required for production of the chemotactic eicosanoids that attract leukocytes to sites of injury *in vivo*¹²³. Because the underlying nuclear lamina is substantially stiffer than the nuclear membranes, it mechanically shields the nuclear membranes from large forces. At the same time, the nuclear lamina can tolerate substantially larger area strains than lipid membranes^{115,116}. Thus, nuclear envelope composition and organization could markedly modulate the stretch response of the nuclear membrane. Furthermore, because the nuclear membranes are continuous with the endoplasmic reticulum, stretching of the nuclear membrane is expected to increase the membrane tension in the adjacent rough endoplasmic reticulum¹²⁴. It will be interesting to determine whether increased membrane tension on the nucleus can alter the organization of the rough endoplasmic reticulum, and possibly the distribution of membrane-bound proteins in the endoplasmic reticulum¹²⁵. For example, polysomes are enriched in the sheets rather than tubules of the endoplasmic reticulum¹²⁶, thus reducing membrane curvature could increase their exposure to the cytosol (Fig. 2).

An extreme form of nuclear mechanotransduction is force-induced nuclear membrane rupture. Compressive forces on the nucleus generated by actomyosin contractility can increase intranuclear pressure and result in nuclear membrane blebbing and transient loss of nuclear envelope integrity (that is, nuclear envelope rupture)^{79,127–130}. Although these phenomena were first observed in cells that were deficient in lamin A/C, carried lamin A/C mutations⁹¹ or had lower levels of B-type lamins⁷⁹, as well as in cancer cells with a compromised nuclear lamina⁵⁴, it is now apparent that all cells regularly exhibit transient nuclear envelope rupture. Defects in

the nuclear lamina, increased actomyosin contractility and external confinement can markedly increase the incidence of nuclear envelope rupture from a few per cent to the majority of cells^{79,131}. Cells typically restore nuclear envelope integrity and remain viable, but loss of nuclear envelope integrity results in uncontrolled exchange of cytoplasmic and nuclear proteins^{91,128}, mislocalization of organelles⁵⁴ and DNA damage^{128,129}. The effect of nuclear envelope rupture on cell signalling, chromatin organization, gene expression and long-term outcomes remain incompletely understood and are topics of active investigation. Transcriptome analysis of nuclear rupture induced by severe cell compression revealed activation of DNA-damage response pathways, metabolism and nucleolar RNA production¹³². Recent findings additionally point to an important function of cGAS, a cytoplasmic DNA-binding protein first recognized for its activation of the STING pathways when encountering viral DNA in the cytoplasm¹³³. The latest findings indicate that cGAS can also be activated when exposed to genomic DNA after nuclear envelope breakdown of micronuclei^{134–137}.

Increased nuclear membrane tension could also potentiate cytoplasmic signalling pathways by altering the permeability of NPCs (Fig. 2). Current models generated from the atomic structures of NPC components suggest that the NPCs can undergo conformational changes that constrict or dilate the NPC in response to mechanical force^{138–140}. Force-mediated alterations to NPC conformations could arise from an increase in nuclear membrane tension or force transmission through LINC-complex proteins and nuclear lamins. Both Sun1 and lamin A/C interact with NUP153^{141,142}, a protein that comprises a portion of the NPC

basket¹⁴³. In support of this mechanism, recent work by the Rocas-Cusachs group found that direct force application to the nucleus is sufficient to promote nuclear entry of YAP, a mechanosensitive transcription factor⁷³. The increase in nuclear YAP localization occurs through increased nuclear import of YAP, mediated by an increase in the permeability of the NPC for larger proteins, and the partial unfolding of YAP to further promote transition through the NPC⁷³. Besides an increase in NPC permeability, other nuclear envelope proteins may modulate the import or export of mechanosensitive transcription factors, such as YAP/TAZ and MKL1^{47,57,144}, through additional mechanisms (Fig. 2). Lamin A/C has also been shown to sequester transcription factors, such as retinoblastoma protein^{145,146} and Fos⁴⁴, at the nuclear periphery and thereby control their activity within the nucleus. Through these mechanisms, the nuclear lamina may further modulate gene expression and cell behaviour.

Whereas short-term force application has been shown to rapidly induce transcription^{28,43}, long-term force application (12 h) can result in a global increase in heterochromatin and transcriptional repression⁴, suggesting that there may be a different response to force application that depends on the duration of stimulation. Future studies will also need to consider differences in the response across cell types, as certain cell types may have an increased susceptibility to chromatin stretching that results from differences in lamin A/C expression⁶¹. Lastly, although it appears that chromatin stretching can rapidly increase gene activation and PolII recruitment (Fig. 2), prolonged mechanical stimulation probably activates mechanoresponsive feedback mechanisms that further influence gene expression, nuclear organization and nucleo-cytoskeletal force transmission. Notably, mechanical force application to isolated nuclei through nesprins results in lamin-A/C recruitment and emerin phosphorylation, causing nuclear stiffening²⁶. Thus, biochemical signalling pathways activated by mechanoresponsive genes could result in similar feedback loops that alter the responsiveness of the cell to further mechanical forces.

Technologies to study nuclear mechanotransduction

One major challenge in the field of nuclear mechanotransduction is uncoupling changes in nuclear structure, organization and transcription that are directly due to force application to the nucleus from those that are secondary to changes in cytoplasmic mechanosensitive signalling pathways. Addressing this challenge requires (1) improvements in the temporal resolution of nuclear events to distinguish between immediate and downstream consequences; (2) enhanced detection of force-induced changes in chromatin organization and local transcription; (3) direct measurements of intranuclear and perinuclear forces; and (4) experimental approaches that can physically separate contributions of mechanotransduction on the nucleus and cytoplasm.

One method to study the force-induced relocation of genes within the nucleus, or the local stretching and unfolding of chromatin loops within a single chromosomal region, is to insert arrays of LacO sequences into specific genomic loci and then fluorescently label these sites with GFP–LacI²⁸ (Fig. 3). This reporter system enables the assessment of how effective chromatin stretching, measured by an increased distance between adjacent GFP–LacI loci, corresponds to changes in gene expression of the reporter gene, which can be quantified by fluorescence in situ hybridization of the RNA transcript. Recent developments in the labelling of specific genomic regions of endogenous genes using CRISPR–Cas9 and related systems could help to overcome the challenge of having to insert large LacO arrays or using bacterial artificial chromosome reporters, and may even enable multi-colour imaging by using dCas9 constructs from different bacterial species, each tagged with a different fluorophore^{147,148} (Fig. 3). Measuring changes in the 4D nucleome could be further aided by the use of

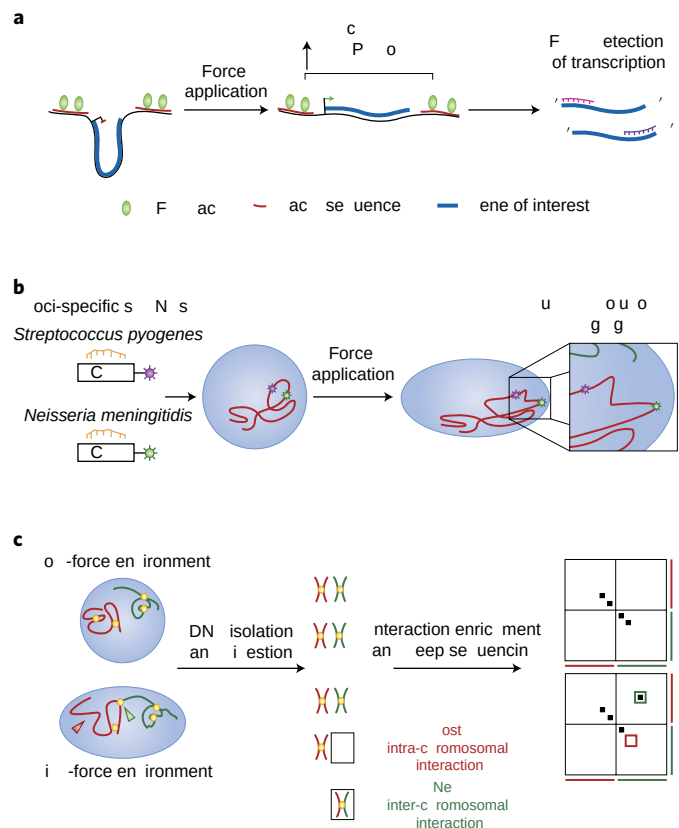


Fig. 3 | Technologies to study the effect of force transmission to the nucleus on genome organization and gene regulation. **a**, Schematic of a reporter transgene that measures chromatin stretching.

The transgene is flanked by two fluorescently labelled regions of DNA. An increase in the distance between the fluorescent spots indicates effective chromatin stretching. Changes to the level of transcription of the transgene can be assessed by RNA fluorescence in situ hybridization (FISH), enabling analysis of the correlation between force-induced chromatin stretch and changes in transgene expression. **b**, Specific endogenous DNA loci can be fluorescently labelled using CRISPR–dCas9 from different species. Changes to the positioning and spacing between adjacent loci following force application can be determined with high resolution by fluorescence microscopy. **c**, Hi-C maps genome-wide chromatin interactions using deep sequencing, with changes to the interaction profile being displayed using heat maps. Interactions appear as hot spots off the diagonal.

super-resolution microscopy, which allows resolving of features that are 20–100 nm in intact cells¹⁴⁹ (Table 1). In addition to optical microscopy-based approaches, changes to the arrangement of chromosomes can be studied using sequence-based technology, such as Hi-C, which is based on chromosome conformation capture-based methodology¹⁵⁰ (Fig. 3). Hi-C can detect chromatin interactions across the entire genome, both within and between chromosomes, by covalently crosslinking protein–DNA complexes in their in situ configuration followed by deep sequencing. Whereas Hi-C is traditionally performed on large cell numbers (approximately 10⁶ cells), approaches are currently under development that extend this technique to smaller cell numbers and even single cells¹⁵¹. Changes in the accessibility of DNA regions may provide additional information on force-induced changes in chromatin organization that could modulate transcriptional activity. One exciting approach is the assay for transposase-accessible chromatin using sequencing (ATAC-seq), which identifies accessible chromatin regions on the basis of the insertion of a hyperactive transposase and subsequent genome

Table 1 | Microscopy techniques: examples of super-resolution microscopy and their application to study nuclear processes and structures

Type of microscopy	Mechanism of action	Application to imaging nuclear structures
Stimulated emission depletion.	Enhances resolution by depleting fluorescence in specific regions of the sample while leaving a center focal spot active to emit fluorescence. This is achieved by generating a 'doughnut' around the focal spot using a second depletion laser beam.	γ -H2AX foci colocalizing with Ku foci ¹⁶⁰ . Mobility of proteins being imported into the nucleus ¹⁶¹ .
Spatially modulated illumination.	Spatially modulated illumination microscopy achieves higher spatial resolution by modulating the illuminating light along the optical axis, after which the sample is moved through a standing wave field at precise axial steps. This technique provides improved z-axis resolution for each of the fluorophores ¹⁶² .	Chromatin compaction of specific loci ¹⁶³ . Live-cell measurements of a Tet-operator repeat insert in U2OS cells ¹⁶⁴ .
Structured illumination microscopy.	Similar to spatially modulated illumination microscopy in that it generates a spatially modulated illumination pattern; however this occurs along the object plane (x, y) rather than the optical (z) plane ¹⁶⁵ . Multiple images are acquired and then computationally combined to generate an image with twice the resolution than traditional wide-field microscopy ¹⁶⁵ .	RecA bundle formation and localization ¹⁶⁶ . NPC colocalization with channels in the lamin network and peripheral heterochromatin ¹⁶⁷ .
Photo-activated localization microscopy and stochastic optical reconstruction microscopy.	Identify precise locations of individual fluorophores by using photoswitchable fluorophores to achieve optical isolation of the signal ¹⁶⁸ .	Volume of chromatin in different epigenetic states ¹⁶⁹ . H2B localization in interphase cells ¹⁷⁰ .

fragmentation and sequencing¹⁵². Applying Hi-C and ATAC-seq analyses to cells in high- and low-force environments, or to cells before and after nuclear force application, should provide detailed information on how external forces alters the spatial interactome of chromatin, which could be further coupled with RNA-sequencing analyses to determine whether chromatin changes correspond to changes in gene transcription.

Molecular tension sensors can provide insights into the forces applied across specific cellular structures. Biophysical measurements of intact cells and isolated nuclei indicate that approximately 1–10 nN are required to induce substantial nuclear deformation^{116,153,154}. The recent development of a nesprin tension biosensor has enabled the first measurements of forces transmitted across the LINC complex^{25,155}. Using an artificial nesprin-2 giant construct containing a Förster resonance energy transfer (FRET)-based tension module, it was shown that force transmission changed with both myosin activity and cell elongation, and that the basal and apical sections of the nucleus are exposed to different forces²⁵. Potential limitations of the current version of the tension sensor include a low signal-to-noise ratio, the insertion site of the FRET tension module, and a limit of the force range of about 6 pN^{156,157}, encouraging further work in this area.

Lastly, one way to circumvent the confounding cytoplasmic signalling events that arise from applying force at the cell surface is to study isolated nuclei or to use micromanipulation to apply force in close proximity to the nucleus^{158,159}. Using magnetic beads bound to the cytoplasmic domain of nesprins allows studying the role of the LINC complex in nuclear mechanotransduction and targeting specific nesprin isoforms²⁶. One limitation of using isolated nuclei is that the isolation procedure may perturb nuclear structure, as well as the chemical composition of the nuclear interior (for example, ion concentrations, ATP levels and molecular crowding), which could affect nuclear mechanics and other nuclear processes⁴². Furthermore, working with isolated nuclei limits experiments to studying factors that originate within the nucleus and excludes studying the import of cytoplasmic factors. Disrupting the LINC complex in intact cells allows exchange of biochemical molecules and can help to identify events that require force transmission to the nucleus and nuclear deformation¹⁵⁹. However, external force application may still induce nuclear deformation through LINC-complex independent mechanisms.

Future perspective

The field of mechanobiology has substantially evolved and advanced in the past two decades, greatly increasing our knowledge of how mechanical cues govern cell behaviour. It is now well-recognized that nuclear envelope proteins have an important role in the cellular response to mechanical stimuli, and that forces are transmitted from the cell surface and cytoskeleton to the interior of the nucleus. Findings suggest that the nucleus can act as a cellular mechanosensor. Nonetheless, many questions remain, including to what extent the nucleus itself responds to mechanical forces, where such nuclear mechanotransduction processes occur, and whether these nuclear processes complement or act in parallel or downstream of cytoplasmic signalling pathways. To further untangle the strong interplay between the nucleus, cytoskeleton and cell surface will require an integrative approach that uses biophysical assays, genetic manipulation, high-throughput genomics and proteomics, and live-cell imaging with high spatial and temporal resolution. Furthermore, experimental approaches must be used that attempt to uncouple nuclear changes due to indirect mechanisms (that is, cytoplasmic signals that modulate chromatin organization and transcription) from force-induced, nucleus-intrinsic events, for example, by utilizing models in which nuclear force transmission is disrupted while other cytoplasmic mechanosensitive pathways remain intact. Unravelling the force-sensitive molecular regulatory networks controlled by the nucleus and the nuclear lamina will not only increase our understanding of cellular mechanotransduction, but may also encourage the development of novel therapeutical approaches to treat the currently incurable diseases that arise from impaired nuclear mechanics and mechanotransduction.

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Competing interests

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Elevated nuclear lamin A is permissive for granulocyte transendothelial migration but not for motility through collagen I barriers

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Summary sentence: Differential effects of nuclear stiffness on chemokine-driven leukocyte squeezing through endothelial and extracellular collagenous barriers

Running title: Role of lamin A in leukocyte migration

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Abbreviations

dHL-60, differentiated HL-60 cells; EC, Endothelial Cells; HDMVEC, Human Dermal Micro-Vascular Endothelial Cells; HBSS, Hank's Balanced Salt Solution; LBR, lamin B receptor; OE, Overexpressing; SEM, scanning electron microscopy; TEM, Transendothelial migration; 2D, Two Dimensional; 3D, Three Dimensional

Abstract

Transendothelial migration (TEM) of lymphocytes and neutrophils is associated with the ability of their deformable nuclei to displace endothelial cytoskeletal barriers. Lamin A is a key intermediate filament component of the nuclear lamina which is downregulated during granulopoiesis. When elevated, lamin A restricts nuclear squeezing through rigid confinements. To determine if the low lamin A expression by leukocyte nuclei is critical for their exceptional squeezing ability through endothelial barriers, we overexpressed this protein in granulocyte-like differentiated HL-60 cells. A 10-fold higher lamin A expression did not interfere with chemokinetic motility of these granulocytes on immobilized CXCL1. Furthermore, these lamin A high leukocytes exhibited normal chemotaxis towards CXCL1 determined in large pore transwell barriers, but poorly squeezed through 3- μ m pores towards identical CXCL1 gradients. Strikingly, however, these leukocytes successfully completed paracellular TEM across inflamed endothelial monolayers under shear flow, albeit with a small delay in nuclear squeezing into their sub-endothelial pseudopodia. In contrast, CXCR2 mediated granulocyte motility through collagen I barriers was dramatically delayed by lamin A overexpression due to a failure of lamin A high nuclei to translocate into the pseudopodia of the granulocytes. Collectively our data predict that leukocytes maintain a low lamin A content in their nuclear lamina in order to optimize squeezing through extracellular collagen barriers but can tolerate high lamin A content when crossing the highly adaptable barriers presented by the endothelial cytoskeleton.

Introduction

The nucleus is the largest cellular organelle and is mechanically stabilized by a constitutive network of laminar proteins [1]. Nucleus deformation is the rate-limiting step for cells to pass through constrictions that are smaller than the nucleus size [1-5]. The mechanical stability of the nucleus, particularly for large deformations, is dictated by lamins, intermediate filaments proteins that form a network underlying the inner nuclear membrane [4, 6, 7]. Lamin A and its spliced variant lamin C impart the nucleus with its mechanical stability whereas the lamin B1 and B2 intermediate filaments are ubiquitously expressed but appear to be less important in mechanical stability of nuclei [8]. The nuclei of both circulating T-cells and neutrophils are soft due to a low content of lamin A/C and B in their lamina [9]. Furthermore, in contrast to epithelial and mesenchymal cells and solid tumors, which usually keep their stiff nuclei at their rear, motile leukocytes translocate their soft nucleus to their leading edge (pseudopodia) irrespective of the barriers they cross [10]. However, the relationship between lamin composition, nuclear stiffness and nucleus location and the impact of each of these parameters on the squeezing ability of leukocytes and other cells remains obscure.

It has been traditionally argued that leukocyte squeezing through endothelial barriers and extracellular barriers composed of collagen fibers involves passage through sub-micron wide gaps. Recent reports suggest, however, that leukocyte TEM involves a considerable widening of paracellular endothelial junctions as well as transcellular endothelial channels by the squeezing leukocyte [10, 11]. This leukocyte driven widening involves large

displacement of endothelial stress fibers rather than active endothelial contractility [10, 11]. Our real-time imaging of nuclei in transmigrating leukocytes also suggested that the endothelial gap widening by squeezing leukocytes is driven by nuclear lobes, which are either preexistent or de novo formed by the deformable nuclei of T-cells [10]. This recent analysis of nuclear squeezing dynamics also raised the possibility that nuclear deformation determines both the gap size generated by squeezing leukocytes during TEM and the dynamics of nuclear squeezing [10]. Leukocyte nuclei appear to function as mechanical “drillers” that displace and collapse different actin assemblies within the endothelial cytoskeleton [10]. Nevertheless, a direct molecular demonstration that nuclear deformation and mechanical stiffness determine leukocyte passage through endothelial barriers and control gap sizes has been missing.

To address these standing questions, we have used a model system based on differentiated HL-60, human promyelocytic leukemia cells, which upon in vitro differentiation give rise to either granulocyte-like leukocytes or macrophages [12]. Whereas genetic manipulation of freshly isolated neutrophils is difficult and can result in side effects caused by the procedures required for ectopic gene expression, HL-60 cells can be readily transfected or transduced with target vectors prior to their differentiation into short-lived granulocyte-like cells. This line has therefore been widely used for structural and functional assays of leukocytes [12-15]. Forced expression of lamin A in a similar HL-60 system, differentiated using all-trans-retinoic acid (ATRA) stimulation for 5 days, impaired nuclear lobulation during differentiation and inhibited serum triggered leukocyte perfusion through narrow channels and

migration through rigid pores [16]. We used a similar system of DMSO differentiated HL-60 to generate granulocyte-like cells with 10-fold higher expression of lamin A. Having validated that the ability of these cells to transit through rigid micron-scale constrictions is severely compromised, we further investigated the ability of these leukocytes to squeeze through inflamed endothelial monolayers as well as through distinct fibrous barriers. Our results indicate that granulocytes overexpressing lamin A successfully adhered to and squeezed through confluent inflamed endothelial cell monolayers and successfully completed TEM in response to chemotactic signals, displacing the endothelial cytoskeleton and translocating their body underneath the monolayer, albeit with slightly delayed rates and creating larger pores in the endothelial layer. On the other hand, these lamin A enriched cells exhibited major defects in their ability to migrate through collagen I barriers in response to similar chemotactic signals. Our results suggest that chemokine-guided leukocyte squeezing through different cytoskeletal and extracellular matrix barriers is restricted to different extents by a given alteration in the stiffness of the leukocyte nuclear lamina.

Materials and Methods

Reagents and antibodies

Human CXCL1 was purchased from Peprotech (Rocky Hill, NJ, USA). PE-anti-human CD11a, PE-anti-human/mouse CD11b (M1/70), PE-anti-human CD29 and PE-anti-human CXCR2 antibodies were purchased from Ebioscience-Thermo Fisher Scientific (Waltham, MA, USA). HECA-452 was purchased from BD Bioscience Pharmingen (Franklin Lakes, New Jersey, USA). Anti CD18 mAb (TS1/18) was a kind gift from D. Staunton (ICOS, Bothell, WA, USA). Anti lamin A/C mAb was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Rabbit polyclonal anti-human Lamin B1 was a kind gift from E. Gomes (University of Lisbon). Alexa 647 anti VE-cadherin mAb was purchased from Biolegend (San Diego, CA). R-Phycoerythrin AffiniPure F(ab')₂ Fragment Goat Anti-Rat IgM (μ chain specific) and Goat Anti-Mouse IgG (H+L) antibodies were purchased from Jackson ImmunoResearch Laboratories, INC. (West Grove, PA, USA). Bovine Serum Albumin (BSA; fraction V), Hoechst, HEPES, CaCl₂, MgCl₂, and Hank's Balanced Salt Solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The CXCR2-HL-60 cell variant line was described elsewhere [17]. Both parental HL-60 [16 1531] and the CXCR2-HL-60 variants were grown in RPMI-1640 medium supplemented with 20mM HEPES, 10% fetal calf serum, L-glutamine and Pen-Strep-Amphotericin. Cells were maintained at less than 1×10^6 cells/ml and differentiated into granulocyte-like cells (dHL-60) by culturing for 7 days in culture medium supplemented with 1.3% (V/V) Dimethyl Sulfoxide (DMSO).

Granulocyte-like appearance was confirmed by upregulation of CD11a and CD11b (Suppl. Fig. 1A, B). HDMVECs (C-12211; PromoCell, Heidelberg, Germany) were grown in PromoCell EC medium MV (C-22020), according to manufacturer's protocol and were used at passages 2-3.

Retroviral transduction

Stably modified lamin A overexpressing (LaminA-OE) CXCR2-HL-60 cells were generated by retroviral transduction with a bicistronic vector pRetroX-PrelaminA-IRES-ZsGreen1 as described [16]. A ZsGreen1 retroviral vector was used to generate the mock control cells. Retroviruses were produced by transfecting Phoenix cells using Lipofectamine® 2000 reagent following manufacturer's protocol. Retrovirus containing supernatant was collected at 48 hrs post transfection and used fresh to infect CXCR2-HL-60 cells in the presence of 4 mg/ml polybrene (Sigma). Cells were either sorted and maintained at >90% purity or taken as a mixture of lamin A-ZsGreen1 expressing and non-expressing cells for differentiation into granulocyte like cells using DMSO.

Flow Cytometry

For analysis of integrin or chemokine surface expression, cells were incubated with primary fluorescence-labeled mAb (CD11a, CD29, CD11b: 10 µg/ml) or unlabeled mAb (CXCR2, CD18, HECA-452: 10 ug/ml) followed by secondary antibodies (1:100, Jackson ImmunoResearch) for 20 min at 4°C per incubation. For intracellular stainings, cells were fixed in chilled 80% methanol for 5 min, followed by 3 washes and permeabilization with tween (0.1%, PBS, 20 min at

RT). Cells were incubated with anti-CD16/CD32 (10 µg/ml diluted in 10% goat serum) for 20 min RT for blockage of Fc receptors. Cells were then incubated with primary antibody (αLamin A/C or mIgG isotype control) diluted in 10% goat serum for 30 min at RT, washed, and stained with phycoerythrin-labeled goat anti-mouse secondary antibodies (1:100) for 30 min at RT in the dark. Antibody stainings and washes were carried out in fluorescence-activated cell sorting (FACS) buffer (Ca²⁺ and Mg²⁺ free PBS (PBS-/-), 1% BSA, 5mM EDTA, and 0.01% sodium azide). Stained cell suspensions were washed and resuspended in PBS+/+ prior to analysis by CytoFlex flow cytometer (Beckman Coulter). Data were acquired with CytExpert software (Beckman Coulter) and post-acquisition analysis was performed using FlowJo software (Tree Star, Inc.).

Cell proliferation assay

250,000 cells/ml were plated (Day 0) in a 10 cm culture plate. Cell count was determined every 24 hours over the course of four days by forward and side scattering and by ZsGreen1 fluorescence in a Cytoflex Flow Cytometer (Beckman Coulter Life Sciences, USA).

Analysis of leukocyte migration under shear flow

Primary HDMVECs were plated at confluence on either plastic or glass bottom 60 mm petri dishes coated with 2 µg/ml fibronectin (cat. # F0895; Sigma Aldrich, USA). A day later, cells were stimulated for 3 hrs. with IL-1β (2 ng/ml). Endothelial cell coated plates were assembled in a flow chamber and washed extensively with the binding medium HBSS (Hank's balanced-salt solution containing 2 mg/mL BSA and 10 mM HEPES, pH 7.4, supplemented with 1 mM

CaCl₂ and 1 mM MgCl₂). Neutrophil-like cells were perfused over the monolayer in binding medium for 40 s at 1.5 dyn/cm², and were then subjected to a shear stress of 5 dyn/cm² for 10 min. Images were acquired at an interval of 15 sec using IX83, Olympus microscope equipped with 20× or 60× phase contrast objectives. Cells were tracked individually using ImageJ and categorized in at least 3 fields of view (~50 cells per field) as previously described [18, 19].

For analysis of migratory categories, leukocytes accumulated during the accumulation phase (40 seconds) were individually tracked throughout the assay by time-lapse microscopy and categorized as fractions of leukocytes originally accumulated at the end of this accumulation phase. Crawling leukocytes were defined as cells moving a distance of at least 30 µm from their initial point of arrest. Transmigrating leukocytes were defined as either arrested or crawling cells which translocated their entire body through the endothelial monolayer. To monitor nuclear translocation and shape changes during TEM, cells were labelled with Hoechst 33342 as described [10] shortly before their introduction into the flow chamber. The location of pseudopodia sent by individual transmigrating dHL-60 cells and their respective nuclei were manually determined from time-lapse recordings (images were captured at 15 sec intervals). Gap size generated by transmigrating Hoechst labeled dHL-60 cells was determined as described by the displacement of a non blocking Alexa 647 anti-VE- cadherin mAb [10] (incubated at 2 µg/ml, 10 min prior to the beginning of the TEM assay). Since the majority of the gaps were oval, an average of both the long and short axes was calculated for each gap generated by transmigrating granulocyte-like dHL-60 cells.

Transwell migration assay

Differentiated HL-60 cells were washed with PBS (-/-) containing 5 mM EDTA, resuspended at a density of 2×10^6 cells/ml in binding medium HBSS (described above), and seeded in the upper chamber of 24-well transwells with 3 or 5- μ m pore sizes (BD Biosciences, San Jose, CA, USA). The bottom chambers were filled with the corresponding media supplemented with and without CXCL1 (50 ng/ml) and incubated at 37°C in 5% CO₂ for 30 min. The transwell inserts were removed, the cells recovered from the bottom chambers were collected, and their numbers were determined by FACS analysis using the CytoFlex flow cytometer (Beckman Coulter).

Nuclear shape analysis and location

Hoechst 33342 labelled granulocyte-like HL-60 cells were allowed to settle for 15 min at 37°C on poly-L-Lysine (PLL) coated glass surface prepared by incubating PLL (0.01% w/v in ddH₂O) for 30 min at 37°C. Images were acquired using a 20 $\times$ objective (IX83, Olympus, Shinjuku, Tokyo, Japan). Nuclear circularity index was determined using ImageJ. Similarly, nuclear circularity index was determined for Hoechst labeled granulocyte like HL-60 cells migrating over immobilized CXCL1. Thirty consecutive frames were captured for each granulocyte at 15 sec intervals, and the circularity index values in each frame were averaged for each cell. Nuclear location in each of these cells was manually determined. The nuclear locations of polarized and motile granulocytes were classified manually using CellSens Dimension Desktop software (Olympus) as either anterior and posterior based on whether the nuclei

remained confined to either the leading or trailing edges, respectively for at least half of the total assay period.

