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Androgen Deprivation Therapy through
Spatial Genomics and Imaging Heterogeneity

PRINCIPAL INVESTIGATOR: Dr. Scott Wilkinson

CONTRACTING ORGANIZATION: The Geneva Foundation, Tacoma, WA

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14. ABSTRACT Objective: Patients enrolled in our clinical study of GnRH agonist plus enzalutamide for six months prior to radical prostatectomy have shown a diversity of responses, including differential responses within the same patient (i.e. one region of tumor responds and another region does not). Based on these initial clinical outcomes, we hypothesize that the specific genomic alterations that arise during tumor development can distinguish exceptional from poor responders to this intense neoadjuvant ADT. We will also test the hypothesis that patients who fail to respond to this intense therapy do so through either pre-existing genomic alterations, adapt to survive with lower levels of androgen receptor activity, or a combination of both. Impact: Further understanding of the genetics of prostate cancer underlying response to treatment is critical to guide patient-specific treatments based on genetic status to improve overall survival. By understanding the genetic factors responsible for either exceptional or failed response to treatment, we will define the biology of lethal prostate cancer to reduce death, while also developing treatments that improve outcomes for men with lethal prostate cancer.					
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1. INTRODUCTION:

Although a large number of prostate cancer patients exhibit a non-aggressive (indolent) form of disease, among those with aggressive disease, treatment options include radiation therapy targeted to the pelvis or radical prostatectomy survey to remove the prostate. Given that newer agents such as abiraterone and enzalutamide have shown promise in the metastatic setting, there is increasing interest in treatment with hormone therapy (neoadjuvant androgen deprivation therapy followed by surgery) while definitive therapy may still be curative. Although several factors, including age, family history, PSA levels, and, increasingly, genetic or genomic test are considered before deciding on surgery, there is currently no clear indication what can predict how a patient might respond to neoadjuvant therapy. This study uses correlative samples from a clinical trial of neoadjuvant enzalutamide at the National Cancer Institute, in which men enrolled on the study receive multiparametric MRI (mpMRI) screening and undergo a pre-treatment targeted biopsy to regions identified on mpMRI. A second mpMRI is performed after treatment, before surgery. The first objective of this study is to ask whether genetic features identified in a pre-treatment biopsy would indicate whether a tumor is likely to respond to treatment. The second objective is to use mpMRI imaging with tissue analysis to create a prostate cancer profile that would allow clinicians to better screen patients prior to treatment.

2. KEYWORDS:

Prostate cancer, androgen deprivation therapy, ADT, neoadjuvant, genomics, tumor heterogeneity, evolution, diversity, immunohistochemistry, enzalutamide

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aims: The aims of the study are to:

- 1) Assess genetic features of exceptional response and resistance to neoadjuvant anti-androgen therapies
- 2) Correlate mpMRI features to multiplex histopathological analysis of pre- and post-treatment tissue

What was accomplished under these goals?

Specific Aim 1: Assess genetic features of exceptional response and resistance to neoadjuvant anti-androgen therapies			
Major Task 1: Biopsy Tumor Mapping and DNA Analysis	Original Timeline	NCI	Status:
Subtask 1: IRB approval and HRPO review/approval	1-4	Dr. Wilkinson	Complete
Subtask 2: Acquisition of ≥ 37 biopsy and corresponding RP specimens from patients enrolled in neoadjuvant enzalutamide trial	5-18	Dr. Wilkinson	Complete
Subtask 3: IHC and tumor mapping on cases	5-18	Dr. Wilkinson	Complete

<i>Milestone Achieved: Tumor mapping for Specific Aim 1</i>	18		Complete
Subtask 4: Tissue microdissection and sequencing analysis	5-20	Dr. Wilkinson	Complete
<i>Milestones Achieved: Identification of genomic marks that predict response to anti-androgen therapy; publication of 1-2 peer-reviewed papers</i>	5-20		Complete
Specific Aim 2: Correlate mpMRI features to multiplex histopathologic analysis of primary prostate disease			Complete
Major Task 2: Mapping RP to corresponding biopsy, IHC	Months	NCI	Complete
Subtask 1: Correlate immunohistologic profile of targeted pre-treatment biopsy with mpMRI lesions	5-18	Dr. Wilkinson	Complete
Subtask 2: Predict efficacy of neoadjuvant ADT by tuning mpMRI to histologic features of RP specimens	12-24	Dr. Wilkinson	Complete
<i>Milestones Achieved: Correlate mpMRI tumor burden with histological tumor burden; publication of 1-2 peer-reviewed papers</i>	5-24	Dr. Wilkinson	Complete

Major Activities:

The framework of this proposal relied upon a correlative study to a then-ongoing clinical trial examining men with intermediate and high-risk features of prostate cancer that were treated with enzalutamide + GnRH antagonist for 6 months. The trial completed accrual in late 2019. Through the duration of the award, we worked with radiologist Dr. Baris Turkbey to map tumor burden and correlate pre-treatment targeted biopsies with post-treatment residual tumor. We performed IHC to characterize both pre-treatment biopsies and post-treatment residual tumor, before performing laser capture microdissection to isolate ultra-pure tumor populations for whole exome sequencing and RNAseq. Copy number alterations, mutation calling, percent genome altered, and genotypic and phenotypic heterogeneity were identified. Tumors were stratified into Exceptional Responders and Incomplete/Non-Responders, and a 4 factor model was developed to predict tumor treatment response based on pre-treatment biopsy specimens.

Specific Objectives:

The objectives of the project are to 1) Assess genetic features of exceptional response and resistance to neoadjuvant anti-androgen therapies; and 2) Correlate mpMRI features to multiplex histopathologic analysis of pre- and post-treatment tissue.

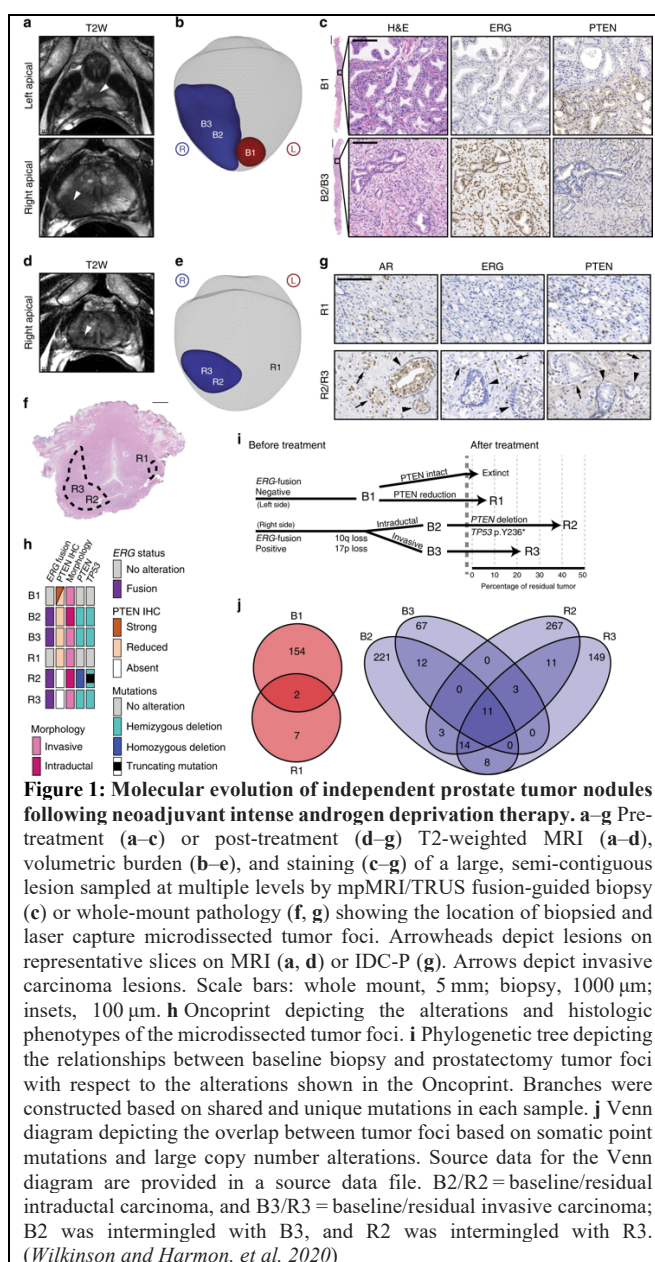
The first objective seeks to examine tumor specimens from patients enrolled in a clinical trial of neoadjuvant androgen deprivation therapy, where pre-treatment targeted biopsy specimens and post-treatment radical prostatectomy are analyzed to determine genomic features unique to tumor samples that exhibit exceptional response to therapy (measured by a residual cancer burden/RCB of $< 0.05\text{cc}$), or incomplete/non-responders ($\text{RCB} > 0.05\text{cc}$). By identifying features unique to these separate cohorts, we are able to better predict whether a tumor sample is likely to

respond to neoadjuvant androgen deprivation therapy.

The second objective aims to correlate histology of pre-treatment biopsies with mpMRI lesions. While mpMRI is a non-invasive method to detect prostate lesions, the resolution provided is inadequate to detect minor lesions. By using our histopathology generated from this trial, we have been able to compare histology with mpMRI lesions in an attempt to better define these lesions. This objective aims to develop a novel multiplex immunofluorescence staining panel to stain six proteins on one slide, which will then be scanned to determine distinct staining patterns. We would then register each stain to its corresponding mpMRI lesion, which would allow detection of potential subclonal tumor populations that are not visible on mpMRI imaging alone.

Significant Results:

By mapping pre-treatment biopsy to post-treatment residual cancer burden, we were able to utilize a combination of genomics, histology, and imaging to track individual tumor clones over the course of treatment. In one patient, we were able to combine these multiple features to identify multiple, independent tumor subclones at baseline, which exhibited divergent response to androgen deprivation therapy (Wilkinson and Harmon, 2020). Of the pre-treatment clones, one clone exhibited nuclear ERG staining on histology and TMPRSS2:ERG fusion, while the other clone did not. The non-responsive tumor clone also exhibited chromosome 10q and 17p loss. During the course of treatment, the resistant clone developed *PTEN* deletion and a *TP53* mutation, resulting in a residual tumor burden of 0.35 cc of the resistant clone. For the tumor clone that was responsive to ADT, TMPRSS2:ERG fusion was never identified. During the course of treatment, the responsive clone separated into two subclones: one with *PTEN* intact, which went extinct during treatment, while the other subclone exhibited *PTEN* reduction (but not deletion). This reduction in *PTEN* status resulted in a resistant tumor, although with a markedly reduced residual tumor burden of 0.01 cc (Figure 1). These findings indicate that spatiogenomic variability is a major confounder for personalized treatment of patients with prostate cancer.

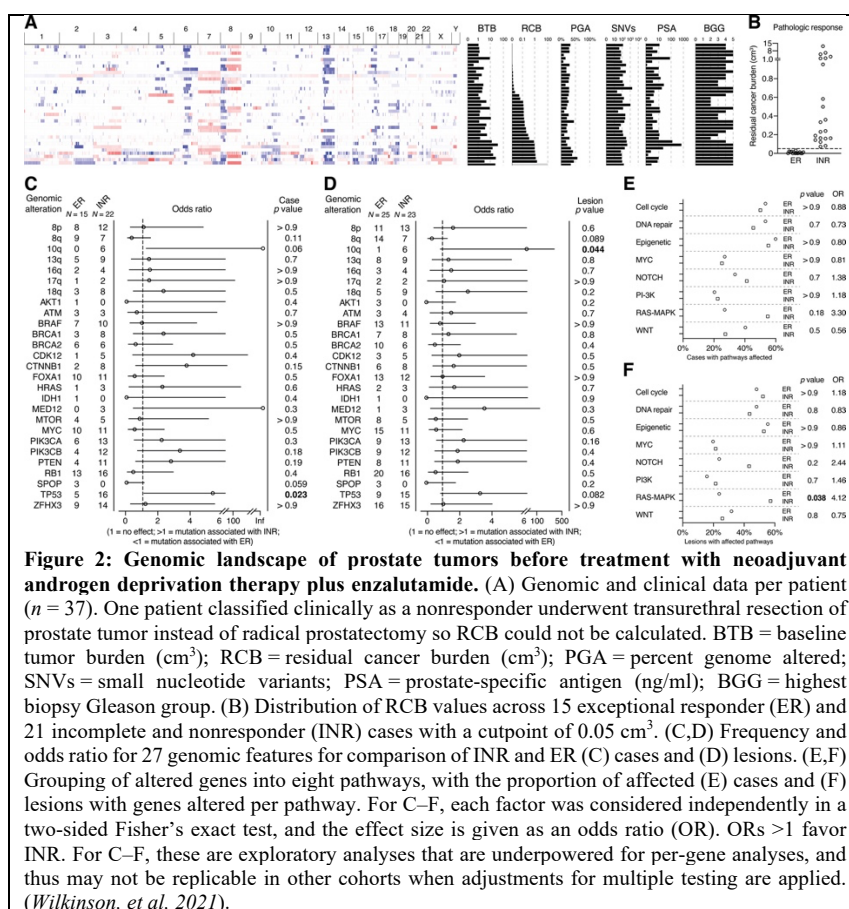


Across the entire cohort, we utilized the combination of genomics, histology, and imaging to identify tumors as “Exceptional Responders” (ER) or Incomplete/Non-Responders (INR). We examined 141 tumor foci across 48 distinct MRI-contoured lesions from 37 patients who completed the study (Wilkinson, 2021). Using a panel of 27 common genomic alterations in prostate cancer, we examined specific characteristics between the exceptional vs incomplete/non-responder patients. At a patient level (using the index lesion for each patient), this panel showed significant alterations in genomic

TP53 status between the two cohorts ($p = 0.023$) (Fig 2). Although not significantly different between the two cohorts at a patient level, when examining these alterations at the lesion level, loss of chromosome 10q (containing the *PTEN* locus) was significantly altered between the two cohorts ($p = 0.044$) (Figure 2). Taken together, we see very few genomic characteristics indicative of aggressive prostate cancer significantly altered between the two cohorts at baseline that would predict response to therapy.

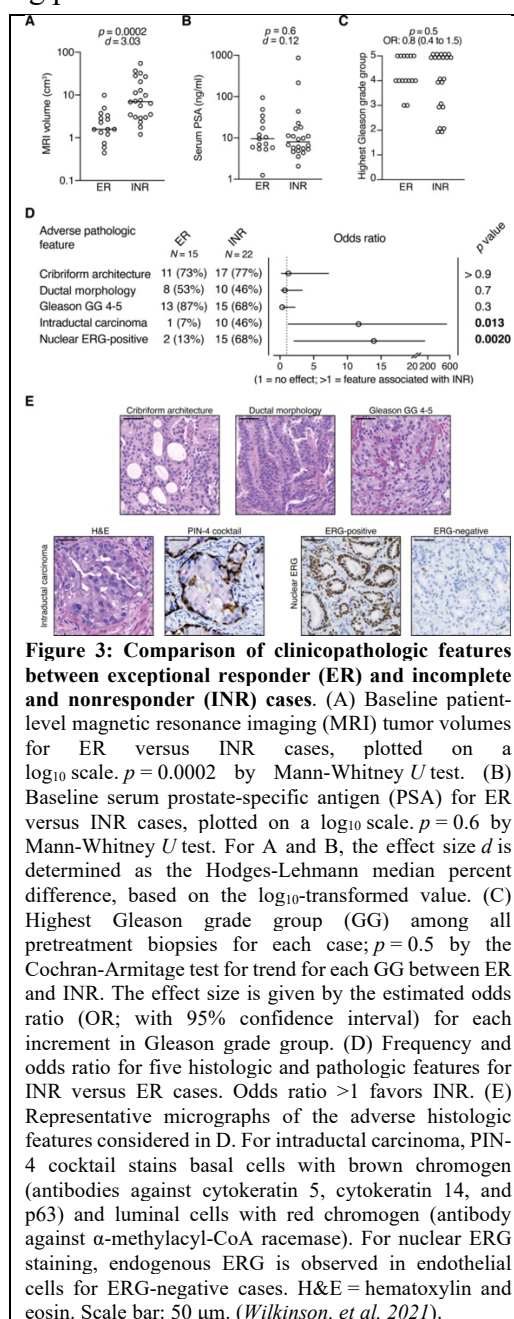
To further examine potential features predictive of therapeutic response, we compared clinicopathologic features between exceptional responders and nonresponders. Pre-treatment MRI volume was significantly enhanced in INR patients ($p = 0.0002$), while serum PSA and highest Gleason grade group exhibited no significant differences ($p = 0.6$, $p = 0.5$, respectively). No changes in cribriforming architecture ($p > 0.9$), ductal morphology ($p = 0.7$), or Gleason grade 4-5 ($p = 0.3$) were identified. However, the presence of intraductal carcinoma and nuclear ERG-positive tumor staining were significantly enriched in incomplete/non-responders ($p = 0.013$ and $p = 0.0020$, respectively) (Figure 3). When combined with the genomic features highlighting *TP53* alterations, these features suggest a combination of genomic and histologic analysis is predictive of response to therapy.

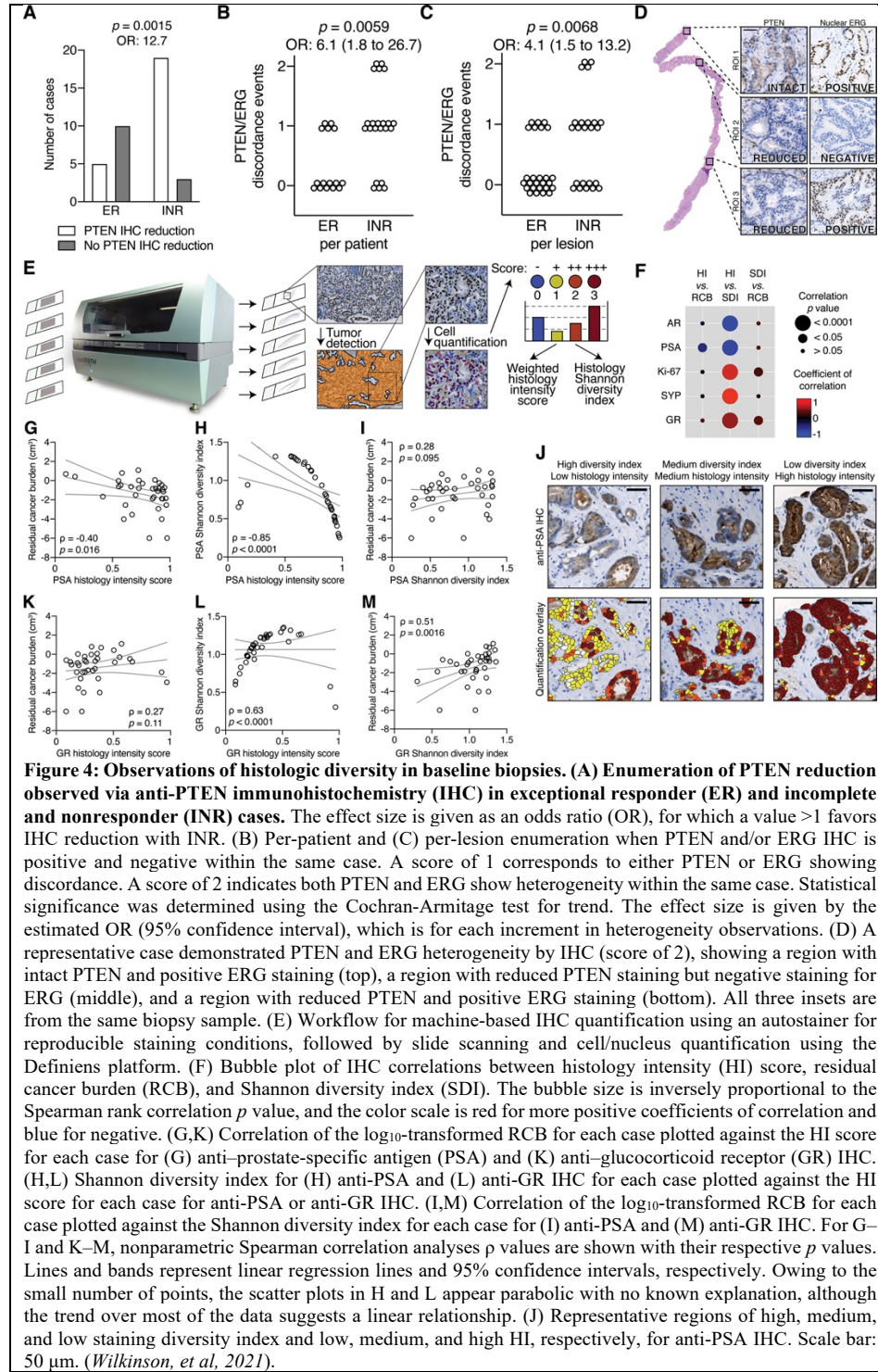
We next examined histological diversity of the pre-treatment biopsies across the cohort. Utilizing an autostainer and automated image analysis to produce robust and reproducible staining across the cohort, we examined PTEN and ERG staining intensity and heterogeneity. On IHC, PTEN reduction was significantly enhanced in the INR cohort ($p = 0.0015$), while discordance (uniformly positive or uniformly negative compared to divergence of staining) was enhanced in INR patients ($p = 0.0059$). Given the *TMPRSS2:ERG* fusion and *PTEN* loss tend to occur early



in tumorigenesis, we hypothesized they may enrich for heterogeneity at baseline. We then extended our histological analysis to include five additional stains: AR, PSA, GR, synaptophysin, and Ki-67. In patients with multiple lesions, we were able to identify tumor staining intensity and heterogeneity, and developed a Shannon diversity index (SDI) to quantify this heterogeneity. We witnessed a significant negative correlation between PSA staining intensity and RCB ($p = 0.016$), and PSA staining intensity and SDI ($p < 0.0001$) (Figure 4). Together, these data indicate that INR samples displaying more PSA heterogeneity at baseline may represent a cohort of subpopulations less similar to their parent clone, reflecting potential tumor resistance.

Given the heterogeneity identified at a histological level, we next examined genomic heterogeneity in patients with more than one pre-treatment lesion. Across a defined set of 69 amplifications and 111 deletions, a significant negative correlation was identified between correlation coefficient and RCB. This heterogeneity likely reflects divergent subclones within these tumor systems. We examined SNVs and SCNAs from each LCM focus to identify tumor subpopulations after reconstructing tumor phylogeny, and observed two clonal structures: A linear evolution with few subclones; or a branched phylogenetic tree with multiple subclones. Branched trees were identified more in INR cases (86% vs 73%), while the number of nodes was positively correlated with RCB ($p = 0.039$). Percent genome altered (PGA) was positively correlated with this genomic diversity ($p = 0.03$) and RCB ($p = 0.11$) (Figure 5). Taken together, these data suggest that tumors resistant to neoadjuvant androgen deprivation therapy may harbor a greater number of pre-existing subclones, conferring a greater resistance when compared to tumors with a simpler evolution history. We utilized this data to develop a 4 factor multivariate model to predict treatment response (Figure 6). Using significantly enriched features (*TMPRSS2:ERG* fusion, chromosome 10q deletion, alterations to *TP53*, and presence of intraductal carcinoma (IDC-P)), we were able to identify up to 16 possible combinations and probabilities of the tumor being an INR patient. This model shows an area under the curve of 0.89 ($p < 0.0001$). Utilizing a cutpoint of 60% correctly classified 30 of the 37 cases in this cohort correctly, highlighting the potential utility for use in diverse tumor profiles.





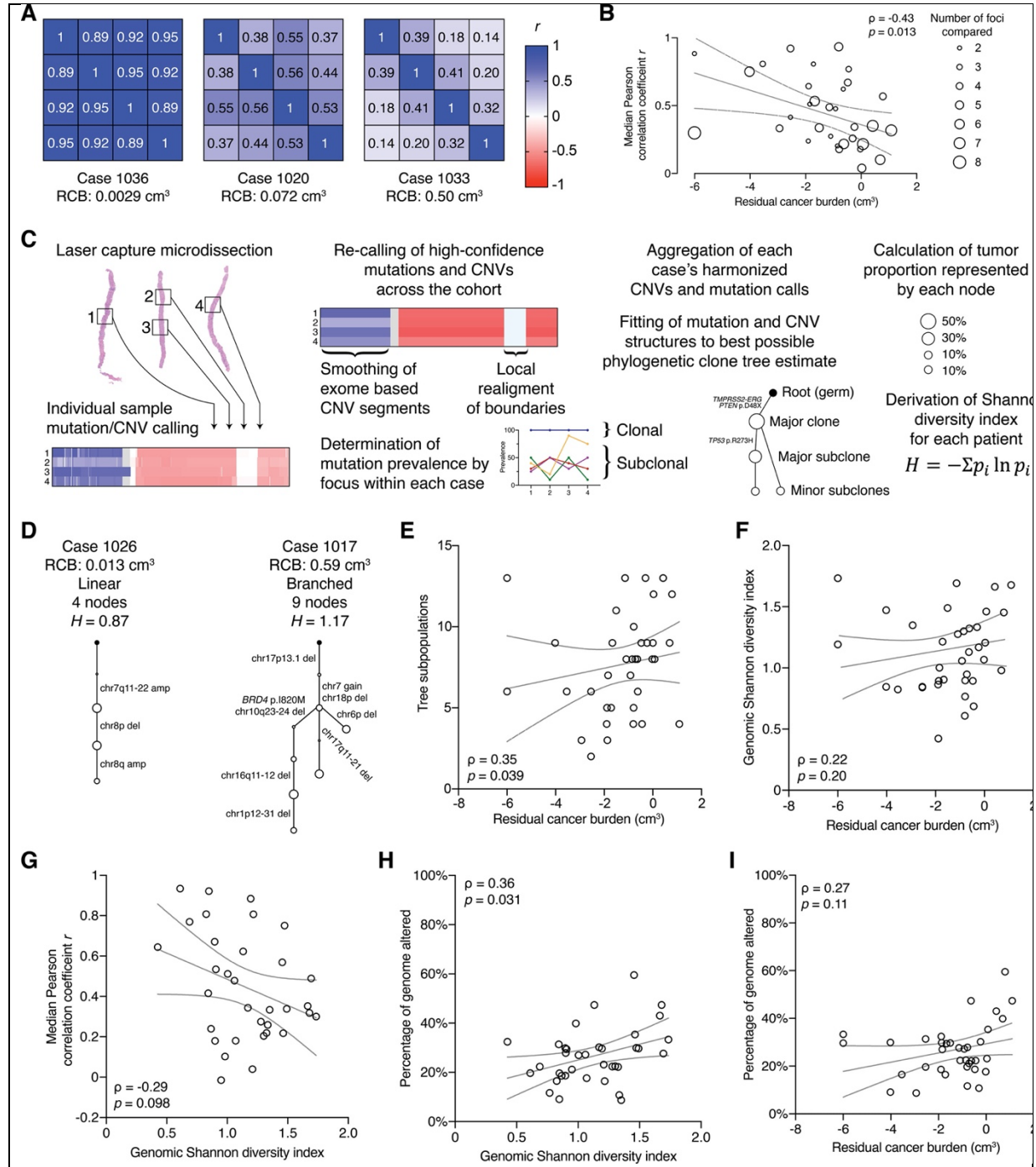
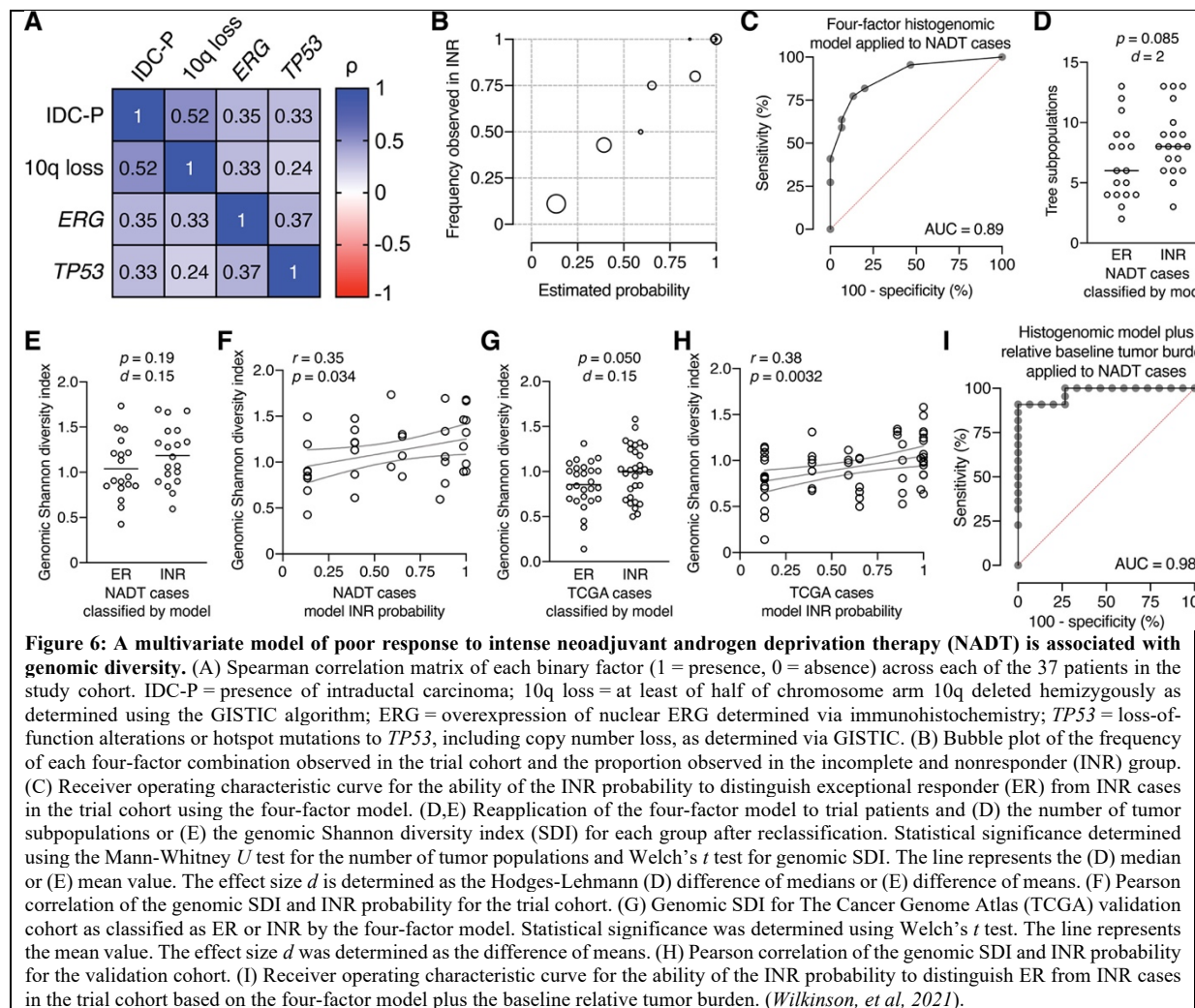


Figure 5: Measures of genomic diversity. (A) Correlation matrices for three representative cases with four tumors each, showing the range of similarities among genomic gains and losses across 180 predefined regions. (B) Median Pearson correlation coefficient for each multiregion case as a function of the log₁₀-transformed residual cancer burden (RCB) value. The number of foci dissected for each case is depicted by the size of the circle. (C) Schematic showing the workflow to harmonize multiregion sampling data and processing for phylogenetic clone tree reconstruction. CNV = copy number variation. (D) Representative linear and branched tree structures. (E) Scatter plot of the number of nodes in each phylogenetic tree versus the log₁₀-transformed RCB for each case. (F) Scatter plot of the genomic Shannon diversity index (SDI) for each case versus the corresponding log₁₀-transformed RCB. (G) Median Pearson correlation coefficient for each multiregion case from B versus the genomic SDI for each case. (H) Percentage of the genome altered (PGA) plotted against the genomic SDI for each case. (I) PGA versus the log₁₀-transformed RCB for each case. For B and E–I, nonparametric Spearman correlation analyses ρ values are shown with the respective p values. Lines and bands represent linear regression lines and 95% confidence intervals, respectively. (Wilkinson, et al, 2021).



