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14. ABSTRACT Our objective was to develop a novel approach to distinguish indolent from aggressive prostate cancer, based on the differential non-random spatial organization of the genome. Individual genes occupy preferred positions within interphase nuclei, with the position of some genes altering in diseased states. We previously identified several genes that occupy different nuclear positions in cancerous prostate and breast tissue, compared to normal tissues. Here, we extend our studies for prognostic purposes, to identify genes that differentially localize in aggressive prostate cancer compared to indolent. Such markers are highly sought after by clinicians, to reduce the growing problem of overtreatment. We find that directional repositioning of <i>SP100</i> and <i>TGFB3</i> gene loci stratifies prostate cancers of differing Gleason scores. A more peripheral position of <i>SP100</i> and <i>TGFB3</i> in the nucleus, compared to benign tissues, is associated with low Gleason score cancers, whereas more internal positioning correlates with higher Gleason scores. Conversely, <i>LMNA</i> is more internally positioned in many non-metastatic prostate cancers, while its position is indistinguishable from benign tissue in metastatic cancer. The false positive rates were relatively low, whereas, the false negative rates of single or combinations of genes were high, limiting the clinical utility of this assay in its current form. Additionally, the positioning patterns of <i>SATB1</i> did not correlate with prognostics clinical features. Nevertheless, our results provide the first proof-of-principle that the spatial positioning of the genes can be used for clinal prognostic purposes.					
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1. INTRODUCTION:

Prostate cancer is one of the most prevalent cancers in the United States, where there are more than 164,000 new cases diagnosed each year and 1 in 9 men develop prostate cancer during their lifetime (Siegel et al. 2018) (for reference list see *Appendix 1*). Over 29,000 men die annually from prostate cancer, accounting for 9% of all male cancer deaths (Siegel et al. 2018). Diagnosis of prostate cancer relies on histological analysis of biopsies, which uses subjective morphological and histological criteria. Currently there is a lack of reliable prognostic markers to accurately distinguish latent prostate cancers, where a watch-and-wait approach is the best strategy for the patient, from aggressive tumors, which require aggressive treatment as early in the cancers life as possible. During the DoD Prostate Cancer Idea Award funding period, our objective is to test the feasibility of using the non-random spatial organization of the genome as the basis of a novel, molecularly defined, prognostic test. Our method is based on the fact that genomes are non-randomly organized within interphase nuclei, with individual chromosomes and genes occupying preferred spatial positions (Ferrai et al. 2010; Meaburn et al. 2016a; Misteli 2007). Changes to these positioning patterns can occur in cancer (Meaburn and Misteli 2007; Zink et al. 2004; Meaburn 2016 [*Appendix 2*]; Adriaens et al. 2018 [*Appendix 3*]). For example, in pancreatic cancer, chromosome 8 shifts to a more peripheral location (Wiech et al. 2005). Similarly, chromosomes 18 and 19 change nuclear location in several cancers types, including cervical, thyroid and colon (Cremer et al. 2003; Murata et al. 2007). We have previously discovered several genes that are repositioned in prostate and/or breast cancer, but, importantly, not in non-cancerous diseases or normal prostate and breast tissues (Leshner et al. 2016; Meaburn 2016; Meaburn et al. 2016b; Meaburn et al. 2009). Such changes in spatial positioning patterns are not a reflection of a global reorganization of the genome during carcinogenesis and are instead locus-specific, with the position of many genomic regions remaining conserved in cancer (Meaburn et al. 2009; Meaburn and Misteli 2008; Wiech et al. 2005). We are now expanding these studies to identify differential positioning patterns between cancers, particularly between aggressive and indolent prostate cancer, with the goal of exploiting spatial genome reorganization events for the prognosis of prostate cancer (Meaburn and Misteli 2019 [*Appendix 4 and 5*]). Feasibility of gene positioning to stratify cancer subtypes is supported by the fact that the position of a gene can vary between cancers derived from the same organ, and also between cancers from different organs (Leshner et al. 2016; Meaburn et al. 2016b; Meaburn et al. 2009; Wiech et al. 2009).

2. KEYWORDS:

Cancer; Prognosis; Aggressive Prostate Cancer; Indolent Prostate Cancer; Spatial Gene Positioning; Nuclear Architecture; Cancer Biomarkers; Gleason Score; *LMNA*; *SP100*; *TGFB3*; *SATB1*.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Identification of gene positioning biomarkers (GPBs) for the detection of high-risk prostate cancer

Major Task 1: Screening for high-risk prostate cancers GPBs based on radial and relative positioning

Aim 1: Milestone(s): Identification of candidate markers to distinguish high-risk from low risk cancers, based on relative gene positioning

Estimated completion (from approved SOW): Month 12 (Sept 2016)

% of completion: 65%

Major Task 2: Large scale validation of high-risk prostate cancer GPBs

Milestone(s): Large scale validation of candidate genes that distinguish high-risk from low-risk cancers based on spatial positioning patterns; establishment of false positive and negative rates for each marker of high-risk or low-risk prostate cancer.

Estimated completion: Month 14 (Nov 2016)

% of completion: 65%

Specific Aim 2: Identification of GPBs to distinguish indolent from aggressive cancer

Major Task 3: Identification of GPBs as predictors of aggressive cancer

Milestone(s): Obtained PCBN TMAs; identification of markers of aggressive prostate cancer.

Estimated completion: Month 26 (Nov 2017)

% of completion: 15%

Major Task 4: Identification of GPBs as predictors of risk of developing cancer

Milestone(s): identification of markers of risk of developing prostate cancer; identification of putative markers to predict treatment outcome.

Estimated completion: Month 26 (Nov 2017)

% of completion: 5%

Specific Aim 3: Large scale validation of high-risk and aggressive cancer GPBs

Major Task 5: Establishment of cancer specificity for the candidate GPBs

Milestone(s): Determination of cancer-specificity of the repositioning of the candidate marker genes.

Estimated completion: Month 32 (June 2018)

% of completion: 50%

Major Task 6: Retrospective and blinded validation of GPBs

Milestone(s): Validation of GPBs; publication of 2-3 peer reviewed papers.

Estimated completion: Month 36 (Sept 2018).

% of completion: 5%

Approved No Cost Extension (NCE) Major Task 7: Annual and final reports

Milestone(s): Reports generated to comply with funding

Estimated completion: Month 48 (Sept 2019).

% of completion: 100%

NCE Major Task 8: Validation of high-risk prostate cancer GPBs

Milestone(s): validation of candidate genes that distinguish high-risk from low-risk cancers based on spatial positioning patterns; establishment of false positive and negative rates for each marker of high-risk or low-risk prostate cancer

Estimated completion: Month 41 (Feb 2019).

% of completion: 100%

NCE Major Task 9: Validation of GPBs as predictors of aggressive cancer

Milestone(s): determine if the spatial positioning pattern of SATB1, SP100 and TGFB3 can be used as markers of aggressive prostate cancer.

Estimated completion: Month 44 (May 2019).

% of completion: 100%

NCE Major Task 10: Establishment of cancer specificity for the candidate GPBs

Milestone(s): Determination of cancer-specificity of the repositioning of the candidate marker genes. Validation of GPBs as potential prognostic markers for prostate cancer; publication of 1-2 peer reviewed papers

Estimated completion: Month 48 (Sept 2019).

% of completion: 100%

What was accomplished under these goals?

Aims and methodology:

The overall aim of this project was to identify genes that occupy distinct intra-nuclear positions between aggressive/high risk prostate cancers and indolent/low risk prostate cancers and to explore the usability of these markers for prognostic purposes. To this end we performed fluorescence *in situ* hybridization (FISH) to detect individual genes (*SP100*, *TFGB3*, *LMNA*, *SATB1*) in 4-5µm thick formalin fixed, paraffin embedded human prostate tissue, as described in (Meaburn and Misteli 2019 [Appendix 4]), using protocols we have previously established for tissues (Leshner et al. 2016; Meaburn et al. 2016b; Meaburn et al. 2009). The tissues used comprise a wide range of well characterized prostate samples sections (Supplementary Table 1 of Meaburn and Misteli 2019 [Appendix 5]). The radial position of any gene of interest was determined using a previously developed image analysis method (Leshner et al. 2016; Meaburn and Misteli 2019; Meaburn et al. 2016b; Meaburn et al. 2009). Radial positioning describes the location of a gene locus along the axis from the center of the nucleus to the nuclear periphery (edge). To account for the fact that nuclei are not always of regular elliptical shape, a method was developed in which the binary Euclidean distance transform (EDT) was computed for each nucleus (Leshner et al. 2016; Meaburn and Misteli 2019; Meaburn et al. 2016b; Meaburn et al. 2009). The EDT is a morphological operation that assigns each pixel in a nucleus a value that equals the shortest distance to the edge of the nucleus. To account for variations in nuclear size, the EDT of the geometric gravity center of a FISH signal is normalized to the maximum EDT value for the given nucleus. Using this method, no assumption regarding nuclear shape is made when determining the radial position of a gene, allowing accurate comparisons between tissues, even if there are differences or irregularities in nuclear shape or size. All alleles in a nucleus were included in the analysis and nuclei were included regardless of the number of alleles present, unless no gene signals were present in a nucleus. For each sample, data from ~130 epithelial nuclei, which were acquired from multiple randomly selected regions of the tissue sample, were analyzed and combined to determine the cumulative relative radial distribution (RRD) for each gene in a tissue. The RRD is a standard measure of a genes position in a population and is defined as the statistical distribution of the radial position of all alleles in a cell population. RRDs were statistically compared to each other using the two-sample 1-D Kolmogorov-Smirnov test (KS; $P < 0.01$) as previously described (Leshner et al. 2016; Meaburn and Misteli 2019; Meaburn et al. 2016b; Meaburn et al. 2009). To determine if the positioning pattern of a gene was different between different subgroups of cancers tissues we performed two different types of analysis. 1) we performed individual pairwise cross comparisons between individual cancer tissues, to enable us to detect if any statistical grouping of cancers based on the nuclear position of a gene corresponded to a feature of a cancer (e.g. to Gleason grade). 2) since in our experience, cross-comparisons between individual tissues can lead to ambiguous results we also compared the position of any given gene in a tissue to normal tissue (individual normal tissue and a standardized normal), and then asked if only specific subset of cancers were significantly different to normal tissues, and if difference to normal (or not) could be used as a way to distinguish between different subsets of cancer tissues. The standard normal distribution, which we call “pooled normal distribution” (PND), for

each gene is generated by pooling the gene location information from multiple known normal samples (Leshner et al. 2016; Meaburn and Misteli, 2019; Meaburn et al. 2016b; Meaburn et al. 2009). We use between 6-8 normal tissues to generate a PND, since we have found the distribution curve changes very little with the addition of added normal tissues after 5-6 normal tissues and we do not use more than 8 tissues for the pooled normal, since the difference in the number of nuclei being compared between the pooled normal and an individual tissue becomes large and may bias the statistical result.

Currently, clinicians base therapeutic decisions for the treatment of prostate cancer on stratifying cancers by risk factors, principally using serum prostate-specific antigen (PSA) levels, Gleason score, clinical T stage, and patient age (Chang et al. 2014; Thompson et al. 2007). These factors are, largely empirically, used to predict which cancers are most likely to be aggressive and will lead to morbidity, metastasis and/or death. These subjective assessments have significant ramifications for the selection of most appropriate treatment strategies. High-risk cancers are generally defined as having serum PSA levels of >20ng/ml or a Gleason score of 8-10 or a clinical T stage of T2c and higher (Chang et al. 2014; Thompson et al. 2007). Low-risk cancers (PSA <10ng/ml and Gleason score of 2-6, clinical T stage T2a or lower) are generally classified as indolent cancers, which are expected to remain asymptomatic and as such the use of active surveillance/watchful waiting is recommended (Chang et al. 2014; Cooperberg et al. 2010; Thompson et al. 2007). Gleason score 7 or clinical stage T2b suggest an intermediate risk, and generally indicates treatment, such as radical prostatectomy and/or radiation treatment, as opposed to watchful waiting (Chang et al. 2014; Cooperberg et al. 2010; Thompson et al. 2007). However, the best markers of high-risk prostate cancer are not objective and can be subject to inter-observer variability (Gleason score, T stage) or lack specificity and sensitivity (PSA) (Chang et al. 2014). Improved markers are needed to better distinguish indolent from high-risk cancers and particularly to assess intermediate-risk cancers, with the goal of reducing overtreatment and optimizing therapeutic strategies.

The aim of this study is to identify markers of high and low risk prostate cancer, and to aid the classification of intermediate risk cancers. We compiled a set of cancer tissues based on several criteria (Meaburn and Misteli 2019). Firstly, we cross-compared cancers for risk simply based only on Gleason score. For this analysis, Gleason scores of 6 and below were classified as low Gleason score, 7 was classified as intermediate, and Gleason score 8-10 was classified as a high Gleason score. Secondly, Since Gleason scores are made up of the 2 most prominent Gleason grades, which can be a mix of high and low grade, we also classified tissues by Gleason grade. Thirdly, we also grouped cancers risked based on a combination of Gleason score, clinical T stage and known metastatic status. For most of the tissues we have currently analyzed no PSA data was available, and the clinical T stage of T2 status was not further characterized as a, b or c (Supplementary Table 1 of Meaburn and Misteli 2019 [Appendix 5]). Therefore, we used Gleason score and clinical T stage to classify risk (high-risk: Gleason score 8-10, Gleason grade 5 present, and/or T stage 3 or 4 (Or T2c if known); low-risk: Gleason score 2-6 (unless Gleason grade 5 present), T1/T2, N0 and M0. Additionally, any cancer with known metastasis (N1/M1) was classified as high risk, since they are known aggressive cancers. Fourthly, we compared known metastatic cancers (N1/M1) cancers with non-metastatic cancers (N0/M0). Finally, to ensure the cancer specificity if any repositioning events, we also compared gene spatial positioning patterns between cancerous and non-cancers (normal and benign disease) prostate tissues.

Significant Results and Key Outcomes:

Due to the ongoing problems/delays during the grant period (see *Actual or anticipated problems or delays and actions or plans to resolve them* section below), we focused on Tasks 1, 2, 3, 5 and all NCE tasks (tasks 7-10) during the funding period.

The results generated from our work, conducted over the entire research period, are published in Meaburn and Misteli 2019 [Appendices 4 and 5]. A full, detailed, description of our results are provided in Meaburn and Misteli 2019, attached to this report as *Appendix 4* (published article) and *Appendix 5* (supplemental data for the published article). Significant results and key outcomes are resented below:

- **Determination of the proof-of-concept for the potential usefulness of spatial gene positioning for prognostic applications** (*combined data from all tasks*).

We find subtype-specific intra-nuclear positioning patterns for the genes *SP100*, *TGFB3* and *LMNA*, which are specific to cancer. Although the sensitivity of this assay is currently too low to be clinically useful, our findings of subtype-specific gene positioning patterns in prostate cancer provides evidence for the potential of spatial genome organization as a novel prognostic biomarker. See key results listed below, plus for full details see Meaburn and Misteli 2019, [Appendix 4 and 5]).

- **Determination of the best method to cross-compare gene positioning patterns between tissues subtypes** (*All tasks*).

It was difficult to spot trends between subgroups of prostate cancers when individual cancers were cross-compared to each other (see Supplementary Tables 2-5 of Meaburn and Misteli 2019 [tables attached in *Appendix 5*], and for further discussion see the “*significant results and key outcome*” section of our previous annual reports (Y1-3; Oct 2016-18). For the most part, there was a similar proportion of cross-comparisons between cancers that were significantly different to each other within clinically relevant subgroups as there was between subgroups (Supplementary Tables 2-5 of Meaburn and Misteli 2019 [Appendix 5]). For example, *SP100* was in a significantly different position in 49.1% (27/55) of cross-comparisons amongst Gleason score 2-6 prostate cancers, and 53.4% (47/88) of cross-comparisons when Gleason score 2-6 cancers were compared to Gleason score 7 cancers, and 50% (44/88) of cross-comparisons between Gleason score 2-6 and Gleason score 8-10 cancers (Supplementary Table 2 of Meaburn and Misteli 2019 [Appendix 5]). We found that, at least with the number of tissues we analyzed, assessing the positioning of a gene based on positioning in a given cancer tissue compared to the PND, and then assessing between groups of cancers was a more effective method, than cross comparing positioning directly between individual cancer tissues, to identify marker genes.

- **Identification of a differential gene positioning patterns between individual cancer tissues** (*Tasks 1, 2, 3, NCE 8, NCE 9*).

We initially assessed the repositioning rates for the candidate genes in the assorted set of prostate cancer samples. Compared to the PND, *SP100* was in a statistically significantly different radial position in 44.4% (12/27) prostate cancer specimens, *LMNA* repositioned in 36.4% (4/11), *SATB1* in 34.8% (8/23), and *TGFB3* in 31.8% (7/22) of prostate cancer tissues (Figure 1, Table 1, Supplementary Tables 2-5 of Meaburn and Misteli 2019 [Appendix 4 and 5]). These results demonstrate that **all four genes repositioned in too few cancers to be of use as prostate cancer diagnostic GPBs** since detecting cancer based on the repositioning of the gene would misclassify 55.6-68.2% of the tumors as not cancerous, depending on the gene (Meaburn and Misteli 2019).

In addition to whether a gene was repositioned, the direction of its repositioning could also vary between cancers (Figure 1, Table 1 of Meaburn and Misteli 2019 [Appendix 4]). Of the 12 prostate cancers in which *SP100* was repositioned, the gene was more internally positioned compare to the PND in five cancer tissues (5/12; 41.7%) and more peripherally positioned in seven (58.3%). Similarly, *TGFB3* was more internally positioned in three (3/7; 42.9%) cancers and more

peripherally positioned in four (57.1%). *SATB1* was more internally positioned in five of the eight cancers where the gene was repositioned (62.5%) and more peripherally positioned in three cancers (37.5%). Conversely, *LMNA* repositioned to a more internal nuclear location in all four cancer specimens in which the gene was repositioned (Figure 1, Table 1 of Meaburn and Misteli 2019 [Appendix 4]).

- **Identification of Gene Positioning Biomarkers (GPB) of prostate cancer risk** (*Tasks 1, 2, NCE 8*).

Specifically:

- o ***SP100* and *TGFB3* as GPBs of Gleason score** (*Tasks 1, 2, NCE 8*).

We sought to determine if the differences in gene repositioning patterns between prostate cancers correlated with clinicopathological features. We first compared positioning patterns to Gleason score. In line with the clinical risk assessment of prostate cancers (Thompson et al., 2007), we classified Gleason scores of 2-6 as a low Gleason score, Gleason score 7 as intermediate, and scores of 8-10 as a high Gleason score. There was a modest increase in the proportion of cancer specimens with either *SATB1* or *LMNA* repositioned, compared to the PND, with increasing Gleason score, however, in the case of *LMNA* this may be due to the small sample size (Table 3 of Meaburn and Misteli [Appendix 4]). *SATB1* was in a statistically different nuclear position in 25% (2/8) of low Gleason score cancers, 33.3% (3/9) Gleason score 7 cancers and 50% (3/6) high Gleason score cancers. Similarly, *LMNA* repositioned in 33.3% of low (2/6) and intermediate (1/3) Gleason score cancers, and 50% (1/2) of high Gleason score cancers. Inclusion of the direction of repositioning did little to aid stratification (Table 3 of Meaburn and Misteli [Appendix 4]). Thus, we concluded that neither *SATB1* nor *LMNA* are biomarkers of Gleason score. The proportion of cancers in which *SP100* and *TGFB3* repositioned also did not stratify Gleason score groups. *SP100* was slightly more frequently repositioned in low Gleason score cancers specimens, repositioning in 54.5% (6/11) of low Gleason score cancers, 37.5% (3/8) of intermediate Gleason score cancers, and 37.5% (3/8) of high Gleason score cancers. *TGFB3* repositioned in 30% (3/10) low Gleason score cancer tissues, 20% (1/5) of intermediate Gleason score cancers, and 42.9% (3/7) of high Gleason score cancers. On the other hand, in cancer specimens in which either gene repositioned, the direction of repositioning correlated with Gleason score (Supplementary Figure 2A, Table 4, of Meaburn and Misteli [Appendix 4 and 5]). Both *SP100* and *TGFB3* shifted to a more peripheral position in 100% of the low Gleason score cancers in which these genes showed an altered radial position. In contrast, *SP100* and *TGFB3* were in a more internal position in 83.3% (5/6) and 75.0% (3/4), respectively, of the Gleason score 7 and higher cancers in which they repositioned. Intermediate/high Gleason score cancer tissue repositioning is not exclusively more internal, since for both genes a more peripheral positioning was detected in a Gleason score 9 prostate cancer (Supplementary Figure 2A, Tables 1 and 4 of Meaburn and Misteli [Appendix 4 and 5]). The positioning patterns of *SP100* and *TGFB3* could not distinguish intermediate Gleason score cancer tissues from high Gleason score cancer tissues (Supplementary Figure 2A, Tables 1 and 4 of Meaburn and Misteli [Appendix 4 and 5]). Nevertheless, this does not necessarily rule them out as useful clinical markers since low Gleason score cancers are less likely to receive treatment than intermediate or high Gleason score cancers (Thompson et al., 2007; Cooperberg et al., 2010; Leshner et al., 2016). Given that there is inter-and intra-observer variability when scoring cancers (Montironi et al., 2005), additional markers that can clarify if a cancer is Gleason score 6 (low) or 7 (intermediate) would be useful in guiding therapeutic choices.

- o **Multiplexing improved the specificity of *SP100* and *TGFB3* as GPBs** (*Tasks 1, 2, NCE 8*).

Although both *SP100* and *TGFB3* displayed differential positioning patterns between low and intermediate/high Gleason score cancer specimens, the sensitivity for subgrouping prostate cancers by Gleason score based on positioning patterns is low, with multiple cancers with repositioning of the gene. We have previously demonstrated that the sensitivity of diagnostic GPBs can be improved by multiplexing (Meaburn et al., 2009; Leshner et al., 2016). We therefore evaluated if combining positioning data from *SP100* and *TGFB3* would increase the number of cancers classified as low or intermediate/high Gleason score based on gene positioning patterns. Importantly for multiplexing to improve the sensitivity, *SP100* and *TGFB3* would need to be frequently repositioned in different cancer specimens. Of a subset of 19 cancer tissues in which both genes were positioned, 10 (52.6%) had differential repositioning patterns for *SP100* to that of *TGFB3* (Table 1, Supplementary Table 7 of Meaburn and Misteli [*Appendix 4 and 5*]). However, multiplexing the two genes did not improve the sensitivity to detect low Gleason score cancers above using *SP100* alone. *SP100* was more peripherally positioned in all five cancers where at least one of the two genes repositioned (Table 1, Supplementary Table 7 of Meaburn and Misteli [*Appendix 4 and 5*]). Nevertheless, multiplexing increased the sensitivity of detecting intermediate/high Gleason score cancers (Table 1, Supplementary Table 7 of Meaburn and Misteli [*Appendix 4 and 5*]). At least one gene repositioned in eight of the 11 (72.7%) Gleason score 7 and higher cancers. Both *SP100* and *TGFB3* contributed to the increased proportion of cancer specimens with repositioning events. Of the seven cancers with only one of the two genes more internally repositioned, *SP100* was more internally repositioned in four and *TGFB3* more internally positioned in three cancers (Table 1, Supplementary Table 7, of Meaburn and Misteli [*Appendix 4*]).

- o ***SP100* and *TGFB3* as markers of Gleason grade** (*Tasks 1, 2, NCE 8*).

We also assessed if positioning patterns correlated with Gleason grade. For all four genes, increasing Gleason grade did not correlate with the percentage of cancer specimens in which the genes repositioned (Tables 3 and 4 of Meaburn and Misteli [*Appendix 4*]). Consistent with Gleason score, the direction that *SATB1* and *LMNA* repositioned did not aid in stratifying cancers by Gleason grade (Table 3 of Meaburn and Misteli [*Appendix 4*]), but the direction of repositioning did correlate with Gleason grade for *SP100* and *TGFB3* (Table 4 of Meaburn and Misteli [*Appendix 4*]). As with Gleason score, in the more highly differentiated cancers (Gleason grades 1-3) *SP100* and *TGFB3* repositioned towards the nuclear periphery in 100% (5 and 3 cancers, respectively) of the cancers in which these genes repositioned. Yet, in the poorly differentiated cancers (Gleason grade 4 and 5) both genes preferentially repositioned towards the nuclear interior. *SP100* was more internally positioned in 80% (4/5) of Gleason grade 4 and 5 cancer in which *SP100* repositioned and *TGFB3* was more internally positioned in 66.7% (2/3) of the Gleason grade 4 and 5 cancers in which *TGFB3* was repositioned, compared to the PND (Table 4 of Meaburn and Misteli [*Appendix 4*]). The similarity between Gleason grade and Gleason score positioning patterns are not surprising given that Gleason score is the sum of the two most prominent Gleason grades. An important caveat to be noted is that while the subgrouping of the cancers was based on the most predominant Gleason grade of the tissue, it is not necessarily the predominant Gleason grade of the nuclei analyzed from each specimen.

o **LMNA is a marker of low risk prostate cancer** (*Tasks 1, 2, NCE 8*).

Having determined the biomarker potential of the candidate genes for subgrouping prostate cancers by Gleason score, we compared their positioning patterns to other clinical markers of poor patient outcome. The TNM staging system is commonly used to aid the prediction of the aggressiveness of the cancer and the risk of poor patient outcome (Thompson et al., 2007). Positioning patterns for *SP100* and *SATB1* were similar in low and high T stage cancer specimens and could not be used to distinguish the different T stage group cancers from each other (Tables 3 and 4 of Meaburn and Misteli [Appendix 4]). *TGFB3* and *LMNA* were both more frequently repositioned in low T stage cancers to that of high T stage cancers, but with high false positive rates (Tables 3 and 4 of Meaburn and Misteli [Appendix 4]). *TGFB3* repositioned in 41.7% (5/12) of T1/2 cancers, 11.1% (1/9) of T3/4 cancers, and 7.7% (1/13) of benign tissues, compared to the PND (Tables 1, 4 and 6, of Meaburn and Misteli [Appendix 4]). This equates to a false negative rate of 58.3% (7/12) and a false positive rate of 9.1% (2/22) for using the reposition of *TGFB3* to detect low T stage prostate cancer. Similarly, *LMNA* repositioned in 42.9% (3/7) of low T stage cancer, 25% (1/4) of high T stage cancers and 12.5% (1/8) of benign tissues (Tables 3 and 6, of Meaburn and Misteli [Appendix 4]), making the false negative and positive rates of using the repositioning of *LMNA* to demark low T stage cancers 57.1% (4/7) and 16.7% (2/12), respectively.

Clinically, multiple factors are combined to determine risk of poor outcome. Therefore, we also compared gene positioning patterns with a multifactorial determinant of risk using standard clinical risk assessment criteria (Thompson et al., 2007; Chang et al., 2014) with the exception of PSA levels, since no information on serum PSA were available for our specimens. Moreover, we included N1 and/or M1 cancers in the high-risk group, since they are known aggressive cancers. We classified low risk cancer as Gleason score 2-6 and T1/2 and N0M0 cancers; intermediate risk cancers as Gleason score 7 and T1/2 and N0M0; high risk as Gleason score 8-10 and/or T3/4 and/or N1 and/or M1 prostate cancers. The positioning patterns of *SP100*, *TGFB3* and *SATB1* were similar in all three risk groups, and thus could not be used as markers of risk (Tables 3 and 4, of Meaburn and Misteli [Appendix 4]). Conversely, *LMNA* was more frequently repositioned in low risk prostate cancer, since it repositioned in 66.7% (2/3) of low risk cancers, 0% (0/2) of intermediate risk and 33.3% (2/6) of high-risk groups (Table 3, of Meaburn and Misteli [Appendix 4]).

• ***SP100* and *TGFB3* cannot distinguish metastatic from non- metastatic low Gleason score cancers** (*Tasks 3 and NCE 9*).

Gleason score is not a perfect measure of risk. Given the variability in positioning patterns within the same Gleason group, we asked if the positioning patterns could be useful to distinguish aggressive low Gleason core cancers from non-aggressive low Gleason score cancers. Such a marker would aid treatment decisions. However, *SP100* and *TGFB3* were repositioned in a similar proportion of low Gleason score cancers with or without metastasis (Table 1 of Meaburn and Misteli 2019 [Appendix 4]). *SP100* repositioned in 50% (2/4) of low Gleason score cancers that had metastasized and 57.1% (4/7) of low Gleason score cancers without metastases. Likewise, *TGFB3* repositioned in 33.3% (1/3) of metastatic low Gleason score cancer specimens and 28.8% (2/7) of non-metastatic low Gleason score cancer specimens Table 1 of Meaburn and Misteli 2019 [Appendix 4]). Thus, in addition to the high false negative rate for Gleason score, *SP100* and *TGFB3* cannot distinguish aggressive low Gleason score cancers from non-aggressive low Gleason score cancers, limiting their clinical potential.

- LMNA as a marker of non-aggressive prostate cancer (Tasks 3 and NCE 9).**
 As a direct measure of the aggressiveness of a cancer we compared non-metastatic cancers (N0M0) and metastatic (N1/M1) prostate cancers (Supplementary Figure 2B, Table 1, 3 and 4 of Meaburn and Misteli 2019 [Appendix 4 and 5]). **No GPBs were identified that identified aggressive (metastatic) prostate cancers.** *SP100* repositioned, compared to the PND, in a similar proportion of N0M0 (47.4%; 9/19) and metastatic (42.9%; 3/7) cancer specimens. Furthermore, the direction of repositioning was similarly mixed in both groups of cancer (Table 4, of Meaburn and Misteli 2019 [Appendix 4]). The remaining three genes repositioned more frequently in non-metastatic cancers. For *TGFB3* and *SATB1* this difference is small, with the genes repositioned in ~33.3% (5/15 and 6/16 respectively; false negative rate ~66.7%) of non-metastatic cancers and 16.7% (1/6) of metastatic cancers (Table 3 and 4, Meaburn and Misteli 2019 [Appendix 4]). **LMNA was the best marker of non-metastatic cancers.** *LMNA* repositioned in 57.1% (4/7) of non-metastatic prostate cancer specimens and was not repositioned in metastatic (0/4) cancer tissues (Supplementary Figure 2B, Table 3, Meaburn and Misteli 2019 [Appendix 4 and 5]). In 100% of the cancers in which *LMNA* repositioned, it was more internally positioned than the PND (Table 1 of Meaburn and Misteli [Appendix 4]).
- Identification of the gene specificity of prognostic GPBs (Tasks 1, 2, 3, NCE 8, NCE 9).**
 The reorganization events between the different subtypes of prostate cancer appear to be gene specific. The repositioning pattern of *SP100* and *TGFB3* correlated with Gleason score, whereas the repositioning pattern of *LMNA* occurred in non-metastatic cancers, as described above. However, *LMNA* and *SATB1* positioning patterns were not able to stratify prostate cancers by Gleason score and *SP100*, *TGFB3* and *SATB1* were not accurate markers of risk or aggressiveness of the cancer. (Tables 1, 3 and 4 of Meaburn and Misteli 2019 [Appendix 4]. Indeed, **the positioning pattern of *SATB1* was not a useful prognostic maker** at all (Table 1, Table 3 of Meaburn and Misteli 2019 [Appendix 4]).
- Conservation of positioning patterns between non-cancerous tissues (Tasks 5 and NCE 10).**
 We positioned *SP100*, *TGFB3*, *SATB1* and *LMNA* in non-cancerous (normal and benign disease) prostate tissues (Figure 2, Supplementary Figure 1, Tables 5 and 6, Supplementary Tables 2-5 of Meaburn and Misteli 2019 [Appendices 4 and 5]). For all four genes we found that the positioning patterns were highly similar between benign tissues. For *SP100*, *TGFB3* and *SATB1*, only 9.1%-14.1% of comparisons between the individual non-cancerous tissues reached significance. There was a little more variability between benign tissues for *LMNA*, where 25% of cross-comparisons between benign tissues were significantly different (Figure 2, Table 2, Supplementary Table 2-5, of Meaburn and Misteli 2019 [Appendices 4 and 5]).
- Cancer specificity of the gene repositioning events (Tasks 5 and NCE 10).**
 There was little repositioning of the four genes in benign tissues when compared to the PND, with repositioning in 7.7%-16.7% of benign tissues, depending on the gene (Figure 2, Tables 5 and 6, of Meaburn and Misteli 2019 [Appendices 4 and 5]). *SP100* was statistically similarly positioned in all seven normal tissues, compared to the PND, but was significantly repositioned in 40% (2/5) of benign disease tissue (Figure 2, Tables 5 and 6, of Meaburn and Misteli 2019 [Appendices 4 and 5]). The positioning patterns of *SP100* were distinct in benign disease and low Gleason score cancer, since it was more internally localized in the two benign disease tissues, yet more peripherally located in low Gleason score cancers (Tables 1 and 5, of Meaburn and Misteli 2019 [Appendices 4 and 5]). We conclude that the repositioning we detect in the cancer tissues is cancer specific since it occurs so infrequently in non-cancerous tissues.

- **Low false positive rates for the GPBs (*Tasks 5 and NCE 10*).**

A false positive was classified as a gene repositioning event associated with a specific subgroup of prostate cancer occurring in a non-cancerous tissue. In keeping with our finding of cancer specificity of reposition events, we identified low false positive rates for our marker genes. When using more peripheral positioning, compared to the PND, as a marker of low Gleason score prostate cancer, the false positive rate for *SP100* is very low, at 3.6%, since it is more peripherally positioned in only one of 28 normal, benign disease and higher Gleason score cancer tissues (Tables 1 and 5, of Meaburn and Misteli 2019 [Appendix 4]). Similarly, *TGFB3* was repositioned in a single normal tissue (12.5%; 1/8) and in none of the benign disease tissues (0/5), compared to the PND (Figure 2, Tables 5 and 6 of Meaburn and Misteli 2019 [Appendix 4]). Unlike *SP100*, the direction *TGFB3* reposition in the normal tissue was the same as in low Gleason score cancer. However, the false positive rate for using a more peripheral positioning of *TGFB3* was relatively low at 8%, because it was more peripherally positioned in two of the 25 benign tissues and higher Gleason score cancers (Tables 1 and 5, of Meaburn and Misteli 2019 [Appendix 4]). The false positive rate of using a more internal position of *SP100* or *TGFB3* to detect intermediate/ high Gleason score cancers is also low, at 8.7% and 0%, respectively, since *SP100* was more internally repositioned in only two of the 23 benign tissues and low Gleason score cancer specimens and *TGFB3* was not more internally repositioned in these groups of tissues (N=23; Table 1 and 5, of Meaburn and Misteli 2019 [Appendix 4]). Similarly, the false positive rate of the positing pattern of *LMNA* is the false positive rate is relatively low at 8.3% (1/12) (Tables 1, 3 and 5 of Meaburn and Misteli 2019 [Appendix 4]).