Chemokine mediated 2D and 3D cell motility assays

Ibidi chamber slides (μ -Slide VI 0.4, Ibidi) were coated with 50 ng/ml CXCL1. Granulocyte-like dHL-60 cells were washed and resuspended in binding solution, injected into the Ibidi chamber and allowed to settle for 10 min at 37°C, and images were acquired with a 20× or 60× objective (IX83, Olympus). Velocity calculations were performed using ImageJ and trajectory analysis was performed using Imaris 9.0.0 (Bitplane, Belfast, UK) software. For 3D migration assays, granulocyte-like dHL-60 cells were washed and resuspended in cold matrigel solution (BD-356234, stock solution mixed with binding medium at 1:1 ratio) or in cold collagen I solution (rat tail, Corning, 3.2 mg/ml in binding medium) and injected into the Ibidi chamber at 4°C. The cells were sedimented at 50×g for 3 minutes and incubated under the matrigel or collagen I solutions at 37°C for 30 min to allow collagen polymerization. Images were acquired with a 20× or 60× objective (IX83, Olympus) for 30 min at intervals of 60 sec. Nuclear location and granulocyte velocities were determined with ImageJ software.

Confocal reflectance microscopy

Collagen I and matrigel solutions were prepared as described above, plated in 35-mm petri dishes and incubated for 30 minutes at 37°C in a humidified incubator. Gels were imaged using a Leica TCS SP5 confocal microscope by a

63×, 0.9 NA water immersion lens. Samples were illuminated with 488 nm Argon laser light and the meta channel of the microscope was set to detect wavelengths between 474 and 494 nm to allow reflectance mode [20].

Scanning electron microscopy of collagen matrices

Samples were fixed using 2.4% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hr at RT. After fixation, samples were rinsed repeatedly with PBS-/- (without Ca^{2+} or Mg^{2+} , pH 7.4) and then treated with guanidine-HCl/tannic acid (4:5) solution (2%) for 1 hr. at RT. Samples were rinsed repeatedly with PBS-/- and then dehydrated in a graded series of 50%, 70%, 80%, 90% and 100% ethanol/water (v/v) for 10 minutes each. The residual ethanol was then removed using a series of 50%, 75% and 100% (x3) Freon solutions in ethanol for 10 min each. Finally, the samples were air dried for few seconds. The dried samples were mounted on aluminum stubs, sputter coated with carbon and, viewed with the SEM (FEI Quanta 250 FEG, OR, USA).

Statistical Analysis

All the data are reported as the sample mean $\pm$ SD or SEM, as indicated, and means of different groups were compared pairwise using two-tailed, unpaired student's t-test. The difference between two datasets was considered significant for p values below 0.05.

Results

Lamin A overexpression in granulocyte-like cells does not affect their chemokinesis but restricts migration through small, rigid pores

Primary neutrophils as well as granulocyte-like differentiated HL-60 cells (dHL-60) downregulate expression of the nuclear lamina proteins lamin A and C during differentiation [9]. In our experiments, we used a variant of HL-60 that stably expresses CXCR2 levels comparable to those in primary neutrophils [21] in order to facilitate migration of these leukocytes across CXCL1-producing inflamed endothelial cell monolayers. This introduced CXCR2 expression was critical for the ability of these cells to cross IL-1 β -stimulated HDMVEC, a well-studied low permeability endothelial barrier, which supports robust neutrophil accumulation and TEM under physiological shear flow [10, 19] (Figure 1A and data not shown). To overexpress lamin A in these HL-60 variants, we infected them with an IRES containing retroviral vector co-encoding the pre lamin A precursor and a ZsGreen1 reporter as previously described [16]. ZsGreen1 expressing cells were found to have 10-fold higher levels of lamin A than control CXCR2-HL-60 cells, when differentiated with DMSO into granulocyte-like cells (Figure 1B). Importantly, lamin A overexpression in these granulocyte-like leukocytes did not impair their proliferation rates, nor alter Lamin B1 expression, CXCR2 levels or change the levels of major integrin members or the expression of E-selectin carbohydrate ligands (Figure 1C-H and Suppl. Fig. 1C, D). Lamin A overexpression also did not affect chemokinetic motility of the differentiated granulocyte-like CXCR2-HL-60 cells (henceforth referred to as CXCR2-dHL-60) measured on a 2D surface coated with the CXCR2 chemokine CXCL1 (Figure 1I). The lamin A overexpressing granulocyte-like dHL-60 cells also

normally crossed through 5 μ m pore transwells towards a CXCL1 gradient, comparable to non-modified control cells (Figure 2A). However, when compared for their ability to squeeze towards identical CXCL1 gradients through transwells containing 3 μ m pores, which are substantially smaller than their nuclear diameter, only a minute fraction of laminA-OE CXCR2-dHL-60 successfully passed through these smaller pores, whereas control CXCR2-dHL-60 cells showed efficient migration through these pores, albeit less than in the larger pores (Figure 2A). Notably, lamin A overexpression did not change the nuclear shape of these differentiated HL-60 cells as assessed by the average circularity of the nuclei in these granulocyte-like cells settled on a poly-L-lysine substrate (Figure 2B,C). Given that lamin A overexpression had only a moderate or no effect on nuclear circularity (Figure 2B-D), we conclude that the impaired migration of lamin A overexpressing CXCR2-dHL-60 cells through 3- μ m pores is caused by the increased nuclear stiffness due to higher lamin levels, and not an increase in nuclear circularity. These migration results are consistent with previous findings in dHL-60 granulocyte-like cells obtained by ATRA treatment [16].

To further validate that the ZsGreen1 reporter on its own is inert in our various assays, the ZsGreen1 gene alone was introduced into CXCR2-dHL-60 cells by an identical viral vector, and both ZsGreen1 expressing and non-expressing CXCR2-dHL-60 cells were compared for their ability to cross rigid transwell pores and transmigrate across inflamed endothelial barriers. As expected, the ZsGreen1 expressing granulocyte-like CXCR2-dHL-60 normally crossed through small rigid pores towards CXCL1 gradients (Supplemental Figure 1E). Thus, high-level expression of the ZsGreen1 reporter, on its own,

does not alter the mechanical properties and squeezing capacity of CXCR2-dHL-60 cells. This observation also suggested that the ZsGreen1 negative granulocyte-like CXCR2-dHL-60 cells are practically identical to ZsGreen1 expressing granulocyte-like CXCR2-dHL-60 cells with respect to their mechanical nuclear properties. Hence, in all the rest of the experiments we assessed the motility and squeezing of the granulocyte-like CXCR2-dHL-60 cells co-expressing high ZsGreen1 and high lamin A levels to those of similarly co-cultured and co-differentiated granulocyte-like CXCR2-dHL-60 cells which did not overexpress lamin A.

Lamin A overexpression in granulocyte-like cells is permissive for protrusion and TEM but slows down nuclear squeezing through endothelial junctions

To further elucidate the effect of lamin A overexpression on dHL-60 migration across endothelial barriers, we next determined the adhesive and migratory capacities of ZsGreen1-LaminA overexpressing granulocyte-like CXCR2-dHL-60 (green cells) to CXCR2-dHL-60 cells which did not express the ZsGreen1-lamin A construct. When both populations were perfused under shear flow on low permeability confluent monolayers of HDMVECs stimulated by IL-1 β [10], lamin A overexpressing dHL-60 cells normally resisted detachment by continuously applied shear forces (Figure 3A). Both groups of dHL-60 cells arrested nearby the endothelial junctions they eventually transmigrated through while exhibiting negligible lateral crawling on the apical endothelial surfaces (Figure 3A). Surprisingly, although lamin A overexpressing granulocyte-like dHL-60 cells failed to squeeze through 3- μ m diameter rigid pores (Figure 2B),

comparable fractions of lamin A overexpressing and control CXCR2-dHL-60 transmigrated through IL-1 β inflamed HDMVECs (Figure 3A, TEM category). Nevertheless, kinetic analysis of the TEM of these leukocytes revealed a statistically significant delay of the lamin A overexpressing cells compared to the control cells (Figure 3B,C). In order to further elucidate at a single cell level the basis for this delay in successful TEM, we analyzed the kinetics of leukocyte protrusion through the endothelial monolayer, as well as the overall time spent by either individual control or high lamin A expressing dHL-60 cells subsequent to this initial protrusion underneath the endothelial monolayer until the completion of TEM. While the rate of protrusion and extension of a basolateral pseudopodium (leading edge) underneath the endothelial monolayer was indistinguishable between control and lamin A overexpressing CXCR2-dHL-60 cells (Figure 3D,E), the lamin A overexpressing CXCR2-dHL-60 cells had an over 30% increase in the average period required for TEM completion compared with control CXCR2-dHL-60 cells (Figure 3F). Notably, the periods required for TEM completion of individual granulocytes was highly variable for both lamin A low and high CXCR2-dHL-60 cells, reflecting the multiple barriers these leukocytes had to overcome to successfully cross the inflamed endothelial monolayers.

Further comparison of Hoechst labeled control and lamin A high CXCR2-dHL-60 cells indicated that the main step delayed for the lamin A overexpressing CXCR2-dHL-60 cells was the time it took the nuclei of these cells to completely pass through the endothelial barrier (Figure 4A,B and Video 1). In contrast, the rate of retraction of the uropod of these cells was comparable (data not shown). These results collectively suggest that the nuclei of

granulocytes overexpressing lamin A are delayed in their insertion into the otherwise normal basolateral pseudopodia generated by these granulocytes in response to chemotactic CXCR2 signals. Thus, their ability to lift the endothelial cells engaged by their basolateral pseudopodia and open gaps between neighboring endothelial cells is impaired, resulting in considerably larger sub-endothelial pseudopodia (Figure 4A and Video 1).

We have recently shown that leukocyte nuclear squeezing through transcellular routes of TEM is associated with pore widening to about 5 μm in diameter [10]. We therefore asked if granulocyte-like cells that overexpress lamin A and are delayed in their squeezing through endothelial gaps exceed this upper limit of endothelial gaps. To address this question, we probed gap formation by VE-cadherin displacement by our different granulocyte-like CXCR2-dHL-60 cells. Remarkably, the average gap size generated by lamin A overexpressing CXCR2-dHL-60 cells was 20% larger than the average gap diameter generated by control CXCR2-dHL-60 cells (Figure 4C) consistent with the higher circularity of the nuclei of lamin A overexpressing CXCR2-dHL-60 cells polarized on immobilized CXCL1 (Figure 2D). These larger openings created in the endothelial layer may explain why lamin A overexpressing CXCR2-dHL-60 cells have similar TEM efficiency as control cells, despite their rounder and less deformable nuclei. Notably, both lamin A overexpressing and control CXCR2-dHL-60 cells readily displaced the stress fibers of the individual endothelial cells they squeezed between, without rupturing these fibers (data not shown).

Lamin A overexpression in granulocyte-like cells restricts chemokine driven motility through and crossing of collagen I barriers

Extravasating leukocytes must cross additional extracellular matrix barriers upon exiting blood vessels and crossing the basement membrane deposited by both the endothelial cells and their neighboring pericytes [22]. To mimic the barriers encountered by leukocytes during interstitial motility, we designed a new readout, which combines chemokine driven leukocyte motility on a rigid 2D surface with a mechanical barrier exerted by a 3D matrix composed of distinct collagen containing fibrous barriers. To this end, both lamin A overexpressing and control CXCR2-dHL-60 cells were settled on a glass slide coated with CXCL1 in the presence of medium alone or medium supplemented with either 50% matrigel, which was allowed to undergo in situ polymerization for 20-30 min at 37°C, or medium supplemented with collagen I allowed to undergo identical in situ polymerization (Figure 5A). Matrigel is an extract derived from mice harboring tumors, and is rich in laminin and collagen IV and is therefore sometimes used as a surrogate basement membrane [23]. While both collagen I and matrigel are commonly used models of cell invasion, they have distinct biomechanical properties. Matrigel is characterized by small pore sizes and high deformability, while collagen I matrices at comparable collagen concentrations have larger pore sizes but are less deformable [23]. Comparison of these two types of barriers using confocal reflective microscopy revealed that the collagen I barrier is composed of thick fibers as opposed to the matrigel (Figure 5B). SEM analysis of these matrices [24] further supported the notion that the collagen I matrix is much denser than the matrigel matrix (Suppl. Fig. 2).

We next compared both the nucleus location and the motility of either control or lamin A overexpressing CXCR2-dHL-60 cells settled on CXCL1 in the presence of these different media (Figure 6A-D). Notably, in aqueous media, dHL-60 with high lamin A content underwent normal polarization on CXCL1 coated 2D surfaces (Figure 6A, Video 2), the nuclei of both granulocytes were readily translocated into their leading edges (Figure 6C, and Video 2), and both granulocytes locomoted at comparable velocities on this 2D substrate (Figure 6D and Video 2). As expected, the granulocyte-like CXCR2-dHL-60 cells locomoted more slowly on immobilized CXCL1 when embedded inside the matrigel relative to when the same cells were settled on the immobilized CXCL1 in medium only (Figure 6B, D and Video 3). Nevertheless, in the presence of the matrigel, lamin A overexpression modestly affected nuclear translocation into the leading edge of migrating granulocytes (Figure 6C and Video 3) and reduced granulocyte motility by only ~20% (Figure 6D). These results indicate that the stiffer nuclei of lamin A overexpressing granulocyte-like CXCR2-dHL-60 cells only marginally restrict the 2D chemokinetic motility when the cells are embedded inside a soft matrigel barrier and do not restrict the nuclear translocation and motility of these cells in aqueous medium. Since none of the lamin A high granulocyte-like CXCR2-dHL-60 cells were arrested by the matrigel barrier, and the majority of their nuclei readily translocated into the leading edge of motile leukocytes (Figure 6B-D, Video 3), these results indicate that when the porous barrier is sufficiently deformable, most lamin A overexpressing, stiff leukocyte nuclei can override the low resistance imposed by such a barrier.

To evaluate the contrasting scenario of a less deformable extracellular

matrix network, we assessed the impact of leukocyte nuclear stiffness on squeezing through a distinct porous barrier that closely resembles the collagen I rich interstitial tissue encountered by leukocytes once they enter peripheral tissues [25]. We therefore compared the ability of lamin A overexpressing and control CXCR2-dHL-60 cells settled on immobilized CXCL1 and embedded in a matrix of pure collagen I to cross this barrier. Notably, the vast majority of lamin A high granulocyte-like CXCR2-dHL-60 cells failed to translocate their nuclei to their protrusive leading edges in spite of their normal ability to extend filopodia-like protrusions (Figure 7A, B and Video 4). Consequently, lamin A overexpressing cells showed a substantially reduced motility when embedded in the collagen I matrix compared with control granulocyte-like CXCR2-dHL-60 cells (Figure 7C), and their ability to successfully move through the collagen, although inherently variable, was significantly impaired (Figure 7D). Interestingly, polymerized collagen I did not slow down the motility of normal granulocyte-like CXCR2 dHL-60 cells more than did the matrigel barrier (i.e. a mean velocity of ± 6.1 in collagen I compared to a mean velocity of ± 6.6 in matrigel), indicating that it imposed a major barrier selectively on the migration of lamin A overexpressing granulocyte-like cells, which have stiffer nuclei [16], whereas control granulocyte-like cells with their highly deformable nuclei can still easily penetrate the collagen I matrix. The dramatically slower motility of these granulocytes through the collagen I gel and retarded nuclear translocation to the leading edge of these leukocytes was not the result, however, of augmented integrin mediated adhesiveness of the lamin A overexpressing CXCR2-dHL-60 cells to the collagen I fibers because the motility of these granulocytes was not affected by exclusion of Mg^{++} (data not shown). Thus, integrin-independent

CXCR2 mediated granulocyte motility through collagen I barriers is dramatically delayed by lamin A overexpression due to a failure of the stiffer, lamin A high nuclei to translocate into the pseudopodia of granulocytes crossing this poorly deformable barrier.

Discussion

The lamin cortex of all mammalian nuclei is a thin, elastic shell encoded by three functionally nonredundant genes, *LMNA*, encoding lamins A/C, and *LMNB1* and *LMNB2*, encoding lamin B1 and lamin B2, respectively [26]. Lamins exist in dynamic equilibrium between the nucleoplasm and the lamina network [27]. The ratio between the *LMNA* gene products lamin A and its shorter spliced variant lamin C and the other lamins is proportionally related to nuclear stiffness in both mesenchymal and hematopoietic cells [28]. In addition to their roles in structure and nuclear stability, the various lamins are also involved in transcription, chromatin organization and DNA replication and their mutations are associated with multiple pathologies [29, 30]. Nevertheless, the lamin A overexpression in our model HL-60 cell system did not affect the expression of the canonical surface markers associated with this differentiation or of key functional receptors involved in leukocyte TEM and motility such as integrins and myeloid GPCRs. Furthermore, lamin A overexpression did not affect the migratory properties of dHL-60 cells under different conditions such as chemokine driven motility on 2D surfaces or chemotaxis through large rigid pores, suggesting that their differentiation and major cytoskeletal machineries were not functionally affected by lamin A overexpression.

Circulating leukocytes express very low lamin A levels, which keep their nuclei soft and thereby presumably allowing these cells to readily squeeze through vascular barriers [1]. Strikingly, however, our granulocyte-like cells genetically manipulated to express 10-fold higher levels of lamin A could still readily cross the low permeability endothelial barriers used in our in vitro TEM setups. These results suggest that leukocytes with stiff nuclei can open up

sufficiently large gaps in between neighboring ECs, orders of magnitude larger than the gaps these cells normally maintain to squeeze their nuclei through these openings and lift these endothelial cells microns above the basement membrane they normally deposit [31]. Thus, while lamin A overexpressing cells take slightly longer to transmigrate through an endothelial cell monolayer, they eventually successfully transmigrate through this barrier, and this is accomplished by opening larger gaps in between neighboring endothelial cells that allow transit of the more rigid nuclei in the lamin A overexpressing cells.

Our data indicate that the size of endothelial gaps generated by squeezing leukocytes is probably dictated by the dimension of the leukocyte nuclei more than by the resistance of the endothelial cytoskeleton to leukocyte squeezing. The endothelial cells were traditionally proposed to facilitate gap enlargement via myosin driven contraction, yet we and other groups have recently ruled out this alternative mechanism for endothelial gap formation [10, 11, 32]. Our new results further suggest that endothelial gap formation is simultaneously regulated by both the leukocyte nuclei and by the endothelial cytoskeleton. In addition to restricting gap widening by squeezing leukocytes, the endothelial cytoskeleton restricts the ability of the endothelial cell to be lifted above the slide by the squeezed leukocyte nucleus. Our results indicate that even the dramatic stiffening introduced to the nuclear lamina of granulocytes by lamin A overexpression only moderately affects this endothelial lifting, without which the leukocyte cannot complete its squeezing through endothelial monolayers.

The nuclear stiffening introduced by lamin A overexpression in our model granulocyte-like HL-60 cells exerted dramatic effects on the ability of these cells

to squeeze and migrate through 3D collagen I matrices in response to chemokinetic CXCL1 signals. At the same time, lamin A high granulocytes successfully crossed the much softer matrigel barriers in response to identical chemokinetic CXCL1 signals. Notably, these cells also normally translocated their stiff nuclei to their lamellipodia and protrusions, further indicating that nucleus stiffening and lamin A overexpression, on their own, do not alter the position of the nucleus in migrating leukocytes. Rather, the translocation of the nucleus in motile leukocytes to the leukocyte lamellipodia is determined both by its relative stiffness and the relative mechanical resistance of the external environment surrounding the motile leukocyte. Why then did the same nuclear stiffening introduced by lamin A overexpression not affect granulocyte squeezing through matrigels? Whereas the components of matrigel are chemically similar to the major components of endothelial basement membranes, polymerized matrigel is mechanically softer than polymerized collagen I [33, 34]. Our structural analysis of 3D matrigel and 3D collagen I gels also indicates the presence of thick collagen fibers in the collagen I gels and absence in polymerized matrigels. This is attributed to the molecular differences between fiber-forming collagen I as opposed to the network assembly of collagen IV together with laminin within matrigels. The 3D collagen I barriers constructed by us in the present work appear to better mimic the physiologically relevant barrier leukocytes encounter during their interstitial motility in tissues. An open question of interest is the crossing ability of leukocytes with stiff nuclei through endothelial basement membrane barriers given that some basement membranes are discontinuous around post capillary venules at some sites of inflammation [35, 36].

One of the most surprising results of our study is the efficient squeezing of the lamin A high nuclei across endothelial junctions and underneath endothelial monolayers. This reflects the remarkable ability of the endothelial barrier to adapt its cytoskeleton to stiff lamin A high nuclei. This adaptive nature of the endothelial cytoskeleton sharply contrasts the mechanical resistance of collagen I fibers to the squeezing of identically stiff nuclei [37]. We thus predict, that unlike these fibers, both the endothelial actin filaments and microtubules that construct the main mechanical barriers of the endothelial cytoskeleton can likely bend, get displaced and undergo remodeling when crossed by either soft (i.e., lamin A low) or stiff (i.e., lamin A high) nuclei. The endothelial resistance to nuclear squeezing is low, likely because of the high elasticity of the endothelial stress fibers and the fast turnover of the short actin filaments interlaced in between these actin bundles [10, 32]. Our results also elude to the possibility that the pulling and pushing forces normally exerted by the leukocyte actomyosins are sufficiently high to propel even the stiffer nucleus of lamin A overexpressing leukocytes into the leukocyte pseudopodia and thereby override the low mechanical resistance imposed by the endothelial cytoskeleton. These actomyosin derived forces are also strong enough to squeeze the stiff lamin A enriched nuclei through thin and porous barriers such as those imposed by matrigels. Similar forces are, on the other hand, insufficient to override the indefinitely high mechanical resistance imposed on the same nuclei by rigid pores and by the high resistance imposed by stiff collagen I fibers.

In summary, our results highlight the permissive nature of the endothelial cytoskeleton, which is highly dynamic and can actively remodel and rapidly

change its fine structure, compared to extracellular matrices, which are relatively passive materials. The remarkable adaptive nature of endothelial cytoskeletal barriers to nuclear squeezing warrants future in vivo analysis of the relative abilities of other leukocytes with variable content of nuclear lamin A and stiffness to squeeze through distinct vascular beds and their different basement membranes. Such future studies should provide additional insights as to why most leukocytes maintain a low lamin A content of their nuclei, while most other cells, including leukocyte precursors in the bone marrow and subsets of activated lymphocytes, benefit from high lamin A content of their nuclei [28, 38, 39].

Authorship

S.K.Y. performed most of the experiments, analyzed data, and assisted in manuscript preparation and writing; S.W.F. assisted in FACS analyses and manuscript writing; F.R. performed some of the real time fluorescence microscopy experiments. JL provided reagents, expertise, and assisted in the interpretation of results and editing of the manuscript. R.A. designed and supervised experiments and wrote the manuscript.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Lamin A overexpression in CXCR2 expressing granulocyte-like dHL-60 cells does not affect the surface expression of receptors involved in trans-endothelial migration and does not alter CXCL1 driven chemokinesis. (A) Adhesive and migratory phenotypes of granulocyte-like DMSO differentiated HL-60 (dHL-60) variants deficient in CXCR2 or stably expressing CXCR2 crossing inflamed endothelial monolayers under shear flow. The two dHL-60 cells were perfused over monolayers of confluent HDMVECs which had been stimulated with IL-1 β for 3 hrs to induce E-selectin, integrin ligands and multiple CXCR2 and CCR2 chemokines. (B) Intracellular FACS staining of lamins A/C in permeabilized CXCR2 dHL-60 cells stably expressing the pRetroX-PrelaminA-IRES-ZsGreen1 construct (green) or control CXCR2 dHL-60 cells (black). Dashed line depicts cell staining with an isotype matched control mAb. (C-H) FACS analyzed surface staining of CXCR2, the E-selectin carbohydrate ligand carrying the HECA-452 epitope, and the integrin subunits CD18, CD11a, CD11b and CD29 on sham (black) vs. ZsGreen1-lamin A/C overexpressing (green) dHL-60 cells. Cells were labeled as described in the Materials and Methods section with either PE conjugated anti-human mAbs or with unlabeled primary antibodies followed by a PE or APC conjugated secondary Ab. (I) Migration tracks of control and lamin A overexpressing dHL-60 cells settled on immobilized CXCL1 analyzed with Imaris 9.0.0 software. The migration tracks are plotted with a common origin (central black dot) and the color code depicts the start and end time points of each track. The average total distance mean $\pm$ SD travelled by individual granulocyte-like cells within the indicated experimental groups interacting with immobilized CXCL1 is depicted

in parenthesis.

Figure 2. Lamin A overexpression in granulocyte-like HL-60 cells restricts chemotaxis via small rigid pores. (A) Chemotaxis of control and lamin A overexpressing (LaminA-OE) granulocyte-like CXCR2 expressing dHL-60 cells towards a CXCL1 gradient across transwell membranes with either 3 or 5 micron pore sizes. Cells were collected 30 mins after introduction to the upper wells. The assays were performed in triplicate. Results are representative of two independent experiments. (B) Representative images of Hoechst labeled CXCR2 control (black) and ZsGreen1-lamin A overexpressing (green, LaminA OE cells) dHL-60 cells settled on a PLL coated surface. (C,D) Scatter plots of nuclear circularity index determined for Hoechst labeled control and lamin A overexpressing dHL-60 cells attached to PLL coated surface (C) or immobilized CXCL1 (D). For more details, refer to the Materials and Methods section. The experiments in C and D are each representative of three. Error bars represent mean $\pm$ SD. * $p < 0.02$.

Figure 3. Lamin A overexpression in granulocyte-like HL-60 cells is permissive for TEM across an inflamed IL-1 β stimulated HDMVEC monolayer. (A) Migratory phenotypes of control and lamin A overexpressing (Lamin A-OE) granulocyte-like differentiated CXCR2 expressing HL-60 cells interacting with HDMVECs stimulated for 3 hrs with IL-1 β under shear flow. Values represent the mean $\pm$ SD of three fields in each experimental group. The experiment shown is representative of three. (B) The percentage of granulocyte-like CXCR2 dHL-60 cells that completed

transmigration across the inflamed HDMVEC monolayer at the indicated time points following the accumulation phase. Values represent the mean $\pm$ SEM of three fields in each experiment. The experiment shown is representative of three. * $p < 0.02$ for $t = 3$ mins. (C) Images of a representative Hoechst-labeled sham (control) and lamin A overexpressing (LaminA-OE) granulocyte-like dHL-60 cell during paracellular TEM taken 3 mins after the end of the accumulation phase. The green outline depicts the basolateral leading edge of the transmigrating dHL-60 cell. (D) The percentage of dHL-60 cells that projected a protrusive sub-endothelial leading edge underneath the monolayer at the indicated time points prior to nucleus crossing. (E) Images of a representative Hoechst-labeled sham (control) and lamin A overexpressing (LaminA-OE) dHL-60 cell taken 15 sec after the end of the accumulation phase. The green outline depicts the basolateral leading edge generated during the early stage of TEM. (F) TEM kinetics of individual control vs. lamin A overexpressing (LaminA-OE) granulocyte-like CXCR2 dHL-60 cells that crossed the inflamed HDMVEC monolayer measured from the first detectable protrusion of a sub-endothelial leading edge to the final detachment of the dHL-60 uropod from the apical endothelial aspect. Values were determined in multiple fields taken from three independent experiments. Error bars represent mean $\pm$ SD. ** $p < 0.002$.

Figure 4. Lamin A overexpressing granulocyte-like dHL-60 cells exhibit slower nuclear squeezing and generate larger endothelial gaps during paracellular TEM. (A) Images taken from Supplemental Video 1 recording a representative Hoechst-labeled sham (control) and lamin A overexpressing (LaminA-OE) granulocyte-like CXCR2 dHL-60 cell squeezing through

paracellular EC junctions. The green outline depicts the basolateral leading edge of the transmigrating dHL-60 cell and the red outline depicts the nuclear lobes inserted underneath the endothelial monolayer. The red circumference of the nucleus is highlighted in white dots at the first time point at which the entire nucleus of each of the transmigrating dHL-60 cells has completed its passage underneath the endothelial monolayer. The yellow asterisks denote the leukocyte uropods at the time of TEM completion. Time intervals are depicted in each image. Scale bar= 5 μ m. (B) Nuclear passage duration in individual control and lamin A overexpressing (LaminA-OE) CXCR2 dHL-60 cells transmigrating across inflamed HDMVECs monolayers. Values represent cells from multiple fields taken from three independent experiments. Error bars represent mean $\pm$ SD. *p < 0.03. (C) The diameter of endothelial gaps generated by crossing granulocyte-like CXCR2 dHL-60 (control vs. LaminA-OE) cells determined as described in Materials and Methods. Values for cells from multiple fields were collected in three independent experiments. Error bars represent the mean $\pm$ SD. *p < 0.03.