What opportunities for training and professional development did the project provide? How were the results disseminated to communities of interest?

This project has provided training and professional development activities, as summarized by major tasks:

Training-specific tasks:

Major Task 1: Broaden Knowledge in Prostate Cancer Bioinformatics.

NCI has routine bioinformatics seminars through the Bioinformatics Training and Education Program (BTEP). These courses include topics ranging from Whole Genome Sequencing to single-cell RNAseq and the interpretation/analysis of data. These seminars have continued in a digital format during COVID-19, and I have attended several as part of the Training-Specific Tasks (Major Task 1: Broaden Knowledge in Prostate Cancer Bioinformatics). Further, NCI and NIH continue to offer courses and workshops on a wide variety of topics, including bioinformatic analysis, multiplexed immunofluorescence, image registration, and automated/quantitative image analysis. I have attended many of these workshops during the duration of the award.

The Prostate Cancer Foundation has continued to offer routine webinars on a wide variety of topics, including bioinformatic analysis of single-cell RNAseq (scRNAseq) and spatial transcriptomics utilizing barcoded fluorophores and subsequent transcriptomic analysis. I have attended many of these webinars, as well as the virtual Prostate Cancer Foundation scientific retreat in 2020 and 2021, to further enhance my understanding of advanced bioinformatics techniques with prostate cancer.

Major Task 2: Enhance Familiarity with Prostate Cancer Biology, Pathology, Oncology, and Basic Cancer Biology

Before the COVID-19 pandemic, routine weekly seminars on oncology were offered throughout NCI, including Grand Rounds, distinguished invited speakers, departmental invited speakers, and departmental seminars. These have largely shifted to a digital format, but continue in a routine manner. As part of this project, I routinely attend these seminars and our weekly journal club. In addition, I have attended the Prostate Cancer Foundation's Scientific Retreat in 2019, as well as the virtual retreats in 2020 and 2021. I presented my research at PCF in 2019 as an accepted abstract, and networked with colleagues to identify potential new research opportunities and collaborations. Further, I attended GU-ASCO in February 2020, where I presented my research based on this project as an accepted abstract. At all these meetings, I participated in activities for "young investigators", including the PCF Young Investigator Day, and a Young Investigator networking luncheon at GU-ASCO. These sessions included networking sessions, mentorship lectures, and discussions about transitioning to a tenure-track position in academia. Together, these conferences and seminars have increased my knowledge of cancer biology and prostate cancer oncological sciences.

Major Task 3: Development of Key Professional Skills

As part of my attendance at scientific conferences, I presented my research in poster sessions at PCF in 2019, and GU ASCO in 2020. These presentations allow development of presentation skills, while also encouraging active discussion regarding the interpretation of data and hypotheses leading to future directions. In addition, I routinely attend our departmental seminar series, and presented my research in an hour-long presentation in July 2020.

To further develop skills as a future independent investigator, I participated in grant writing workshops offered by NCI and the American Society for Cell Biology. I submitted a K99/R00 application during the award, which was ultimately not selected for funding. I plan to enroll in a formal grant writing workshop offered by NCI in early 2022, in preparation for an NCI K22 application in June 2022. I will also consider submitting a DOD Idea Development award and other applicable grants directed towards senior postdocs transitioning to tenure-track academic positions. In December 2021, I received the NCI CCR Excellence in Postdoctoral Research Transition Award, a competitive application awarded to the top two postdocs in the NCI intramural research program. This award provides full stipend support for up to two years, S&S funding, a postbaccalaureate fellow, and NCI support for a K22 application. Based on the receipt of this award, I will seek to apply to tenure-track academic positions for the 2022-2023 academic cycle.

I continue to develop my professional and leadership skills through active participation in scientific societies. I have maintained an active Associate Membership in AACR since 2015, and submitted an abstract for the AACR Prostate Cancer meeting and AACR regular meeting in 2020. AACR Prostate was canceled due to COVID-19, while the abstract submitted to AACR was withdrawn due to concerns of the COVID-19 pandemic. I have also maintained active

membership in the American Society for Cell Biology (ASCB) since 2013. I am currently (2019-2022) serving as the Chair of the student/postdoc leadership committee (COMPASS). I was on the Public Policy Committee from 2016-2019 and 2021-2022, the Governing Taskforce in 2019-2020, the Nomination Committee in 2020 and 2022, the Finance Committee in 2020, and am serving on the Honorific Awards Taskforce for 2020-2022. My participation active participation in the ASCB has provided immense networking opportunities and training in leadership and mentorship, while also introducing me to leaders in the cell biology scientific community.

How were the results disseminated to communities of interest?

Results have been disseminated to communities through presentations at scientific societies and manuscript publications. This research was presented as a poster at the 2019 Prostate Cancer Foundation Retreat, 2019 ASCB, 2020 GU ASCO, and was anticipated to be shared at AACR Prostate Cancer and AACR, prior to their cancelation due to the COVID-19 pandemic. Unfortunately, additional abstracts for scientific conferences were disrupted due to the continuing impact of the COVID-19 pandemic.

Scientific publications are also being prepared to disseminate this data to the broader scientific community. Of note, one first-author manuscript was published in *Nature Communications* in 2020 (Wilkinson and Harmon, et al). An additional first-author manuscript was recently published in *European Urology* (Wilkinson et al, 2021). A co-authored manuscript was published reporting the results of the clinical trial at *Clinical Cancer Research* (Karzai and Walker, et al, 2021). Additional manuscripts utilizing data generated during this project were published in *Oncogene* (Whitlock, et al, 2020), *Nature Cancer* (Morel, et al, 2021), *Briefings in Bioinformatics* (Ku, Wilkinson, and Sowalsky, 2021), with an additional manuscript at *Molecular Cancer Research* (Dong, et al, 2022), and a further one in revision at *Journal of Urology* (Bright, et al, 2022).

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

Nothing to Report.

4. IMPACT:

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

These data highlight the critical nature of tumor heterogeneity at diagnosis. Our work highlights that more-evolved tumors correlate with worse response to androgen deprivation therapy, which would suggest that identifying heterogeneity and evolution pre-treatment could better guide therapy. To do this, clinicians would need to consider the importance of sampling more than just the index lesion, or multiple sampling and analysis of tumor subclones. If a tumor system is predicted to respond poorly to ADT (highly evolved or non-androgen driven

tumor), the patient may be guided to a better-tailored therapy. By better researching this diversity and evolution of aggressive localized tumors, we can better predict which patients will respond to therapy.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

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:

The COVID-19 pandemic resulted in a shutdown of NCI labs for approximately three months. We had recently completed acquisition of the pre-treatment biopsy specimens and conducted a majority of the histology staining and imaging by this point. We were able to continue the bioinformatics analysis and image analysis/quantification during this period. Upon returning to the labs in summer 2020, we were able to resume the remaining research-specific tasks to conclude Major Task 2 (Mapping RP to corresponding biopsy, IHC), utilizing appropriate PPE and safety measures for all lab personnel.

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.

Significant changes in use or care of human subjects:

Nothing to Report.

Significant changes in use or care of vertebrate animals:

Nothing to Report.

Significant changes in use of biohazards and/or select agents:

Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations

Presentations:

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2020). Multiple primary prostate tumors exhibit divergent responses to intense neoadjuvant androgen deprivation therapy. 2020 AACR Prostate Cancer meeting. Denver, CO.

*Conference was canceled due to COVID-19 pandemic.

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2020). Multiple primary tumors with differential drug sensitivity. 2020 GU ASCO Symposium. San Francisco, CA.

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2019). Multiple primary tumors with differential drug sensitivity. 2019 ASCB Annual Meeting. Washington, DC.

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2019). Integrated radiologic, genomic and pathologic analysis revealed intratumoral divergent responses to neoadjuvant anti-androgen therapy. 26th Annual Prostate Cancer Foundation Scientific Retreat. Carlsbad, CA.

S. Harmon, **S. Wilkinson**, H. Ye, F. Karzai, N. V. Carrabba, R. Atway, N. Terrigino, J. Bright, R. Lake, D. VanderWeele, P. Choyke, P. Pinto, W. L. Dahut, A. G. Sowalsky, B. Turkbey. (2019). Baseline

multiparametric MRI characteristics of exceptional pathologic response to neoadjuvant enzalutamide for high-risk, localized prostate cancer. 2019 RSNA Annual Meeting. Chicago, IL.

Journal publications:

Authors: J. R. Bright, R. T. Lis, A. T. Ku, N. T. Terrigino, S. Y. Trostel, N. V. Carrabba, S. A. Harmon, B. Turkbey, **S. Wilkinson**, A. G. Sowalsky.

Title: Prostate-specific membrane antigen is a biomarker for residual disease following neoadjuvant intense androgen deprivation therapy in prostate cancer.

Journal: *Journal of Urology*. In Revision. (2022)

Acknowledgement of federal support: Yes

Authors: X. Dong, H. Xue, F. Mo, Y. Lin, D. Lin, N. Wong, Y. Sun, **S. Wilkinson**, A. Ku, J. Hao, X. Ci, R. Wu, A. Haegert, R. Silver, M. Taplin, S. Balk, J. Alumkal, A. G. Sowalsky, M. Gleave, C. Collins, Y. Wang.

Title: Modeling prostate cancer dormancy induced by androgen deprivation therapy and its clinical implications.

Journal: *Molecular Cancer Research*.

Issue: DOI: 10.1158/1541-7786.MCR-21-1037. (2022)

Acknowledgement of federal support: Yes

Authors: A. T. Ku, **S. Wilkinson**, A. G. Sowalsky.

Title: Comparison of approaches to transcriptomic analysis in multi-sampled tumors.

Journal: *Briefings in Bioinformatics*.

Issue: DOI: 10.1093/bib/bbab337. (2021)

Acknowledgement of federal support: Yes

Authors: **S. Wilkinson**, H. Ye, F. Karzai, S. Harmon, N. T. Terrigino, D. VanderWeele, J. Bright, R. Atway, S. Trostel, N. Carrabba, N. Whitlock, S. Walker, R. Lis, H. Sater, B. Capaldo, R. Madan, J. Gulley, G. Chun, M. Merino, P. Pinto, D. Salles, H. Kaur, T. Lotan, D. Venson, P. Choyke, B. Turkbey, W. L. Dahut, A. G. Sowalsky.

Title: Nascent prostate cancer heterogeneity drives evolution and resistance to intense hormonal therapy.

Journal: *European Urology*.

Issue: S0302-2838(21)00207-4. DOI: 10.1016/j.eururo.2021.03.009. (2021)

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Title: EZH2 inhibition activates a dsRNA–STING–interferon stress axis that potentiates response to PD-1 checkpoint blockade in prostate cancer.

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Authors: F. Karzai*, S. M. Walker*, **S. Wilkinson**, R. A. Madan, J. H. Shih, M. J. Merino, S. Harmon, D. VanderWeele, L. M. Cordes, N. V. Carrabba, J. R. Bright, N. T. Terrigino, G. Chun, M. Bilusic, A. Couvillon, A. Hankin, M. N. Williams, R. T. Lis, H. Ye, P. L. Choyke, J. L. Gulley, A. G. Sowalsky, B. Turkbey, P. A. Pinto, W. L. Dahut.

Title: Sequential prostate MRI imaging in newly diagnosed high-risk prostate cancer treated with neoadjuvant enzalutamide prior to surgery.

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Authors: **S. Wilkinson***, S. Harmon*, N. T. Terrigino, K. Karzai, P. A. Pinto, R. A. Madan, D. J. VanderWeele, R. Lake, R. Atway, J. R. Bright, N. V. Carrabba, S. Y. Trostel, R. T. Lis, G. Chun, J. L. Gulley, M. J. Merino, P. L. Choyke, H. Ye, W. L. Dahut, B. Turkbey, A. G. Sowalsky.

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Authors: N.C. Whitlock, S.Y. Trostel, **S. Wilkinson**, N.T. Terrigino, S.T. Hennigan, R. Lake, N.V. Carrabba, R. Atway, E.D. Walton, B.E. Gryder, B.J. Capaldo, H. Ye, A.G. Sowalsky.

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Authors: **S. Wilkinson**, H. Ye, R. T. Lis, A. G. Sowalsky.

Title: Reply to Alessia Cimadamore, Liang Cheng, Antonio Lopez-Beltran, Marina Scarpelli, and Rodolfo Montironi's Letter to the Editor re: Scott Wilkinson, Huihui Ye, Fatima Karzai, et al. Nascent Prostate Cancer Heterogeneity Drives Evolution and Resistance to Intense Hormonal Therapy.

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Authors: **S. Wilkinson**, A. G. Sowalsky.

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Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Scott Wilkinson
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2613-8425
Nearest person month worked:	12 months
Contribution to Project:	PI, Performed experiments, attended workshops and trainings
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Name:	
Project Role:	
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A case report of multiple primary prostate tumors with differential drug sensitivity

Scott Wilkinson^{1,10}, Stephanie A. Harmon^{2,3,10}, Nicholas T. Terrigino¹, Fatima Karzai⁴, Peter A. Pinto⁵, Ravi A. Madan⁴, David J. VanderWeele^{1,8}, Ross Lake¹, Rayann Atway¹, John R. Bright¹, Nicole V. Carrabba¹, Shana Y. Trostel¹, Rosina T. Lis¹, Guinevere Chun⁴, James L. Gulley⁴, Maria J. Merino⁶, Peter L. Choyke², Huihui Ye^{7,9}, William L. Dahut⁴, Baris Turkbey^{2,11} & Adam G. Sowalsky^{1,11}✉

Localized prostate cancers are genetically variable and frequently multifocal, comprising spatially distinct regions with multiple independently-evolving clones. To date there is no understanding of whether this variability can influence management decisions for patients with prostate tumors. Here, we present a single case from a clinical trial of neoadjuvant intense androgen deprivation therapy. A patient was diagnosed with a large semi-contiguous tumor by imaging, histologically composed of a large Gleason score 9 tumor with an adjacent Gleason score 7 nodule. DNA sequencing demonstrates these are two independent tumors, as only the Gleason 9 tumor harbors single-copy losses of *PTEN* and *TP53*. The *PTEN/TP53*-deficient tumor demonstrates treatment resistance, selecting for subclones with mutations to the remaining copies of *PTEN* and *TP53*, while the Gleason 7 *PTEN*-intact tumor is almost entirely ablated. These findings indicate that spatiogenetic variability is a major confounder for personalized treatment of patients with prostate cancer.

¹Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, NIH, 37 Convent Drive, Bethesda, MD 20892, USA. ²Molecular Imaging Program, National Cancer Institute, NIH, 10 Center Drive, Bethesda, MD 20892, USA. ³Clinical Research Directorate, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, 8560 Progress Drive, Frederick, MD 21701, USA. ⁴Genitourinary Malignancies Branch, National Cancer Institute, NIH, 10 Center Drive, Bethesda, MD 20892, USA. ⁵Urologic Oncology Branch, National Cancer Institute, NIH, 10 Center Drive, Bethesda, MD 20892, USA. ⁶Laboratory of Pathology, National Cancer Institute, NIH, 10 Center Drive, Bethesda, MD 20892, USA. ⁷Department of Pathology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, USA. ⁸Present address: Department of Medicine, Feinberg School of Medicine, 420 E. Superior Street, Chicago, IL 60611, USA. ⁹Present address: Department of Pathology and Department of Urology, University of California Los Angeles, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA. ¹⁰These authors contributed equally: Scott Wilkinson, Stephanie A. Harmon. ¹¹These authors jointly supervised this work: Baris Turkbey, Adam G. Sowalsky. ✉email: adam.sowalsky@nih.gov

Localized prostate cancers are uniquely genetically variable and frequently multifocal. Retrospective DNA sequencing studies have demonstrated that 80–90% of prostate cancers comprise multiple spatially separate (distinct) regions, with occasional development of two independent clones within the same tumor^{1,2}. This multifocality is a well-recognized problem for diagnosis, and to date there has been no understanding of whether this variability can influence personalized management decisions. Conventional management of high-risk localized prostate cancer includes radical prostatectomy or external beam radiation therapy with androgen deprivation therapy (ADT)³. The target of ADT is the transcription factor androgen receptor (AR), which mediates terminal differentiation of luminal epithelium in benign prostatic tissue, but drives cell proliferation in prostatic adenocarcinoma. Observations that AR-targeting agents (such as abiraterone and enzalutamide) improve overall and metastasis-free survival in patients with hormone-sensitive and nonmetastatic disease, respectively, suggests that earlier intense inhibition of AR may further improve outcomes^{4–6}. To this end, the effect of introducing intense androgen deprivation therapies in the neoadjuvant setting has been explored in a series of clinical trials at several cancer centers⁷. Although overall survival benefits associated with neoadjuvant intense ADT are yet unknown, complete pathologic responses were reported in ~10% of patients, and minimal residual disease (MRD) has been observed in ~30% of patients⁸. Surgical specimens from ~90% of patients show treatment effect, with substantial residual disease in ~20% of cases, which are likely due to driver mutations present at baseline, such as mutations to *AR* itself⁹, or selection for subclones with losses to tumor suppressors, including *PTEN* and *TP53*¹⁰. Despite evidence demonstrating repeated sampling of multifocal prostate cancer gives rise to discordant scores on prognostic tissue tests, the treatment outcomes of independent prostate cancer foci in a patient with a polyclonal or polytumor phenotype remain unknown¹¹.

Multiparametric magnetic resonance imaging (mpMRI) has become an established imaging technique for diagnosis and staging of localized prostate cancer, especially in detection and sampling of clinically significant disease while decreasing detection of indolent, low-risk tumors^{12–14}. In our study of neoadjuvant ADT plus enzalutamide, we hypothesized that mpMRI prior to surgery would assess radiographic tumor response compared with baseline imaging, while also providing biopsy targets for molecular and histologic examination of pre-treatment foci. Given the high-risk population for whom neoadjuvant intense ADT is suggested to benefit, patients on our study with larger lesions on mpMRI undergo saturation biopsy of imaging targets, to more accurately assess tumor heterogeneity^{15,16}.

In this report, we describe a patient who presented with high-risk prostate cancer that exhibited differential intratumoral response to a clinical trial of neoadjuvant intense ADT. Histologic and genomic analyses reveal the presence of a polytumor, in which one nodule was mostly sensitive to treatment and a separate, clonally independent nodule was resistant. While comprehensive molecular profiling of multiple specimens from a single patient is uncommon for many biomarker-driven therapies, we propose that success of genomically driven trials and treatment strategies may require complete assessment of larger tumors prior to application of precision therapies.

Results

Identification of two primary prostate tumors at baseline. In our phase 2 clinical study of neoadjuvant ADT and enzalutamide, patients with localized intermediate to high-risk prostate cancer undergo mpMRI, which is used for guiding the acquisition of biopsy tissue. Patients then receive intense hormonal therapy

(goserelin plus enzalutamide) for 24 weeks, followed by a second mpMRI and radical prostatectomy (RP). The final results for this trial have yet not been reported.

In a single case from this trial, a 66-year-old patient's pre-treatment mpMRI (see Supplementary Table 1) identified a semi-contiguous tumor that extended from the right apical-mid peripheral zone to the left distal apical peripheral zone, although imaging alone did not provide sufficient evidence of two independent tumors during the initial mpMRI interpretation (Fig. 1a). Due to the extension of tumor across the midline with differential T2-weighted and *b2000* MRI characteristics in the distal apical portion of the prostate (Supplementary Fig. 1a), the left- and right-sided tumor extent were separately sampled by MR/ultrasound (US)-fusion transrectal biopsy (Fig. 1b; Supplementary Fig. 1b). These biopsies showed markedly differing histologies (Fig. 1c). The left-sided tumor (B1) was Grade Group (GG) 3 (Gleason score 4 + 3 = 7) with intermediately to well-differentiated adenocarcinoma. In contrast, the right-sided tumor (B2/B3) was GG5 (Gleason score 4 + 5 = 9) poorly differentiated carcinoma with a component of intraductal carcinoma (IDC-P) (Supplementary Fig. 1c) and perineural invasion.

The TMPRSS2:ERG fusion, which occurs in 40–50% of prostate cancer patients and results in ERG expression, is an early genomic event during tumor development^{17,18}. *PTEN* loss, represented by significant reduction or complete loss of PTEN expression, is another common alteration in prostate cancer pathogenesis and tends to co-occur after an ETS family fusion^{18,19}. We therefore performed anti-ERG and anti-PTEN immunohistochemistry on serial sections of biopsy tissue (Fig. 1c; Supplementary Fig. 1c). The left-sided tumor was ERG-negative with reduced PTEN in a subset of tumor cells (B1), while the right-sided tumor stained positively for ERG and had homogeneous reduction in PTEN expression in both the IDC-P (B2) and invasive (B3) components.

To determine if the genetic basis of reduced PTEN expression in the left- and right-sided tumors was due to the same genomic event, we performed laser capture microdissection (LCM) of tumor foci and adjacent normal tissue, and then performed whole-exome sequencing (WES) to assess somatic copy number alterations (SCNAs) and point mutations. As shown in Supplementary Fig. 2a and Supplementary Table 2, both foci from the right-sided tumor (B2 and B3) showed an arm-level deletion of chromosome 10q encompassing *PTEN* and a focal deletion in chromosome 17p encompassing *TP53*. However, the left-sided tumor (B1) showed no genomic hits to *PTEN* or *TP53* (Supplementary Fig. 2a), suggesting a noncoding or epigenetic driver of reduced PTEN expression. In addition to these mutually exclusive SCNAs, the B2 and B3 foci harbored shared losses of chromosome 16 not found in the B1 focus, and B1 carried a gain of chromosome 7 not detected in B2 or B3 (Supplementary Fig. 2a).

Examination of somatic point mutations resolved by WES revealed that the vast majority were harbored by only a single tumor focus, although nine point mutations were shared by the B2 and B3 foci (Supplementary Table 2). No mutations were shared by all three foci. Given the relatively shallow coverage from WES, we verified that these mutations were also definitive markers distinguishing the B1 and B2/B3 tumors by performing additional focused sequencing to an average depth of ~50,000×. We observed 29 point mutations that demonstrated mutual exclusivity between the left- and right-sided tumor foci (Supplementary Table 2), confirming the clonal independence of these two tumors.

Identification of independent tumors after treatment. Following baseline imaging and biopsy, the patient received 6 months of neoadjuvant intense ADT with goserelin and enzalutamide per

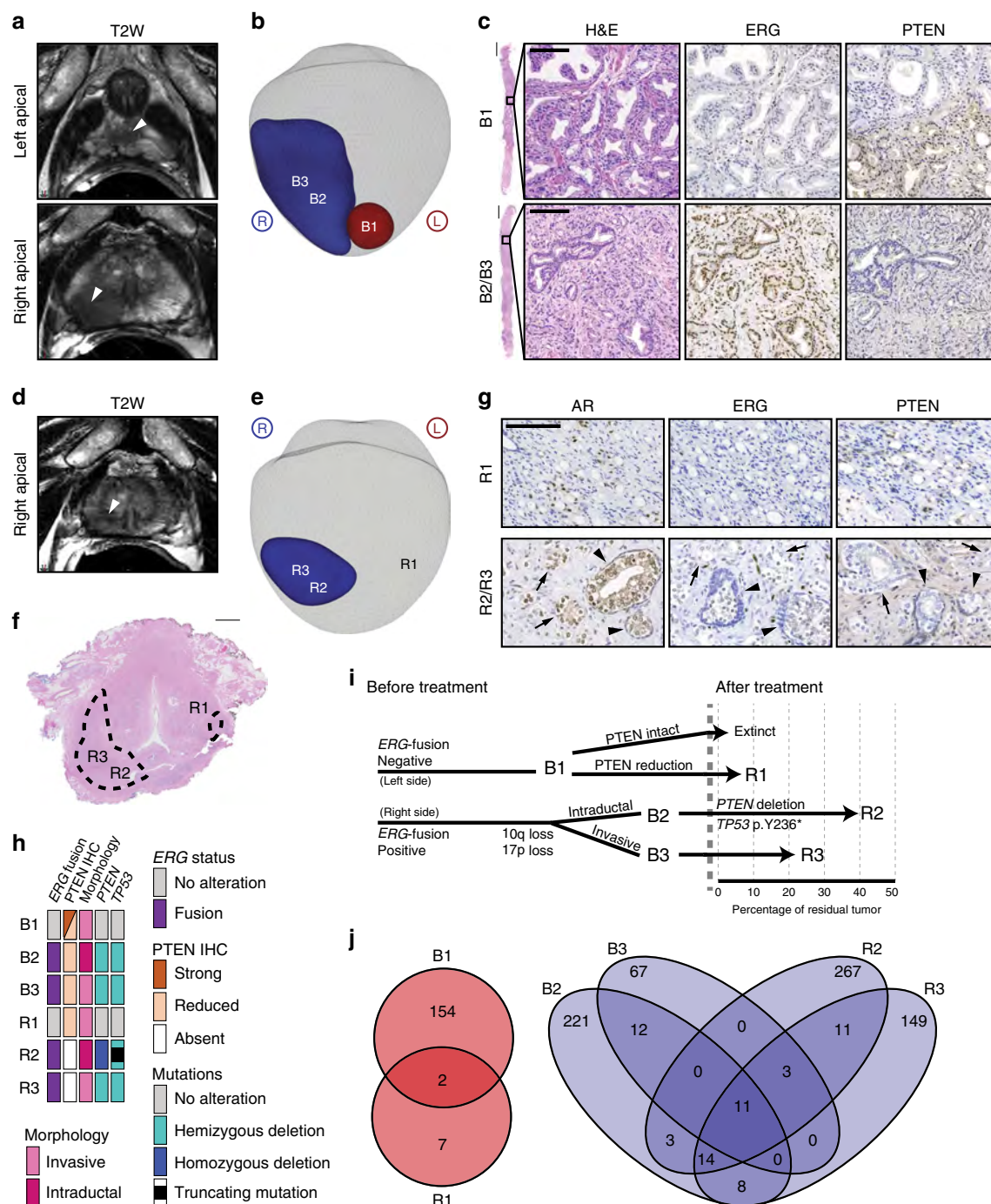


Fig. 1 Molecular evolution of independent prostate tumor nodules following neoadjuvant intense androgen deprivation therapy. **a-g** Pre-treatment (**a-c**) or post-treatment (**d-g**) T2-weighted MRI (**a-d**), volumetric burden (**b-e**), and staining (**c-g**) of a large, semi-contiguous lesion sampled at multiple levels by mpMRI/TRUS fusion-guided biopsy (**c**) or whole-mount pathology (**f, g**) showing the location of biopsied and laser capture microdissected tumor foci. Arrowheads depict lesions on representative slices on MRI (**a, d**) or IDC-P (**g**). Arrows depict invasive carcinoma lesions. Scale bars: whole mount, 5 mm; biopsy, 1000 μ m; insets, 100 μ m. **h** Oncoprint depicting the alterations and histologic phenotypes of the microdissected tumor foci. **i** Phylogenetic tree depicting the relationships between baseline biopsy and prostatectomy tumor foci with respect to the alterations shown in the Oncoprint. Branches were constructed based on shared and unique mutations in each sample. **j** Venn diagram depicting the overlap between tumor foci based on somatic point mutations and large copy number alterations. Source data for the Venn diagram are provided in a source data file. B2/R2 = baseline/residual intraductal carcinoma, and B3/R3 = baseline/residual invasive carcinoma; B2 was intermingled with B3, and R2 was intermingled with R3.

protocol, and then underwent a second mpMRI prior to RP. The post-treatment mpMRI displayed reductions both to total prostate volume (33 cc to 20 cc) and tumor size (2.8–1.7 cm) (Fig. 1d, e). The right-sided tumor exhibited a partial response to therapy and was visible on both MR imaging and microscopic

examination, whereas the left-sided tumor displayed an excellent response and was only detected microscopically (Fig. 1f; Supplementary Fig. 3). Per protocol, the prostatectomy specimen was grossed and sectioned in the same three-dimensional plane as the mpMRI (Supplementary Fig. 3a), and comparison of residual

tumor on the left side revealed that left-sided tumor was likely too hypocellular and scarce to be detected by mpMRI. While diagnostic performance of mpMRI after neoadjuvant treatment is not well-characterized in the literature, mpMRI of untreated prostate cancer is known to have a poor detection rate for clinically insignificant disease^{20,21}.

Because reconstitution of AR nuclear localization and AR activity are common mechanisms of resistance to neoadjuvant intense ADT¹⁰, we examined nuclear AR expression using immunohistochemistry in treatment-resistant tumor foci in whole-mount RP tissues, as well as anti-PTEN and anti-ERG immunohistochemistry (Fig. 1g; Supplementary Fig. 3b). As the right-sided tumor foci (R2 and R3) displayed reduced but positive ERG staining consistent with suppression of AR activity by ADT plus enzalutamide, we confirmed using immunofluorescence that the right-sided tumor was ERG-positive and distinct from the left-sided tumor (R1), which was ERG-negative (Supplementary Fig. 3c). The left-sided tumor, which showed focal PTEN reduction before treatment, displayed homogeneous PTEN reduction in the minimal residual disease, whereas residual tumor on the right side displayed complete loss of PTEN expression in both IDC-P (R2) and invasive tumor (R3) foci.