- **The high false negative rates limit the clinical usefulness of the identified GPBs (*Tasks 2, 3 NCE 8, NCE 9*).**

False negative rates are important for clinical tests as they reveal the sensitivity of the assay by giving the proportion of cancers that would be not included (i.e. missed) as a specific subgroup of cancer. In our assay, a false negative is classified as when a specific repositioning pattern (e.g. repositioned more internally) was not detected within a cancer of a specific subgroup, thereby falsely classifying cancer as a different subgroup of cancer. The false negative rates for *SP100* and *TGFB3* as markers of Gleason score were high, at 45-70%, and multiplexing the two genes left false negative rates high (36-45%) (Table 1 and supplementary Table 7 in Meaburn and Misteli 2019 [Appendices 4 and 5]). Collectively, these observations demonstrate that while positioning patterns performed less well than the Gleason system at stratifying cancers, we identify differential gene positioning patterns between subgroups of prostate cancers. Similarly, the false negative rate of *LMNA* as a marker of non-metastatic cancer was high, at 43% (Table 1, [Appendix 4]). For a full discussion of false negative rates, see the results section in Meaburn and Misteli 2019 [Appendix 4]. We conclude that the observed high false negative rates reduce marker strength and the utility of these genes for prognostic purposes.

- **Demonstration that genomic instability does not influence gene repositioning potential and thus does not inhibit the use of spatial gene positioning patterns for prognostic tests (*Tasks 1, 2, 3, NCE 8, NCE 9*).**

The expected number of copies of a given gene in any cell nucleus is two, however cancer is associated with genomic instability and altered copy numbers of genes. While prostate cancer is associated with a lower level of genomic instability than many types of cancer, we found both gains and losses of copy numbers of our candidate genes in some cancers, and found variability in the copy number of each gene between cancers (Supplementary Table 6 of Meaburn and Misteli 2019 [Appendix 5]). However, the likelihood of a gene repositioning in a cancer did not correlated

with gene copy number (Figure 1, Supplementary Table 6 of Meaburn and Misteli 2019 [Appendix 4 and 5]). Importantly for a clinical test, we found even in the background of variable genomic instability between cancers, it is still possible to use gene positioning to stratify cancers into clinically distinct subgroups, as demonstrated in this study (Meaburn and Misteli 2019).

- **Publication of peer reviewed papers (Tasks 6 and NCE 10).**
One original research paper in *Frontiers in Genetics* (Meaburn and Misteli 2019 [Appendices 4 and 5]), and two reviews (Meaburn 2016, *Frontiers in Genetics* [Appendix 2]; Adriaens et al. 2018, *Histochem Cell Biol* [Appendix 3]) were published during this grant funding period, as a direct result of the studies performed for this grant.
- **No relative positioning biomarkers were identified (All tasks).** See Year 1-3 annual reports, and for information on difficulties in performing this aspect of the SOW approved work, see the section *Actual or anticipated problems or delays and actions or plans to resolve them* section below).

(Task NCE 7 – all annual reports and the final report were complete).

Conclusion

Our findings of subtype-specific gene positioning patterns in prostate cancer **provides proof-of-concept for the potential usefulness of spatial gene positioning for prognostic applications** and encourage further exploration of spatial gene positioning patterns to identify novel clinically relevant molecular biomarkers, which may aid treatment decisions for cancer patients.

We did not meet our goal for the number of genes or tissues screened, as our automated analysis software was not as robust as anticipated, meaning analysis had to be performed semi-automated, significantly slowing down throughput. This was compounded by the fact we also had problems getting good FISH signals in our tissues during Year 2/3, which required significant troubleshooting. See the section “*Actual or anticipated problems or delays and actions or plans to resolve them*” below for more details.

What opportunities for training and professional development has the project provided?

The project has provided the key worker (Dr. Karen Meaburn) professional development by allowing her to present the project in seminars on the NIH campus and at a national meeting (Predictive Cancer Biomarkers, Next Generation Diagnostics Summit, Washington, DC, USA. August 2016), and to collaborate with a prostate cancer pathologist (Dr. True, University of Washington). Both have fostered useful discussions with experts in various fields. In addition, attending the conference on biomarkers, which focused on new and upcoming diagnostic approaches, the talks and discussion provided valuable knowledge around the subject of biomarker development.

How were the results disseminated to communities of interest?

Results have been presented at various scientific workshops, conferences and seminar series, nationally and internationally (see listing below). We have published our findings as an original research article in *Frontiers in Genetics* (Meaburn and Misteli 2019 [Appendices 4 and 5]), a journal which is free for all to read immediately after publication, without a subscription (open access). To allow faster dissemination of our findings, upon submission for review at *Frontiers in Genetics*, we also uploaded our manuscript to the open access preprint service *Biorxiv* (doi: <https://doi.org/10.1101/728972>;

<https://www.biorxiv.org/content/10.1101/728972v1>). Additionally, we have published two reviews highlighting the need of such projects to use genome organization to find alternative biomarkers for cancer diagnostics and prognostics (Meaburn 2016 [Appendix 2]; Adriaens et al. 2018 [Appendix 3]).

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report (Final report).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have previously shown that the human genome is highly organized within the interphase nucleus, and that highly specific changes to this organization occur during carcinogenesis. For example, the gene *FLI1* positions closer to the edge of the nucleus in prostate cancer tissue compared to normal tissue (Leshner et al. 2016). Our marker genes are able to accurately distinguish cancerous tissues from normal in 56 to 92% of cases, depending on the gene, and up to 100% of cancers if genes were combined, with very low false positive and false-negative detection rates (Leshner et al. 2016; Meaburn et al. 2016b). This provided the first direct evidence that the spatial organization of the genome could have diagnostic applications. Using a similar technique, we have now extended our studies to prostate cancer prognosis. Our results demonstrate for the first time a proof-of-principle that spatial gene positioning can be developed into a prognostic tool for prostate cancer (Meaburn and Misteli 2019). We find that directional repositioning of *SP100* and *TGFB3* gene loci stratifies prostate cancers of differing Gleason scores. A more peripheral position of *SP100* and *TGFB3* in the nucleus, compared to benign tissues, is associated with low Gleason score cancers, whereas more internal positioning correlates with higher Gleason scores (Meaburn and Misteli 2019). Conversely, *LMNA* is more internally positioned in many non-metastatic prostate cancers, while its position is indistinguishable from benign tissue in metastatic cancer (Meaburn and Misteli 2019). The false positive rates were relatively low (Meaburn and Misteli 2019), which is necessary for a diagnostic test. However, since the false negative rates of single or combinations of genes were high, it limits the clinical utility of the genes we analyzed (Meaburn and Misteli 2019). Nevertheless, because our observations do provide the first proof-of-principle that the spatial organization of genes in the interphase nucleus can be used to stratify cancers originating from the same organ, our findings encourage further exploration, by us and others in the field, of spatial gene positioning patterns to identify novel clinically relevant molecular biomarkers, which may aid treatment decisions for cancer patients. It is also likely these studies will provide knowledge that will help other scientists in the field when designing experiments to understand the biological function of genome organization, since we provide a well characterized example of a specific repositioning event.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

If our findings, that the spatial organization of the genome in interphase cells can be used for a prostate cancer prognostic, holds true after further validation in an increased number of prostate tissues, they would form the bases of a patent and could lead to the development of a novel cancer prognostic clinical test.

Such a test, particularly if already protected by a patent, would be of significant interest to a commercial company.

What was the impact on society beyond science and technology?

Our results demonstrate a proof-of-principle that spatial genome organization can be developed into a prognostic tool for prostate cancer. If validated, this would represent a significant impact for clinicians and patients diagnosed with prostate cancer. There is currently a high level of overtreatment for men diagnosed with cancer (in the range of 20-80% of cancers) (Meaburn 2016), because there is a lack of markers to accurately distinguish aggressive cancer from indolent cancer. Men with aggressive cancer require urgent and aggressive treatment. However, since indolent cancer is slow growing, generally asymptomatic and unlikely to be lethal, active surveillance is a better option of men with indolent cancer. Taken together, this means due to overtreatment in the range of 1:5-4:5 prostate cancer patients are currently receiving treatment they do not require and are suffering unnecessarily because of it. Markers that can identify “clinically relevant” prostate cancers would aid physician’s decision making when planning effective treatment options for their patients, would reduce unnecessary treatments, and the debilitating side effects that accompany them, and reduce health care costs. Moreover, since specific gene repositioning events have been detected in many diseases, including several cancers (Meaburn 2016), once validated for prostate cancer this approach could be applied to other cancers where over-diagnosis is a problem, for example breast cancer.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Throughput

We had several problems/delays during the grant period. First, the throughput issue that impacted our research in all years (as fully described in the *Actual or anticipated problems or delays and actions or plans to resolve them* sections in our Annual reports for Year 1-3 (Oct 2016, 2017 and 2018 reports)). Briefly, a major bottleneck in the analysis of these samples is image analysis. We have relied on a semi-manual method to identify nuclei and FISH signals in the tissue sample, the large number of individual nuclei (100 per tissue sample, several hundred tissues) exceeded our analysis capacity. In this semi-automated analysis nuclei must be manually identified and segmented. To try to overcome this problem, we have, in close collaboration with Dr. Stephen Lockett, NCI, attempted to develop a novel image analysis tool, which automatically segments nuclei (with high accuracy) from microscopy images. However, so far, we have been unable to develop a tool that could segment nuclei with a high enough precision for our use. Radial positioning measurements are highly dependent on the outline of the nucleus, inaccuracies in segmenting nuclei would have a profound effect on the quality of our positioning data. Since the automated software was unable perform at a high enough level, we were unable to perform high-throughput automated image analysis as planned and had to only use manual analysis. This significantly slowed down our throughput, for all SOW tasks.

FISH

FISH on FFPE human tissues was required for all SOW approved tasks. Although we have successfully performed FISH on FFPE human tissues (normal, cancerous, benign disease, in both breast and prostate tissues) for well over 10 years and are experts in the technique (Leshner et al. 2016; Meaburn et al. 2016b; Meaburn et al. 2009; Meaburn and Misteli 2019), during Year 2 we suffered problems with the assay, which extended into Year 3. These issues are fully discussed in Annual reports for Year 2 and 3 (Oct 2017 and 2018 reports). Briefly, we experienced a gradual decline in FISH signal quality resulting in difficulty obtaining primary data (lack of FISH signals/very pale FISH signals/background issues, damaged nuclei). After a long period of troubleshooting and re-optimizing conditions (see Year 2 and 3 annual reports (Oct 2017 and 2018)), we were able to get the assay working fully again. Due to these issue, for a proportion of Year 2 we were unable to generate any FISH data and for much of Year 3 we were only able to perform FISH for a single gene per slide of tissue, instead of our usual dual-color FISH (where two genes can be visualized in the same nucleus). This greatly reduced our throughput and made mapping relative positioning of genes (distance between alleles of two different genes) impossible.

For full details of the systematic troubleshooting of our FISH protocol see Year 2 annual report (Specifically the *Actual or anticipated problems or delays and actions or plans to resolve them* section and *Appendix 3* of the Year 2 report), which including the reduction in time and temperate for the denaturation of DNA step, using an alternative hot block and optimizing the tissue digestion steps, reduced the incidence of damaged nucleus. A major challenge was to keep the balance of not over-digesting the tissues (which leads to damaged nuclei) yet not under-digesting the tissues (where probes are not able to enter the nuclei (resulting in no or pale FISH signals and increased cytoplasmic background)). Further, as described in our Year 3 annual report, we changed the labeled used to visualize our probes to improve FISH signal quality. By switching to DY457-dUTP (Dyomics; directly fluorescently labeled dUTP), in place of biotin-16-dUTP (Roche, used for indirect labeling of probe DNA), to fluorescently labeled probe DNA, for visualization of a given genes location within a nucleus, we were able to robustly, reliably and clearly detect FISH signals in red. However, using a variety of different labeled dUTPs we were unable to visualize FISH signals in green. Using an alternative antibody to detect digoxigenin (DyLight-488 anti-digoxigenin (Vector)), resulted in good FISH signals in the green channel, without a background speckle problem, was used in combination with DY457-dUTP probes to enable us to perform dual-color FISH again (NCE period).

Microscope

Finally, during Year 2 was that the microscope system used for this project (IX70 (widefield; Olympus), Deltavision (Applied Precision), 60x 1.42 numerical aperture oil objective (Olympus), auxiliary magnification 1.6, 0.5 μ m Z step size)) developed a fault that was too costly to repair (Year 2 annual report). Due to this we had to switched to an alternative, similar, widefield microscope in our imaging facility: IX71 (Olympus), Deltavision Elite (Applied Precision), 60x 1.42 numerical aperture oil objective (Olympus), 100x 1.40 numerical aperture oil objective (Olympus)). We ensured images produced on the new microscope system yielded data that was directly comparable to data generated from the old system. We re-imaged on the new microscope system a tissue (slide) we had previously imaged on the old system and compared the gene positioning data generated from both sets of images, and the results between the two microscope systems were statistically similar ($P = 0.79-0.86$, KS test; see *Appendix 2* of the our Year 2 annual report, Oct 2017; and *Methods and Materials* in Meaburn and Misteli 2019). This test demonstrated that our gene positioning results can be accurately reproduced and compared, regardless on which imaging system the microscope images were obtained from, thus old and new data can accurately be compared to each other for assessment of gene repositioning between different subsets of prostate tissues. They also

demonstrate the high level of reproducibility in the analysis method, and that the positioning distribution is not dependent on microscope system used. Both of which are highly important requirements of a test to be used clinically.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

6. PRODUCTS:

• Publications, conference papers, and presentations

Journal publications.

- Meaburn, K. J. (2016). Spatial genome organization and its emerging role as a potential diagnosis tool. *Front. Genet.* 7, 134. doi: 10.3389/fgene.2016.00134.

Published

Acknowledgement of federal support: *Yes.*

(Included as appendix 2)

- Adriaens C, Serebryanny LA, Feric M, Schibler A, Meaburn KJ, Kubben N, Trzaskoma P, Shachar S, Vidak S, Finn EH, Sood V, Pegoraro G, Misteli T. (2018) Blank spots on the map: some current questions on nuclear organization and genome architecture. *Histochem Cell Biol.*, 150(6):579-592. doi: 10.1007/s00418-018-1726-1.

Published

Acknowledgement of federal support: *Yes.*

(Included as appendix 3)

- Meaburn KJ and Misteli T (2019) Assessment of the Utility of Gene Positioning Biomarkers in the Stratification of Prostate Cancers. *Front. Genet.* 10:1029. doi: 10.3389/fgene.2019.01029

Published

Acknowledgement of federal support: *Yes.*

(Included as appendix 4, supplementary data as appendix 5)

All three publications were peer reviewed before publication.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Presentations:

Year 1 (30 Sept 15-29 Sept 16):

- Predictive Cancer Biomarkers, Next Generation Diagnostics Summit, Washington, DC, USA.
- Department of Pathology, Harvard University, Boston.
- Annual Meeting of the Society for Investigative Dermatology (keynote), Phoenix, AZ.
- Genomes in Space and Time, International Center for Physics, Trieste, Italy.
- 12th International Congress of Cell Biology, Prague, Czech Republic.
- EMBO workshop on “Cell nucleus and stem cell fate”, Kyllini, Greece.

Year 2 (30 Sept 16-29 Sept 17):

- Genetics Branch, NCI, NIH, Bethesda, Maryland, USA
- LRBGE Branch, NCI, NIH, Bethesda, Maryland, USA
- Workshop on “Genome Architecture”, Trieste, Italy
- Catholic University, Washington DC
- Keystone Symposium “Epigenetics and Disease”, Seattle, WA
- Laboratory of Genitourinary Cancer Pathogenesis, NCI, Bethesda, MD
- University of Maryland Cancer Center, Baltimore, MD
- Hebrew University, Jerusalem, Israel
- Paul Allen Institute, Seattle, WA
- Laboratory of Molecular Biology, NCI, Bethesda, MD
- Mayburry Lecture, Northwestern University, Chicago, IL
- University of Calgary, Calgary, Canada
- LXXXII Cold Spring Harbor Symposium on Quantitative Biology, Cold Spring Harbor, NY
- Gordon Conference on “Genome Architecture and Cell Fate”, Hong Kong
- Rockefeller University, Anderson Cancer Symposium, New York, NY

Year 3 (30 Sept 17-29 Sept 18):

- The Stowers Institute, Kansas City, MI
- UC Santa Cruz, Santa Cruz, CA
- NCI-Institute Curie workshop, Paris, France
- Mendel Lecture, Brno, Slovakia

NCE year (30 Sept 18-29 Sept 19):

- LRBGE Branch, NCI, NIH, Bethesda, Maryland, USA
- Hood College Symposium, Frederick Maryland, USA
- University of Michigan, Ann Arbor, Michigan, USA
- Georgetown University, Medical School, Washington DC, USA
- MD Anderson Cancer Center, Houston, Texas, USA
- City of Hope Cancer Center, Duarte, California, USA

• **Website(s) or other Internet site(s)**

Nothing to report

• **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

- **Personnel receiving pay from the research effort**

Dr Karen Meaburn (Research scientist)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

1) PI

Name:	Tom Misteli
Project Role:	PI
Researcher Identifier (e.g., ORCID ID):	orcid.org/0000-0003-3530-3020
Nearest person month worked:	1
Contribution to Project:	Dr. Misteli supervised this project.
Funding Support:	National Institutes of Health Intramural Research Program funding.

2) Key personnel

Name:	Karen Meaburn
Project Role:	Research Scientist
Researcher Identifier (e.g., ORCID ID):	orcid.org/0000-0002-1327-5957
Scopus Author ID:	15758132700
Nearest person month worked:	12
Contribution to Project:	Dr. Meaburn performed all the experimental work and analysis presented.
Funding Support:	This grant (W81XWH-15-1-0322).

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

<u>Collaborator:</u>	Dr. Lawrence True.
<u>Organization Name:</u>	University of Washington
<u>Location of Organization:</u>	Seattle, WA
<u>Partner's contribution to the project (identify one or more)</u>	

Collaboration.

Dr. True provided some prostate tissues for the work, plus provides expertise on prostate cancer and prostate tissue pathology.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

Appendix 1:

References cited in this report

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Appendix 2:

- Meaburn, K. J. (2016). Spatial genome organization and its emerging role as a potential diagnosis tool. Front. Genet. 7, 134. doi: 10.3389/fgene.2016.00134

Appendix 3:

- Adriaens C, Serebryanny LA, Feric M, Schibler A, Meaburn KJ, Kubben N, Trzaskoma P, Shachar S, Vidak S, Finn EH, Sood V, Pegoraro G, Misteli T (2018) Blank spots on the map: some current questions on nuclear organization and genome architecture. Histochem Cell Biol. 150(6):579-592. doi: 10.1007/s00418-018-1726-1

Appendix 4:

- Meaburn KJ and Misteli T (2019) Assessment of the Utility of Gene Positioning Biomarkers in the Stratification of Prostate Cancers. Front. Genet. 10:1029. doi: 10.3389/fgene.2019.01029

Appendix 5:

- Supplemental data for: Meaburn KJ and Misteli T (2019) Assessment of the Utility of Gene Positioning Biomarkers in the Stratification of Prostate Cancers. Front. Genet. 10:1029. doi: 10.3389/fgene.2019.01029



Spatial Genome Organization and Its Emerging Role as a Potential Diagnosis Tool

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In eukaryotic cells the genome is highly spatially organized. Functional relevance of higher order genome organization is implied by the fact that specific genes, and even whole chromosomes, alter spatial position in concert with functional changes within the nucleus, for example with modifications to chromatin or transcription. The exact molecular pathways that regulate spatial genome organization and the full implication to the cell of such an organization remain to be determined. However, there is a growing realization that the spatial organization of the genome can be used as a marker of disease. While global genome organization patterns remain largely conserved in disease, some genes and chromosomes occupy distinct nuclear positions in diseased cells compared to their normal counterparts, with the patterns of reorganization differing between diseases. Importantly, mapping the spatial positioning patterns of specific genomic loci can distinguish cancerous tissue from benign with high accuracy. Genome positioning is an attractive novel biomarker since additional quantitative biomarkers are urgently required in many cancer types. Current diagnostic techniques are often subjective and generally lack the ability to identify aggressive cancer from indolent, which can lead to over- or under-treatment of patients. Proof-of-principle for the use of genome positioning as a diagnostic tool has been provided based on small scale retrospective studies. Future large-scale studies are required to assess the feasibility of bringing spatial genome organization-based diagnostics to the clinical setting and to determine if the positioning patterns of specific loci can be useful biomarkers for cancer prognosis. Since spatial reorganization of the genome has been identified in multiple human diseases, it is likely that spatial genome positioning patterns as a diagnostic biomarker may be applied to many diseases.

Keywords: genome organization, nuclear architecture, spatial positioning, gene positioning, disease, cancer, diagnosis

Abbreviations: FISH, fluorescence *in situ* hybridization; HGPS, Hutchinson-Gilford progeria syndrome; ICE, immunodeficiency, centromere instability and facial anomalies syndrome; NE, nuclear envelope; NPC, nuclear pore complex; PSA, prostate-specific antigen; TAD, topologically associating domain.

A SHORTAGE OF QUANTITATIVE CANCER BIOMARKERS

Cancer is a major public health issue. In 2012 alone, across the globe approximately 14.1 million people were diagnosed with cancer and 8.2 million people lost their lives to it (Torre et al., 2015). In developed countries a third to a half of the population will be diagnosed with cancer during their lifetime (Ahmad et al., 2015; Siegel et al., 2016). Not only is cancer a leading cause of morbidity, it is also a leading cause of mortality. In fact, only heart disease accounts for more deaths than cancer; although cancer is the most frequent cause of death for 40–79 year olds (Siegel et al., 2016). Cancer is a heterogeneous collection of diseases. The most prevalent cancers are lung, breast, prostate and colorectal carcinomas, which, when combined account for ~43% of all new cancer diagnoses (Torre et al., 2015; Siegel et al., 2016). These four cancers are also leading causes of cancer-related mortality, contributing ~40% of the total (Torre et al., 2015; Siegel et al., 2016). Even cancers derived from the same organ can be quite heterogeneous from each other, and are further stratified based on morphological patterns, clinical features and/or molecular characteristics. This sub-grouping is important clinically because the appropriate treatment and the risk of recurrence or lethality differ between subgroups. For example, HER2 (also known as ERBB2) positive breast cancers have a poorer prognostic outlook than luminal breast cancers (Sorlie et al., 2001; Ferte et al., 2010), and many HER2-positive breast cancers respond well to trastuzumab (Herceptin) treatment, while endocrine therapy is an effective treatment option for the majority of luminal breast cancers (Ferte et al., 2010; Schnitt, 2010).

A key to the successful treatment of any disease is accurate diagnosis, preferably at an early stage in disease progression when treatments are generally more effective and less aggressive. However, early detection of cancer, particularly by population-based cancer screenings such as mammography or serum PSA levels, often comes at the cost of overdiagnosis (Welch and Black, 2010; Bleyer and Welch, 2012; Sandhu and Andriole, 2012). Overdiagnosis is the detection of indolent cancers that will remain asymptomatic during the patient's lifetime and, as such, do not require treatment. Most commonly, cancer diagnosis is made following histological examination of biopsy or surgical tissue specimens. Unfortunately, in most instances there is a limit to the prognostic information that can be gleaned from histological analysis, and the pathologist is unable to distinguish indolent from aggressive cancers with a high degree of certainty. Presently, due to a lack of additional markers to identify aggressive cancer, many overdiagnosed patients are being overtreated since they are receiving unnecessary therapies. Not only are these patients receiving no therapeutic benefit, as their cancer would never have significantly progressed, they are actually being harmed by the cancer treatment itself, because treatments can have both short-term and long-term effects that cause illness, reduce quality of life, create large financial burdens (for example by loss of income), and can even lead to death (Cooperberg et al., 2010; Welch and Black, 2010; Bleyer and Welch, 2012; Sandhu and Andriole, 2012). It has become a major focus of research to find markers that can distinguish between

indolent and aggressive cancers, to aid the determination of the best possible treatment plan for the patient.

TARGETING THE NUCLEUS IN THE SEARCH FOR CLINICALLY RELEVANT BIOMARKERS

Molecular markers, by their very nature, tend to be highly quantifiable, and are a good potential source of diagnostic and prognostic cancer biomarkers (Ferte et al., 2010). However, few molecular markers are currently in routine clinical practice (Ferte et al., 2010). An exciting area for exploration to identify novel biomarkers is cancer-related changes in the architecture of the cell nucleus (Veltri and Christudass, 2014). This is not without precedence and changes in nuclear structure, such as nuclear size, shape, prominence of nucleoli and chromatin texture, have long been used by pathologists as part of their criteria to identify cancerous cells (Zink et al., 2004b; Veltri and Christudass, 2014). Moreover, identifying chromosomal translocations is important for diagnosing hematological cancers, as specific translocations characterize the type of leukemia or lymphoma, are associated with distinct clinical features and are often indicative of the appropriate treatment (Mitelman et al., 2007). For example, chronic myeloid leukemia is diagnosed after the cytogenetic detection of *t*(9;22)(q34;q11), the Philadelphia chromosome, which results in a *BCR-ABL* fusion protein. Tyrosine kinase inhibitors (Imatinib/Gleevec) inhibit the activity of the resultant oncogenic fusion protein and use of this targeted therapy results in exceptionally high rates of remission for chronic myeloid leukemia patients (Hehlmann et al., 2007). FISH, a technique used to visualize selected sequences of DNA within interphase nuclei (Figures 1A,B) or on mitotic chromosomes, is one method used in clinical practice to detect the presence of specific translocations (Muhlmann, 2002; Zink et al., 2004b; Hehlmann et al., 2007). FISH is also used clinically to detect other chromosomal aberrations in solid and hematological cancers, such as amplifications of the *ERBB2* locus in breast cancer, to aid diagnosis or as a prognostic marker (Muhlmann, 2002; Lambros et al., 2007; Hastings, 2010). Changes in gene expression profiles can also be useful diagnostically. For instance, there are several commercially available assays with prognostic value for various sub-types of breast cancer based on the gene expression profiles of between 2 and 97 genes (Dai et al., 2015).

Beyond gene expression changes and genomic aberrations, there are multiple other aspects of nuclear structure and function that are deregulated in cancer and could be exploited clinically. For example, alteration in nuclear shape, size, chromatin texture, global levels and patterns of heterochromatin and/or histone modifications during carcinogenesis have been shown to be predictive of cancer progression (Zink et al., 2004b; Nielsen et al., 2008; Veltri and Christudass, 2014; Hveem et al., 2016). Aberrant expression of A- and/or B- type lamins is a common feature of many types of cancer, including lung, breast, prostate, colorectal, skin, and gut carcinomas (Broers et al., 1993; Moss et al., 1999; Venables et al., 2001; Willis et al., 2008; Belt et al., 2011; Kong et al., 2012; Wazir et al., 2013; Broers and Ramaekers,

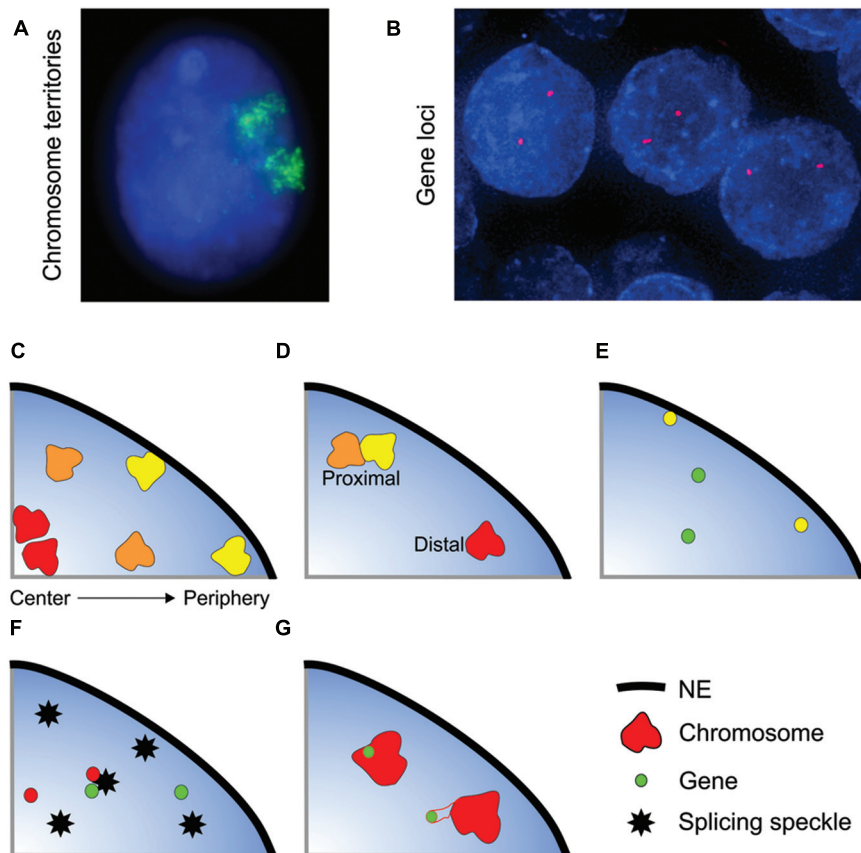


FIGURE 1 | Non-random organization of the genome. (A,B) Whole chromosomes and genes can be visualized within interphase nuclei using FISH.

(A) Chromosome 11 (green) in an interphase nucleus (blue) of the breast epithelial cell line MCF10A. **(B)** *SPDEF* (red) gene loci in normal prostate tissue (formalin-fixed paraffin embedded tissue) nuclei (blue). The spatial organization of the genome is characterized in several ways: **(C)** Radial positioning describes the position of a locus relative to the center and periphery of the nucleus. Gene-poor chromosomes (yellow) generally locate to the nuclear periphery, whilst gene-rich chromosomes have a preference for the nuclear interior. **(D–G)** Relative positioning describes the position of a locus relative to another nuclear landmark. Association frequencies can either be measured on a per cell basis (i.e., the number of nuclei in a population with at least one copy of a gene or chromosome in close proximity to the given landmark) or on a per allele basis. Examples of types of relative positioning include: **(D)** The position of a locus relative to another locus. The yellow and orange chromosomes are in close spatial proximity, whereas the orange and red chromosomes are distal. **(E)** Association with the nuclear envelope (NE). Some genes preferentially co-localize with the NE (yellow gene). **(F)** Association with a nuclear body such as a splicing speckle. **(G)** The position of gene relative to its own chromosome territory. Some gene rich loci loop out of the bulk of the chromosome territory when highly expressed.

2014; Saarinen et al., 2015). The majority of lamin proteins form the nuclear lamina, which underlies the NE, and an additional pool of intranuclear lamins exists (Dittmer and Misteli, 2011). Although highly variable between individual cancers and cancer sub-types, a broad generalization suggests cancers with lower expression levels of A-type lamins tend to have poorer outcomes and more aggressive phenotypes (Belt et al., 2011; Wazir et al., 2013; Saarinen et al., 2015). This is in line with findings that cells with a deficiency in A-type lamins can migrate more easily through narrow constrictions (Davidson et al., 2014), which may aid metastatic progression, and that reduced lamin A has been linked to a higher cellular proliferation rate (Venables et al., 2001). However, the associations between reduced lamin expression and prognosis tend to be weak or based on a low number of patients, and there are conflicting studies that find higher levels of A-type lamin expression to be predictive of a poor outcome (Willis et al., 2008; Kong et al., 2012). Since difference in lamin expression can

vary between sub-groups of cancers, for example between small cell lung cancer and non-small cell lung cancer (Broers et al., 1993), future studies with more defined cohorts of patients may prove enlightening. It is not only changes in expression, mislocalization of lamins to the cytoplasm (Broers et al., 1993; Moss et al., 1999), or the nucleolus (Venables et al., 2001) have also been observed in some cancer specimens. The reduction and/or loss of lamin isoforms may be an early event in carcinogenesis as, at least in cervical cancer, it can occur before neoplasia is detectable (Capo-Chichi et al., 2016). Other nuclear bodies, including the nucleolus and PML bodies, are also disrupted in cancer, with the specific aberration detected dependent on the type of cancer (Zink et al., 2004b).

While loss of genomic integrity, such as mutations, amplifications, deletions and translocations, are hallmarks of cancer and cancer progression (Mitelman et al., 2007; Hanahan and Weinberg, 2011), an additional feature of the

genome, the spatial positioning of individual genetic loci within the interphase nucleus, may also have the potential to become a prominent cancer biomarker.

NON-RANDOM SPATIAL ORGANIZATION OF THE GENOME IN INTERPHASE NUCLEI

The potential of the spatial organization of the genome to be used as a biomarker for cancer diagnosis has been suggested by the fact that specific genes reproducibly occupy alternative nuclear positions within cancerous tissues compared to normal, yet are positioned in very similar patterns between individuals in non-diseased cells (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. et al., 2016; Meaburn K. J. et al., 2016). Before discussing the spatial reorganization of the genome in disease states, this review will first focus on what is known about how the genome is spatially organized within interphase nuclei and its role in correct nuclear function.

The genome is highly spatially organized within the nucleus, with, for the most part, each gene and chromosome occupying preferred nuclear positions (Cremer and Cremer, 2001; Ferrai et al., 2010; Dekker and Misteli, 2015; Meaburn K. et al., 2016). Although the spatial organization of the genome is non-random it does not mean that each allele in each cell within a population has an identical positioning pattern. In fact, spatial genome organization is probabilistic in nature with considerable cell-to-cell variability, and while in the majority of cells a certain position (e.g., internal, proximal to a nuclear speckle, etc.) will be favored, within the population of cells a specific genomic region can be found in any position within the cell (Parada et al., 2003; Bolzer et al., 2005; Goetze et al., 2007; Berger et al., 2008; Tjong et al., 2012; Dekker and Misteli, 2015; Kind et al., 2015). Moreover, the two alleles within the same cell can adopt different positions (Figures 1E–G), for example the clustering of chromosomes 12–14–15 in mouse spleenocytes most often only involves 1 copy of each chromosome per cell, with the second copy of each of these chromosomes not clustering together (Parada et al., 2002).

The spatial positioning of genomic loci can be characterized in several ways, the most common being radial positioning and relative positioning (Figure 1). Radial positioning describes the position of a locus along a central axis between the nuclear periphery and the center of a nucleus (Figure 1C). For instance, in many cell types human chromosome 18 preferentially locates to the nuclear periphery, and, conversely, chromosome 19 is positioned centrally (Croft et al., 1999; Boyle et al., 2001; Cremer et al., 2003). On the other hand, relative positioning measures the position of a gene or chromosome relative to another nuclear landmark, such as to another gene or chromosome, association with the NE or with a nuclear body, including a transcription factory or a splicing speckle, or the position of a gene with respect to the rest of the chromosome it is located on (Figures 1D–G). Relative positioning can measure either the frequency of close spatial proximity (either on per cell or per allele basis) or the distance between the two objects. As an example of relative positioning, in human intermediate

erythroblasts cells the α -globin gene locus frequently loops out of its chromosome territory (Figure 1G), and is often in close spatial proximity to the β -globin gene at splicing factor-enriched nuclear speckles (Figure 1F) (Brown et al., 2006). Traditionally radial and proximal positioning is measured after FISH. More recently, biochemical methods have been developed to map the proximity of a locus to either other genomic regions (a range of chromatin conformation capture techniques, e.g., 3C, 4C, 5C, GCC, HiC) or to nuclear structures (e.g., DamID, ChIA-PET) (Rodley et al., 2009; van Steensel and Dekker, 2010; Bickmore and van Steensel, 2013; Dekker and Misteli, 2015). These traditional and new methods are highly complementary, and it is their combined use that will drive forward our understanding of genome organization. Unlike FISH, the biochemical methods are population-based and the heterogeneity inherent in positioning, either between individual cells or between the two alleles of a gene within a single cell, is not captured. Conversely, as opposed to many of the biochemical methods, FISH is relatively low-throughput and candidate genes are required for analysis. Thus, there exist multiple complementary methods for examining nuclear organization. It should be noted that due to current cost and complexity, sequencing-based methodologies may be less suitable at present for clinical use in countries with developing economies. DNA FISH-based microscopic methods are a potentially universal and flexible technology with relatively straightforward analysis protocols that can likely be performed in many more countries.