Figure 5. A setup for leukocyte motility on a chemokine-coated surface in the presence of distinct 3D collagenous barriers. (A) A scheme depicting the experimental model used to assess leukocyte crossing of distinct collagenous barriers. Leukocytes suspended in cold matrigel or collagen I solutions were sedimented for 3 mins and incubated under the distinct collagen solutions for 30 min at 37°C to allow collagen polymerization. Leukocyte motility was recorded for 30 additional minutes. (B) Representative confocal reflectance images of matrigel (50% solution) and collagen 1 matrices (3.2

mg/ml) which underwent polymerization for 30 mins at 37°C. Scale bars= 10 μ m.

Figure 6. Lamin A overexpression in granulocyte-like cells does not affect their chemokine driven motility through a permissive matrigel barrier.

Images from Supplemental Videos 2 and 3 depicting representative control (A) vs. lamin A overexpressing (LaminA-OE, B) Hoechst-labeled granulocyte-like dHL-60 cells migrating on immobilized CXCL1 either in aqueous medium (left) or when embedded inside a polymerized matrigel matrix (right). Time codes are depicted and scale bars= 5 μ m. (C) Nuclear locations in control (Cont.) and lamin A (LaminA-OE) overexpressing Hoechst labeled granulocyte like dHL-60 cells migrating over immobilized CXCL1 either in medium or embedded in polymerized matrigel (50% solution). Results were determined for 40-50 cells from 3 independent experiments. (D) The velocities of individual control (Cont.) and lamin A overexpressing (LaminA-OE) granulocyte-like dHL-60 cells migrating over immobilized CXCL1 alone or when embedded in the polymerized matrigel. Values represent cells from multiple fields taken from three independent experiments. Error bars represent mean $\pm$ SD. *p < 0.05; **p < 0.007.

Figure 7. Lamin A overexpression in granulocyte-like cells restricts nucleus squeezing and chemokine driven motility through a dense collagen I barrier. (A) Images from Supplemental Video 4 depicting representative control vs. lamin A overexpressing (LaminA-OE) Hoechst-labeled granulocyte-like dHL-60 cells migrating on immobilized CXCL1 while

being embedded inside a polymerized collagen I matrix (B). Time codes are depicted for each image. Scale bar= 5 μ m. Yellow arrows depict the direction of granulocyte motility over the chemokine coated 2D surface. The red asterisk indicates a retracting leading edge. (B) Nuclear locations in control and lamin A overexpressing (LaminA-OE) Hoechst labeled granulocyte like dHL-60 cells migrating over immobilized CXCL1 through the collagen I barrier. Results were determined for 40-50 cells from 3 independent experiments. (C) Scatter plot of velocities of individual control and lamin A/C overexpressing (LaminA-OE) granulocyte-like dHL-60 cells migrating through polymerized collagen I matrices. Values were collected from three independent experiments. Error bars represent mean $\pm$ SD. **p < 0.004. (D) Kinetics of collagen I barrier crossing of individual control and lamin A overexpressing (LaminA-OE) granulocyte-like dHL-60 cells. The numbers of cells within each experimental group that successfully crossed a 20 μ m long barrier of collagen I as a function of time. Values represent the mean $\pm$ SEM of three fields. ** p< 0.01 for t= 9 mins. The experiment shown is a representative of three.

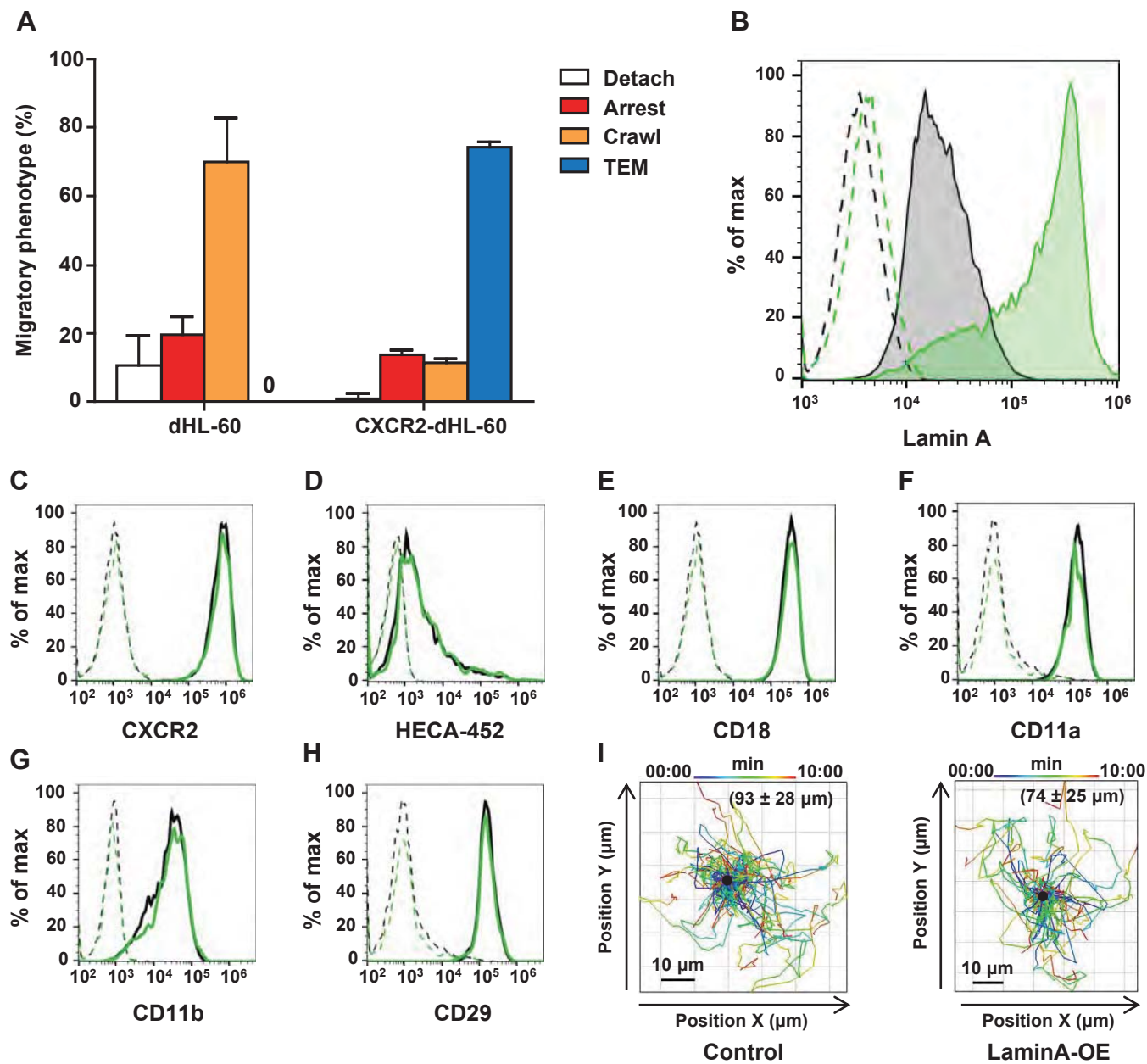


Figure 1

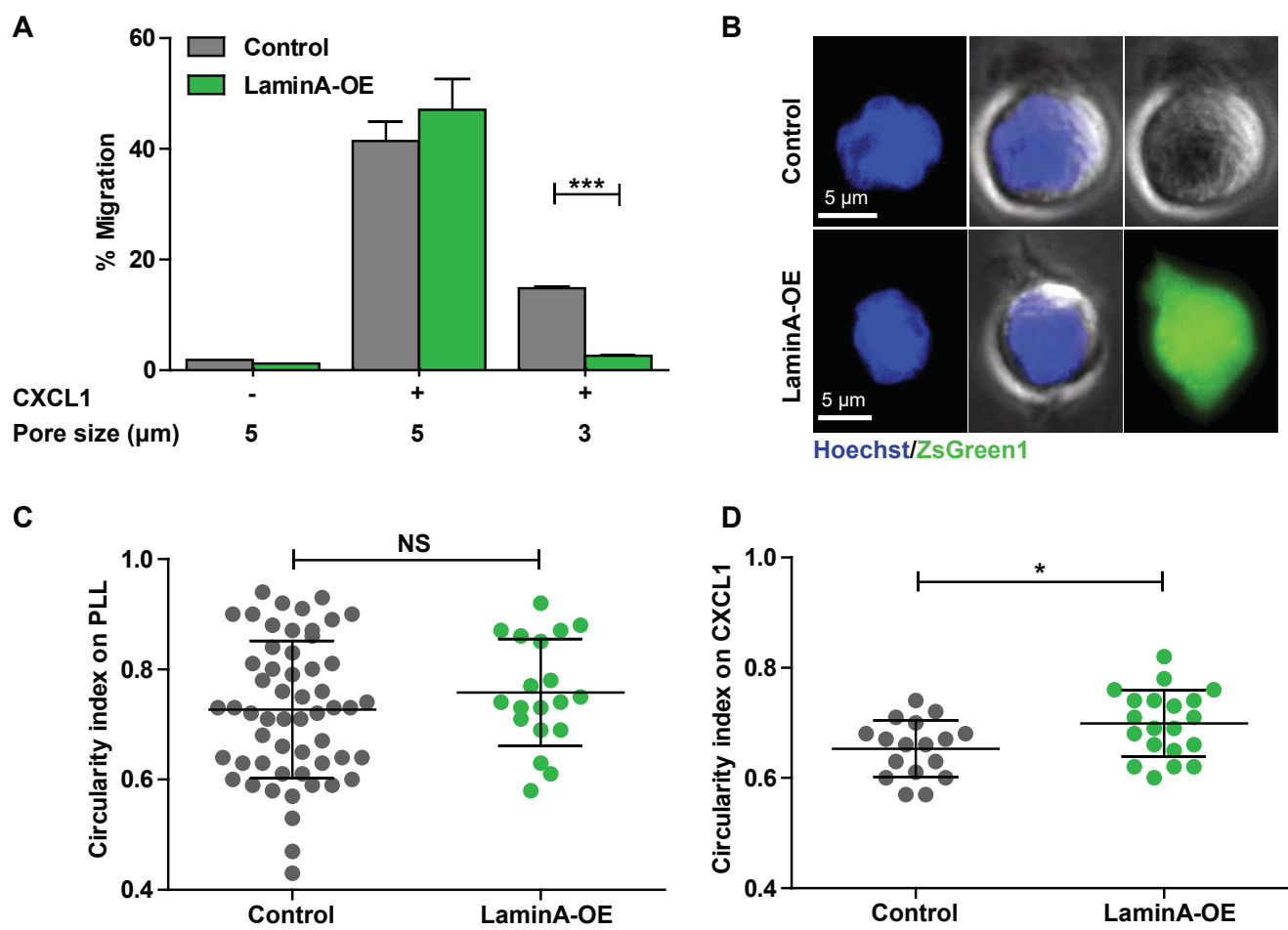


Figure 2

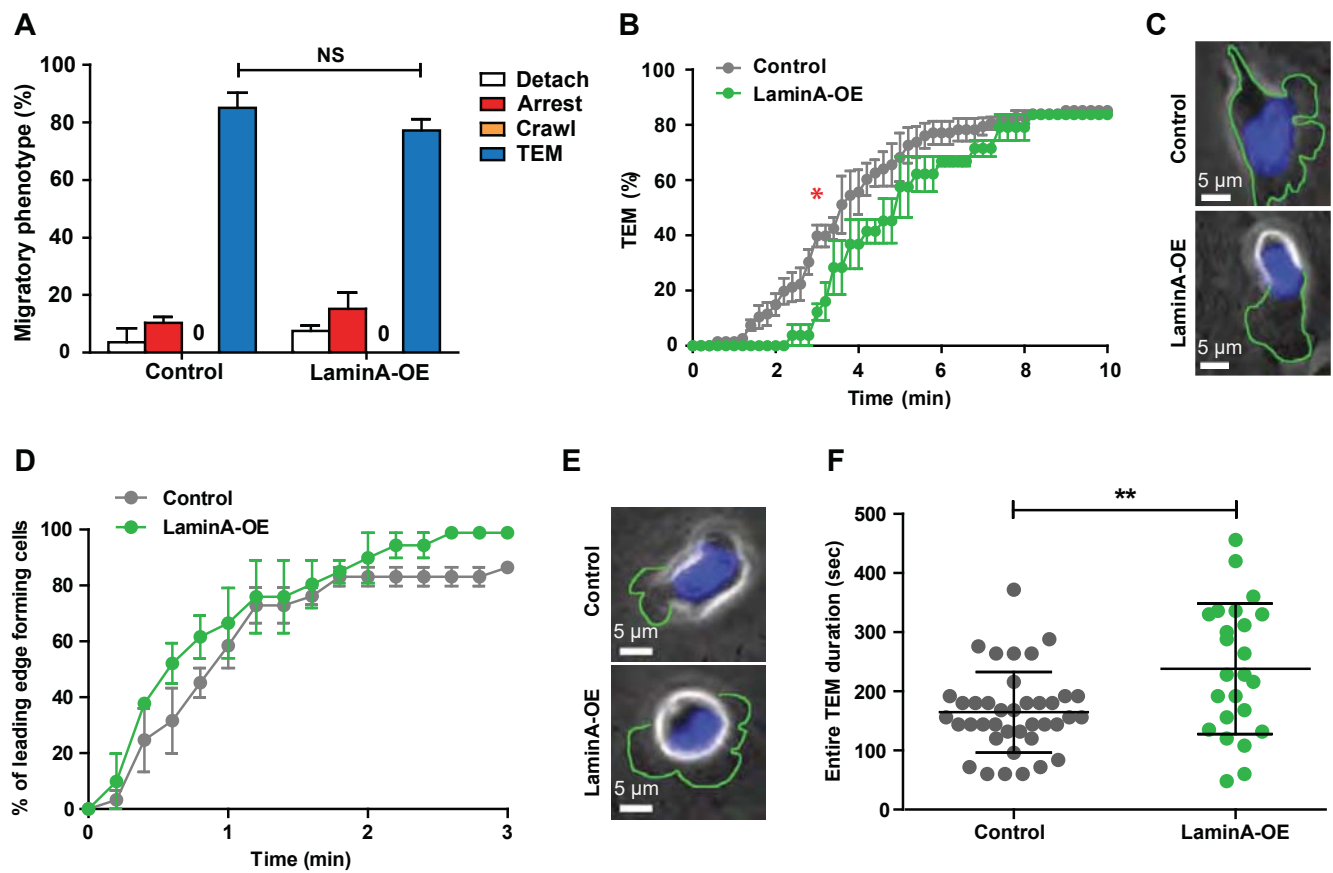


Figure 3

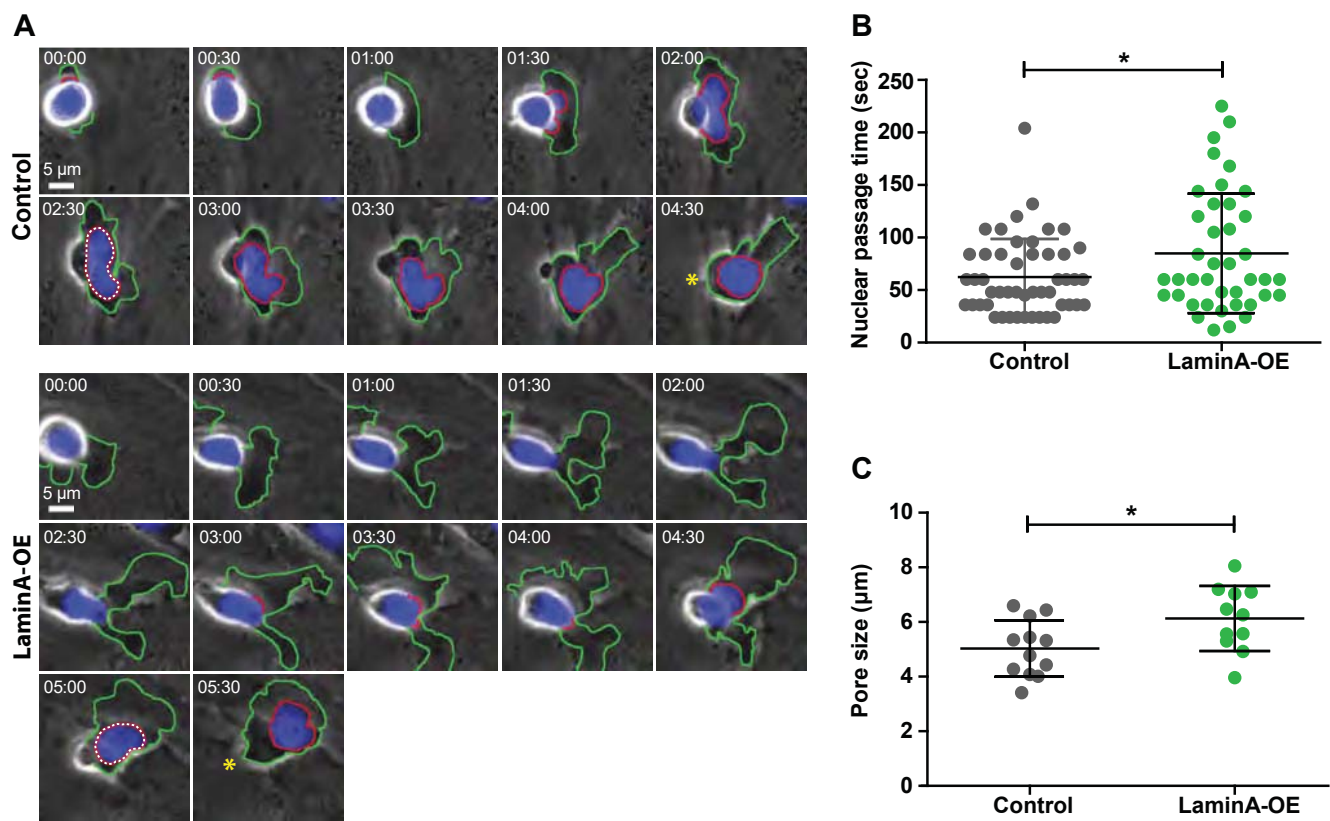
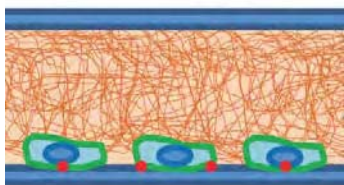
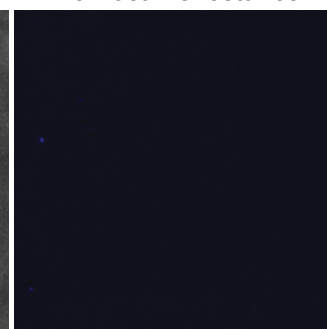
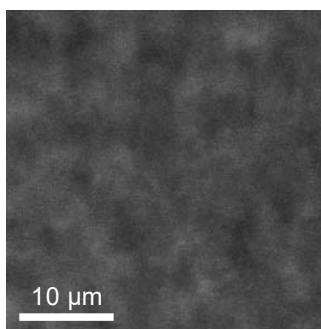
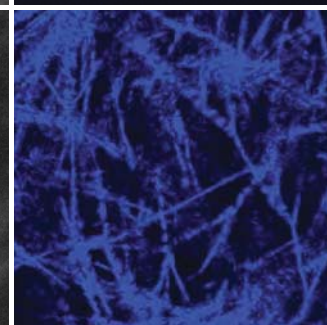
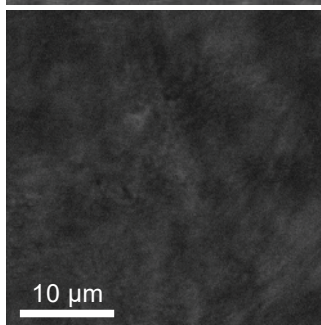


Figure 4

A**Immobilized
CXCL1****Matrigel****Collagen I****B****DIC****Confocal reflectance****Matrigel****Collagen I****Figure 5**

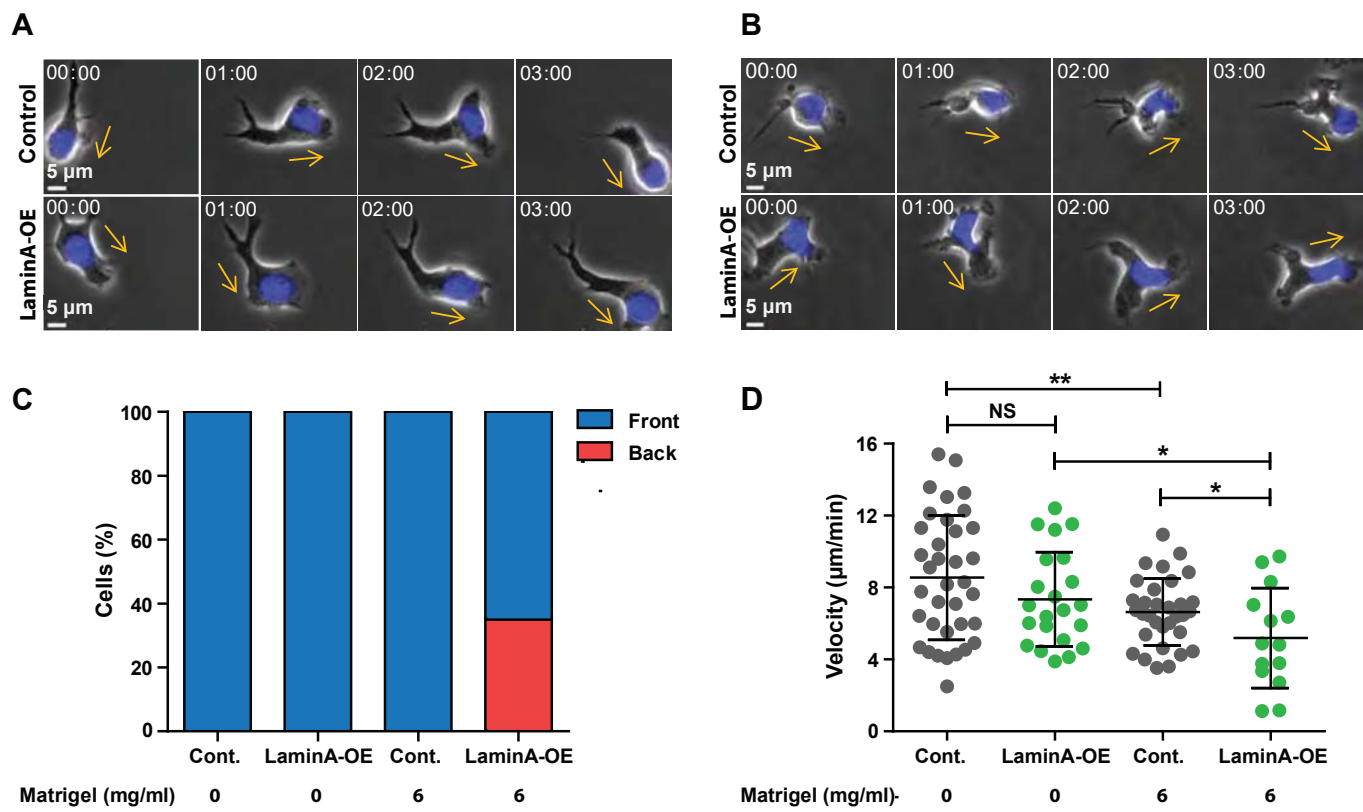


Figure 6

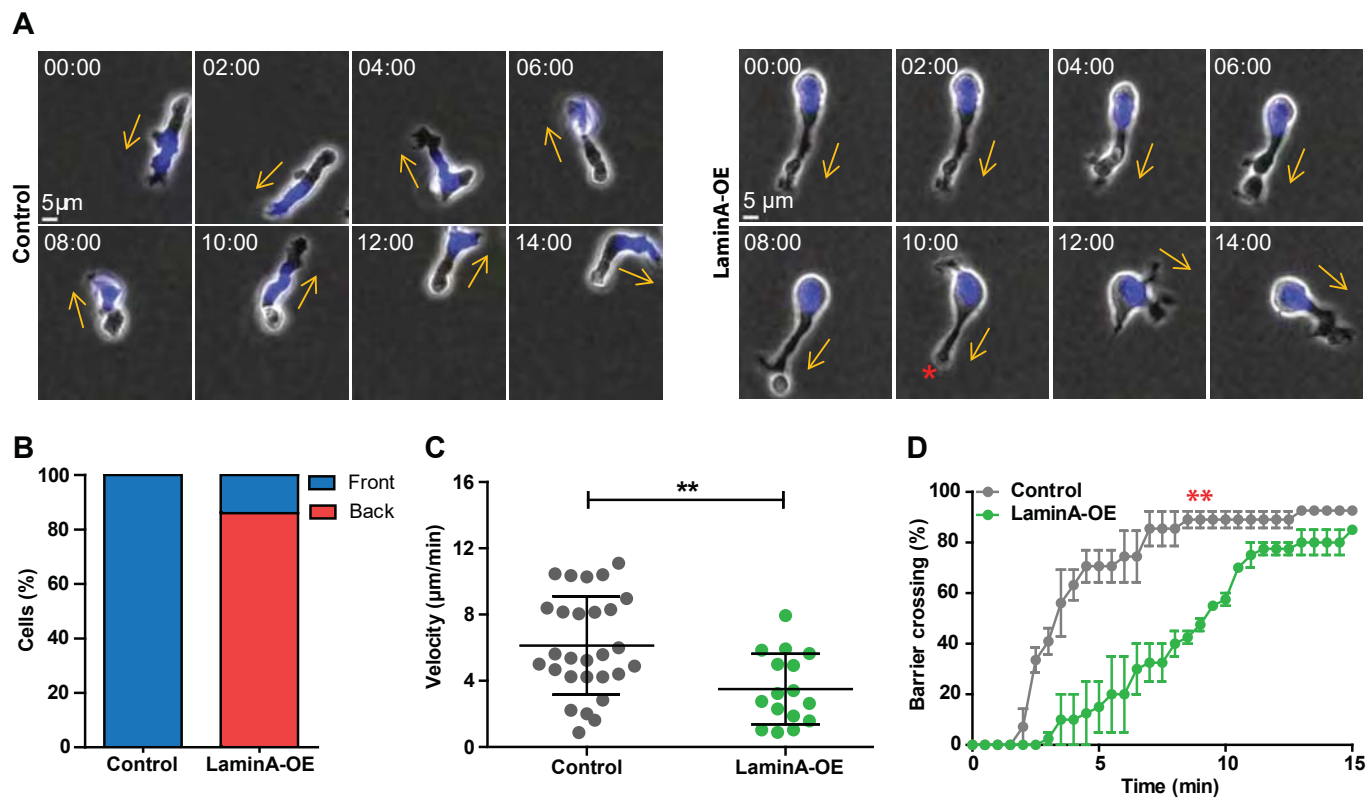
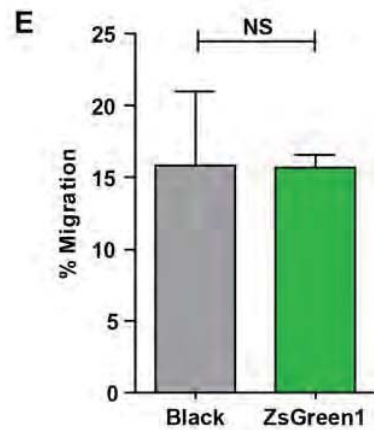
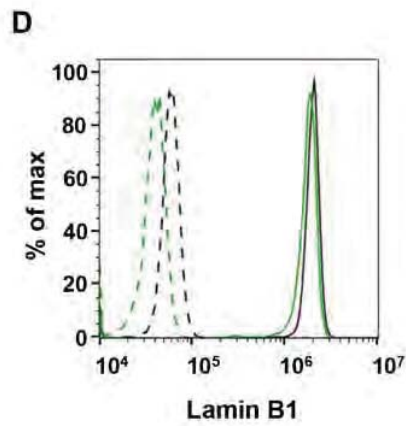
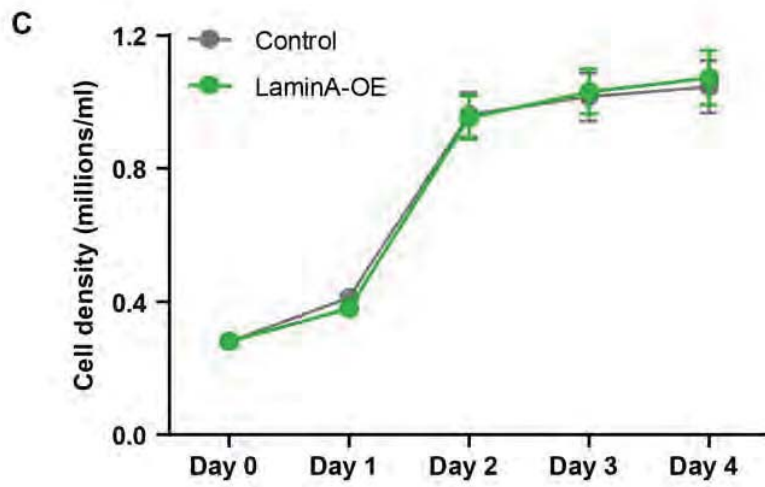
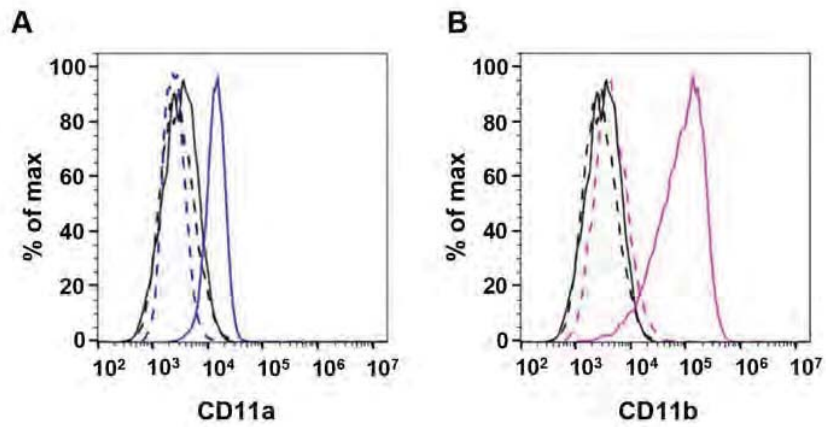


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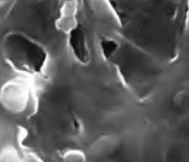


Figure 10 shows the surface morphology of the $\tilde{\text{AF}}$ membrane. The surface is highly porous and interconnected, with a network of fibers and numerous circular and oval pores. A scale bar is present in the bottom right corner.