Taking the same approach as with the biopsy tissue, we performed LCM and WES on residual tumor foci from the right and left sides, sampling tissue from three separate levels (Supplementary Fig. 3a). Residual IDC-P (depicted as R2) on the right side had two copy losses of *PTEN*: the same single-copy loss observed on biopsy plus an additional focal loss at 10q23.31 (Supplementary Fig. 2a, b). The residual invasive tumor (R3) on the right side showed the same single-copy loss of *PTEN*, but no additional loss. Copy number analysis of the residual IDC-P also revealed the same focal loss of chromosome 17p13.1 (Supplementary Table 2) observed on biopsy plus an additional nonsense mutation to *TP53* rendering the IDC-P negative for both PTEN and p53. R2 and R3 also harbored the same loss to chromosome 16 detected in the B2 and B3 foci. In contrast, none of these alterations were observed in the residual tumor cells on the left side (R1), which despite being PTEN-deficient by IHC did not harbor any detectable SCNAs (Supplementary Fig. 2a).

Evolution of treatment-resistant tumor. In order to visualize the spatial, genomic, and temporal relationships between micro-dissected pre-treatment biopsies and post-treatment residual tumor, we constructed an oncoprint plot and phylogenetic tree (Fig. 1h, i). Using the TMPRSS2:ERG fusion as a putative early driver of tumorigenesis, based on its expression in all right-sided tumor foci (B2, B3, R2, and R3), we modeled that the IDC-P and invasive foci identified in the RP descended from IDC-P and invasive foci sampled on biopsy. Presumably, a small subpopulation of the IDC-P at baseline (B2) also harbored the second loss to *PTEN* and the nonsense mutation to *TP53*, as these were observed in the IDC-P on the right side (R2), which in turn comprised the largest volume of all residual tumor.

Deep focused resequencing of a subset of mutations at ~50,000× coverage confirmed that of 27 point mutations harbored by R2 and/or R3, no somatic mutations were shared by both R1 and R2 and/or R3 (Supplementary Table 4). Moreover, one point mutation detected in R1 was shared by B1, but neither B2 nor B3, and of six SCNAs observed in R1, one was shared by B1 (Fig. 1j). In addition, 21 mutations were confirmed as shared by B2/R2 and B3/R3. Further strengthening the relationship of the IDC-P and invasive post-treatment foci to their pre-treatment counterparts was the observation that when point mutations were observed in either B2 or B3, but both R2 and R3 (Supplementary Table 4), the frequency of the mutation

in the matching histologic tumor was generally greater. Combined with shared and distinct somatic copy number alterations (Supplementary Fig. 2a), these data collectively confirm that the right-sided and left-sided residual tumors were clonally independent and descended from the tumor foci sampled on biopsy (Fig. 1j). The absence of any genomic hits to *PTEN* on the left side suggests alternative mechanisms of suppressing PTEN expression, which may include epigenetic silencing. As all three foci of residual tumor displayed reduced or absent PTEN expression by IHC (Supplementary Fig. 3b), the left- and right-sided tumors independently underwent phenotypic convergence, selecting for subclones with dysregulated PTEN.

Discussion

Here, we describe a patient with high-risk prostate cancer treated by neoadjuvant intense ADT and surgery. Retrospective analysis of imaging and tissue data revealed that there were two potentially lethal-independent cancers present at baseline, and the two tumors responded differently and demonstrated different status for the tumor suppressor genes *PTEN* and *TP53*.

These molecular findings are consistent with large cohort studies of metastatic castration-resistant prostate cancer (mCRPC) previously treated with enzalutamide, in which loss of function of p53 is the most common and enriched alteration relative to untreated primary disease^{22,23}. In contrast, *PTEN* loss was abundant in primary disease and mCRPC, and thus likely contributed to early-stage disease initiation or progression. Treatment of this patient with intense ADT selected for PTEN- and p53-null subclones as they represented the majority of residual disease, with cells harboring epigenetically silenced PTEN or single-copy losses of *PTEN* and *TP53* displaying less of a growth advantage. Meanwhile, the cells with intact PTEN were fully sensitive to intense ADT, as they were not present in the post-treatment specimen.

Multiple lines of histologic and genomic data contributed to the evidence that the left- and right-sided tumors were independent. First, the incidence of the TMPRSS2:ERG fusion is documented to occur early during tumorigenesis, even in pre-cancerous high-grade prostatic intraepithelial neoplasia (PIN), such that it is rarely a subclonal event²⁴. We have previously showed that when low- and high-grade tumor foci were sampled from the same index tumor, they were 100% concordant for ERG expression and when positive harbored the same genomic breakpoints¹⁷. Based on these existing evidence, and morphologic and spatial findings in our study, the left- and right-sided tumors are most likely two juxtaposed independent tumor nodules even though we cannot completely eliminate the possibility that the ERG-positive tumor diverged early from the ERG-negative clone.

Second, our exome sequencing and high-coverage resequencing did not identify somatic variants in common between the left- and right-sided tumor foci, but did confirm the lineage of pre-treatment to post-treatment tumor foci in these tumors. By segmented copy number analysis, the right-sided tumor carried clonal losses to 17p (*TP53*) and 10q (*PTEN*), as well as 16q, which carries multiple putative tumor suppressors²⁵. In contrast, these were absent from all tumor foci on the left side. The left-sided tumor at baseline harbored a subclonal gain to most of chromosome 7, and these chromosome 7 alterations were absent from all right-sided foci. This chromosome 7 gain may have been associated with the PTEN-intact portion of that left-sided tumor, which completely responded to treatment, potentially explaining in part why it was not detected in the residual tumor from the left side. A limitation of this analysis was the low cellularity of the left-sided residual tumor, which may explain in part why somatic mutations and copy number alterations were infrequent but still

present, distinguishing the sampled cells from adjacent benign tissue.

Third, the ERG-negative and ERG-positive tumors displayed different behaviors in the context of neoadjuvant intense ADT. We observed that the ERG-negative/*PTEN*-intact subclone was the most sensitive to treatment, and the ERG-positive/*PTEN*-negative IDC-P subclone was the most resistant in this patient. This pattern is consistent with results from a recent trial of neoadjuvant ADT plus abiraterone and enzalutamide, in which IDC-P, ERG positivity, and *PTEN* loss were associated with the highest residual tumor volumes⁷.

Notably, the tumor focus at baseline with IDC-P histology demonstrated the most resistance to therapy, harboring homozygous inactivation of both *TP53* and *PTEN*. Recent reports have also linked prostate ductal and intraductal pathologies to somatic and/or germline alterations affecting the homologous recombination genes *ATM*, *BRCA1*, and *BRCA2*^{26–28}. The patient did not carry somatic or germline alterations to these three genes, although the IDC-P focus at baseline did harbor a deletion to chromosome 11 which encompassed *ATM* (see Supplementary Table 2). As this deletion was not observed in its descendent post-treatment tumor (R2), we conclude that the effects of this alteration on *ATM* expression were not required for the maintenance, resistance or progression of the IDC-P tumor focus.

The previous study of neoadjuvant ADT plus abiraterone and enzalutamide also concluded that no tumor with both ERG positivity and *PTEN* loss responded to the point of MRD⁷. MRD has been defined by either RCB <0.25 cc, or as the greatest dimension of residual tumor <0.5 cm. Using this latter criterion, a meta-analysis of three recent trials demonstrated that MRD was a surrogate for 5-year biochemical recurrence-free survival; 11 out of 11 patients in those trials with MRD ≤ 0.5 cm were recurrence-free with median follow-up of 3.4 years⁸. These patients tended to have lower tumor volumes at baseline, but biopsy GG alone did not predict response. In this study, the response of the left-sided tumor met the criteria for MRD despite both the left- and right-sided tumors harboring predominantly Gleason pattern 4 disease.

Integrating the imaging, histologic, and genomic data available to us, we conclude that this patient harbored a polytumor, defined by two completely independent primary tumor systems in the same organ². Evolutionarily, polyclonal or multiple independent prostate tumors trend toward higher risk, because increased diversity of tumor cells increases fitness and thus may increase aggressiveness and treatment resistance². Evolutionary trajectories that resulted in tumor heterogeneity in this case likely contributed to our observation that two independent prostate tumor nodules with distinct genetic alterations responded differently to neoadjuvant intense ADT.

In previous neoadjuvant clinical trials, it was suggested that multiple clones developed in a subset of higher-risk patients prior to therapy, but the absence of targeted biopsies precluded reconstruction of the evolutionary events leading to heterogeneously resistant tumors^{9,10}. Our analysis of this patient reveals that genomic profiling of a single biopsy would not have predicted the overall response observed following treatment. It is likely that the spatiogenetic variability of localized prostate tumors will continue to be a major confounder for genetically driven trials in localized prostate cancer, and efforts to integrate multiparametric MRI with histopathology to direct genomic studies in the context of neoadjuvant therapies are needed to characterize independent clones at baseline for optimal clinical management.

Methods

Study approval. This clinical and laboratory studies described in this paper were conducted according to the principles of the Declaration of Helsinki. Informed

written consent was received from the study participant prior to inclusion in the study. This clinical study was approved by the National Institutes of Health Institutional Review Board (protocol number 15-c-0124), and was registered with ClinicalTrials.gov (registration number NCT02430480).

Study subject. The patient is a 66-year-old man who presented to the National Institutes of Health Prostate Multidisciplinary Clinic to discuss treatment options for newly diagnosed high-risk prostate cancer (clinical stage T3a). His PSA had risen slowly over the past 8 years (1.65–5.53 ng/ml), and a templated 6-core biopsy with his primary urologist demonstrated Gleason score 9 and Gleason score 7 cancer. Apical and basal biopsy cores showed evidence of perineural invasion, and conventional imaging (CT, bone scan) did not show any evidence of metastatic disease.

mpMRI of the prostate showed a single semi-contiguous lesion encompassing the right apical–mid peripheral zone also affecting the left distal apical peripheral zone with concern for extraprostatic extension (PIRADS score 5/5), and axial T1-weighted MRI of the abdomen showed no nodal disease. Using right apical and left apical MRI targets, MR/ultrasound-fusion-targeted biopsy of the prostate was performed and confirmed the presence of adenocarcinoma in both lobes: Gleason patterns 4 and 5 in the right apex and right mid regions, and Gleason patterns 3 and 4 in the left apex and right apex regions. Treatment options, including RP and EBRT with ADT, were discussed with the patient along with clinical trial options. The patient enrolled in an IRB-approved phase 2 clinical study of neoadjuvant ADT and enzalutamide (ClinicalTrials.gov #NCT02430480).

The patient was administered two injections of goserelin 12 weeks apart with 160 mg enzalutamide orally daily. After 24 weeks of treatment, his total testosterone declined from 193 ng/dl to 26.8 ng/dl, and his PSA declined from 5.53 ng/ml to <0.02 ng/ml. He then received a second multiparametric MRI, which demonstrated substantial reduction in total prostate volume and a decrease in size of the right apical–mid peripheral zone lesion. No disease was noted on the left side, and no extraprostatic extension or nodal disease was observed on MR. A three-dimensional mold was constructed based on the MR, and following robotic-assisted prostatectomy, the prostate was grossed in the mold to create tissue slices in the same plane as the MRI. Pathologic examination revealed extensive residual disease (cellularity corrected volume of residual cancer burden, RCB) of 0.35 cc on the right side corresponding to the MR-visible lesion, with features of intraductal carcinoma. A much smaller, hypocellular region (RCB 0.01 cc) was also observed on the left side. No additional ADT or RT was performed, and 2 years following surgery, PSA levels remain undetectable.

Multiparametric MRI acquisition. MR images were acquired at 3-Tesla (Achieva 3.0T-TX, Phillips Healthcare, Best, Netherlands) using a combination of the anterior half of a 32-channel cardiac SENSE coil (InVivo, Gainesville, FL, USA) and an endorectal coil (BPX-30, Medrad, Pittsburgh, PA, USA) filled with 45 ml of fluorinert (3 M, Maplewood, MN, USA). The mpMRI protocol included acquisition of T2-weighted (T2W) sequences acquired separately in axial, coronal, sagittal plane, two diffusion-weighted imaging (DWI) sequence acquisitions consisting of one 5 b-value acquisition (0–750 s/mm²) for Apparent Diffusion Coefficient (ADC) estimation and one high b-value (2000 s/mm²), and Dynamic Contrast Enhanced (DCE) MRI. DCE MRI images were obtained in 5.6 s intervals following a single dose of gadopentetate dimeglumine 0.1 mmol/kg at 3 ml/s. Axial T1-weighted MR images of the abdomen were also obtained. Full acquisition parameters are listed in Supplementary Table 1.

Multiparametric MRI interpretation. Pre-treatment mpMRI imaging was interpreted by single expert genitourinary radiologist with more than 10 years' experience (B.T.) following the Prostate Imaging–Reporting and Data System version 2 (PI-RADSv2) guidelines. Five distinct regions within the large apical–mid PZ lesion affecting right side of prostate and extending to left side of the prostate in distal component were identified for biopsy targeting. These five targets included: left distal apical PZ, right distal apical PZ, right posterior apical PZ, right anterior apical PZ, and right mid PZ. Post-treatment imaging was interpreted by the same expert radiologist for assessment of residual tumor burden.

MR/ultrasound-fusion biopsy. mpMRI was performed upon study entry and again prior to surgery. Biopsy planning and procedure was completed using a commercially available MRI/TRUS fusion-targeting platform (UroNav, Invivo Corp., Gainesville, Florida). MR/ultrasound-fusion biopsies were targeted to regions identified on the first mpMRI. For each MR target, the urologist (P.P.) acquired a biopsy in the axial and sagittal plane of the patient. The axial and sagittal biopsies of the right-sided tumor were concordant for high-grade cancer, perineural invasion, and intraductal carcinoma. Invasive and intraductal tumor foci were separately isolated, and genomic material was pooled across both biopsies by histology. The axial biopsy of the left-sided tumor-only sampled fibromuscular tissue. The sagittal biopsy of the left-sided tumor was used for this study.

Histology. After sampling, biopsy tissue was placed into formalin and processed into paraffin blocks using standard methods. Following surgery, whole-mount prostate specimens were processed with patient-specific MRI-based 3D-printed

molds for image-pathology correlation²⁹. Briefly, the prostate specimen was serially sectioned within the mold from apex to base at 6-mm intervals corresponding to axial planes on mpMRI images. The mold was placed in formalin overnight, the prostate was sliced for whole mounting, and slices were placed in cassettes for processing into paraffin blocks using standard methods.

Biopsy and prostatectomy tissues were sectioned at 5 µm thickness onto charged slides and stained with hematoxylin and eosin using standard methods. Serial sections of biopsy and RP tissues were stained with anti-AR (Cell Signaling, Cat# 5153S, 1:100 into SignalStain diluent), anti-ERG (Abcam, Cat# ab92513, 1:500 into SignalStain diluent), anti-PTEN (Cell Signaling, Cat# 9188L, 1:100 into SignalStain diluent), and PIN-4 cocktail (Biocare Medical, Cat# PPM225DS, ready-to-use) antibodies. SignalStain diluent was from Cell Signaling. Slides were baked for 15 min at 60 °C, except for PIN-4 staining which was baked overnight at 45 °C, deparaffinized through xylenes and rehydrated through graded alcohols. Antigen retrieval was performed using a NxGen Decloaker (Biocare Medical), for 15 min at 110 °C in Tris-EDTA (Abcam, Cat# ab93684) for PTEN, and for 15 min at 110 °C in Diva Decloaker (Biocare Medical, Cat# DV2004MX) buffers. Sections were blocked with hydrogen peroxide (Sigma Aldrich, Cat# 216763) for 5 min, blocked with Background Sniper (Biocare Medical, Cat# BS966) for 10 min for PIN-4 or VectaStain Elite ABC HRP kit (Vector Laboratories, Cat# PK-6101) for PTEN, AR, and ERG, and incubated with primary antibody for overnight at 4 °C (AR, ERG, PTEN) or 1 h (PIN-4). Secondary labeling was performed using Mach 2 Double Stain for PIN-4 (Biocare Medical, Cat# MRC525) or the VectaStain Elite ABC HRP kit for AR, ERG and PTEN for 30 min. Avidin-biotin complexing was then performed for 30 min for AR, ERG, and PTEN. Colorimetric detection was achieved using DAB Peroxidase HRP (Vector Laboratories, Cat# SK4100) for AR, ERG, and PTEN, or Vulcan Red Fast Chromogen (Biocare Medical, Cat# FR805) and Betazoid DAB (Biocare Medical, Cat# BDB2004) for PIN-4. Counterstaining was performed using Mayer's Hematoxylin Solution (Sigma Aldrich, Cat# MHS16). PIN-4 stained slides were air-dried. AR, ERG, and PTEN-stained slides were dehydrated through graded alcohol and cleared in xylenes. Slides were mounted using Permunt (Thermo Fisher).

ERG expression of post-treatment specimens was assessed using Opal Multiplex Immunohistochemistry (Akoya Biosciences, Cat# NEL811001KT). Antigen retrieval was performed using a NxGen Decloaker, for 15 min at 110 °C in Opal AR 9 antigen retrieval buffer (Akoya Biosciences, Cat# AR9001KT). Sections were blocked with 2.5% NGS (ImmPRESS HRP Anti-Rabbit IgG polymer kit, Vector Laboratories, Cat# MP-7451) for 10 min, and incubated with anti-ERG (Abcam, Cat# ab92513) primary antibody at 1:500 for 30 min at room temperature. ImmPRESS HRP anti-rabbit IgG polymer was added for 15 min, then an anti-HERP-conjugated Opal 520 fluorophore (Akoya Biosciences, Cat# FP1487001KT) was applied at 1:500 for 10 min. DAPI (4', 6-diamidino-2-phenylindole, Thermo Fisher, Cat# 1306) was applied at 350 nM for 10 min before slides were mounted using ProLong Glass Antifade Mountant (Thermo Fisher, Cat# P36980).

H&E-, bright-field IHC-, and immunofluorescent IHC-stained slides were scanned using the 20× objective (Plan-Apochromat, NA 0.8) with bright-field or LED fluorescent illumination on an Axio Scan.Z1 (Zeiss) with slide loaders to accommodate standard (25 mm × 75 mm) slides and whole-mount double-wide (50 mm × 75 mm) slides. Immunofluorescent images were uniformly contrast enhanced using ImageJ (NIH). The merged image of immunofluorescence and H&E was generated by removing the background of the fluorescent images in Photoshop CC 2019 (Adobe) and overlaying onto the bright-field image.

Residual cancer burden calculations. Residual tumor was concordantly identified by three board-certified genitourinary pathologists (M.M., R.L., and H.Y.). Residual cancer burden (RCB)³⁰ was measured and calculated by multiplying the number of slices through which each residual tumor extended by the largest cross-sectional width and length and block thickness (0.6 cm). Volume was further corrected by multiplying by 0.4 and the estimated tumor cellularity. Precise length and width measurements were performed on scanned slides using Zen Blue 2012 (Zeiss) with objective/magnification and pixel:distance calibrations recorded within the scanned CZI file. The right-sided lesion extended through four blocks. With a length of 2.09 cm, a width of 1.73 cm, and 10% cellularity, the RCB was 0.35 cc. The left-sided lesion extended through two blocks. With a length of 0.62 cm, a width of 0.35 cm, and 10% cellularity, the RCB was 0.01 cc.

Genomic analysis. Regions of residual tumor identified in whole-mount prostatectomy specimens were registered with post-treatment mpMRI, and co-registered with pre-treatment mpMRI using imaging landmarks for reference. Biopsy paths were superimposed on pre-treatment MR targets to indicate the spatial relationship between biopsy tissue and RP tissue. Serial sections of tumor tissue (and benign regions uninvolved with tumor) were cut onto metal frame PEN-membrane slides (MicroDissect GmbH), stained with Paradise Plus stain (Thermo Fisher), and laser capture microdissected using an ArcturusXT Ti microscope (Thermo Fisher) onto CapSure Macro LCM Caps (Thermo Fisher). Serially stained slides of PIN-4, anti-AR, and anti-ERG immunostaining were used as references.

Using the QIAamp DNA FFPE Tissue Kit (Qiagen), microdissected cells adhered to caps were lysed and DNA was purified according to the manufacturer's instructions. DNA yields were quantified using Picogreen reagent (Thermo Fisher). For exome sequencing, 10–50 ng of DNA were sheared (Covaris), and assembled into

whole-exome libraries using the SureSelect Human All Exon V7 Low Input Exome (Agilent). Samples were sequenced to an average depth of 35× on-target coverage on an Illumina HiSeq4000 at 150 cycles paired-end, eight indexing cycles, and ten molecular barcoding cycles. Pass-filter FASTQ files were trimmed using SureCall Trimmer 4.0.1 (Agilent), aligned with the Burrows Wheeler Aligner BWA-MEM version 0.7.17³¹ to version hg19 of the human genome (b37 with decoy chromosomes), duplicate-marked using LocatIt 4.0.1 (Agilent), and quality score recalibrated using version 4.0.5.2 of the Genome Analysis Toolkit (GATK). Somatic point mutations were called on intervals from the Agilent bait design BED files using MuTect2 (part of the GATK4 package), first by running MuTect2 in tumor-only mode on all BAM files generated by our group using the same Agilent library preparation method from normal (not tumor) FFPE DNA and merging the resultant VCF files using the CreateSomaticPanelOfNormals module of GATK. MuTect2 was run in somatic mode on each tumor BAM paired with the normal BAM from this patient and the somatic panel of normal with af-of-alleles-not-in-resource set to 0.0000025 disable-read-filter set to MateOnSameContigOrNoMappedMateReadFilter. GetPileupSummaries and CalculateContamination were used on each tumor BAM file, and the resultant contamination table was used to filter somatic mutations using FilterMutectCalls. CollectSequencingArtifactMetrics and FilterByOrientationBias were used to further filter mutations for 8-oxoG artifacts using the settings -AM G/T -AM C/T. These pass-filter mutations were then functionally annotated using Oncotator version 1.9.70 (database version April052016). Mutations were then further filtered on a per-sample basis by requiring any locus with a somatic mutation be covered at least half of the mean coverage for the sample and by no fewer than 16 reads and at least 2 unique counts of the alternate allele. Mutations resulting from obvious read bias were removed by running HaplotypeCaller on the reassembled BAM output of MuTect2 in somatic mode and using the directionality of the reads in the strand direction-annotated VCF file. All remaining pass-filter SNVs and indels were then evaluated against the output of MuTect2 from the other samples. For loci where mutations were called confidently in one sample but filtered out, those mutations were backfilled. For loci where no mutations were called, the read depth of the reference allele was backfilled using Pysamstats version 0.24.3 in Python 2.7.

Somatic copy number alterations and aCGH-style plots were generated using Nexus Copy Number version 9 (BioDiscovery). All BAM files generated by our group using the same Agilent library preparation method from normal (not tumor) FFPE DNA were loaded into the Multiscale Reference Builder, specifying the Agilent bait design BED file for selecting regions of the genome to estimate copy number. All default settings were used. After generating the pooled reference, each tumor BAM file was separately loaded with the following analysis settings: systematic correction: quadratic correction; recenter probes: median; analysis: SNP-FASST2 segmentation; significance threshold: 1.0E-6; max contiguous probe spacing (Kbp): 1000.0; percent outliers to remove: 3.0. Because Nexus copy number relies on arbitrary cutoffs for calling gains and losses, we imputed SCNA calls from GATK4 for determining whether deviation from a copy number ratio of 0 was a meaningful alteration. To restrict analysis to only those regions covered by the exome library, the Agilent library design BED file was preprocessed with PreprocessIntervals with bin-length set to 0 and interval-merging-rule set to OVERLAPPING_ONLY. The BED file was also annotated with GC content using AnnotateIntervals. These interval files were used in all GATK4 copy number calling steps. To obtain SCNA calls with GATK4, read counts were first obtained from all BAM files using CollectReadCounts, including from all BAM files generated by our group using the same Agilent library preparation method from normal (not tumor) FFPE DNA. The normal read count files were compiled into a panel of normal using CreateReadCountPanelOfNormals, and the panel of normal was used to smooth read counts across all samples using DenoiseReadCounts. CollectAllelicCounts was also performed on all denoised BAM for identifying regions of potential LOH. ModelSegments used tumor smoothed read counts and the paired normal/tumor allelic counts for generating copy number estimates that were then called using CallCopyRatioSegments.

To determine whether an unbiased assessment of all SNVs and copy number alterations would identify clonal somatic events based on grouped analysis of all six tumor samples, we employed CLONET (version 1.0.0 revision 20171016) with the v2 method for estimating error³². SCNAs jointly called by both Nexus and GATK were reformatted into SEG files, and each tumor BAM file was filtered for informative SNPs using ASEQ version 1.1.1.1. We then parsed the point mutation and SCNA clonality tables for events marked “clonal” or “subclonal” by CLONET, which would indicate that CLONET had identified molecular evidence of a single tumor cell population with expected allelic fractions in interrelated subclones. However, CLONET marked all events as “subclonal.uncertain” or “notanalysed” which occurs when segments and mutations do not fit the assumption of the expected model for identifying subclonal populations³².

Based on pass-filter SNVs and indels identified from exome sequencing, 50 targets were selected randomly from 871 total calls for additional amplicon-based sequencing, with the exception of the TP53 Y236X nonsense alteration, which was subsequently included for verification^{33,34}. Prior to nomination for targeted sequencing, each selected genomic coordinate was inspected in IGV and mutation calls falling in MAPQ < 10 were excluded. Four of the randomly selected mutations were excluded by this vetting process, resulting in 47 total targets. Using parameters to limit the amplicon size to 140 nucleotides, we used the Ion AmpliSeq Designer [<https://www.ampliseq.com>] tool which identified 46 multiplex primer pairs that were compatible with our intended targets in a single pool. All multiplex

primer panel designs were cross-checked using In-Silico PCR [<https://genome.ucsc.edu/cgi-bin/hgPcr>] to prevent production of unintended amplicons for each patient-specific batch of targets. The forward and reverse primer sequences identified above were appended with adaptor sequences³³ at the 5' end, complementary to Illumina Nextera full-length primers (IDT). Dual-barcoded sequencing adaptors were modified³³ to contain longer regions of complementarity and were ordered with NGSO-4 purity from Sigma. Primers were resuspended in 1 × TE buffer (pH 8.0, 100 μM). A designated pre-PCR workstation was used for the first reaction. A mixture of all forward primers was prepared such that each primer was represented at 2 μM concentration. An aliquot of the primer mix (2.5 μL) was combined with uracil N-glycosylase (0.3 U, 2 μL), cDNA (8 μL), 2 × KAPA HiFi HotStart Uracil + ReadyMix (15 μL), and dUTP (3.5 μL, 2.5 μM). Samples were incubated in a thermal cycler at 37 °C for 2 min, 98 °C for 5 min, 65 °C for 2 min, and 72 °C for 7 min. Products from this reaction were cleaned using AMPure XP SPRI beads (54 μL for each 30 μL tagging reaction), following the manufacturer's recommended protocol for mixing, magnetic separation, ethanol washes, and drying. Elution was performed with deionized nuclease-free water (10 μL) to recover the purified tagged product. Purified tagged products (8 μL) were combined with ddATP (2 μL, 0.2 mM), 10 × CoCl₂ (2 μL), 10 × terminal deoxynucleotidyl transferase (Tdt) buffer (2 μL), low TE buffer (4.4 μL), and Tdt enzyme (1.6 μL of 20 U) for a total volume of 20 μL. Samples were incubated for 90 min at 37 °C and 15 min at 75 °C. Samples were held at 4 °C until clean-up, which was performed using the Select-A-Size Clean & Concentrator Kit (Zymo) according to the manufacturer's instructions for a 100 bp one-sided cutoff with the following modifications: the last wash step was centrifuged at maximum speed for 1 min, and elution was performed with 10 μL Zymo elution buffer with a 5 min incubation. 8 μL of purified product were recovered. For the amplification of targets using the reverse primers, a mixture of reverse oligonucleotides was created with 1 μL of each 100 μM primer in a total volume of 100 μL TE, for a final concentration of 1 μM each primer. To 1.25 μL of this primer mix, 3.5 μL of 2.5 μM dUTP, 1 μL of TE, 8 μL of product from the terminal transferase reaction, 1.25 μL of a 2 μM stock of i5 barcoded sequencing adaptor³³, and 15 μL of 2 × KAPA HiFi HotStart Uracil + ReadyMix were added. Samples were incubated in a thermal cycler at 98 °C for 5 min initially and followed by 20 cycles of PCR (98 °C–65 °C–72 °C for 30 s each with 7 min of final extension at 72 °C). Clean-up was performed using the Select-A-Size Clean & Concentrator Kit using the guidelines for a 200 bp one-sided cutoff according to the manufacturer's protocol with the following modifications: the last wash step was centrifuged at maximum speed for 1 min, and elution was performed with 10 μL Zymo elution buffer following a 5 min incubation. In total, 8 μL of purified product were recovered. Final construction of each sequencing library was performed by additional amplification using both the NGSO-4 i5 and i7 sequencing adaptors to prime PCR. All 8 μL of product from the previous step was mixed with 4 μL of 4 μM i7 adaptor stock, 4 μL of 2 μM i5 adaptor stock, 4 μL of TE, and 20 μL of 2 × KAPA HiFi HotStart Uracil + ReadyMix. Samples were incubated in a thermal cycler at 98 °C for 5 min and followed by 10–30 cycles of PCR (98 °C–72 °C for 30 s each with 7 min of final extension at 72 °C). The exact number of cycles of PCR to use were determined empirically by substituting control FFPE genomic DNA in the first step of the library preparation protocol, substituting 4 μL of 6 × SYBR green in the final PCR step above, and performing qPCR on a QuantStudio 3 (Thermo Fisher). The number of cycles was determined by cycle threshold value corresponding to the ½-maximum of amplification saturation on a linear curve. Clean-up was performed using the Select-A-Size Clean & Concentrator Kit with a 200 bp one-sided cutoff as described above, but with a 20 μL elution. Quality control was performed on the completed library using D1000 ScreenTapes on a TapeStation looking for a peak size between 250 bp and 350 bp with primer dimer comprising <10% of the library. The library was then quantified for functional concentration using the KAPA Illumina Quantification Kit for NGS, using a 1:50,000 dilution of the library per the manufacturer's protocol. The library was adjusted to a final concentration of 10 μM.

The amplicon library was sequenced on a MiSeq (Illumina) instrument with the v2 Reagent Kit (500-cycle). 20% Phi-X was added to the final library pool. Sequencing was performed with 195 cycles paired-end and dual 8-cycle indexing reads on the P5 and P7 adaptors. This sequencing strategy intentionally sequenced through the entire insert molecule and into the adaptor on the other side, reading the i5 and i7 indexing adaptor an additional time. Individually barcoded libraries were recovered and filtered for quality using bcl2fastq (Illumina). Pass-filter FASTQ files were trimmed with Trimmomatic version 0.36, preserving the trimmed-off sequences and parsing them for the expected i5 and i7 adaptor based on bcl2fastq binning. Comparing each library to the expected adaptor pair, read pairs corresponding to the incorrect adaptors were discarded. Remaining read pairs in FASTQ files were aligned to b37-decoy using BWA-MEM version 0.7.10. BAM files were sorted and indexed using samtools version 1.9, and unique base calls were identified by running using Pysamstats version 0.24.3 in Python 2.7 over the defined intervals for each target, and all calls were manually inspected using IGV version 2.4.19.