CORRELATIONS BETWEEN SPATIAL GENOME ORGANIZATION AND TRANSCRIPTION

The exact molecular mechanisms that regulate the spatial organization of the genome are currently not well elucidated. Moreover, it is not clear if the spatial organization of the genome is important to regulate proper nuclear function or if it is simply a byproduct of nuclear processes. Part of the difficulty in resolving this question is that it is virtually impossible to completely separate the various nuclear functions from each other and, therefore, determine cause and effect. It is also likely that different genes are regulated by different mechanisms; for every rule suggested to govern spatial genome organization there are multiple examples of exceptions. As such, there are multiple layers of nuclear function that ultimately may influence gene positioning in normal and diseased cells. There are several lines of evidence that suggest functional importance to spatial genome organization. The first hint comes from the fact that gene-rich regions of the genome are spatially separated from gene-poor regions (Boyle et al., 2001; Boutanaev et al., 2005; Clemson et al., 2006; Shopland et al., 2006; Simonis et al., 2006; Lieberman-Aiden et al., 2009; Boyle et al., 2011; Pichugina et al., 2016). For instance, gene-rich chromosomes are generally internally located whereas gene-poor chromosomes are enriched at the nuclear periphery (Boyle et al., 2001) and within individual chromosomes gene-poor and gene-rich chromatin are spatially separate, even at the Mb scale (Boutanaev et al., 2005; Shopland

et al., 2006; Lieberman-Aiden et al., 2009). This suggests that the spatial organization of the genome is functionally important for gene expression, or is at least dictated by gene expression. This hypothesis is supported by the observation of spatial clustering of some co-regulated genes, e.g., tRNA genes (Thompson et al., 2003), KLF1-regulated genes (Schoenfelder et al., 2010), STAT-regulated genes (Hakim et al., 2013) or olfactory receptor genes (Clowney et al., 2012). Further, genomic regions that are gene-poor and/or contain mostly inactive genes tend to cluster together, for example at the nuclear lamina, around the edge of the nucleolus or with peri-centromeric heterochromatin (Pickersgill et al., 2006; Guelen et al., 2008; Nemeth et al., 2010; Peric-Hupkes et al., 2010; van Koningsbruggen et al., 2010; Kind et al., 2013; Wijchers et al., 2015). Moreover, the spatial organization of the genome is different in cells with differing gene expression profiles, for example the genome reorganizes during differentiation and is different between different cell- and tissue-types (Kosak et al., 2002; Parada et al., 2004; Solovei et al., 2009; Peric-Hupkes et al., 2010; Foster et al., 2012; Hakim et al., 2013; Rao et al., 2014; Meaburn K. J. et al., 2016). While specific loci often change nuclear positions, there is not a global spatial rearrangement of the genome between cell types. Positioning patterns are remarkably conserved between cell types and stages of differentiation, with only specific genomic regions in alternative positions. For instance, in human fibroblasts and lymphocytes most chromosomes are in similar radial positions and only the position of chromosomes 8, 20, and 21 differ (Boyle et al., 2001; Meaburn et al., 2008). Similarly, of the genomic regions that associated with the nuclear lamina in mouse embryonic stem cells and astrocytes, approximately 20% are lamina-associated in only one of the two cell types (Peric-Hupkes et al., 2010).

Most saliently, genes can relocate to different nuclear locations with changes in their transcriptional output (Fraser and Bickmore, 2007; Ferrai et al., 2010). For example, active *Gfap* and *Irf4* alleles are generally more internally located than their silent counterparts (Takizawa et al., 2008a) and *CFTR*, *Mash1* and *IgH* display lower association with the nuclear periphery upon activation (Kosak et al., 2002; Zink et al., 2004; Williams et al., 2006). Conversely, genes, such as *INO1*, *HXX1* and *GAL1* move to the NPC, at the periphery of the nucleus, upon activation (Brickner and Walter, 2004; Taddei et al., 2006; Brickner et al., 2007; Berger et al., 2008) and the “gene territories” occupied by loci such as *GAL1* increase when activated (Berger et al., 2008). It is not only changes in radial position that are detected to correlate with gene expression, for example, some highly expressed gene-rich regions of the genome, including the *major histocompatibility complex*, *epidermal differentiation complex* and *HoxB* loci loop out of the bulk of their chromosome territory when active (Volpi et al., 2000; Williams et al., 2002; Chambeyron and Bickmore, 2004) and changes to the cell-cycle phase specific clustering of genes sets in yeast correlated well with changes in gene expression, particularly for genes that down regulate during the transition of cell cycle phase (Grand et al., 2014). Not all changes in nuclear location coincide with the exact time of changes in expression. Some loci reposition before changes in gene expression, adopting poised positions in the days leading up

to developmentally regulated activation of expression (Ragoczy et al., 2003; Chambeyron and Bickmore, 2004; Peric-Hupkes et al., 2010), suggesting, at least for some regions, changes in nuclear positions are not simply a consequence of the activation of gene expression. On the other hand, some genes move only after changes in expression, for example, during erythroid differentiation the mouse β -globin gene is activated prior to relocation away from the nuclear periphery (Ragoczy et al., 2006). In this case the movement of the locus might help modify the magnitude of transcription since expression is further increased with the repositioning (Ragoczy et al., 2006). It is not only upon activation genes alter nuclear location. Relocation to blocks of heterochromatin is associated with gene repression for some genes (Brown et al., 1997, 1999; Francastel et al., 1999; Wijchers et al., 2015); however, this is not a general rule as other silenced gene loci do not associate with blocks of heterochromatin (Moen et al., 2004; Takizawa et al., 2008a).

LACK OF CORRELATIONS BETWEEN SPATIAL GENOME ORGANIZATION AND TRANSCRIPTION

In addition to the discrepancy amongst genes on the timing of spatial repositioning in relation to altered transcription, there are a large number of studies demonstrating that for many genes changes in nuclear position and gene expression are uncoupled (Scheuermann et al., 2004; Williams et al., 2006; Kumaran and Spector, 2008; Meaburn and Misteli, 2008; Takizawa et al., 2008b; Morey et al., 2009; Harewood et al., 2010; Hakim et al., 2011; Shachar et al., 2015). For instance, when a breast epithelial cell line, MCF10A, was grown under four different growth conditions there was no correlation between change in gene expression and change radial positioning pattern for any of the eleven genes analyzed (Meaburn and Misteli, 2008). There could be several reasons to explain this. Firstly, radial positioning is not an ideal measure to assess function (Takizawa et al., 2008b). With the exception of the NE, all radial locations within the nucleus are approximately equal with respect to function and gene expression. Transcription factories and active genes are equally distributed throughout the nucleus (Wansink et al., 1993; Takizawa et al., 2008a; Paz et al., 2015). Thus, changes in radial position do not necessary represent biologically relevant changes, as they do not correlate with moving a locus into a more or less transcriptionally active environment. Moreover, radial positioning can miss biologically relevant changes to positioning if it does not shift the locus to a different radial zone. Proximity to and association with nuclear bodies is likely to be far more important for gene expression and RNA processing than radial positioning. Indeed, for many genes changes in gene expression accompany changes in association with nuclear sub-compartments, including the lamina, nucleolus, transcription factories, heterochromatin, polycomb bodies, NPCs, Cajal bodies and nuclear speckles (Ahmed et al., 2010; Ferrai et al., 2010; Bickmore and van Steensel, 2013; Khanna et al., 2014; Dekker and Misteli, 2015; Wang et al., 2016). Furthermore, transient co-localization of X chromosomes during the establishment

of X inactivation and co-localization of imprinted genes with imprinting control region loci suggest a critical role for spatial genome organization in establishing and maintaining mono-allelic expression in these regions (Fraser and Bickmore, 2007; MacDonald et al., 2016). However, radial positioning vs. relative positioning cannot be the full story in explaining the differences between the studies because some genes spatially reposition in the absence of a change in expression. For example, *Mash1* relocates to a more internal nuclear position upon activation during neuronal differentiation, however, the repositioning is not specific to *Mash1* and several chromosomally neighboring genes also reposition despite remaining transcriptionally silent (Williams et al., 2006). This is not limited to transcriptionally silent genes; active genes can also change nuclear positions without modulating transcription. As an example, all four genes (*AKT1*, *BCL2*, *ERBB2* and *VEGF*) identified to change radial position after *in vitro* induction of early breast cancer were expressed at similar levels in the normal as compared to tumor cells (Meaburn and Misteli, 2008).

Why might these differences in nuclear positioning exist between different genes? It could be that some genes are more sensitive to their nuclear position for expression than others, and the genes that require specific positioning patterns for their correct expression drive the relocation of neighboring regions by “pulling” them along. Consistent with this, when loci are forced into a different nuclear environment, either by being artificially tethered or due to a chromosomal translocation, the expression of some but not all genes is affected (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008; Harewood et al., 2010; Wijchers et al., 2015). Additionally, the type of expression change may be an important factor. Most of the genes that have been identified to alter nuclear location in parallel with changes in gene expression are genes that are being activated or silenced during development and differentiation, i.e., are genes associated with a more or less permanent change in expression, and although these expression changes occur in a coordinated manner, it does not need to be rapid. Thus, it could be that a specific nuclear environment is required for this activation/repression, and time can be taken for a locus to be relocated to this environment, but once a gene is permanently induced or silenced the environment can change without affecting the expression of that region. In support, transient homologous pairing of *Oct4* alleles occurs during mouse ESC differentiation only as *Oct4* becomes permanently repressed (Hogan et al., 2015). It is also likely that ongoing transcription is not required to maintain spatial positioning patterns, since long-range DNA interactions of active β -globin and *Rad23a* genes are maintained after the inhibition of RNA polymerase II transcription (Palstra et al., 2008). Precise spatial positioning, especially radial positioning, may not have a significant role for all genes beyond switching on/off. For genes that turn on and off rapidly in response to changing environmental signals, for example upon stimulation by a hormone or stress signals, rapid induction may be enhanced by proximal spatial positioning of sets of co-regulated genes prior to the stimulation, i.e., in a permanently poised position. In keeping with this, glucocorticoid receptor responsive genes cluster in the absence of glucocorticoid, and (re)stimulation

with glucocorticoid does not significantly change positioning patterns (Hakim et al., 2011). Direct evidence for the concept of a more rapid activation of expression for genes already in the correct nuclear environment comes from yeast studies. The NPC associates with active regions of the genome, with specific genes recruited to the NPC upon activation (Egecioglu and Brickner, 2011; Liang and Hetzer, 2011). Even after the stimulation has been removed and the expression from these genes has been repressed several genes, including *HXX1*, *GAL1* and *INO1*, remain at the nuclear periphery and these peripheral loci are activated more quickly upon future re-stimulation (Brickner et al., 2007; Tan-Wong et al., 2009).

ADDITIONAL PLAYERS AND FUNCTIONS

Additionally, remodeling of chromatin is intimately linked to gene expression. Thus, it is possible that chromatin remodelers and the resulting alterations to histones and chromatin compaction are an important part of the molecular machinery that reorganizes the genome, rather than gene transcription itself. Emerging evidence suggests this may well be the case. Decondensation of chromatin in the absence of altered gene expression, even for silent genes, is sufficient for a locus to spatially reposition (Chambeyron and Bickmore, 2004; Therizols et al., 2014). Similarly, modifications to histones are important for anchoring loci to the nuclear periphery, independently of a role in gene repression these modifications are associated with (Harr et al., 2016). For example, histone acetyltransferase SAGA (Dultz et al., 2016), histone deacetylases (Zullo et al., 2012; Demmerle et al., 2013; Dultz et al., 2016) and histone H3K9 di- and tri-methylation by histone methyltransferase (Towbin et al., 2012; Bian et al., 2013; Kind et al., 2013; Harr et al., 2015) are required to localize at least some genes to the nuclear periphery. It is most likely that chromatin remodelers work in tandem and that it is specific combinations of histone marks that are required to target a genomic region to the nuclear periphery (Harr et al., 2016). It is also likely that chromatin remodelers play a role in targeting genes within the nucleoplasm, away from the NE, since chromatin remodelers EZH2 and SUV39H1 have been demonstrated to be critical for local chromatin clustering into sub-domains (Wijchers et al., 2016) and knocking down a range of chromatin remodelers and histone modifiers affected the position of several non-NE associated genes (Shachar et al., 2015).

In addition to gene expression and chromatin state, many other nuclear factors have been linked to a role in correct spatial organization of the genome, these include actin, myosin (Chuang et al., 2006; Dundr et al., 2007; Mehta et al., 2010), NE proteins, include A-type lamins (Meaburn et al., 2007; Mewborn et al., 2010; Mehta et al., 2011; Kubben et al., 2012; Solovei et al., 2013; Harr et al., 2015), B-type lamins (Malhas et al., 2007; Ranade et al., 2016), lamin B receptor (Clowney et al., 2012; Solovei et al., 2013), emerin (Demmerle et al., 2013) and several other NE transmembrane proteins (Zuleger et al., 2013), NPC proteins (Ahmed et al., 2010; Dultz et al., 2016) and non-coding RNAs (Hacisuleyman et al., 2014).

Interestingly, different genes can be targeted to the same nuclear compartment via different protein complexes and pathways, and the use of the different mechanisms may add an additional layer of control over the speed of inducing or enhancing gene expression (Randise-Hinchliff et al., 2016). Moreover, different genes require different proteins complexes for their correct nuclear location. SiRNA screening of 669 nuclear proteins, combined with mapping the radial position of 3–4 genes by FISH, identified 50 proteins with roles in spatially organizing the genome. However, of these proteins, only knocking down one, *CACNG1*, repositioned all four genes studied. Moreover, half of the hits affected the position of only a single gene (Shachar et al., 2015).

At some level underlying DNA sequence must play a role in the spatial positioning of the genome. Transcription factors bind to specific sequences in the genome, thereby, activating a specific set of genes, and genes regulated by specific transcription factors can spatially cluster (Schoenfelder et al., 2010; Hakim et al., 2011; Hakim et al., 2013). Some specific DNA sequences have been demonstrated to have a direct role in targeting genes to specific locations within the nucleus. In *Saccharomyces cerevisiae*, two short gene recruitment sequences, within gene promoters, are required to target inducible genes to the NPC, allowing an enhanced transcriptional output (Ahmed et al., 2010). It has recently been proposed that the association between repetitive sequences, in particular homotypic associations between short interspersed retrotransposable elements (SINEs), which are enriched in gene-rich regions of the genome, or long interspersed retrotransposable elements (LINEs), which are enriched in gene-poor regions, may be a key underlying factor responsible for the spatial separation of gene-rich and gene-poor regions of the genome (Cournac et al., 2016; Solovei et al., 2016). However, spatial positioning patterns cannot be an intrinsic feature of a piece of DNA, since, as discussed above, genes and chromosomes can alter position without changes in DNA sequence, e.g., between cell types or upon activation. Additionally, some human chromosomes, including chromosome X, adopt different nuclear positions when they are in a mouse nucleus compared to when they are within their endogenous human cell (Meaburn et al., 2008). It is also unlikely all repeat sequences direct spatial organization, as pericentromeric satellite repeats migrate to existing heterochromatin compartments during differentiation rather than drive the aggregation of heterochromatin (Wijchers et al., 2015).

Replication has also been linked to the spatial organization of the genome. Genomic regions alter their nuclear location as they change replication timing during development and disruptions to replication can affect the radial position of some genes (Hiratani et al., 2008; Shachar et al., 2015). Similarly, mitosis and NE reformation in early G₁ are important for some loci to establish nuclear location, especially for gene-poor and inactive regions when associating with the NE (Bridger et al., 2000; Kumaran and Spector, 2008; Kind et al., 2013) or during muscle differentiation (Neems et al., 2016). However, once more, these cannot be general mechanisms for all genes. Firstly, several genes and chromosomes reposition as cells exit the cell cycle and become quiescent or senescent, which clearly occurs in the absence of

replication, mitosis and NE reformation (Bridger et al., 2000; Solovei et al., 2004; Meaburn et al., 2007; Meaburn and Misteli, 2008). Secondly, an alternative mechanism must be required for genes that move rapidly after stimulation. For example, relocation to the NPC occurs within 15–60 min of activation in 60% of nuclei (Randise-Hinchliff et al., 2016). This timing is too fast for replication, mitosis or NE reformation to play a major role.

The spatial organization of the genome may also have a role in DNA repair, since whole chromosomes can reposition after DNA damage (Mehta et al., 2013; Ioannou et al., 2015), however, given that in mammalian cells broken DNA ends do not significantly move (Kruhlak et al., 2006; Soutoglou et al., 2007; Roukos et al., 2013), the function of this repositioning is not clear. What is clear, however, is the role the spatial organization plays in the partner choice when oncogenic chromosomal translocations form, after the DNA breaks have occurred. Translocations preferentially form between loci that are in close spatial proximity prior to damage (Lukasova et al., 1997; Neves et al., 1999; Roix et al., 2003; Lin et al., 2009; Mani et al., 2009; Mathas et al., 2009; Chiarle et al., 2011; Klein et al., 2011; Hakim et al., 2012; Roukos et al., 2013). In a similar vein, the spatial organization of the genome also helps dictate the integration sites of HIV-1 virus DNA into the human genome after infection. HIV-1 integrates preferentially into highly transcribing genes that associated with the NPC (Lelek et al., 2015; Marini et al., 2015).

Most studies on the spatial organization of the genome focus on its role for the correct function of the genome. However, there is evidence to suggest the spatial organization of the genome may have additional structural roles that aid cellular function in a non-genetic manner (Bustin and Misteli, 2016). For example, the clustering of condensed chromatin to the nuclear periphery helps the nucleus withstand mechanical forces, while the redistribution of heterochromatin to the center of the nucleus in the rod cells of nocturnal mammals aids night vision by optimizing the diffraction of light through the retina (Solovei et al., 2009; Bustin and Misteli, 2016).

While trends are emerging as to how and why the genome is spatially organized, no “one size fits all model” has yet to clearly present itself. This is not surprising given the complexity of the nucleus. Most likely it will be a combination of molecular strategies working in concert, and differing depending on the gene and the cellular context, that optimizes correct nuclear function and organization. Until recently, due to practical constraints, loci could only realistically be studied in virtual isolation. With the rapidly reducing cost of sequencing and the advent of multiple biochemical methods to analyze genome organization on a larger scale, the tools are now in place to study the spatial organization of chromatin on a genome-wide scale. It will be important to compare these positioning maps to gene expression data, epigenetic maps, proteome data, non-coding RNA, replication timings etc., in combination. Moreover, it will be important to not only ask what is changing at the level of an individual locus, it will be vital to consider the chromosomal neighborhood at large. It will be critical to study candidate regions, identified by population-based biochemical methods, in greater detail using single cell FISH analysis. This will answer questions the biochemical methods can not, such

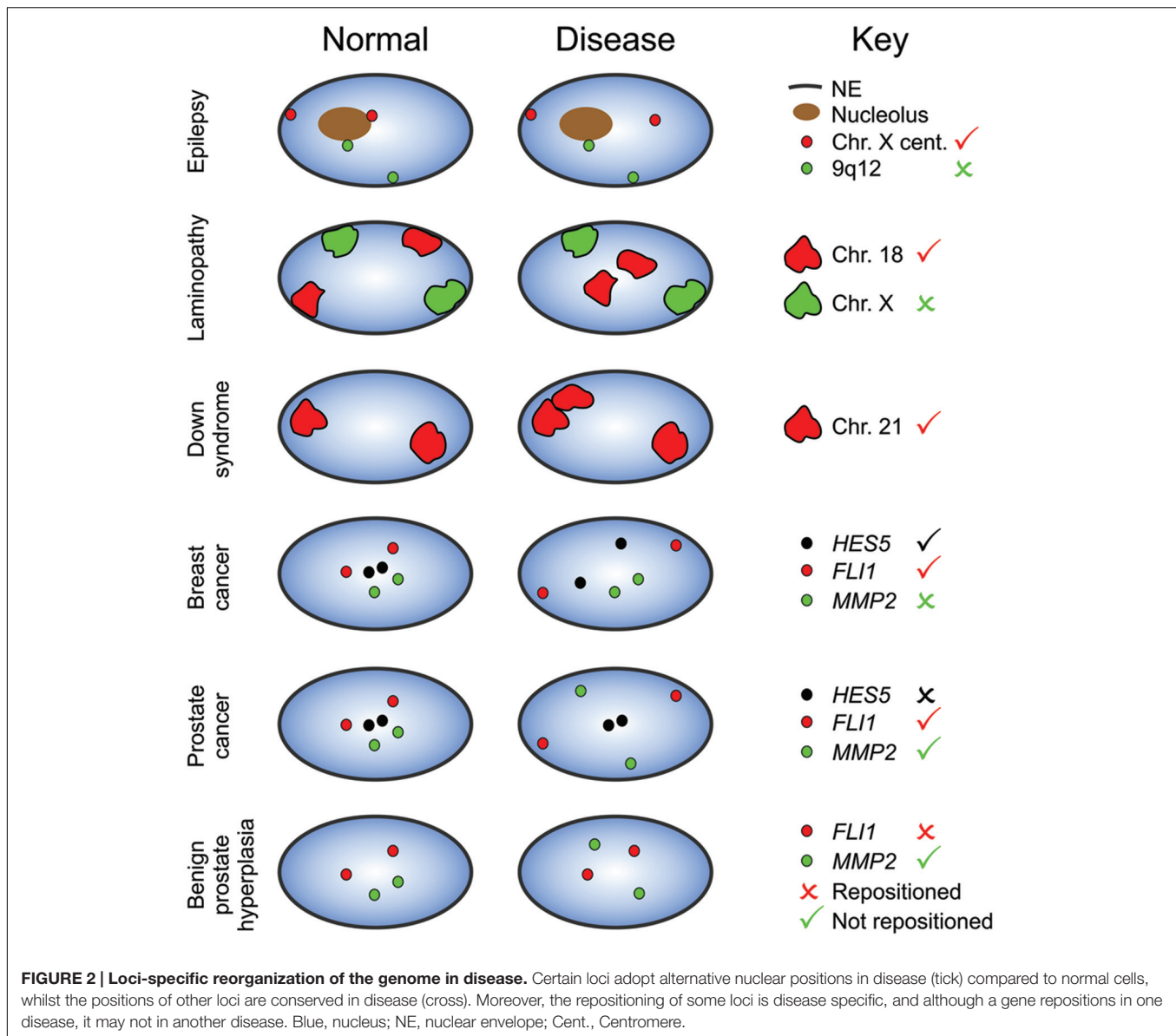
as, in how many cells is a given positioning patterns found? Do both alleles of a gene behave in the same manner? Are positioning patterns found in the population mutually exclusive at the single cell level? From here, loci or nuclear factors can then be manipulated to determine the true functional relevance, beyond correlation.

SPATIAL REORGANIZATION OF THE GENOME IN NON-MALIGNANT DISEASE

Even though many mechanistic and functional aspects of the spatial organization of the genome have yet to be resolved, these data do indicate that discrete and quantifiable changes to spatial positioning patterns exist between cells which, if they occur specifically in disease states could be exploited as biomarkers. In a wide variety of diseases, including cancer, individual genes, genomic regions and/or whole chromosomes are known to reposition (**Figure 2**) (Ferrai et al., 2010; Bridger et al., 2014; Meaburn K. et al., 2016). This phenomenon was first identified in the 1980s in brain tissues of epilepsy patients (Borden and Manuelidis, 1988). The centromere of chromosome X reproducibly redistributed away from the NE or edge of nucleoli in neurons from the epileptic seizure focus as compared to normal neurons from unaffected areas of the brain from the same individuals (**Figure 2**) (Borden and Manuelidis, 1988). This repositioning is not common to other heterochromatin regions, as neither the pericentromeric heterochromatin regions on chromosomes 1 (1q12) and 9 (9q12) nor the large heterochromatin block mapping to Yq12 reposition (**Figure 2**) (Borden and Manuelidis, 1988). Generally speaking, different loci have been positioned in different diseases, with very few loci studied in more than one affliction, making it unclear how general the disease-related repositioning of a given locus is. At least some repositioning events are different between different diseases, however. While 1q12 is not repositioned in epilepsy, in the lymphocytes from patients with endometriosis 1q12 is more peripherally located, compared to control individuals, (Mikelsaar et al., 2014) and more internally located in ICF patient lymphocytes (Jefferson et al., 2010; Dupont et al., 2012). Additional genomic regions were also found to reposition in ICF, including a reduced association of chromosome 16's pericentromeric heterochromatin region with the NE, and an increased incidence of *human pseudoautosomal region 2* genes looping out of their chromosome territories (chromosome Y and the inactive X) (Matarazzo et al., 2007; Jefferson et al., 2010). Similarly to epilepsy, loci-specific repositioning was observed in ICF, with other regions on chromosome 1 (*BTG2*, *CNN3*, *ID3*, and *RGS1*) and the pericentromeric heterochromatin of chromosome 9 not repositioning in ICF (Jefferson et al., 2010; Dupont et al., 2012). Pericentromeric loci are not the only repeat sequences to be spatially affected in disease. Aberrant telomere clustering has been identified in several diseases, including cancer and Alzheimer's, often due to telomere dysfunction, and the degree of clustering can reflect the aggressiveness of the disease (Chuang et al., 2004; Gadji et al., 2010; Mathur et al., 2014).

Loci-specific repositioning also occurs in laminopathies (**Figure 2**) (Meaburn et al., 2007; Taimen et al., 2009; Mewborn et al., 2010; Mattout et al., 2011; Mehta et al., 2011; McCord et al., 2013). Laminopathies are a wide ranging group of rare diseases characterized by mutations in lamin A/C, and includes Emery-Dreifuss muscular dystrophy and the premature aging disease HGPS (Dittmer and Misteli, 2011). The organization of chromosomes in proliferating laminopathy fibroblasts cells mimics that of normal quiescent cells, with chromosomes 13 and 18 shifted to a more internal position, chromosome 10 repositioning towards the nuclear periphery, however, chromosomes 4 and X remaining at the nuclear periphery (**Figure 2**) (Meaburn et al., 2007; Mewborn et al., 2010; Mehta et al., 2011). Moreover, there is evidence to suggest that the spatial reorganization of the genome in laminopathies is not limited to the repositioning of a few specific chromosomes, and may evolve as the cells increase in passage. Compared to proliferating normal fibroblasts, a dramatic genome-wide reduction in the spatial separation of active and inactive chromatin was observed in senescent HGPS fibroblasts (McCord et al., 2013). Some of these differences in interaction frequencies were senescence- rather than disease-related, since they were mostly not detected in proliferating HGPS cultures (McCord et al., 2013) yet were detected in normal senescent fibroblasts (Chandra et al., 2015). Yet, differences between normal senescent and senescent HGPS cells were detected (Chandra et al., 2015). It remains unclear how disease related these differences are, as it could be more a reflection of the type of senescence being compared. The HGPS cells used had reached (early) replicative senescence by continued culture (McCord et al., 2013), whereas senescence was induced in the normal cells by oncogene activation, which, unlike replicative senescence, forms senescence-associated heterochromatin foci (Chandra et al., 2015). The redistribution of the genome in progeria may well be progressive, as is pointed to by the findings that the abnormal clustering of centromeres associated with the *LMNA* E145K mutation only occurs in nuclei with aberrant shapes, and the prevalence increases with increased time in culture (Taimen et al., 2009).

The specific functional relevance of the spatial repositioning of the genome to the various disease phenotypes present in the different laminopathies is unclear, and appears to result more generally from the presence of aberrant A-type lamin rather than the specific disease *per se*. Generally, the spatial reorganization of the genome is very similar between different laminopathy patients, regardless of the disease or mutation present (Meaburn et al., 2007; Mewborn et al., 2010), the exception being that chromosome 13 is more peripheral in patients with $\Delta 303$ or D596N *LMNA* mutations (Mewborn et al., 2010) and the spatial organization of the genome in asymptomatic laminopathy carriers is very similar to the laminopathy patients (Meaburn et al., 2007). The release of chromosome 13 and 18 from the nuclear periphery in laminopathy fibroblasts may be linked to an altered ability of the mutant A-type lamin proteins to bind specific chromatin regions (Meaburn K. et al., 2016) and/or changes in chromatin status, since there is a reduction of heterochromatin-specific



markers, including HP1 α and histone H3K9 methylation, in HGPS fibroblasts (Scaffidi and Misteli, 2005), or it may be due to an uncoupling of cell-cycle regulation and spatial positioning patterns (Meaburn et al., 2007). Interesting, while few differences in positioning patterns have been detected between fibroblast cell lines of different laminopathies, the laminopathy-related spatial positioning patterns are tissue-specific, suggesting they may contribute to the disease phenotype. Chromosomal positioning abnormalities have not been detected in laminopathy patient lymphoblasts, a cell-type that does not require A-type lamins (Boyle et al., 2001; Meaburn et al., 2005). Further, in a *Caenorhabditis elegans* model of Emery-Dreifuss muscular dystrophy, a muscle-specific promoter was retained at the NE in the muscles of diseased animals, however, the release from the NE of a gut-specific promoter was not affected in gut tissue (Mattout et al., 2011).

In addition to the repositioning of gene poor regions of the genome in disease, several genes have been identified to occupy disease-specific positions. In benign prostate hyperplasia tissues *MMP2* shifts to a more peripheral position than normal prostate tissue (Figure 2) (Leshner et al., 2016). Benign prostate hyperplasia is not associated with a general reorganization of the genome, since the positions of *MMP9* and *FLI1* are unaffected (Figure 2) (Leshner et al., 2016). In lymphocytes from Down syndrome patients, two (or three) copies of chromosome 21 clusters together more frequently than in diploid control cells (Figure 2) (Paz et al., 2013, 2015). Moreover, *SOD1*, which is located on chromosome 21, is more peripherally located in Down syndrome (Paz et al., 2015). This altered position is not simply a consequence of the presence of an additional chromosome 21, however, as the radial position of *DYRK1A*, which also maps to chromosome 21, is unaffected (Paz et al.,

2015). It is not only chronic diseases that are associated with a spatial reorganization of the genome. Short-term repositioning of some, but not all, genes or chromosomes occurs after viral or parasitic infection (Li et al., 2010; Knight et al., 2011; Arican-Goktas et al., 2014). Chromosome 17, but not 18, relocates to a more peripheral position in human lymphocytes within 24 h of Epstein-Barr virus infection (Li et al., 2010). Similarly, dynamic radial repositioning of *actin*, *ferritin* and *hsp70*, occurs in both cultured snail cells and in intact live snails within hours of active parasitic infection, with the repositioning correlating to changes in gene expression (Knight et al., 2011; Arican-Goktas et al., 2014).

In most cases the link between gene repositioning in disease and the disease phenotype has not clear. In a few cases a direct link between altered positioning patterns and disease relevant gene expression has been demonstrated. The spatial clustering of *FTO* and *IRX3* is implicated in obesity, by altering the expression of *IRX3* expression and consequently mis-regulating body mass (Smemo et al., 2014). Local disruptions to hierarchical genome organization can also be important for disease progression. High-resolution mapping of the 3D genome has revealed that beyond a spatial separation of gene-rich and gene-poor chromatin, chromosomes further fold into a succession of ten to hundreds of kb long self-associating globular domains, known as TADs (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). Individual TADs are stably conserved between species and cell types and are separated by sharp boundaries that are enriched in histone marks associated with active genes, SINEs, binding sites for the chromatin-binding protein CTCF and housekeeping genes (Dixon et al., 2012). TAD boundaries are believed to be important for gene regulation, by acting as insulators, inhibiting the expansion of either heterochromatin or active chromatin into a neighboring TADs and by increasing the frequency of specific regulatory interactions by confining certain promoters and enhances into a single TAD (Dixon et al., 2012; Sexton et al., 2012; Narendra et al., 2015). Consistent with this, genes within TAD domains are co-expressed during differentiation and disruption of TAD boundaries results in a partial or complete fusion of neighboring TADs leading to altered gene expression and in some cases, disease phenotypes (Nora et al., 2012; Lupianez et al., 2015; Narendra et al., 2015; Hnisz et al., 2016). Disruption to the TAD boundary near the *EPHA4* locus by deletion, duplication or inversion of DNA sequences at the boundary results in limb deformation syndromes (Lupianez et al., 2015). In normal limb development *EPHA4* is active but genes in neighboring TADs are silent. The loss of the TAD boundary allows enhancer-promoter interactions between regulatory sequences normally separated into different TADs, and consequently results in the spuriously activation of the neighboring genes in the mis-fused TAD (Lupianez et al., 2015). Similarly, cancer-associated micro-deletions or mutations at TAD boundaries can activate oncogenes, and may represent a common mechanism of gene mis-regulation in multiple cancers (Hnisz et al., 2016). The study of TADs is still in its infancy, and most likely disruption to TAD boundaries will be detected as functionally important in many other diseases.

SPATIAL GENOME ORGANIZATION IN CANCER

Given the urgent need for additional diagnostic and prognostic tests for cancer, reproducible differences in the spatial positioning patterns of the genome detected between normal/benign tissues and cancer, or between different cancer subtypes could be invaluable clinically. As with non-malignant disease, some genes and chromosomes occupy distinct nuclear positions in cancerous cells compared to their normal or benign counterparts (**Figure 2**) (Cremer et al., 2003; Murata et al., 2007; Meaburn and Misteli, 2008; Meaburn et al., 2009; Wiech et al., 2009; Zeitz et al., 2013; Leshner et al., 2016; Meaburn K. J. et al., 2016). For example, in breast cancer cell lines there is a significant change to the set of genes that are close spatial neighbors of *IGFBP3* (Zeitz et al., 2013). Moreover, ten genes (*FLI1*, *HES5*, *MMP9*, *HSP90AA1*, *TGFB3*, *MYC*, *ERBB2*, *FOSL2*, *CSF1R* and *AKT1*) have been identified to reproducibly radially reposition in epithelial cells of breast cancer tissues (Meaburn et al., 2009; Meaburn K. J. et al., 2016) and *FLI1*, *MMP9* and *MMP2* occupy alternative radial locations in prostate cancer (Leshner et al., 2016). Also in line with non-malignant diseases, and despite the high level of genomic instability associated with many cancers, which could conceivably be expected to result in a large-scale disruption to the spatial organization of the genome, the repositioning is loci-specific, with a general conservation of spatial positioning patterns in cancer, with the majority of genes and chromosomes not altering nuclear position (Kozubek et al., 2002; Parada et al., 2002; Cremer et al., 2003; Meaburn and Misteli, 2008; Meaburn et al., 2009; Timme et al., 2011; Zeitz et al., 2013; Leshner et al., 2016; Meaburn K. J. et al., 2016). For example, 44 of 47 (94%) genes spatially mapped in human prostate tissues do not alter radial position in prostate cancer and the radial positions of 13 out of 23 (57%) genes are conserved in breast cancer (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016).