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Opinion

Beyond Tissue Stiffness and Bioadhesivity:
Advanced Biomaterials to Model Tumor
Microenvironments and Drug ResistanceAnkur Singh,^{1,2,3,*} Ilana Brito,¹ and Jan Lammerding^{1,4}

Resistance to chemotherapy and pathway-targeted therapies poses a major problem in cancer research. While the fields of tumor biology and experimental therapeutics have already benefited from *ex vivo* preclinical tissue models, these models have yet to address the reasons for malignant transformations and the emergence of chemoresistance. With the increasing number of *ex vivo* models poised to incorporate physiological biophysical properties, along with the advent of genomic sequencing information, there are now unprecedented opportunities to better understand tumorigenesis and to design therapeutic approaches to overcome resistance. Here we discuss that new preclinical *ex vivo* models should consider – in addition to common biophysical parameters such as matrix stiffness and bioadhesivity – a more comprehensive milieu of tissue signaling, nuclear mechanics, immune response, and the gut microbiome.

Chemoresistance and Changes in Biophysical Factors

Therapeutic resistance in cancer often arises through genetic mutations that enhance drug metabolism, inactivate apoptotic pathways, and activate prosurvival signals [1–3]. The underlying genetic mutations are accompanied and sometimes preceded by changes in the biochemical and biophysical properties of the surrounding tissue. The biophysical factors, such as bioadhesivity, porosity, confinement, and stiffness, have been extensively studied as the response of individual cells to these factors and are vital for cellular functioning and tissue development. Cells cope with biophysical stimuli through integrated mechanosignaling by physically interconnected proteins starting from extracellular matrix (ECM) adhesion molecules (integrins), focal adhesion plaques, actin fibers, and structural components of the cells' nuclei, among others. The mechanotransduction response includes the activation of mechanosensitive transcription factors and downstream genes as well as the rearrangement of cellular structure and organization to adjust to the physical environment [4,5]. Mutations in cellular proteins and alterations in the cellular microenvironment aberrantly engage mechanosignaling networks in cancer cells, either by perturbing the mechanical input or by altering the signaling network itself, which can promote cell growth, invasion, migration, and probably chemoresistance. For example, integrin signaling has been shown to increase epidermal growth factor secretion and receptor tyrosine-protein kinase erbB-2 (ERBB2) clustering in breast cancer cells, resulting in resistance to the ERBB2 inhibitor trastuzumab [6]. Increased tumor and stroma stiffness has also become a hallmark of cancer, as evident from the use of palpation for detection of breast tumors and cancerous lymph nodes in the case of lymphomas. Increased tissue stiffness in the liver, pancreas, prostate, and lung has also been shown to be a positive indicator of disease progression in the corresponding cancers [7–10]. Nevertheless, how chemoresistance and changes in biophysical and biochemical factors relate to one another is poorly understood.

Highlights

Matrix stiffness influences the phenotype and epigenetics of tumor cells and influences chemoresistance across solid, palpable, and liquid tumors.

Biomaterials that independently modulate matrix stiffness from composition and architecture reveal that, in normal mammary epithelial cells, increasing matrix stiffness alone induces malignant phenotypes.

Healthy and malignant cells migrating through narrow confinements undergo nuclear deformation, which can result in transient loss of nuclear envelope integrity, herniation of chromatin across the nuclear envelope, DNA damage, and redistribution of mobile nuclear proteins.

An immune-privileged microenvironment could selectively impair the recognition of tumor antigens by cytotoxic T cells.

A commensal microbiota promotes the efficacy of cancer therapies.

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While genomic studies have benefitted from direct patient sample analysis, exploring the role that the stiffness of the microenvironment plays in cellular function has become possible only through the use of atomic force microscopy (AFM), microindenters, and engineered tissues. *Ex vivo* preclinical models that recapitulate the tumor microenvironment have been critical in improving our understanding of tumorigenic growth and resistance. In the case of mammary tumors, changes in tissue stiffness are associated with increased deposition and crosslinking of collagen type I, and the stiffness can increase from 100–400 Pa up to 1–5 kPa when comparing normal and cancerous mammary tissue [9,11]. It is now well accepted that matrix stiffness perturbs epithelial morphogenesis by clustering integrins to enhance extracellular signal-regulated kinase (ERK) activation and increase Rho-associated protein kinase (ROCK)-generated contractility and focal adhesions. Integrin signaling and stiffness are involved not only in chemoresistance in solid tumors but also in palpable lymphoid malignancies, as shown by us [12], as well as in liquid tumors [13]. Recent work from Shin and Mooney demonstrated that matrix softening leads to resistance against standard chemotherapy in myeloid leukemias [13]. More recently, matrix softness was shown to influence the histone methylation and epigenetics of tumor-repopulating cells [10], which exhibit high chemoresistance to conventional chemotherapeutic drug treatment. To better understand the role of tissue stiffness in cancer, we refer the reader to excellent recent reviews [11,14]. Nonetheless, these *ex vivo* models have yet to successfully address the reasons for the emergence of tumor resistance. This is because most *ex vivo* tissues focus on bioadhesive signaling and stiffness. Although extensively investigated, cell adhesion- and stiffness-mediated drug resistance is not the only factor that contributes to chemoresistance *in vivo*. Here we discuss that, in addition to matrix stiffness, cellular, biochemical, and biophysical parameters such as stress relaxation, adhesion, spatiotemporal protein signaling, and porosity/confinement need to be considered. For *ex vivo* models, it will thus be important to incorporate these various biophysical parameters, ideally in a modular fashion to maximize control of cell fate and the drivers of oncogenic transformations. We propose several new areas of technological advancement needed for the building of better *ex vivo* cancer models to understand tumor resistance. These topics cover the integration of biomaterials-based engineering with emerging forefronts of tissue mechanics, nuclear mechanics, the immune response, and the gut microbiome.

Integrating Independent Control of Biomechanical and Spatiotemporal Signaling of Tumors

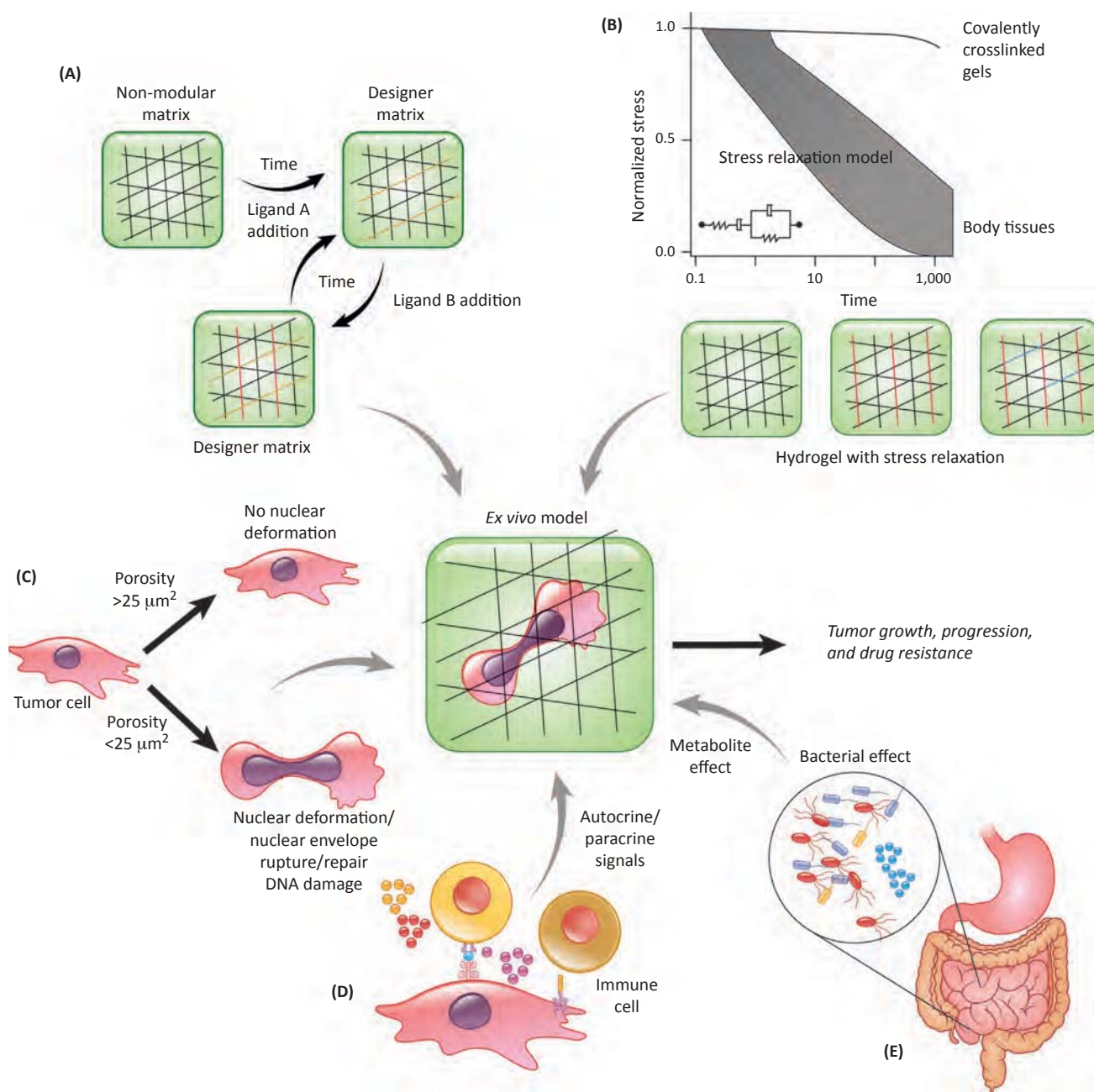
The plasticity of cancer cells to evolve different drug-resistant phenotypes is encoded by the organization and spatiotemporal dynamics of signal transduction networks. This plasticity allows them to adapt to challenging microenvironments, remodel them in their own favor, and withstand highly toxic therapeutic assaults. A recent review discusses the rich molecular signaling dynamics and their impact on cancer cell proliferation, survival, invasiveness, and drug resistance [15]. Most prior studies in cancer 3D modeling used hydrogels or scaffolds as hydrated networks of motif-containing bulk proteins or their peptidomeric forms. These peptides are either short peptides that represent adhesive binding motifs (e.g., fibronectin- or vitronectin-derived RGD) or hydrogel crosslinking peptides that are matrix metalloproteinase (MMP) degradable and allow matrix remodeling. While numerous studies have shown the utility of these hydrogels in spread, mesenchymal-like cells, we have engineered modular hydrogels to show that integrin ligands and matrix degradability can serve as prosurvival signals in B and T cell lymphomas, modulating their 3D aggregation and response to therapeutics [12]. Using RGD-presenting hydrogels and complementary studies in patient-derived xenograft mouse models, we have further shown that integrin $\alpha v \beta 3$ acts as a membrane receptor for thyroid hormones to mediate angiogenesis in malignant T cells [16]. This particular study led us to discover a novel mechanism for the endocrine modulation of T cell lymphoma pathophysiology.

However, the flow of information between tumor cells and the surrounding ECM is bidirectional and functions in a spatiotemporal manner. The adhesion process involves dynamic interactions occurring over multiple time and length scales, from seconds for nanoscale integrin receptor–ECM ligand binding to days and weeks for meso/macroscale ECM remodeling and cancerous tissue organization. Similar to the dynamic nature of cell–ECM interactions, the engineering of materials to elicit desired tumor cell responses will require precise and independent, multidimensional control over matrix spatiotemporal bioligand presentation, structural porosity, and the mechanical properties of the materials (Figure 1A, Key Figure). Recent biomaterials designs now allow spatiotemporal control, as reported by several research groups across various cell–tissue models [17–21]. Nonetheless, current hydrogels have yet to demonstrate that the same biomaterials can provide independent control over all of the bioadhesivity, spatiotemporal signaling, stiffness, and porosity of the material. In a major advance, chemical strategies that allow external manipulation of ligand presentation in real time were recently developed. A new class of hydrogels was reported that used an addition–fragmentation–chain transfer chemistry and permitted repeated exchange of biochemical ligands in a non-destructive manner [22]. Such advances afford powerful designer tools in material engineering to study cancer cell processes.

Another emerging trend is the understanding of stress relaxation *in vivo* and *ex vivo* and is increasingly becoming a crucial parameter for biomaterials design. Using bead displacement methods, Legant and colleagues showed deformations of 20–30% peak principal strain in the hydrogel surrounding the cell [78]. In 2D culture of cells on acrylamide gels, strains of 3–4% (ratio of traction to elastic modulus) are typically observed [23]. The ability of *ex vivo* scaffolds to either store (purely elastic) or dissipate (viscoelastic) forces generated by cells in contact with these surfaces can influence a cell's interaction with its surrounding [24] and we believe they may in turn regulate spread, growth, migration, and possibly chemoresistance. Stem cell growth and differentiation are enhanced in hydrogels with fast stress relaxation characteristics, as reported recently by Chaudhuri and colleagues [24]. Most non-degradable, synthetic hydrogels are purely elastic, whereas many naturally derived matrices and tissues are viscoelastic (Figure 1B), can be degraded/remodeled by cells, and often exhibit partial stress relaxation when a constant strain of 15% is applied [24]. For example, collagen and fibronectin matrices exhibit a decrease in the storage or elastic modulus over time when a constant strain is applied. This is likely to occur from the unbinding of weak hydrophobic and electrostatic interactions that hold the fibers in a network [25,26]. The elasticity of these materials is also nonlinear. On reconstituted natural matrices, the resistance to cellular traction forces is expected to relax over time due to flow and remodeling of the matrix, dissipating the energy that cell-generated forces impart into the material. Substrates with stress relaxation enhance cell spreading at a low initial elasticity, which is mediated through $\beta 1$ integrin, actin polymerization, and actomyosin contractility and is associated with increased Yes-associated protein (YAP) nuclear localization and proliferation [26,27]. This suggests that increased stress relaxation can compensate for matrices with a lower stiffness. Since several mechanosignaling network components are involved, we suspect that changes in stress relaxation and the resulting cellular response could be key to chemoresistance. Therefore, it is imperative that advanced tissue models for cancer (and also for regenerative medicine) research should consider stress relaxation beyond, and independent of, stiffness and bioadhesivity. Some of the questions to be considered are: can stress relaxation potentially program tumor cells into a more resistant phenotype? Can stress relaxation crosstalk with genetic mutations? What is the role of time-dependent viscoelastic properties in tumorigenesis and the infiltration of immune cells? What categories of tumors depend on stress relaxation? These questions can be answered by using engineered biomaterials that incorporate stress relaxation behaviors

Key Figure

Ex Vivo Cancer Tissues with Multidimensional Control of Biophysical and Biochemical Properties



Trends in Cancer

Figure 1. The schematic depicts the integration of new strategies into existing tumor tissue models. (A) Time-dependent, controlled, reversible exchange of biochemical ligands. (B) Stress relaxation, which models the viscoelastic behavior of tissues compared with current, covalently crosslinked matrices. Simple covalently crosslinked hydrogels (black-lined network) with stress relaxation can be designed by including ionic bridges (red lines) and chemical spacers (e.g., polyethylene glycol; blue lines). (C) 3D niche porosity to model the nuclear deformability of cancer cells. (D,E) Integrating the cell-cell and autocrine/paracrine effect of the immune system and the gut microbiome.

and comparing them with purely elastic materials. The challenge will be in developing such materials where stress relaxation, bioadhesivity, porosity, stiffness, and topography are independently tunable.

Designing 3D Niche Porosity to Accommodate Nuclear Deformability of Cells

In vivo cells often have to transit through narrow constrictions smaller than their nuclear diameter during migration; for example, when passing through interstitial spaces or endothelial layers during intra- and extravasation. This concept applies to all migrating cells, including immune cells, fibroblasts, invasive cancer cells, and possibly even tissue stem cells. Recent work suggests that the biophysical properties of the nucleus can play a crucial rate-limiting role during cell migration in 3D environments: For pores substantially smaller than the nuclear cross-section, migration efficiency decreases, and cells eventually stall completely when the pore size reaches the ‘nuclear migration limit’ [28]. The ability of cells to pass through such confined spaces is mostly determined by nuclear size, deformability, and cytoskeletal contractility, which are affected by nuclear and cytoskeletal composition and organization [28–31]. As these parameters can vary widely between cell types, porosity and confinement should be key considerations in engineered microenvironments. Although most engineered niches have considered the porosity of the scaffold from the crosslinking density perspective, better material designs are needed to account for nuclear deformability, as some applications may favor designs that prevent (specific) cells from entering while other designs may benefit from having large enough pores to allow cells to enter the material. In addition to limiting the motility of cells, moving a large cell nucleus through small pores can have other biological consequences. Migrating through such tight spaces places substantial physical stress on the nucleus, which can result in transient loss of nuclear envelope integrity, herniation of chromatin across the nuclear envelope, DNA damage, and redistribution of mobile nuclear proteins *in vitro* and *in vivo* [32–34]. Recent *in vitro* work by the Discher group further supports the concept that migration through tight spaces results in increased genomic stability [34,35], but direct observations of migration-induced DNA damage *in vivo* and resulting genomic instability remains outstanding. Nuclear deformation may also alter chromatin organization and gene expression, which could further modulate tumor progression and resistance to therapy. Nuclear envelope rupture and migration-induced DNA damage may emerge as a potential novel therapeutic approach to specifically target metastatic cells. Supporting this idea, combined inhibition of nuclear envelope repair and DNA damage repair resulted in significant death of cancer cells during migration through confined environments [32]. Finally, our recent work further links nuclear envelope rupture in micronuclei and an inflammatory response, explaining how chromosomal instability drives metastasis through a cytosolic DNA response [36].

Given the importance of cell migration in many biomedical applications, ranging from tissue engineering to prosthetic device coating and cancer therapy, these recent findings highlight the importance of considering pore sizes and the nuclear deformability of the relevant cells in the design process of biomaterials-based scaffolds. In general, when designing new biomaterials-based *ex vivo* tissue-engineered models for development, wound healing, tumors, and even the coating of prosthetic devices, one should consider tissue scaffold pore sizes of $<25\ \mu\text{m}^2$ in cross-section (Figure 1C). These considerations will vary with regard to tumor type and patient-specific attributes. Infiltrating immune cells, such as leukocytes, neutrophils, and dendritic cells, can squeeze through much smaller pores, down to around $1\text{--}2\ \mu\text{m}^2$. We suggest that the scaffold pore size is an important design parameter, especially since small pores could not only slow migrating cells but also induce nuclear rupture and deformation. We suspect that nuclear rupture and deformation can contribute to DNA damage and genomic instability, promoting

drug resistance through genomic rearrangements or mutations that increase cell proliferation and/or abnormal signaling. Nevertheless, a generalized approach in determining pore size is not feasible because of wide differences in the biophysical characteristics of tumor cells. Applications for different cells will require different considerations for pore size, and the degradability of the material must also be considered, as many cells can remodel the ECM, for example, by secretion of MMPs.

Integrating the Immune System and the Effect of the Gut Microbiome

In addition to changes in cell and tissue stiffness, other hallmarks of cancer include chronic inflammation and altered immune responses [37]. While modeling of the tumor immune interaction in engineered tissues is important, another point of view is that tumor-infiltrating immune cells differentiate into phenotypes that support each step of the metastatic cascade and thus are novel targets for therapy. *In vivo* tumor-infiltrating T cells (including regulatory T cells), B cells, immunosuppressive myeloid-derived suppressor cells (MDSCs), and macrophages continuously interact with tumor cells through direct cell contact or by the secretion of a milieu of proinflammatory cytokines and chemokines. This immune-privileged microenvironment could selectively impair the recognition of tumor antigens by cytotoxic T cells and also protect residual tumor cells against cytotoxic destruction [38–41]. Immune cell populations, like T cells and natural killer cells, can control metastases of cancer cells by either restricting them to the primary tumor niche or promoting migration away from the primary tumor site [42,43]. Even macrophages, the cells of the innate immune system, adapt to the tumor microenvironment and polarize to the M1 extreme and secrete high levels of interleukins (e.g., interleukin-8 and -10) and granulocyte colony-stimulating factor, which suppress immunity [44]. The secretion of cytokines is dependent on tumor subtype.

The introduction of these immune components and recapitulation of the immune–cancer interaction complex in an *ex vivo* engineered system is ideal but non-trivial (Figure 1D). One of the main challenges is that immune cells, when cultured *ex vivo*, undergo rapid apoptosis over time unless rescued by antiapoptotic signals and replenished with a fresh supply of immune cells [45]. Newer approaches that recapitulate the continuous replenishment of immune cells *in vivo* can be achieved by integrating microfluidic platforms [46] with engineered tissues, where immune cells could be added to the feeding lymphatic or vascular networks. Simple encapsulation of immune cells in collagen or Matrigel is suboptimal, as these biomaterials may not provide the necessary survival signals and functional immune cells. Alternatively, advanced biomaterials-based scaffolds that support immune cell growth [47–51] could be integrated with the existing tumor niches and other on-chip approaches [52]. The development of such tissues can further be applied to study the efficacy of immunotherapy and the effect of immunomodulatory drugs on immune cells.

In addition to immune cells, tumor progression and the efficacy of antitumor therapies are now known to be affected by the microbiome. For example, *Fusobacterium nucleatum*, among other bacteria, is enriched in colorectal cancer patients' microbiomes [53,54] and *Helicobacter pylori* has been shown to be abundant in patients with non-cardia gastric cancers [55]. These interactions can be local, as *Clostridium*, *Bifidobacterium*, and *Salmonella* (reviewed in [56]) have been shown to grow in the tumor microenvironment and elicit immune reactions that can assist in tumor suppression. Similarly, in the colon, microbial biofilms have been implicated in the progression of colon cancer [57]. Direct impacts of the microbiome may also be systemic. Recently, the production of a microbe-derived carcinogen, deoxycholic acid, was shown to directly contribute to liver cancer [58]. Although the mechanisms that causally link microbes and tumorigenicity have not been established [59], several mechanisms have been proposed,

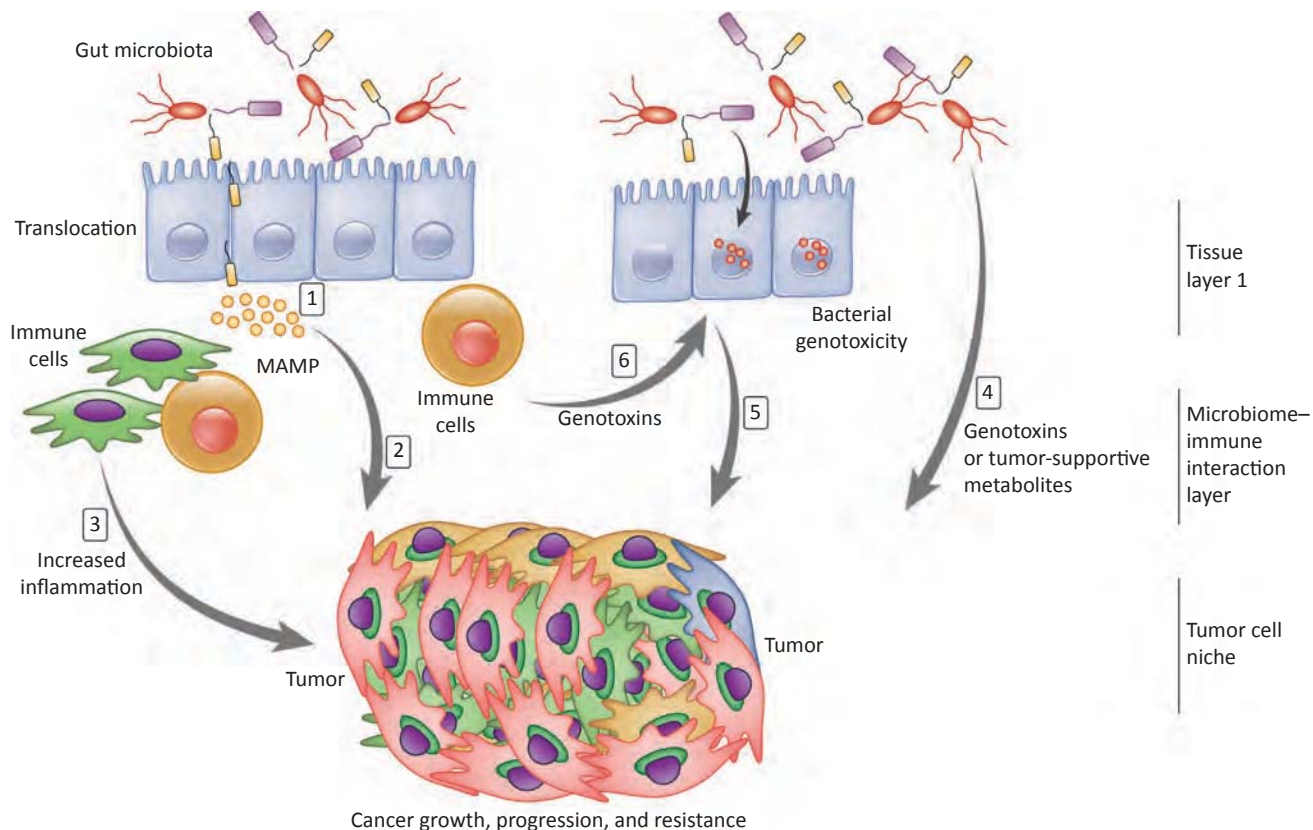
including metabolic signaling to promote proliferation or angiogenesis pathways [60]. Microbes may also produce reactive oxygen species, which may in turn contribute to DNA damage and, ultimately, tumor resistance. Manipulating these systems *in vivo* can be technically challenging due to the complexity of the communities and the difficulty of working with germ-free or monocolonized mice. An outstanding question is how to best integrate the effects of the microbiome into current *in vitro* engineered model systems for cancer and tumor resistance to create a more efficient cancer ecosystem (Figure 1E).

The longest-standing evidence for a link between the microbiome and cancer is in relation to colon cancer. *In vitro* models of colon cancer utilize organoid models, which are extremely useful for testing drug delivery, genetic manipulation, and the incorporation of biopsied tissue. Bacteria can be administered in these models by injecting them directly into the organoid cavity [61], but integration of biofilms into the organoid model of colon cancer remains to be done. An alternative to organoids for intestinal cancer models are 3D scaffold models of intestinal tissue. Several studies have been completed with co-culture of organisms on these scaffolds [62,63], but these experiments are limited in scope to a handful of organisms and are constrained by the difficulties of co-culturing intestinal cells with organisms that are normally found in an anaerobic environment. There are additional engineering challenges in introducing the range of biologically relevant cell types, which would both enhance mucous production and maintain barrier integrity. More attention is warranted in the areas of understanding the microscale interactions of these organisms and cancer cells and the shear stress caused by the laminar flow of intestinal contents, peristalsis, and large-scale mechanical reflexes and cellular apoptosis.

The mechanistic effects of the microbiome in colon cancer may be direct (e.g., signaling and small-molecule delivery directly to intestinal stem cells, affecting their proliferation) or indirect, via immune system function. The most striking example of this relates the microbiome and the efficacy of cancer immunotherapies in rodent models of colon cancer and melanoma [64,65]. It is currently thought that the microbiome alters the function of the immune system, specifically through changes in immune cell composition, maturation, and the inflammatory milieu. Several questions remain: do *in vitro* models of cancer lacking microbes adequately recapitulate *in vivo* malignancies or must we include microbes? Are there effects on tumors or the immune system that are specific to certain microbes or microbiome compositions? Are microbiome-mediated effects on cancer progression direct or predominantly mediated through the immune system? Can microbiome-mediated effects on cancer progression be modeled by simply introducing metabolites, cellular lysate, or supernatant to tumor cell culture or do we need to co-culture bacterial cells via *ex vivo* gut reactors integrated with immune and cancer tissues to elicit real-time feedback? Additionally, this feedback is likely to impact the composition of the microbiome as well as its function. The promise of the integration of complex microbial communities is that it will permit bidirectional analysis of microbiome effects on cancerous cells and the effects of tumorigenic cells on those bacteria. Single-cell analysis of individual bacteria [66] will provide information on selective pressures exerted on bacteria in the tumor microenvironment; metagenomic and strain-level analyses of microbial communities [67] co-cultured with tumor cells can be used to examine the function of these cells, including information on the metabolites they produce that might in turn influence tumorigenic progression and the formation of resistance. *In vitro* systems may prove fruitful in testing the effects of these different communities on immune or cancer tissue.