Of the 46 targets, 1 target was in a region of poor complexity and was removed from future consideration. One target failed to amplify in all samples, while five targets preferentially demonstrated bias toward the alternate allele in the normal sample and were also removed. Based on counts of the alternate allele determined a priori from exome sequencing, a sample was determined to harbor the mutation in

question if (1) the frequency of the mutation in the tumor sample was three times greater than in the benign reference; (2) if the alternate absolute allele read count was greater than 50; and (3) if the read count for the base in question was at least three times greater than the average alternate allele for the remainder of the locus (i.e., above the background error rate of library preparation and sequencing).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

For the protection and privacy of the human subject in this study, the raw whole-exome and targeted sequencing data have been deposited in NCBI Database of Genotypes and Phenotypes (dbGaP), a controlled-access database, under the accession ID [PHS001938.v1.p1](https://www.ncbi.nlm.nih.gov/submitter/study/PHS001938.v1.p1). All the other data supporting the findings of this study are available within the article and its supplementary information files, and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. The source data underlying Fig. 1j, Supplementary Table 3, and Supplementary Table 4 are provided as a Source Data file.

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Author contributions

S.W., S.H., H.Y., B.T., and A.G.S. designed the research study. P.A.P., P.L.C., and W.L.D. designed the clinical study. F.K., P.A.P., R.A.M., D.J.V., G.C., J.L.G., M.J.M., and B.T. were involved in patient care and acquired patient data. S.W., D.J.V., R.T.L., R.L., R.A., J.R.B., N.V.C., S.Y.T., M.J.M., H.Y., and A.G.S. performed histopathological analyses. S.H. and B.T. performed imaging analyses. S.W., N.T.T., S.Y.T., and A.G.S. performed genomics analyses. S.W., S.H., D.J.V., H.Y., R.T.L., B.T., and A.G.S. interpreted results. S.W., S.H., and A.G.S. prepared figures. S.W., S.H., N.V.C., H.Y., and A.G.S. drafted the paper. All authors approved the final paper.

Competing interests

H.Y. and R.T.L. perform consulting in an advisory role for Janssen Pharmaceuticals. The remaining authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.G.S.

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Prostate Cancer

Nascent Prostate Cancer Heterogeneity Drives Evolution and Resistance to Intense Hormonal Therapy

Scott Wilkinson^a, Huihui Ye^{b,c}, Fatima Karzai^d, Stephanie A. Harmon^{e,f}, Nicholas T. Terrigino^a, David J. VanderWeele^{a,g}, John R. Bright^a, Rayann Atway^a, Shana Y. Trostel^a, Nicole V. Carrabba^a, Nichelle C. Whitlock^a, Stephanie M. Walker^e, Rosina T. Lis^a, Houssein A. Sater^d, Brian J. Capaldo^a, Ravi A. Madan^d, James L. Gulley^d, Guinevere Chun^d, Maria J. Merino^h, Peter A. Pintoⁱ, Daniela C. Salles^j, Harsimar B. Kaur^j, Tamara L. Lotan^j, David J. Venzon^k, Peter L. Choyke^e, Baris Turkbey^e, William L. Dahut^d, Adam G. Sowalsky^{a,*}

^a Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, Bethesda, MD, USA; ^b Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA, USA; ^c Department of Pathology and Department of Urology, University of California-Los Angeles, Los Angeles, CA, USA; ^d Genitourinary Malignancies Branch, National Cancer Institute, Bethesda, MD, USA; ^e Molecular Imaging Program, National Cancer Institute, Bethesda, MD, USA; ^f Clinical Research Directorate, Frederick National Laboratory for Cancer Research, Frederick, MD, USA; ^g Department of Medicine, Feinberg School of Medicine, Chicago, IL, USA; ^h Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA; ⁱ Urologic Oncology Branch, National Cancer Institute, Bethesda, MD, USA; ^j Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD, USA; ^k Biostatistics and Data Management Section, National Cancer Institute, Rockville, MD, USA

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Abstract

Background: Patients diagnosed with high risk localized prostate cancer have variable outcomes following surgery. Trials of intense neoadjuvant androgen deprivation therapy (NADT) have shown lower rates of recurrence among patients with minimal residual disease after treatment. The molecular features that distinguish exceptional responders from poor responders are not known.

Objective: To identify genomic and histologic features associated with treatment resistance at baseline.

Design, setting, and participants: Targeted biopsies were obtained from 37 men with intermediate- to high-risk prostate cancer before receiving 6 mo of ADT plus enzalutamide. Biopsy tissues were used for whole-exome sequencing and immunohistochemistry (IHC).

Outcome measurements and statistical analysis: We assessed the relationship of molecular features with final pathologic response using a cutpoint of 0.05 cm³ for residual cancer burden to compare exceptional responders to incomplete and nonresponders. We assessed intratumoral heterogeneity at the tissue and genomic level, and compared the volume of residual disease to the Shannon diversity index for each tumor. We generated multivariate models of resistance based on three molecular features and one histologic feature, with and without multiparametric magnetic resonance imaging estimates of baseline tumor volume.

Results and limitations: Loss of chromosome 10q (containing *PTEN*) and alterations to *TP53* were predictive of poor response, as were the expression of nuclear ERG on IHC and the presence of intraductal carcinoma of the prostate. Patients with incompletely and nonresponding tumors harbored greater tumor diversity as estimated via phylogenetic

* Corresponding author. Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, 37 Convent Drive, Bethesda, MD 20892, USA. Tel. +1 240 7607118.
E-mail address: adam.sowalsky@nih.gov (A.G. Sowalsky).



tree reconstruction from DNA sequencing and analysis of IHC staining. Our four-factor binary model (area under the receiver operating characteristic curve [AUC] 0.89) to predict poor response correlated with greater diversity in our cohort and a validation cohort of 57 Gleason score 8–10 prostate cancers from The Cancer Genome Atlas. When baseline tumor volume was added to the model, it distinguished poor response to NADT with an AUC of 0.98. Prospective use of this model requires further retrospective validation with biopsies from additional trials.

Conclusions: A subset of prostate cancers exhibit greater histologic and genomic diversity at the time of diagnosis, and these localized tumors have greater fitness to resist therapy.

Patient summary: Some prostate cancer tumors do not respond well to a hormonal treatment called androgen deprivation therapy (ADT). We used tumor volume and four other parameters to develop a model to identify tumors that will not respond well to ADT. Treatments other than ADT should be considered for these patients.

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1. Introduction

The vast majority of prostate cancers are detected and treated early while potentially curable by surgery or radiation [1]. By contrast, localized prostate tumors that progress despite definitive therapy are characterized by extensive evolutionary heterogeneity at the time of initial treatment and later at recurrence, and therapy resistance in metastatic castration-resistant prostate cancer (mCRPC) can be associated with multiple subclones that emerged from a common ancestor [2,3]. Numerous studies have identified subsets of human tumors, including prostate cancers, with a higher risk of recurrence because of greater intratumoral heterogeneity, and evidence suggests that active mutational processes are more likely to give rise to a daughter clone with resistance in more evolutionarily complex cancer systems [4–6]. For patients with prostate cancer, who may go for more than a decade between initial therapy and development of radiographically confirmed metastases, intervening chemohormonal treatments and their effects on tumor progression are based on biochemical recurrence with rare opportunities for sampling tumors by biopsy until later in the disease course. Consequently, little is known about the relationship between the evolutionary trajectory of treatment-naïve localized prostate tumors and their propensity to resist direct treatment.

The androgen receptor (AR) remains a molecular target for prostate cancer therapy via inhibition of the release of testicular androgen (androgen deprivation therapy, ADT) and newer-generation inhibitors including abiraterone and enzalutamide. These agents are the standard of care for treatment of mCRPC, but recent clinical trials have demonstrated their efficacy in nonmetastatic CRPC as well as in de novo metastatic hormone-sensitive prostate cancer [7–10]. Although the rationale for applying these drugs earlier in the disease course is that AR activity is critical for progression, it is logical that inhibition of AR activity can ablate organ-confined disease or prevent it from spreading.

Neoadjuvant ADT (NADT) and, more recently, intense NADT (ie, ADT plus one or more AR-targeted agents) has been tested in a series of phase 2 clinical trials in efforts to

decrease intratumoral testosterone levels and diminish the volume of residual disease before surgery [11–13]. Analyses of these cohorts have demonstrated that greater baseline tumor volume is a significant predictor of greater final tumor volume [14]. A recent meta-analysis of several studies also indicated that patients with less residual disease at the end of treatment experience longer durations of biochemical recurrence-free survival [15]. With pathologic complete response rates varying across cohorts, analyses of post-treatment surgical specimens have indicated that molecular alterations driving resistance are subclonal and are present before treatment, pointing to a relationship between divergence from the truncal clone for a given tumor and greater fitness associated with subclonal heterogeneity in a hormone-sensitive state [16,17].

We thus hypothesized that evolutionary principles relating diversity and heterogeneity to clinical outcome could explain in part why a heterogeneous cohort of high-risk tumors at baseline could result in diverse resistance mechanisms after treatment. We present an integrated histologic and genomic analysis of 141 tumor exomes sampled via laser capture microdissection and representing 110 baseline biopsy tissues from 37 patients with intermediate- to high-risk prostate cancer who were enrolled in a single-arm phase 2 clinical trial of NADT intensified with enzalutamide [14]. Using both tissue- and DNA-based analyses, we observed that the volume of residual disease at the end of therapy increases proportionally with tumoral diversity at baseline. Localized prostate cancers with more complex evolutionary trajectories are thus more likely to be resistant to intense systemic hormonal therapy and have a lower chance of relapse-free survival. To predict the likelihood of nonresponse, we demonstrate that a straight-forward four-factor histogenomic model recapitulates the relationship between nonresponse and greater baseline subclonal diversity. Baseline tumor volumes estimated via multiparametric magnetic resonance imaging (mpMRI) are complementary to diversity measures and further improve prediction. Thus, our results indicate that the greater fitness of more diverse tumors contributes, at least in part, to treatment resistance in high-risk localized prostate cancer.

2. Patients and methods

2.1. Biospecimen procurement

All patients provided informed consent to join National Cancer Institute study 15-c-0124 (NCT02430480), approved by the National Institutes of Health Institutional Review Board in accordance with the Declaration of Helsinki [14].

Patients underwent baseline mpMRI, and MRI/ultrasound fusion biopsies were targeted to one or more mpMRI lesions. A 12-core template biopsy was also obtained in a subset of patients. A sample of saliva (Oragene OG-500; DNA Genotek, Ottawa, Canada) or blood, from which genomic DNA was further derived, was also collected before treatment initiation. Following 6 mo of ADT plus enzalutamide, patients underwent a second mpMRI before radical prostatectomy (RP).

2.2. Sample processing

Serial sections (5 μ m) of formalin-fixed, paraffin-embedded biopsy tissues were stained with hematoxylin and eosin, stained with antibodies via immunohistochemistry (IHC), or cut onto polyethylene naphthalate membrane slides (MicroDissect GmbH, Herborn, Germany) for laser capture microdissection (LCM). LCM was performed to sample 141 histologically distinct tumor foci from 110 biopsies (median of four foci per patient), plus regions of benign tissue. DNA was extracted, assembled into libraries for whole-exome sequencing, and analyzed for detection of somatic mutations and copy number alterations. IHC was performed with an IP FLX autostainer (Biocare Medical, Pacheco, CA, USA) using AR, prostate-specific antigen (PSA), glucocorticoid receptor (GR), ERG, PTEN, synaptophysin, PIN-4, Ki-67, NKX-3.1, CAM5.2, p53, and p63 antibodies. The Supplementary material provides complete descriptions of the sampling and processing steps.

2.3. Statistical analysis

Statistical analyses were performed with GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA), Microsoft Excel for Mac version 16 (Microsoft Corporation, Redmond, WA, USA), and SAS/STAT software version 14.1 (SAS Institute, Cary, NC, USA). Null hypothesis tests of associations (enrichments) between response and individual dichotomous factors were performed using a two-sided Fisher's exact test at a per-patient and a per-lesion level. Estimated odds ratios were calculated with 95% confidence intervals (CIs) derived from inverted one-sided exact tests. Associations between factors were measured using Pearson or Spearman correlations. Comparisons of single factors between exceptional responder (ER) and incomplete and nonresponder (INR) cases were performed using Student or Welch's *t* tests or the Wilcoxon rank sum test. Comparisons of covariates within single factors between ER and INR cases were performed using the Cochran-Armitage test for trend. The four features used in the histogenomic model were selected according to the top performing features from genomic and histologic univariate analyses with odds ratios favoring INR. Logistic regression models including the four factors were fitted to the data. Estimated probabilities were compared to the observed response proportions for the factor combinations. The accuracy of the dichotomized estimated probabilities was examined using receiver operating characteristic curves. Response probabilities were estimated as the same function of the factor values in an independent validation population. Genomic Shannon diversity index (SDI) scores were tested for Pearson correlations with these probabilities. Owing to the small cohort size, corrections for multiple comparisons were not performed.

3. Results

3.1. Genomic alterations consistent with high-risk prostate cancer

We analyzed a spectrum of somatic copy-number alterations and gene mutations frequently reported for human tumors in 141 LCM foci from baseline biopsies using whole-exome sequencing (Supplementary Fig. 1 and Supplementary Table 1). Germline alterations to cancer-related genes were detected via whole-genome sequencing of DNA from saliva or buffy coats (Supplementary material). These 141 foci, based on different histologies or biopsy blocks, were sampled from 48 distinct MRI-contoured lesions (Supplementary Fig. 2 and Supplementary Table 2) from all 37 patients who completed our clinical study. There was variable genomic heterogeneity between foci from the same MRI-defined lesion and between lesions in the same patient (Supplementary Figs. 1 and 2). We curated 28 somatic alterations for deeper analysis on the basis of their frequent reporting in large prostate cancer cohort studies [18–21]. When collapsed to single data points for each patient (Fig. 1A and Supplementary Table 3) on the basis of the index MRI lesion, the landscape of these alterations was not significantly different from that for 188 high-risk, Gleason pattern 4 and 5 tumors from The Cancer Genome Atlas (TCGA) PRAD [19] data set (Supplementary Fig. 3A and Supplementary Table 4). Individually, 27 of these features did not significantly differ (χ^2 test) between our clinical trial and the TCGA cohort (Supplementary Fig. 3B; $p=0.098$, Wilcoxon matched-pairs test), validating the frequency of these alterations overall.

Following treatment, we used RP specimens to assess local responses to therapy by calculating the residual cancer burden (RCB), a validated intermediate endpoint for systemic therapy that has clinical significance for predicting recurrence after surgery [13,15]. On the basis of minimal residual disease rates in prior neoadjuvant studies, we preselected 0.05 cm³ as the cutpoint (Fig. 1B) between ER and INR cases at the patient level, resulting in 15 ER and 22 INR patients. One patient whose tumor expanded during therapy underwent transurethral resection of prostate tumor instead of RP and thus RCB could not be calculated. Within the INR group, registration of pretreatment biopsies and imaging to final MRI and pathology identified 12 additional biopsy-confirmed lesions of which ten exhibited an exceptional pathologic response, for a total of 25 ER and 24 INR MRI-visible lesions (23 INR lesions with genomic data).

We then compared the frequency of 27 recurrent genomic events between the two groups in univariate analysis (Fig. 1C). Arm-level loss (encompassing $\geq 50\%$) for chromosome 10q was exclusive to the INR group and hotspot mutations to *SPOP* were only observed in the ER group. Somatic copy-number losses, frameshifts, and hotspot mutations to *TP53* were significantly enriched in the INR group ($p=0.023$, Fisher's exact test) at baseline (Supplementary material). Similar trends were also observed at the lesion level (Fig. 1D) and loss of chromosome

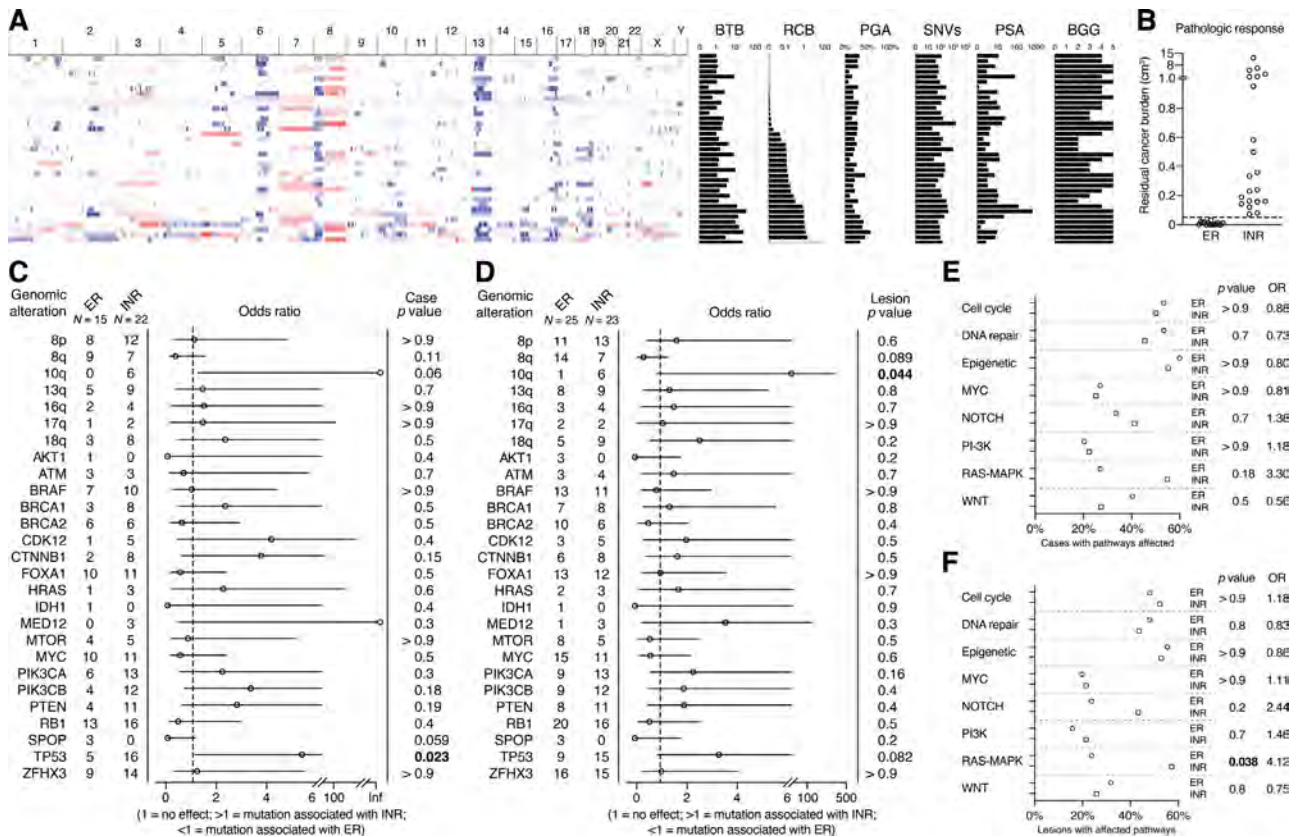


Fig. 1 – Genomic landscape of prostate tumors before treatment with neoadjuvant androgen deprivation therapy plus enzalutamide. (A) Genomic and clinical data per patient ($n=37$). One patient classified clinically as a nonresponder underwent transurethral resection of prostate tumor instead of radical prostatectomy so RCB could not be calculated. BTB=baseline tumor burden (cm^3); RCB=residual cancer burden (cm^3); PGA=percent genome altered; SNVs=small nucleotide variants; PSA=prostate-specific antigen (ng/ml); BGG=highest biopsy Gleason group. (B) Distribution of RCB values across 15 exceptional responder (ER) and 21 incomplete and nonresponder (INR) cases with a cutpoint of 0.05 cm^3 . (C,D) Frequency and odds ratio for 27 genomic features for comparison of INR and ER (C) cases and (D) lesions. (E,F) Grouping of altered genes into eight pathways, with the proportion of affected (E) cases and (F) lesions with genes altered per pathway. For C–F, each factor was considered independently in a two-sided Fisher's exact test, and the effect size is given as an odds ratio (OR). ORs >1 favor INR. For C–F, these are exploratory analyses that are underpowered for per-gene analyses, and thus may not be replicable in other cohorts when adjustments for multiple testing are applied.

10q was significantly associated with poor response to NADT ($p=0.044$). Although the significance levels for these alterations may not be conclusive because of multiple testing, further exploration is warranted for these top genomic features associated with poor response.

After grouping gene alterations into pathways [22], we found that a greater proportion of INR tumors accumulated somatic alterations to RAS/RAF/MAPK pathway genes, which was statistically significant at the lesion level (Fig. 1E,F). Germline frameshift mutations to DNA homologous repair genes (*BRCA1*, *BRCA2*, and *PALB2*) were observed in an ER case and two INR cases (Supplementary Table 3), but neither group was significantly enriched for alterations in DNA damage repair genes, consistent with similar mutation-type proportions and signatures between ER and INR patients (Supplementary Fig. 4A–D). Thus, both ER and INR tumors demonstrated features of high-risk tumors and significantly altered genomes, but there were relatively few differences between the groups.

3.2. Pathologic and histologic features distinguish ER from INR tumors

With a broad range of pathologic responses across the cohort, we explored whether clinicopathologic parameters can explain, at least in part, differences in treatment response. Although INR patient tumor burdens generally were greater at baseline (Table 1, Fig. 2A, and Supplementary Fig. 5A), there was no difference between ER and INR patients in baseline serum PSA levels (Fig. 2B), highest Gleason grade group for pretreatment biopsies per patient (Fig. 2C) and per mpMRI-visible lesion (Supplementary Fig. 5B), or the number of mpMRI-visible lesions (Supplementary Fig. 5C). On univariate analysis, the proportion of cases or lesions harboring cribriform architecture, ductal morphology, or Gleason pattern 4 or 5 did not distinguish ER from INR, but nuclear ERG and intraductal carcinoma (IDC-P) were significantly enriched in INR cases (Fig. 2D,E and Supplementary Fig. 5D; $p=0.0020$ and $p=0.013$, respectively).

Table 1 – Patient characteristics

Parameter	Exceptional responders	Incomplete or nonresponders	p value
Patients (n)	15	22	
Median age, yr (IQR)	63 (58–71)	62 (56–68)	0.20
Race, n (%)			0.60
White	11 (73)	14 (64)	
Black	3 (20)	3 (14)	
Other	1 (7)	5 (23)	
Median baseline PSA, ng/ml (IQR)	9.58 (5.90–21.37)	8.05 (5.37–15.38)	0.55
Median baseline PSA density, ng/ml/ml (IQR)	0.22 (0.16–0.40)	0.21 (0.15–0.36)	0.96
Median prostate volume, ml (IQR)	43.0 (35.0–56.5)	36.5 (31.6–47.9)	0.14
Median MRI tumor burden, cm ³ (IQR)	1.58 (1.27–3.07)	6.99 (3.13–18.53)	0.0002
Pretreatment clinical T stage, n (%)			0.15
cT1	2 (13)	2 (9)	
cT2	5 (33)	1 (5)	
cT3a	5 (33)	10 (45)	
cT3b	1 (7)	6 (27)	
cT4	2 (13)	3 (14)	
Pretreatment cN1 stage, n (%)	3 (20)	8 (36)	0.47
Biopsy ISUP grade group, n (%)			0.52
2	0 (0)	4 (18)	
3	2 (13)	3 (14)	
4	7 (47)	4 (18)	
5	6 (40)	11 (50)	

IQR = interquartile range; ISUP = International Society of Urological Pathology; MRI = magnetic resonance imaging; PSA = prostate-specific antigen.

As suggested by the frequency of *PTEN* genomic alterations (Fig. 1C), reductions in *PTEN* on IHC staining were enriched in INR cases (Fig. 3A; $p=0.0015$, Fisher's exact test). We also observed intratumoral heterogeneity in the co-occurrence of *PTEN*-intact and -reduced, or ERG-positive and -negative, tumors. Most cases (Fig. 3B) or lesions (Fig. 3C) were uniformly positive or negative for both *PTEN* and ERG (score of 0), but the frequency of tumors with either heterogeneous *PTEN* or ERG staining (score of 1) or both *PTEN* and ERG heterogeneous staining (score of 2) was greater in the INR group (Fig. 3D; $p=0.0059$ and $p=0.0068$, respectively, Cochran-Armitage test for trend).

Because *TMPRSS2-ERG* fusion and *PTEN* loss tend to occur early during prostate tumorigenesis [23], our observation of their heterogeneity enriched among INR tumors at baseline raised the possibility of increased treatment resistance being due to a greater number of tumor subclones. As these subclones may display a wider array of phenotypes apparent in situ, we extended our histologic analyses to five additional immunostains of pretreatment biopsy tissues: AR, PSA, GR, synaptophysin (SYP), and Ki-67. Automated staining within the linear range of colorimetric development for each antibody, coupled with machine-learning-based image analysis, resolved up to four subpopulations of tumor cells on the basis of staining intensity, which in turn was used to derive weighted histology intensity (HI) scores and histology heterogeneity measures based on the SDI (Fig. 3E). All stains demonstrated significant associations between HI and SDI (Fig. 3F), indicating staining homogeneity in areas of high (AR, PSA) or low (Ki-67, SYP, GR) intensity. The HIs for PSA were negatively correlated with RCB (Fig. 3G) and SDI (Fig. 3H), but PSA SDI and RCB were positively correlated (Fig. 3I), indicating that INR cases displayed more PSA heterogeneity as HI decreased proportional to RCB (Fig. 3J). Thus, ER cases

were more uniformly strong for PSA staining, with lower SDI at lower RCB. Unlike PSA, the HI for GR was positively correlated with RCB (Fig. 3K) and SDI (Fig. 3L), yet diversity was still correlated with RCB (Fig. 3M). The heterogeneity of these markers in a subset of tumors at baseline may represent subpopulations less similar to the truncal clone, and reflecting future treatment resistance.

3.3. Resistance to neoadjuvant enzalutamide is associated with greater genomic diversity at baseline

We observed greater within-patient similarities when examining the whole-genome somatic copy-number alteration (SCNA) data for individual foci from ER than from INR cases (Supplementary Fig. 1). Across a defined set of 69 amplifications and 111 deletions, correlation matrices revealed greater coefficients for patients with lower residual cancer burdens (Fig. 4A), irrespective of foci number. Across all cases with two or more foci, there was a statistically significant negative correlation ($p=-0.43$, 95% CI -0.68 to -0.086 ; $p=0.014$) between the median correlation coefficient for each case and its RCB (Fig. 4B) showing more intratumoral homogeneity in ERs. Although this could reflect variable tumor purity in the INR group, heterogeneity could also reflect increasingly divergent subclones within the tumor systems. We therefore considered all callable, high-confidence small nucleotide variants (SNVs) and boundary-harmonized SCNAs from every LCM focus to identify tumor subpopulations via bioinformatic reconstruction of tumor phylogenies (Fig. 4C). We observed two general types of clone tree structures (Fig. 4D): (1) a linear evolution path with few subclones inferred from nodes; and (2) a branched phylogenetic tree with multiple subclones. A depiction of each tree and supporting SNV/SCNA assignments is provided in Supplementary Figure 6.

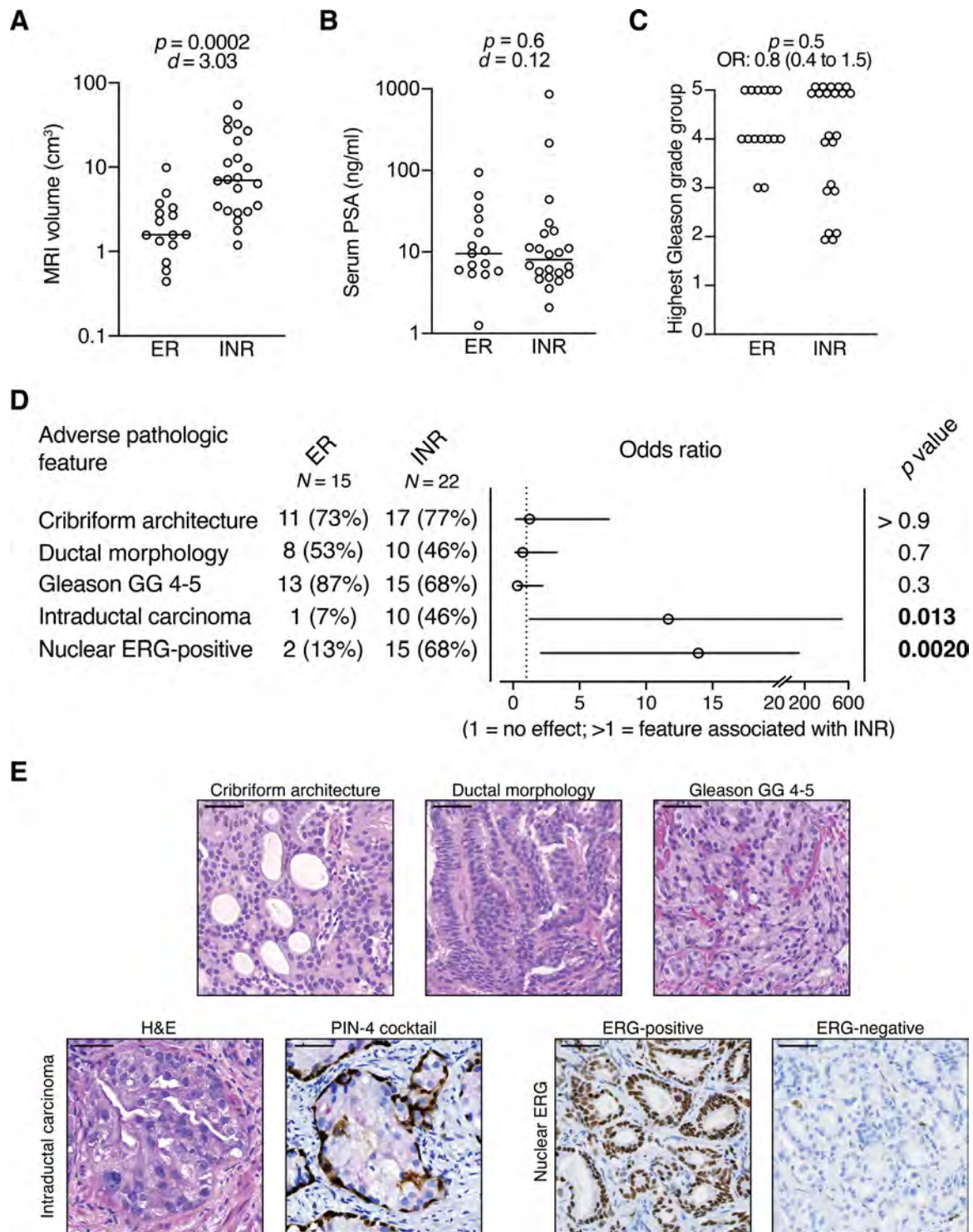


Fig. 2 – Comparison of clinicopathologic features between exceptional responder (ER) and incomplete and nonresponder (INR) cases. (A) Baseline patient-level magnetic resonance imaging (MRI) tumor volumes for ER versus INR cases, plotted on a log₁₀ scale. $p = 0.0002$ by Mann-Whitney U test. (B) Baseline serum prostate-specific antigen (PSA) for ER versus INR cases, plotted on a log₁₀ scale. $p = 0.6$ by Mann-Whitney U test. For A and B, the effect size d is determined as the Hodges-Lehmann median percent difference, based on the log₁₀-transformed value. (C) Highest Gleason grade group (GG) among all pretreatment biopsies for each case; $p = 0.5$ by the Cochran-Armitage test for trend for each GG between ER and INR. The effect size is given by the estimated odds ratio (OR; with 95% confidence interval) for each increment in Gleason grade group. (D) Frequency and odds ratio for five histologic and pathologic features for INR versus ER cases. Odds ratio >1 favors INR. (E) Representative micrographs of the adverse histologic features considered in D. For intraductal carcinoma, PIN-4 cocktail stains basal cells with brown chromogen (antibodies against cytokeratin 5, cytokeratin 14, and p63) and luminal cells with red chromogen (antibody against α -methylacyl-CoA racemase). For nuclear ERG staining, endogenous ERG is observed in endothelial cells for ERG-negative cases. H&E = hematoxylin and eosin. Scale bar: 50 μ m.