Unlike laminopathies, the patterns of spatial reorganization differ between different cancers. While the radial positions of chromosomes 18 and 19 are affected in many cancer types, including thyroid, colon and cervical cancer, with a tendency for chromosome 18 to become more internally located and chromosome 19 to shift away from the center of the nucleus (Cremer et al., 2003; Murata et al., 2007; Wiech et al., 2009), in some cervical cancers, chromosome 18 becomes more internally located (Wiech et al., 2009). Moreover, of the eleven genes so far reported with altered radial position in either breast or prostate cancer, only *MMP9* and *FLI1* robustly reposition in both types of cancer (**Figure 2**) (Leshner et al., 2016; Meaburn K. J. et al., 2016). This suggests cancer-type specific repositioning, with different sets of genes repositioning in breast and prostate cancer. Similarly, *BCL2* repositions in a *BCL2*-positive cervical squamous carcinoma, but not in a *BCL2*-negative cervical squamous carcinoma (Wiech et al., 2009), nor in breast (Meaburn et al., 2009) or prostate cancer (Leshner et al., 2016). When taken together with the findings discussed above that a genomic region can behave differently, with respect to repositioning between different non-cancerous diseases, it suggests that the overall organization of the genome in disease is, mostly, specific to

the disease and not a consequence of a more general “stress” response. In keeping with this, of the genes that occupy distinct radial positions in breast and/or prostate cancer, only *MMP2* and *ERBB2* are also commonly repositioned in non-malignant breast or prostate disease; but even for these gene it cannot be a general disease response as *MMP2* repositions in prostate but not breast cancer and visa versa *ERBB2* does not reposition in prostate cancer (**Figure 2**) (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016). Thus, there appear to be an exploitable minority of gene repositioning events that could be used to distinguish cancerous tumors from normal tissue and between different cancer types.

It is interesting to note that many of the factors implicated in influencing gene positioning patterns, as discussed earlier in this review, are often altered in cancer, e.g., lamins, gene expression, proliferation status, chromatin status and histone modifications. However, the mechanisms involved in the mis-organization of the genome in cancer are unclear. It would seem that, for the radially repositioned genes at least, transcription of the locus itself is not the key to the repositioning. No correlation was found for the repositioning of a given gene and its transcriptional output in a model of early cancer (Meaburn and Misteli, 2008). Moreover, cancer-associated genes, that are known to have altered expression in cancer, often did not reposition in breast or prostate cancer (Meaburn and Misteli, 2008; Meaburn et al., 2009; Leshner et al., 2016). Even for the cancer-associated genes that do reposition in cancer, it may not be altered transcription of the loci that is driving the alternative positioning pattern. For example, although elevated expression of *MMP9* and *MMP2* is a marker of poor prognosis in prostate cancer, and linked to metastasis, their expression is from tumor-associated stroma cells and not from the cancerous epithelial cells, where the gene repositioning was detected (Egeblad and Werb, 2002; Leshner et al., 2016). This does not rule out gene expression as a factor in repositioning, and further studies are required that measure gene expression changes in extended chromosomal regions of the repositioned gene. However, it does suggest that clinical diagnostic tests based on spatial gene positioning would be independent of gene expression and would not be a more convoluted way to get to the same result as a gene-expression based diagnostic test, and instead would be an independent biomarker that could be used in combination with existing diagnostic and prognostic strategies.

An increase in proliferation is a hallmark of cancer yet spatial positioning patterns are can also be influenced by proliferation. Again, the spatial reorganization of the genome in cancer seems to be independent of this, and does not seem to simply be a reflection of a transition from quiescent normal breast or prostate tissue to a proliferating tumor. The spatial genome organization patterns in cancer cells do not mimic proliferating (or quiescent) breast epithelial cells (Meaburn and Misteli, 2008). This is not surprising, given the fact that the majority of cells within a cancerous tumor are actually not proliferating. Ki-67 is commonly used as a marker of cellular proliferation, since it is present in all stages of the cell cycle with the exception of G₀. Breast cancers that contain at least 30% Ki-67 positive cells are classified as highly proliferative cancers, and the majority of breast cancers, almost 60%, have only 10% or less of cells positive

for Ki-67 (de Azambuja et al., 2007; Jonat and Arnold, 2011). In fact, no feature of a genomic locus has yet been identified that enables the prediction of whether a gene will reposition or not in cancer (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016), again suggesting spatial positioning patterns will be independent of other biomarkers.

CLINICAL BIOMARKER POTENTIAL

Regardless of why the genome is spatially reorganized in the nuclei of cancer cells, the small scale studies so far performed suggest that these differences in positioning patterns have the potential to be exploited for diagnostic use (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016). Distinguishing cancer from normal tissue by spatial gene positioning patterns could practically be integrated into clinical use because it requires only a small tissue sample for analysis (100–1000s of cells), meaning it will not require additional invasive procedures for the patient. Moreover, FISH is currently already used in diagnostic labs, thus, the infrastructure for such analysis already exists in the clinic, at least in many developed countries. It remains to be seen the practicality of such a diagnostic technology for all developing economies. Importantly for clinical applications, for most genes tested, at least in the small scale studies so far performed, false positive rates were low since for the vast majority of genes spatial positioning patterns are highly similar between individuals in non-diseased tissue (Borden and Manuelidis, 1988; Wiech et al., 2005; Murata et al., 2007; Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016) and repositioning events did not generally occur in non-malignant breast and prostate disease (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016). There were a few notable exceptions, *MMP2* and *ERBB2* repositions in both cancer and non-malignant disease (**Figure 2**), and the radial position of a few genes, such as *CCND1* and *TIMP3*, were highly variable between normal prostate tissues. This highlights that each candidate marker gene must be rigorously tested to eliminate genes with high false positive rates, as is standard practice for any marker brought to the clinic. Critically, particularly when genes are used in combination, spatial positioning patterns can detect breast or prostate cancer with high accuracy (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016). When genes are used as markers singularly, between 64 and 100% of samples are correctly identified as cancer based on radial positioning patterns. Multiplexing genes improved the accuracy of cancer detection for genes with lower sensitivities, reducing false negative rates (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016). Repositioning events are independent of variations in copy number and even in the background of highly variable, and in some cases large scale, genomic instability between cancer specimens, it is possible to use gene positioning to accurately distinguish cancers from control tissue-specimens (Meaburn and Misteli, 2008; Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016).

Although these studies represent the largest analysis of genome organization in cancerous tissues performed to date,

the eleven genes identified as potential breast or prostate cancer biomarkers were analyzed in a low number of individuals (11–19 cancers, 6–10 normal tissues and 5 non-malignant disease tissues), in tissues that were known to be cancerous or benign and were performed by the same group (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016). Moreover, these studies were not performed on tissue biopsies. This makes comparing the accuracies of gene-positioning based diagnostics with current diagnostic strategies impossible currently. The accuracy of current diagnostic tests reported are based on 100s and 1000s of biopsy specimens, from multiple testing sites, and will include sampling errors (e.g., no cells in the sample, the biopsy missing the tumor and collecting only normal tissue, the protocol failing to stain etc.). Larger scale follow-up studies are required to assess the sensitivity and specificity of these spatial positioning gene markers, to determine if they are strong enough for clinical use. It will be important to compare gene-positioning biomarkers to existing diagnostic methodologies and to establish if using the various methods in combination, or alone, improves diagnostic accuracy.

PROGNOSTIC POTENTIAL FOR SPATIAL GENOME ORGANIZATION?

Additionally, the original studies were not designed to determine if the spatial organization of the genome in cancer has prognostic value. Yet prognostic markers are the most urgent cancer biomarkers required to distinguish aggressive cancer from indolent, thereby reducing overtreatment caused by overdiagnosis. It is difficult to accurately assess the current level of overdiagnosis, since directly measuring overdiagnosis involves monitoring the progress of the cancer and the cause of death of cancer patients, without treating the individuals and is also dependent on the precise definition of “clinically relevant” cancer. Nevertheless, for breast and prostate cancer, estimates suggest overdiagnosis is in the range of 22–67% of cancers (Draisma et al., 2009; Welch and Black, 2010; Bleyer and Welch, 2012; Sandhu and Andriole, 2012; Miller et al., 2014). Using prostate cancer mortality as an endpoint, as many as 84% of PSA screen-detected prostate cancers may be overdiagnosed (McGregor et al., 1998). Breast and prostate cancers are highly prevalent, with almost 1.7 million women and 1.1 million men worldwide diagnosed with these cancers, respectively, in 2012 (Torre et al., 2015). In the USA, where population-based screening for breast and prostate cancer is common, over 240,000 women and more than 180,000 men per year are diagnosed, respectively (Siegel et al., 2016). Given these numbers, even taking the low estimate of overdiagnosis of 1 in 5 cancers additional prognostic markers could benefit 100s of 1000s of patients every year and reduce health care costs considerably.

Histological assessment of tissue specimens can be beneficial in the clinical setting to reduce overtreatment. For example, pathologists classify histological patterns within prostate cancer tissue according to the Gleason grading system, which broadly correlates with the differentiation status of the cancerous tissue (Montironi et al., 2005; Epstein et al., 2016). Gleason scores,

particularly when used in combination with other clinical factors, including PSA level and clinical tumor stage, are the strongest markers of prostate cancer prognosis and progression currently available (D’Amico et al., 1998; Montironi et al., 2005; Cooperberg et al., 2009; Chang et al., 2014; Epstein et al., 2016). Part of the strength of the Gleason scoring system is that it takes into account intra-individual tumor heterogeneity (Van der Kwast, 2014; Epstein et al., 2016). A low Gleason score (Gleason score 6 or below) is highly indicative of an indolent cancer, and often monitoring the tumor (watchful-waiting/active surveillance) is recommended in place of treatment (Thompson et al., 2007). However, additional markers are required as both under- and over-treatment remain an issue (Cooperberg et al., 2010). Additionally, histological analysis of tissues is, by its very nature, subjective, which can lead to inter- and intra-observer variations. Taking the example of prostate cancer again, two pathologists will agree on the Gleason score of an individual cancer tissue 36–81% of the time (Montironi et al., 2005). Similarly, the same pathologist will derive an identical Gleason score for the same tissue specimen 43–78% of the time (Montironi et al., 2005). Most frequently, the difference in score varies by a single Gleason grade, suggesting these small variations would make little difference to most patients. However, for men with Gleason score 6 or 7 cancers this could mean the difference between receiving treatment or not. The addition of quantitative diagnostic/prognostic markers, to be used alongside histological analysis, would reduce inter- and intra-observer variations, and thus reduce overtreatment.

Given the fact that the position of a gene can vary between cancers derived from the same organ, and between cancers from different organs (Meaburn et al., 2009; Wiech et al., 2009; Leshner et al., 2016; Meaburn K. J. et al., 2016), it suggests the possibility that the spatial organization of the genome can be used to sub-type cancers for prognostic purposes. It is “simply” a matter of finding the right genes. Due to the selection criteria to identify a diagnostic spatial positioning biomarker, which is required to repositioning in the majority of prostate cancer specimens (compared to normal), it is likely different gene loci will be useful prognostic biomarkers than for diagnostic markers. For example, *FLII* repositions in 100% (10/10) of breast cancers and 92.9% (13/14) of prostate cancers (Leshner et al., 2016; Meaburn K. J. et al., 2016), rates far too high to suggest it can be used to stratify indolent and aggressive cancers. Future screening studies are required, using a highly defined set of indolent and aggressive cancers. Since spatial reorganization of the genome has been identified in multiple human diseases, it is likely that the same principle of using spatial genome positioning patterns as a diagnostic biomarker could be applied to any disease.

SUMMARY

The genome is highly spatially organized, and reorganizes in disease. Accumulating evidence suggests that these repositioning patterns can be exploited for diagnostic use. Currently, there is no way to predict which genes will reposition in cancer. As we gain a better understanding of how spatial positioning is regulated in

a normal cell, it may become easier to predict regions that will be diagnostically interesting. The reverse is also true. Comparing positioning patterns between normal and diseased cells will inform our understanding of spatial genome organization. While it is exciting that proof-of-principle studies have identified spatial positioning of the genome as a potentially sensitive and specific biomarker of cancer, much work is yet to be done. Large-scale follow-up studies are required to determine the feasibility and usefulness of spatial genome organization-based diagnostic in the clinical setting. Moreover, while additional diagnostic tests have their use, there is urgent need for biomarkers that can distinguish aggressive cancers from indolent ones. These markers would have a tremendous impact on cancer patients, by guiding clinicians to the best treatment regime for an individual and reducing both under and over-treatment. As such, as future studies relating to genome organization for clinical use should focus on prognostics rather than simply diagnostics.

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AUTHOR CONTRIBUTIONS

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Blank spots on the map: some current questions on nuclear organization and genome architecture

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Abstract

The past decades have provided remarkable insights into how the eukaryotic cell nucleus and the genome within it are organized. The combined use of imaging, biochemistry and molecular biology approaches has revealed several basic principles of nuclear architecture and function, including the existence of chromatin domains of various sizes, the presence of a large number of non-membranous intranuclear bodies, non-random positioning of genes and chromosomes in 3D space, and a prominent role of the nuclear lamina in organizing genomes. Despite this tremendous progress in elucidating the biological properties of the cell nucleus, many questions remain. Here, we highlight some of the key open areas of investigation in the field of nuclear organization and genome architecture with a particular focus on the mechanisms and principles of higher-order genome organization, the emerging role of liquid phase separation in cellular organization, and the functional role of the nuclear lamina in physiological processes.

Keywords Genome organization · Nuclear architecture · Phase separation · Nuclear lamina

Introduction

The genomic material in each eukaryotic cell is organized in a complex and hierarchical fashion in the cell nucleus (Misteli 2007; Bickmore 2013). DNA, which serves as the primary carrier of genetic information, is wrapped around a histone octamer to form nucleosomes which in turn are arranged in a beads-on-a-string-like manner into higher-order chromatin fibers. Recent observations using electron tomography methods suggest that the chromatin polymer

exists predominantly as a 5–24 nm disordered fiber (Ou et al. 2017). At a higher level of organization, the fiber folds into ~500 Mb domains, referred to as topologically associating domains (TADs), which are defined biochemically based on the preferential physical interactions of sites within a TAD compared to regions outside of these domains (Dixon et al. 2016). The linear arrangement of TADs ultimately gives rise to chromosome territories which denote the physical volume occupied by a single chromosome in the nucleus (Cremer and Cremer 2001). Intriguingly, the location of individual chromosomes is non-random within the nucleus and a loose correlation between gene activity and proximity to the nuclear periphery has been noted with inactive genome regions in many cell types localized closer to the nuclear edge (Takizawa et al. 2008; van Steensel and Belmont 2017).

The nuclear space is further compartmentalized into distinct bodies (Dundr 2012). These are non-membranous structures that likely form via phase separation of nuclear proteins and nucleic acids (Banani et al. 2017; Shin and Brangwynne 2017). Nuclear bodies often serve as sites of particular nuclear functions such as the transcription of ribosomal genes in the nucleolus or the processing of nascent

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histone transcripts in the histone locus body (Dundr 2012). Alternatively, they may serve as storage sites for functionally related proteins such as splicing speckles, which are enriched in pre-mRNA splicing factors (Dundr 2012; Galganski et al. 2017).

A major landmark, and functionally relevant entity, in metazoan nuclei is the nuclear lamina. This anastomosed network of lamin proteins, which are members of the intermediate filament family, lines the inner nuclear membrane (INM) and protects the genome from mechanical forces that act on the nucleus (Dittmer and Misteli 2011). In addition, the lamina serves as a platform for the association of specific chromatin domains to the nuclear periphery. These lamina-associated domains (LADs) are typically gene poor, structurally condensed and enriched in repressive histone modifications (van Steensel and Belmont 2017), in line with longstanding observations of enrichment of heterochromatin at the nuclear periphery of most cell types (Lemaître and Bickmore 2015). Of relevance to human health, mutations in lamin proteins cause a wide range of tissue-specific diseases, including muscular dystrophies, lipodystrophies, neuropathies, and systemic premature aging syndromes (Gruenbaum and Foisner 2015; Vidak and Foisner 2016).

The past two decades have brought dramatic progress in the detailed description of nuclear structure. Traditional imaging approaches have been refined to provide unprecedented spatial and temporal resolution, and the use of in vivo imaging methods has led to an appreciation for the highly dynamic nature of many nuclear structures and processes. These methods have been complemented and extended by the development of biochemical techniques, so called 3C technologies, to map chromatin–chromatin interactions at a genome-wide scale, leading to a comprehensive description of the chromatin landscape in the cell nucleus (Grob and Cavalli 2018). Furthermore, the recent realization of phase separation as a fundamental organizational principle of nuclear organization has provided a framework for how membraneless suborganelles can exist in the nuclear space (Banani et al. 2017; Shin and Brangwynne 2017), and the description of diseases caused by mutations in lamin genes has highlighted the critical nature of the nuclear periphery (Dittmer and Misteli 2011; Vidak and Foisner 2016). However, many questions regarding the structure and function of the cell nucleus remain. We discuss here a select, yet neither comprehensive nor objective, set of unanswered questions that we consider of high priority in the field.

Genome organization

The fact that genomes are organized in a hierarchical fashion and in non-random patterns is now well established (Misteli 2007). Some of the most relevant questions in the field

center around the functional role of genome organization and the molecular mechanisms involved in establishing and maintaining higher-order genome organization.

What is the regulatory function of genome organization?

Genome organization has been shown to be conserved during evolution (Dixon et al. 2012) to be cell-type specific (Dixon et al. 2015) and to be disrupted in disease (Lupiáñez et al. 2015; Valton and Dekker 2016), suggesting a functional role in gene regulation. However, while some features of genome organization, such as the position of a gene relative to the nuclear periphery, have been correlated with transcription, a causative role for these patterns is limited at best (Takizawa et al. 2008), raising the crucial question of whether genome organization is of functional relevance or is simply a reflection of the chromatin state.

With respect to the position of a gene relative to the nuclear periphery, the notion of a repressive role of the nuclear edge has long been supported by the observation that gene-poor regions are often enriched at the nuclear envelope (Bickmore 2013). In addition, association with the nuclear periphery is often correlated with transcriptional repression (Kosak et al. 2002; Dietzel et al. 2004; Zink et al. 2004a). However, this correlation does not apply to all genes; for example, the *PLP* gene is highly expressed and peripherally located in oligodendrocytes (Nielsen 2002) and *T-bet* is silenced without repositioning to the periphery in T helper cells (Hewitt et al. 2004). Furthermore, tethering to the nuclear periphery is sufficient to silence some, but not all, reporter genes (Finlan et al. 2008; Harr et al. 2015). Studies in mice showed that while either decompacting chromatin or independently inducing transcription of an endogenous gene was sufficient to move that gene from the periphery to the center of the nucleus, decompaction was required for repositioning, but transcription was not (Therizols et al. 2014). This observation suggests that peripheral positioning does not provide an independent means of silencing chromatin, but instead is, like transcriptional status, controlled by chromatin state. Similarly, in worms, loss of heterochromatin–lamina association is not sufficient on its own to cause widespread misregulation, although it does decrease the efficiency of differentiation (Gonzalez-Sandoval et al. 2015). It thus appears that positioning at the nuclear periphery is correlated with, but not directly causative of, gene repression.

Similarly, disrupting the boundaries of chromatin domains, particularly TADs, has been shown to sometimes, but not always, result in functional consequences, particularly aberrant interactions between promoters and enhancers (Lupiáñez et al. 2015; Rodríguez-Carballo et al. 2017). In support of a functional role of TADs is the fact that they are evolutionarily conserved (Dixon et al. 2012) and have

been observed to change during differentiation (Fraser et al. 2015a; Ji et al. 2016). However, the interpretation of these correlations is complicated by the fact that the molecular mechanisms underlying TAD formation are unclear. In *Drosophila*, TAD boundaries are marked by active genes (Ulianov et al. 2016), whereas in mammals they are marked by domains enriched for CTCF binding sites (Nora et al. 2012; Tang et al. 2015), although CTCF binding is neither necessary (Barutcu et al. 2018) nor sufficient (Rao et al. 2014) for TAD formation. These observations suggest that rather than being discrete entities with a single conserved mechanism and function, TADs may merely be a recurring shape with limited functional relevance. Fortunately, recent studies identifying several proteins involved in TAD formation in mammalian cells suggest avenues for studying their function on a global level (Wutz et al. 2017). Determining whether the widely observed genome organization patterns, such as the non-random 3D position of a gene or the formation of chromatin domains, has functional consequences is of prime importance and will have profound implications for understanding the biology of the cell nucleus and of genomes.

What are the molecular mechanisms underlying 3D genome organization?

Next generation sequencing (NGS) and 3C technologies have greatly improved our understanding of hierarchical 3D genome organization (Nora et al. 2012; Rao et al. 2014; Bonev and Cavalli 2016). However, the question of what the underlying molecular mechanisms responsible for higher-order genome organization are is only beginning to be explored (Shachar et al. 2015; Guo et al. 2015; Sanborn et al. 2015). Cohesins and CTCF have been identified as two major determinants of higher-order genome structure (Bouwman and de Laat 2015), but it seems likely that other regulators of genome architecture exist. One reason for the scarcity of information is the difficulty in experimental approaches to identify them in an unbiased fashion.

One promising approach to systematically dissect mechanisms of nuclear architecture is the use of imaging-based RNAi screens (Joyce et al. 2012; Shachar et al. 2015; Pegoraro and Misteli 2017) and CRISPR/Cas9 screens (Shalem et al. 2014; Wang et al. 2014; Tan and Martin 2016; Henser-Brownhill et al. 2017; de Groot et al. 2018), in which changes in various nuclear and genome features are assessed, often by imaging after knockdown of specific cellular factors. When compared to RNAi screens, the use of CRISPR/Cas9 is more robust to off-target effects (Evers et al. 2016; Morgens et al. 2016), can be used to scan structural non-coding elements (Gasperini et al. 2017), and can be adapted to knock out or induce the expression of cellular genes (Gilbert et al. 2014).

Screening approaches will be instrumental to address a variety of questions related to genome organization. In the short term, high-throughput microscopy-based assays, in conjunction with arrayed libraries of CRISPR/Cas9 reagents, and with programmable OligoPaint protocols (Beliveau et al. 2012, 2015), are expected to lead to the identification of molecular pathways involved in the establishment of chromatin domains, which are relatively large and can be readily visualized with fast, diffraction limited microscopy methods. Similarly, this kind of approach may be used to dissect the mechanisms regulating the preferential positioning of certain genomic regions to nuclear landmarks such as the nuclear envelope (NE) (Guelen et al. 2008), speckles, or nucleoli (Quinodoz et al. 2018). In the long term, the adaptation of large single-cell Hi-C protocols (Ramani et al. 2017) to single-cell pooled CRISPR screens (Adamson et al. 2016; Dixit et al. 2016) will potentially lead to the genome-wide measurement of smaller 3D genome structures, such as TADs, in hundreds or thousands of perturbations in parallel. Altogether, these large-scale functional genomics approaches will enable the discovery and understanding of the molecular rules underlying genome organization and its role in regulating other nuclear processes.

What mechanisms underlie the dynamic nature of genome organization?

Assessing genome organization by high-throughput sequencing methods belies the dynamic nature of the genome (Parfrey et al. 2008; Mishra and Hawkins 2017). In live cells, the genome is dynamic at different levels of organization: TADs dissolve during mitosis and reappear at G1, intra-TAD loops change along with expression of the associated genes during differentiation, and gene expression in turn re-organizes nuclear compartments (Denholtz et al. 2013; Smith et al. 2016; Freire-Pritchett et al. 2017; Nagano et al. 2017; Shachar and Misteli 2017). However, the mechanistic basis of these rearrangements is unclear. These changes both reflect and affect changes in gene expression; thus understanding the principles underlying the dynamic organization of genomes is essential for a complete picture of gene regulation (Geyer et al. 2011).

A key question in this area is what factors drive the intra-TAD loops that juxtapose enhancers to cognate promoters. The primary function of intra-TAD loops is thought to bring cognate enhancers and promoters in close proximity in 3D space and enable their interaction (Schoenfelder et al. 2015; Freire-Pritchett et al. 2017). While some of the major drivers of TAD organization have been identified as the chromatin proteins CTCF, cohesin and condensin, little is known about regulators of intra-TAD organization (Bouwman and de Laat 2015). Recent studies show that induction of genes can lead to the formation of pronounced enhancer–promoter

loops or a diffuse increase in intra-TAD contacts (Ron et al. 2017; Vian et al. 2018), suggesting that both the enhancer and promoter loci show dynamic association. However, this leaves us with the question: what cellular factors guide the specificity and the strength of these dynamic interactions?

A further question regarding chromatin dynamics is the issue of what mechanisms relocalize genes to and from the nuclear periphery. In higher eukaryotes, genes associated with the nuclear lamina move to the interior region upon induction (Kind and van Steensel 2010). In yeast, genes often associate with nuclear pores upon activation (Casolari et al. 2004; Takizawa et al. 2008). It is unclear how these directional movements are brought about. In the case of yeast, components of the RNA export pathways are believed to direct the transcribing gene to the nuclear periphery (Steglich et al. 2013). However, such translocations can occur even in the absence of transcription (Brickner et al. 2007). DNA zip codes bound by transcription factors have been implicated in yeast and a close parallel has been reported in human B cells (Ahmed et al. 2010; Zullo et al. 2012). To conclusively resolve mechanisms that directly relocalize genes would require following the movement of native genes in live cells coupled with quick conditional depletion of regulators of chromatin and transcription.

What is the structure of chromatin in the intact cell?

One of the long-standing, and still remarkably poorly understood, fundamental questions in chromatin biology is how the 2 m of DNA is packaged into the $\sim 10\ \mu\text{m}$ -sized nucleus. New imaging methods, particularly superresolution and modified electron microscopy methods, have recently provided some insights.

Traditional light imaging methods have a resolution limit of $\sim 250\ \text{nm}$, far below what is needed to visualize chromatin fibers. Recent superresolution techniques achieve considerably better resolution, for example structured illumination microscopy (SIM) has a resolution of 100–130 nm and stimulated emission depletion (STED) microscopy ~ 20 –70 nm in the XY plane (Yamanaka et al. 2014). However, these methods are still not sufficient to clearly visualize the chromatin fiber which has a diameter in the order of 5–30 nm. One of the most promising approaches to probe chromatin is localization microscopy based on the detection of single molecules using special fluorophores with a capacity for stochastic blinking (Lakadamyali and Cosma 2015). The resolution of photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) can be as low as 10–30 nm in the XY plane, and, if combined with additional approaches such as optical astigmatism or dual objectives systems, can be reduced down to 10–75 nm in the Z direction (Yamanaka et al. 2014). A notable application of 3D-STORM is the study of the structure of

active, inactive, and Polycomb-repressed chromatin domains ranging from 10 to 500 kb in *Drosophila* cells (Boettiger et al. 2016). Using a high-resolution approach and specific OligoPaint probes that enable labeling of multiple, distinct regions of the chromatin fiber, different levels of chromatin folding for different epigenetic states become evident (Boettiger et al. 2016). Furthermore, using superresolved images of histones and computational approaches, the number of nucleosomes spanning the chromatin fiber was counted in human cells (Ricci et al. 2015), and it was shown that nucleosomes aggregate in groups of different sizes called “clutches” in intact cells. These methods should eventually also become applicable for high-resolution studies of specific regions such as individual genes and chromatin domains in vivo.

A complementary approach is the use of advanced electron microscopy. A method termed ChromEMT (Chromatin Electron Tomography) uses a photoconvertible DNA-intercalating dye combined with 3D tomography of serial sections to visualize the chromatin fiber in intact cells (Ou et al. 2017). ChromEMT reveals that chromatin exists predominantly as a 5–24 nm fiber and that the difference between heterochromatin and euchromatin is largely due to different packaging densities of the fiber, rather than differences in the folding patterns. Further application and development of this method promises to reveal the fine structure of chromatin in the intact cell.

An overarching critical question in the field is how biochemical 3C methods relate to single-cell imaging methods (Nagano et al. 2013; Dekker and Misteli 2015; Fraser et al. 2015b; Giorgetti and Heard 2016; Stevens et al. 2017). This is a pertinent question since 3C methods report averaged contact frequencies, whereas fluorescent in situ DNA hybridization measures spatial distances in single cells and, even more promisingly, in case of superresolution microscopy, has the potential to reveal the structure of chromatin regions. Superresolution visualization, especially combined with biochemical approaches or CRISPR/Cas9 in vivo tagging, will bring us closer to understanding genome organization in the interphase nucleus in physiological and pathological conditions.

Do post-translational modifications regulate genome organization?

One potential mechanism for modulating chromatin structure and genome organization is post-translational modifications (PTMs). Support for this notion comes from a high-throughput imaging screen to identify factors which affect the position of genes in the human cell nucleus (Shachar et al. 2015). Of 50 factors which altered the position of four tested target genes, 11 were components of post-translational modifying complexes or the modifying enzymes themselves,

and 10 of the 11 identified in the screen targeted histones as substrates (Shachar et al. 2015). These data suggest that specific histone modifications may target genome locations to precise positions within the nucleus. This model is also supported by findings in *C. elegans* where transcriptionally repressed chromatin is targeted to the nuclear periphery via histone H3-lysine 9 mono-, di-, and trimethylation (Towbin et al. 2012; Gonzalez-Sandoval et al. 2015). Furthermore, the histone H3K9-methyl reader CEC-4 was identified and found to independently localize to the nuclear membrane and anchor heterochromatin to the nuclear periphery by binding histones directly in a histone H3 K9 methyl-dependent manner.

An additional, and most likely complementary, possibility is that post-translational modifications of non-histone proteins play a role in genome organization. Such proteins would likely regulate genomic organization through changes in protein–protein interactions caused by the PTMs. A potential set of substrates which could affect gene positioning through the addition of PTMs are the lamin proteins (Simon and Wilson 2013). Lamins A, B1, B2, and C all interact with chromatin and are all extensively modified with PTMs such as farnesylation, acetylation, phosphorylation, ubiquitination, and sumoylation, among others (Simon and Wilson 2013). In addition, a plethora of lamin-binding proteins have already been identified suggesting that these interactions could be regulated by PTMs (Dittmer et al. 2014). Of physiological interest is the fact that many post-translational modifying enzymes and complexes have been implicated in various diseases including cancer (Butler et al. 2012). Identifying how post-translational modifying components regulate gene positioning will lead to a better understanding of how the cell organizes its genome and how these regulatory mechanisms are disrupted in human disease.

How heritable is nuclear organization?

Spatial genome organization is relatively invariable in a given cell type, but can be altered quite dramatically during tissue development, differentiation, and malignant transformation (Krumm and Duan 2018). During interphase, chromatin is constrained in mobility and usually does not move over long distances; however, following cell division during the first 2 h of the G1 phase, global nuclear organization needs to be re-established to allow for diverse nuclear functions (Vazquez et al. 2001; Walter et al. 2003; Thomson et al. 2004; Strickfaden et al. 2010). Evidently, the processes taking place in early G1 have a significant impact on the outcome of nuclear organization, but to date it is unclear what kind of nuclear structures, forces, and factors causally underlie these processes. The mechanisms by which chromatin compartments are lost during mitosis and re-established in

G1 or how loci form and lose association with nuclear substructures in daughter nuclei are unknown.

One way to shed light on the players that contribute to re-formation of organization is to use an experimental system that allows tracking of the location of large genomic regions in live cells over multiple cell divisions, similar to the one described for Lamin B1-contacting regions (Kind et al. 2013). This assay could be combined with a CRISPR-based screen to knock out various genes and to discover new organization candidates that function in early G1. Another possible approach to address this question would be to use a well-characterized developing model organism such as the fly or the worm and to generate lines that carry a fluorescent reporter in an endogenous locus, allowing simultaneous visualization of the genomic region and transcriptional outcome at the single cell level (Chen et al. 2018). Such systems are now available and can be used to follow the position of a locus in a developing embryo and to correlate position with gene activity over multiple cell cycles. Combined with genetic screens, factors that affect positioning and/or activity in various stages of development should become apparent. Identification of these mechanisms will shed light on how nuclear memory of genome organization is generated, how it affects transcription and cell fate, and how heritable genome organization is.

Can spatial genome reorganization be used for diagnostic and prognostic purposes in the clinical setting?

Annually, ~ 14.1 million people worldwide are diagnosed with cancer (Torre et al. 2015), yet there are few biomarkers available to aid oncologists in prescribing the most beneficial treatment for a patient or to identify indolent cancers, which in the absence of treatment the patient would remain asymptomatic (Ferté et al. 2010; Welch and Black 2010; Meaburn 2016). Consequently, there is an urgent need for additional clinically relevant cancer biomarkers. While the functional relevance of spatial organization of the genome within an interphase nucleus and the mechanisms that choreograph these positioning patterns currently remain elusive, it is likely that the spatial organization of the genome could be exploited for clinical purposes (Meaburn 2016; Meaburn et al. 2016b).

The rationale for the use of genome organization as a clinical tool is the fact that the non-random organization of various genome features changes in pathological conditions (Meaburn 2016). Specific genes and chromosomes occupy alternative spatial positions in disease, including in epilepsy (Borden and Manuelidis 1988; Walczak et al. 2013), laminopathies (Meaburn et al. 2007; Taimen et al. 2009; Mewborn et al. 2010), Trisomy 21 (Paz et al. 2013, 2015; Kemeny et al. 2018), and cancer (Cremer et al. 2003; Wiech

et al. 2009; Meaburn et al. 2016a; Taberlay et al. 2016). An indication for the feasibility of spatial genome organization as a diagnostic tool comes from a set of small-scale retrospective studies in which cancer tissues were accurately identified using spatial positioning patterns (Meaburn et al. 2009, 2016a; Leshner et al. 2016; Meaburn 2016). For example, the *FLII* gene repositioned in 100% (10/10) of breast cancers (Meaburn et al. 2016a) and 92.9% (13/14) of prostate cancers (Leshner et al. 2016), and *HES5* was repositioned in 100% (13/13) of breast cancers (Meaburn et al. 2009). Moreover, genes that repositioned in a lower frequency of cancers can be used in combination to improve their sensitivity in detecting cancer (Meaburn et al. 2009; Leshner et al. 2016). Critically, positioning biomarkers have high specificity since repositioning rates are low in non-cancerous tissues (Meaburn et al. 2009, 2016a; Leshner et al. 2016). The most likely application of spatial genome biomarkers will be in combination with routinely used pathology indicators, such as gross morphology of tissues and nuclei, to refine the current diagnostic methods (Zink et al. 2004b; Meaburn 2016).