Last, because we know that the tumor microenvironment is permissive to bacterial colonization, can we study the effects of tumor-targeting synthetic bacteria *in vitro*? Din and colleagues

engineered a side-trap array microfluidic platform to co-culture human cervical cancer HeLa cells with *Salmonella typhimurium* [68]. Ingber and colleagues have also reported a simple biomimetic ‘human gut-on-a-chip’ microdevice that recapitulates epithelium polarization and villus-like folds and can be used for co-culture of a normal intestinal microbe (*Lactobacillus rhamnosus* GG) for extended periods of time (>1 week) without compromising epithelial cell viability [69]. Yissachar *et al.* [70] have taken an alternative approach whereby segments of intestinal tissue are excised and cultured *in vitro*, linking intestinal inputs and outputs directly to cultured immune and neuronal cells. This system may prove valuable in linking microbiome outputs to other *in vitro* models of tumor progression. The next challenge will be in determining how to integrate the diversity of the microbiome so that we can study its effect on overall tumor progression, tumor resistance, and drug targeting while modulating other aspects of the tumor microenvironment crosstalk (Figure 2).



Trends in Cancer

Figure 2. Ex Vivo Cancer Models with Possible Microbiome–Tumor Interactions. The figure shows an example of a three-layer ex vivo model. (1) Alterations in gut microbiota may result in increased bacterial translocation. (2) Increased abundance of microorganism-associated molecular patterns (MAMPs) directly influences tumor cells through local or distant mediators. (3) MAMPs stimulate Toll-like receptors on immune and other niche cells, leading to an increased tumor-supportive milieu of cytokines (e.g., interleukins) and growth factors. (4) The microbiota mediates tumor suppression through the generation of short-chain fatty acids and biological activation of cancer-preventing phytochemicals. (5) Bacterial genotoxins, after being delivered to the nuclei of host cells, actively induce DNA damage in organs that are in direct contact with the microbiome, such as the gastrointestinal tract. Other genotoxic components include reactive oxygen species, reactive nitrogen species released from inflammatory cells, and hydrogen sulfide from the microbiota. (6) Gut-mediated metabolites may result in: (i) activation of genotoxins such as acetaldehyde; (ii) activation of the metabolism of hormones; or (iii) alterations in the metabolism of bile acids.

Concluding Remarks

Engineered microenvironments have already emerged as important and useful tools to study tumor cells *in vitro*. A key advantage of *ex vivo* models is that the number of features (and correspondingly, the number of variables in the system) is constrained so that the effects of specific factors can be more clearly delineated. Although the most comprehensive models may not necessarily be the best model for a particular application, there remains a need to address unresolved questions of cellular, molecular, and microenvironment complexity (see Outstanding Questions). In addition to conventional bioadhesive matrices, decellularized matrices and glycosaminoglycans (GAGs) offer alternative choices for fine-tuning the 'right' characteristics in the model in terms of both pore size and matrix relaxation. Most previous *ex vivo* models have focused on stiffness and biochemical ligand presentation as the predominant design parameters and tumor invasion as the primary readouts. We propose that additional parameters should be considered in the design of such models, including viscoelastic properties, physical pore sizes, and the microbiome. Additional aspects of tumor microenvironments could include cancer-associated fibroblasts, stromal cells, immune cells, the role of fluid flow, matrix heterogeneity, and interactions with the vascular and lymphatic circulations [71–73]. In addition, genomic and epigenetic evolution of cancer cells should be considered as key drivers of therapy resistance, and the effect of the physical microenvironment on these processes should be investigated in more detail. We believe that careful modeling of *ex vivo* niches will result in improved understanding of epigenetic, metabolic, and signaling patterns in cancer, therefore leading to the development of new therapeutics [16,74–77]. Modular approaches that enable independent tuning of individual biophysical and biochemical parameters will facilitate more systematic studies. Technical advances that permit co-culture of bacteria with cancer cells can not only lead to better understanding of the tumor–microbiome interaction but also drive new directions to systemically deliver an antitumor toxin using synthetically engineered bacteria. Finally, newer *ex vivo* models that recapitulate complete selective aspects of the tumor immune microenvironment interactome are needed to maximize our effort towards a tumor microenvironment-driven precision medicine strategy and evaluate synergies between combination therapies to overcome resistance. Such innovative approaches will increase the 'predictive power' of preclinical inhibitors, provide potential biomarkers for correlative studies in new inhibitor clinical trials, and provide clues towards mechanisms that induce resistance to therapeutic inhibitors by more faithfully representing patient biological features and creating clinically relevant treatment regimens.

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Outstanding Questions

How do chemoresistance and changes in biophysical and biochemical factors relate to one another?

Can stress relaxation potentially program tumor cells into a more resistant phenotype? Can stress relaxation crosstalk with genetic mutations? What categories of tumors depend on stress relaxation?

What is the role of time-dependent viscoelastic properties in tumorigenesis and infiltration of immune cells?

How can we engineer *ex vivo* models with cell-specific consideration of pore size?

Is it sufficient to leave out the microbes from *in vitro* models of cancer? Are there effects of specific microbes or is it mainly the triggers they provide to the immune system?

Can microbiome-mediated effects on cancer progression be modeled by simply introducing metabolites, cellular lysate, or supernatant to tumor cell culture or do we need to co-culture bacterial cells via *ex vivo* gut reactors integrated with immune and cancer tissues to elicit real-time feedback?

How can we best integrate the effects of the microbiome into current *in vitro*-engineered model systems of cancer and tumor resistance to create a more efficient cancer ecosystem?

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Assembly and use of a microfluidic device to study cell migration in confined environments

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Abstract

Cells migrating in tissues must often pass through physical barriers in their surroundings in the form of fibrous extracellular matrix or other cells. To improve our understanding of how cells move in such confined microenvironments, we have designed a microfluidic device in which cells migrate through a series of three-dimensional polydimethylsiloxane (PDMS) constrictions with precisely controlled geometries that mimic physiological pore sizes. The migration device offers an experimental platform that combines a well-defined three-dimensional (3D) environment with a set-up well suited for imaging confined cell migration at high spatial and temporal resolution. In this protocol, we describe the fabrication and use of these devices using standard soft lithography techniques and light microscopy. Analysis of live-cell time-lapse series of cells with fluorescently labeled nuclear and/or cytoskeletal structures migrating in the devices can reveal new insights into the molecular processes required for confined migration, including the role of the linker of nucleoskeleton and cytoskeleton (LINC) complex, which has been implicated in 3D migration.

1. Introduction

Cell migration represents a crucial step in a variety of biological processes, including cancer metastasis, inflammation, and wound healing. In the *in vivo* tissue environment, interstitial spaces, extracellular matrix networks, and other cells constitute a confined environment for migrating cells. Dense fibrous tissue matrix and layers of endothelial cells form narrow constrictions, which measure 0.1-30 μm in diameter [1,2]. To move through such tight spaces, cells must produce significant intracellular forces to compress their nucleus, as it is typically the largest and stiffest organelle [3-5]. Prior work has suggested that the linker of nucleus and cytoskeleton (LINC) complex facilitates the transmission of forces from contractile actomyosin fibers to the nucleus during confined migration [6-10]. Consequently, depletion of LINC complex proteins such as Nesprin-2 and Nesprin-3 reduces migration speed through confining environments, and impairs the cell's ability to deform its nucleus [6-9]. Since the dimensionality of the cell environment modulates migratory behavior, the study of cell migration *in vitro* requires model systems that faithfully reproduce the 3D confinements of biological tissue (Fig. 1A-B) [11]. Additionally, to elucidate the dynamic nuclear and cytoskeletal processes that mediate nuclear translocation in confined spaces, these model systems must also enable the detailed observation of cells by time-lapse microscopy by confocal or wide field microscopy.

Many tools have been developed for the study of confined migration *in vitro* [12,13]. One popular group of devices are transwell invasion assays, such as the Boyden chamber. However, these systems are limited by a limited ability to directly observe cells during migration, as cells in these chambers migrate perpendicular to the imaging plane. Another approach for studying confined migration are extracellular matrix scaffolds (e.g., collagen or Matrigel). These scaffolds are generated through random self-assembly of matrix fibers, which produces an environment that closely mimics biological tissue, but provides only limited control over the size of individual pores. In recent years, microfluidic devices have been developed using soft lithography techniques to reproduce the physical confinements of the *in vivo* tissue environment [12,14,15]. These devices feature channels of defined geometries through which the cells must migrate, either spontaneously or following a chemotactic gradient. These microfluidic platforms are quite versatile as they

provide the user with flexibility in the design and layout of constrictions.

In this protocol, we describe the design and use of a polydimethylsiloxane (PDMS) microfluidic device with a series of tight constrictions located between two larger chambers (Fig. 2B, C)[14]. Cells are seeded in one of the chambers and then migrate through a section 5 μm in height containing constrictions between 1 and 2 μm in width, formed by 30 μm wide circular pillars (Figs. 1C and 2). Additionally, there is a set of 15 μm wide constrictions, which cells can migrate through without deforming their nucleus, and which serve as an important control to assess effects independent of nuclear confinement. Unlike other microfluidic devices, in which cells often move through long, continuously confining channels, the confinement in the device described here is limited to a very short segment, i.e., cells move through a single, tight constriction, enter a less confined region, and then encounter the next constriction. This design seeks to reproduce the varied, discontinuous sequence of pores and confinement which cells are subjected to during in vivo migration, such as matrix fibers, or endothelial cell layers [1,16]. These devices have already aided in our initial studies of dynamic processes that occur during confined migration, such as the rupture of the cell nucleus and the essential role of perinuclear myosin IIB in moving the nucleus through narrow constrictions [17,7].

To produce these devices, SU-8 microfluidic features are formed onto a silicon wafer through photolithography. Next, a PDMS replica is cast from the SU-8 features, the PDMS is cut into individual devices, bound to a glass slide, functionalized with extracellular matrix (ECM) solution, and seeded with cells. Following a period of incubation to allow cells to enter the constrictions, analysis of cell migration can be performed by live-cell imaging or standard immunofluorescence techniques. This protocol will outline the procedures necessary for both producing these devices and using them for the study of confined migration (Fig. 3). The protocol assumes basic familiarity with SU8 and PDMS soft lithography. For users new to soft lithography, we recommend Qin et al (2010) as a good starting point [18].

2. Materials

2.1 Photolithography

1. CZ silicon wafer, 4 inch diameter, type N, 525 μm thick, <1-0-0> orientation (Silicon Quest International)
2. Chrome photomask on a quartz substrate, 5" $\times$ 5" $\times$ 0.090" (Telic, Valencia, CA)
3. Heidelberg DWL 2000 mask writer (Heidelberg Instruments, Heidelberg, Germany)
4. Long-pass filter for near UV light (PL-360LP from Omega Optical, or equivalent.)
5. AutoCAD software (Autodesk, Mill Valley, CA) or equivalent
6. Mask aligner system (ABM, San Jose, CA)
7. Molecular vapor deposition system (SPTS Technologies, Newport, RI)
8. Kapton polyimide film (Dupont, Wilmington, DE)
9. Oven suitable for temperatures up to 150°C
10. CEE Model 100 spin coater, or equivalent (Brewer Sciences, Rolla, MO)
11. SU-8 2005 photoresist (MicroChem, Newton, MA)
12. SU-8 100 photoresist (MicroChem, Newton, MA)
13. SU-8 developer (MicroChem, Newton, MA)
14. Semiconductor grade acetone
15. Cleanroom swab (Texwipe TX761 Alpha Swab with long handle, or equivalent)
16. CMOS grade isopropyl alcohol (IPA)
17. Deionized (DI) water
18. >95% (1*H*,1*H*,2*H*,2*H*-perfluorooctyl)trichlorosilane (FOTS) (Gelest Inc, Morrisville, PA)

2.2 Casting of migration devices in PDMS

1. 150 mm Petri dish
2. Sylgard 184 silicone elastomer base and curing agent (Dow Corning, Midland, MI)
3. Stirring rod for mixing elastomer base and curing components

4. Vacuum pump (Oerlikon Trivac D2.5E)
5. Vacuum desiccator (Catalog #Fisher 08-594-16C)
6. Oven suitable for temperatures of 65°C (e.g., VWR Gravity Convection Oven, Catalog #414005-108, or equivalent)

2.3 Mounting and seeding of devices

1. Biopsy punches (1.2 mm and 5mm)
2. 24 mm x 60 mm Number 1.5 Micro Cover Glass (VWR, Radnor, PA, Catalog # 48393-251)
stored overnight in 0.2 M hydrochloric acid
3. Isopropyl alcohol (IPA)
4. DI water
5. 70% ethanol
6. Oxygen plasma cleaner (Harrick Plasma, Catalog# PDC-001)
7. Type I collagen (50 µg/mL in 0.02M glacial acetic acid) or fibronectin (5 µg/mL in PBS) solution
8. Dulbecco's Phosphate Buffered Saline (DPBS), no calcium, no magnesium (Thermo Fisher, Waltham, MA, Catalog# 14200-075, or equivalent)
9. Cells of interest (see Note 1)
10. Cell culture media appropriate for cells of interest
11. Pipettes and tips for loading devices (20 µL and 200 µL pipettes work well)

2.4 Microscopy and Analysis

1. Inverted fluorescence microscope. Microscope should have objective with 20× magnification and fluorescence excitation/emission filters for GFP and/or other fluorophores of interest.
2. Stage-top incubation chamber for microscope to maintain temperature at 37 °C. Humidity control is optional, as microfluidic devices can be sealed.
3. Microscope-mounted CCD or CMOS camera for image acquisition

4. Image acquisition software, such as ZEN BLUE (Zeiss), Micromanager, or others
5. ImageJ, FIJI, MATLAB, or other software for image analysis

3. Methods

3.1 Photolithography

All photolithography steps should be performed in a dedicated clean room facility under standard clean-room conditions, with protective equipment and sufficient ventilation underneath a fume hood. The development of SU-8 microfluidic features onto a silicon wafer is described below.

1. Generate a design for the mask using CAD software and the downloaded CAD files. The device design and geometry is described in detail in Davidson et al.(2015) [14]. The CAD files for the device can be downloaded at <http://lammerding.wicmb.cornell.edu/>.
2. Using a Heidelberg DWL 2000 Mask Writer, develop the chrome photomask based on the CAD design layout. This photomask will be used to expose the design features in SU-8 photoresist onto the silicon wafer using near ultraviolet (UV) light.
3. Clean silicon wafer using standard metal oxide semiconductor (MOS) cleaning procedures (see Note 2) and bake overnight at 90° C to dehydrate.
4. Coat wafer with ~2 mL of SU-8 2005 (MicroChem) and spin down using a spin coater (CEE Model 100 spin coater, or equivalent) at 3000 RPM for 30 seconds (see Note 3) to obtain a 5 µm thick layer, which will be used for the design of the first layer with the 5 µm tall features.
5. Allow the SU-8 to relax for 10 minutes at room temperature (RT). Then remove 5 mm of excess SU-8 from the edge of the wafer perimeter using a cleanroom swab soaked in acetone.
6. Bake the wafer on a hot plate from RT to 65 °C at a rate of 2.0°C per minute, hold at 65°C for 10 minutes, then remove from plate and allow to cool back to RT (see Note 4).
7. Expose the wafer to near UV light at 365nm using an ABM contact aligner with a long pass filter for 40 seconds (see Note 5).
8. An hour after exposure, bake the wafer on a hot plate from RT to 95°C at a rate of 2.0°C per minute, hold at 95°C for 1 minute, and then leave to cool back to RT.
9. Place wafer in SU-8 developer overnight to remove unexposed SU-8.

10. Rinse wafer with fresh SU-8 developer, then rinse with isopropyl alcohol and deionized water two times each to clean.
11. Bake wafer in an oven from RT to 150°C for 20 minutes. Then shut off oven, and allow wafer to cool to RT. This step is a “hard bake” step, which solidifies the first SU-8 layer, and prevents unwanted merging with the second layer.
12. Dehydrate wafer overnight by baking at 90°C.
13. Cover the alignment marks on the wafer for the 5µm layer using Kapton tape (see Note 6).
14. For a thickness of 200µm, coat wafer with ~2 mL SU-8 100 and spin at 1500 RPM for 60 seconds (see Note 7).
15. Allow the SU-8 to relax for 10 minutes and then remove 5mm of excess SU-8 from the edge of the wafer perimeter using a cleanroom swab soaked in acetone.
16. Bake the wafer on a hot plate with a lid (any cover is sufficient; we use a Pyrex petri dish cover) from RT to 55°C at a rate of 2.0°C per minute for 14 hours, then increase to 60°C at a rate of 2.0°C per minute for 14 hours, then leave to cool back to RT.
17. Score the edges of the Kapton tape with a razor and gently remove from the wafer.
18. Expose the wafer on the contact aligner with a long pass filter for one minute, six times with one minute of rest in-between (see Note 8).
19. Twenty minutes after exposure, bake the wafer on a hot plate with a lid. Increase the temperature from RT to 95°C at a rate of 1.5°C per minute, hold at 95°C for 1 minute, and then leave to cool back to RT.
20. Leave wafers in SU-8 developer overnight.
21. Rinse wafer with fresh SU-8 developer, followed by a wash with isopropyl alcohol and deionized water, two times each.
22. Bake wafers from RT to 60°C for two hours in an oven on an aluminum plate to remove moisture and leave to cool in the oven to RT.
23. Coat wafers with FOTS using a Molecular Vapor Deposition (MVD) tool.

24. Wafers can now be removed from the clean room and used for casting into PDMS (Fig. 2ii, iv).

3.2 Casting of migration devices in PDMS

From this point on, all components should be handled with nitrile gloves to minimize risk of contamination of devices. All steps may be performed on a lab bench, until devices are assembled, sprayed with ethanol, and moved into a cell culture hood.

1. Place the silicon wafer with SU-8 features facing upward into a 150 mm petri dish (or use secondary plastic mold, see Note 9).
2. Add PDMS base and curing agents into a standard plastic cup at a 10:1 ratio (typically 50 grams of base and 5 grams of curing agent is sufficient for a set of 12 device chips) and stir vigorously for 5 minutes to fully combine. The stirring of these components will cause many air bubbles to form in the mixture, and these must be removed through degassing prior to curing of the PDMS.
3. Place PDMS mixture into a vacuum desiccator at 30 psi for 20 minutes to eliminate bubbles and accelerate degassing of the polymer.
4. Pour PDMS mixture over wafer (or secondary device mold), and allow 5 to 10 minutes to set at room temperature.
5. Using a very light stream of pressurized air, blow directly over the surface of the PDMS in order to eliminate all remaining bubbles. After this step, PDMS should be completely clear.
6. Preheat an oven to 65°C, and then bake PDMS mixture in oven for at least 2 hours (see Note 10).
7. Remove mold from oven and allow PDMS to cool to room temperature (see Note 11).
8. Using a razor, cut around edges of PDMS mold, ensuring that there is sufficient space in between the cut-edge and device features.
9. Carefully peel PDMS out of the mold, ensuring that PDMS does not tear during removal. The molded PDMS will contain 12 device “chips,” each of which contains 2 migration devices

(Fig. 1A).

10. Using a razor, cut PDMS into 12 device chip segments (Fig. 2v).
11. Place PDMS devices onto clean packing tape, feature-side down, to protect devices from dust during storage.
12. Store devices at room temperature, or proceed immediately to mounting onto glass slides for use with cells.

3.3 Mounting and seeding of devices

1. Prior to mounting, store glass slides overnight in 0.2 M hydrochloric acid (see Note 12).
2. Remove a device chip from packing tape and immediately prepare for cleaning.
3. Cut out holes for seeding ports and media reservoir into PDMS using biopsy punches (Fig. 1A, i and ii). Location of holes is marked on PDMS as part of the mask design. Media reservoirs are cut out using a 5 mm punch, while the seeding inlets should be cut out with a 1.2 mm punch (see Note 13).
4. Hold device with forceps and rinse with isopropyl alcohol (IPA), followed by deionized water. Repeat once (see Note 14).
5. Using pressurized air, dry device thoroughly, and then place inside of plasma cleaner, feature side up.
6. Repeat washing and drying steps on cover glass slides and place inside of plasma cleaner alongside device (see Note 15).
7. Close plasma cleaner and turn on pump and power switches. Wait a few minutes to allow the plasma cleaner to warm up, and then turn on the RF level (which regulates the intensity of plasma within the chamber) to High.
8. Plasma treat the PDMS devices and glass cover slides for five minutes, adjusting the air intake in order to keep the plasma active (a bright pink color) throughout the treatment procedure (see Note 16).

9. Turn off the plasma cleaner and release the air pressure very slowly.
10. Remove the treated glass slide and PDMS device from the plasma cleaner, and place the device onto the glass slide, feature-side down (see Note 17).
11. Using your thumb, press the device down firmly onto the glass slide. Press around the device to ensure that the whole device is firmly bonded to the glass cover slide (see Note 18).
12. To improve adhesion of the PDMS to the glass, place the bonded device onto a hot plate at 95°C for 5 minutes (see Note 19).
13. Remove the device from the hot plate and allow the device to cool for a few minutes, before spraying the outside of the device completely with ethanol, and moving the device into a cell culture hood.
14. Fill the media reservoirs with ethanol and allow the device to incubate for 10 minutes at RT for sterilization.
15. Remove ethanol from the device and rinse the media reservoirs three times with PBS for 5 minutes each. Each device will hold between 150 and 200 μL of fluid.
16. At this stage, the inside surfaces of the device can be functionalized with various biologically relevant coating, depending on the cell line to be used and the experimental goals. We use fibronectin or collagen coatings for most cell lines. To functionalize the inside of the device, fill devices with protein solution through seeding ports (Figure 1A, ii) and allow the coatings several hours to set to the device surface (see Note 20, Table 1).
17. Remove coating solution and rinse the inside of the device three times using 180 μL of cell culture media applied to one of the reservoirs, allowing 5 minutes of incubation between each rinse.
18. Prepare cells for seeding into devices (see Note 21, Table 1).
19. Completely aspirate all media from devices, ensuring that bypass channels and device features are clear.
20. Pipette 6 μL of cell suspension into the seeding port on the same side of the device as the bypass

channel (Fig. 1A). Seed cells into the right port on left device, and left port on right device (see Note 22).

21. Check seeding of the cells underneath a bright field microscope. The cells should be distributed evenly across the front of the device constriction channels (Fig. 3A). If the cells are biased towards one end of the constrictions (Fig. 3B), aspirate cells from the device and repeat seeding process.
22. Slowly add 180 μ L of cell culture media into the media reservoir at the end of the device opposite from where cells were seeded (Fig. 1A, into top reservoirs). When media is added to one reservoir, it will flow through the constrictions and bypass channels to fill the reservoir on the opposite side. By adding media on the reservoir on the end of the device opposite the seeding channels, this prevents the inflow of media from pulling cells off of the glass and potentially pushing them prematurely into the constrictions.
23. Check cell seeding under microscope after this step to ensure that addition of media did not move cells from their original, uniformly seeded position.
24. Place the device with cells into a 37°C cell culture incubator and incubate until ready to image.

3.4 Time-Lapse Imaging and Analysis of Cell Migration

Time-lapse imaging of cells requires a microscope with an incubation chamber to maintain optimal cell culture conditions (i.e., adequate temperature, CO₂ concentration, humidity) throughout the imaging process (see Note 23). When using fluorescence microscopy, the experiments may require some troubleshooting to determine suitable excitation intensity, imaging intervals, exposure times, and imaging duration to avoid phototoxicity caused by repeated imaging throughout the experiment. Here, we will briefly outline our analysis protocol for studying nuclear transit in confined migration, after a time series of migrating cells within these devices has been collected. Alternatively, cells can also be fixed within migration devices and processed with standard immunofluorescence staining techniques for further analysis of nuclear and cytoskeletal elements involved in nuclear translocation (see Note 24).

1. Place the migration device in the microscope incubation chamber and bring the cells into focus.

We find that a single region of constrictions is best visualized under 20× magnification to measure transit times, but higher magnification may be required to capture subcellular dynamics.

2. Let the microscope with mounted migration device thermally equilibrate for 15-30 minutes to avoid drift of focus.
3. Set up image acquisition software to capture region of interest within the device at regular time intervals (minimum of 1 frame every 10 minutes, more frequent imaging may be necessary for faster migrating cells). Each device has 6 regions to capture, representing areas with different constriction sizes (three with $1 \times 5 \mu\text{m}^2$ constrictions, two with $2 \times 5 \mu\text{m}^2$ constrictions, and one with $15 \times 5 \mu\text{m}^2$ constrictions), and each chip contains two devices.
4. Acquire time-lapse image series of all regions of interest overnight (see Note 25).
5. Using ImageJ, or an equivalent image analysis software, define the nuclear perimeter of each cell throughout the time series of images. If using cells modified to express fluorescently labeled proteins within the nucleus, you may define the nuclear perimeter using an intensity threshold (see Fig. 4).
6. For each cell attempting to pass through a constriction, define the time point at which the cell has “committed” to enter the constriction. This can be done by noting when the front of the nucleus crosses an imaginary line parallel to the center of the constriction (see Fig. 4, and Note 26).
Similarly, define the time point at which the nucleus has exited the constriction, either when the rear of the nucleus crosses a second imaginary line towards the rear of the constriction (successful pass, see Fig. 4), or when the nucleus backs out of the constriction, i.e., the front of the nucleus is no longer inside the region between the imaginary lines (unsuccessful attempt). For successful passes, the ‘transit time’ is defined as the time between the entry and exit point.
7. Repeat this process for cells in the $15 \mu\text{m}$ wide channels. This measurement is crucial when comparing different cell lines or treatment conditions, which may affect the overall motility of

cells, regardless of nuclear confinement (see Note 27).

8. Normalize the transit time of cells moving through the 1 μm and 2 μm wide constrictions to the average transit time of cells under the same condition (genotype, treatment) moving through the 15 μm wide channels. This is considered the 'normalized transit time' and describes the effect of the nuclear confinement on the migration efficiency.
9. Compare normalized transit times between different constriction sizes (e.g. 1 μm vs. 2 μm wide constrictions), genotype, or treatment (e.g., LINC complex disruption vs. mock control) for analysis of migration times throughout the device.

4. Notes

1. Most migratory cell lines should work well in these devices. We have had success with HT1080 fibrosarcoma cells, mouse embryonic fibroblasts, differentiated HL60 neutrophil-like cells, MDA-MB-231 metastatic breast cancer cells, and a variety of other invasive breast cancer cell lines.
2. MOS Cleaning is a 10 minute base dip in 1 part NH_4OH , 1 part H_2O_2 and 6 parts water for 10 minutes, DI water rinse and an acid dip in 1 part HCl , 1 part H_2O_2 and 6 parts water for another 10 minutes with a final DI water rinse.
3. It is important to slowly ramp up, and ramp down from the 3000 RPM top speed to ensure even spreading of the SU-8. The spin protocol we have found to be successful is: ramp up to 500 RPM at 100 RPM/sec for 10 sec, increase to 3000 RPM at 300 RPM/sec for 30 sec , then ramp down to 100 RPM at 100 RPM/sec for 30 sec before stopping.
4. This pre-exposure baking step removes excess solvents, improves the photoresistive profile of the SU-8, and prevents adhesion to the contact mask.
5. ABM Contact Aligner dose with long pass filter: 8.8 mW/cm^2 at 365 nm wavelength and 12.9 mW/cm^2 at 405 nm wavelength. Long pass filter is a PL-360LP from Omega Optical, or equivalent.
6. Tape may be forgone by using a cleanroom wipe with acetone to remove the freshly spun SU-8 from the alignment marks.
7. Same as in Note 2: ramp up to 500 RPM at 100 RPM/sec for 10 sec, increase to 1500 RPM at 300 RPM/sec for 30 sec, and ramp down to 100 RPM at 100 RPM/sec for 15 sec before stopping.
8. It may be possible to use shorter exposure times, but 1 minute exposure is typically sufficient to achieve good SU-8 feature sizes. The UV lamp intensity can decrease over its lifetime, and therefore the optimal exposure time for this step may vary. Over- or underexposure will affect the dimensions of the SU-8 features and of the final PDMS device. Thus, constriction dimensions

should be validated for new devices. This can be done by imaging the SU-8 features, or by filling the assembled PDMS devices with fluorescently labeled dextran or other fluorescent solution and acquiring confocal image stacks of the constriction channels.