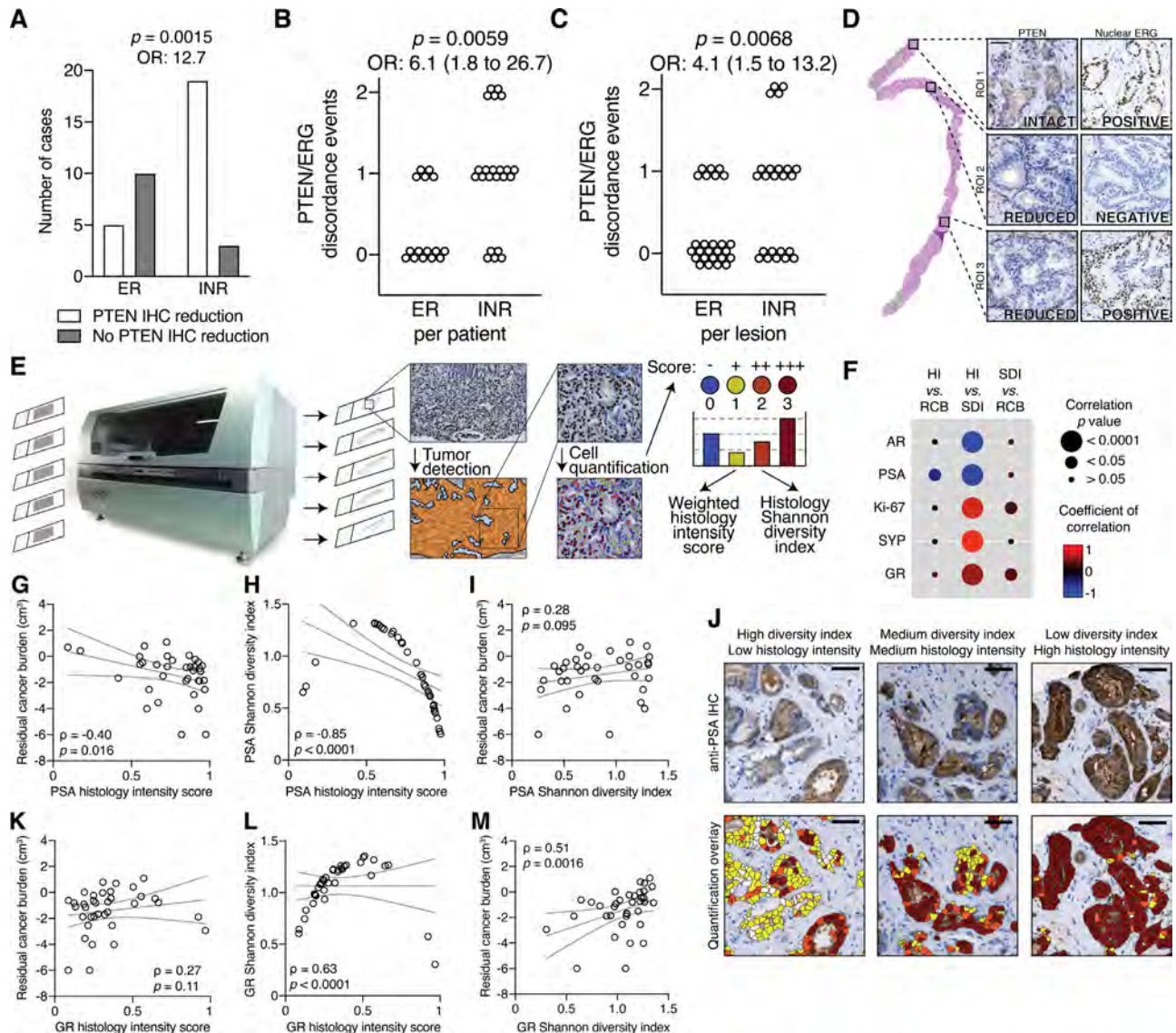


Fig. 3 – Observations of histologic diversity in baseline biopsies. (A) Enumeration of PTEN reduction observed via anti-PTEN immunohistochemistry (IHC) in exceptional responder (ER) and incomplete and nonresponder (INR) cases. The effect size is given as an odds ratio (OR), for which a value > 1 favors IHC reduction with INR. (B) Per-patient and (C) per-lesion enumeration when PTEN and/or ERG IHC is positive and negative within the same case. A score of 1 corresponds to either PTEN or ERG showing discordance. A score of 2 indicates both PTEN and ERG show heterogeneity within the same case. Statistical significance was determined using the Cochran-Armitage test for trend. The effect size is given by the estimated OR (95% confidence interval), which is for each increment in heterogeneity observations. (D) A representative case demonstrated PTEN and ERG heterogeneity by IHC (score of 2), showing a region with intact PTEN and positive ERG staining (top), a region with reduced PTEN staining but negative staining for ERG (middle), and a region with reduced PTEN and positive ERG staining (bottom). All three insets are from the same biopsy sample. (E) Workflow for machine-based IHC quantification using an autostainer for reproducible staining conditions, followed by slide scanning and cell/nucleus quantification using the Definiens platform. (F) Bubble plot of IHC correlations between histology intensity (HI) score, residual cancer burden (RCB), and Shannon diversity index (SDI). The bubble size is inversely proportional to the Spearman rank correlation p value, and the color scale is red for more positive coefficients of correlation and blue for negative. (G,K) Correlation of the $\log_{10}$ -transformed RCB for each case plotted against the HI score for each case for (G) anti-prostate-specific antigen (PSA) and (K) anti-glucocorticoid receptor (GR) IHC. (H,L) Shannon diversity index for (H) anti-PSA and (L) anti-GR IHC for each case plotted against the HI score for each case for anti-PSA r anti-GR IHC. (I,M) Correlation of the $\log_{10}$ -transformed RCB for each case plotted against the Shannon diversity index for each case for (I) anti-PSA and (M) or anti-GR IHC. For G–I and K–M, nonparametric Spearman correlation analyses p values are shown with their respective p values. Lines and bands represent linear regression lines and 95% confidence intervals, respectively. Owing to the small number of points, the scatter plots in H and L appear parabolic with no known explanation, although the trend over most of the data suggests a linear relationship. (J) Representative regions of high, medium, and low staining diversity index and low, medium, and high HI, respectively, for anti-PSA IHC. Scale bar: 50 μm .

We observed more branched trees among INR cases (86% vs. 73%); the number of nodes per tree (Fig. 4E) was positively correlated with RCB ($p = 0.35$, 95% CI 0.0095–0.62; $p = 0.039$). Using the prevalence of each tumor subpopulation to calculate a tree SDI, we observed a modest positive correlation ($p = 0.22$, 95% CI –0.13 to 0.52; $p = 0.20$) between

baseline SDI and post-treatment RCB (Fig. 4F). A modest inverse correlation was observed between SCNA interfocal homogeneity and genomic SDI ($p = -0.29$, 95% CI –0.59 to 0.067; $p = 0.098$) associating genomic heterogeneity with processes of evolutionary diversity (Fig. 4G). Moreover, the percentage genome altered (PGA), a metric associated with

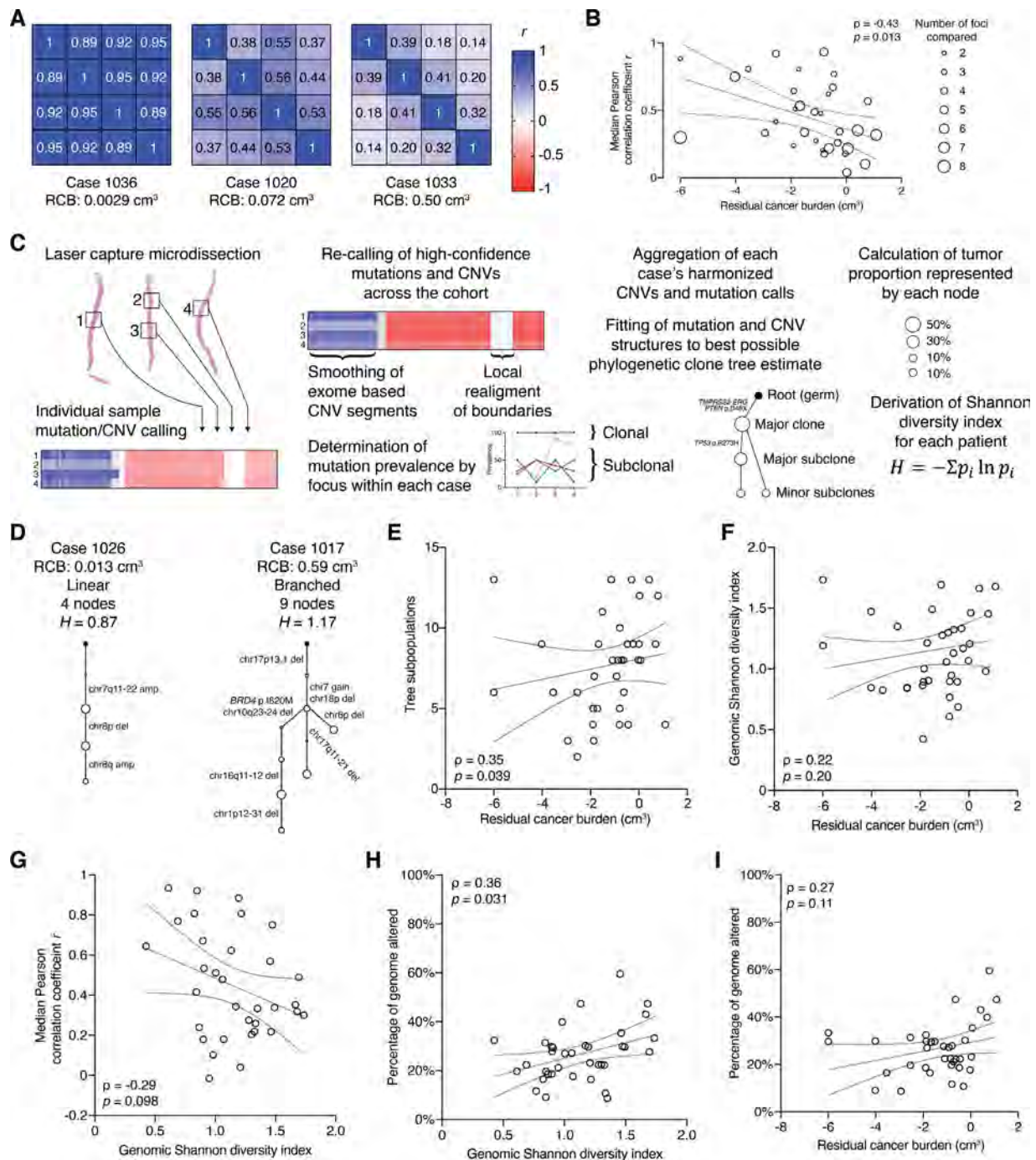


Fig. 4 – Measures of genomic diversity. (A) Correlation matrices for three representative cases with four tumors each, showing the range of similarities among genomic gains and losses across 180 predefined regions. (B) Median Pearson correlation coefficient for each multiregion case as a function of the $\log_{10}$ -transformed residual cancer burden (RCB) value. The number of foci dissected for each case is depicted by the size of the circle. (C) Schematic showing the workflow to harmonize multiregion sampling data and processing for phylogenetic clone tree reconstruction. CNV = copy number variation. (D) Representative linear and branched tree structures. (E) Scatter plot of the number of nodes in each phylogenetic tree versus the $\log_{10}$ -transformed RCB for each case. (F) Scatter plot of the genomic Shannon diversity index (SDI) for each case versus the corresponding $\log_{10}$ -transformed RCB. (G) Median Pearson correlation coefficient for each multiregion case from B versus the genomic SDI for each case. (H) Percentage of the genome altered (PGA) plotted against the genomic SDI for each case. (I) PGA versus the $\log_{10}$ -transformed RCB for each case. For B and E–I, nonparametric Spearman correlation analyses p values are shown with the respective p values. Lines and bands represent linear regression lines and 95% confidence intervals, respectively.

tumor aggressiveness, was positively correlated with genomic SDI ($p=0.36$, 95% CI 0.031–0.62; $p=0.03$) and RCB ($p=0.27$, 95% CI –0.071 to 0.56; $p=0.11$; Fig. 4H,I). We therefore conclude that tumors resistant to NADT may harbor a greater number of major preexisting subclones,

and that sensitivity to NADT, even in tumors with driver alterations, is conferred by lower fitness associated with the simpler evolutionary history of these tumors.

To validate these findings, we selected four histogenomic factors according to their performance in univariate

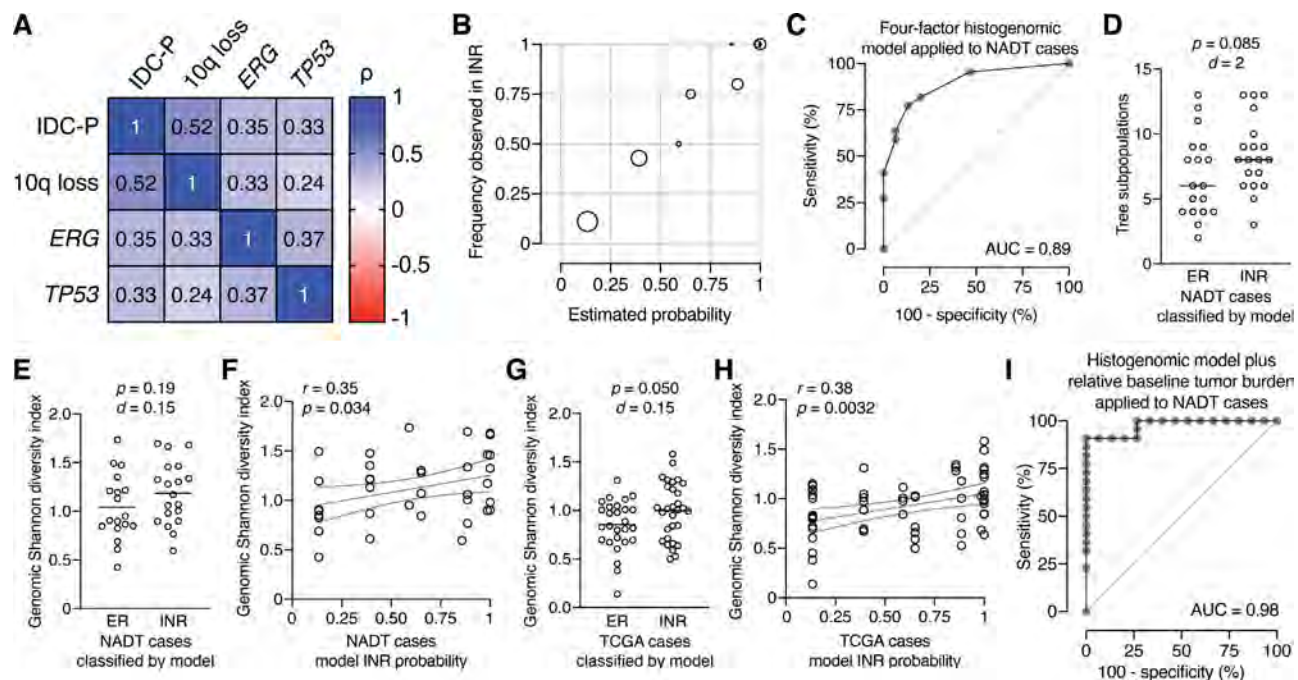


Fig. 5 – A multivariate model of poor response to intense neoadjuvant androgen deprivation therapy (NADT) is associated with genomic diversity. (A) Spearman correlation matrix of each binary factor (1 = presence, 0 = absence) across each of the 37 patients in the study cohort. IDC-P = presence of intraductal carcinoma; 10q loss = at least of half of chromosome arm 10q deleted hemizyously as determined using the GISTIC algorithm; ERG = overexpression of nuclear ERG determined via immunohistochemistry; TP53 = loss-of-function alterations or hotspot mutations to TP53, including copy number loss, as determined via GISTIC. (B) Bubble plot of the frequency of each four-factor combination observed in the trial cohort and the proportion observed in the incomplete and nonresponder (INR) group. (C) Receiver operating characteristic curve for the ability of the INR probability to distinguish exceptional responder (ER) from INR cases in the trial cohort using the four-factor model. (D,E) Reapplication of the four-factor model to trial patients and (D) the number of tumor subpopulations or (E) the genomic Shannon diversity index (SDI) for each group after reclassification. Statistical significance determined using the Mann-Whitney U test for the number of tumor populations and Welch's t test for genomic SDI. The line represents the (D) median or (E) mean value. The effect size d is determined as the Hodges-Lehmann (D) difference of medians or (E) difference of means. (F) Pearson correlation of the genomic SDI and INR probability for the trial cohort. (G) Genomic SDI for The Cancer Genome Atlas (TCGA) validation cohort as classified as ER or INR by the four-factor model. Statistical significance was determined using Welch's t test. The line represents the mean value. The effect size d was determined as the difference of means. (H) Pearson correlation of the genomic SDI and INR probability for the validation cohort. (I) Receiver operating characteristic curve for the ability of the INR probability to distinguish ER from INR cases in the trial cohort based on the four-factor model plus the baseline relative tumor burden.

analyses that showed imperfect associations with each other (Fig. 5A) to construct a multivariate model for poor response using logistic regression (Supplementary Table 6). These features (TMPRSS2:ERG fusion/nuclear ERG expression, deletion of $\geq 50\%$ of chromosome 10q, loss-of-function or hotspot alterations to TP53, and IDC-P) allow up to 16 possible combinations and probabilities of being INR (Fig. 5B). This model (Fig. 5C) has an area under the receiver operating characteristic curve (AUC) of 0.89 ($p < 0.0001$), and a cutpoint of 60% offered the best discrimination, classifying 30 of 37 NADT cases correctly. This cutpoint also recapitulated the trend towards greater diversity in the number of nodes per tree and greater genomic SDI for each case with higher INR probability (Fig. 5D–F), demonstrating potential utility in our cohort of diverse tumor profiles.

Our validation group for this four-factor histogenomic model consisted of 57 cases of Gleason score 8–10 tumors from the prostate TCGA cohort with evaluable genomic and histologic data. The 60% INR cutpoint resulted in 30 cases classified as INR that harbored a significantly higher genomic SDI than ER ($p = 0.050$, Welch's t test; Fig. 5G), and the probability of INR across the group was positively correlated ($r = 0.38$, 95% CI 0.14–0.59; $p = 0.0032$) with

genomic SDI (Fig. 5H), suggesting that greater genomic diversity in a subset of untreated prostate tumors is associated with a higher likelihood of predicted treatment resistance.

As mpMRI estimates of normalized baseline tumor volume were predictive of treatment response at AUC = 0.89 [14], we investigated whether addition of relative volume as a fifth factor would improve the fit of our model in classifying ER versus INR cases. MRI volume measurements were independent of tumor heterogeneity or diversity (Supplementary material), so adding baseline relative tumor burden to the model improved the AUC to 0.98 (Fig. 5I). Thus, genomic diversity and tumor volumes provide complementary data to inform on tumor behavior when predicting outcome following NADT.

4. Discussion

Patients diagnosed with intermediate- to high-risk localized prostate cancer have variable outcomes, and in this clinical trial we found that pathologic responses to intense NADT did not trend with classical clinicopathologic

parameters observed at baseline, such as Gleason score and clinical stage. We used whole-exome sequencing and IHC to identify potential molecular differences between ER and INR cases that could be exploited to further sensitize resistant tumors or select the best trial candidates in future studies. Despite extensive multiregion sampling, we identified relatively few genes that were significantly altered in solely one group; nearly all cases demonstrated hallmarks of aggressive tumors. However, among ER cases, which we defined as harboring RCB of $<0.05\text{ cm}^3$, these somatic alterations were clonal and histologic phenotypes were homogeneous. By contrast, tumors that demonstrated therapeutic resistance had greater subclonal heterogeneity, and phylogenetic reconstruction indicated more complex, branched evolutionary paths, such that both genomic and histologic diversity at baseline positively correlated with residual tumor. At the extreme end of this phenomenon was one patient in our study who harbored two completely independent tumors that exhibited differential responses to therapy [24].

The long-recognized variability and heterogeneity of localized prostate cancer demonstrate that tumor branching occurs at key times during its development, including on transition from high-grade prostatic intraepithelial neoplasia to cancer, from low- to high-grade cancer, and from localized disease to metastasis, with concomitant heterogeneity in IHC marker expression and somatic mutation profiles [3,25–28]. Although some earlier studies demonstrated selection bias in the analysis of larger tumors that were more conducive to multiregion sampling and sequencing, more recent cohort-based molecular analyses have demonstrated strong relationships between tumor evolution and aggressiveness [4,5]. Here, we attempted to subdivide otherwise high-risk tumors on the basis of their reconstructed evolution paths before treatment. While tumors with greater diversity were more likely to resist therapy, they were not necessarily larger, despite larger tumors presumably having undergone more cell divisions since tumor initiation. Indeed, even smaller tumors in our study were sampled comprehensively and exhibited a spectrum of diversity values, such that our bioinformatic reconstruction of tumor phylogenies was not biased by less complex data for ER cases. Meanwhile, more homogeneous tumors were more sensitive, but not necessarily smaller, and still harbored features of advanced cancer such as higher Gleason grade and a cribriform glandular architecture.

The range of residual tumor volumes lends itself to several possible strategies for patient management determined according to prognostic factors at baseline. Combined with MRI, which is increasingly common for staging and guiding biopsies of primary prostate cancer [29], and a series of straightforward immunostains readily available in most hospital laboratories, use of a commercial cancer sequencing panel would provide enough information to determine the probability of response to NADT according to the five-factor model. For patients in the INR group, those with the largest residual tumor volumes often harbored three or four histogenomic factors, and thus may benefit

from either immediate surgery (with or without adjuvant radiotherapy) or systemic chemotherapy before surgery. INR patients with intermediate RCBs (Fig. 1B) may represent incomplete responders and thus may have responded further to androgen annihilation via ADT in conjunction with abiraterone and an AR antagonist.

This study has several limitations. Until future studies with similar analyses of baseline tissue are performed, we will be unable to validate the interaction of these factors with RCB following intense NADT. As many of the factors we identified were based on exploratory studies, they are underpowered and require confirmation in larger studies. In particular, the features selected for generating the four-factor model were not selected on the basis of their likelihood to remain noteworthy after correction for multiple testing, but rather largely because of their emergence as top distinguishing features among those tested and that they did not strongly correlate with each other. In addition, while the selected factors are predicted to correlate with poor response to intense NADT, the presence of these factors needs to be confirmed in post-treatment tissue specimens from this study and other trials to confirm their status as drivers of resistance. Such analyses are ongoing.

The four factors selected to develop a multivariate model of INR probability independently correlated with genomic diversity and offered even stronger discrimination between ER and INR when combined with baseline volume. Prospective assessment of patients lacking these factors despite harboring high-risk prostate cancer may select for a greater proportion of ER cases in future studies.

5. Conclusions

Outcomes for patients with intermediate- to high-risk prostate cancer remain variable, but in the context of prior studies [4,26,27], connecting short-term responses to treatment with extensive histogenomic analyses has revealed that the evolutionary history of a tumor is clearly related to its fitness. Patients with smaller tumors harboring more linear evolution paths exhibited responses to preoperative intense NADT, which is expected to delay or prevent recurrence. By contrast, patients with more diverse tumor systems may harbor oligometastatic disease, for whom sensitive molecular imaging modalities such as PSMA positron emission tomography could identify opportunities for radiation therapy and treatment regimens other than ADT. A study using intense NADT with imaging and serial prostate biopsies to evaluate intraprostatic tumor responses at multiple time points is currently in progress (NCT02430480).

Author contributions: Adam G. Sowalsky had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Ye, VanderWeele, Dahut, Sowalsky.

Acquisition of data: Wilkinson, Carrabba, Whitlock, Bright, Atway, Trostel, Lotan, Turkbey, Harmon, Walker.

Analysis and interpretation of data: Wilkinson, Ye, Terrigino, Capaldo, Bright, Lis, Sater, Merino, Salles, Kaur, Lotan, Choyke, Pinto, Gulley, Madan, Sowalsky.

Drafting of the manuscript: Wilkinson, Terrigino, Carrabba, Lotan, Venzon, Sowalsky.

Critical revision of the manuscript for important intellectual content: Karzai, VanderWeele.

Statistical analysis: Venzon.

Obtaining funding: Dahut.

Administrative, technical, or material support: Chun.

Supervision: Choyke, Dahut, Sowalsky.

Other: None.

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Data sharing statement: All raw sequencing data have been deposited in the Database of Genotypes and Phenotypes (dbGaP) at accession number phs001938.v2.p1. The custom code used for processing data is available at <https://github.com/CBIIT/lgcp>. Slides used for histologic analysis have been deposited in The Cancer Imaging Archive. The complete study protocol and associated forms are available on request to the corresponding author.

CRediT authorship contribution statement

Scott Wilkinson: Methodology, Validation, Investigation, Data curation, Writing - original draft, Visualization, Project administration, Funding acquisition. **Huihui Ye:** Conceptualization, Investigation, Data curation. **Fatima Karzai:** Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing. **Stephanie A. Harmon:** Investigation, Data curation, Writing - review & editing. **Nicholas T. Terrigino:** Validation, Formal analysis, Investi-

gation, Data curation, Writing - original draft, Visualization. **David J. VanderWeele:** Conceptualization, Resources, Writing - original draft. **John R. Bright:** Investigation. **Rayann Atway:** Investigation. **Shana Y. Trostel:** Investigation. **Nicole V. Carrabba:** Methodology, Investigation, Writing - original draft. **Nichelle C. Whitlock:** Investigation. **Stephanie M. Walker:** Investigation, Data curation. **Rosina T. Lis:** Validation, Data curation. **Houssein A. Sater:** Investigation, Data curation. **Brian J. Capaldo:** Methodology, Software, Formal analysis. **Ravi A. Madan:** Resources. **James L. Gulley:** Resources. **Guinevere Chun:** Resources. **Maria J. Merino:** Investigation, Resources. **Peter A. Pinto:** Resources. **Daniela C. Salles:** Investigation. **Harsimar B. Kaur:** Investigation. **Tamara L. Lotan:** Investigation, Writing - original draft. **David J. Venzon:** Software, Formal analysis, Data curation, Writing - original draft, Visualization. **Peter L. Choyke:** Resources, Supervision. **Baris Turkbey:** Methodology, Resources, Writing - review & editing, Supervision, Project administration. **William L. Dahut:** Resources, Supervision, Funding acquisition. **Adam G. Sowalsky:** Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.eururo.2021.03.009>.

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Modeling androgen deprivation therapy-induced prostate cancer dormancy and its clinical implications

Xin Dong^{1,2,3}, Hui Xue^{1,2,3}, Fan Mo^{2,3,4,5}, Yen-yi Lin^{2,3}, Dong Lin^{1,2,3}, Nelson K. Y. Wong¹, Yingqiang Sun⁵, Scott Wilkinson⁶, Anson T. Ku⁶, Jun Hao^{1,2,3}, Xinpei Ci^{1,2,3}, Rebecca Wu¹, Anne Haegert^{2,3}, Rebecca Silver⁷, Mary-Ellen Taplin⁷, Steven Balk⁸, Joshi J. Alumkal⁹, Adam G. Sowalsky⁶, Martin Gleave^{2,3}, Colin Collins^{2,3}, Yuzhuo Wang^{1,2,3}

¹Department of Experimental Therapeutics, BC Cancer Agency, Vancouver, BC, Canada. ²Vancouver Prostate Centre, ³Department of Urologic Sciences, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada. ⁴College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang, China. ⁵Hangzhou AI-force therapeutics, Hangzhou, Zhejiang, China. ⁶Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, Bethesda, MD, USA. ⁷Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. ⁸Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA. ⁹Division of Hematology-Oncology, Department of Internal Medicine, Rogel Cancer Center, University of Michigan, Ann Arbor, MI, USA.

Note: Xin Dong and Hui Xue contributed equally to this work

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Corresponding author:

Yuzhuo Wang, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada. Phone: 604-675-8013; Fax: 604-675-8019; E-mail: ywang@bccrc.ca.

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Key words: Androgen deprivation therapy (ADT); Patient-derived xenograft (PDX); Predictive gene signature; Prostate cancer (PCa); Subrenal capsule grafting; Tumor Dormancy.