Although additional biomarkers for the diagnosis of cancers would be welcome, prognostic biomarkers are in even higher demand. The question remains whether spatial positioning of the genome could yield more nuanced information and be used to sub-type cancers. For example, are there genes, or sets of genes, whose positioning patterns can be used to distinguish aggressive cancers from indolent ones? This remains to be formally addressed, but the fact that there are different spatial positioning signatures between different diseases, including between cancers originating in different organs, suggests a promising potential for the spatial organization of the genome to also provide prognostic biomarkers (Meaburn 2016).

The use of the spatial organization of the genome as a clinical biomarker is a relatively understudied topic and requires further studies on large cohorts of patients. Identification of useful biomarker genes is hindered by a lack of knowledge of what drives the reorganization of the genome, making it currently impossible to predict which genes may be useful spatial biomarkers, meaning large numbers of genes will need to be screened to find promising biomarkers and will require high-throughput imaging and analysis approaches applicable to tissues.

Principles of nuclear organization

The nucleus is like no other cellular organelle in that it is characterized by the presence of not just protein, but large amounts of nucleic acids, and contains a large number of subcompartments which are not defined by membranes (Dundr 2012). These features point to unique organizational

principles of the nuclear space. One principle that has emerged recently is the paradigm that non-membrane bound organelles spontaneously arise from phase separation of cellular components (Banani et al. 2017; Shin and Brangwynne 2017).

Is the nucleus an emulsion of phase-separated droplets of nucleic acids and proteins?

Phase separation is likely relevant for nuclear organization. Several nuclear organelles such as nucleoli and nuclear speckles appear as discrete micron-sized bodies that remain compositionally distinct from the surrounding nucleoplasm without the use of a membraned divider (Dundr 2012). Moreover, many of these nuclear bodies share remarkably similar behavior with that of liquid droplets (Brangwynne et al. 2011; Hyman et al. 2014) and their macroscopic properties are governed by specific molecular interactions (Feric et al. 2016). These and other assemblages of nucleic acids and proteins are collectively known as biomolecular condensates (Banani et al. 2017).

Recently, several nuclear proteins, not associated with canonical nuclear bodies, have also been shown to behave as biomolecular condensates. For example, the ability of heterochromatin protein 1a (HP1a) to condense DNA into liquid-like droplets provides a simple, yet robust mechanism for gene silencing of heterochromatin (Narlikar et al. 2017; Strom et al. 2017). By extension, phase separation may play a role in faithfully compartmentalizing chromosomes into discrete territories or sub-compartments within the nucleus (Erdel and Rippe 2018). Furthermore, the clustering of super-enhancer elements and transcription factors along DNA into defined condensates (Hnisz et al. 2017; Chong et al. 2018; Sabari et al. 2018) and the interplay between other liquid-like components, such as the Mediator transcriptional coactivator and RNA polymerase II, may represent a physical means to control gene expression (Cho et al. 2018). Speculatively, other nuclear processes may also involve the phase separation of nucleic acids and proteins into functional interaction hubs, such as the cohesion of proteins to telomeres to prevent the degradation of chromosome ends; the spontaneous formation of DNA damage repair foci in response to damage; and the interaction of intrinsically disordered tails of histone proteins in promoting chromatin compaction.

Together, these results pose an intriguing question: is the genome organized as an emulsion of many phase separated droplets of DNA and protein? If so, what determines the sites and timing of droplet assembly and disassembly? Furthermore, how do the emergent properties of these condensates affect gene expression, and, importantly, how do anomalies in phase separation of the genome contribute to

disease progression, such as in neurodegenerative disorders and cancer?

Answering these questions will require multidisciplinary methods that bridge the physics of phase separation with the specific biological players involved in organizing the nuclear genome. A top-down approach of using high-throughput screening and imaging methods to assay for phenotypic changes in condensate morphology can be complemented by a bottom-up approach of evaluating proteins for phase separation behavior in vitro. Furthermore, systematic mutations and deletions will lead to the identification of the essential sequences and regions that promote formation or partitioning into droplets (Wang et al. 2018). Ultimately, differences in phenomenological properties of biological condensates between disease states may shed light on which molecular interactions therapeutic strategies should target. This future work will provide insight into how information encoded by molecular interactions becomes propagated via phase separation across larger length scales within the cell.

It is intriguing to speculate that phase separation may also have played a role in enabling the origin and evolution of life. In this view, RNA may have acted as a scaffold for the formation of early membrane-less compartments that served to concentrate biochemical reactions by providing the necessary spatial and chemical diversity (Poudyal et al. 2018). This model would explain the large number of non-coding RNAs observed in higher organisms, and the idea is consistent with the prominent role of RNA in early life and the presence of RNA in many phase-separated structures including nuclear paraspeckles (Hennig et al. 2015; Yamazaki et al. 2018) and cytoplasmic ribonucleoprotein granules (Van Treeck and Parker 2018). Furthermore, dysregulation of nuclear RNA-mediated phase separation leads to disease as shown in neuromuscular disorders where repeat expansions of RNA cause aberrant gelation (Aguzzi and Altmeier 2016; Jain and Vale 2017). To address this model, key phase separating molecules from primitive to complex organisms need to be biochemically dissected to determine the conditions that favor compartmentalization. A prediction from this model is that many non-coding RNAs are indispensable in the context of liquid phase-separated structures in the nucleus.

The nuclear envelope and the lamina

The nuclear lamina is a major structural feature of the nucleus of metazoan cells (Serebryanny and Misteli 2018). Its primary function has long been assumed to provide physical stability to the nucleus and to protect the genome from mechanical forces transmitted through the cytoplasm. However, recent observations suggest more diverse functions for the lamina, including anchoring specific genome

regions to the nuclear periphery, controlling the intranuclear concentration of proteins by their regulated sequestration to the lamina, serving as a platform for nuclear signaling events and possibly other cellular functions (Serebryanny and Misteli 2018). Numerous important questions regarding the function of the nuclear lamina, particularly with regard to its physiological and pathological functions, remain.

Is the nuclear lamina a site of protein degradation?

The nuclear envelope is a double membrane consisting of the outer nuclear membrane (ONM) and the INM, connected at the sites of nuclear pores (Hetzer 2010; Boban and Foisner 2016). The ONM is continuous with the endoplasmic reticulum (ER). Protein homeostasis in this compartment is ensured by endoplasmic reticulum-associated protein degradation (ERAD) pathways (Khmelniskii et al. 2014); however, little is known about possible protein quality control at the INM.

Some indications for a role of the INM in protein degradation come from studies in the yeast *Saccharomyces cerevisiae*, where the Asi complex, containing a RING finger (RNF) domain ubiquitin E3 ligases Asi1 and Asi3, has been shown to target integral proteins of the INM for proteasomal degradation (Foresti et al. 2014; Khmelniskii et al. 2014). Although Asi components are not conserved outside of yeast, emerging evidence has suggested the existence of transmembrane RNF-containing E3 ubiquitin ligases in eukaryotic cells (Nakamura 2011). In line with this, the RNF123 and HECW2E3 E3 ubiquitin ligases have been implicated in proteasomal degradation of several nuclear proteins, including lamin B1 and mutants of lamin A/C associated with Emery–Dreifuss muscular dystrophy (EDMD) (Khanna et al. 2018; Krishnamoorthy et al. 2018). Apart from the ubiquitin–proteasome-dependent protein degradation, autophagy-mediated degradation of nuclear envelope proteins has recently been described in mammalian cells, demonstrating that the autophagy protein LC3 interacts with lamin B1 and mediates its clearance by macroautophagy in response to oncogenic stress (Dou et al. 2015). In addition to lamin B1, macroautophagy has been shown to be involved in downregulation of INM proteins emerin, lamin B receptor (LBR), and SUN1 in oncogene-induced senescent cells (Lenain et al. 2015). Although the mechanisms of lamin A/C degradation remain an open question in the field, recent studies suggest that the disease-causing lamin A mutant isoform progerin is partially degraded by macroautophagy (Cao et al. 2011; Cenni et al. 2011; Gabriel et al. 2015; Vidak and Foisner 2016). Progerin is the causative agent in the premature aging disorder Hutchinson–Gilford progeria syndrome (HGPS) and progerin, unlike the wild-type lamin A, is permanently farnesylated and thus tightly associated with the INM (Dechat et al. 2007; Reddy and Comai 2012).

The observed partial degradation of progerin coupled with the observation that wild-type lamin A/C is not degraded by macroautophagy in senescent cells (Lenain et al. 2015) suggests another pathway may be involved in the degradation of lamin A/C in mammalian cells. One possible mechanism is a selective form of autophagy called chaperone-mediated autophagy (CMA) that has recently been implicated in the degradation of nuclear proteins (Park et al. 2015; Tekirdag and Cuervo 2018). One of the distinctive features of CMA is that proteins are selected through a penta-peptide recognition motif in their amino acid sequences, biochemically similar to KFERQ (Cuervo and Wong 2014) present both in wild-type lamin A/C and progerin, making them strong candidates for degradation by CMA. Furthermore, the NFE2L2/NRF2 transcription factor has recently been identified as an important regulator of CMA activity (Pajares et al. 2018). Interestingly, progerin has been shown to sequester NRF2, resulting in impaired NRF2 transcriptional activity (Kubben et al. 2016), allowing for a possible scenario where impaired regulation of NRF2 in progerin-expressing cells could lead to decreased CMA activity and contribute to increased stability and accumulation of proteins at the nuclear periphery. An intriguing possibility is that CMA might represent a missing link to understand pathways involved in the degradation of lamin A/C in health and disease. Taken together, these emerging observations point to an underappreciated role of the nuclear lamina in the control of protein homeostasis.

Does nuclear envelope-associated chromatin maintain cell specificity and differentiation?

Just as the mechanisms that regulate protein homeostasis at the NE remain poorly understood, so are the functional consequences of changes in its protein composition. The NE serves as a selective barrier for the regulation of nuclear import of signaling factors and of mechanical force transduction (Uhler and Shivashankar 2017; Kirby and Lammerding 2018). A combination of microscopy, DNA adenine methyltransferase identification (DamID), chromatin immunoprecipitation (ChIP), and next-generation sequencing approaches have suggested that NE-associated proteins also organize hundreds of genes into transcriptionally silenced domains at the nuclear periphery (van Steensel and Belmont 2017). Although the basic tenets of NE-associated chromatin organization such as the transcriptional repression of genes associated with the nuclear lamina and preferential localization of heterochromatin to the nuclear periphery appear to be conserved (Kind et al. 2015; van Steensel and Belmont 2017), drastic changes in the association of chromatin with the lamina occur upon differentiation and correlate with alterations in histone modifications as well as the tissue-dependent expression of NE-associated proteins

(Peric-Hupkes et al. 2010; Solovei et al. 2013; Swift et al. 2013; Robson et al. 2016; Atlasi and Stunnenberg 2017). These correlations may suggest a role for the nuclear lamina in defining genome organization patterns associated with differentiation. Furthermore, observations in *C. elegans* suggest that the differentiation state may be maintained by the sustained association of chromatin with the NE (Gonzalez-Sandoval et al. 2015), since mutation of the methylated H3K9-binding protein, CEC-4, primarily affects perinuclear gene anchoring and impedes proper cell differentiation but does not alter gene expression (Towbin et al. 2012; Gonzalez-Sandoval et al. 2015). Therefore, delineating the relationship between cell identity and NE-associated chromatin organization will have wide-reaching implications in our understanding of development (Peric-Hupkes et al. 2010) and aging-associated diseases (Berman et al. 2011; Timp and Feinberg 2013; Meaburn 2016; Serebryanny and Misteli 2018).

To elucidate the role of the NE in differentiation, it will be important to comprehensively identify NE-associated protein candidates and changes in chromatin conformation during differentiation processes and mutant proteins must then be experimentally reintroduced to test their effect on cell fate and plasticity. Identifying NE-associated proteins responsible for cell identity may be complicated by the large number of proteins localized to the NE (Korfali et al. 2012), the changes in chromatin organization mediated by mechanoregulation of the nucleus (Uhler and Shivashankar 2017), and the functional redundancy between NE-associated proteins (Amendola and van Steensel 2015). However, unbiased screening approaches to identify functional proteins (Shachar et al. 2015) or analysis of RNA and protein expression differences between cell types may narrow the pool of candidates (Robson et al. 2016). Further clues may be ascertained by studying chromatin reorganization in the presence of disease-causing nuclear envelope protein mutants or loss of NE-associated proteins (Dittmer and Misteli 2011; Chen et al. 2014; Elzeneini and Wickström 2017; Briand et al. 2018; Whitton et al. 2018). To determine if changes in organization have functional implications, inducible protein dimerization systems or protein targeting domains may be used to relocate proteins within the cell (Stanton et al. 2018), whereas artificial tethering arrays, specific targeting sequences, and dCas9 can be employed to reposition chromatin within the nucleus (Reddy et al. 2008; Harr et al. 2015; Morgan et al. 2017). These types of detailed investigations into the functional role of chromatin compartmentalization to the nuclear envelope should reveal general principles of genome regulation and their likely relevance to differentiation and cell fate decisions.

Is disruption of the nuclear lamina architecture a prerequisite for the development of aging-associated diseases?

Nearly half of human deaths are caused by chronic aging-associated diseases, including cancer, diabetes, cardiovascular and neurodegenerative pathologies, as well as chronic lung and kidney diseases (Lopez et al. 2006). At the tissue level, all of these diseases are characterized by an increased accumulation of aged cells (Kubben and Misteli 2017). The functional decline of aged cells has been attributed to the manifestation of various cellular defects, so-called hallmarks of aging, including a disruption of the nuclear lamina architecture, and loss of (epi)genomic, proteostatic and metabolic integrity (López-Otín et al. 2013). While disruption of the nuclear lamina architecture is known to give rise to other hallmarks of cellular aging and to be overall sufficient to trigger cellular aging and several aging-associated pathologies, it remains unknown whether loss of the nuclear lamina structural integrity is a prerequisite for cellular aging and the development of aging-associated diseases (Kubben et al. 2016; Kubben and Misteli 2017). Establishing that disruption of the lamina structure is essential in aging-associated diseases etiology would have major therapeutic implications for the discovery and targeting of cellular pathways that serve to protect the nuclear lamina architecture in the treatment of most leading aging diseases.

A cellular hallmark of aging is the increased lobulation and blebbing of the nuclear lamina, which has been observed across cells of various tissues and these architectural malformations have been observed with increased frequency in various aging-associated diseases, including cancer, Parkinson's disease, and atherosclerosis (Olive et al. 2010; Thaller and Patrick Lusk 2018). No studies have been performed to date to systematically quantify the degree of structural disruption of the nuclear lamina across prevalent aging-associated diseases or to determine the timing of their occurrence in relation to other pathological and clinical hallmarks of these diseases (Kubben and Misteli 2017). It is anticipated that if structural disruption is a requirement for the development of aging-associated diseases, lobulations and nuclear blebbing occur relatively early in the disease process and that the degree of nuclear disruption has predictive power for the severity of pathological developments.

Mouse models provide a powerful complementary experimental strategy to directly assess the role of nuclear lamina architecture in aging-associated disease (Stewart et al. 2007). Age-related nuclear lamina deformities have been attributed to the accumulation of the alternatively spliced lamin A protein progerin in the premature aging disorder HGPS (Scaffidi and Misteli 2006) which is characterized by numerous phenotypes that recapitulate physiological aging pathologies, including atherosclerotic cardiovascular defects

(De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003). It would be important to generate a novel mouse model in which the ability to express progerin is eliminated through mutating its alternative splice site, and to determine whether the loss of progerin is sufficient to improve life- and health span and delays the onset and severity of aging-associated pathologies, when crossed with aging-disease specific mouse models.

Regardless of disease relevance, more insight is needed regarding the pathways that prevent or repair structural damage to the nuclear lamina. A potential strategy to identify such pathways is to perform genome-wide CRISPR screens for genes that modulate nuclear shape across various cell types as well as prevent the formation of aging defects and nuclear dysmorphology upon expression of nuclear structure-disrupting proteins such as progerin. Identified drug-gable pathways that strengthen the nuclear architecture may represent an attractive new class of therapeutics for many aging-associated diseases.

Conclusions

The questions of how the cell nucleus and its genome are organized and the corresponding functional implications are of fundamental importance. We have seen remarkable progress in this still relatively new area of cell biology. As expected for a developing field, much of the effort over the past years has been on describing the structural features of the nucleus and of genomes at various levels using novel imaging and biochemical mapping methods. The future of the field will be in the exploration of how the structural features of the nucleus and genomes relate to their functions. Of particular relevance is the question of what the functional role of the most prominent nuclear features is. Furthermore, while numerous patterns of protein and chromatin organization have been described in the nucleus, the molecular mechanisms that create them are in most cases unknown. Finally, and possibly most importantly, the relevance of nuclear architecture and genome organization to physiological and pathological events needs to be fully elucidated. As is often the case when surveying a field of research, one marvels at the wealth of tantalizing insight that has been generated in recent years from efforts to understand nuclear structure and genome organization, and at the same time one is humbled by how much still remains to be discovered.

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Assessment of the Utility of Gene Positioning Biomarkers in the Stratification of Prostate Cancers

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There is a pressing need for additional clinical biomarkers to predict the aggressiveness of individual cancers. Here, we examine the potential usefulness of spatial genome organization as a prognostic tool for prostate cancer. Using fluorescence *in situ* hybridization on formalin-fixed, paraffin embedded human prostate tissue specimens, we compared the nuclear positions of four genes between clinically relevant subgroups of prostate tissues. We find that directional repositioning of *SP100* and *TGFB3* gene loci stratifies prostate cancers of differing Gleason scores. A more peripheral position of *SP100* and *TGFB3* in the nucleus, compared to benign tissues, is associated with low Gleason score cancers, whereas more internal positioning correlates with higher Gleason scores. Conversely, *LMNA* is more internally positioned in many non-metastatic prostate cancers, while its position is indistinguishable from benign tissue in metastatic cancer. The false positive rates were relatively low, whereas, the false negative rates of single or combinations of genes were high, limiting the clinical utility of this assay in its current form. Nevertheless, our findings of subtype-specific gene positioning patterns in prostate cancer provides proof-of-concept for the potential usefulness of spatial gene positioning for prognostic applications, and encourage further exploration of spatial gene positioning patterns to identify novel clinically relevant molecular biomarkers, which may aid treatment decisions for cancer patients.

Keywords: spatial genome organization, spatial gene positioning, gene positioning biomarkers, prostate cancer, cancer stratification

INTRODUCTION

The genome is highly spatially organized within the interphase nucleus (Cremer and Cremer, 2001; Bickmore, 2013). Most chromosomes, genes, and individual non-coding regions of the genome occupy preferred nuclear positions relative to the center of the nucleus or to other nuclear landmarks, such as associations with other genomic loci or nuclear bodies (Takizawa et al., 2008b; Bickmore and van Steensel, 2013; Meaburn, 2016). Some loci alter their position under different physiological conditions, for example, between cell/tissue types (Boyle et al., 2001; Parada et al., 2004; Peric-Hupkes et al., 2010; Meaburn et al., 2016) or between different proliferation states (Bridger et al., 2000; Meaburn and Misteli, 2008; Chandra et al., 2015). Spatial reorganization of the genome is also

Abbreviations: BAC, bacterial artificial chromosome; EDT, Euclidean distance transform; FISH, fluorescence *in situ* hybridization; FFPE, formalin-fixed, paraffin embedded; GPB, gene positioning biomarker; HSA, human chromosome; KS test, Kolmogorov-Smirnov test; NAT, normal adjacent to tumor; PND, pooled normal distribution; PSA, serum prostate specific antigen; RRD, relative radial distribution; TMA, tissue microarray.

a common feature of disease, and has been documented in a wide range of pathologies, including epilepsy (Borden and Manuelidis, 1988), Down syndrome (Paz et al., 2015), laminopathies (Meaburn et al., 2007; Mewborn et al., 2010), viral and parasitic infections (Li et al., 2010; Knight et al., 2011), and cancer (Meaburn, 2016; Mai, 2018). Repositioning events are loci-specific and do not reflect global genome reorganization events (Meaburn, 2016).

Although the spatial organization of the genome has been studied for decades, how gene positioning patterns are established and maintained remains largely elusive. It is also unclear if the nuclear position of a locus is important for function or is largely a consequence of nuclear activities (Meaburn, 2016). Most often, a functional link is drawn between spatial genome organization and gene expression (Brown et al., 1997; Brickner and Walter, 2004; Williams et al., 2006; Takizawa et al., 2008a; Peric-Hupkes et al., 2010), however, there are also many instances where changes in gene expression and nuclear position of a locus are unrelated (Scheuermann et al., 2004; Williams et al., 2006; Kumaran and Spector, 2008; Meaburn and Misteli, 2008; Harewood et al., 2010; Meaburn, 2016). Most likely, there are multiple mechanisms in play to determine the spatial organization of the genome (Shachar et al., 2015; Meaburn, 2016; Randise-Hinchliff et al., 2016). In addition to gene expression, chromatin modifications, even in the absence of changes in gene expression (Towbin et al., 2012; Therizols et al., 2014; Harr et al., 2016; Cabianca et al., 2019; Falk et al., 2019), replication timing (Hiratani et al., 2008) and a variety of structural nuclear proteins (Dundr et al., 2007; Meaburn et al., 2007; Solovei et al., 2013; Zuleger et al., 2013; Shachar et al., 2015) have been implicated in the positioning of genomic loci.

While the mechanisms governing spatial positioning patterns are unclear, the fact that the genome is spatially reorganized in disease begs the question of whether spatial positioning patterns can be exploited for clinical purposes (Meaburn, 2016; Mai, 2018). We have previously demonstrated that the positioning patterns of some genes can be used to reproducibly and accurately discriminate benign breast and prostate tissues from cancerous ones (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016). For instance, the positioning patterns of *HES5* and *FLI1* are highly indicative of cancer, with both *HES5* and *FLI1* repositioned in 100% of breast cancers and *FLI1* repositioned in 92.9% of prostate cancers, compared to benign tissue controls (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016). High repositioning rates result in low false negative detection rates. Crucially for diagnostic applications, many of the genes that reproducibly reposition in cancer show limited variability between morphologically normal tissues and do not reposition in benign disease, yielding low false positive detection rates (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016). Given the sensitivity and specificity for the positioning patterns of several genes in detecting cancer, these small-scale studies suggest gene positioning biomarkers (GPBs) could be a useful addition to cancer diagnostics.

Prostate cancer is a leading cause of cancer and cancer-related deaths (Bray et al., 2018). As with most cancers, while there is value in additional diagnostic biomarkers, there is also a critical need for additional prognostic biomarkers, to predict best treatment options, including to reduce overtreatment in patients whose cancer would have remained asymptomatic

during their lifetime without treatment (Welch and Black, 2010; Sandhu and Andriole, 2012). Currently, the cornerstone of predicating a patient's outcome is the Gleason grading system, which is based on histological assessment (Epstein et al., 2005; Brimo et al., 2013). In this system the architectural structure of the prostate tissue is graded from Gleason grade 1, which represents a well differentiated tissue morphology, to the very poorly differentiated Gleason grade 5. The two most prominent Gleason grades in a given tumor/biopsy are summed to give a Gleason score (Epstein et al., 2005; Epstein, 2018). Low Gleason score cancers are more likely to be indolent, whereas higher Gleason scores correlates with poor outcomes (Albertsen et al., 1998; Pound et al., 1999; Brimo et al., 2013). However, further markers are required as there is a range in outcomes for patients with the same Gleason score.

To improve on the Gleason system, additional clinical factors, most commonly serum prostate-specific antigen (PSA) levels, T stage (size of tumor/spread to nearby tissues), percentage of cancer positive biopsy cores, and patient age are taken into account (D'Amico et al., 1998; Thompson et al., 2007; Cooperberg et al., 2009; Chang et al., 2014). ~15% of patients are diagnosed with high-risk (likely to cause morbidity, recur, metastasize and/or be lethal) prostate cancer, based on PSA levels of >20ng/ml and/or Gleason score of 8-10 and/or T stage of either T2c-T4 or T3a-T4, depending on the classification system (Thompson et al., 2007; Cooperberg et al., 2009; Chang et al., 2014). Low-risk cancers (PSA <10ng/ml, Gleason score 2-6, and T stage T1-T2a) are generally predicted to remain asymptomatic during the patient's lifetime and the use of active surveillance/watchful waiting is often recommended, as opposed to active treatment (Thompson et al., 2007; Cooperberg et al., 2009). Conversely, intermediate risk (Gleason score 7 or T stage T2b/c) patients generally receive treatment (Thompson et al., 2007; Cooperberg et al., 2010). Gleason scores can be subject to inter- and intra-observer variability, usually of just a single Gleason score (Montironi et al., 2005), but for patients at the border of low and intermediate risk this may make the difference of receiving treatment or not. Moreover, with the current clinical criteria to stratify risk, both over- and under-treatment remains a concern for all prostate cancer risk groups (Cooperberg et al., 2010; Punnen and Cooperberg, 2013). Improved markers are needed to better distinguish indolent from high-risk prostate cancers and to aid classification of intermediate-risk cancers, to reduce overtreatment and optimize therapeutic strategies.

There is a growing number of genomic prognostic biomarkers for prostate cancer, including several commercial assays based on the DNA methylation status of a small number of genes or on gene expression (Kornberg et al., 2018). Additionally, changes to nuclear size and shape and gross chromatin texture, which are not considered in Gleason scoring, provide additional predictive power to detect aggressive prostate cancers (Veltri and Christudass, 2014; Hveem et al., 2016). Few studies have assessed the prognostic potential of the spatial organization of the genome. The most compelling evidence for prognostic GPBs comes from telomeres, where increased telomeric aggregation correlates with progression and risk in several types of cancers (Mai, 2018). Similarly, in a single acute myeloid leukemia patient, HSA8 and 21 became more proximal to each other while the patient was in

remission, prior to a disease relapse and re-emergence of t(8;21) in the patient's bone marrow (Tian et al., 2015).

Here, we explore the utility of spatial gene positioning patterns to identify clinically distinct subgroups of prostate cancer. We find subtype-specific positioning for *SP100*, *TGFB3* and *LMNA*. The direction in which *SP100* and *TGFB3* reposition, compared to benign tissue, distinguishes low and intermediate/high Gleason score cancers, whereas *LMNA* repositions in many non-metastatic cancers but not in metastatic cancers. Although the sensitivity of this assay is currently too low to be clinically useful, our findings of subtype-specific gene positioning patterns in prostate cancer provides additional evidence for the potential of spatial genome organization as a novel prognostic biomarker.

MATERIALS AND METHODS

Tissue Fluorescence *In Situ* Hybridization (FISH)

4–5 µm thick normal, benign disease (hyperplasia and chronic prostatitis), and cancerous formalin-fixed, paraffin embedded (FFPE) human prostate tissues were obtained from US Biomax Inc, Imgenex Corporation, BioChain Institute, or the University of Washington (Prof. Lawrence True) under the guidelines and approval of the Institutional Review Board of the University of Washington (#00-3449) (**Supplementary Table 1**). Patient tissues were de-identified before receipt.

To generate probe DNA for FISH, bacterial artificial chromosome (BAC) clones were labeled with biotin- (Roche), digoxigenin- (Roche) or DY-547P1- (Dyomics GmbH) conjugated dUTPs by nick translation (Meaburn, 2010). The following BACs were used: RP11-727M18 (to position *SP100*, chromosome location: 2q37.1); RP11-270M14 (*TGFB3*, 14q24.3); RP11-1021J5 (*SATB1*, 3p24.3); RP11-35P22 (*LMNA*, 1q22) (BACPAC resource center). Single- or dual-probe FISH experiments were performed as previously described (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016), with the following modifications: for most tissues a 1 hour 60°C bake step was performed, prior to the xylenes (Macron Fine Chemicals) deparaffinization step; 40 µg yeast RNA (Life Technologies) was used in place of tRNA; DyLight 488 labeled anti-digoxigenin (Vector Laboratories) was occasionally used to detect digoxigenin-labeled probe DNA; and no probe detection steps were required for the fluorescently labeled DY-547P1-dUTP FISH probes.

Image Acquisition

Epithelial nuclei were randomly imaged throughout the tissue, unless benign and malignant glands were present in the same tissue section. In such cases, care was taken to image and analyze the different morphologies separately, whilst still acquiring epithelial cell nuclei randomly within the benign or malignant regions to capture as much diversity within the cancer (or benign tissue) as possible. Image acquisition was performed as previously described, using an IX70 (Olympus) Deltavision (Applied Precision) system, with a 60x 1.42N oil objective lens (Olympus), an auxiliary magnification of 1.6, and a X-Y pixel size of 67.25 nm (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016) or with a similar imaging regime

using an IX71 (Olympus) Deltavision (Applied Precision) system, 100x 1.40N oil objective lens (Olympus), with an X-Y pixel size of 64.6 nm. Image stacks were acquired to cover the thickness of the tissue section, with a 0.5 µm or 0.25 µm step interval along the Z axis, respectively. All image stacks were deconvolved and converted to maximum intensity projections using SoftWoRx (Applied Precision). The change in acquisition approach did not affect the resulting positioning data from the image datasets. We obtained similarly statistically identical distributions for the position of a gene in a given tissue using the two different acquisition methods ($P = 0.79$ – 0.86 , Kolmogorov-Smirnov (KS) test), as from repeat analysis of tissues using an identical acquisition method ($P = 0.65$ – 0.99 (Meaburn et al., 2009), unpublished data).

Image Analysis

Image analysis to determine the radial position of a gene within a tissue was performed as previously described (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016). Briefly, 96–167 interphase epithelial nuclei were manually segmented in Photoshop (Adobe) for each gene in each tissue, except for *TGFB3* in tissue C10 where 88 nuclei were segmented. To map the radial position of the gene loci, nuclei were run through custom image analysis software scripts, using MATLAB (The Mathworks Inc.), with DIPImage and PRTools toolboxes [Deft University, P. Gudla and S Lockett (NCI/NIH); (Meaburn et al., 2009)]. Euclidean distance transform (EDT) was computed for each nucleus, to assign every pixel within the nucleus its distance to the nearest nuclear boundary. The software then determined the nuclear EDT position of the geometric gravity center of the automatically detected FISH signals. To normalize for variations in nuclear size and shape between specimens, the EDT of a FISH signal was normalized to the maximal nuclear EDT for that nucleus, with 0 denoting the nuclear periphery and 1 the nuclear center. The normalized FISH signal EDTs for a given gene in each specimen was then combined to produce a relative radial distribution (RRD), and a cumulative frequency distribution was generated. All detected alleles in a nucleus were included, regardless of the number present. In the case of the pooled normal distributions (PNDs), the normalized FISH EDTs from all allele in all the normal tissues analyzed, for a given gene, were combined into a single dataset (**Supplementary Figure 1**). The number of nuclei and tissues used in each PND were as follows: the *SP100* PND contained 845 nuclei from 7 normal tissues; *TGFB3*, 996 nuclei from 8 normal tissues; *SATB1*, 874 nuclei from 7 normal tissues; and *LMNA*, 725 nuclei from 6 normal tissues. Finally, to statistically compare a gene's positioning patterns, RRDs between tissues, or between specimens and the PND, were cross-compared using the nonparametric two-sample 1D KS test, where $P < 0.01$ was considered significant.

Some previously reported RRDs were included in the current analysis [**Supplementary Table 1**; (Leshner et al., 2016; Meaburn et al., 2016)], which were compared to an update PND. The four PNDs used in this study included normal tissues N6 and N7, in addition to the normal tissues previously reported, which did not affect the RRDs ($P = 0.83$ – 1 , 1D KS test). RRDs for *TGFB3* in tissues C25, C27, B9, N3, N4, N11–14 were previously reported in

(Meaburn et al., 2016), and the RRDs of *SP100* in C11, C12, C13, C25, N1, N2, N6-10, *SATB1* in C11, C12, C13, C25, B9, N1, N2, N6-10, and *LMNA* in C11, C18, C19, N5, N10 and N15-16 were reported in (Leshner et al., 2016).

RESULTS

Mapping of Candidate Genes in Prostate Tissues

We have previously identified genes that radially reposition in breast and/or prostate cancer and have demonstrated their potential as diagnostic biomarkers (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016). Here, we sought to extend these studies to determine if candidate genes occupied distinct nuclear positions between different subgroups of prostate cancer, with the goal of assessing their utility for cancer prognostics. To identify prognostic candidate genes we took advantage of our previous studies, in which we had screened the radial positions of 47 genes in a panel of prostate cancers (Leshner et al., 2016; Meaburn et al., 2016). From that gene set we chose two genes, *SATB1* and *LMNA*, for further assessment as potential biomarkers of high-risk prostate cancer because both genes repositioned in a single high-risk T3 stage cancer, but not in two intermediate risk T2 cancers, or a low risk T2 cancer (Leshner et al., 2016). We also selected *SP100* to test its potential as a marker of low risk, since we previously found it to reposition in a low risk Gleason score 6 prostate cancer, but not in three intermediate or high-risk Gleason score 7 cancers (Leshner et al., 2016). Finally, we

selected *TGFB3* for further analysis since it repositioned in one of two low risk Gleason score 6 prostate cancers, but not in two prostate cancers of unknown Gleason score and TNM stage (Meaburn et al., 2016), representing a potential low-risk/indolent prostate cancer biomarker.

To determine whether the positioning patterns of these genes were able to stratify prostate cancers into clinically relevant subgroups, we performed FISH on a panel of 4-5 μ m thick FFPE human prostate tissues, which included a diverse group of 32 prostate cancer specimens covering a range of Gleason scores and T stages, with and without known metastases, and 25 benign prostate tissues (for details see **Supplementary Table 1**). To map the spatial positioning pattern of a gene in a given tissue, we measured the radial position, normalized for nuclear size and shape, of each locus in ~120 epithelial interphase nuclei as previously described (see Materials and Methods; (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016)). The normalized radial position of each gene was determined and the cumulative RRDs were statistically compared to a PND, a standardized normal distribution created by pooling all nuclei from normal tissues for a given gene, or individual tissues using the 1D KS test, with $P < 0.01$ considered significant (see Materials and Methods, (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016), **Supplementary Figure 1**).