9. In order to protect the silicon wafer and delicate SU-8 features from damage during regular device production, we recommend that the first set of PDMS cast migration devices is used to produce a secondary plastic mold. This plastic mold should then be used for subsequent reproduction of migration devices. Detailed notes on producing a secondary plastic mold can be found in Desai et al. (2009) [19].
10. Ensure that the mold sits completely flat in the oven to prevent PDMS from tilting during curing. Tilted devices can still be used; however the devices will vary in thickness and therefore will not be able to hold the same volume of cell media.
11. When using a secondary plastic mold to make devices, the PDMS can be immediately removed following baking. When using the silicon wafer as your device mold, be sure to allow the device to cool completely before proceeding to avoid damaging SU-8 features.
12. 0.2M hydrochloric acid (HCl) solution should be replaced on a weekly basis. Over time, HCl will evaporate, and devices will not properly adhere to glass slides.
13. During this step, be very careful with the placement of the punch-outs. The 1.2 mm punch sits between the bypass channel and the constrictions; interference with either will likely affect your results. It is also advised that you punch through the PDMS with the feature-side up to prevent poorly located cut-outs.
14. When rinsing device, hold device upward, above your hands and pour IPA and water downwards onto device. Otherwise, run-off of material from gloves may fall onto device and prevent adhesion between PDMS and the glass slide.
15. During drying of cover glass with pressurized air, hold glass firmly and slowly increase air pressure, blowing parallel to the length of the glass to prevent bending and breaking of the glass.
16. This will activate the surface of the PDMS, allowing better adhesion between the device features

and the glass slide. Pay close attention and adjust air intake to keep plasma bright pink throughout cleaning, as we have found that poor device adhesion often results from poor plasma cleaning technique.

17. At this stage, ensure that you do not touch the surface of the glass slide nor the device-side surface of the PDMS. Additionally, check orientation of the device constrictions and place very carefully, as you will not be able to adjust the orientation after it contacts the glass. Orientation of devices is entirely up to the user, but if mounting multiple devices on a single glass slide, analysis is generally easier if all cells are migrating in the same direction. Generally, we align the devices so that the cells will migrate “upward” (i.e., in the y-direction) while observing under a microscope.
18. This step may take some trial and error: if the PDMS device is not pressed sufficiently firmly against the glass, some device features may not fully adhere to the glass. In this case, cells can migrate underneath the PDMS pillars (Figure 3D). If the PDMS device is pressed too hard against the glass, the features may collapse, preventing cells from migrating through the device. We have found that gently pressing around the device perimeter, then evenly rolling one finger across the whole device with very light pressure works best.
19. At this step, the device can be inspected underneath a bright field microscope to see if the PDMS features are fully adhered. There will be a notably different color coming through regions of the device that are not bonded to the glass.
20. When coating, check the inside of the device for pockets of air bubbles, which may form during addition of protein solutions into the device. These air bubbles can form around constrictions, which can prevent the protein from coating these features. To eliminate the bubbles, vigorously pipette protein solution through the device. Optimal seeding densities of cells and concentrations of ECM solutions that we have determined are found in Table 1.
21. The exact number of cells to be seeded will be dependent on the specific cell line, and may require optimization depending on what cell density is desired for the experiments. We have

found that seeding 30,000 MDA-MB-231 cells per device works well for live-imaging experiments. In order to concentrate this number of cells into 6 μ L of cell suspension, cells should be suspended in media at a concentration of 5 million cells per mL.

22. Cells must be seeded slowly and steadily with the pipette. Pushing the cells into the device too quickly may lead to the cells spreading unevenly across the device (Fig. 3B). Additionally, when seeding cells, do not dispense the pipette past the first point of resistance; injection of air behind the cells results in the formation of bubbles within the device and can hinder cell migration (Fig. 3C).
23. If necessary, HEPES or other buffers may be added to cell culture media to maintain pH balance, particularly for longer duration studies. To prevent evaporation of cell media, devices should be sealed with cover glass laid across the media reservoirs before moving to microscope. In experiments that run longer than 12 hours, it may be necessary to change cell culture media on a regular basis (typically every 24 hours).
24. Fixation and staining of cells can be performed using standard immunofluorescence protocols; however it is recommended that longer times are used for each incubation and washing step to ensure that reagents have sufficient time to distribute completely throughout the microfluidic device. For immunofluorescence studies, cells should be seeded at lower densities, as large numbers of cells can block up the entry to the constrictions channels, preventing staining reagents from reaching some cells within the devices. Alternatively, experiments can be performed with devices without covalently attaching the PDMS to the glass slides (i.e., without plasma treatment). In this case, the PDMS can be removed after fixing the cells inside the devices, and staining can be performed on cells adhering to the glass slide. The fixation step may require some optimization to identify conditions that promote cells to preferentially adhere to the glass cover slide and not the PDMS features.
25. Total imaging time is up to the user and depends on the cell line being used. For most cell lines, imaging overnight (12-14 hours) is sufficient to yield a good number of cells passing through

constrictions (10 – 30 cells per region of interest). For longer studies, it may be necessary to periodically replace cell media to keep cells healthy.

26. We have found that the most consistent measurement for a ‘point of commitment’, i.e. when a cell is attempting to pass through a constriction, is an imaginary line drawn 7 μm away from the centerline of the constrictions, towards the entry side (Fig. 4). Another imaginary line can be drawn on the opposite side centerline to define when the nucleus has fully passed through the constriction. It is also necessary to check image sequences for signs of potential issues with the constrictions, which would result in exclusion of the affected cells. For example, if it appears that some part of the nucleus or the cell body passes underneath the device pillars during migration, instead of in between the pillars that form the constriction, this indicates that the PDMS pillars were not sufficiently bonded to the glass and the nucleus is not fully confined.
27. As in Note 26, is also important to establish criteria for exclusion of particular cells migrating through the 15 μm channels. For example, when determining the migration speed or nuclear transit time of cells in the 15- μm wide channels, it may be necessary to exclude cells that spontaneously switch their migration direction, as this would affect the results.

5. Figure legends

Figure 1. Overview of the migration device. **(A)** Top-down view of migration device, with food coloring added to enhance details. Cell culture media is added into the media reservoirs (i). Cells are seeded into the devices through seeding ports (ii) that lead to the 5 μm tall area with the constrictions (iii). Larger bypass channels (iv) allow rapid equilibration of media reservoir levels to prevent flow through constrictions, facilitating formation of a chemotactic gradient by diffusion if serum or growth factors are added to one of the reservoirs. **(B)** Image of the 5 μm tall area of the device with 2 μm wide constrictions without cells present. Scale bar 25 μm . **(C, D)**. Images of constriction area containing MDA-MB-231 breast cancer cells expressing an mCherry-actin chromobody and an mNeonGreen-H2B histone label. Panel **(C)** depicts a cell as it starts to pass through a constriction. Scale bar 25 μm .

Figure 2. Overview of migration device fabrication. **i)** SU-8 microfluidic features are developed onto a silicon wafer through photolithography. **ii)** A PDMS replicate of these features is formed using a 2-part elastomer curing-base mixture. **iii)** Optionally (but recommended), a secondary plastic mold can be cast from the initial PDMS replicate, which will be used to form additional PDMS devices. **iv)** Using a similar process to step **ii**, a PDMS replicate is formed from the secondary plastic mold. **v)** The PDMS replicate is cut into 12 device “chips.” Each “chip” contains 2 independent microfluidic devices, each with the features shown in Figure 1. **vi)** Media reservoirs (labeled **i** in Figure 1A) and seeding ports (labeled **ii** in Figure 1A) are cut out using biopsy punches. **vii)** Devices are cleaned with IPA and DI water, treated using a plasma cleaner, and then covalently bound to glass slides. **viii)** Devices are functionalized with extracellular matrix proteins prior to adding cells through the seeding ports (labeled **ii** in Figure 1A) and filling reservoirs with cell media. At this point, devices may be incubated until ready for analysis using live cell imaging or immunofluorescence.

Figure 3. Recognizing potential issues with device loading and bonding. **(A)** Properly loaded cells with even distribution across the device. Scale bar 200 μm . **(B)** Uneven loading of cells in front of constrictions; bottom constrictions section has no cells at entrance (arrow). Scale bar 200 μm . **(C)** Air bubbles in cell media formed within the device, blocking entry into the constriction channels. Scale bar 200 μm . **(D)** Cells migrating underneath constriction pillars (arrows), indicating insufficient bonding of PDMS pillars to glass. Scale bar 25 μm .

Figure 4. Analysis of nuclear transit time through constriction. Nuclear transit time is defined as the time for a cell to completely translocate its nucleus through a single constriction. The most robust metric for this measurement is the time from when the nucleus “commits” to enter a constriction (i.e., crossing an imaginary dashed line, located 7 μm outside the constriction center, top dashed line) and begins to deform, to when it has fully passed the constriction (i.e., the trailing edge of the nucleus has passed an imaginary line 7 μm past the constriction center, bottom dashed line). This analysis can be performed manually or using automated particle tracking programs. The example shows an MDA-MB-231 breast cancer cell expressing an mCherry-actin Chromobody and an mNeonGreen-H2B histone label. Scale bar 10 μm .

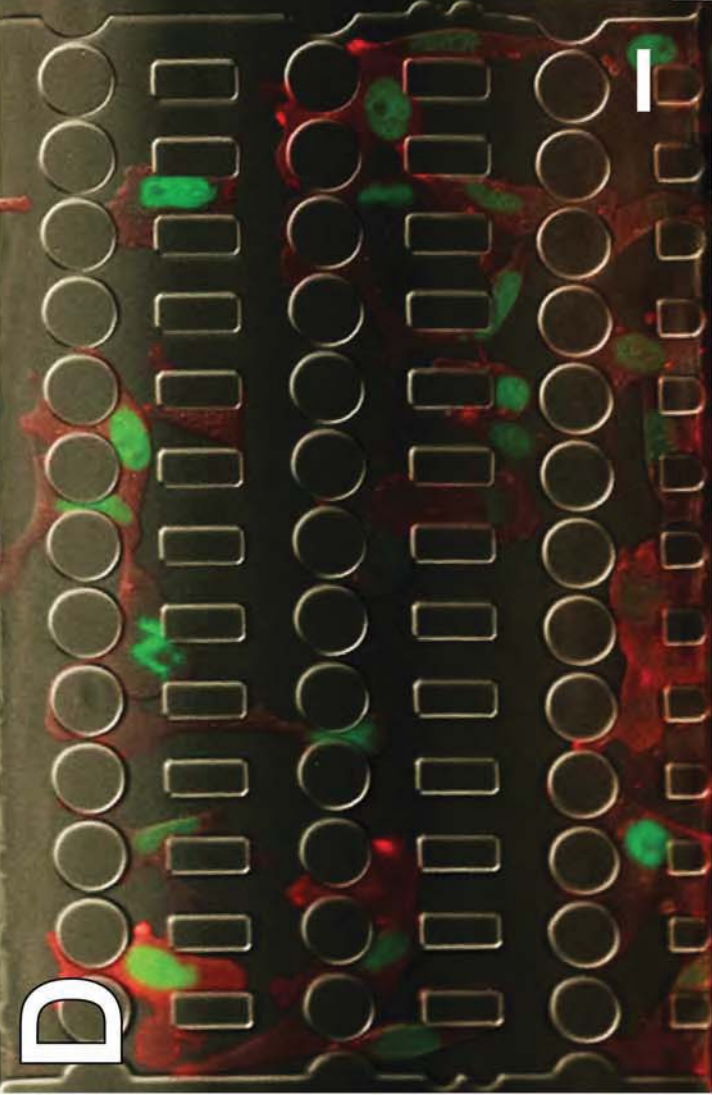
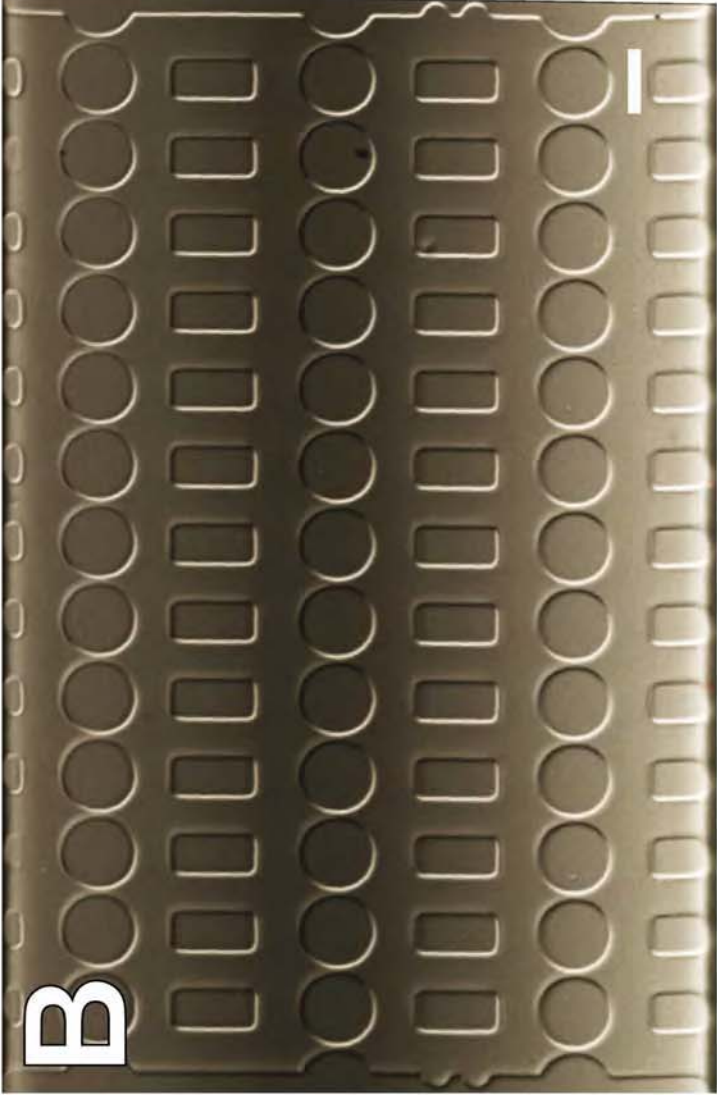
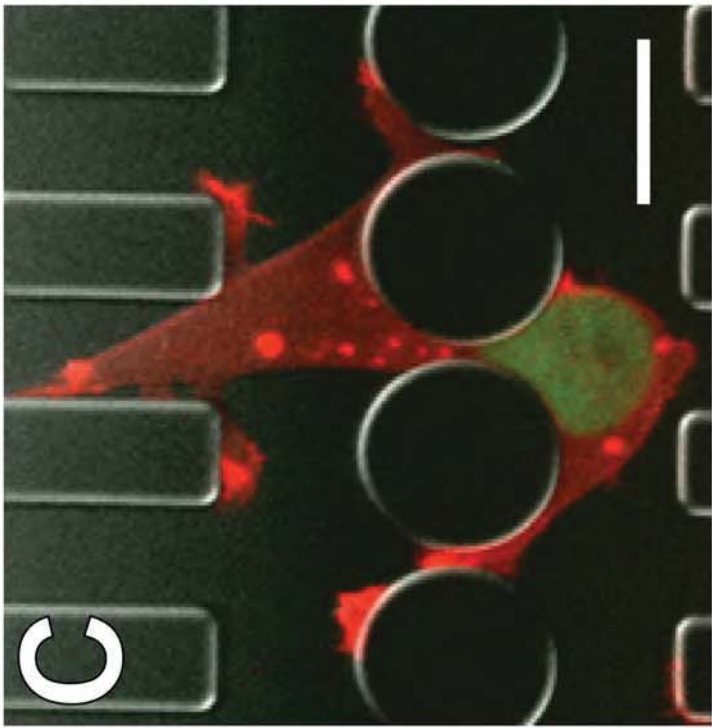
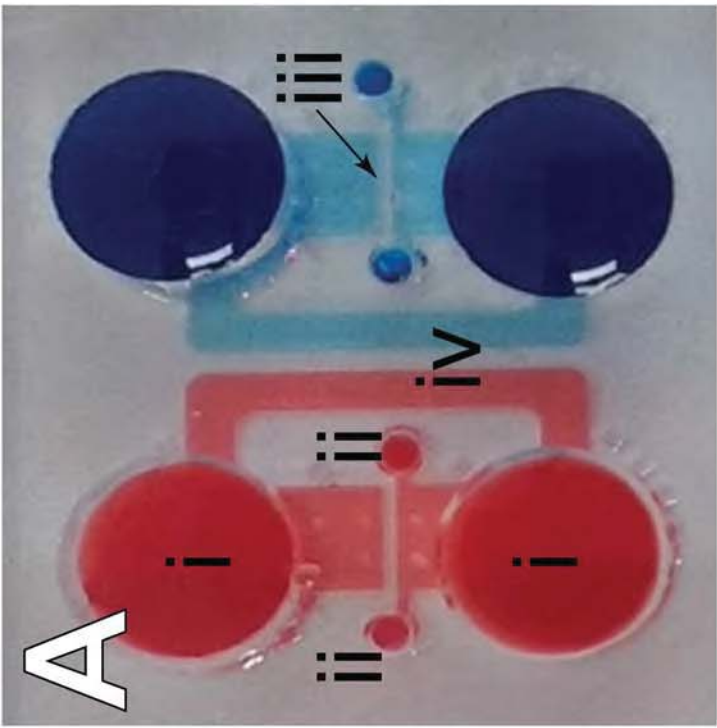
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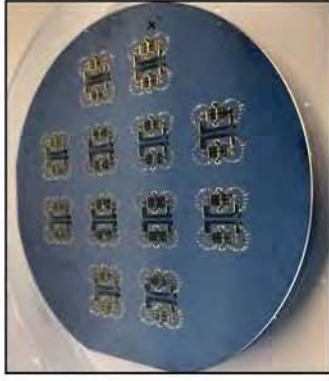
Table 1: Optimal cell seeding densities and concentrations of ECM coating to prepare migration devices

Cell Line	Cells Seeded per Device	ECM Coating	ECM Incubation
HT1080	80,000 cells seeded minimum 2 hours before imaging	50 µg/mL Corning 354236 Rat Tail Collagen Type I diluted in 0.02M Acetic Acid	4°C overnight or longer
MDA-MB-231 on collagen	50,000 seeded minimum 2 hours before imaging	50 µg/mL Corning 354236 Rat Tail Collagen Type I diluted in 0.02M Acetic Acid	4°C overnight or longer
MDA-MB-231 on fibronectin	30,000 seeded 24 hours before imaging	5 µg/mL Millipore FC010 Human Plasma Fibronectin diluted in PBS	4°C overnight or longer, or 4 hours at 37°C
Human fibroblasts	30,000 seeded 24 hours before imaging	3 µg/mL Millipore FC010 Human Plasma Fibronectin diluted in PBS	4°C overnight

Migration Direction



i. Form SU-8 features



ii. Create PDMS replicate



iii. Create plastic mold



iv. Create 2nd PDMS replicate



viii. Functionalize with ECM coating and seed cells



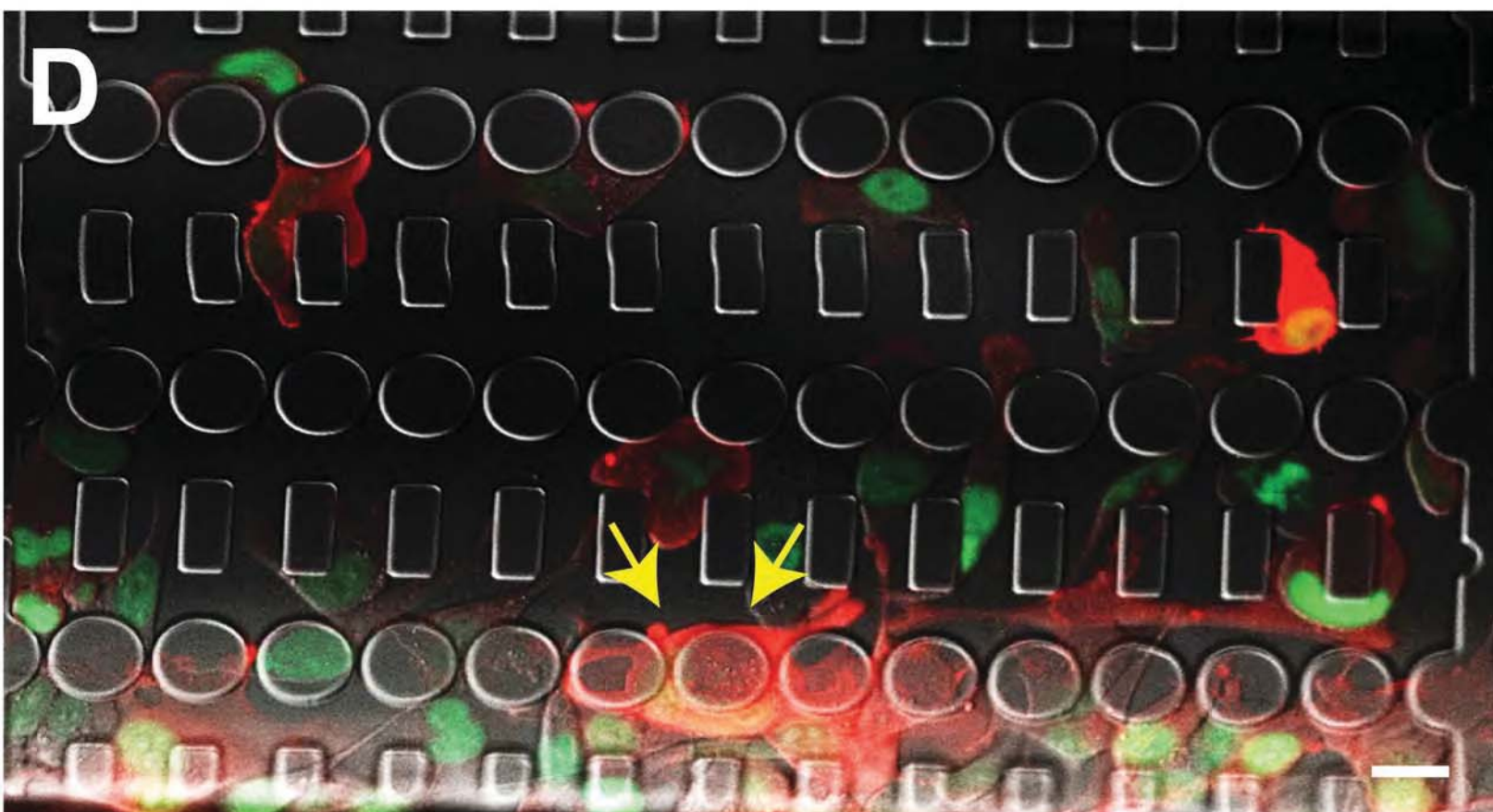
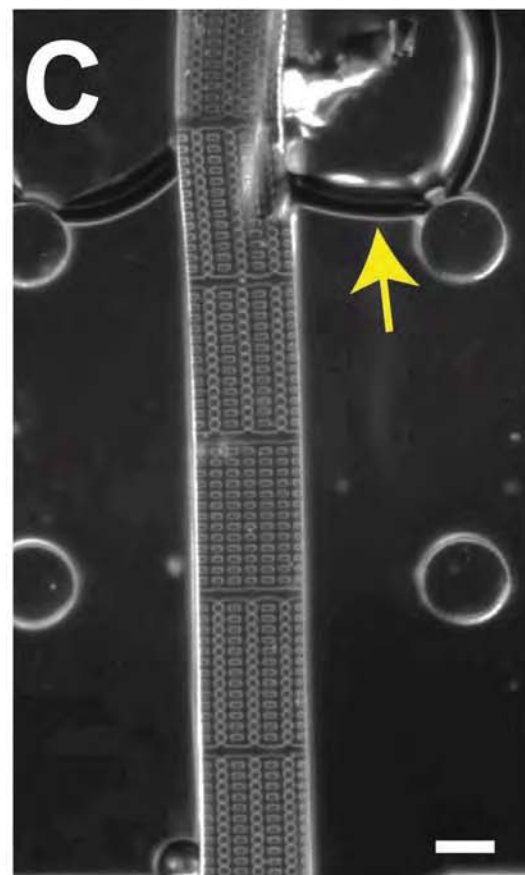
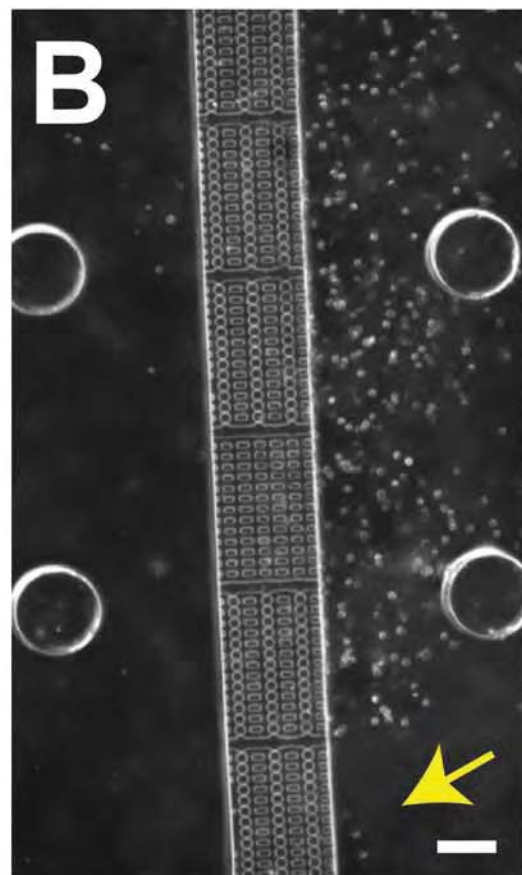
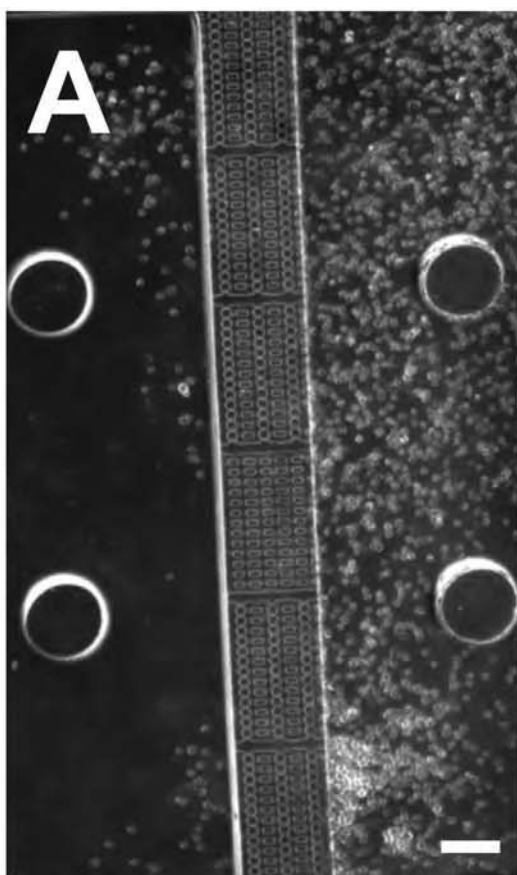
vii. Plasma treat and bind devices to cover glass