ABSTRACT

Treatment-induced tumor dormancy is a state in cancer progression where residual disease is present but remains asymptomatic. Dormant cancer cells are treatment-resistant and responsible for cancer recurrence and metastasis. Prostate cancer (PCa) treated with androgen-deprivation therapy (ADT) often enters a dormant state. ADT-induced PCa dormancy remains poorly understood due to the challenge in acquiring clinical dormant PCa cells and the lack of representative models. In this study, we aimed to develop clinically relevant models for studying ADT-induced PCa dormancy. Dormant PCa models were established by castrating mice bearing PCa patient-derived xenografts (PDXs) of hormonal naïve or sensitive PCa. Dormancy status and tumor relapse were monitored and evaluated. Paired pre- and post-castration (dormant) PDX tissues were subjected to morphological and transcriptome profiling analyses. As a result, we established eleven ADT-induced dormant PCa models that closely mimicked the clinical courses of ADT-treated PCa. We identified two ADT-induced dormancy subtypes that differed in morphology, gene expression, and relapse rates. We discovered transcriptomic differences in pre-castration PDXs that predisposed the dormancy response to ADT. We further developed a dormancy subtype-based, predisposed gene signature that was significantly associated with ADT response in hormonal naïve PCa and clinical outcome in castration-resistant PCa treated with ADT or androgen-receptor pathway inhibitors.

Implications: We have established highly clinically relevant PDXs of ADT-induced dormant PCa and identified two dormancy subtypes, leading to the development

66 of a novel predicative gene signature that allows robust risk stratification of PCa
67 patients to ADT or androgen-receptor pathway inhibitors.

68

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INTRODUCTION

Prostate cancer (PCa) survival and growth are largely dependent on androgens (1). Androgen deprivation therapy (ADT) has been a cornerstone of treatment for locally advanced and metastatic PCa (2). While ADT initially leads to rapid apoptotic or atrophic responses of PCa (3), more than 50% of ADT-treated cases experience recurrence within two years (4,5), indicating the presence of residual cancer cells that are either insensitive to treatment or in a temporarily dormant stage.

In the clinical context, tumor dormancy is the extensive period of time in which tumors remain in a growth arrest in the primary site or in metastatic dissemination, and patients remain asymptomatic before relapse (6-10). During tumor dormancy, the residual tumor cells are often undetectable by routine diagnostic methods such as medical imaging and biomarkers. The dormant tumor cells are thought to contribute to therapeutic resistance, evade immune surveillance, and lead to future recurrence. Tumor dormancy can be further subdivided into various categories/subtypes at different levels, including the clinical level (e.g., metastatic dormancy, treatment-induced dormancy), cell population level (e.g. cellular dormancy, tumor mass dormancy), and mechanistic level (e.g., angiogenic dormancy, immunologic dormancy) (6-10).

ADT-induced PCa dormancy remains poorly understood. To study ADT-induced PCa dormancy, cancer cells must be characterized during the dormant state. However, it is challenging to acquire sufficient clinical dormant PCa tissues and establish representative experimental models. As a result, the effects of ADT-induced tumor dormancy on disease progression remain unknown, as are

clinically useful predictive markers for patients treated with ADT. Therefore, mechanistic understanding of ADT-induced PCa dormancy is critically important, and reliable biomarkers that can predict ADT response and stratify patients for recurrence risks are urgently needed.

We have previously established a large collection of PCa patient-derived xenograft (PDX) models (www.livingtumorlab.com), mostly from primary hormone-naïve PCa. These models retain major characteristics of the patients' original tumors, including gene expression and treatment response (11-13). Therefore, these models are clinically relevant platforms for PCa discovery (14-18), personalized cancer therapy (19-21), predictive marker development (19,22), and drug development (23-26).

In this study, we aimed to model ADT-induced dormant PCa, to explore ADT-induced dormant PCa at biological, cellular, and gene expression levels, to study the clinical association of the findings in PDXs, and to develop a predictive gene signature for risk-stratification in patients treated with ADT. Herein, using a panel of hormonal naïve or sensitive PCa PDXs, we established eleven ADT-induced dormant PCa PDX models, including 9 from hormone-naïve PCa. Our PDX models revealed two ADT-induced PCa dormancy subtypes differing greatly in morphology, gene expression, and biological recurrence. We discovered transcriptomic predispositions in the pre-castration PDXs of the two dormancy subtypes. We further developed a dormancy subtype-based, predisposed gene signature that was strongly associated with ADT treatment response in hormone-naïve PCa. This gene signature was also an independent risk factor for patients with castration-resistant PCa (CRPC) treated with androgen-receptor pathway

inhibitors. These findings provide unique insights of PCa dormancy and will help improve the management of the disease.

MATERIAL AND METHODS

Materials and animals

Chemicals, solvents and solutions were obtained from Sigma-Aldrich, Oakville, ON, Canada, unless otherwise indicated. Six- to 8-week old NRG mice were purchased from the Animal Resource Centre of the BC Cancer Research Institute, Vancouver, Canada. Animal experiments were conducted according to a protocol (A17-0165) approved by the UBC Animal Care Committee. Human PCa specimens used for PDXs were obtained from patients with informed written consent, according to a protocol (H09-01628) approved by the Institutional Review Board of the University of British Columbia (UBC).

PDXs and castration-induced PCa dormancy

Tissues from 11 PDX models were grafted at the sub-renal capsule grafting sites (SRC) of NRG mice (n=55) as previously described (13,27). In brief, cryopreserved PDX seeds were recovered with 6- to 8-week old NRG mice by SRC implantation (13,20). Expanded PDX tissues were then re-grafted into NRG mice supplemented with testosterone to ensure a human-equivalent serum testosterone level was reached. For each tumor line, when tumor volume reached an approximate volume of 150 mm³ by palpation and micro-ultrasound imaging, the mice were randomly assigned into 3 subgroups: Pre-Castration (PRE), Dormancy (CX), and Long-term Monitoring (LM) groups. PRE PDX tissues were harvested without host castration. Mice in the CX and LM groups were surgically

castrated by removing testes and testosterone pellets. PDX tissues of the CX group were harvested at Week 12 post-castration. This time point was determined by a pilot study where both tumor volume and serum levels of Prostate Specific Antigen (PSA) remained stable at a lower or undetectable level. The remaining 33 mice (3/tumor line) were monitored for post-castration relapse for up to 42 weeks. Relapsed tumors were collected when tumor volumes reached 300 mm³ or when hosts reached humane endpoint due to aging or sickness. All mice bearing PDXs without a sign of relapse were terminated at Week 42 post-castration.

Histology, immunohistochemistry and quantification

Harvested xenografts were bisected through the longest dimension, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Preparation of formalin-fixed-paraffin-embedded tissue sections, routine hematoxylin and eosin (H&E) staining, and immunohistochemistry were conducted as previously described (20). Residual tumor volumes in dormant PCa (12 weeks post-castration time point) were determined by microscopic imaging analyses as previously described (20).

For immunohistochemistry, a rabbit monoclonal anti-AR antibody (1:100, Abcam, Cambridge, MA), a rabbit monoclonal anti-PSA antibody (1:200, Abcam, Cambridge, MA), a monoclonal mouse anti-human Ki-67 antibody (1:25, Dako, Santa Clara, CA) and a rabbit monoclonal anti-Caspase 3 antibody (1:50, Cell Signalling, Danvers, MA) were used as primary antibodies. For quantification of Ki-67 or caspase-3 immunohistochemical stains, positive cells per 5000 cancer cells were counted by two independent investigators.

RNA microarray gene profiling

RNA from frozen PDX tissues were extracted and purified according to standard protocols. Total RNA samples were processed using Agilent's One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling v6.0. An input of 100 ng of total RNA was used to generate cyanine-3–labeled cRNA. Samples were hybridized on Agilent SurePrint G3 Human GE 8 x 60K Microarray. Arrays were scanned with the Agilent DNA Microarray Scanner at a 3-mm scan resolution and data were processed with Agilent Feature Extraction 10.10. Processed signal was quantile normalized with Agilent GeneSpring 11.5.1. The RNA microarray data generated in this study are publically available on Gene Expression Omnibus (GEO) at GSE193500.

Bioinformatics and statistical analysis

Student-t test was used to detect differences in abundance levels among the groups studied. Hierarchical clustering of gene expression data was performed using distance metrics calculated from pairwise correlation coefficients.

Gene Set Enrichment Analysis (GSEA) (<http://software.broadinstitute.org/gsea/index.jsp>) was used as previously described (15) to determine whether a defined set of genes (Hallmark Gene Sets) showing significant and consistent differences between two biological phenotypes (e.g., PRE vs. CX, or tumor mass dormancy vs. cellular dormancy). All GSEA analyses in this study used whole transcriptomic data without expression level cut-off as expression datasets. False discovery rate (FDR) q values were calculated using 1000 permutations. A gene

set was considered significantly enriched if its normalized enrichment score (NES) has an FDR q below 0.25.

Ingenuity pathway analysis (IPA) was performed as previously described (11,17). Differentially expressed gene list was generated using fold change (≥ 2) and P values ($P < 0.05$) between samples of two groups as cut-offs.

Clinical cohorts

Data from High-low clinical trial cohort and Takeda clinical trial cohort (28) were acquired from Dr. Colin Collins. Data from TCGA cohort (PanCancer Atlas) and SU2C cohort (PNAS 2019) were accessed through cBioPortal (29,30). Data of Sowalsky neoadjuvant-hormonal-therapy (NHT) cohort (31) were acquired from Dr. Adam Sowalsky. Data of the Alumkal cohort (32) were acquired from Dr. Joshi Alumkal. In all cohorts, only patients that received preoperative or post-surgery/post-biopsy ADT or androgen-receptor inhibitors were included in this study.

Gene signature development and validation

Candidate genes that are differentially expressed in pre-castration PDXs of different dormancy subtypes were identified by applying stringent filters to ensure the reliability of results. The PDX-based candidate genes were developed into a predictive gene signature by using the TCGA cohort as a training cohort. The predictive gene signature was further validated in the Sowalsky NHT cohort for its predictive power in predicting ADT response. Another two independent cohorts, SU2C and Alumkal, were used to validate the prognostic value of the predictive gene signature in predicting clinical outcome for patients with

castration refractory PCa treated with AR inhibitors. Methods used to develop and validate the predictive gene signature were explained below in details.

The gene read counts from TCGA cohort were converted by log-transformation. Values of zeros were replaced with the minimal value of the cohort to avoid negative infinity. Based on availabilities in clinical follow-ups, the primary outcome for each cohort was as follows: for TCGA the primary outcome was 5-year progression-free survival (PFS); for SU2C the primary outcome was 3-year overall survival (OS) and for Alumkal 3-year PFS. The gene candidates were ranked based on the contribution to the patient stratification in TCGA training cohort using the method of Information Gain and Signal Noise Ratio. Top ranked gene candidates were selected for fusion-cross validation. A balanced-mode logistic regression model was used to build a predictive gene signature. The binary prediction results were presented as “1” and “0”. Patients indicated as “1” were predicted to have a high risk in developing recurrence compared to ones indicated as “0”. The performance of gene signature was evaluated by AUC (Area under the ROC curve). Kaplan-Meier (KM) and Cox proportional hazard regression model were employed to validate the prediction in survival outcome and to perform multivariate analyses. All statistical calculations were performed using R (version 3. 4. 1; <http://www.rproject.org/>), GraphPad Prism version 8 (GraphPad Software, USA), and SPSS (IBM SPSS Statistics for Windows, Version 25.0., USA). All *P* values were two-sided, and a value of less than 0.05 was considered statistically significant.

RESULTS

Eleven dormant PCa PDX models were established and resembled the clinical course of ADT treatment response

Eleven castration-induced dormant PCa PDX models were established from 11 parental hormone naïve or sensitive PDX tumor lines. The biological and genetic background of the 11 parental PDX tumor lines is summarized in Supplementary table S1. All PDXs exhibited a marked response to host castration by 12 weeks post-castration: average tumor volume decreased by 88.35% and host serum PSA levels were at an undetectable level (Fig. 1A-B). Consistent with our pilot study, the low tumor volume were held at a low but a steady state with undetectable serum PSA---a state mimicking the clinical tumor dormancy period seen in patients with PCa after ADT treatment. The PSA nadir was consistent with the low tumor volumes of the dormant xenografts, indicating the attainment of dormant state in PCa PDXs as compared to the acute castration effects where serum PSA is still detectable and tumor volumes constantly decreasing. The castration-induced PCa dormancy in PDX was further evaluated at morphological and genetic levels, as described in the following sections.

PDXs recapitulated morphological characteristics of ADT-induced dormant PCa in the clinic

Light microscopy revealed residual carcinoma in all post-castration PDXs. The residual PCa cells demonstrated morphological changes previously reported in clinical ADT-treated samples (e.g., reductions of glandular structures, hyalinised stroma, cytoplasmic vacuolization, and nuclear pyknosis) [27, 28] (Fig. 1C). Mitotic figures were rarely seen. AR nuclear protein expression was decreased, and PSA protein expression was undetectable or weakly expressed in tumor foci (Fig. 1C).

Compared to pre-castration PDXs, cell proliferation and apoptosis in post-castration dormant PDXs, as measured by anti-Ki67 or anti-cleaved Caspase 3 immunostaining, were significantly reduced ($P=0.0001$ and $P=0.0013$, respectively, Fig. 1D), indicating decreased cellular activities in both proliferation and apoptosis in the PDXs 12 weeks after castration, as opposed to the presence of high proliferative and apoptotic activities in the pre-castration PDXs. The low level of apoptotic activity in the 12-week post-castration PDXs differentiates the dormant tumors from those in acute castration response that involves prominent degree of cell death.

Gene expression analyses revealed broad changes of dormant PCa PDXs in AR signaling and cell proliferation

Global transcript profiling of eleven pairs of pre-castration and post-castration (dormant) PDXs demonstrated that each dormant PDX clustered closely with its paired pre-castration PDXs (Fig. 2A). Gene Set Enrichment Analysis (GSEA) using whole transcriptomic data showed that 12 hallmark gene sets related to androgen response, DNA repair, cell cycle progression, and protein synthesis were significantly enriched in pre-castration PDXs ($FDR<0.25$, Fig. 2B), suggesting these gene sets are downregulated in dormant PDXs. An unsupervised hierarchical clustering analysis based on GSEA Leading Edge Genes demonstrated a split between the pre-castration and the post-castration dormant PDXs (Fig. 2C). Ingenuity Pathway Analysis (IPA) on differential-expression genes (fold-change of ≥ 2 , $P<0.05$) between pre-castration and dormant PDXs showed that, consistent with GSEA, the most significantly downregulated pathways in dormant PDXs were typically related to cell cycle progression (435 genes), cell survival (315 genes),

cellular assembly (205 genes), and cell growth (739 genes) (Fig. 2D). Out of 58 differentially expressed genes that were identified by IPA to have direct interactions with androgen receptor (AR), 53 were down-regulated in dormant PDXs (Fig. 2E). These results were in line with the morphological findings, indicating PCa dormancy in post-castration PDXs.

Two biologically different ADT-induced PCa dormancy subtypes were associated with disease progression

We observed two dormancy subtypes in post-castration dormant PDXs. As revealed by morphological analysis, mitotic figures were absent in 8 out of 11 post-castration dormant PDX models, with Ki67 < 5% (Fig. 1C-D), indicating a status of *cellular dormancy* that involves a state of temporary cell cycle arrest in tumor cells (7,10). In contrast, the other three dormant PDX models (LTL-467CX, LTL-331CX, and LTL-484CX) showed mitotic figures, with Ki67 percentages at 5-15% (Fig. 1C-D), indicating a state of *tumor mass dormancy* where overall tumor growth is stopped due to likely an equilibrium between cell death and cell proliferation (7,10). Since quantification using Ki67 alone has limitations in uniformity and consistency (33), we adopted a quantified histopathological scoring system for ADT-treated clinical PCa samples (34) to evaluate dormant PCa comprehensively (Supplementary Table S2). Three PDX models with score 0-2 were considered as tumor mass dormancy, and five with score 7-10 as typical cellular dormancy (Table 1). Three PDX models with score 3-6 appeared to be less responsive than the typical cellular dormancy PDXs, with the former harboring more residual cancer cells and demonstrating cytological hints of active biosynthesis (e.g. large conspicuous nucleoli and abundant eosinophilic

cytoplasm). However, these three models were still considered as cellular dormancy since mitotic figures were absent and Ki-67 positivity was low (<5%).

GSEA using transcriptomic profiles of 3 tumor mass dormancy and 5 typical cellular dormancy demonstrated that 10 hallmark gene sets were significantly enriched in tumor mass dormancy (FDR<0.25), including those related to cell cycle, glycolysis, Wnt/ β -catenin, DNA repair, and PI3K-AKT-MTOR signaling (Supplementary Table S3). These results coincided with the relatively higher degree of activities of the residual tumor cells in tumor mass dormancy.

We further analyzed GSEA Leading Edge genes and generated a 42-gene set (Fig. 3A, Supplementary Table S4). Based on this gene set, Principal Component Analysis (PCA) exerting 11 pairs of pre-castration and dormant PDXs showed that the three tumor mass dormancy PDXs were separated from the rest of the dormant PDXs, grouping closely with the proliferative pre-castration PDXs (Fig. 3B). The result suggested that this gene set, similar to the histopathological score system, reflected dormant status in post-castration PDXs.

To investigate whether the dormancy status functionally impacted disease progression in PDXs, we castrated male mouse hosts bearing 11 PDX tumor lines (3 mice/line) and monitored for tumor relapse. Seven out of the 11 PDX lines developed relapsed tumors within 42 weeks post-castration (Supplementary table S5), including 6 AR+/PSA+ CRPC and one neuroendocrine PCa. The proliferative status of relapsed tumors was also confirmed by H&E and Ki67 staining (Fig. 1C). PDXs displaying tumor mass dormancy had a significantly shorter time to relapse (P=0.0001, Fig. 3C) and had much higher relapse frequency than those exhibiting

cellular dormancy (8 out of 9 mice (88.8%), and 5 out of 24 mice (20.83%), respectively; $P=0.0001$; Supplementary table S5).

To determine if the Leading Edge gene set that segregated dormancy subtypes can be applied to clinical PCa samples and distinguished the dormancy subtypes post neo-adjuvant hormone therapy (NHT), we further analyzed transcriptomic profiles from 18 patients who were exposed to preoperative NHT (High-low and Takeda clinical cohorts, Supplementary Table S6)(28). Based on the same Leading Edge gene set used for PDX analysis, PCA showed that the 18 cases were separated into two subgroups (Fig. 3D). One group tended to have a shorter progression-free survival compared to the other group, although the Kaplan-Meier survival analysis did not reach statistical significance due to small sample size ($P=0.13$, Fig. 3E),

Transcriptomic differences in pre-castration PDXs predisposed the dormancy response to ADT

Since dormancy is resultant of acute cellular injury brought about by androgen ablation, we hypothesized that there was genomic and/or transcriptomic predisposition that enabled the tumors to enter one of the two dormancy subtypes after ADT. Upon analyzing the RNA sequencing data of *pre-castration* PDXs (11), we did not observe significant differences in genetic configuration (Supplementary table S1). Transcriptomic analyses showed that a total of 1619 genes were differentially expressed between *pre-castration* PDXs of tumor mass dormancy and typical cellular dormancy (cut-offs: fold change ≥ 2 and $\log(\text{baseline expression intensity}) \geq 6$). IPA showed that the *pre-castration* PDXs entering tumor mass dormancy after ADT had significantly decreased expression

in Wnt/ β -catenin, Myc-mediated Apoptosis, and p38-MARK signaling and increased expression in integrin and EMT signalling (Supplementary table S7). These results suggested a presence of transcriptomic predisposition before host castration, which dictated the type of dormancy in response to ADT.

A dormancy subtype-based, predisposed gene signature predicted ADT response and clinical outcomes in independent hormone-naïve or castration-resistant PCa clinical cohorts.

Reliable biomarkers are urgently needed for prediction of ADT response and risk stratification of PCa patients receiving ADT. Based on the aforementioned findings, we hypothesized that a predictive gene signature derived from the dormancy subtype-related, transcriptomic predisposition in *pre-castration* PDXs could be used for predicting ADT response in patients prior to the administration of treatment and for risk stratification. To this end, top 234 differentially-expressed genes between the *pre-castration* PDXs of tumor mass dormancy and typical cellular dormancy (cut-offs: $P < 0.01$, $\text{Log}(\text{baseline gene expression intensity}) \geq 6$) were used as candidate genes for signature development. Four clinical cohorts (Supplementary Table S8) were employed for development and validation of the gene signature. Using the TCGA cohort (29,30) as a training cohort ($n=69$, only primary hormone-naïve PCas subsequently treated with ADT were included), we developed the PDX candidate genes into a 34-gene signature (hereafter referred to as, Pre-disposed Gene Signature, PGS. Supplementary Table S9) that separated patients with or without recurrence.

We next validated the PGS using the Sowalsky cohort (31) ($n=37$, Supplementary table S8), in which 37 patients with localized primary hormone-

naïve PCa were treated with pre-operative ADT (6 months of Lupron plus enzalutamide). Pre-ADT treatment biopsies were profiled for RNA expression, and ADT response (non-responders, incomplete, and exceptional responders) was defined by post-treatment residual cancer volumes (31,35). We performed single-sample GSEA using the PGS and generated a PGS enrichment score for each pre-ADT tumor. We observed a strong proportional relationship between the PGS score and residual cancer volumes ($r(35)=0.74$, $P<0.00001$, Fig. 4A). The non-responders had a significantly higher PGS score compared to the exceptional responders ($P<0.0001$) and incomplete responders ($P<0.0001$) (Fig.4B). These results strongly suggest that PGS is significantly enriched in intrinsically resistant tumors in primary PCa.

Since the pre-disposed PGS reflects intrinsic resistances to ADT, we further investigated whether this signature could predict clinical outcomes for patients with more advanced stages of PCa. The PGS was applied to PCa patients in two independent CRPC cohorts: SU2C (29,30,36)($n=60$) and Alumkal (32) ($n=25$), separately (Supplementary table S8). All patients received post-biopsy/surgery AR inhibitors. The binary prediction results were presented as “1” and “0”. In agreement with the initial discovery TCGA cohort, patients with PGS=1 had significantly shorter OS or PFS on AR inhibitors compared to those with PGS=0 (SU2C: $P=0.0002$; Alumkal: $P=0.004$; Fig. 4C-D). Multivariate Cox Regression analysis revealed that the PGS was an independent risk factor in both SU2C and Alumkal cohorts (Table 2, $P=0.003$ and 0.006 , respectively). Panther Protein Classification and IPA analysis of the PGS revealed that the 34 genes mainly associated with cell growth, proliferation, and transcriptional regulation (Fig. 4E).

Discussion

In this study, we study ADT-induced dormancy in PCa using highly clinically-relevant PDX models. Treatment-induced tumor dormancy is a common phenomenon after systemic treatment, such as chemotherapy and hormonal therapy, and has been used to explain post-treatment tumor relapse owing to the regrowth of drug-resistant, dormant cancer cells (10). Modeling ADT-induced PCa dormancy has been a major challenge in studying post-ADT dormancy in primary PCa. Experimental cellular dormancy in cell lines is usually achieved by highly artificial culture conditions (37). PCa PDX models are usually developed from highly aggressive PCa, mostly CRPC tissues, instead of primary hormone-naïve PCa (13). We therefore established 11 PDX models of castration-induced dormant PCa, 9 of which were from hormone-naïve PCa. One arresting benefit of using these PDXs to model PCa dormancy is that the dormant PCa PDX models simplify intricate clinical scenarios (e.g. treatment duration and tolerance, and various collecting time points) during the collection of dormant clinical samples.

We adopted the original clinical definition for tumor dormancy: the extensive period of time in which tumors remain in a growth arrest and patients remain asymptomatic before relapse (6-10). In the context of our PDX models, we used serum PSA and tumor volume as clinically relevant indicators for the castration-induced dormancy. The attainment of post-castration tumor dormancy was determined by tumor volumes reaching the lowest point without further change and the serum PSA reaching nadir. In addition, the dormant PCa PDXs demonstrated characteristics similar to those observed in post-ADT clinical PCa samples, including changes in morphology and marker expressions, and biological

recurrence (38,39). Moreover, these dormancy PDX models demonstrated various
 ki67 index after host castration, which is consistent with the observation in
 clinical NHT cohorts (40). In view of these, these dormancy models provide unique
 clinically relevant research tools for ADT-induced PCa dormancy, recapitulating
 different features of ADT-induced PCa dormancy in the clinic.

In 1970-80s, experimental models of dormancy have revealed that tumor
 dormancy may also result from a balance between cell proliferation and death so
 that the tumor mass maintains at a constant size (41-43). Tumor dormancy is thus
 divided into two subtypes at the cell population level: *cellular dormancy* (non-
 proliferative tumor cells persisting over a long period of time without dying) and
tumor mass dormancy (populations of cancer cells with cell proliferation balanced
 by cell death) (6-10,44,45). Our results demonstrated that these two ADT-induced
 PCa dormancy subtypes were significantly different in morphology and gene
 expressions. PDXs typically in *cellular dormancy* were characterized by no
 proliferative activities (no mitoses figures, negative for Ki67 stains); and those in
tumor mass dormancy might be achieved by a low, but balanced, levels of cell
 proliferation and apoptosis (indicated by positive Ki67 and Caspase-3 stains in
 limited number of residual cancer cells). The two dormancy subtypes showed
 significant difference at the transcriptomic level in hallmark gene sets such as cell
 cycle, glycolysis, PI3K-AKT-MTOR, and DNA repair. We further demonstrated that
 the dormancy subtypes functionally impacted recurrence in PDXs, indicating that
 post-ADT PCa dormancy status is associated with tumor latency, and could be a
 useful indicator for ADT response and outcome prediction. Currently, large
 collections of post-ADT PCa samples with dormancy-subtype characterization and
 followup information are not available. Future validation with such cohorts will

help better understand the relationship between dormant subtypes and disease progression in ADT-treated PCa.

Notably, the dormancy period investigated in this study is different from the acute castration effects (e.g., drastic cell death, rapid tumor shrinkage, and microenvironment changes). In our pilot study, we found that the acute castration effects are most drastic during the first week after castration. After 4-6 weeks post-castration, host PSA generally reached nadir, and tumor volumes remained unchanged. Our study revealed that at 12 weeks post-castration, the residual dormant cancer cells had re-established a stable, non-progressing state either by cell cycle arrest or by the balance between cell death and proliferation. The cellular activities including both proliferation and apoptosis are decreased in dormant cancer cells, as indicated by low levels of Ki67 and Caspase-3 expressions, respectively. The low level of apoptotic activity in the 12-week post-castration PDXs differentiates the dormant tumors from those in acute castration response that involves prominent cell death. This dormant period in PDX models can last months before proliferative, relapsed tumors re-emerge. This period of tumor dormancy could be considered as part of the chronic castration effect because the androgen levels stayed at nadir through this period. However, given the later development of relapsed tumors, it suggests that, at the cellular levels, cancer cells in the dormancy period are undergoing dynamic changes that will enable them to resist the chronic suppressive effect of androgen deprivation. These changes perhaps cannot be attributed solely to the chronic castration effect.

In addition to tumor dormancy, other terms have been used to describe the growth arrest state of cancer cells, such as cellular quiescence. Quiescence is a

reversible, non-proliferative state of cells at G0 phase in response to environmental stress and changes (46). Although quiescent cells share many similar characteristics with the cells in tumor dormancy (e.g., growth stagnation, decreased metabolism, survival advantage, and resistance to adjuvant therapies), cancer cell quiescence differs from tumor dormancy in a number of aspects. First, although quiescence is often used to describe the state of G0/G1 cell cycle arrest in tumor cells in cellular dormancy, it does not reflect the cells in tumor mass dormancy where growth equilibrium resulted from a balance between cellular proliferation and death is present. Second, tumor dormancy is primarily derived from clinical observations, describing the tumor as a whole, while quiescence describes the G0-arrest state of cancer cells at cellular level. Third, quiescent cells are in a delay of growth or differentiation and can immediately resume growth or differentiation once the environmental stress is removed. Dormant cells, however, may need to develop new mechanisms in order to exit the dormant state and grow again in a persistently unfavorable environment (47). In this study, although cancer cells in the xenografts in *cellular dormancy* demonstrate a quiescent, non-proliferate state, the cells in *tumor mass dormancy* remain certain levels of proliferation activities and therefore are not in a quiescent state. Moreover, in spite of the consistently low androgen level in the castrated hosts, a significant number of PDX models that exhibited *cellular dormancy* or *tumor mass dormancy* have conquered the low-androgen environment and developed recurrent tumors within a period of 42 weeks. One of the PDX models, LTL-331, even undergone a neuroendocrine transdifferentiation and re-emerged as a neuroendocrine prostate cancer that recapitulated the donor patient's clinical disease progression. The development of recurrent tumors in a continuously androgen-suppressed

environment, especially in the PDX models in *cellular dormancy*, suggest that cancer cells might have developed adaptive mechanisms enabling them to grow in unfavorable environment. In view of this, tumor dormancy is a more accurate description for this study than quiescence, reflecting dynamic changes of the ever-evolving dormant cancer cells.

Predicting PCa patients' response to ADT has remained a major challenge in clinical management. We herein revealed that predisposed transcriptomic differences in the *pre-castration* PDXs might contribute to the two distinctive dormancy subtypes. These transcriptomic predispositions were linked to Wnt/ β -catenin, apoptosis, p38-MAPK, integrin, and EMT signalling. These findings suggest that *de novo* gene expression signatures may lead to tumor mass dormancy after ADT, thus impact survival. We further developed and validated a 34-gene pre-disposed gene signature (PGS) for predicting ADT response in primary PCa. The 34-gene PGS was unbiasedly generated using a mathematic model, based on the top gene candidates differentially expressed between *pre-castration* PDXs that gave rise to tumor mass dormancy and those gave rise to cellular dormancy after host castration. The reason for using *pre-castration* PDXs was to mimic the potential availability of the patient samples before receiving ADT. The generated PGS will then allow us to predict the ADT response prior to the administration of treatment. We further validated the PGS in multiple independent clinical cohorts. In the Sowalsky cohort (hormone-naïve PCa), the PGS was proportionally correlated with the residual tumor volume, being significantly higher in non-responders compared to exceptional responders. The results indicate that the PGS is strongly enriched in intrinsically resistant tumors in primary PCa and is a strong indicator for *de novo* response to ADT treatment in

primary hormone-naïve PCa, suggesting a causal relationship between predisposition to ADT as a cause of tumor dormancy and the development of treatment-refractory disease. Moreover, we demonstrated that PGS was predictive of clinical outcome and was an independent risk factor in two independent castration-resistant PCa cohorts (SU2C and Alumkal) treated with AR pathway inhibitors. These results suggest that PGS is an important determinant of patient outcome and response to subsequent AR pathway inhibitor therapy even after development of castration resistance. Taken together, these findings are of immense clinical significance, as it has the potential to aid in the clinical management of PCa by predicting therapeutic response in cancer patients. For patients with high PGS, there might be a necessity in combining other adjuvant therapies with ADT or AR pathway inhibitors to eliminate proliferative cells in the tumor mass dormancy. Further prospective study with patients harboring hormone-naïve localized high-risk and advanced PCa will validate the clinical significance of the studies. In addition, understanding factors that mediate this gene signature may lead to new approaches to target the disease.