We initially assessed the repositioning rates for the candidate genes in the assorted set of prostate cancer samples. Compared to the PND, *SP100* was in a statistically significantly different radial position in 44.4% (12/27) prostate cancer specimens (**Figure 1**, **Table 1**, **Supplementary Table 2**). Similarly, *LMNA*

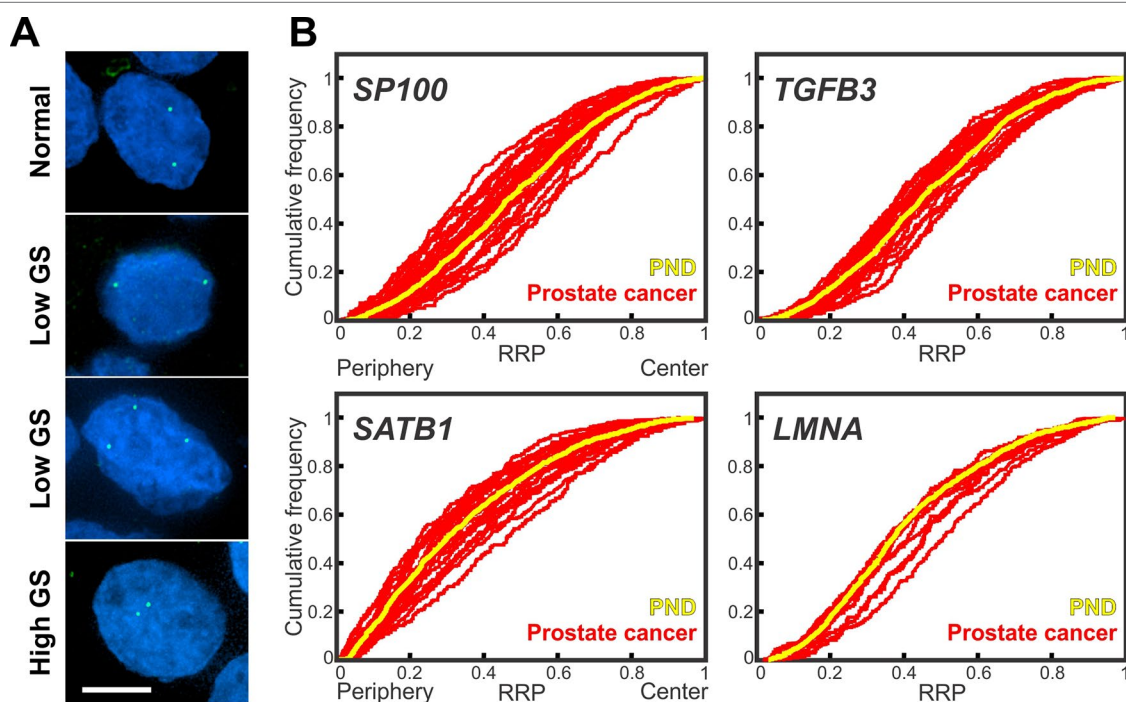


FIGURE 1 | Gene positioning in prostate cancer tissues **(A)** Gene loci were detected by FISH in FFPE prostate tissue sections. *SP100* gene loci (green) in normal and cancerous prostate tissues. GS, Gleason score. Projected image stacks shown. Nuclei were counterstain with DAPI (blue). Scale bar, 5 μ m. **(B)** Cumulative RRDs for the indicated genes in prostate cancer (red) and the pooled normal distribution (PND; Yellow). RRP, relative radial position.

TABLE 1 | Spatial repositioning of target genes in prostate cancer.

Tissue	SP100	TGFB3	SATB1	LMNA	Gleason score	Gleason Grade	TNM	Risk
C1	I				9 (4 + 5)	4	T3N0M1	High
C2					9 (5 + 4)	4-5	T2N1M1c	High
C3			I		9 (5 + 4)	5	T3aN0M0	High
C4	I				8 (4 + 4)	4	T3N0M0	High
C5				I	9 (4 + 5)		T2N0M0	High
C6	P	I	P		9 (4 + 5)		T2N0M0	High
C7		I			9 (4 + 5)		T2N0M0	High
C8		P	P		8 (4 + 4)	4	Unknown	High
C9					7 (3 + 4)	4	T3N0M1b	High
C10					7 (3 + 4)	3	T4N1M1c	High
C11			I	I	7	3	T3N0	High
C12					7	3	T2N0	Intermediate
C13					7	3	T2N0	Intermediate
C14		I	P		7 (3 + 4)		T2N0M0	Intermediate
C15	I		I		7 (3 + 4)	4	T2N0M0	Intermediate
C16	I				7 (3 + 4)	4	T2N0M0	Intermediate
C17	I				7 (3 + 4)		T1N0M0	Intermediate
C18					7		T2N0	Intermediate
C19					7		T2N0	Intermediate
C20	P		I		4 (2 + 2)	2	T4N1M1	High
C21	P	P			3 (1 + 2)	2	T3N1M1	High
C22					6 (2 + 4)	4	T3N0M1b	High
C23					6 (3 + 3)	3	T3N1M0	High
C24	P				6 (3 + 3)	3	T3N0M0	High
C25	P	P			6 (3 + 3)	3	T2N0M0	Low
C26	P	P		I	6 (3 + 3)	3	T2N0M0	Low
C27					6		T2N0	Low
C28	P			I	5 (1 + 4)		T2N0M0	Low
C29					5 (2 + 3)	3	T2N0M0	Low
C30					5 (2 + 3)	3	T2N0M0	Low
C31			I		4 (2 + 2)	2	T2N0M0	Low
C32					3 (1 + 2)	1	T1N0M0	Low

Statistical comparisons of the RRD of a gene in individual cancer tissues to the PND, using the two-sample 1D KS test. Red, significantly different ($P < 0.01$); Blue, statistically similar position ($P > 0.01$). I, a more internal position in the cancer, compared to the PND; P, a more peripheral positioned in the cancer tissue; Red text: mark of aggressive/high risk cancer; blue text: mark of low risk cancer; purple text, intermediate Gleason score. Low risk, Gleason score 2-6 and T1/2 and N0M0; intermediate risk, Gleason score 7 and T1/2 and N0M0; high risk, Gleason score 8-10 and/or T3/4 and/or N1 and/or M1.

repositioned in 36.4% (4/11), *SATB1* in 34.8% (8/23), and *TGFB3* in 31.8% (7/22) of prostate cancer tissues (**Figure 1**, **Table 1**, **Supplementary Tables 3–5**). The repositioning rates are slightly higher than in the previous smaller scale studies (25–33.3%) (Leshner et al., 2016; Meaburn et al., 2016). However, in keeping with previous findings, all four genes repositioned in too few cancers to be of use as prostate cancer diagnostic GPBs since detecting cancer based on the repositioning of the gene would misclassify 55.6–68.2% of the tumors as not cancerous, depending on the gene. The likelihood of a gene repositioning in a cancer did not correlated with gene copy number (**Figure 1**, **Supplementary Table 6**).

In addition to whether a gene was repositioned, the direction of its repositioning was also determined (**Figure 1**, **Table 1**). Of the 12 prostate cancers in which *SP100* was repositioned, the gene was more internally positioned compare to the PND in five cancer tissues (5/12; 41.7%) and more peripherally positioned in seven (58.3%). Similarly, *TGFB3* was more internally positioned in three (3/7; 42.9%) cancers and more peripherally positioned in four (57.1%). *SATB1* was more internally positioned in five of the eight cancers where the gene was repositioned (62.5%) and more peripherally positioned in three cancers (37.5%). Conversely,

LMNA repositioned to a more internal nuclear location in all four cancer specimens in which the gene was repositioned (**Figure 1**, **Table 1**). The direction of repositioning accounted for most of the differences in the positioning patterns for a given gene between the cancer tissues in which repositioning occurred. There was little variation between the RRDs of a gene between the cancers in which the gene was more internally positioned. Similarly, there was little statistical variation in RRDs among cancers in which the gene was more peripherally positioned, with the exception of *SATB1* (**Table 2**, **Supplementary Tables 2–5**). Taken together, we find heterogeneity in the radial positioning patterns for all four candidate genes between prostate cancers.

SP100 and TGFB3 Exhibit Differential Positioning Patterns Between Low and Intermediate/High Gleason Score Cancers

Next, we sought to determine if the differences in gene repositioning patterns between prostate cancers correlated with clinicopathological features. Comparing RRDs between individual cancer tissues was not useful for subgrouping cancers. For the most part, there was a similar proportion of

TABLE 2 | Cross-comparisons between individual tissues % (number) of significantly different cross-comparison among.

	SP100	TGFB3	SATB1	LMNA
Individual cancer tissues	46.7% (164/351)	33.8% (78/231)	42.3% (107/253)	32.7% (18/55)
More I cancers	0% (0/10)	0% (0/3)	0% (0/10)	N/A
More P cancers	9.5% (2/21)	0% (0/6)	66.7% (2/3)	33.3% (2/6)
Individual normal vs cancer tissues	33.3% (63/189)	28.4% (50/176)	22.4% (36/161)	24.2% (16/66)
Individual normal tissues	0.0% (0/21)	21.4% (6/28)	19.0% (4/21)	26.7% (4/15)
Individual benign disease tissues	20% (2/10)	10.0% (1/10)	0.0% (0/10)	0.0% (0/1)
Individual normal vs benign disease	14.3% (5/35)	10.0% (4/40)	5.7% (2/35)	25.0% (3/12)
Individual benign tissues	10.6% (7/66)	14.1% (11/78)	9.1% (6/66)	25.0% (7/28)

Significantly different, based on a KS test, $P < 0.01$; More I cancers, cancers in which the gene is more internally positioned than the PND ($P < 0.01$); More P cancers, cancers in which the gene is more peripherally positioned than the PND ($P < 0.01$); N/A, not applicable.

cross-comparisons between cancers that were significantly different to each other within clinically relevant subgroups as there was between subgroups (for P -values see **Supplementary Tables 2-5**). For example, *SP100* was in a significantly different position in 49.1% (27/55) of cross-comparisons amongst Gleason score 2-6 prostate cancers, and 53.4% (47/88) of cross-comparisons when Gleason score 2-6 cancers were compared to Gleason score 7 cancers, and 50% (44/88) of cross-comparisons between Gleason score 2-6 and Gleason score 8-10 cancers (**Supplementary Table 2**).

In contrast, the behavior of a gene in a cancerous tissue compared to the PND was a better indicator to detect differential positioning between subgroups (**Figure 1**, **Supplementary**

Figure 2, **Tables 1**, **3** and **4**, **Supplementary Tables 2-5**). We first compared positioning patterns to Gleason score. In line with the clinical risk assessment of prostate cancers (Thompson et al., 2007), we classified Gleason scores of 2-6 as a low Gleason score, Gleason score 7 as intermediate, and scores of 8-10 as a high Gleason score. There was a modest increase in the proportion of cancer specimens with either *SATB1* or *LMNA* repositioned, compared to the PND, with increasing Gleason score, however, in the case of *LMNA* this may be due to the small sample size (**Table 3**). *SATB1* was in a statistically different nuclear position in 25% (2/8) of low Gleason score cancers, 33.3% (3/9) Gleason score 7 cancers and 50% (3/6) high Gleason score cancers. Similarly, *LMNA* repositioned in 33.3% of low (2/6) and intermediate

TABLE 3 | Positioning patterns for *SATB1* and *LMNA* by prostate cancer subgroups.

Direction of repositioning:	SATB1 % (number) of cancers SD to the PND			LMNA % (number) of cancers SD to the PND		
	Any	Internal	Peripheral	Any	Internal	Peripheral
All cancers	34.8% (8/23)	62.5% (5/8)	37.5% (3/8)	36.4% (4/11)	100% (4/4)	0% (0/4)
GS 2-6	25.0% (2/8)	100% (2/2)	0% (0/2)	33.3% (2/6)	100% (2/2)	0% (0/2)
GS 7-10	40.0% (6/15)	50.0% (3/6)	50.0% (3/6)	40.0% (2/5)	100% (2/2)	0% (0/2)
GS 7	33.3% (3/9)	66.7% (2/3)	33.3% (1/3)	33.3% (1/3)	100% (1/1)	0% (0/1)
GS 8-10	50.0% (3/6)	33.3% (1/3)	66.7% (2/3)	50.0% (1/2)	100% (1/1)	0% (0/1)
GG1/GG2	66.7% (2/3)	100% (2/2)	0% (0/2)	0% (0/3)		
GG3	12.5% (1/8)	100% (1/1)	0% (0/1)	100% (2/2)	100% (2/2)	0% (0/2)
GG4/GG5	44.4% (4/9)	50.0% (2/4)	50.0% (2/4)	33.3% (1/3)	100% (1/1)	0% (0/1)
Unknown GG	33.3% (1/3)	0% (0/1)	100% (1/1)	33.3% (1/3)	100% (1/1)	0% (0/1)
T1/T2	30.8% (4/13)	50.0% (2/4)	50.0% (2/4)	42.9% (3/7)	100% (3/3)	0% (0/3)
T3/T4	33.3% (3/9)	100% (3/3)	0% (0/3)	25.0% (1/4)	100% (1/1)	0% (0/1)
Unknown T stage	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/0)		
NOM0	37.5% (6/16)	50.0% (3/6)	50.0% (3/6)	57.1% (4/7)	100% (4/4)	0% (0/4)
N1/M1	16.7% (1/6)	100% (1/1)	0% (0/1)	0% (0/4)		
Unknown N/M status	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/0)		
T1/T2 NOM0	30.8% (4/13)	50.0% (2/4)	50.0% (2/4)	50.0% (3/6)	100% (3/3)	0% (0/3)
T3NOM0	66.7% (2/3)	100% (2/2)	0% (0/2)	100% (1/1)	100% (1/1)	0% (0/1)
T4/N1/M1	16.7% (1/6)	100% (1/1)	0% (0/1)	0% (0/4)		
Unknown T/N/M	100% (1/1)	0% (0/1)	100% (1/1)	0% (0/0)		
Low risk	25.0% (1/4)	100% (1/1)	0% (0/1)	66.7% (2/3)	100% (2/2)	0% (0/2)
Int. risk	33.3% (2/6)	50.0% (1/2)	50.0% (1/2)	0% (0/2)		
High risk	38.5% (5/13)	60.0% (3/5)	40.0% (2/5)	33.3% (2/6)	100% (2/2)	0% (0/2)

SD, significantly different, based on 1D KS test ($P < 0.01$); GS, Gleason score; GG, Gleason grade; low risk, Gleason score 2-6 and T1/2 and NOM0; int. risk, intermediate risk (Gleason score 7 and T1/2 and NOM0); high risk, Gleason score 8-10 and/or T3/4 and/or N1 and/or M1.

(1/3) Gleason score cancers, and 50% (1/2) of high Gleason score cancers. Inclusion of the direction of repositioning did little to aid stratification (**Table 3**). Thus, we concluded that neither *SATB1* nor *LMNA* are biomarkers of Gleason score. The proportion of cancers in which *SP100* and *TGFB3* repositioned also did not stratify Gleason score groups. *SP100* was slightly more frequently repositioned in low Gleason score cancers specimens, repositioning in 54.5% (6/11) of low Gleason score cancers, 37.5% (3/8) of intermediate Gleason score cancers, and 37.5% (3/8) of high Gleason score cancers. *TGFB3* repositioned in 30% (3/10) low Gleason score cancer tissues, 20% (1/5) of intermediate Gleason score cancers, and 42.9% (3/7) of high Gleason score cancers. On the other hand, in cancer specimens in which either gene repositioned, the direction of repositioning correlated with Gleason score (**Supplementary Figure 2A, Table 4**). Both *SP100* and *TGFB3* shifted to a more peripheral position in 100% of the low Gleason score cancers in which these genes showed an altered radial position. In contrast, *SP100* and *TGFB3* were in a more internal position in 83.3% (5/6) and 75.0% (3/4), respectively, of the Gleason score 7 and higher cancers in which they repositioned. Intermediate/high Gleason score cancer tissue repositioning is not exclusively more internal, since for both genes a more peripheral positioning was detected in a Gleason score 9 prostate cancer (**Supplementary Figure 2A, Tables 1 and 4**). The positioning patterns of *SP100* and *TGFB3* could not distinguish intermediate Gleason score cancer tissues from high Gleason score cancer tissues (**Supplementary Figure 2A, Tables 1 and 4**).

We also assessed if positioning patterns correlated with Gleason grade. For all four genes, increasing Gleason grade did not correlate with the percentage of cancer specimens in which the genes repositioned (**Tables 3 and 4**). Consistent with Gleason score, the direction that *SATB1* and *LMNA* repositioned did not aid in stratifying cancers by Gleason grade (**Table 3**), but the direction of repositioning did correlate with Gleason grade for *SP100* and *TGFB3* (**Table 4**). As with Gleason score, in the more highly differentiated cancers (Gleason grades 1-3) *SP100* and *TGFB3* repositioned towards the nuclear periphery in 100% (5 and 3 cancers, respectively) of the cancers in which these genes repositioned. Yet, in the poorly differentiated cancers (Gleason grade 4 and 5) both genes preferentially repositioned towards the nuclear interior. *SP100* was more internally positioned in 80% (4/5) of Gleason grade 4 and 5 cancer in which *SP100* repositioned and *TGFB3* was more internally positioned in 66.7% (2/3) of the Gleason grade 4 and 5 cancers in which *TGFB3* was repositioned, compared to the PND (**Table 4**). The similarity between Gleason grade and Gleason score positioning patterns are not surprising given that Gleason score is the sum of the two most prominent Gleason grades (Epstein et al., 2005; Epstein, 2018). An important caveat to be noted is that while the subgrouping of the cancers was based on the most predominant Gleason grade of the tissue, it is not necessarily the predominant Gleason grade of the nuclei analyzed from each specimen.

Collectively, these observations demonstrate that while positioning patterns performed less well than the Gleason system

TABLE 4 | Positioning patterns for *SP100* and *TGFB3* by prostate cancer subgroups.

Direction of repositioning:	<i>SP100</i> % (number) of cancers SD to the PND			<i>TGFB3</i> % (number) of cancers SD to the PND		
	Any	Internal	Peripheral	Any	Internal	Peripheral
All cancers	44.4% (12/27)	41.7% (5/12)	58.3% (7/12)	31.8% (7/22)	42.9% (3/7)	57.1% (4/7)
GS 2-6	54.5% (6/11)	0% (0/6)	100% (6/6)	30.0% (3/10)	0% (0/3)	100% (3/3)
GS 7-10	37.5% (6/16)	83.3% (5/6)	16.7% (1/6)	33.3% (4/12)	75.0% (3/4)	25.0% (1/4)
GS 7	37.5% (3/8)	100% (3/3)	0% (0/3)	20.0% (1/5)	100% (1/1)	0% (0/1)
GS 8-10	37.5% (3/8)	66.7% (2/3)	33.3% (1/3)	42.9% (3/7)	66.7% (2/3)	33.3% (1/3)
GG1/GG2	50.0% (2/4)	0% (0/2)	100% (2/2)	33.3% (1/3)	0% (0/1)	100% (1/1)
GG3	33.3% (3/9)	0% (0/3)	100% (3/3)	28.6% (2/7)	0% (0/2)	100% (2/2)
GG4/GG5	45.5% (5/11)	80.0% (4/5)	20.0% (1/5)	30.0% (3/10)	66.7% (2/3)	33.3% (1/3)
Unknown GG	66.7% (2/3)	50.0% (1/2)	50.0% (1/2)	50.0% (1/2)	100% (1/1)	0% (0/1)
T1/T2	43.8% (7/16)	42.9% (3/7)	57.1% (4/7)	41.7% (5/12)	60.0% (3/5)	40.0% (2/5)
T3/T4	50.0% (5/10)	40.0% (2/5)	60.0% (3/5)	11.1% (1/9)	0% (0/1)	100% (1/1)
Unknown T stage	0% (0/1)			100% (1/1)	0% (0/1)	100% (1/1)
NOM0	47.4% (9/19)	44.4% (4/9)	55.6% (5/9)	33.3% (5/15)	60% (3/5)	20.0% (2/5)
N1/M1	42.9% (3/7)	33.3% (1/3)	66.7% (2/3)	16.7% (1/6)	0% (0/0)	100% (1/1)
Unknown N/M status	0% (0/1)			100% (1/1)	0% (0/1)	100% (1/1)
T1/T2 NOM0	46.7% (7/15)	42.9% (3/7)	57.1% (4/7)	41.7% (5/12)	60.0% (3/5)	40.0% (2/5)
T3NOM0	50.0% (2/4)	50% (1/2)	50.0% (1/2)	0% (0/3)		
T4/N1/M1	42.9% (3/7)	33.3% (1/3)	66.7% (2/3)	16.7% (1/6)	0% (0/1)	100% (1/1)
Unknown T/N/M status	0% (0/1)			100% (1/1)	0% (0/1)	100% (1/1)
Low risk	50.0% (3/6)	0% (0/3)	100% (3/3)	33.3% (2/6)	0% (0/2)	100% (2/2)
Int. risk	50.0% (3/6)	100% (3/3)	0% (0/3)	33.3% (1/3)	100% (1/1)	0% (0/1)
High risk	40.0% (6/15)	33.3% (2/6)	66.7% (4/6)	30.8% (4/13)	50% (2/4)	50% (2/4)

SD, significantly different, based on 1D KS test ($P < 0.01$); GS, Gleason score; GG, Gleason grade; low risk, Gleason score 2-6 and T1/2 and NOM0; int. risk, intermediate risk (Gleason score 7 and T1/2 and NOM0); high risk, Gleason score 8-10 and/or T3/4 and/or N1 and/or M1.

at stratifying cancers, we identify differential gene positioning patterns between subgroups of prostate cancers.

Multiplexing *SP100* and *TGFB3* Improves Detecting Intermediate and High Gleason Score Cancers

Although both *SP100* and *TGFB3* displayed differential positioning patterns between low and intermediate/high Gleason score cancer specimens, the sensitivity for subgrouping prostate cancers by Gleason score based on positioning patterns is low. Using a more peripheral positioning of *SP100* compared to the PND as a marker of low Gleason score cancers, the false negative rate (percentage of cancers without a more peripheral positioning) is 45.4% (5/11 low Gleason score cancers; **Table 1**). For *TGFB3* the false negative rate is even higher at 70% (7/10; **Table 1**). Additionally, using this criterion, false positive cancers were identified for both genes. More peripheral positioning was detected in one high Gleason score specimen for both *SP100* and *TGFB3*, resulting in a false positive rate of 6.3% (1/16) and 8.3% (1/12), respectively, for intermediate and high Gleason score cancers (**Table 1**). Neither gene was more internally positioned in low Gleason score cancers (**Table 1**). Using a more internal positioning pattern as a biomarker of intermediate and high Gleason score cancers resulted in a false negative rate of 62.5% (10/16) and 66.7% (8/12) for *SP100* and *TGFB3*, respectively (**Table 1**).

We have previously demonstrated that the sensitivity of diagnostic GPBs can be improved by multiplexing (Meaburn et al., 2009; Leshner et al., 2016). We therefore evaluated if combining positioning data from *SP100* and *TGFB3* would increase the number of cancers classified as low or intermediate/high Gleason score based on gene positioning patterns. Importantly for multiplexing to improve the sensitivity, *SP100* and *TGFB3* would need to be frequently repositioned in different cancer specimens. Of a subset of 19 cancer tissues in which both genes were positioned, 10 (52.6%) had differential repositioning patterns for *SP100* to that of *TGFB3* (**Table 1**, **Supplementary Table 7**). For six of these cancers *SP100* was repositioned but *TGFB3* was not, whereas in three cancers only *TGFB3* was repositioned. For one cancer sample, both genes were repositioned, compared to their PND, but they relocated in opposite directions, with *SP100* being more peripherally positioned, while *TGFB3* was more internally positioned (**Table 1**, **Supplementary Table 7**). However, multiplexing the two genes did not improve the sensitivity to detect low Gleason score cancers above using *SP100* alone. *SP100* was more peripherally positioned in all five cancers where at least one of the two genes repositioned (**Table 1**, **Supplementary Table 7**). Nevertheless, multiplexing increased the sensitivity of detecting intermediate/high Gleason score cancers (**Table 1**, **Supplementary Table 7**). At least one gene repositioned in eight of the 11 (72.7%) Gleason score 7 and higher cancers. Both *SP100* and *TGFB3* contributed to the increased proportion of cancer specimens with repositioning events. Of the seven cancers with only one of the two genes more internally repositioned, *SP100* was more internally repositioned in four and *TGFB3* more internally positioned in three cancers (**Table 1**, **Supplementary Table 7**).

Using a more internal position of at least one of *SP100* or *TGFB3* the false negative rate for detecting intermediate or high Gleason score cancers was reduced to 36.4% (4/11). While most of the repositioning events in intermediate/high Gleason score cancers were to a more internal position, in one cancer the only repositioning event resulted in a more peripheral location of *TGFB3* and in another cancer tissue, there was both a more peripheral and more internal repositioning events, with *SP100* more peripherally position and *TGFB3* more internally located (**Table 1**, **Supplementary Table 7**).

Gleason score is not a perfect measure of risk. Given the variability in positioning patterns within the same Gleason group, we asked if the positioning patterns could be useful to distinguish aggressive low Gleason core cancers from non-aggressive low Gleason score cancers. Such a marker would aid treatment decisions. However, *SP100* and *TGFB3* were repositioned in a similar proportion of low Gleason score cancers with or without metastasis (**Table 1**). *SP100* repositioned in 50% (2/4) of low Gleason score cancers that had metastasized and 57.1% (4/7) of low Gleason score cancers without metastases. Likewise, *TGFB3* repositioned in 33.3% (1/3) of metastatic low Gleason score cancer specimens and 28.8% (2/7) of non-metastatic low Gleason score cancer specimens (**Table 1**). Thus, in addition to the high false negative rate for Gleason score, *SP100* and *TGFB3* can not distinguish aggressive low Gleason score cancers from non-aggressive low Gleason score cancers, limiting their clinical potential.

Low Gleason Score Cancer Gene Positioning Patterns Are Distinct From Benign Disease

Given the fact that low Gleason score cancers are fairly well differentiated tissues, it is possible that low Gleason score cancers have a similar genome organization to benign disease. We therefore sought to determine the cancer-specificity of the repositioning events. We positioned *SP100*, *TGFB3*, *SATB1* and *LMNA* in non-cancerous prostate tissues (**Figure 2**, **Supplementary Figure 1**, **Tables 5** and **6**, **Supplementary Tables 2–5**). For all four genes we found that the positioning patterns were highly similar between benign tissues. For *SP100*, *TGFB3* and *SATB1*, only 9.1%–14.1% of comparisons between the individual non-cancerous tissues reached significance. There was a little more variability between benign tissues for *LMNA*, where 25% of cross-comparisons between benign tissues were significantly different (**Figure 2**, **Table 2**, **Supplementary Table 2–5**). There was also little repositioning of the four genes in benign tissues when compared to the PND, with repositioning in 7.7%–16.7% of benign tissues, depending on the gene (**Figure 2**, **Tables 5** and **6**). *SP100* was statistically similarly positioned in all seven normal tissues, compared to the PND, but was significantly repositioned in 40% (2/5) of benign disease tissue (**Figure 2**, **Tables 5** and **6**). However, the positioning patterns of *SP100* were distinct in benign disease and low Gleason score cancer, since it was more internally localized in the two benign disease tissues, yet more peripherally located in low Gleason score cancers (**Tables 1** and **5**).

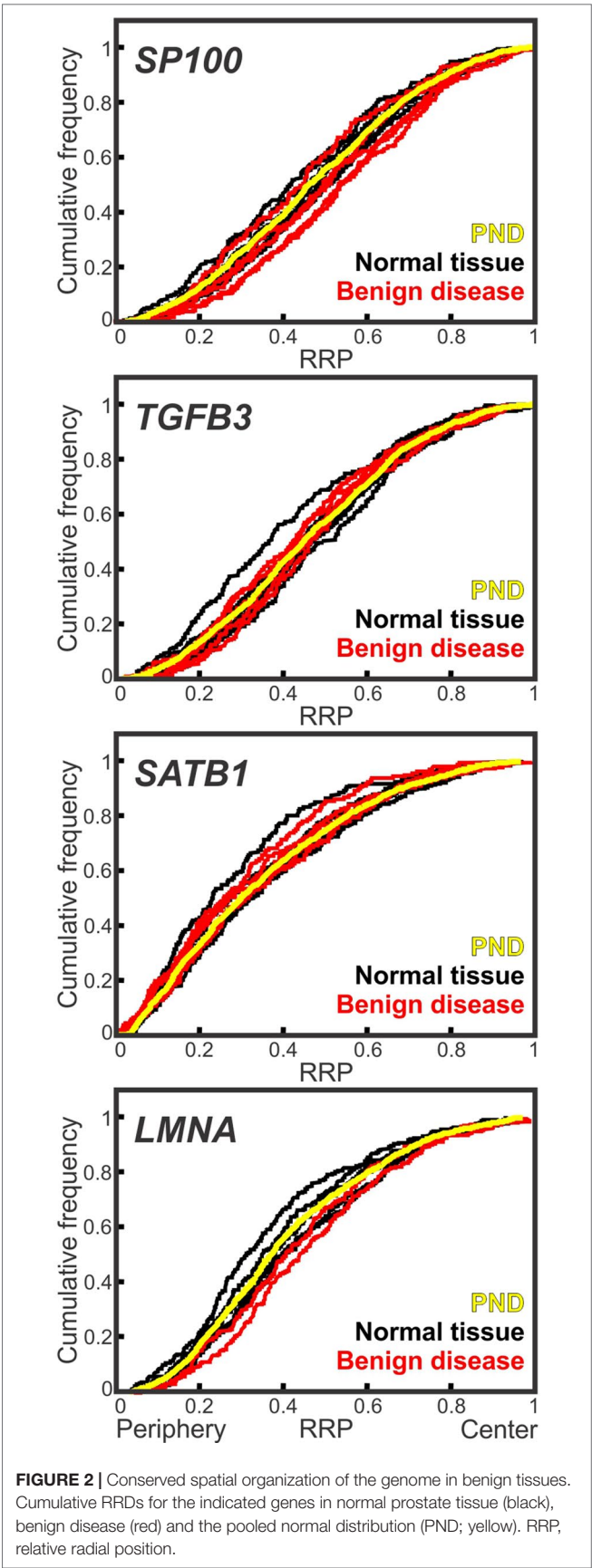


TABLE 5 | Conservation of positioning between normal prostate tissues and in benign disease.

Tissue	SP100	TGFB3	SATB1	LMNA
N1				
N2			P	
N3		P		
N4				
N5				
N6				
N7				
N8				
N9				
N10				
N11				
N12				
N13				
N14				
N15				
N16				
B1	I			
B2				
B3	I			I
B4				
B5				
B6				
B7				
B8				
B9				

Statistical comparisons of the RRD of a gene in individual benign tissues and to the PND, using the two-sample 1D KS test. Red, significantly different ($P < 0.01$); Blue, statistically similar position ($P > 0.01$). N1-16, normal prostate tissue; B1-9, benign disease tissues; I, a more internal position in the benign, compared to the PND; P, a more peripheral positioning in the benign tissue.

Inclusion of the direction of repositioning in the analysis further confirmed the specificity of the repositioning events to the different Gleason score subgroups. When using more peripheral positioning, compared to the PND, as a marker of low Gleason score prostate cancer, the false positive rate for *SP100* is very low, at 3.6%, since it is more peripherally positioned in only one of 28 normal, benign disease and higher Gleason score cancer tissues (Tables 1 and 5). Similarly, *TGFB3* was repositioned in a single normal tissue (12.5%; 1/8) and in none of the benign disease tissues (0/5), compared to the PND (Figure 2, Tables 5 and 6). Unlike *SP100*, the direction *TGFB3* repositioning in the normal tissue was the same as in low Gleason score cancer. However, the false positive rate for using a more peripheral positioning of *TGFB3* was relatively low at 8%, because it was more peripherally positioned in two of the 25 benign tissues and higher Gleason score cancers (Tables 1 and 5). The false positive rate of using a more internal position of *SP100* or *TGFB3* to detect intermediate/high Gleason score cancers is also low, at 8.7% and 0%, respectively, since *SP100* was more internally repositioned in only two of the 23 benign tissues and low Gleason score cancer specimens and *TGFB3* was not more internally repositioned in these groups of tissues ($N = 23$; Table 1 and 5). Taken together, we find the spatial organization of the genome is generally conserved between benign tissues, and benign disease tissue have a distinct genome organization to both low and intermediate/high Gleason score cancers.

TABLE 6 | Comparison of individual benign tissue to the pooled normal.

	<i>SP100</i>	<i>TGFB3</i>	<i>SATB1</i>	<i>LMNA</i>
Normal tissues	0.0% (0/7)	12.5% (1/8)	14.3% (1/7)	0.0% (0/6)
Benign disease	40.0 (2/5)	0.0% (0/5)	0.0% (0/5)	50.0% (1/2)
Total benign tissues	16.7% (2/12)	7.7% (1/13)	8.3% (1/12)	12.5% (1/8)

% (number) of benign tissues where the RRD is significantly different to the PND ($P < 0.01$; KS test).

LMNA Repositions in Low Risk and Non-Metastatic Cancers

Having determined the biomarker potential of the candidate genes for subgrouping prostate cancers by Gleason score, we compared their positioning patterns to other clinical markers of poor patient outcome. The TNM staging system is commonly used to aid the prediction of the aggressiveness of the cancer and the risk of poor patient outcome (Thompson et al., 2007). In stage T1 and T2 prostate cancers the tumor is contained within the prostate, whereas in stages T3 and T4 the cancer has spread from the prostate into the surrounding tissue. T1 and T2 cancers are lower risk cancers and respond better to treatment than T3 and T4 prostate cancers (Thompson et al., 2007; Chang et al., 2014). The N and M score indicates whether the cancer has spread beyond the surrounding tissue. In N0 cancers, no cancer cells are detected in the regional lymph nodes, whereas N1 denotes that the cancer has spread into the regional lymph nodes. For M0 cancers, no distant metastasis are detected, while distant metastases, to non-regional lymph nodes or organs, have occurred in M1 cancers (Thompson et al., 2007).

Positioning patterns for *SP100* and *SATB1* were similar in low and high T stage cancer specimens and could not be used to distinguish the different T stage group cancers from each other (Tables 3 and 4). *TGFB3* and *LMNA* were both more frequently repositioned in low T stage cancers to that of high T stage cancers, but with high false positive rates (Tables 3 and 4). *TGFB3* repositioned in 41.7% (5/12) of T1/2 cancers, 11.1% (1/9) of T3/4 cancers, and 7.7% (1/13) of benign tissues, compared to the PND (Tables 1, 4 and 6). This equates to a false negative rate of 58.3% (7/12) and a false positive rate of 9.1% (2/22) for using the reposition of *TGFB3* to detect low T stage prostate cancer. Similarly, *LMNA* repositioned in 42.9% (3/7) of low T stage cancer, 25% (1/4) of high T stage cancers and 12.5% (1/8) of benign tissues (Tables 3 and 6), making the false negative and positive rates of using the repositioning of *LMNA* to demark low T stage cancers 57.1% (4/7) and 16.7% (2/12), respectively.

Clinically, multiple factors are combined to determine risk of poor outcome. Therefore, we also compared gene positioning patterns with a multifactorial determinant of risk using standard clinical risk assessment criteria (Thompson et al., 2007; Chang et al., 2014) with the exception of PSA levels, since no information on serum PSA were available for our specimens. Moreover, we included N1 and/or M1 cancers in the high-risk group, since they are known aggressive cancers. We classified low risk cancer as Gleason score 2-6 and T1/2 and N0M0 cancers; intermediate risk cancers as Gleason score 7 and T1/2 and N0M0; high risk as Gleason score 8-10 and/or T3/4 and/or N1 and/or M1 prostate

cancers. The positioning patterns of *SP100*, *TGFB3* and *SATB1* were similar in all three risk groups, and thus could not be used as markers of risk (Tables 3 and 4). Conversely, *LMNA* was more frequently repositioned in low risk prostate cancer, since it repositioned in 66.7% (2/3) of low risk cancers, 0% (0/2) of intermediate risk and 33.3% (2/6) of high-risk groups (Table 3).