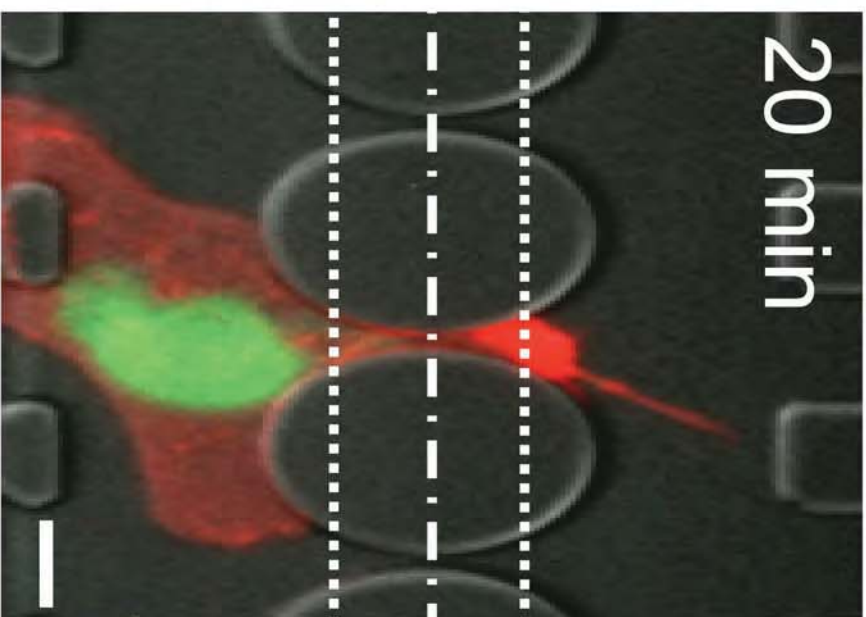
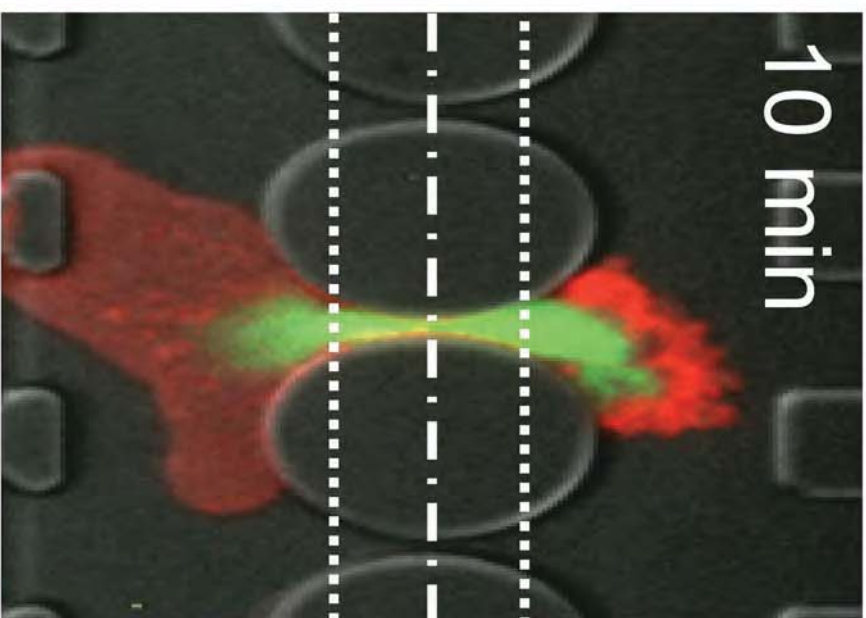
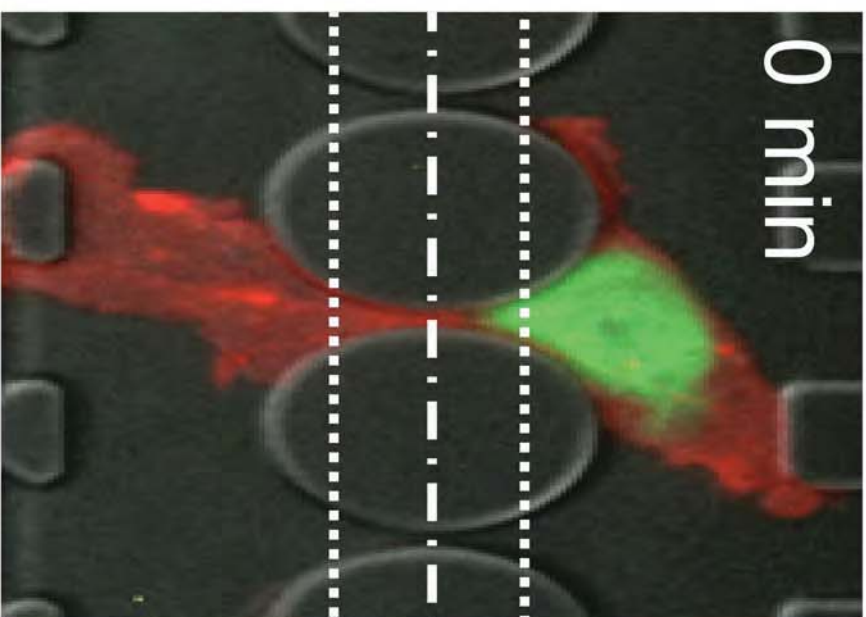
vi. Cut out seeding ports and media reservoirs



v. Cut PDMS into individual device "chips"



Migration Direction
←



Automated analysis of cell migration and nuclear envelope rupture in confined environments

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9 **Abstract**

10 Recent *in vitro* and *in vivo* studies have highlighted the importance of the cell nucleus in
11 governing migration through confined environments. Microfluidic devices that mimic the narrow
12 interstitial spaces of tissues have emerged as important tools to study cellular dynamics during
13 confined migration, including the consequences of nuclear deformation and nuclear envelope
14 rupture. However, while image acquisition can be automated on motorized microscopes, the
15 analysis of the corresponding time-lapse sequences for nuclear transit through the pores and events
16 such as nuclear envelope rupture currently requires manual analysis. In addition to being highly
17 time-consuming, such manual analysis is susceptible to person-to-person variability. Studies that
18 compare large numbers of cell types and conditions therefore require automated image analysis to
19 achieve sufficiently high throughput. Here, we present an automated image analysis program to
20 register microfluidic constrictions and perform image segmentation to detect individual cell nuclei.
21 The MATLAB program tracks nuclear migration over time and records constriction-transit events,
22 transit times, transit success rates, and nuclear envelope rupture. Such automation reduces the time
23 required to analyze migration experiments from weeks to hours, and removes the variability that
24 arises from different human analysts. Comparison with manual analysis confirmed that both
25 constriction transit and nuclear envelope rupture were detected correctly and reliably, and the
26 automated analysis results closely matched a manual analysis gold standard. Applying the program
27 to specific biological examples, we demonstrate its ability to detect differences in nuclear transit
28 time between cells with different levels of the nuclear envelope proteins lamin A/C, which govern
29 nuclear deformability, and to detect an increase in nuclear envelope rupture duration in cells in
30 which CHMP7, a protein involved in nuclear envelope repair, had been depleted. The program
31 thus presents a versatile tool for the study of confined migration and its effect on the cell nucleus.

Introduction

Cell migration is necessary for a number of important physiological processes including immune response, wound healing, and cancer metastasis. Cell migration is particularly important in the context of cancer metastasis, which is responsible for the vast majority of cancer-related deaths, including over 90% of breast cancer deaths [1]. For cancer cells to metastasize, they must first migrate away from the site of the primary tumor (invasion), enter blood or lymphatic vessels (intravasation) through which they are transported to distant parts of the body, and then exit the vessels (extravasation) and migrate to new sites, where they may grow into secondary tumors [1, 2]. The migration behavior of cancer cells is a good indicator of patient prognosis, as more migratory cells form metastases at higher rates. Preventing or reducing cancer cell migration could significantly improve cancer patient outcomes, and present a key step in reducing metastasis-related mortality.

During the processes of tissue invasion and intra- and extravasation, cancer cells have to squeeze through small spaces between other cells and within the extracellular matrix (ECM). Recent findings point to an important role of the cell nucleus in the migration of cells through such confined environments [3]. Deformation of the nucleus, which is the largest and stiffest cellular organelle, determines the ability of cells to pass through constrictions smaller than the nuclear cross-section [3-5]. Cells with less deformable nuclei take longer to pass through microscopic pores than cells with more deformable nuclei [6-8]. One of the primary determinants of nuclear deformability is the expression of lamins A and C, intermediate filament proteins that form a dense protein network (nuclear lamina) underneath the inner nuclear membrane [9, 10]. Intriguingly,

expression of lamin A/C is decreased in many cancers [11-15], which could contribute to increased metastatic potential of tumor cells by facilitating both invasion and intra- and extravasation.

In addition to modulating transit efficiency through confined environments, migration through tight spaces places substantial physical stresses on the nucleus, which can lead to a transient loss of nuclear envelope (NE) integrity during interphase, referred to as NE rupture [16, 17]. NE rupture, which allows uncontrolled exchange of cytoplasmic and nuclear proteins, along with protrusion of chromatin into the cytoplasm, could result in increased genomic instability and promote cancer progression [18]. Cells can restore NE integrity using components of the endosomal sorting complexes required for transport-III (ESCRT-III) machinery [16, 17]. Inhibiting NE repair, when combined with inhibition of DNA damage repair pathways, results in substantially increased cell death after NE rupture [16, 17], pointing to potential treatment approaches to specifically target metastatic cancer cells.

These findings have motivated a rapidly growing interest in studying nuclear deformation and NE rupture, particularly during confined migration [10, 18-22]. Microfluidic devices with precisely defined constrictions that mimic interstitial spaces *in vivo* have emerged as powerful tools to study the role of nuclear deformation and NE rupture in cell migration [7, 16, 17, 20, 23-28]. Although the walls of such devices are more rigid than the *in vivo* spaces through which cells migrate, confined migration and NE rupture results obtained in these microfluidic devices closely match those obtained in collagen matrices and from intravital imaging studies [16, 17], and the devices enable time-lapse imaging of single-cell migration under precisely defined conditions. In such experiments, nuclei are often identified by fluorescently labeled DNA (e.g., staining with

Hoechst 33342) or histones (e.g., expression of H2B-tdTomato). NE rupture events can be detected by monitoring the intracellular localization of a green fluorescent protein containing a nuclear localization sequence (NLS-GFP) [16, 17, 29]. NLS-GFP is normally contained within the nucleus but spills into the cytoplasm during NE rupture and is gradually re-imported into the nucleus upon NE repair (Fig. 1). Time-lapse experiments using cancer cells typically cover 6 to 24 hours, with multi-color (fluorescence and transmitted light) images acquired every 2 to 10 minutes, resulting in large (>40 GB per experiment), multi-dimensional data sets that take several days to weeks to manually analyze. Such low throughput image analysis provides a substantial challenge when studying large sets of experimental conditions. Furthermore, manual analysis by different observers can add substantial variability to the experimental data.

Figure 1. Cell migration through microfluidic constrictions. (A) Cells expressing NLS-GFP and H2B-tdTomato migrating through a microfluidic device. Scale bar: 50 μm . (B) Time series of a nucleus squeezing through a constriction. Scale: bar 20 μm . (C) Time series of a NE rupture event. NLS-GFP leaks into the cytoplasm upon NE rupture and is reimported into the nucleus as the NE is repaired. Scale bar: 20 μm .

To address these issues, we developed a MATLAB program to perform the image analysis in an automated, reproducible, and robust process. The program is capable of tracking individual cells/nuclei as they migrate through microfluidic constriction channels and compute transit times for individual constrictions. While primarily intended to study cell migration in confined environments, the program can also be used to study cells migrating on unconfined 2-D substrates. The program can also reliably detect NE rupture events and their duration. The program

automatically recognizes dividing cells, resulting in increased robustness and accuracy comparable to expert manual analysis, but with substantially increased efficiency.

Automated analysis algorithm

Overview

Automated image analysis begins by locating the constrictions in the first image (Fig. 2). The image is then processed to reduce noise and to detect fluorescently labeled nuclei. For each subsequent image in the sequence, image stabilization is performed to account for small shifts in the field of view during image acquisition. Each image is then subject to the same processing as above to reduce noise and detect nuclei. Identified nuclei are tracked from the previous image to the current one. All nuclei are then observed for incidences of constriction passage and NE rupture. After the full sequence has been analyzed, the tracking results are exported to a spreadsheet and presented to the user for manual validation (Fig. 2). The program was implemented in MATLAB 2016a and runs on all MATLAB supported platforms, version 2016a and newer. It can be downloaded at the following URL: <https://github.com/Lammerding/MATLAB-CellTracking>.

Figure 2. Flowchart of automated analysis steps. Steps the program takes when analyzing an image sequence are detailed, including image processing (top) and post-processing (bottom).

Locating constrictions

Constriction location is performed by identifying the round pillars in the microfluidic devices that form the three rows of constrictions (Fig. 1A and Suppl. Fig. S1). This is accomplished by applying a circular Hough transform (a technique that identifies circles in an image) to a transmitted light image of the device. All images are then rotated to align the rows of constrictions horizontally. Virtual boundaries are defined at a specific distance above and below the constriction centerline to determine nucleus entry and exit for each row of constrictions. (Suppl. Fig. S1). This approach can be adjusted for devices with different designs.

Image pre-processing and stabilization

To reduce the noise in the fluorescence images and enhance the contrast between the nuclear signals and the background, a 10×10 pixel Gaussian filter is applied to the images of the fluorescence channels. Image stabilization is then performed via normalized 2D cross-correlation between an image and its predecessor. The obtained spatial offset values are applied to the transmitted light as well as the fluorescence image channels.

Nucleus detection

Nuclei are identified by binarizing images with a locally adaptive threshold based on their H2B-tdTomato signal and applying connected component analysis. Local thresholding, while more computationally expensive, provides better results than global thresholding, especially for unevenly illuminated images. To separate touching nuclei into distinct objects, further segmentation is necessary. Since nuclei are generally oval-shaped, the program uses watershed segmentation based on the distance transform of the identified nuclei. Watershedding segments an image based on “watershed lines”, which separate the image into different

“catchment basins”. Figuratively, the image is treated like a topographic map, with image intensities representing the height. This means image areas containing low pixel intensity values are grouped together and separated from other groups if there are high pixel intensity values between them (the “watersheds”). The resulting regions are converted into binary images and further processed to distinguish single nuclei, which form a single catchment basin, from multiple touching nuclei, which form multiple basins separated by a watershed line. Additional imaging process steps are applied to prevent over-segmentation. A visual guide to this algorithm is provided in Suppl. Fig. S2. Nuclei inside of constrictions are excluded from this segmentation since they take on a dumbbell shape (Fig. 1B, 0:50 h) and otherwise may be incorrectly split into two objects. After image segmentation, identified objects are deleted if their properties, such as size and circularity, suggest that they are not nuclei (Fig. 3A, B).

Figure 3. Examples of nuclear identification and tracking. (A) Merged image of the transmitted light and tdTomato channels. Nuclei (red) can be seen in the migration device. Scale bar: 50 μ m. (B) Binarized version of red channel of image A. Each nucleus is identified as a separate object (white). (C) Example of tracking results. Nuclei (red) have been identified, and their centroid positions during migration are shown as yellow tracks. For clarity, tracks displayed here are limited to data for only the last six hours.

Nucleus tracking

After nuclei have been identified, they are tracked over time by recording their centroid position at each time point. The following error function is applied to every possible pairing of a nucleus in one image and a nucleus in the following image:

$$E = distance^2 + 2 * |\Delta fluorescent\ intensity| + 2 * |\Delta area|$$

This error function is related to the likelihood that two objects are the same nucleus and is based on the square of the distance between the centroids of the two objects. Since comparing only the distances between objects produces inaccurate results when multiple nuclei are in close proximity, the error function also includes the change in each object's area and its average H2B-tdTomato fluorescent intensity. These values are expected to remain relatively constant over time for individual nuclei, but vary between different nuclei. The object from the previous time point and the object in the current time point that together have the lowest error function value are paired with one another and marked as unavailable for other pairings. Pairs can only be made if the centroids of the objects are within 40 μm of one another, which is the maximum distance a cell typically travels within the chosen time interval. If necessary, the user can later correct this during the manual validation and editing stage, but we found that such instances are rare. Object pairing and marking availability status continues until no further object pairings are available. Upon completion, time-resolved data for each identified nucleus include centroid position, bounding box, area, and fluorescence intensity, which can be displayed for each nucleus (Fig. 3C) and used for further analysis.

Detection of cell transit through constrictions

The passage of nuclei through constrictions is evaluated as follows: if the top of a nucleus' bounding box is above the lower boundary of a constriction and the bottom of the nucleus' bounding box is below the upper boundary of the constriction, the nucleus is considered to be

attempting to pass through the constriction (Suppl. Fig. S3A). When an attempting nucleus moves completely above the upper bounding box, it is recorded as having successfully passed the constriction (Suppl. Fig. S3B). A nucleus that is attempting to traverse a constriction but then moves back out of the boundary of the constriction is recorded as failing to pass through the constriction (Suppl. Fig. S3C). Nuclei that only briefly (1 time point) attempt to enter a constriction are excluded from the analysis. Such instances can occur when a cell moves parallel to the row of constrictions and a part of the nucleus crosses the boundary of the constriction, without the cell attempting to pass through the constriction.

NE rupture detection

NE rupture is detected by monitoring the inverse ratio of the nuclear NLS-GFP signal to the H2B-tdTomato signal. Since the total amount of NLS-GFP per cell stays approximately constant over time, and NLS-GFP spills into the cytoplasm during NE rupture, the average NLS-GFP nuclear intensity decreases during NE rupture. In contrast, the H2B-tdTomato signal remains nearly constant, allowing for normalization to the H2B-tdTomato signal. The normalization accounts for variations due to photobleaching and other image acquisition effects. The ratio of average H2B-tdTomato signal to average nuclear NLS-GFP signal [$H2B/NLS$] is measured for each nucleus at every time point. If the difference in ratio between two consecutive time points [$\Delta(H2B/NLS)$] exceeds 20% of the previous time point's ratio, or if the $H2B/NLS$ ratio increases continuously over the course of at least 5 consecutive time points, then NE rupture is determined to have begun (Fig. 4A, red arrow). As the NE is repaired, NLS-GFP re-enters the nucleus, and $\Delta(H2B/NLS)$ becomes negative. The NE rupture event is completed when the $H2B/NLS$ ratio returns close to its pre-rupture value and $\Delta(H2B/NLS)$ returns to zero (Fig. 4C-D).

Figure 4. Detection of NE rupture. (A) During NE rupture (arrow), NLS-GFP (green) spreads throughout the cytoplasm, causing the nuclear NLS-GFP signal to lose intensity. In contrast, the H2B-tdTomato signal (red) remains approximately constant. (B) Normalizing these two signals against one another (H2B/ NLS) significantly reduces the effects of noise and allows for more accurate NE rupture detection. (C) Steep increases in the H2B/NLS ratio, which correspond to high values of $\Delta(\text{H2B/NLS})$, plotted in (D), indicate the start of a NE rupture event. The data shown here are for a representative cell.

Since NE breakdown also occurs during mitosis, it is important to distinguish loss of nuclear NLS-GFP signals between mitotic cells and those exhibiting interphase NE rupture to avoid false positive detection of NE rupture. Mitosis and NE rupture both begin with NLS-GFP spreading into the cytoplasm and are initially indistinguishable from one another. However, during mitosis, two daughter nuclei form from one initial nucleus. Thus, if a nucleus is detected as undergoing NE rupture, and a new nucleus appears in its vicinity in the next time point, the event is reclassified as mitosis, and not NE rupture (Suppl. Fig. S4).

Manual verification

Automated data analysis can occasionally misidentify events; therefore, a video for manual verification is generated for every image sequence analyzed. The video is displayed on a graphical user interface, and the user can manually select individual nuclei and events to make corrections as necessary. Recorded data is exported to a file after manual validation.

Automated analysis results

Comparison of automated and manual analysis

To assess the accuracy of the program, we acquired two image sequences of BT-549 breast cancer cells migrating through a microfluidic device with 2- μm wide constrictions. The two image sequences were manually analyzed by four trained observers, recording constriction entry and exit times for each nucleus. One image sequence (Suppl. Vid. S1) was used to “train” the automated image analysis program to define the boundaries that mark entry and exit of the nucleus into/out of the constrictions. The program analyzed the video for six conditions, with constriction boundary lines placed either 5, 6, 7, 8, 9, or 10 μm from the constriction centerline. Comparing the program’s results with the manual results revealed that placing the boundaries 7 μm above and below the center produced the best agreement with the manual analysis.

The other image sequence (Suppl. Vid. S2) was then used to “test” the program and the 7- μm constriction boundaries (Fig. 5). Results of the automated image analysis for each nuclear transit event were compared to a manual analysis gold-standard, defined as the average result from four expert reviewers who assess nuclear transit times based on the visible deformation of the nucleus as it enters and exists the constriction. Comparison of the constriction transit times of individual cells determined by the program and four expert observers showed excellent agreement between the program and the manual gold-standard (Fig. 5A). Similarly, the average constriction transit times computed by the program for each of the image sequences closely matched the data of the manual observers. Importantly, the program and all four observers correctly identified that cells that overexpress lamin A (Image sequence 1), and which have less deformable nuclei, had

significantly longer transit times ($p < 0.05$) than mock-modified control cells (Image sequence 2) (Fig. 5B).

Figure 5. Verification of automated image analysis by comparison to manual analysis.

(A) Automated analysis results plotted against manual analysis results (mean $\pm$ s.e.m. from four observers) for individual cells in two separate image sequences, each of which corresponds to a single section of a microfluidic device. For perfect agreement, the regression line plotted through these points would have a slope of one. Only one automated-analysis result substantially deviated from the manual reference, indicated by an asterisk. The manual analysis determined the nucleus to make two attempts to pass through the constriction, failing the first but succeeding the second time. The program identified this as a single, longer attempt. (B) Constriction transit times (mean $\pm$ s.e.m.) determined by four manual analysts and the automated analysis for the cells in the same two image sequences analyzed for panel A. Cells are BT-549 breast cancer and are either overexpressing lamin A (Image sequence 1) or an empty vector (Image sequence 2). Overexpression of lamin A results in less deformable nuclei and longer transit times through narrow constrictions (*, $p < 0.05$; **, $p < 0.001$ as calculated by ANOVA followed by Tukey's multiple comparison test; $n = 23-26$ and $20-24$ (depending on the analyst), respectively).

Out of the 50 verified constriction transit events present and analyzed in the two image sequences, 46 were identified by the program prior to manual verification/correction, resulting in a “miss” rate of 8%. We recorded similar “miss” rates in six separate, independent image

sequences analyzed by the program (data not shown). The four expert analysts had a combined “miss” rate of 10%, identifying an average of 45 events out of the total 50 transit events in the two image sequences. Therefore, even prior to manual verification, the program misses a similar or even lower number of nuclear constriction transit events than the expert analysts. The events missed by the program can be quickly and easily added to the programmatic analysis during the manual verification stage.

In addition to the identification of true nuclear transit events, person-to-person variability also applies to the transit times measured for each event. For any given transit event, the transit times recorded by the four manual analysts varied by greater than 2 image frames on average, ranging from 0 (complete agreement) to a maximum of 6 image frames. In all but one case (identified by the asterisk in Figure 5A) the program determined a transit time within the range of the manual measurements for that event. The single exception occurred in a cell that moved back and forth as it struggled to pass through the constriction, and the event could be identified as either one long attempt (as assessed by the expert analysts) or two shorter attempts (the first of which a failed attempt, followed by a successful attempt), as assessed by the program.

Detection of differences in constriction transit times

To assess whether the program could detect differences in constriction transit times in cell lines other than those used for the ‘training’ and ‘test’ data, we performed experiments with A549 human lung carcinoma cells treated with siRNA against lamin A/C or a non-target control (Suppl. Fig. S5). A previous study found that lamin A/C depletion in A549 cells results in increased transit efficiency through small pores [6]. In another study, lamin A/C-deficient mouse embryo fibroblasts had significantly shortened transit times for passage through small constrictions than

wild-type controls [7]. Consistent with the previous reports, the automated image analysis of our experiments found that lamin A/C-depleted cells passed faster through 1- and 2- μ m wide constrictions than the non-target controls ($p < 0.05$). In contrast, both groups had comparable transit times ($p = 0.34$) when passing through 15- μ m wide control channels that do not require nuclear deformation (Fig. 6A).

Figure 6. Application of automated image analysis program. (A) A549 cells depleted for lamin A/C ($n = 40$ cells across 4 microfluidic device sections) pass faster through small constrictions than non-target controls ($n = 26$ cells across 4 device sections). Transit times through larger openings were not statistically different (*, $p < 0.05$ as calculated by t -test; $n = 21, 14$, respectively). (B) HT-1080 cells depleted for CHMP7 ($n = 65$ cells across 3 device sections) took longer to repair their NE and restore nucleo-cytoplasmic compartmentalization than non-target controls (**, $p < 0.01$ as calculated by Mann-Whitney t -test; $n = 48$ cells across 3 device sections). (C) Dynamics of NE rupture and repair visualized by the ratio of nuclear H2B-tdTomato/NLS-GFP fluorescence for the CHMP7-depleted and non-target control cells. The H2B/NLS signal is expressed relative to its value at $t = 0$, i.e., immediately prior to rupture, and normalized to reach a peak value of 1 for each nucleus. CHMP7-depletion results in slower return to baseline, indicating delay in NE repair. Error bars represent mean $\pm$ s.e.m.

Detection of differences in NE rupture durations

The program's ability to detect NE rupture events was verified through manual inspection of analyzed image sequences. To ensure that automated NE rupture detection is both precise and

robust at identifying the durations of NE rupture/repair, we performed experiments with HT-1080 human fibrosarcoma cells treated with siRNA against the ESCRT-III family protein charged multivesicular body protein 7 (CHMP7) (Suppl. Fig. S6). Since ESCRT-III proteins and CHMP7 are crucial for NE repair [16, 17, 29, 30], depletion of CHMP7 is expected to result in increased NE rupture duration. Automated image analysis confirmed that CHMP7-depleted HT-1080 cells experienced significantly longer NE rupture durations than the non-target controls ($p < 0.01$) (Fig. 6B). CHMP7-depleted and non-target control cells showed similar H2B-tdTomato/NLS-GFP ratio values prior to NE rupture ($p > 0.9$) and 2 hours after rupture ($p = 0.28$), while CHMP7-depleted cells were slower to return to this baseline following NE rupture (Fig. 6C).

Discussion

We have developed and validated a MATLAB program for the automated and robust analysis of nuclear activity as cells migrate through microfluidic devices. This automation reduces the amount of time required to analyze an image sequence from multiple days/weeks to ~5 hours for a time-lapse experiment with 24 positions/experimental conditions and over 100 time points per position. Furthermore, the automated analysis removes person-to-person variability in the obtained results. The results produced by the program are in close agreement with expert manual analysis. The program is suitable for a broad range of applications that use microfluidic devices to study the migration of cells through confined environments, including analysis of transit times through pores of different size, or incidence of NE rupture. Previously, collecting data on a large number of cell lines, patient samples, or treatment conditions would have been impractical due to the substantial amount of time required to analyze the image sequences.

While the results presented here are based on a specific microfluidic migration device design, the modular nature of the program can be easily adapted to different design geometries, making it useful for a broad user base. Notably, the implemented automatic alignment and recognition of constrictions is independent of the constriction size and position. Similarly, fluorophores other than H2B-tdTomato and NLS-GFP can be used for the identification of nuclei and NE rupture, respectively.

The automated analysis is precise and robust enough to reach reliable conclusions concerning a population of cells' constriction transit times and NE rupture durations with only minimal user supervision. Furthermore, the program can generate and collect data that would be challenging to obtain through manual analysis. For example, the ability to collect pixel intensity values in specific areas of interest allows the program to monitor the intensity of fluorescence in every nucleus over time. This allows the actual time course of NE repair to be observed, recorded, and compared across populations of cells. In contrast, while manual analysis can record the duration of NE rupture events, it lacks the accuracy required to analyze the extent of repair at earlier time points.

This nucleus tracking program is currently used only to monitor constriction transit times and NE rupture events, but could readily be expanded to a broader array of applications. For example, measurements of nuclear migration persistence, i.e., the tendency of the nucleus to move in a constant direction, could be recorded, since nucleus centroids are determined for each time point. Such an analysis would be extremely tedious and highly time-consuming if done manually. The automated analysis could also be expanded to include cell death detection, for example, based

on the permanent loss of NLS-GFP intensity and unmatched nuclei after cell death. Automated cell death analysis could be useful for screening of drugs that target metastatic cancer cells. Under the conditions used in the current experiments, only few cells died during imaging, and these cases appeared to be the result of confined migration and continuous NE rupture, rather than phototoxicity, consistent with previous reports [13, 16]. Additionally, the object identification and tracking elements of the program could be applied to any other set of time-lapse images, for example, to cells migrating on 2-D substrates or contact-printed micropatterns.

Materials and methods

Creating microfluidic devices

Microfluidic devices were created from a silicon wafer mold fabricated by 2-layer SU-8 photolithography as described previously [20]. Polydimethylsiloxane (PDMS) was created by mixing Sylgard 184 Silicone Elastomer Base and Silicone Elastomer Curing Agent in a 10:1 ratio as per the manufacturer's instructions (Corning). A vacuum chamber was then used to remove air bubbles, and the PDMS was poured into the silicon mold and baked for 2 hours at 65 °C to solidify. After removal from the mold, the PDMS was cut to size, and biopsy needles were used to cut out device reservoirs and perfusion channel inlets/outlets.

Devices and glass slides were then washed with deionized water and isopropyl alcohol, dried, and plasma cleaned for five minutes. Covalent bonding of the devices to the slides then occurred by gentle pressing of the device onto the slide and placing the slide on a hot plate at 95 °C for 5 minutes. Slide-bound devices were then brought to a tissue-culture hood, and rinsed with 70% ethanol followed by deionized water. Device reservoirs were filled with 20 µg/mL of fibronectin in PBS (for A549 and BT-549 cells) or 0.05 mg/mL of collagen in 0.02 M acetic acid

(HT-1080 cells). Devices were kept in a sealed Petri dish at 4 °C overnight to allow binding of the protein to the glass slide and PDMS.

Cell culture

The human lung carcinoma cell line A549 (ATCC) was cultured in F-12K media (Gibco) supplemented with 10% fetal bovine serum (FBS, VWR) and 1% penicillin and streptomycin (pen/strep, Gibco). The human fibrosarcoma cell line HT-1080 (ATCC) was cultured in DMEM supplemented with 10% FBS and 1% pen/strep. BT-549 breast cancer cells (ATCC) were cultured in RPMI media supplemented with 10% FBS and 1% pen/strep. All cells were cultured at 37 °C and 5% CO₂.

Generation of fluorescently labelled cell lines

Cell lines were stably modified with a retroviral vector to express both the NE rupture reporter NLS-GFP, and histone marker H2B-tdTomato (pQCXIP-NLS-copGFP-P2A-H2B-tdTomato-IRES-puro, System Biosciences). The retroviral vector was generated in two steps. LifeAct-GFP was digested out of the pQCXIP-LifeAct-GFP-P2A-H2B-tdTomato vector, and NLS-copGFP was ligated into the vector. NLS-copGFP was obtained from a lentiviral vector (pCDH-CMV-NLS-copGFP-EF1-blastiS) via digestion. The product was then amplified via touchdown PCR, introducing the NotI and AgeI restriction sites, using the following forward and reverse primers, respectively:

5'-CAAGCGGCCGCACCATGACTGCTCCAAAGAAGAAGCG-3' and 5'-GCAACCGGTGCGAGATCCGGTGGAGCCGG-3'.