Conclusion

In conclusion, we have successfully established highly clinically relevant PDX models of ADT-induced dormant PCa. We uncovered two dormancy subtypes that showed significant difference in morphology, transcriptomic predisposition, and disease progression. We developed and validated a novel pre-disposed gene signature for robust risk-stratification for PCa patients receiving ADT or androgen-receptor pathway inhibitors. Future in-depth studies focusing on mechanisms underlying tumor dormancy, clinical impact of the predictive gene signature, and

therapeutic target development will help improve understanding of dormant PCa.
 We may also develop new therapeutic approaches to target dormant PCa.

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716

FIGURE LEGENDS

Figure 1. At Week 12 after host castration, dormant PCas were induced in PDXs. **A**, percentage volumes of dormant (CX) PDXs at 12 weeks post-castration relative to the PDX volumes before host castration (Mean±SD=11.65%±11.18%). **B**, serum PSA of the hosts dropped to undetectable levels on Day 84 (Week 12) after castration. **C**, representative images of H&E and immunohistochemistry in pre-castration, dormancy and relapsed PCa PDXs. **D**, Ki-67 and Caspase-3 (cleaved) percentages in pre-castration and dormant (at 12 weeks post-castration) PDXs. PCa, prostate cancer; PDX, patient-derived xenograft; H&E, hematoxylin and eosin staining; Casp-3, cleaved form caspase-3; AR, androgen receptor; PSA, prostatic specific antigen.

Figure 2. Compared to the pre-castration PDXs, dormant PDXs showed specific changes in gene expressions. **A**, genome-wide unsupervised hierarchical cluster showed most dormant (CX) PDXs clustered with their parental pre-castration (PRE) PDXs. **B**, Gene Set Enrichment Analysis (GSEA) based on whole transcripts without pre-ranking identified 12 significantly enriched gene sets in PRE PDXs (FDR<0.25). **C**, unsupervised hierarchical clustering based on the Leading Edge genes generated by GSEA showed a distinct separation between PRE and most CX PDXs. **D**, Ingenuity Pathway Analysis (IPA) revealed that deregulated pathways were mainly associated with cell cycle, cell death and survival, cellular assembly, DNA repair and cell growth. **E**, 53 out of 58 differentially expressed genes that were indicated by IPA to have a direct interaction with androgen receptor (AR) were downregulated in PDXs of dormant PCa. PDX, patient-derived xenograft; PCa, prostate cancer.

Fig. 3. Two subtypes of castration-induced PCa dormancy were associated with disease progression. **A**, a 42-gene set generated by GSEA Leading Edge analysis (tumor mass dormancy (orange) vs. cellular dormancy (blue)). **B**, principal component analysis (PCA) using the leading edge gene set demonstrated a clear split between 8 PDXs in cellular dormancy and 3 in tumor mass dormancy. The latter were grouped closely to pre-castration samples. PRE, pre-castration PDXs; CX, dormant PDXs. **C**, Kaplan-Meier estimate (KM) of progression-free (PFS) survival in PDXs showed that PDXs displaying tumor mass dormancy had a significantly shorter time to relapse ($P<0.001$). **D**, PCA based on the same Leading Edge gene set separated 18 patients

treated with pre-operative ADT (neo-adjuvant hormonal therapy) into two subgroups (Groups A and B, n=7 and n=11, respectively). E, KM of patients in the two subgroups ($P=0.13$). PCa, prostate cancer; GSEA, Gene Set Enrichment Analysis; PDX, patient-derived xenograft; ADT, androgen deprivation therapy.

Figure 4. Pre-disposed Gene Signature (PGS) predicted ADT response or clinical outcomes in independent hormone-naïve or castration-resistant prostate cancer (PCa) clinical cohorts. A. Single-sample gene set enrichment score of PGS is proportionally related to the residual tumor value ($r(35)=0.74$, $P<0.00001$) in primary hormone-naïve PCa treated with pre-operative ADT (Sowalsky clinical cohort, n=37). **B.** In the Sowalsky cohort, the PGS gene set enrichment score is significantly higher in non-responders (NR) compared to exceptional responders (ER, $P<0.0001$) and incomplete responders (IR, $P<0.0001$). **C-D**, Kaplan-Meier estimate of overall survival or progression-free survival (PFS) segregated according to the PGS score in (A) SU2C (n=60) and (B) Alumkal (n=25) clinical cohorts. In both cohorts, patients with PGS=1 had worse outcome than those with PGS=0 ($P=0.0002$ and 0.006 , respectively). **E**, Panther Protein Classification of the PGS genes revealed that the genes were involved in various biological processes.

TABLES

Table 1. Histopathological quantification results of dormant PCa in PDXs

PDX ID	Contributing factors				Reduction in cell density and stromal changes	Final score
	Pyknosis	Cytoplasmic vacuolization	Mitoses	Ki-67 positivity		
LTL-313B-CX	2	2	2	2	1	9
LTL-313H-CX	2	2	2	2	1	9
LTL-471-CX	2	1	2	2	2	9
LTL-556-CX	2	1	2	2	1	8
LTL-310-CX	2	1	2	2	0	7
LTL-508-CX	1	1	2	2	0	6
LTL-412-CX	0	0	2	2	0	4
LTL-418-CX	0	0	2	1	0	3
LTL-331-CX	0	0	0	0	0	0
LTL-467-CX	0	0	0	0	0	0
LTL-484-CX	0	0	1	0	0	1

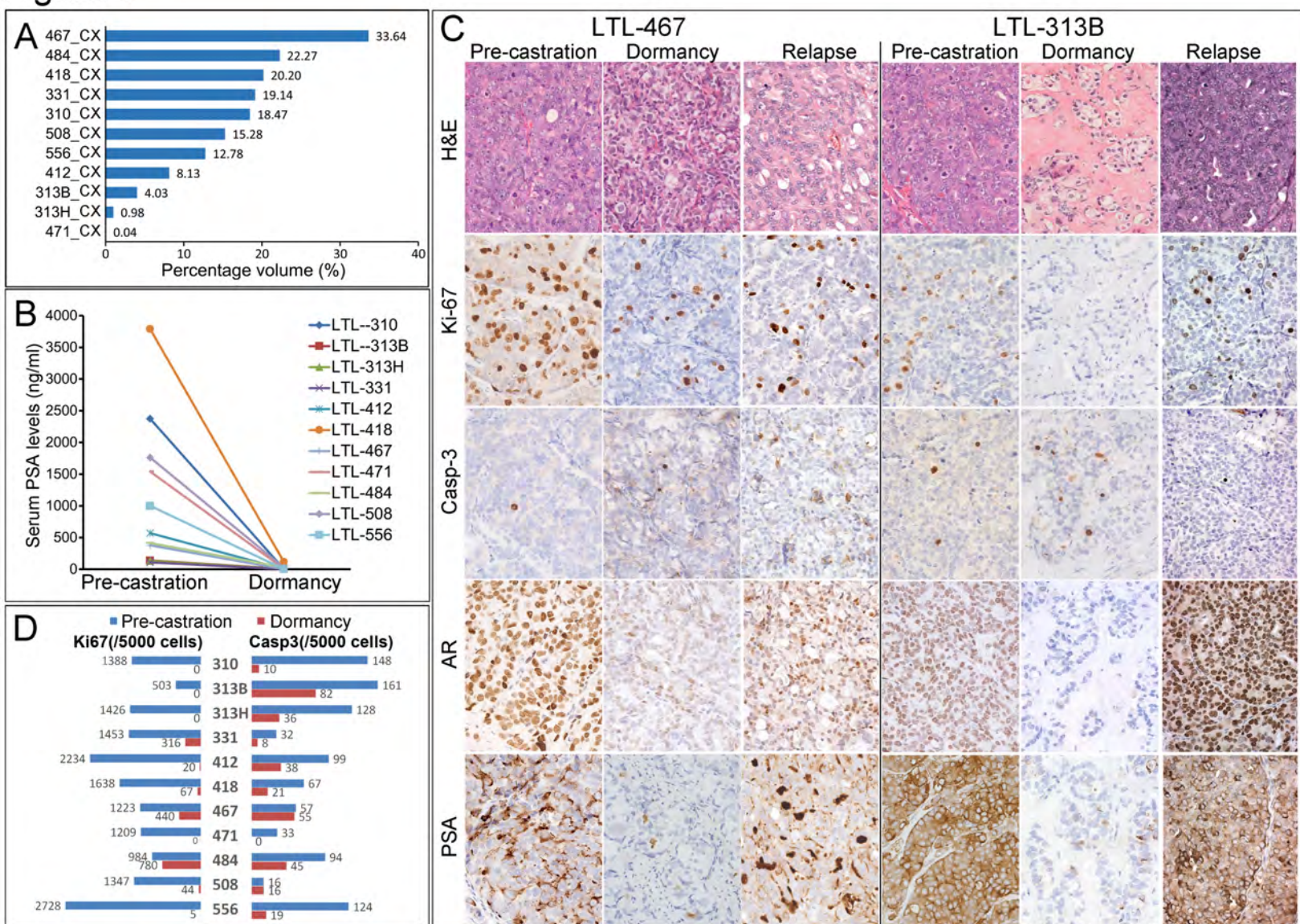
PDX, patient-derived xenograft; CX, 12 week post castration.

764 Table 2. Multivariate Cox Regression analysis of PGS in SU2C and Alumkal clinical cohorts.

Cohort	Covariates	Co- efficients	Standard error	P value	HR	95% CI	
						Lower	Upper
SU2C	Age (<65 vs. ≥65 y/o)	0.791	0.41	0.054	2.205	0.987	4.923
	Gleason score (<7 vs. ≥7)	0.581	0.421	0.168	1.787	0.783	4.078
	PSA at diagnosis (<20 vs. ≥20ug/mL)	0.001	0.001	0.095	1.001	1.000	1.002
	PGS (PGS=1 vs. PGS=0)	1.326	0.449	0.003	3.767	1.561	9.088
Alumkal	Age (<65 vs. ≥65 y/o)	0.994	0.706	0.159	2.702	0.678	10.772
	Gleason score (<7 vs. ≥7)	0.339	0.522	0.516	1.404	0.505	3.905
	PSA at diagnosis (<20 vs. ≥20ug/mL)	0.557	0.596	0.350	1.746	0.543	5.613
	PGS (PGS=1 vs. PGS=0)	1.741	0.61	0.004	5.702	1.726	18.842

765

Figure 1



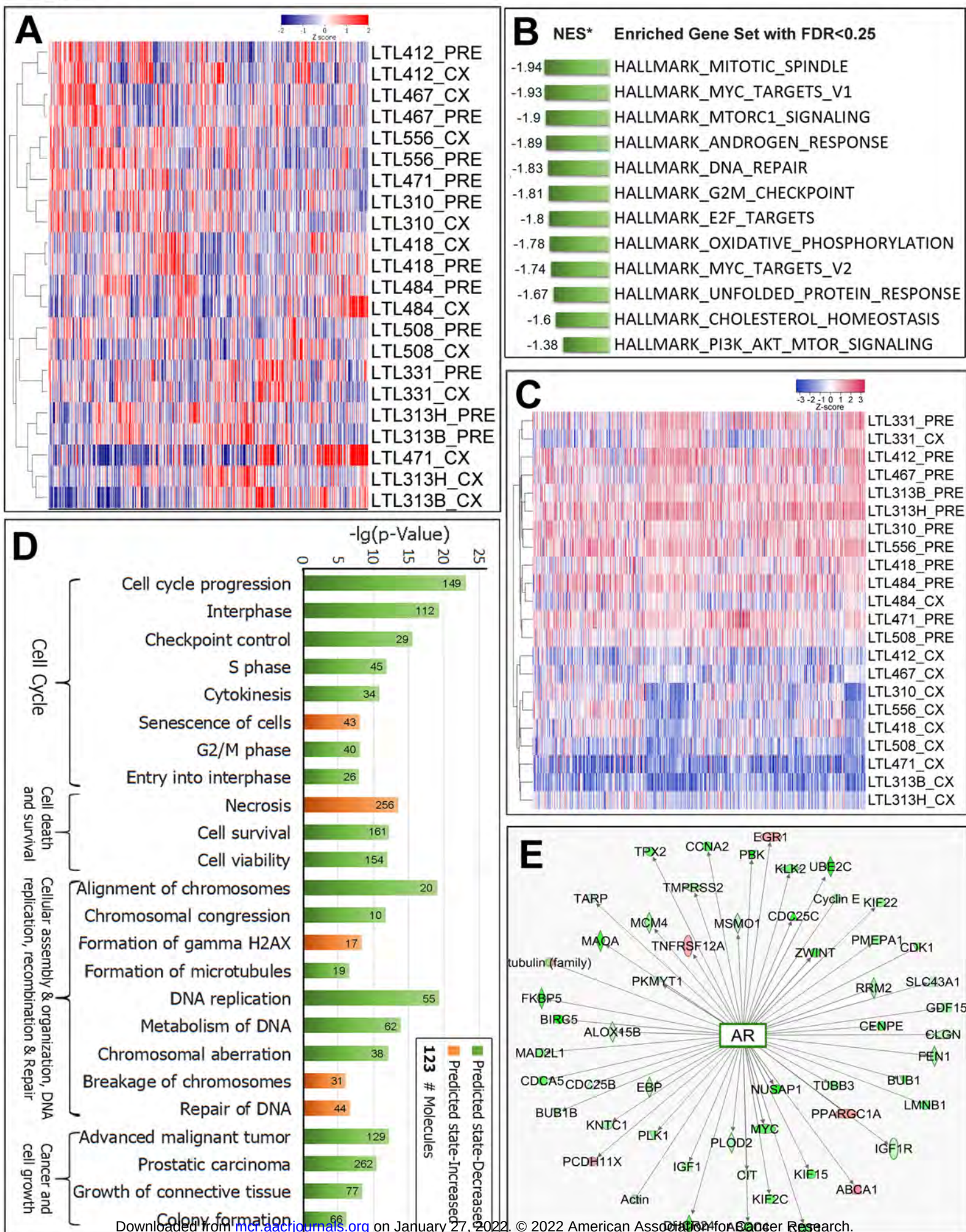


Figure 3

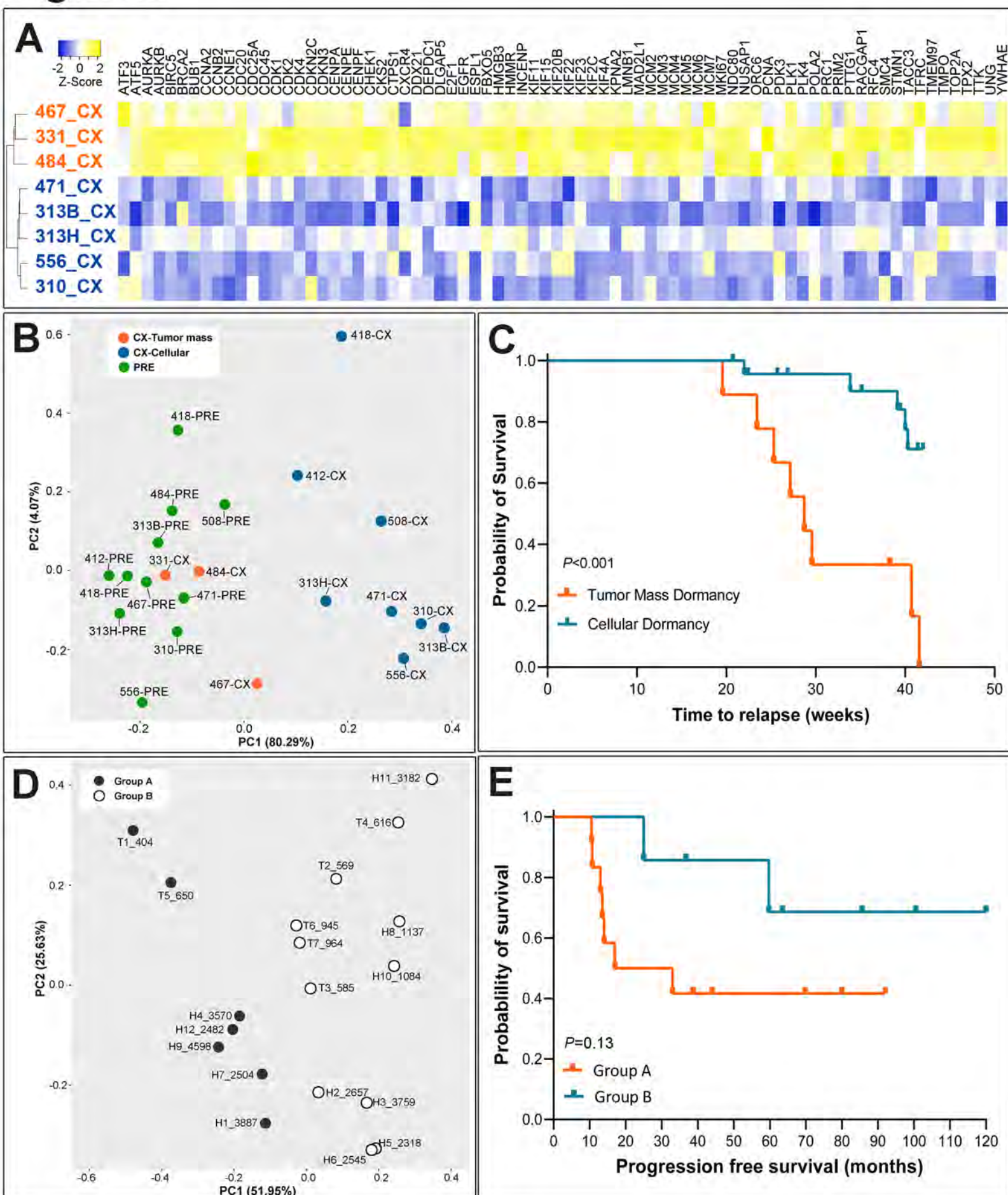
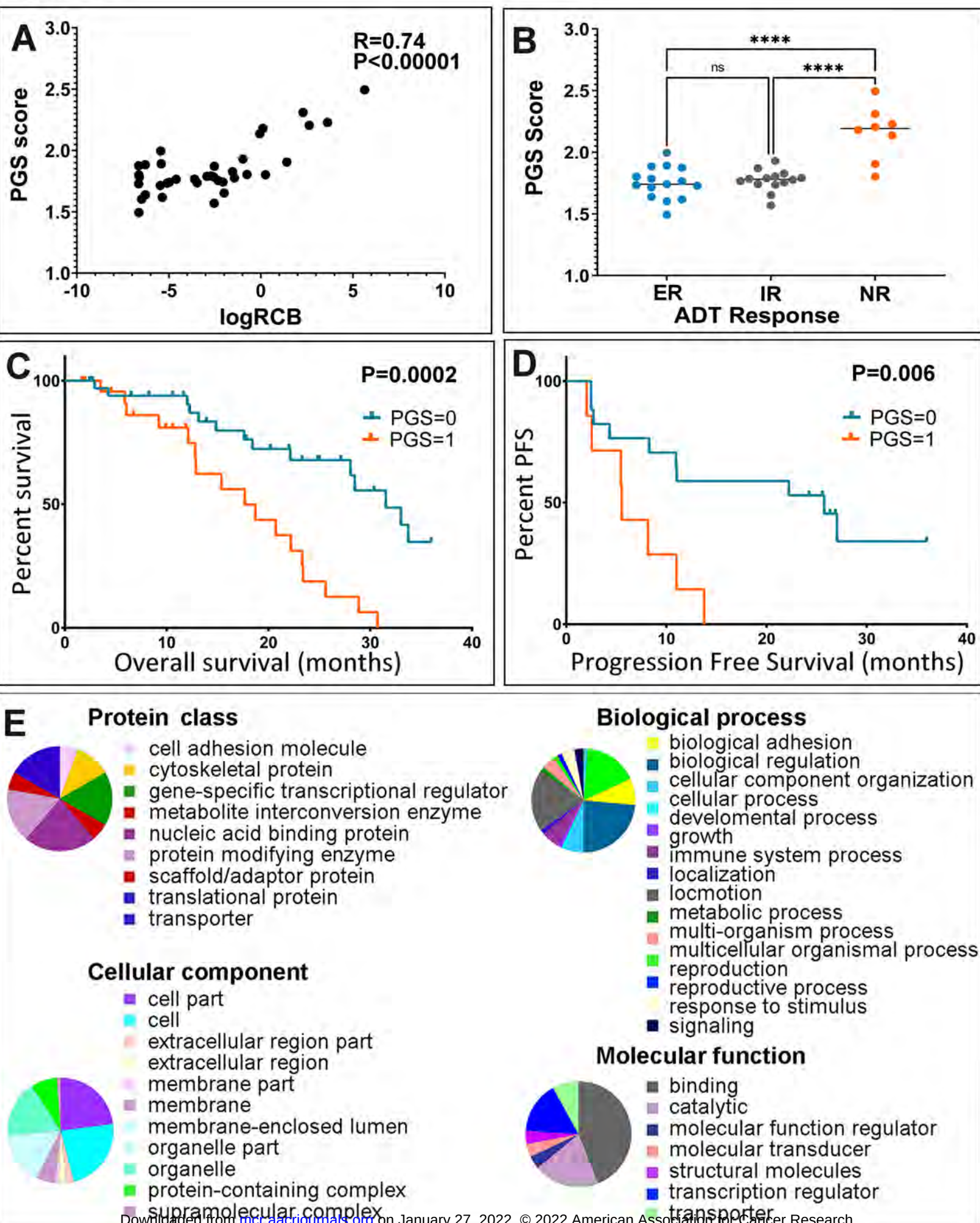


Figure 4



Molecular Cancer Research

Modeling androgen deprivation therapy-induced prostate cancer dormancy and its clinical implications

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Comparison of approaches to transcriptomic analysis in multi-sampled tumors

Anson T. Ku, Scott Wilkinson and Adam G. Sowalsky

Corresponding author: Adam G. Sowalsky, Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, NIH, 37 Convent Drive, Building 37, Room 1062B, Bethesda, MD 20892, USA. Tel.: +1-240-760-7118; E-mail: adam.sowalsky@nih.gov

Abstract

Intratumoral heterogeneity is a well-documented feature of human cancers and is associated with outcome and treatment resistance. However, a heterogeneous tumor transcriptome contributes an unknown level of variability to analyses of differentially expressed genes (DEGs) that may contribute to phenotypes of interest, including treatment response. Although current clinical practice and the vast majority of research studies use a single sample from each patient, decreasing costs of sequencing technologies and computing power have made repeated-measures analyses increasingly economical. Repeatedly sampling the same tumor increases the statistical power of DEG analysis, which is indispensable toward downstream analysis and also increases one's understanding of within-tumor variance, which may affect conclusions. Here, we compared five different methods for analyzing gene expression profiles derived from repeated sampling of human prostate tumors in two separate cohorts of patients. We also benchmarked the sensitivity of generalized linear models to linear mixed models for identifying DEGs contributing to relevant prostate cancer pathways based on a ground-truth model.

Key words: linear mixed model; prostate cancer; transcriptomics; RNA-seq; multiple sampling; variance

Introduction

Large-scale pan-cancer genomic analyses, such as the Pan-Cancer Atlas [1] and the Pan-Cancer Analysis of Whole Genomes [2], have firmly established the heterogeneous and diverse nature of many primary tumors. Indeed, deep sequencing experiments have revealed that most cancers harbor complex phylogenies with distinct driver and passenger alterations such that one region of a cancer may demonstrate a different phenotype than another part of the same tumor [3]. This spatial tumoral heterogeneity not only creates challenges for clinical diagnostics but also adversely impacts clinical outcome [4]. While the clinical convention of biopsying tumors to confirm diagnoses has historically been based on a single sample of tissue for most cancers, the regional histologic heterogeneity of prostate cancer (PCa), in particular, lends itself

to multiple sampling for a final diagnosis [5–8]. Paradoxically, PCa molecular diagnostic tests, like many others, still rely on a single biopsy sample; the delicate balance between expensive and comprehensive testing is justified by treating the most aggressive-appearing tumor when given a choice [9]. Given the extent of tumor heterogeneity, however, sampling for research purposes has recapitulated diverse genomic and genotypic results across multiple samples of the same human tumor, which can similarly be reflected in discordant within-patient diagnostic scores [5–8].

For studies of the tumor transcriptome, measures of differentially expressed genes (DEGs) are used to discover relationships between biological activity and phenotypes of interest, such as response to drug treatment or the impact of genomic alterations.

Anson T. Ku is a postdoctoral fellow at the Laboratory of Genitourinary Cancer Pathogenesis (LGCP) at the National Cancer Institute (NCI). His research interests focus on the contribution of tumor heterogeneity to cancer phenotype and patient outcome.

Scott Wilkinson is a postdoctoral fellow at the LGCP at the NCI. His research interests include histologic assessments of tumor heterogeneity and the development of multiplex diagnostic assays for prostate cancer.

Adam G. Sowalsky is an investigator at the LGCP at the NCI. His work focuses on prostate cancer evolution in response to hormone-directed therapy.

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With multiple-sampling strategies increasingly becoming common due to lower costs of sequencing and computational power, key concerns linger pertaining to the consolidation or aggregation of multiple sources of (valuable) information from the same biological sample (i.e. a patient) with an outcome that is ascribed to the whole patient and thus all samples as well [4, 10–12]. For example, how much variance is acceptable to lose in the process of reconciling an evolutionarily and phenotypically diverse tumor to a discrete outcome? Perhaps, and more importantly, what measures related to outcome harbor intrinsically more variance deemed by repeated measures of the tumor?

For ribonucleic acid (RNA) sequencing (RNA-seq), there is an underlying assumption that biological variability is often much greater between samples of different individuals than from within the same individual. Numerous bioinformatic solutions have been developed to assess the relationship between gene expression and outcome, often within the context of well-defined biological model systems with negligible within-individual variance and highly reproducible analytical measures such as RNA-seq. The most common tools include EdgeR, Limma/Voom and DESeq2, and these tools are frequently used to compare phenotypes with a defined number of biological replicates for each phenotype such that each replicate is given equal weighting using generalized linear models [13–15]. If applied to multiple samples of the same individual, options thus would include aggregating all within-individual data to individual-level values, or weighting each sample equally, which would skew observations. For example, an approach using DESeq2 would collapse pseudo-replicates by summing RNA-seq counts, which reduces the information available to discern fixed effects (variables that are constant across individuals, like treatment outcome) from random effects (categorical variables like somatic mutation status). Although Limma/Voom can accommodate the modeling of a single random effect, this would assume there are no other sources of variance to model, which is rarely the case when working with human biospecimens, especially if batch effects or purity are considered. To wit, when extended to translational cancer research, modeling the transcriptomes of patient samples that are acquired or compared based on innumerable salient phenotypes requires the inclusion of both fixed and random effects, which can be accommodated in linear mixed models (LMMs). The R package for LMMs, lme4 [16], is used by the Dream package [17] for repeated-sampling analysis by RNA-seq. However, its performance with real-world tumor data benchmarked against conventional analyses is unknown.

Here, we consider three potential genotypes based on somatic mutations, which are relevant to PCA (TMPRSS2-ERG fusion, PTEN loss and 18q deletion), the potential interaction between TMPRSS2-ERG fusion and PTEN loss and the sampling of tumors from patients with those genotypes. The TMPRSS2-ERG fusion occurs very early in the natural history of approximately 50% of PCA and frequently co-occurs with losses to PTEN, which manifest subclonally [18]. We explore the impact of five different analytical methods on the differential expression analysis from two PCA cohorts in which repeated-sampling design is a consideration and in which four of the methods collapse the specimens from the same lesion into a single sample (collectively termed aggregation), while the fifth method employs a LMM. By comparing between-sample individuals of each cohort with known heterogeneous cancer somatic phenotypes to robust ‘gold standard’ PCA transcriptomes from The Cancer Genome Atlas (TCGA), we show that LMMs demonstrate superior sensitivity for identifying DEGs when controlling for heterogeneous confounders such as PTEN loss.

Aggregation approaches show similar sensitivity to LMMs when working with fewer samples per individual or when working with phenotypes that result in little within-patient variability. As such, comparing the sensitivity of these methods further reveals sources of variance depending on target phenotypes and is a practical exercise for identifying occult causes of heterogeneity within a cohort. We thus provide this computational code as a resource for translational cancer researchers exploring heterogeneity within their own datasets.

Materials and methods

Patient cohorts

Cohort 1 consists of tumor samples from 37 patients diagnosed with intermediate- to high-risk PCA and subsequently treated with neoadjuvant hormone therapy and radical prostatectomy [8]. These tumor samples were acquired from magnetic resonance imaging (MRI)-targeted biopsies prior to the onset of therapy and were guided by immunohistochemistry prior to laser capture microdissection. The manuscript by Wilkinson et al. [8] describes this cohort and each laser capture microdissected (LCM) tumor focus.

Cohort 2 consists of tumor samples from 39 patients diagnosed with intermediate- to high-risk PCA and subsequently treated by radical prostatectomy [19]. These tumor samples were acquired from tissue punches of radical prostatectomy tissue, guided by a pathologist's determination of low-grade or high-grade cancer regions. The manuscript by Champi et al. [19] describes this cohort and each tumor region.

Raw sequence data processing

Paired-end RNA-seq FASTQ files for 130 tumor samples (cohort 1), 66 tumor samples (cohort 2) and 494 tumor cases (TCGA-PRAD) were downloaded from dbGaP (cohort 1), SRA (cohort 2) and the NCI Genomic Data Commons (TCGA-PRAD) and were trimmed using Trimmomatic [20] version 0.36 using Illumina adaptor sequences with the parameter 3:50:10 MINLEN:36. Gene-level counts were estimated from paired-end reads aligned to version hg19 of the human genome using RSEM [21] version 1.3.2 as a wrapper around STAR [22] version 2.7.0f. Candidate gene fusions were detected using deFuse [23] version 0.8.1.