Finally, as a more direct measure of the aggressiveness of a cancer we compared non-metastatic cancers (N0M0) and metastatic (N1/M1) prostate cancers (Supplementary Figure 2B, Table 1, 3 and 4). *SP100* repositioned, compared to the PND, in a similar proportion of N0M0 (47.4%; 9/19) and metastatic (42.9%; 3/7) cancer specimens. Furthermore, the direction of repositioning was similarly mixed in both groups of cancer (Table 4). The remaining three genes repositioned more frequently in non-metastatic cancers. For *TGFB3* and *SATB1* this difference is small, with the genes repositioned in ~33.3% (5/15 and 6/16 respectively; false negative rate ~66.7%) of non-metastatic cancers and 16.7% (1/6) of metastatic cancers (Table 3 and 4). *LMNA* was the best marker of non-metastatic cancers. *LMNA* repositioned in 57.1% (4/7) of non-metastatic prostate cancer specimens and was not repositioned in metastatic (0/4) cancer tissues (Supplementary Figure 2B, Table 3). As with *SP100* and *TGFB3* as markers of Gleason score, the false negative rate for using *LMNA* positioning as a marker of non-metastatic cancer was high at 42.9% (3/7), and the false positive rate is relatively low at 8.3% (1/12) (Tables 1, 3 and 5).

Taken together, our data suggest that there are distinct spatial gene positioning patterns between some subgroups of prostate cancers, although the false negative rates were generally high, limiting their potential for clinical use.

DISCUSSION

To reduce overtreatment in cancer patients that receive no benefit from medical intervention, there is an urgent need for biomarkers that predict the aggressiveness of a cancer. Here, we assess the feasibility of utilizing the spatial positioning patterns of genes in interphase nuclei for prognostic purposes in prostate cancer. We find a differential enrichment of specific positioning patterns for multiple genes between clinically relevant subgroups of prostate cancers. While the false positive rates for prognostic evaluation are low, the false negative rates are generally high, limiting clinical usefulness. Our results of subtype-specific genome organization patterns suggest that it should be possible to find clinically valuable prognostic GPB by screening additional genes and combinations of genes.

The spatial organization of the genome is altered in diseased cells, and at least some of the changes to genomic spatial positioning

patterns are disease-specific (Meaburn, 2016). For instance, *HES5* repositions in breast cancer, but not in benign breast disease nor prostate cancer (Meaburn et al., 2009; Meaburn et al., 2016). Alternative spatial positioning patterns are not only found in cancers formed in different organs, but there is also heterogeneity in the spatial organization of the genome between individual cancers of the same type (Meaburn et al., 2009; Knecht et al., 2012; Leshner et al., 2016; Meaburn et al., 2016). We hypothesized that heterogeneity within a cancer type may reflect the aggressiveness of a cancer and therefore be of prognostic value. Prognosis-related repositioning of genomic regions in several types of cancer has previously been reported, with increased clustering of telomeres linked to poorer patient outcomes (Mai, 2018). For example, at the time of diagnosis telomeres were more likely to cluster in Hodgkin lymphoma patients whose disease later relapsed or progressed compared to patients who responded well to treatment (Knecht et al., 2012). Currently, Gleason score and the presence or absence of metastasis are key clinicopathological tumor features for predicting the aggressiveness of a prostate cancer. We find that *SP100* and *TGFB3* occupy alternative positions in low Gleason score cancers compared to higher Gleason scored cancers, and that *LMNA* repositions more internally in many non-metastatic and low risk prostate cancers, but infrequently reposition in high risk/aggressive cancers.

Our previous identification of diagnostic GPBs was based on the percentage of cancer specimens in which a gene had an alternative radial position, compared to its distribution in normal tissues (Meaburn, 2016). Interestingly, for *SP100* and *TGFB3* it was not the repositioning itself, but the direction of repositioning that was useful for stratification of prostate cancers. Repositioning of either *SP100* or *TGFB3* towards the nuclear periphery was associated with low Gleason score whereas repositioning towards the interior was a marker of higher Gleason score cancers. The repositioning patterns of *SP100* and *TGFB3* could not distinguish intermediate from high Gleason score cancers. Nevertheless, this does not necessarily rule them out as useful clinical markers since low Gleason score cancers are less likely to receive treatment than intermediate or high Gleason score cancers (Thompson et al., 2007; Cooperberg et al., 2010; Leshner et al., 2016). Given that there is inter- and intra-observer variability when scoring cancers (Montironi et al., 2005), additional markers that can clarify if a cancer is Gleason score 6 (low) or 7 (intermediate) would be useful in guiding therapeutic choices. However, because the positioning patterns of our genes could not separate aggressive, metastatic low Gleason score cancers from non-metastatic low Gleason score cancers, they are unlikely to aid the decision of whether to treat a cancer or not. In keeping with a differential spatial genome organization in cancers above and below the treatment threshold, we previously found *MMP9* to reposition in 20% of low Gleason score cancers compared to 82% of intermediate/high Gleason score cancers (Leshner et al., 2016). Unlike *SP100* and *TGFB3*, the direction *MMP9* repositioned did not aid stratification (Leshner et al., 2016, unpublished data). *MMP9* was positioned predominantly in Gleason score 6 and 7 prostate cancers, making it unclear how specific these positioning patterns are more generally to the different Gleason scores subgroups. *SP100*, *TGFB3* and *MMP9* each map to different chromosomes (HSA 2, 14 and 20, respectively) and therefore represent independent repositioning events within the subgroups of different Gleason score cancers.

In our analysis we find low false positive rates for distinguishing low from intermediate/high Gleason score cancers. In keeping with previous studies (Borden and Manuelidis, 1988; Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016), we find similar positioning patterns for both *SP100* and *TGFB3* amongst normal tissues and between normal and benign disease tissues, highlighting that the gene repositioning in cancer tissues were specific to cancer. Despite the fact that low Gleason score cancers represents fairly well differentiated tissues there were distinct positioning patterns for *SP100* and *TGFB3* between low Gleason score cancers and benign disease, which are considered differentiated tissues. *TGFB3* did not reposition in benign disease and *SP100* was repositioned in only 20% of the benign disease tissues. However, unlike low Gleason score cancers, *SP100* was more internally positioned in benign disease tissues, and therefore does not contribute to the false positive rate when using more peripheral positioning of these genes to detect low Gleason score cancers. Unlike biomarkers used to diagnose cancer, the false positive rate of detecting a subtype of cancer for prognostic purposes is not only generated from non-cancerous tissues, it needs to also include cancers from the alternative subgroups. Even so, the false positive rates of detecting low Gleason score prostate cancers were low because the direction of repositioning for *SP100* and *TGFB3* was mostly specific to the subgroups. In contrast to the false positive rates, the false negative rates for *SP100* and *TGFB3* were high, at 45–70%. We have previously found that for some genes multiplexing reduces the false negative rate and thus increases the sensitivity of detecting cancer (Meaburn et al., 2009; Leshner et al., 2016). Constant with this, we find that multiplexing *SP100* and *TGFB3* reduces the false negative rate of detecting intermediate and higher Gleason score cancers. However, multiplexing with a more peripheral position of either *SP100* or *TGFB3* did not reduce the false negative rate for low Gleason score cancers from that of using *SP100* alone. We conclude that the observed high false negative rates reduce marker strength and the utility of these genes for prognostic purposes.

Even though the positioning patterns of *SP100* and *TGFB3* are inferior to the Gleason system at stratifying cancers, our results reveal subtype-specific genome organization. Similarly, the repositioning of *LMNA* is also subtype-specific, but in this case the repositioning occurs only in non-metastatic cancers, although also with a high false negative rate. Interestingly, the reorganization events between the different subtypes of prostate cancer appear to be gene-specific. *LMNA* and *SATB1* positioning patterns were not able to stratify prostate cancers by Gleason score and *SP100*, *TGFB3* and *SATB1* were not accurate markers of risk or aggressiveness of the cancer. Consistently, the radial repositioning patterns of *FLI1*, *MMP9* and *MMP2* also do not correlate with the risk/aggressiveness of prostate cancer (Leshner et al., 2016). Given that it can take many years after the initial diagnosis of prostate cancer to progress to recurrence, metastasis and/or lethality (Albertsen et al., 1998; D'Amico et al., 1998; Pound et al., 1999; Cooperberg et al., 2009), it will be necessary to analyze specimens with long-term (15+ years) follow-up to accurately assess the potential of spatial positioning for assessment of risk or aggressiveness.

It is unknown what mechanisms lead to the reorganization of the genome in disease, and many processes have been implicated in regulating spatial positioning patterns, including changes in

gene expression, replication timing, chromatin modifications, altered amounts of nuclear proteins, making it likely that the mis-regulations of these cellular functions in cancer cells is related to the spatial mis-organization of the genome (Zink et al., 2004; Meaburn, 2016; Flavahan et al., 2017). The four genes we studied have all been associated with carcinogenesis and have a range of functions. SP100 is a major component of the PML nuclear body and has been implicated in transcription regulation, cellular stress, oxidative stress, telomere length and stability, senescence, apoptosis and DNA damage repair (Lallemend-Breitenbach and de The, 2010). However, most of the evidence for PML bodies role in cancer relates to the PML protein, not SP100, which has not been implicated in prostate cancer. *TGFB3* is a cytokine, with important roles in development, wound healing, the immune response and acts as a tumor suppressor in early cancers but can switch to promoting cancer progression in later stages (Massague, 2008; Lavery et al., 2009). *TGFB3* gene expression levels have been identified as a potential biomarker for prostate cancer, being expressed at lower levels in prostate cancer than normal tissue (Wang et al., 2017). Moreover, *TGFB3* expression levels correlated weakly with both progression-free survival and Gleason score (Wang et al., 2017). SATB1, a nuclear architectural protein that facilitates DNA loop formation and chromatin remodeling (Kohwi-Shigematsu et al., 2013), promotes the progression of many cancers, including prostate cancer, and is overexpressed in high Gleason score cancers compared to low Gleason score cancers and in metastatic compared to non-metastatic prostate cancers (Mao et al., 2013; Shukla et al., 2013; Naik and Galande, 2019). *LMNA* encodes for A-type lamins, proteins that reside predominantly at the nuclear envelope, and have a variety of roles including in nuclear structure, transcription regulation, and spatial genome organization (Dittmer and Misteli, 2011). A-type lamins levels are altered in many types of cancer, with reduced levels often, but not always, linked to a tendency for a poorer prognostic outcome (Meaburn, 2016). It is not currently clear what effect prostate cancer has on A-type lamin protein levels. On the one hand, levels of A-type lamins in prostate cancer have been correlated with poor outcome, with reduced levels associated with an increased risk of lymph node metastasis, and poor outcome in Gleason score 7 and higher prostate cancers (Saarinen et al., 2015). On the other hand, reduced A-type lamin levels in Gleason score 6 cancer compared to high Gleason score cancer, increased A-type lamin levels in cells at the invasive leading edge of prostate cancers, and enhanced migration and invasion in the presence of high A-type lamin levels have been also been reported (Skvortsov et al., 2011; Kong et al., 2012).

Increased cell proliferation is associated with a poor outcome for prostate cancer patients (Berlin et al., 2017), and several genomic loci are differentially positioned between proliferating and non-proliferating cells (Bridger et al., 2000; Meaburn and Misteli, 2008). However, variations in proliferation rate is unlikely to be a major determinant in the gene repositioning we detect. In fact, the vast majority of cells in a prostate cancer tumor are non-proliferating, with a mean of just 6.1% proliferating cells per cancer (Berlin et al., 2017). Furthermore, in a cell culture model of breast cancer, there were distinct genome spatial rearrangements associated with proliferation status to that of carcinogenesis (Meaburn and Misteli, 2008). Similarly, although we find that changes in copy number did not correlated with propensity to reposition (**Supplementary**

Table 6; (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016), we can not fully rule out that structural genomic alterations have not influenced the spatial position of any of the genes in the tissues analyzed since, in some cases, genomic instability can lead to spatial reorganization of the genome (Croft et al., 1999; Taslerova et al., 2003; Harewood et al., 2010; Federico et al., 2019). Regardless, importantly for a clinical test, we find that even in the background of genomic instability it is still possible to use gene positioning to distinguish normal tissue from cancer (Meaburn et al., 2009; Leshner et al., 2016; Meaburn et al., 2016) and to stratify cancers into clinically distinct subgroups, as demonstrated in this study.

Taken together, this study assesses the utility of spatial gene positioning in the stratification of prostate cancers. Our results reveal correlations between gene location and the aggressiveness of a tumor, which may serve as the foundation for prognostic usage of gene positioning. While the genes analyzed here have a relatively high false negative rate of detecting cancer subgroups, our results encourage the exploration of additional candidate genes in larger sample sets for the discovery of spatial genome positioning patterns as prognostic biomarkers.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

KM and TM conceived the study and wrote the manuscript. KM designed the study, performed the experiments, analyzed and interpreted data, and made the figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.01029/full#supplementary-material>.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Figure 1. Pooled normal distribution. Cumulative RRDs for individual normal prostate tissue (black) and the pooled normal distribution (PND; yellow), generated from combining all normal nuclei for a given gene into a single dataset.

Supplementary Figure 2. Subtype specific spatial gene repositioning in cancer. (A) Cumulative RRDs for *SP100* and *TGFB3* in prostate cancer tissues are color coded according to Gleason score: Gleason score 2-6 (blue), Gleason score 7 (purple) and Gleason score 8-10 (red). **(B)** Cumulative RRDs for *LMNA* in prostate cancer tissues are color coded according to metastatic status: Non-metastatic (N0M0) cancers (black), metastatic (N1/M1) cancers (red). In all graphs the pooled normal distribution (yellow) is included for comparison. GS, Gleason score; RRP, relative radial position.

Supplementary Table 1. Characterization of human prostate tissues

Supplementary Table 2. Pairwise statistical comparisons of *SP100* positioning between individual prostate tissues

Supplementary Table 3. Pairwise statistical comparisons of *TGFB3* positioning between individual prostate tissues

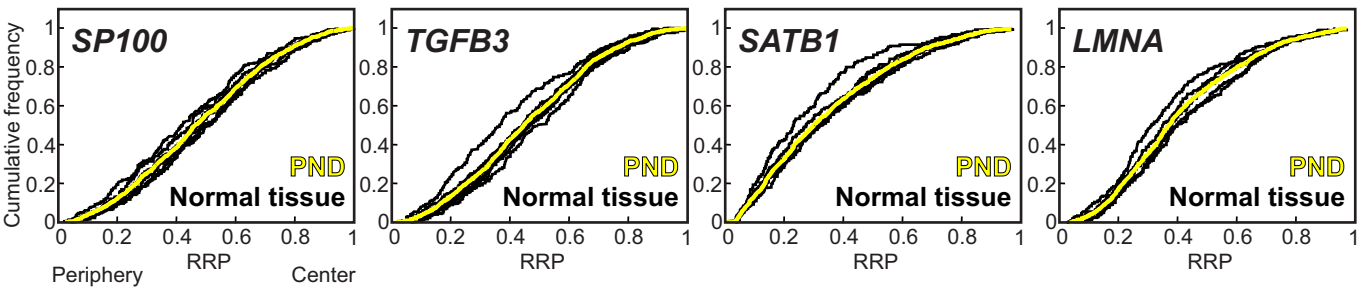
Supplementary Table 4. Pairwise statistical comparisons of *SATB1* positioning between individual prostate tissues

Supplementary Table 5. Pairwise statistical comparisons of *LMNA* positioning between individual prostate tissues

Supplementary Table 6. Repositioning events do not correlate with variations in copy number

Supplementary Table 7. Multiplexing *SP100* and *TGFB3*

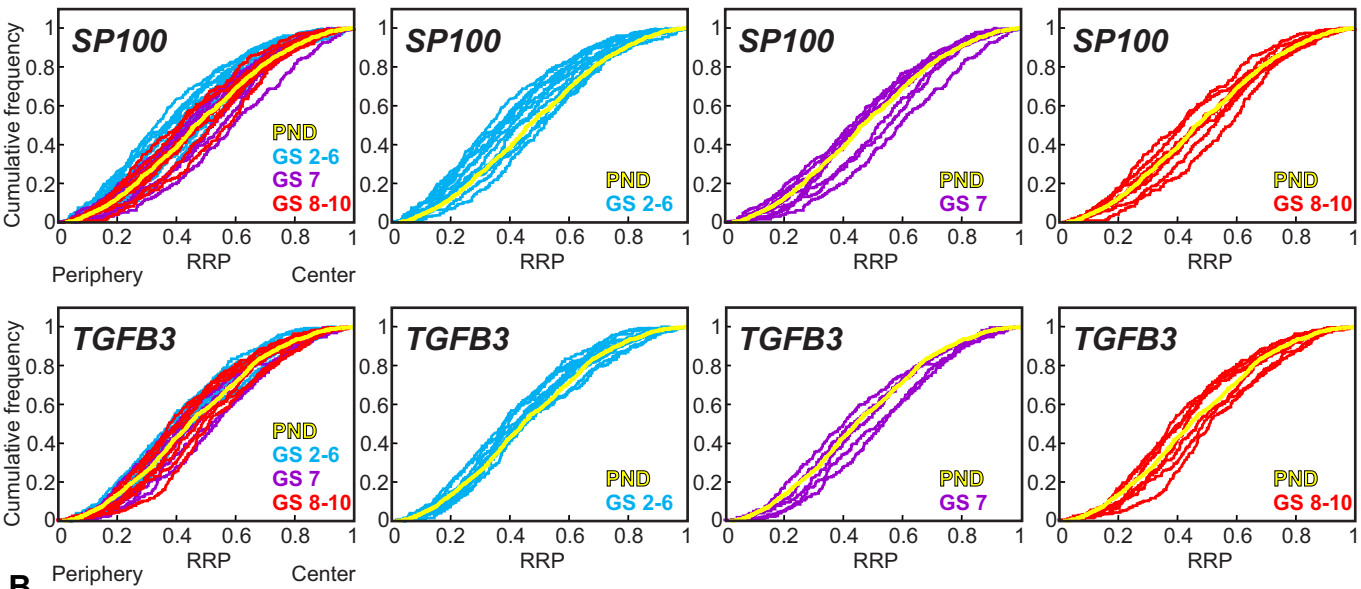
Supplementary Figure 1



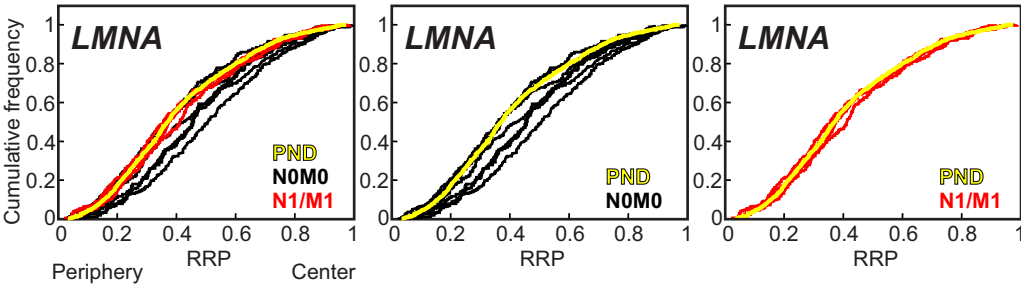
Supplementary Figure 1. Pooled normal distribution. Cumulative RRDs for individual normal prostate tissue (black) and the pooled normal distribution (PND; yellow), generated from combining all normal nuclei for a given gene into a single dataset.

Supplementary Figure 2

A



B



Supplementary Figure 2. Sub-type specific spatial gene repositioning in cancer. (A) Cumulative RRDs for *SP100* and *TGFB3* in prostate cancer tissues are color coded according to Gleason score: Gleason score 2-6 (blue), Gleason score 7 (purple) and Gleason score 8-10 (red). **(B)** Cumulative RRDs for *LMNA* in prostate cancer tissues are color coded according to metastatic status: Non-metastatic (N0M0) cancers (black), metastatic (N1/M1) cancers (red). In all graphs the pooled normal distribution (yellow) is included for comparison. GS, Gleason score; RRP, relative radial position.

Supplementary Table 1 Characterization of human prostate tissues

A: Prostate cancer specimens

Sample ID	Gleason Score	Gleason Grade	TNM	Risk/aggressive	Grade	Stage	Age	Source	TMA	TMA Core/source ID
C1	9 (4+5)	4	T3N0M1	High	3	IV	64	Biomax	PR483c	C7
C2	9 (5+4)	4-5	T2N1M1c	High		IV	75	Biomax	PR243b	C2
C3	9 (5+4)	5	T3aN0M0	High	3	III	66	Biomax	T195e	A4
C4	8 (4+4)	4	T3N0M0	High	2-3	III	70	Biomax	T195e	B6
C5	9 (4+5)		T2N0M0	High	III		47	Biomax	PRC481	E3
C6	9 (4+5)		T2N0M0	High	III		77	Biomax	PRC481	F5
C7	9 (4+5)		T2N0M0	High	III		72	Biomax	PRC481	E2/F2
C8	8 (4+4)	4	Unknown	High	2		82	Biomax	PR808a	B2
C9	7 (3+4)	4	T3N0M1b	High	2	IV	64	Biomax	PR242b	C2
C10	7 (3+4)	3	T4N1M1c	High	2	IV	74	Biomax	PR483c	A1
C11	7	3	T3N0	High			60's	L True	N/A	UW10
C12	7	3	T2N0	Intermediate			50's	L True	N/A	UW9
C13	7	3	T2N0	Intermediate			60's	L True	N/A	UW11
C14	7 (3+4)		T2N0M0	Intermediate	III		72	Biomax	PRC481	F7/E7
C15	7 (3+4)	4	T2N0M0	Intermediate	2	IIA	57	Biomax	PR808a	B9
C16	7 (3+4)	4	T2N0M0	Intermediate	2	IIA	73	Biomax	PR808a	D8
C17	7 (3+4)		T1N0M0	Intermediate	II		60	Biomax	PRC481	D4/C4
C18	7		T2N0	Intermediate			50's	L True	N/A	UW12
C19	7		T2N0	Intermediate			50's	L True	N/A	UW13
C20	4 (2+2)	2	T4N1M1	High	1	IV	75	Biomax	PR242a/b	B2
C21	3 (1+2)	2	T3N1M1	High	1	IV	66	Biomax	PR242a/b	A1/2
C22	6 (2+4)	4	T3N0M1b	High		IV	64	Biomax	PR242a	C2
C23	6 (3+3)	3	T3N1M0	High	2	IV	60	Biomax	PR808a	A7/A8
C24	6 (3+3)	3	T3N0M0	High	2	III	62	Biomax	PR483c	B8
C25	6 (3+3)	3	T2N0M0	Low		II	73	Biomax	N/A	HuCAT371

C26	6 (3+3)	3	T2N0M0	Low		II	58	Biomax	PR242a	B5
C27	6		T2N0	Low			50's	L True	N/A	UW5
C28	5 (1+4)		T2N0M0	Low	I-II		61	Biomax	PRC481	D2/C2
C29	5 (2+3)	3	T2N0M0	Low	1	I	68	Biomax	PR808a	A1/A2
C30	5 (2+3)	3	T2N0M0	Low	1	I	75	Biomax	PR808a	B8
C31	4 (2+2)	2	T2N0M0	Low	1	II	71	Biomax	PR483c	A5
C32	3 (1+2)	1	T1N0M0	Low		I	57	Biomax	T195c	A5

B: Benign prostate tissues

Sample ID	Pathology	Age	Source	TMA	Core/source ID
N1	Normal, absence ca	35	Biomax	N/A	HuFPT143
N2	Normal, absence ca		L True	N/A	UW21
N3	Normal, absence ca	28	Biomax	N/A	HuFPT141
N4	Normal, absence ca	54	Imegenex	N/A	IMH-1035
N5	Normal, absence ca		L True	N/A	UW23
N6	NAT	27	Biomax	PR242a	D4
N7	NAT	25	Biomax	PR242a	D5
N8	NAT	60's	L True	N/A	UW11
N9	NAT	50's	L True	N/A	UW9
N10	NAT	60's	L True	N/A	UW10
N11	NAT		L True	N/A	UW7
N12	NAT		L True	N/A	UW8
N13	NAT	50's	L True	N/A	UW18
N14	NAT	64	Biochain	N/A	T2234201
N15	NAT	50's	L True	N/A	UW12
N16	NAT	50's	L True	N/A	UW13
B1	Chronic prostatitis (inflammation)	54	Biomax	PRC481	B3
B2	Hyperplasia	27	Biomax	PR243b	B3
B3	Hyperplasia	35	Biomax	PR243b	B7
B4	Hyperplasia	84	Biomax	PRC481	A8

B5	Hyperplasia	64	Biomax	PRC481	B7
B6	Hyperplasia	65	Biomax	PRC481	B5/A5
B7	Hyperplasia	65	Biomax	PRC481	B6
B8	Hyperplasia	68	Biomax	PRC481	F1/E1
B9	Hyperplasia		L True	N/A	UW20

Source ID, deidentified specimen identification code from the source of the tissue. Biomax, US Biomax Inc; L.D.T., Dr. Lawrence True; Imgenex, Imgenex corporation; NAT, Normal Adjacent to Tumor; Normal, Normal tissue taken from cancer free prostates; TMA, Tissue microarray. Red: mark of aggressive/high risk cancer. Blue: mark of low risk cancer. Purple, intermediate Gleason score. M1b: metastasis in bone, M1c: Metastasis in another part of the body, with or without bone metastasis. N/A not applicable, single tissue slide. Grey sample ID: RRD's for these tissues previously published (*SP100*, *SATB1*, *LMNA*: Leshner et al., 2016; *TGFB3*: Meaburn et al., 2016), compared to an updated PND (see Materials and Methods).

Sample ID		PND	Normal										Benign disease					Cancer					Cancer																			
			N1	N2	N6	N7	N8	N9	N10	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5	C6	C7	C8	C10	C11	C12	C13	C14	C15	C16	C17	C20	C21	C22	C23	C24	C25	C26	C28	C30	C31	C32	
		1	0.069	0.609	0.318	0.493	0.319	0.589	0.922	9E-04	0.097	0.002	0.141	0.314	1E-05	0.028	0.616	0.008	0.872	0.007	0.636	0.695	0.055	0.258	0.16	0.16	0.163	0.009	3E-04	4E-07	0.001	1E-05	0.018	0.021	6E-05	5E-06	4E-16	5E-06	0.135	0.414	0.527	
Normal	N1	0.069	1	0.063	0.013	0.022	0.656	0.027	0.145	4E-05	0.029	9E-05	0.826	0.015	4E-07	0.228	0.107	8E-05	0.142	0.782	0.099	0.061	0.949	0.01	0.377	0.112	0.641	3E-04	2E-05	7E-08	0.72	0.111	4E-04	0.657	0.165	0.048	1E-04	0.093	0.567	0.028	0.041	
	N2	0.609	0.063	1	0.712	0.748	0.147	0.786	0.824	0.042	0.6	0.081	0.134	0.776	0.007	0.056	0.518	0.239	0.98	0.01	0.845	0.877	0.048	0.596	0.163	0.198	0.161	0.178	0.024	4E-04	0.007	2E-04	0.245	0.048	2E-04	4E-05	7E-09	4E-04	0.131	0.94	0.74	
	N6	0.318	0.013	0.712	1	1	0.704	0.063	0.441	0.278	0.25	0.825	0.083	0.028	0.906	0.017	0.003	0.39	0.377	0.43	0.002	0.191	0.539	0.009	0.1	0.034	0.034	0.051	0.148	0.336	0.005	7E-04	2E-05	0.721	0.007	2E-05	1E-05	2E-11	4E-06	0.058	0.676	0.335
	N7	0.493	0.022	0.748	0.074	0.01	1	0.111	0.684	0.448	0.103	0.692	0.043	0.042	0.807	0.028	0.009	0.572	0.142	0.556	0.004	0.384	0.731	0.024	0.287	0.06	0.035	0.185	0.086	0.173	0.008	0.002	6E-05	0.137	0.007	0.002	2E-05	3E-09	1E-05	0.076	0.746	0.07
	N8	0.319	0.656	0.147	0.063	0.111	1	0.155	0.496	0.922	4E-04	0.017	1E-03	0.929	0.072	4E-05	0.554	0.18	0.009	0.193	0.365	0.039	0.188	0.689	0.114	0.676	0.24	0.778	0.005	6E-04	2E-06	0.385	0.023	0.01	0.734	0.055	0.025	3E-05	0.006	0.876	0.14	0.127
	N9	0.589	0.027	0.786	0.441	0.684	0.155	1	0.551	0.922	0.03	0.172	0.086	0.188	0.962	0.005	0.031	0.367	0.322	0.484	0.005	0.966	0.993	0.038	0.618	0.155	0.329	0.06	0.311	0.028	9E-04	2E-03	0.014	0.518	0.032	1E-04	3E-05	2E-10	5E-04	0.115	0.648	0.504
N10	0.922	0.145	0.824	0.278	0.448	0.496	0.551	1	0.922	0.006	0.109	0.012	0.661	0.37	3E-04	0.172	0.461	0.031	0.665	0.042	0.947	0.749	0.122	0.719	0.646	0.7	0.498	0.041	0.004	2E-05	0.032	9E-04	0.05	0.37	0.002	4E-04	7E-08	0.003	0.443	0.411	0.54	
Benign disease	B1	9E-04	4E-05	0.042	0.25	0.103	4E-04	0.03	0.006	1	0.229	0.68	4E-04	0.058	0.396	6E-06	0.018	0.159	0.013	2E-06	0.007	0.017	2E-05	0.002	2E-04	2E-04	3E-04	0.338	0.43	0.161	3E-07	5E-09	0.119	4E-05	3E-07	2E-09	2E-16	4E-09	7E-05	0.123	0.042	
	B2	0.097	0.029	0.6	0.825	0.692	0.017	0.172																																		

Individual *P*-values for pair-wise statistical comparisons of the RRDs of *SP100* between tissues, using the two-sample 1D KS test. Red denotes a significantly different RRD between the two tissues ($P < 0.01$). Blue denotes a non-significant difference ($P > 0.01$); PND, pooled normal distribution.