Retroviral particles were produced via 293-GPG cell transfection with the plasmid and Lipofectamine 2000 (Invitrogen) following the

manufacturer's protocol. Retrovirus-containing supernatants were collected once every 24 hours for 5 days following transfection and strained through a 0.22 μ m filter. Cells were seeded into 6-well plates to reach 50-60% confluency on the day of infection, and were transduced with viral stock in the presence of 8 μ g/mL polybrene (Sigma-Aldrich) every 24 hours for three days. On the fourth day, the viral solution was replaced with fresh culture medium, and cells were cultured for three days before selection with puromycin. After selection, cells were sorted on a BD FACSARIA FUSION fluorescence activated cell sorter (Cornell University Biotechnology Resource Center), and used for experiments or frozen down.

siRNA mediated depletion of lamin A/C and CHMP7

Lamin A/C depletion in A549 cells and CHMP7 depletion in HT-1080 cells was accomplished using DharmaFECT (Dharmacon) and target-specific siRNA according to the manufacturer's protocol, with final siRNA concentrations of 2.5 nM (*LMNA*) and 100 nM (*CHMP7*). SmartPool siRNA oligonucleotides, containing four target sequences in one mix to reduce off-target effects, were purchased from Dharmacon (GE Healthcare): human LMNA (ON-TARGET plus SMART pool, L-004978-00), human CHMP7 (ON-TARGETplus SMARTpool, L-015514-01), and non-targeting control siRNA (ON-TARGETplus non-targeting pool, D-001810-10).

Generation of cell lines overexpressing lamin A

BT-549 cells were stably modified to express NLS-RFP using a pCDH lentiviral construct (Systems Biosciences). After selection with blasticidin (InvivoGen), cells were modified with a retroviral bicistronic constructs expressing lamin A or a mock control as described previously [31]. Cells were then sorted for RFP- and GFP-expressing cells before being used in experiments.

Seeding cells into microfluidic devices

Cells were trypsinized, centrifuged, counted and resuspended in media to a concentration of 5,000 cells/ μ L. A cell suspension containing 30,000 cells was added to the inlet port of each device. Device reservoirs were then filled with media and kept in an incubator overnight to allow cell attachment to the fibronectin- or collagen-coated devices. In the morning media was removed, and one reservoir was filled with plain media while the other was simultaneously filled with FBS-supplemented media, creating a chemotactic gradient to promote cell migration across the constrictions. Devices were then returned to the incubator until the start of imaging. Just prior to imaging, media was again removed from the devices. The FBS gradient was established in the same manner as before, but with Fluorobrite (Gibco) imaging media containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco) to keep the cells at physiologic pH in the absence of 5% CO₂ during time-lapse imaging. Cover slips were placed over the devices to prevent evaporation of media.

A detailed schematic of the microfluidic device used, including reservoirs, cell seeding ports, and constrictions, can be found elsewhere [20]. The constriction design we used here is the rightmost one presented in Figure 1g of that reference. Designs for the device are available at: <http://lammerding.wicmb.cornell.edu/migration-device-design/>

Imaging

Migration devices were imaged on an inverted Zeiss Observer Z1 microscope with a temperature-controlled stage set to 37 °C at 20 $\times$ magnification (NA 0.8 air objective) and a

CoolSNAP EZ CCD camera (Photometrics). Zen software (Zeiss) was used to automate image acquisition, taking images of specific sections of the migration device every 2 min (HT-1080) or 10 min (A549 and BT-549). Images of the migration device and cells were acquired with differential interference contrast (DIC); fluorescence microscopy was used to capture the NLS-GFP signal (excitation with 450-490 nm light, collection of emission at 500-550 nm; exposure time of 75 ms), and the H2B-tdTomato signal (excitation by 550-580 nm light, collection of 590-650 nm light; exposure time of 400 ms). All images were saved in the Carl Zeiss Image (*.czi) format.

Western blotting

To quantify protein depletion, Western blots were performed with cell lysates. Parallel prepared samples of the siRNA treated cells were lysed at the time of imaging in high salt radioimmunoprecipitation assay (HS RIPA) buffer. Lysates were vortexed for five minutes to shear DNA, heated to 93 °C for two minutes, and centrifuged to pellet DNA. Protein concentrations were determined by Bradford assay. Equal amounts of protein (15 µg) for each sample were loaded into a NuPAGE 10% Bis-Tris gel (Gibco). Gels ran in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at 100 V until the ladder bands began to separate, and then at 175 V until completion. Proteins were then transferred to a polyvinylidene difluoride (PVDF, Millipore) membrane at 16 V for one hour. Gels were stained with Coomassie to observe quality of protein loading. After transfer, the membrane was blocked for at least one hour at room temperature in 5% milk. Primary antibodies (anti-lamin A/C, Santa Cruz, sc-6215, dilution 1:2000; anti-CHMP7, Sigma, HPA036119, dilution 1:200; anti-actin, Santa Cruz, sc-1615 HRP, dilution 1:2000) were added and left on overnight at 4 °C. Secondary antibodies (donkey anti-rabbit 800

488 cw and donkey anti-mouse 680 RD, Licor) were incubated for one hour at room temperature prior
489 to imaging on a LI-COR Odyssey CLx.

491 **Image analysis**

492 Image sequences were analyzed using the custom-written MATLAB program. To allow the
493 program to read the images in the .czi format, the Bio-Formats package was downloaded from the
494 Open Microscopy Environment's webpage and added to the MATLAB search path. Constriction
495 passage times as well as NE rupture events were recorded for all cells. Data was automatically
496 exported into a comma-separated values (*.csv) file for use with Microsoft Excel. To verify the
497 accuracy of the program, results for selected image sequences were compared to results from
498 manual analysis using Zen software.

500 **Statistical analysis**

501 Statistical analysis was performed on GraphPad's Prism software. Distributions of
502 constriction transit times and NE rupture durations were tested for normality, and their means were
503 compared using the appropriate statistical tests. Two-tailed t-tests with Welch's correction for
504 unequal variances were used to compare two normally distributed means. The Mann-Whitney test
505 was used to compare two means if either was not normally distributed. One-way analysis of
506 variance (ANOVA) followed by Tukey's multiple comparison test was used to compare the
507 constriction transit times determined by the program with those determined through manual
508 analysis. In the comparison between manual and automated image analysis, three data points were
509 excluded due to large discrepancies in the manual analysis between the four observers. Two-tailed

510 t-tests with the Bonferroni correction were used to compare the normalized fluorescent intensities
511 during NE rupture events.

512

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Supporting information

Figure S1. Automated detection of microfluidic constrictions. (A) Image of a microfluidic device section prior to rotation. The rows of constrictions are angled upwards. (B) The results of a circular Hough transform have been superimposed onto the original image (red). These circles represent the columns that form the constrictions and their centers are used to determine the angle of rotation. (C) Image of the microfluidic device rotated so that the rows of constrictions are horizontal. (D) A circular Hough transform is applied to the rotated image (red). Now the locations of the circles' centers can be used to place boundaries for entering and exiting a constriction (blue).

Figure S2: Example of watershed segmentation. 1) An image of migrating nuclei is binarized based on the H2B-tdTomato signal. Since the two nuclei shown are touching, they become a single object in the binary image. 2) The binary image is inverted so that the distance transform, which measures the distance from any given pixel to the nearest non-zero (white) pixel, works as needed. 3) The distance transform is applied. Inside of the nuclei, values are higher (closer to white in the image) the farther they are from the nucleus' nearest edge. 4) The image from the distance transform is inverted so that the watershed segmentation works as needed. Now the center of a nucleus is a minimum (closer to black in the image). 5a) A watershed transform is applied. The black lines represent watershed lines, cutting through local maxima to separate all of the image's local minima or catchment basins (each of which is shown as a different shade of gray). 6a) The watershed lines are used to segment the original binary image. Over-segmentation has occurred since the top nucleus has been erroneously split into three separate objects. 5b) An

h-minima transform is applied to the inversion of the distance transform. Local minima that are too shallow are removed from the image to prevent over-segmentation from occurring. **6b)** A watershed transform is applied. Since negligible minima were removed from the image there are now only two catchment basins. **7b)** The watershed line is used to segment the original binary image. Application of the h-minima transform during this process prevented over-segmentation from occurring. Use of the watershed segmentation successfully separated two touching nuclei into distinct objects.

Figure S3: Examples on constriction transit identification. **A)** The two nuclei depicted are identified as attempting to pass through the constrictions by the program. This is because the leading (top) edges of their bounding boxes (shown in blue) are above the lower constriction boundary (both constriction boundaries depicted as dashed green lines), but their bounding box trailing (lower) edges are still below the upper constriction boundary. **B)** Movement of the nucleus as shown here would result in the program recording a successful constriction passage since the trailing edge of the nucleus' bounding box eventually crosses the upper constriction boundary. **C)** Movement of the nucleus as shown here would result in the program recording a failed constriction passage since the leading edge of the nucleus' bounding box recedes below the lower constriction boundary.

Figure S4. Detection of mitotic cells to reduce misclassification of nuclear envelope rupture and incorrect nucleus matching. (A) Example of incorrectly labeled nuclear envelope rupture

(box with the letter R) and unmatched nucleus appearing in the fourth frame (magenta box) when a mitotic cell divides into two daughter cells. (B) Results obtained with the automated mitosis detection feature of the program. The nucleus outlined in cyan is now recorded as undergoing division (signified by the letter D). The nuclei outlined in magenta and gray are recorded as daughters of the cyan-outlined nucleus. Proper distinction between mitosis and nuclear envelope rupture is necessary to prevent recording of false positive nuclear envelope rupture data.

Figure S5. Depletion of lamin A/C by siRNA. (A) Western blot of the A549 cells used in four independent migration experiments. Visual inspection reveals lower lamin A/C expression in the cells that received the knockdown (KD) as compared to the cells that received the non-targeting siRNA (NT). (B) Quantification of lamin A levels, normalized to actin loading control. (C) Quantification of lamin C levels, normalized to actin loading control. *, $p < 0.05$

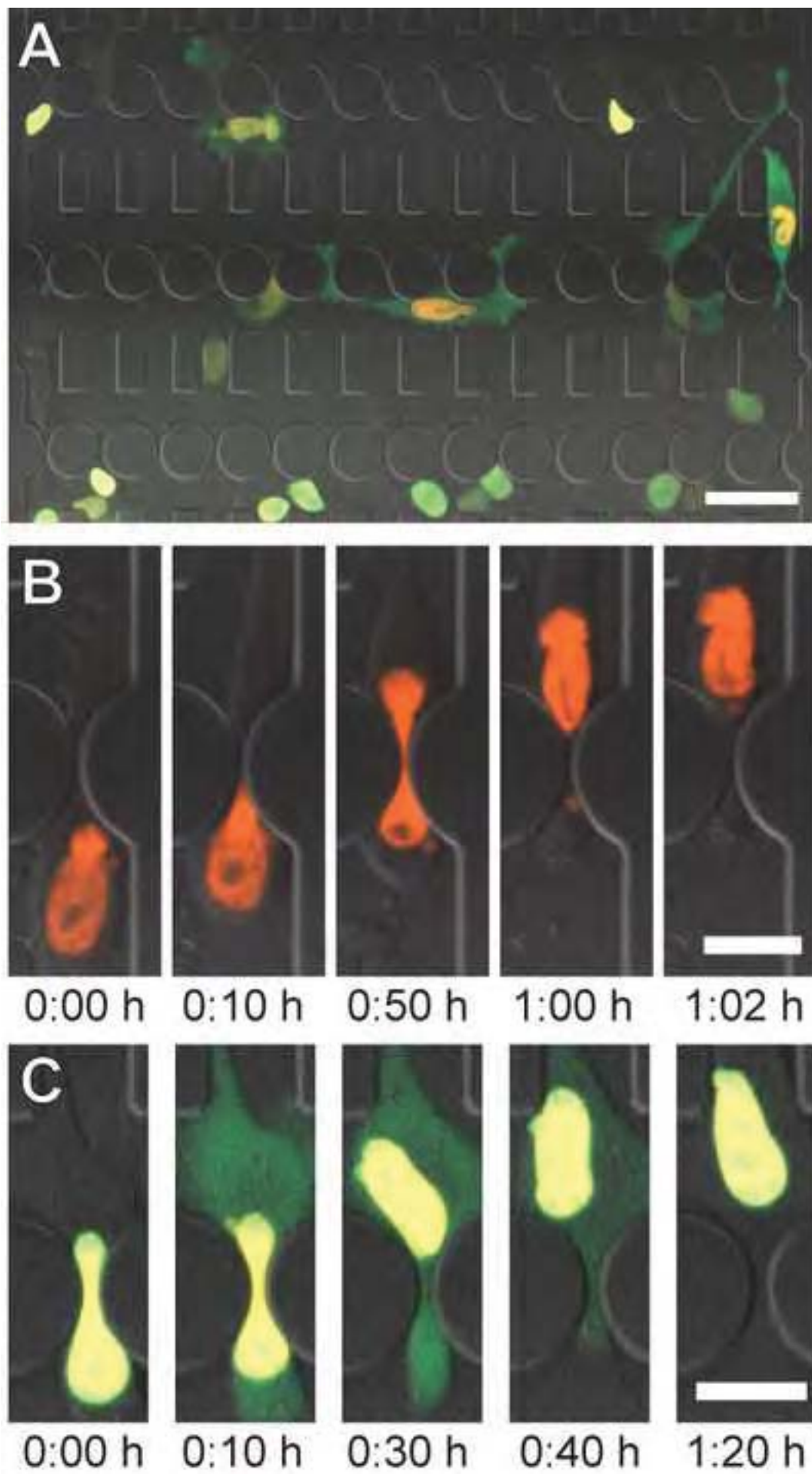
Figure S6. Depletion of CHMP7 by siRNA. Western blot of the HT1080 cells used in three independent migration experiments. Visual inspection confirms lower CHMP7 expression in the cells that received the knockdown (KD) as compared to the cells that received the non-targeting siRNA (NT). (B) Quantification of CHMP7 levels, normalized to actin loading control. ***, $p < 0.001$

Video S1: Video of Migrating Nuclei Corresponding to Image Sequence 1 of Figure 5. Video of BT-549 cells migrating through a microfluidic device with 2- μm wide constrictions,

707 corresponding to Image Sequence 1 in Figure 5. Cells were modified to express NLS-GFP and
708 over-express lamin A, resulting in more rigid nuclei and impaired transit through the constrictions.
709 This video was used as the “training” data for the program.

710

711 **Video S2: Video of Migrating Nuclei Corresponding to Image Sequence 2 of Figure 5.** Video
712 of BT-549 cells migrating through a microfluidic device with 2- μ m wide constrictions,
713 corresponding to Image Sequence 2 in Figure 5. Cells were modified to express NLS-GFP and a
714 mock control vector. This video was used as the “test” data for the program.



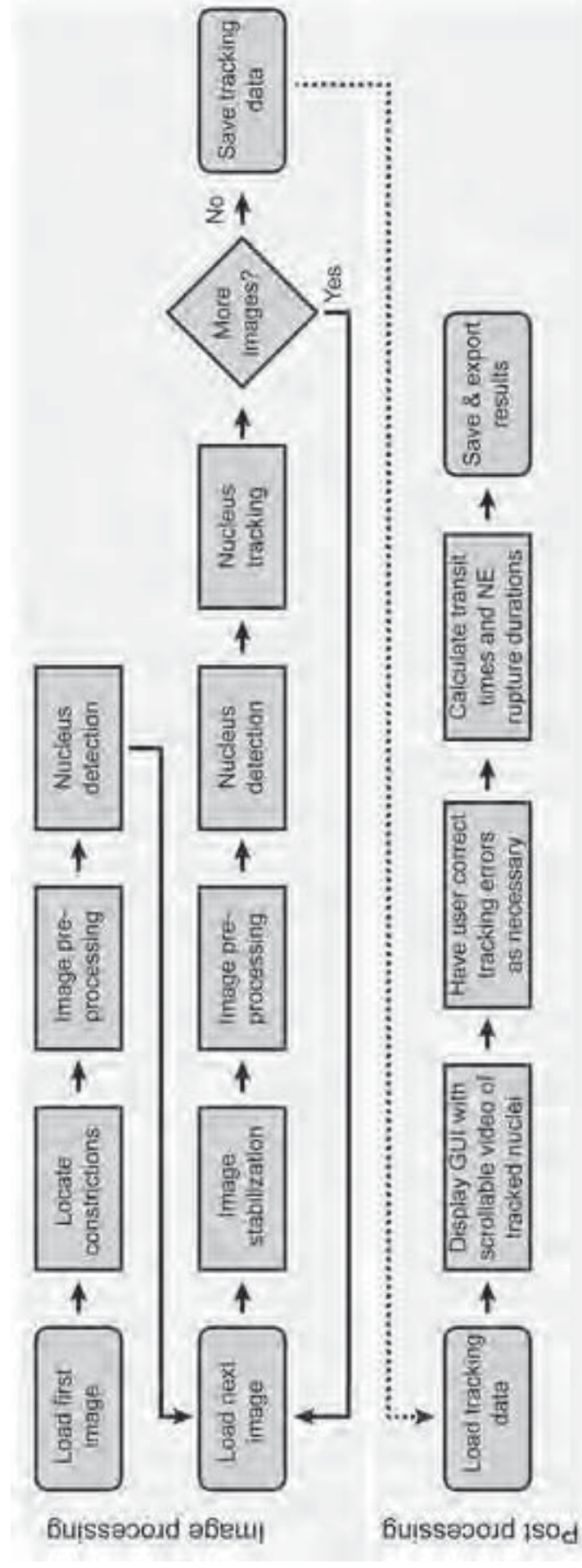
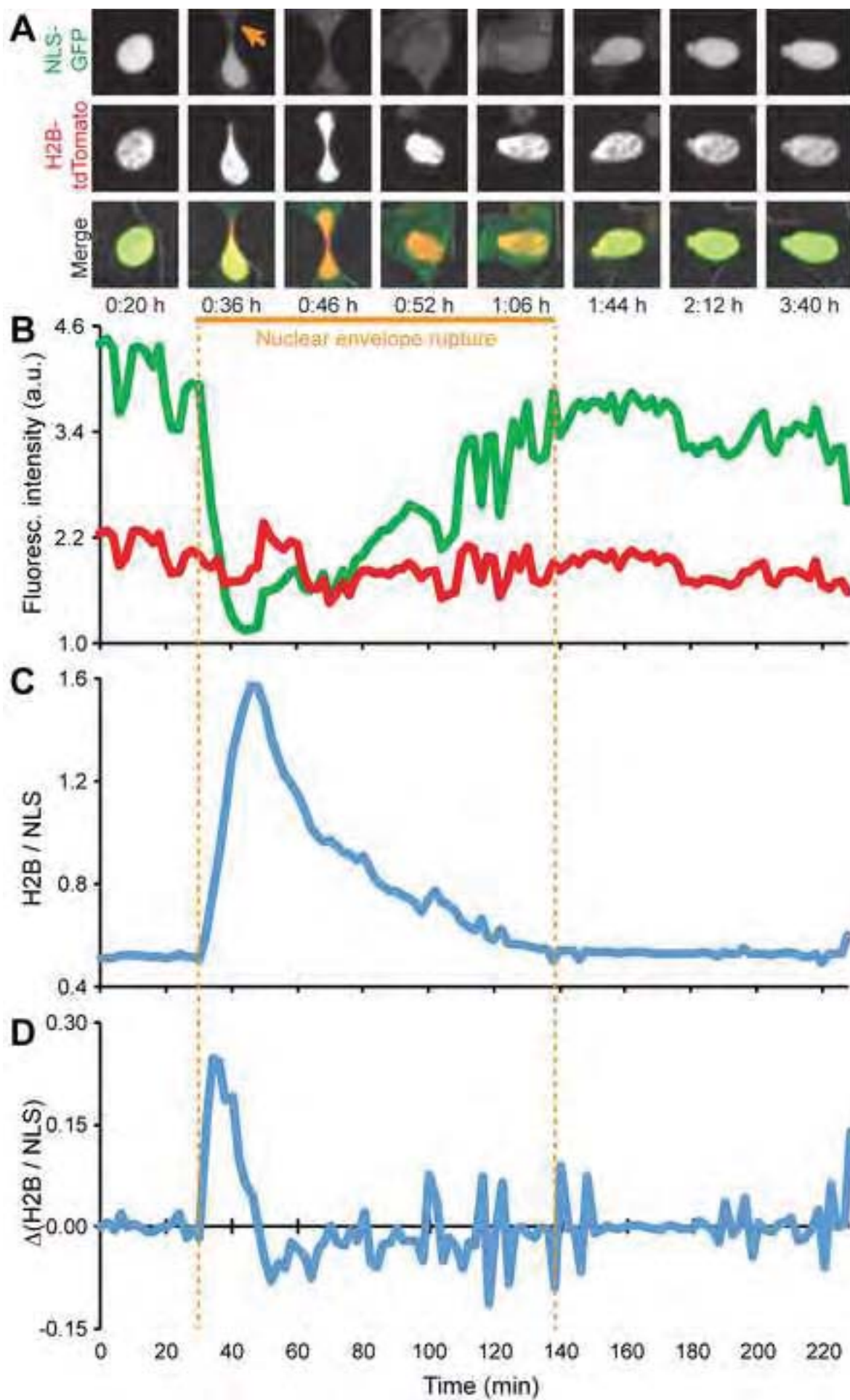
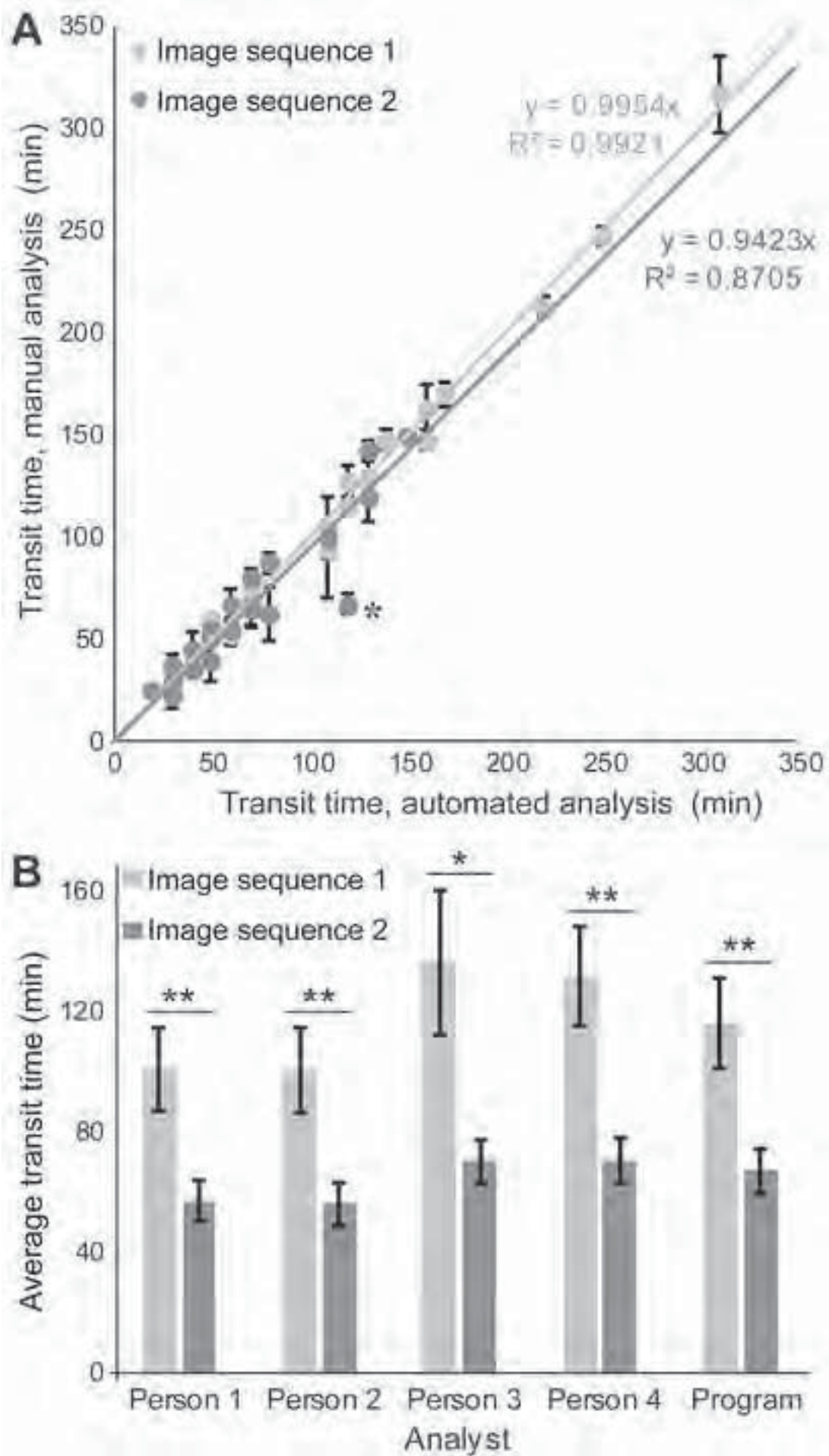


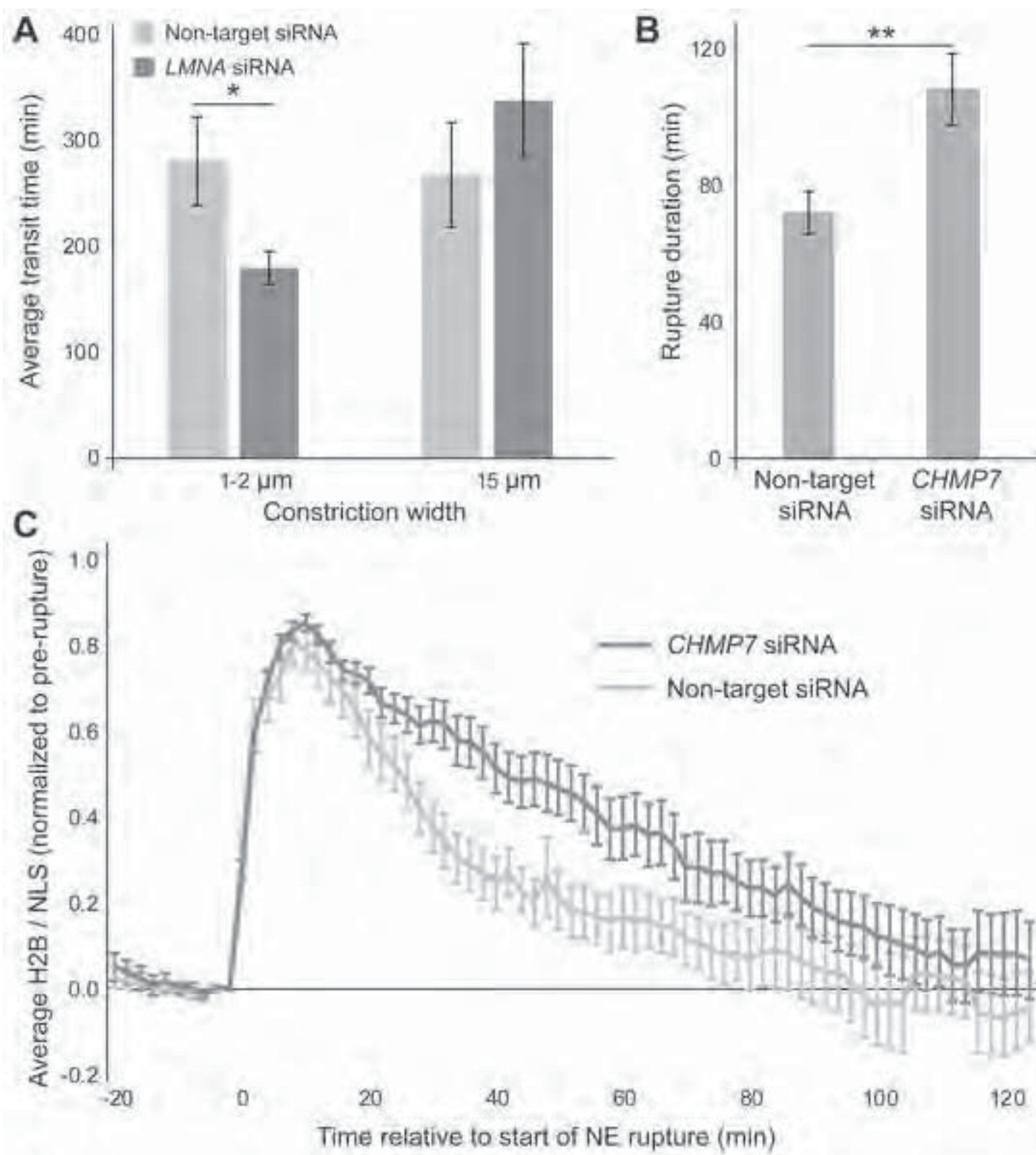


Figure 4

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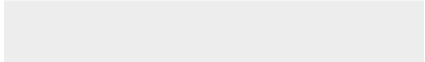








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