Supplementary Table 3: Pairwise statistical comparisons of *TGFB3* positioning between individual prostate tissues

		Normal								Benign disease					Cancer																							
Sample ID	PND	N3	N4	N6	N7	N11	N12	N13	N14	B1	B6	B7	B8	B9	C1	C3	C4	C5	C6	C7	C8	C9	C10	C14	C15	C16	C20	C21	C23	C24	C25	C26	C27	C29	C30	C31		
	PND	1	1E-04	0.851	0.57	0.72	0.467	0.89	0.779	0.044	0.079	0.31	0.216	0.091	0.948	0.048	0.01	0.109	0.089	1E-08	0.001	2E-04	0.314	0.012	2E-05	0.052	0.916	0.134	0.003	0.202	0.621	0.001	3E-05	0.226	0.463	0.229	0.173	
Normal	N3	1E-04	1	6E-04	1E-04	0.023	2E-04	0.004	0.001	2E-06	1E-05	6E-05	0.054	0.022	0.013	0.001	0.196	0.002	5E-06	7E-13	1E-08	0.157	3E-04	4E-06	7E-10	0.387	0.018	3E-05	0.003	0.088	0.012	0.605	0.619	0.004	0.003	0.011	4E-04	
	N4	0.851	6E-04	1	0.8	0.573	0.835	0.704	0.866	0.393	0.499	0.905	0.127	0.068	0.643	0.035	0.011	0.071	0.55	5E-04	0.095	0.005	0.945	0.196	0.008	0.097	0.731	0.1	0.011	0.137	0.554	0.006	0.003	0.772	0.945	0.231	0.283	
	N6	0.57	1E-04	0.8	1	0.479	0.895	0.529	0.987	0.199	0.604	0.999	0.112	0.141	0.548	0.142	0.053	0.111	0.136	0.003	0.348	4E-04	0.512	0.032	0.004	0.026	0.428	0.145	0.004	0.075	0.39	0.003	1E-04	0.333	0.508	0.1	0.696	
	N7	0.72	0.023	0.573	0.479	1	0.279	0.422	0.46	0.082	0.056	0.319	0.879	0.494	0.937	0.339	0.261	0.234	0.03	5E-06	0.01	0.06	0.194	0.009	5E-04	0.485	0.807	0.136	0.105	0.762	0.672	0.067	0.028	0.081	0.184	0.641	0.353	
	N11	0.467	2E-04	0.835	0.895	0.279	1	0.618	0.939	0.39	0.654	0.791	0.065	0.084	0.713	0.067	0.028	0.125	0.639	3E-04	0.098	0.004	0.982	0.223	0.011	0.028	0.409	0.189	0.007	0.043	0.292	0.003	4E-04	0.431	0.725	0.171	0.19	
	N12	0.89	0.004	0.704	0.529	0.422	0.618	1	0.827	0.086	0.236	0.32	0.213	0.088	0.744	0.03	0.014	0.081	0.201	2E-05	0.023	0.004	0.393	0.039	0.004	0.118	0.783	0.083	0.01	0.262	0.678	0.012	0.002	0.267	0.308	0.136	0.346	
	N13	0.779	0.001	0.866	0.987	0.46	0.939	0.827	1	0.157	0.741	0.882	0.295	0.158	0.891	0.303	0.172	0.293	0.537	0.002	0.109	0.035	0.873	0.07	0.003	0.088	0.792	0.613	0.062	0.233	0.648	0.046	0.011	0.654	0.813	0.45	0.403	
	N14	0.044	2E-06	0.393	0.199	0.082	0.39	0.086	0.157	1	0.081	0.182	0.006	0.002	0.094	0.004	2E-04	0.004	0.597	0.061	0.755	5E-06	0.396	0.553	0.135	0.003	0.079	0.004	2E-04	0.005	0.097	7E-05	6E-06	0.226	0.202	0.004	0.014	
Benign Disease	B1	0.079	1E-05	0.499	0.604	0.056	0.654	0.236	0.741	0.081	1	0.851	0.015	0.008	0.165	0.125	0.004	0.044	0.263	0.003	0.113	0.003	0.878	0.017	0.003	0.004	0.119	0.321	0.016	0.013	0.113	0.002	6E-05	0.123	0.274	0.113	0.262	
	B6	0.31	6E-05	0.905	0.999	0.319	0.791	0.32	0.882	0.182	0.851	1	0.07	0.142	0.389	0.1	0.035	0.05	0.326	0.009	0.486	3E-04	0.546	0.051	0.022	0.022	0.259	0.135	0.099	0.468	1	0.727	0.186	0.08	0.079	0.184	0.841	0.068
	B7	0.216	0.054	0.127	0.112	0.879	0.065	0.213	0.295	0.006	0.015	0.07	1	0.992	0.652	0.142	0.519	0.657	0.038	5E-08	0.001	0.257	0.1	0.002	2E-05	0.664	0.797	0.099	0.403	1	0.727	0.186	0.08	0.079	0.184	0.841	0.068	
	B8	0.091	0.022	0.068	0.141	0.494	0.084	0.088	0.158	0.002	0.008	0.142	0.992	1	0.612	0.142	0.797	0.323	0.047	1E-06	0.004	0.1	0.054	6E-04	1E-05	0.729	0.56	0.041	0.247	0.98	0.649	0.216	0.033	0.133	0.075	0.644	0.083	
	B9	0.948	0.013	0.643	0.548	0.937	0.713	0.744	0.891	0.094	0.165	0.389	0.652	0.612	1	0.487	0.243	0.724	0.083	4E-05	0.014	0.08	0.569	0.021	4E-04	0.284	0.989	0.623	0.189	0.602	0.979	0.1	0.027	0.181	0.35	0.922	0.648	
Cancer	C1	0.048	0.001	0.035	0.142	0.339	0.067	0.03	0.303	0.004	0.125	0.1	0.142	0.142	0.487	1	0.087	0.213	0.016	5E-05	0.004	0.007	0.085	3E-04	2E-05	0.052	0.403	0.711	0.097	0.152	0.398	0.021	0.005	0.04	0.029	0.281	0.675	
	C3	0.01	0.196	0.011	0.053	0.261	0.028	0.014	0.172	2E-04	0.004	0.035	0.519	0.797	0.243	0.087	1	0.302	0.013	5E-08	2E-04	0.799	0.043	4E-05	9E-07	0.786	0.198	0.049	0.274	0.47	0.229	0.544	0.493	0.051	0.022	0.576	0.067	
	C4	0.109	0.002	0.071	0.111	0.234	0.125	0.081	0.293	0.004	0.044	0.05	0.657	0.323	0.724	0.213	0.302	1	0.025	7E-08	7E-04	0.118	0.155	6E-04	8E-06	0.068	0.648	0.373	0.697	0.395	0.62	0.026	0.012	0.188	0.072	0.69	0.066	
	C5	0.089	5E-06	0.55	0.136	0.03	0.639	0.201	0.537	0.597	0.263	0.326	0.038	0.047	0.083	0.016	0.013	0.025	1	0.006	0.386	4E-05	0.382	0.303	0.041	0.008	0.186	0.046	0.002	0.027	0.025	4E-04	1E-05	0.482	0.435	0.018	0.017	
	C6	1E-08	7E-13	5E-04	0.003	5E-06	3E-04	2E-05	0.002	0.061	0.003	0.009	5E-08	1E-06	4E-05	5E-05	5E-08	7E-08	0.006	1	0.061	2E-12	0.001	0.034	0.396	2E-09	8E-06	2E-06	2E-09	7E-08	2E-05	3E-10	6E-14	9E-05	3E-05	8E-08	0.004	
	C7	0.001	1E-08	0.095	0.348	0.01	0.098	0.023	0.109	0.755	0.113	0.486	0.001	0.004	0.014	0.004	2E-04	7E-04	0.386	0.061	1	3E-08	0.217	0.119	0.214	1E-04	0.011	0.001	2E-06	3E-04	0.01	2E-06	2E-09	0.05	0.039	2E-04	0.09	
	C8	2E-04	0.157	0.005	4E-04	0.06	0.004	0.004	0.035	5E-06	0.003	3E-04	0.257	0.1	0.08	0.007	0.799	0.118	4E-05	2E-12	3E-08	1	0.002	3E-06	2E-10	0.681	0.167	0.02	0.182	0.205	0.073	0.752	0.654	0.017	0.004	0.207	0.004	
	C9	0.314	3E-04	0.945	0.512	0.194	0.982	0.393	0.873	0.396	0.878	0.546	0.1	0.054	0.569	0.085	0.043	0.155	0.382	0.001	0.217	0.002	1	0.059	0.014	0.025	0.447	0.185	0.019	0.103	0.304	0.01	1E-03	0.358	0.623	0.206	0.096	
	C10	0.012	4E-06	0.196	0.032	0.009	0.223	0.039	0.07	0.553	0.017	0.051	0.002	6E-04	0.021	3E-04	4E-05	6E-04	0.303	0.034	0.119	3E-06	0.059	1	0.34	7E-04	0.048	4E-04	3E-05	0.002	0.008	7E-06	1E-06	0.035	0.089	0.001	0.002	
	C14	2E-05	7E-10	0.008	0.004	5E-04	0.011	0.004	0.003	0.135	0.003	0.022	2E-05	1E-05	4E-04	2E-05	9E-07	8E-06	0.041	0.396	0.214	2E-10	0.014	0.34	1	2E-06	4E-04	3E-06	1E-08	4E-05	6E-04	1E-08	8E-11	0.001	5E-04	1E-05	0.001	
	C15	0.052	0.387	0.097	0.026	0.485	0.028	0.118	0.088	0.003	0.004	0.022	0.664	0.729	0.284	0.052	0.786	0.068	0.008	2E-09	1E-04	0.681	0.025	7E-04	2E-06	1	0.297	0.013	0.154	0.852	0.279	0.353	0.5	0.24	0.175	0.535	0.018	
	C16	0.916	0.018	0.731	0.428	0.807	0.409	0.783	0.792	0.079	0.119	0.259	0.797	0.56	0.989	0.403	0.198	0.648	0.186	8E-06	0.011	0.167	0.447	0.048	4E-04	0.297	1	0.6	0.178	0.802	0.787	0.09	0.089	0.59	0.746	0.917	0.334	
	C20	0.134	3E-05	0.1	0.145	0.136	0.189	0.083	0.613	0.004	0.321	0.135	0.099	0.041	0.623	0.711	0.049	0.373	0.046	2E-06	0.001	0.02	0.185	4E-04	3E-06	0.013	0.6	1	0.24	0.104	0.59	0.005	0.001	0.082	0.138	0.29	0.477	
	C21	0.003	0.003	0.011	0.004	0.105	0.007	0.01	0.062	2E-04	0.016	0.003	0.468	0.247	0.189	0.097	0.274	0.697	0.002	2E-09	2E-06	0.182	0.019	3E-05	1E-08	0.154	0.178	0.24	1	0.358	0.126	0.079	0.026	0.019	0.01	0.268	0.034	
	C23	0.202	0.088	0.137	0.075	0.762	0.043	0.262	0.233	0.005	0.013	0.041	1	0.98	0.602	0.152	0.47	0.395	0.027	7E-08	3E-04	0.205	0.103	0.002	4E-05	0.852	0.802	0.104	0.358	1	0.804	0.404	0.081	0.108	0.075	0.888	0.085	
	C24	0.621	0.012	0.554	0.39	0.672	0.292	0.678	0.648	0.097	0.113	0.291	0.727	0.649	0.979	0.398	0.229	0.62	0.025	2E-05	0.01	0.073	0.304	0.008	6E-04	0.279	0.787	0.59	0.126	0.804	1	0.114	0.04	0.071	0.16	0.657	0.407	
	C25	0.001	0.605	0.006	0.003	0.067																																

Supplementary Table 4: Pairwise statistical comparisons of SATB1 positioning between individual prostate tissues

		Normal								Benign disease					Cancer																						
Sample ID	PND	PND 1	N1	N2	N6	N7	N8	N9	N10	B1	B6	B7	B8	B9	C1	C3	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C20	C21	C23	C24	C25	C26	C28	C31
			0.826	0.004	0.662	0.832	0.846	0.939	0.28	0.197	0.921	0.598	0.081	0.351	0.654	8E-04	0.645	0.005	0.09	0.004	0.53	0.531	0.002	0.036	0.173	0.002	8E-04	0.028	0.101	1E-06	0.014	0.195	0.029	0.358	0.286	0.077	8E-08
Normal	N1	0.826	1	0.01	0.985	0.997	0.503	0.629	0.897	0.167	0.994	0.837	0.047	0.266	0.626	0.055	0.93	0.011	0.708	0.004	0.282	0.79	0.113	0.052	0.188	0.004	0.083	0.03	0.598	0.008	0.039	0.857	0.023	0.761	0.25	0.297	5E-04
	N2	0.004	0.01	1	0.003	0.003	0.048	0.032	0.004	0.009	0.044	0.005	0.238	0.121	0.004	4E-06	0.018	4E-04	6E-05	0.316	0.169	0.012	5E-06	0.147	0.207	0.504	2E-06	0.073	0.001	5E-09	0.023	6E-04	0.816	0.01	0.022	7E-04	7E-10
	N6	0.662	0.985	0.003	1	0.994	0.475	0.365	0.682	0.234	0.975	0.53	0.054	0.362	0.817	0.023	0.806	0.003	0.874	0.026	0.208	0.579	0.07	0.036	0.073	0.013	0.033	0.064	0.423	0.005	0.041	0.992	0.04	0.408	0.239	0.791	2E-04
	N7	0.832	0.997	0.003	0.994	1	0.539	0.566	0.794	0.225	0.871	0.869	0.046	0.222	0.875	0.05	0.912	0.009	0.726	0.022	0.261	0.735	0.072	0.03	0.098	0.01	0.056	0.035	0.471	0.002	0.017	0.944	0.024	0.63	0.24	0.341	2E-04
	N8	0.846	0.503	0.048	0.475	0.539	1	0.702	0.232	0.849	0.794	0.402	0.409	0.924	0.368	0.001	0.533	0.043	0.145	0.218	0.954	0.578	0.005	0.274	0.55	0.183	0.002	0.646	0.065	1E-04	0.307	0.258	0.376	0.307	0.947	0.154	4E-06
	N9	0.939	0.629	0.032	0.365	0.566	0.702	1	0.366	0.288	0.561	0.584	0.194	0.482	0.609	0.032	0.641	0.102	0.146	0.064	0.627	0.604	0.038	0.075	0.261	0.009	0.004	0.07	0.199	2E-04	0.047	0.149	0.064	0.466	0.444	0.09	9E-06
	N10	0.28	0.897	0.004	0.682	0.794	0.232	0.366	1	0.1	0.901	0.775	0.081	0.108	0.458	0.101	0.864	0.009	0.839	1E-03	0.327	0.614	0.259	0.014	0.072	6E-04	0.144	0.008	0.69	0.017	0.014	0.935	0.011	0.545	0.17	0.32	0.001
Benign Disease	B1	0.197	0.167	0.009	0.234	0.225	0.849	0.288	0.1	1	0.458	0.178	0.077	0.615	0.47	7E-04	0.284	0.012	0.057	0.352	0.365	0.164	0.002	0.053	0.14	0.106	0.001	0.81	0.029	6E-05	0.069	0.094	0.122	0.07	0.485	0.371	7E-06
	B6	0.921	0.994	0.044	0.975	0.871	0.794	0.561	0.901	0.458	1	0.847	0.064	0.597	0.866	0.035	0.972	0.034	0.429	0.039	0.31	0.917	0.078	0.16	0.235	0.026	0.086	0.094	0.581	0.005	0.183	0.791	0.184	0.75	0.655	0.404	4E-04
	B7	0.598	0.837	0.005	0.53	0.869	0.402	0.584	0.775	0.178	0.847	1	0.163	0.249	0.686	0.021	0.915	0.09	0.338	0.003	0.354	0.812	0.051	0.062	0.204	0.005	0.016	0.049	0.534	5E-04	0.053	0.343	0.032	0.835	0.325	0.211	5E-05
	B8	0.081	0.047	0.238	0.054	0.046	0.409	0.194	0.081	0.077	0.064	0.163	1	0.767	0.067	6E-04	0.13	0.039	0.009	0.106	0.603	0.261	7E-04	0.293	0.966	0.255	3E-04	0.222	0.02	5E-07	0.348	0.007	0.535	0.257	0.299	0.003	1E-07
	B9	0.351	0.266	0.121	0.362	0.222	0.924	0.482	0.108	0.615	0.597	0.249	0.767	1	0.222	6E-04	0.307	0.024	0.071	0.196	0.69	0.219	0.001	0.287	0.737	0.141	0.002	0.867	0.049	2E-05	0.292	0.134	0.635	0.124	0.796	0.083	3E-07
Cancer	C1	0.654	0.626	0.004	0.817	0.875	0.368	0.609	0.458	0.47	0.866	0.686	0.067	0.222	1	0.064	0.446	0.016	0.762	0.083	0.264	0.502	0.071	0.024	0.148	0.008	0.124	0.097	0.198	0.007	0.011	0.894	0.022	0.348	0.114	0.68	1E-03
	C3	8E-04	0.055	4E-06	0.023	0.05	0.001	0.032	0.101	7E-04	0.035	0.021	6E-04	6E-04	0.064	1	0.03	2E-05	0.162	2E-05	0.005	0.002	0.927	6E-06	5E-04	8E-07	0.679	7E-06	0.163	0.31	4E-05	0.011	8E-06	0.009	1E-04	0.029	0.024
	C5	0.645	0.93	0.018	0.806	0.912	0.533	0.641	0.864	0.284	0.972	0.915	0.13	0.307	0.446	0.03	1	0.044	0.605	0.004	0.733	0.314	0.058	0.057	0.391	0.005	0.038	0.033	0.453	0.005	0.128	0.533	0.03	0.944	0.226	0.437	1E-04
	C6	0.005	0.011	4E-04	0.003	0.009	0.043	0.102	0.009	0.012	0.034	0.09	0.039	0.024	0.016	2E-05	0.044	1	7E-04	2E-05	0.185	0.079	2E-05	0.012	0.089	2E-04	1E-06	0.006	0.009	6E-10	0.301	7E-04	0.002	0.056	0.009	1E-04	5E-10
	C7	0.09	0.708	6E-05	0.874	0.726	0.145	0.146	0.839	0.057	0.429	0.338	0.009	0.071	0.762	0.162	0.605	7E-04	1	8E-04	0.077	0.182	0.168	0.002	0.009	4E-04	0.23	0.004	0.298	0.031	0.002	0.803	0.004	0.205	0.027	0.263	8E-04
	C8	0.004	0.004	0.316	0.026	0.022	0.218	0.064	1E-03	0.352	0.039	0.003	0.106	0.196	0.083	2E-05	0.004	2E-05	8E-04	1	0.072	0.002	6E-06	0.011	0.125	0.692	2E-05	0.497	2E-04	1E-08	0.006	0.006	0.626	0.002	0.075	0.051	3E-08
	C9	0.53	0.282	0.169	0.208	0.261	0.954	0.627	0.327	0.264	0.005	0.733	0.185	0.077	0.264	0.005	0.733	0.185	0.077	0.072	1	0.663	0.004	0.272	0.86	0.078	0.004	0.328	0.073	2E-05	0.527	0.071	0.281	0.436	0.884	0.028	1E-06
	C10	0.531	0.79	0.012	0.579	0.735	0.578	0.604	0.614	0.164	0.917	0.812	0.261	0.219	0.502	0.002	0.314	0.079	0.182	0.002	0.663	1	0.003	0.159	0.436	0.013	0.005	0.051	0.076	1E-04	0.222	0.329	0.062	0.691	0.378	0.078	4E-06
	C11	0.002	0.113	5E-06	0.07	0.072	0.005	0.038	0.259	0.002	0.078	0.051	7E-04	0.001	0.071	0.927	0.058	2E-05	0.168	6E-06	0.004	0.003	1	2E-05	9E-04	1E-06	0.934	1E-05	0.165	0.435	3E-05	0.043	2E-05	0.018	2E-04	0.024	0.042
	C12	0.036	0.052	0.147	0.036	0.03	0.274	0.075	0.014	0.053	0.16	0.062	0.293	0.287	0.024	6E-06	0.057	0.012	0.002	0.011	0.272	0.159	2E-05	1	0.374	0.049	1E-05	0.237	0.001	7E-08	0.212	0.007	0.224	0.063	0.244	0.007	1E-09
	C13	0.173	0.188	0.207	0.073	0.098	0.55	0.261	0.072	0.14	0.235	0.204	0.966	0.737	0.148	5E-04	0.391	0.089	0.009	0.125	0.86	0.436	9E-04	0.374	1	0.064	9E-05	0.211	0.027	7E-06	0.721	0.013	0.379	0.24	0.731	0.011	7E-08
	C14	0.002	0.004	0.504	0.013	0.01	0.183	0.009	6E-04	0.106	0.026	0.005	0.255	0.141	0.008	8E-07	0.005	2E-04	4E-04	0.692	0.078	0.013	1E-06	0.049	0.064	1	2E-06	0.4	2E-04	6E-10	0.016	0.003	0.719	0.006	0.026	0.003	3E-10
	C15	8E-04	0.083	2E-06	0.033	0.056	0.002	0.004	0.144	0.001	0.086	0.016	3E-04	0.002	0.124	0.679	0.038	1E-06	0.23	2E-05	0.004	0.005	0.934	1E-05	9E-05	2E-06	1	2E-05	0.031	0.293	3E-05	0.015	2E-05	0.004	2E-04	0.043	0.059
	C16	0.028	0.03	0.073	0.064	0.035	0.646	0.07	0.008	0.81	0.094	0.049	0.222	0.867	0.097	7E-06	0.033	0.006	0.004	0.497	0.328	0.051	1E-05	0.237	0.211	0.4	2E-05	1	0.003	2E-08	0.068	0.012	0.504	0.024	0.146	0.094	1E-08
	C17	0.101	0.598	0.001	0.423	0.471	0.065	0.199	0.69	0.029	0.581	0.534	0.02	0.049	0.198	0.163	0.453	0.009	0.298	2E-04	0.073	0.076	0.165	0.001	0.027	2E-04	0.031	0.003	1	0.002	0.003	0.42	0.002	0.29	0.011	0.212	9E-05
	C20	1E-06	0.008	5E-09	0.005	0.002	1E-04	2E-04	0.017	6E-05	0.005	5E-04	5E-07	2E-05	0.011	4E-05	0.128	0.301	0.002	0.006	0.527	0.222	3E-05	0.212	0.721	0.016	3E-05	0.068	0.003	3E-08	1	0.007	0.157	0.234	0.291	0.009	5E-10
	C21	0.014	0.039	0.023	0.041	0.017	0.307	0.047	0.014	0.069	0.183	0.053	0.348	0.292	0.011	4E-05	0.128	0.301	0.002	0.006	0.527	0.222	3E														

Supplementary Table 5: Pairwise statistical comparisons of *LMNA* positioning between individual prostate tissues

		Normal						Benign disease		Cancer											
Sample ID	PND	N5	N6	N7	N10	N15	N16	B2	B3	C2	C5	C11	C18	C19	C20	C21	C22	C26	C28	C32	
	PND	1	0.244	0.302	0.617	0.211	0.742	0.01	0.113	5E-04	0.918	1E-11	4E-07	0.943	0.488	0.642	0.666	0.074	9E-04	0.001	0.553
Normal	N5	0.244	1	0.594	0.094	0.789	0.677	0.002	0.789	0.315	0.429	4E-04	0.052	0.488	0.336	0.717	0.131	0.692	0.365	0.185	0.192
	N6	0.302	0.594	1	0.169	0.286	0.716	7E-04	0.426	0.02	0.325	5E-07	3E-04	0.491	0.968	0.975	0.122	0.505	0.013	0.016	0.228
	N7	0.617	0.094	0.169	1	0.112	0.418	0.227	0.098	0.001	0.492	4E-09	9E-06	0.55	0.475	0.369	0.842	0.103	0.003	0.002	0.214
	N10	0.211	0.789	0.286	0.112	1	0.428	0.002	0.466	0.035	0.309	3E-04	0.016	0.363	0.199	0.467	0.393	0.615	0.248	0.11	0.27
	N15	0.742	0.677	0.716	0.418	0.428	1	0.008	0.561	0.039	0.877	6E-06	0.003	0.857	0.71	0.964	0.262	0.321	0.039	0.016	0.335
	N16	0.01	0.002	7E-04	0.227	0.002	0.008	1	7E-04	8E-07	0.028	4E-12	2E-08	0.093	0.011	0.004	0.153	6E-04	3E-05	9E-06	0.087
Benign disease	B2	0.113	0.789	0.426	0.098	0.466	0.561	7E-04	1	0.26	0.236	4E-04	0.053	0.286	0.259	0.431	0.071	0.543	0.072	0.268	0.092
	B3	5E-04	0.315	0.02	0.001	0.035	0.039	8E-07	0.26	1	0.006	0.031	0.785	0.018	0.014	0.017	0.001	0.121	0.012	0.936	0.002
Cancer	C2	0.918	0.429	0.325	0.492	0.309	0.877	0.028	0.236	0.006	1	1E-07	1E-04	0.732	0.778	0.752	0.45	0.121	0.034	0.004	0.725
	C5	1E-11	4E-04	5E-07	4E-09	3E-04	6E-06	4E-12	4E-04	0.031	1E-07	1	0.034	1E-05	7E-06	3E-07	2E-07	3E-04	1E-04	0.065	1E-07
	C11	4E-07	0.052	3E-04	9E-06	0.016	0.003	2E-08	0.053	0.785	1E-04	0.034	1	0.004	0.002	4E-04	4E-04	0.04	0.005	0.989	2E-04
	C18	0.943	0.488	0.491	0.55	0.363	0.857	0.093	0.286	0.018	0.732	1E-05	0.004	1	0.751	0.731	0.888	0.372	0.035	0.021	0.692
	C19	0.488	0.336	0.968	0.475	0.199	0.71	0.011	0.259	0.014	0.778	7E-06	0.002	0.751	1	0.857	0.351	0.37	0.015	0.014	0.325
	C20	0.642	0.717	0.975	0.369	0.467	0.964	0.004	0.431	0.017	0.752	3E-07	4E-04	0.731	0.857	1	0.339	0.493	0.031	0.01	0.378
	C21	0.666	0.131	0.122	0.842	0.393	0.262	0.153	0.071	0.001	0.45	2E-07	4E-04	0.888	0.351	0.339	1	0.227	0.038	0.003	0.976
	C22	0.074	0.692	0.505	0.103	0.615	0.321	6E-04	0.543	0.121	0.121	3E-04	0.04	0.372	0.37	0.493	0.227	1	0.197	0.132	0.18
	C26	9E-04	0.365	0.013	0.003	0.248	0.039	3E-05	0.072	0.012	0.034	1E-04	0.005	0.035	0.015	0.031	0.038	0.197	1	0.08	0.045
	C28	0.001	0.185	0.016	0.002	0.11	0.016	9E-06	0.268	0.936	0.004	0.065	0.989	0.021	0.014	0.01	0.003	0.132	0.08	1	0.007
	C32	0.553	0.192	0.228	0.214	0.27	0.335	0.087	0.092	0.002	0.725	1E-07	2E-04	0.692	0.325	0.378	0.976	0.18	0.045	0.007	1

Individual *P*-values for pair-wise statistical comparisons of the RRDs of *LMNA* between tissues, using the two-sample 1D KS test. Red denotes a significantly different RRD between the two tissues ($P < 0.01$). Blue denotes a non-significant difference ($P > 0.01$); PND, pooled normal distribution.

Supplementary Table 6: Repositioning events do not correlate with variations in copy number

A: Cancer

	SP100	TGFB3	SATB1	LMNA
C1	1: 37.8%; 2: 61.4% ; ≥3: 0.8%	1: 34.3%; 2: 59.4% ; ≥3: 5.9%	1: 49.1% ; 2: 49.1%; ≥3: 1.7%	
C2	1: 22.0%; 2: 74.0% ; ≥3: 4.1%			1: 27.6%; 2: 70.1% ; ≥3: 2.3%
C3	1: 60.1% ; 2: 56.5%; ≥3: 0.8%	1: 42.4% ; 2: 55.0%; ≥3: 2.3%	1: 43.1% ; 2: 52.0%; ≥3: 4.9%	
C4	1: 44.3% ; 2: 54.5%; ≥3: 1.2%	1: 27.5%; 2: 70.0% ; ≥3: 2.5%		
C5	1: 37.7%; 2: 55.7% ; ≥3: 6.6%	1: 25.7%; 2: 67.0% ; ≥3: 7.1%	1: 41.0% ; 2: 50.0%; ≥3: 8.7%	1: 26.7%; 2: 52.6%; ≥3: 20.7%
C6	1: 34.5%; 2: 41.8%; ≥3: 23.4%	1: 30.2%; 2: 38.0%; ≥3: 31.8%	1: 21.8%; 2: 33.9%; ≥3: 44.4%	
C7	1: 29.8%; 2: 45.2%; ≥3: 25.0%	1: 12.5%; 2: 24.0%; ≥3: 63.5%	1: 31.8%; 2: 37.2 %; ≥3: 31.0%	
C8	1: 25.6%; 2: 44.8%; ≥3: 29.6%	1: 14.3%; 27.0%; ≥3: 58.7%	1: 33.1%; 2: 45.7%; ≥3: 21.3%	
C9		1: 31.2%; 2: 67.0% ; ≥3: 1.8%	1: 41.2% ; 2: 52.0%; ≥3: 6.9%	
C10	1: 22.6%; 2: 37.1%; ≥3: 40.1%	1: 27.3%; 2: 33.0%; ≥3: 39.8%	1: 28.8%; 2: 44.9%; ≥3: 26.3%	
C11	1: 24.2%; 2: 42.2%; ≥3: 33.6%		1: 38.0% ; 2: 45.0% ; ≥3: 17.1%	1: 30.4%; 2: 32.6%; ≥3: 37.0%
C12	1: 33.6%; 2: 42.2%; ≥3: 24.2%		1: 41.1% ; 2: 33.9%; ≥3: 24.9%	
C13	1: 44.7% ; 2: 51.2%; ≥3: 4.1%		1: 38.0% ; 2: 39.5%; ≥3: 2.5%	
C14	1: 21.5%; 2: 39.3%; ≥3: 39.2%	1: 44.0% ; 2: 46.6%; ≥3: 9.5%	1: 32.5%; 2: 47.9%; ≥3: 19.7%	
C15	1: 50.0% ; 2: 48.4%; ≥3: 1.6%	1: 32.8%; 2: 67.2% ; ≥3: 0.0%	1: 34.6%; 2: 42.3% ; ≥3: 23.1%	
C16	1: 32.9%; 2: 67.1% ; ≥3: 0.0%	1: 35.6%; 2: 64.4% ; ≥3: 0.0%	1: 41.7% ; 2: 55.4%; ≥3: 2.9%	
C17	1: 55.4% ; 2: 44.6%; ≥3: 0.0%		1: 30.8% ; 2: 45.5%; ≥3: 3.8%	
C18				1: 60.7% ; 2: 30.0%; ≥3: 4.3%
C19				1: 64.4% ; 2: 32.8%; ≥3: 0.9%
C20	1: 22.4%; 2: 28.0%; ≥3: 49.6%	1: 7.5%; 2: 29.2; ≥3: 63.3%	1: 15.7%; 2: 33.1%; ≥3: 51.2%	1: 11.7%; 2: 25.8%; ≥3: 45.8%
C21	1: 39.2%; 2: 57.5% ; ≥3: 3.3%	1: 21.7% ; 2: 65.2% ; ≥3: 13.0%	1: 19.8%; 1: 61.0% ; ≥3: 14.4%	1: 35.2%; 2: 61.4% ; ≥3: 6.4%
C22	1: 29.6%; 2: 63.5% ; ≥3: 7.0%			1: 36.8%; 2: 41.0% ; ≥3: 16.0%
C23	1: 19.8%; 2: 57.8%; ≥3: 22.4%	1: 27.6%; 2: 46.5%; ≥3: 25.9%	1: 23.7%; 2: 55.9%; ≥3: 20.4%	
C24	1: 48.1% ; 2: 49.2%; ≥3: 2.3%	1: 31.1%; 2: 68.9% ; ≥3: 0%	1: 40.4% ; 2: 48.2%; ≥3: 2.7%	
C25	1: 25.0%; 2: 74.2% ; ≥3: 0.8%	1: 35.2%; 2: 54.1% ; ≥3: 10.7%	1: 32.5%; 2: 64.4% ; ≥3: 3.2%	
C26	1: 13.1%; 2: 28.5%; ≥3: 58.5%	1: 12.4%; 2: 28.1%; ≥3: 59.5%	1: 17.7%; 2: 30.6%; ≥3: 51.6%	1: 14.7%; 2: 20.2%; ≥3: 65.1%
C27		1: 36.6%; 2: 63.4% ; ≥3: 5.7%		
C28	1: 56.0% ; 2: 42.4%; ≥3: 1.6%		1: 36.2%; 1: 63.1% ; ≥3: 4.7%	1: 69.4% ; 2: 28.6%; ≥3: 2.0%
C29	1: 30.9%; 2: 43.9%; ≥3: 25.2%			
C30	1: 25.8%; 2: 39.5%; ≥3: 34.7%	1: 31.5%; 2: 37.0%; ≥3: 31.5%		
C31	1: 41.0% ; 2: 58.2%; ≥3: 0.0%	1: 41.0% ; 2: 57.0%; ≥3: 0%	1: 51.2% ; 2: 47.9%; ≥3: 0.8%	
C32	1: 16.1%; 2: 79.8% ; ≥3: 8.1%			1: 25.0%; 2: 68.1% ; ≥3: 4.6%

B: Benign tissues

ID	SP100	TGFB3	SATB1	LMNA
N1	1: 37.1%; 2: 58.9% ; ≥3: 4.0%		1: 45.0% ; 2: 49.2%; ≥3: 5.8%	
N2	1: 40.4% ; 2: 56.0 %; ≥3: 3.7%		1: 49.0% ; 2: 46.1%; ≥3: 4.9%	
N3		1: 40.2% ; 2: 51.2%; ≥3: 8.7%		
N4		1: 37.1%; 2: 58.9% ; ≥3: 4.0%		
N5				1: 42.1% ; 2: 41.3%; ≥3: 6.6%
N6	1: 32.8%; 2: 67.2% ; ≥3: 0.0%	1: 30.5%; 2: 68.0% ; ≥3: 1.6%	1: 32.6%; 2: 65.9% ; ≥3: 1.5%	1: 32.8%; 2: 65.0% ; ≥3: 1.6%
N7	1: 38.3%; 2: 60.2% ; ≥3: 1.5%	1: 23.6%; 2: 74.8% ; ≥3: 1.6%	1: 26.5%; 2: 66.7% ; ≥3: 6.8%	1: 32.8%; 2: 63.9% ; ≥3: 3.3%
N8	1: 49.1% ; 2: 50.4%; ≥3: 0.0%		1: 60.5% ; 2: 36.4%; ≥3: 3.1%	
N9	1: 51.0% ; 2: 46.8%; ≥3: 1.6%		1: 54.8% ; 2: 42.9%; ≥3: 2.4%	
N10	1: 27.9%; 2: 69.8% ; ≥3: 2.3%		1: 45.7% ; 2: 52.8%; ≥3: 1.6%	1: 49.6% ; 2: 49.6%; ≥3: 0.8%
N11		1: 39.9%; 2: 59.0% ; ≥3: 3.5%		
N12		1: 41.8% ; 2: 55.6%; ≥3: 3.2%		
N13		1: 51.0% ; 2: 45.2%; ≥3: 3.8%		
N14		1: 33.3%; 2: 66.6% ; ≥3: 6.8%		
N15				1: 49.1% ; 2: 49.1%; ≥3: 1.7%
N16				1: 41.0% ; 2: 53.8%; ≥3: 4.3%
B1	1: 37.0%; 2: 60.6% ; ≥3: 2.4%	1: 11.4%; 2: 88.6% ; ≥3: 0.0%	1: 62.2% ; 2: 36.9%; ≥3: 0.9%	
B2	1: 36.2%; 2: 63.0% ; ≥3: 0.8%			1: 44.0% ; 2: 48.4%; ≥3: 7.1%
B3	1: 22.5%; 2: 69.8% ; ≥3: 7.8%			1: 21.5%; 2: 74.6% ; ≥3: 3.8%
B4	1: 60.0% ; 2: 38.2%; ≥3: 0.9%			
B5	1: 46.2% ; 2: 52.1%; ≥3: 1.7%			
B6		1: 27.0%; 2: 69.0% ; ≥3: 4.0%	1: 63.0% ; 2: 36.0%; ≥3: 1.0%	
B7		1: 21.0%; 2: 78.2% ; ≥3: 0.8%	1: 29.8%; 2: 68.4% ; ≥3: 1.8%	
B8		1: 26.0%; 2: 73.4% ; ≥3: 1.6%	1: 56.0% ; 2: 43.1%; ≥3: 0.9%	
B9		1: 47.7% ; 2: 52.3%; ≥3: 0.0%	1: 49.6%; 2: 48.9% ; ≥3: 1.5%	
PND	1: 38.3%; 2: 60.2% ; ≥3: 1.5%	1: 36.8%; 2: 59.0% ; ≥3: 4.2%	1: 42.8% ; 2: 53.9%; ≥3: 3.3%	1: 43.0% ; 2: 53.9%; ≥3: 3.0%
	7N, 854 nuc	8N, 996 nuc	7N, 874 nuc	6N, 725 nuc

gene SD compared to PND (1D KS test $P < 0.01$)
 gene NSD compared to PND
 1: x%; 2: x%; ≥3: x% % nuclei with the given number of FISH signals

A comparison of the incidence of gene repositioning with the number of FISH signals detect for a gene in a given tissue. Black box: gene was repositioned compared to the PND ($P < 0.01$). Grey box: gene was not repositioned compared to the PND. C1-32: prostate cancer specimens, N1-16, normal prostate tissues and B1-9, benign disease tissues. Copy numbers are denoted by 1: x%; 2: x%; ≥3: x%; where X% of nuclei with the given number of signals (1, 2, or ≥3 FISH signals per nuclei). Yellow text highlights of the tissue had an amplification (>20% of nuclei had 3 or more FISH signals), loss of copy number (>40% of nuclei had only 1 FISH signal), or was diploid (rest of the nuclei), based on the classification system we have previously used (Meaburn et al., 2009; Leshner et al., 2016; Meaburn K.J. et al., 2016). N.B. Incomplete nuclei in X-Y (e.g. nuclei at the edge of the projected image) where not included for analysis, but since 4-5µm thick tissue sections were used the full thickness of some nuclei may not have been imaged.

Supplementary Table 7: Multiplexing *SP100* and *TGFB3*

Sample ID	<i>SP100</i>	<i>TGFB3</i>	Gleason Score	TNM
C1	I		9 (4+5)	T3N0M1
C3			9 (5+4)	T3AN0M0
C4	I		8 (4+4)	T3N0M0
C5			9 (4+5)	T2N0M0
C6	P	I	9 (4+5)	T2N0M0
C7		I	9 (4+5)	T2N0M0
C8		P	8 (4+4)	unknown
C10			7 (3+4)	T4N1M1c
C14		I	7 (3+4)	T2N0M0
C15	I		7 (3+4)	T2N0M0
C16	I		7 (3+4)	T2N0M0
C20	P		4 (2+2)	T4N1M1
C21	P	P	3 (1+2)	T3N1M1
C23			6 (3+3)	T3N1M0
C24	P		6 (3+3)	T3N0M0
C25	P	P	6 (3+3)	T2N0M0
C26	P	P	6 (3+3)	T2N0M0
C30			5 (2+3)	T2N0M0
C31			4 (2+2)	T2N0M0

Positioning patterns of *SP100* and *TGFB3* in cancer tissues (C1-C31), compared to the PND. Only cancers with both genes positioned shown (see Table 1). I denotes a more internal position in the cancer compared to the PND, P denotes a more peripheral position. Red boxes indicate gene is repositioned compared to the PND ($P < 0.01$; KS test). Blue boxes indicate similar radial position to the PND ($P > 0.01$). Yellow boxes highlight the cancers with one of the two genes repositioned. Orange box highlights the cancer in with the two genes reposition, but in different directions.