Paired-end BAM files corresponding to exome capture libraries for the matched samples in cohort 1 and cohort 2 were downloaded and converted to FASTQ files using GATK SamToFastq. FASTQ files were then re-aligned with the Burrows Wheeler Aligner [24] BWA-MEM version 0.7.17 to version hg19 of the human genome (b37 with decoy chromosomes). The SAM alignment files were coordinate-sorted and duplicate-marked using PICARD version 2.18.27 SortSam and MarkDuplicates and then quality score-recalibrated using version 4.1.3.0 of the Genome Analysis Toolkit (GATK) BaseRecalibrator and ApplyBQSR. Mutation calling for ascertaining PTEN status was performed using MuTect2 (part of the GATK4 package), first by running MuTect2 in tumor-only mode on all of the normal tissue BAM files individually, with the parameters disable-read-filter set to MateOnSameContigOrNoMappedMateReadFilter and max-mnp-distance set to 0. The ‘padded’ bait BED file (Agilent Human All Exon V7 for cohort 1 and Agilent Human All Exon V5 + UTR for cohort 2) was used as the interval file of covered regions for all MuTect2 and filtering steps. Each of the output VCF files from this analysis of normal BAMs was merged into a database using GATK GenomicsDBImport

with the setting merge-input-intervals set to true and then by generating a panel of normal from the database using GATK CreateSomaticPanelOfNormals. MuTect2 was run in somatic mode on each tumor-normal BAM pair and the panel of normals with af-of-alleles-not-in-resource set to 0.0000025 to exclude sites present in gnomAD and with disable-read-filter set to MateOnSameContigOrNoMappedMateReadFilter. GetPileupSummaries and CalculateContamination were used on each tumor BAM file, and the resultant contamination table was used to filter somatic mutations using FilterMutectCalls. CollectSequencingArtifactMetrics and FilterByOrientationBias were used to further filter mutations for 8-oxoG artifacts using the settings -AM G/T -AM C/T. These pass-filter mutations were then functionally annotated using Oncotator version 1.9.70 (database version April052016).

PTEN copy number changes were called following whole-genome assessment of somatic copy number alterations (SCNAs) across genomic intervals specified by the Agilent library design BED file, with variable resolution depending on bait spacing. The design BED file was preprocessed with GATK PreprocessIntervals, with bin-length set to 0 and interval-merging-rule set to OVERLAPPING_ONLY. The BED file was also annotated with GC content using AnnotateIntervals. These interval files were used in all copy number calling steps. Read counts were first obtained from all tumor and normal BAM files using GATK CollectReadCounts. The normal read count files were compiled into a panel of normals using GATK CreateReadCountPanelOfNormals, excluding cases that had read depth coverage outside the 95% confidence interval (CI) for the dataset. The panel of normals was used to smooth read counts across all samples using GATK DenoiseReadCounts. Normal BAM files were then processed with GATK CollectAllelicCounts to identify regions of potential LOH. GATK CollectAllelicCounts was also applied to each tumor BAM file. GATK ModelSegments used smoothed read counts from each tumor BAM along with the paired normal/tumor allelic counts for generating copy number estimates.

Calls of PTEN somatic copy number changes were performed using GISTIC 2.0.23 on the GenePattern platform (module version 6.15.28). The SEG file from GATK ModelSegments was used as input with the following parameters: focal length cutoff set to 0.50, gene GISTIC set to yes, confidence level set to 0.90, cap value set to infinite, broad analysis set to on (to call arm-level events), max sample segments set to 10 000, arm peel set to no and gene collapse method set to extreme. Post-GISTIC log₂-copy number ratio threshold values were used for calling discrete gene-level calls as follows: >1.3 = 2-copy gain; 0.1–1.3 = 1-copy gain; –1.3 to –0.1 = 1-copy loss; <–1.3 = 2-copy loss. Chromosome 18q changes followed the same threshold scheme per tumor focus by using the convention that at least 50% of the chromosome arm (noncontiguously) must be affected.

Genotype/phenotype determination

For all TCGA cases, the reported status of TMPRSS2-ERG, PTEN and 18q were used as deposited on cBioPortal for the Pan-Cancer Atlas analysis (comprising 489 tumors). Fusions involving TMPRSS2 or ERG and other genes (such as SLC45A3-ERG) were excluded. Cases with undocumented 18q status, or gains to PTEN or 18q were also excluded. For cohort 1, PTEN status was determined jointly based on the status of anti-PTEN immunohistochemistry reported for each focus [8] and the somatic copy number or mutation calls. For cohort 2, PTEN status was determined based on somatic copy number or mutation calls. For

cohort 1, TMPRSS2-ERG status was determined jointly based on the status of nuclear anti-ERG immunohistochemistry reported for each focus [8] and RNA-seq fusions determined by deFuse. For cohort 2, TMPRSS2-ERG status was determined based on RNA-seq fusions determined by deFuse supplemented with any observed interstitial deletions between ERG and TMPRSS2 on chromosome 21 detected by somatic copy number analysis.

Aggregation of ground-truth phenotypes from foci to lesions and/or patients was as follows: a pre-determined threshold was set for a minimum number of foci to harbor that alteration to call a particular copy number event for the entire lesion. For lesions sampled by 7 or 8 foci, the threshold was 3; for 3, 4, 5 or 6 foci, the threshold was 2 and for 1 or 2 foci, the threshold was 1. For each focus, PTEN or 18q somatic copy number calls from GISTIC (i.e. –1, –2, 0, 1 or 2) were averaged across all foci from each lesion. If the absolute value of that average was greater than the pre-determined threshold, the averaged value was rounded to the nearest integer. For PTEN point mutations or the TMPRSS2-ERG, the lesion (cohort 1) or patient (cohort 2) was considered to harbor the mutation if any number of foci harbored it. Similarly, if any focus harbored PTEN loss or ERG expression by immunohistochemistry, the entire lesion (cohort 1) or patient (cohort 2) was considered to have harbored it. Lesions marked as the index lesion were used as patient-level data for cohort 1.

Sample counts aggregation

The input to the five analysis methods were count data with >3 million reads. Five (cohort 1) or four (cohort 2) different methods of transcriptomic analysis of multiple-sampled tumors were investigated as depicted in Figure 1. In the first method, concatenation, paired-end FASTQ files derived from samples of the same lesion or tumor were concatenated into a single pair of FASTQ files representing the entire lesion or tumor. These FASTQ pairs were then processed as above with RSEM and STAR, arriving at a single counts file per lesion or tumor. The remaining analyses were performed with sample counts obtained after alignment and count estimation as stated above. In the second method, averaging, counts from the same lesion were averaged across genes, creating a representative lesion-level transcriptome profile for each lesion or patient. In method three, RNA weighting, gene counts were normalized based on the weighted average of the specimen RNA input amounts into library preparation as seen in Equation (1).

$$X_{L,g} = \sum_1^J FC_{L,j} \bullet X_{j,g}, \quad (1)$$

where $X_{L,g}$ is the count of gene g , $FC_{L,j}$ is defined as the fractional concentration of specimen j obtained from lesion L , which is calculated as Equation (2).

$$FC_{L,j} = \frac{c_j}{\sum_1^J c_j}. \quad (2)$$

Due to missing data, RNA weighting was only performed for cohort 1. In method four, summation, counts of the same lesion/patient were added across genes to create a high coverage profile of the tumor's transcriptome. Finally, in method five, the LMM was used with fixed and random effects to account for inter-patient and inter-lesion variability.

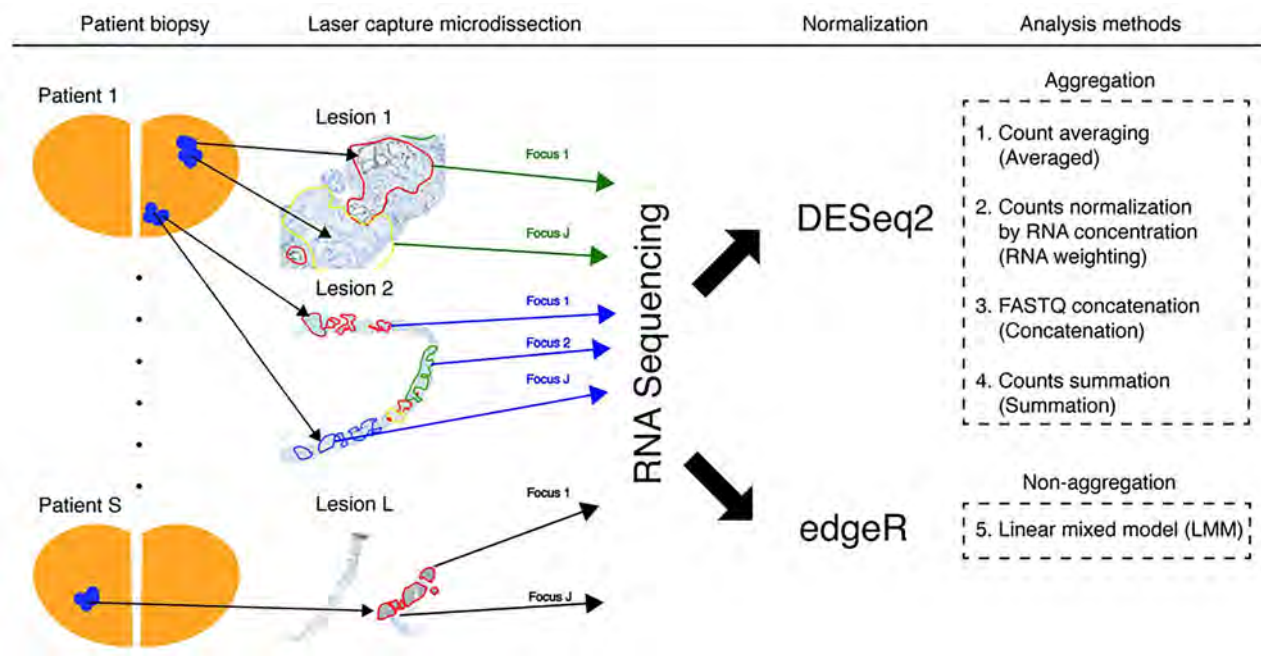


Figure 1. Schematic illustration of the work flow for cohort 1. Biopsies were obtained from one or more lesions per patient. For each lesion, one or more foci were laser capture microdissected (LCM) from biopsied materials. RNA sequencing was performed on each focus and the associated ERG and PTEN protein status were determined by immunohistochemistry of adjacent slides, while PTEN and chromosome 18q status were obtained from exome sequencing data. The gene expression data were analyzed by five different methods: counts averaging, RNA weighting, FASTQ concatenation, counts summation and LMM.

Purity calculation

Determination of tumor sample purity was performed using ESTIMATE version 1.0.13 for R [25], which uses the single sample gene set enrichment algorithm to infer the transcriptomic signal from tumor gene expression datasets. Transcripts per million (TPM) values from RSEM-STAR alignments were filtered for common genes with the ESTIMATE algorithm and the tumor purity score was outputted as a projection interpolated against a priori ground-truth purity percentages. For cohort 1, mean purity was 96.6% (95% CI: 96.1–97.1%) and was thus not factored into any gene expression model. For cohort 2, mean purity was 87.4% (95% CI: 85.2–89.7%) and was included as a covariate.

Differential gene expression analysis

Differential gene expression analysis for aggregated samples in cohorts 1 and 2 was performed using the DESeq2 package available in R [15]. Gene count matrices were prepared as described above using RSEM/STAR. Genes with zero counts in more than half of the samples were removed. Next, counts were normalized by the median ratio method from DESeq followed by differential expression analysis by using the following 5 phenotypic classifications as fixed effect parameters: (1) TMPRSS2-ERG fusion-positive versus TMPRSS2-ERG fusion-negative, (2) PTEN loss versus PTEN intact, (3) TMPRSS2-ERG fusion-positive and PTEN intact versus TMPRSS2-ERG fusion-negative and PTEN intact, (4) TMPRSS2-ERG fusion-positive and PTEN loss versus TMPRSS2-ERG fusion-negative and PTEN intact and (5) chr18q loss versus chr18q intact. Genes with false discovery rate (P_{adj}) < 0.05 and $\log_2$ fold-change < -1 or > 1 were considered to be differentially expressed.

LMM analysis

Differential gene expression analyses using a LMM were performed using the variancePartition package available in R [17]. First, gene counts were normalized by the trimmed mean method in edgeR. Next, the genomic alterations of interest and sample purity (for cohort 2) were modeled as fixed effects, while patients and their lesions were modeled as nested random effects in the LMM model using the formula: $\sim \text{Fixed_effect} + (1|\text{Patient:Lesion})$ for cohort 1. For cohort 2, the genomic alterations of interest were modeled as fixed effects while patients were modeled as a random effect variable using the formula: $\sim \text{Fixed_effect} + \text{purity} + (1|\text{Patient})$. For the aggregation methods (averaged, concatenation and summation), the average purity of the specimens were used as an adjustment to the linear model.

Derivation of PTEN loss, TMPRSS2-ERG and 18q-loss gene sets

Gene count matrices from TCGA-PRAD were annotated with PTEN, TMPRSS2-ERG and 18q status, and differential expression analysis based on each genotype was performed using DESeq2. After removing genes with zero counts in at least half of the samples, genes were normalized by the median of ratios method as described in DESeq2. Gene signatures were generated by performing differential expression analysis using DESeq2 by using each genotype as an input parameter and by accepting DEGs with $P_{adj} < 0.05$ by Wald test.

Statistics

Similarities between groups of samples were determined using unsupervised hierarchical clustering (Euclidean distance) of all genes. Null hypothesis tests of associations between

affected and unaffected samples were performed using two-sided Fisher's exact tests. Generalized linear models using the likelihood ratio test were used to compare gene expression between groups of singly sampled tumors. Unless noted otherwise, alpha of 0.05 was used as a significance threshold for all statistical analyses.

Software

All mathematical analyses were performed using R version 4.0.2 and RStudio 1.3.

Results

Transcriptomic consequences of genomic heterogeneity in prostate tumors

Despite its common genetic background, within-patient tumor heterogeneity manifests itself through variable histologies and genomic alterations, even by geographically proximal clusters of tumor cells. As a framework for assessing salient gene expression as a function of histologic or genomic heterogeneity, we selected three somatic alterations common to PCa: genomic deletion or deleterious alterations to the tumor suppressor *PTEN*, deletion of at least half of chromosome 18q and the fusion of *TMPRSS2* and *ERG* on chromosome 21. Alterations to *PTEN* and 18q were determined by analysis of whole-exome sequencing data from matched samples (compared to benign controls), while *TMPRSS2-ERG* fusion was determined by the expression of nuclear *ERG* by immunohistochemistry.

We assessed the frequency of these alterations in two distinct cohorts. As depicted in Figure 1, cohort 1 was comprised of 130 LCM foci, sampled from 51 different biopsies targeting 50 distinct imaging-visible lesions from 39 patients, with 1–3 lesions per patient and 1–8 foci per patient [8]. Foci in cohort 1 were microdissected based on distinct histologic features, such as gland architecture, proximity to other tumor cells and immunochemistry against common PCa markers, such as anti-*ERG* and anti-*PTEN*. Cohort 2 was comprised of 66 tumor punches from either high-grade or low-grade tumors from prostatectomy samples assessed without imaging, with 1–2 foci per patient [19]. Regions sampled in cohort 2 were determined solely on tumor aggressivity.

We first performed unsupervised clustering on gene expression data from each focus individually, in both cohorts, annotating each sample for *PTEN*, *ERG* and 18q status. Of these three factors, clustering revealed that foci clustered most strongly based on *ERG* fusion status and then by *PTEN* status, both in cohort 1 (Fisher's exact test, adjusted $P = 4.29 \times 10^{-24}$; 5.39×10^{-11} and 1, respectively, $k = 3$; Figure 2A and B) and in cohort 2 (Fisher's exact test, adjusted $P = 2.10^{-5}$; 0.041 and 1, respectively, $k = 2$; Figure 2C and D). As *ERG* fusion and *PTEN* loss showed significant per-focus co-occurrence in cohort 1 but not cohort 2 (Fisher's exact test, $P = 6.30 \times 10^{-9}$ and 0.6, respectively), we assessed the inter- and intra-lesion variations of *ERG*, *PTEN* and chr18q alterations of cohort 1 and the intra-patient variation in cohort 2. *PTEN* loss had the highest inter- and intra-lesion variabilities of the three alterations in cohort 1 (Figure 2A and B) and the highest intra-patient variability in cohort 2 (Figure 2C and D), suggesting that the subclonal nature of major driver alterations, such as *PTEN* loss, might affect tumor phenotypes within a patient.

We thus further subdivided each cohort based on the dual status of *TMPRSS2-ERG* and *PTEN* to examine whether the 'double-hit' frequent co-occurrence of *TMPRSS2-ERG* fusion and

PTEN loss was significantly different than *TMPRSS2-ERG* fusion on a wild-type (intact) *PTEN* background. For both cohorts, the *TMPRSS2-ERG* fusion-positive samples separated distinctly from *TMPRSS2-ERG* fusion-negative samples by the first two principal components (Supplementary Figure S1 available online at <http://bib.oxfordjournals.org/>), with *PTEN* intact versus loss samples showing adjacent grouping with the *TMPRSS2-ERG*-positive structure. In contrast, *TMPRSS2-ERG*-negative cases with *PTEN* loss versus intact are admixed on the first two principal components, suggesting that *PTEN* loss alone is a weak effect on global transcription. These high-level views of these data further suggest that the effect of *PTEN* may be challenging to dissect from the dominant signal arising from *TMPRSS2-ERG* fusion.

Transcriptomic consequences of somatic genomic alterations

Patient response to cancer therapies are often distilled to a binary value: remission or recurrence, response or failure and survival or mortality. Precision medicine workflows to predict such clinical responses most often rely upon a single sample from which various measures are taken. However, assessments of tumor heterogeneity (i.e. multiple region sampling) are exploratory in nature and are for research purposes; given the geometric increase in cost for clinical assays and the challenges associated with interpreting multiple and potentially conflicting results from a single patient, repeated clinical assays of the same sample per patient are not feasible. Thus, the translatability of human oncology research depends upon a definitive outcome wherein the evidence supporting that result takes into account phenotypic variability due to heterogeneity.

We thus considered five different methods for deriving DEGs from our two cohorts when working with tumors that were sampled multiple times: (1) averaging of counts within each case (averaged), (2) weighted averaging of counts based on RNA input amounts into library preparation (RNA weighting), (3) concatenation of FASTQ files prior to a single analysis per case (concatenation), (4) summation of counts (summation) and (5) use of a LMM. DEGs were derived for each cohort and method based on the status of *ERG* (*TMPRSS2-ERG* fusion-positive versus -negative), and *PTEN* (reduced or lost versus intact). Due to the strong potential of interaction between *TMPRSS2-ERG* fusion and *PTEN* in driving changes in gene expression, we modeled two additional subsets: (1) samples with only *TMPRSS2-ERG* fusion-positive and (*PTEN* being intact) versus *TMPRSS2-ERG* fusion-negative (and *PTEN* intact); and (2) combined *TMPRSS2-ERG* fusion-positive and *PTEN* loss versus combined *TMPRSS2-ERG* fusion-negative and *PTEN* intact. Finally, as a negative control, we modeled a highly subclonal event that was predicted to have minimal effects on gene expression: chromosome 18q (lost versus intact). For aggregation methods 1–4, if a single sample was considered altered, the entire case was considered altered. For the LMM (method 5), *TMPRSS2-ERG*, *PTEN* and 18q status were specified as fixed effects, while cases were modeled as random effect. Sample purity, which was highly variable for cohort 2 only, was modeled as an adjustment to the linear model for aggregation methods 1, 3 and 4 and as a fixed effect for the LMM.

Table 1 shows the number of statistically significant DEGs ($P_{adj} < 0.05$). Generally, the number of DEGs was dependent on the genomic alteration and the method of aggregation. However, we noted significant differences for a subset of the DEGs based, in part, on sample segregation observed in the PCA plot (Supplementary Figure S1 available online at

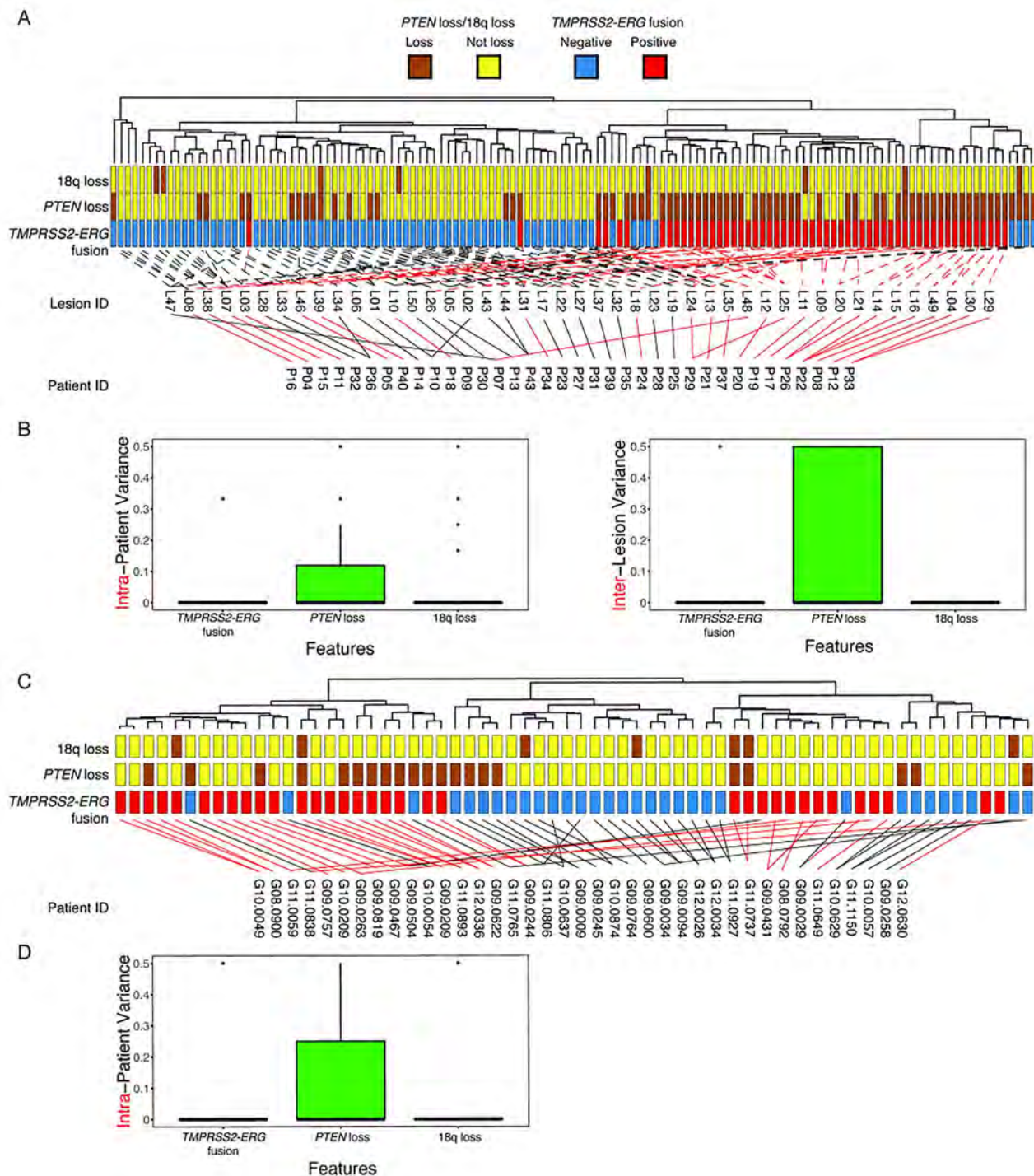


Figure 2. Summary of gene expression profile of cohort 1 and 2. (A) Unsupervised clustering of focus level gene expression data from cohort 1 with TMPRSS2-ERG, PTEN and chr18q loss annotation. The results revealed a strong tendency for focus to group based on TMPRSS2-ERG and PTEN status (Fisher's exact test, adjusted $P = 4.29 \times 10^{-24}$ and 5.39×10^{-11} , respectively) but not chr18q loss ($P = 1$). Corresponding patient identifier and lesion identifier were mapped below the dendrogram. Black lines corresponding to foci or lesions without TMPRSS2-ERG fusion were drawn connecting to lesions and patients, while red lines correspond to foci or lesions with TMPRSS2-ERG fusion foci. (B) Assessment of TMPRSS2-ERG, PTEN and chr18q intra and inter lesion heterogeneity. PTEN loss remains the most variable feature within and between lesions in cohort 1. (C) Unsupervised clustering of focus level gene expression data from cohort 2 with TMPRSS2-ERG, PTEN and chr18q status annotation. The results revealed a strong tendency for foci to group based on TMPRSS2-ERG and PTEN status (Fisher's exact test, adjusted $P = 2.10^{-5}$ and 0.041 , respectively) but not chr18q loss ($P = 1$). (D) Assessment of TMPRSS2-ERG, PTEN and chr18q intra lesion heterogeneity. PTEN loss remains the most variable feature within lesions in cohort 2. Clustering was performed on the basis of total gene expression.

Table 1. The total number of DEGs for each aggregation method per cohort, per genotype

Method	Cohort 1					Cohort 2				
	ERG	PTEN	ERG ^{fus} /PTEN ^{wt}	ERG ^{fus} /PTEN ^{loss}	18q	ERG	PTEN	ERG ^{fus} /PTEN ^{wt}	ERG ^{fus} /PTEN ^{loss}	18q
Count averaging	804	551	69	971	6	869	542	618	1038	2
RNA input weighting	807	580	101	943	4	N/A	N/A	N/A	N/A	N/A
Concatenation	785	557	103	942	6	880	554	625	1056	2
Count summation	840	464	105	949	10	881	562	634	1061	2
LMM	894	17	510	1012	0	415	309	634	616	1

ERG, TMPRSS2-ERG fusion-positive versus TMPRSS2-ERG fusion-negative; PTEN, PTEN loss versus PTEN intact; ERG^{fus}/PTEN^{wt}, TMPRSS2-ERG fusion-positive and PTEN intact versus TMPRSS2-ERG fusion-negative and PTEN intact; ERG^{fus}/PTEN^{loss}, TMPRSS2-ERG fusion-positive and PTEN loss versus TMPRSS2-ERG fusion-negative and PTEN intact; 18q, chr18q loss versus chr18q intact.

<http://bib.oxfordjournals.org/>). TMPRSS2-ERG fusion, a categorically early event in the natural history of prostate tumors, yielded among the largest number of DEGs for all three comparisons (all fusion-positive cases, only fusion-positive cases with PTEN loss and only fusion-positive cases with intact PTEN). Notably, for cohort 1, the greatest number of DEGs was observed when comparing 'double-hit' TMPRSS2-ERG fusion-positive and PTEN loss versus 'double-neutral' TMPRSS2-ERG fusion-negative and PTEN intact cases on account of the majority of individual samples being concordant (positive or negative) for alterations to ERG and PTEN. TMPRSS2-ERG fusion accounting for a majority of transcriptomic variance in cohort 1 (with PTEN loss contributing subtly to the variance) explains the ability of LMM to be sensitive to tracking subclonal ERG and PTEN mutations better than aggregate methods, resulting in the greatest number of DEGs for cohort 1.

In both cohorts, PTEN loss alone contributes very little to global transcriptomic variance by PCA and thus is modeled more accurately by LMM, with the fewest number of genes for both cohorts. In cohort 2, the frequency of co-occurring TMPRSS2-ERG and PTEN mutations is less than that of cohort 1, which accounts for all ERG and double-hit DEGs being fewer than TMPRSS2-ERG only (with intact PTEN) dominating the DEG list. Indeed, when visualized using an UpSet plot ([Supplementary Figure S2](#) available online at <http://bib.oxfordjournals.org/>), we observed the greatest number of shared DEGs within method were between the TMPRSS2-ERG fusion/PTEN loss and the TMPRSS2-ERG fusion genotypes, further suggesting that the greater number of DEGs among aggregation methods versus LMM (and especially for PTEN loss) represents 'spillover' from the TMPRSS2-ERG fusion genotype. The complete lists of DEGs for each genotype are given in [Supplementary Tables S1–S5](#) (cohort 1) and [Supplementary Tables S6–S10](#) (cohort 2) available online at <http://bib.oxfordjournals.org/>.

We next performed set analysis to compare each aggregation or LMM method directly. 467, 7, 24, 616 and 0 DEGs were shared between the five methods for TMPRSS2-ERG fusion, PTEN loss, TMPRSS2-ERG fusion/PTEN intact, TMPRSS2-ERG fusion/PTEN loss and chr18q loss, respectively in cohort 1 ([Figure 3A](#)). Similarly, a total of 331, 182, 316, 482 and 0 genes were common for cohort 2 ([Figure 3B](#)). Within both cohorts, a large number of genes were common to all methods except LMM, given the common generalized lineal models used to identify those DEGs. Interestingly, however, despite the similarities between concatenation and summation (pre-alignment versus post-alignment addition), there were very few DEGs exclusive to just these two aggregation methods.

To determine if the number of DEGs produced by different methods was sensitive to an alpha threshold, the number of unique DEGs obtained from all five methods were pooled and measured as a function of increasing alpha for all five genomic features in cohorts 1 ([Figure 4](#), top) and 2 ([Figure 4](#), bottom). When increasing alpha from 0.05 to 0.2, the number of unique DEGs increased only in cohort 1 for the TMPRSS2-ERG fusion and TMPRSS2-ERG fusion/PTEN loss genotypes, in part, due to the strong effect of TMPRSS2-ERG fusion on global transcription and the concordance of TMPRSS2-ERG and PTEN mutations in these samples ([Figure 2](#)). The lack of convergence for the PTEN and TMPRSS2-ERG fusion/PTEN intact genotypes in cohort 1 likely represents the sensitivity of LMM for handling PTEN heterogeneity as a blocking factor. Because cohort 2 was derived from tissue punches and not microdissected based on ERG and PTEN status like cohort 1, the lack of convergence represents hidden heterogeneity that is incompletely addressed by any model.

The clinical practice of determining an entire cancer being called PTEN-negative due to at least 5% of tumor cells showing PTEN reduction by IHC would be expected to have an effect on heterogeneity when multiple samples from the same tumor are captured without microdissection. Thus, when we assessed the similarities between the five different methods using unsupervised clustering of the differential expression output, we noted that LMM clustered away from other methods for all genotypes, in both cohorts ([Figure 5A](#) and [B](#)). Based on DEGs, we anticipated that PTEN loss would show the greatest difference by unsupervised clustering. However, the effect of PTEN loss was similar to 18q and all three TMPRSS2-ERG fusion genotypes in which accounting for multiple samples from the same individual as random effects has a stronger influence than any other method in which variance from repeated measures is ignored.

Because TMPRSS2-ERG fusion and losses in PTEN were commonly co-occurring in cohort 1, we specifically assessed which DEGs were spilling over from TMPRSS2-ERG into PTEN analysis in all five methods. The result revealed a total of 269, 283, 257, 282 and 5 genes that are common between TMPRSS2-ERG fusion and PTEN DEG analysis from counts averaging, RNA weighted averaging, concatenation of FASTQ files, counts summation and LMM methods, respectively. To better understand the impact of within-patient variability and remaining variance after accounting for each genomic alteration on gene expression, we performed variance decomposition using results from LMM for both cohorts ([Figure 6](#)). Indeed, between-patient variance was a significant source of transcriptomic heterogeneity, explained by a median of 31–38% percent of variance in TMPRSS2-ERG

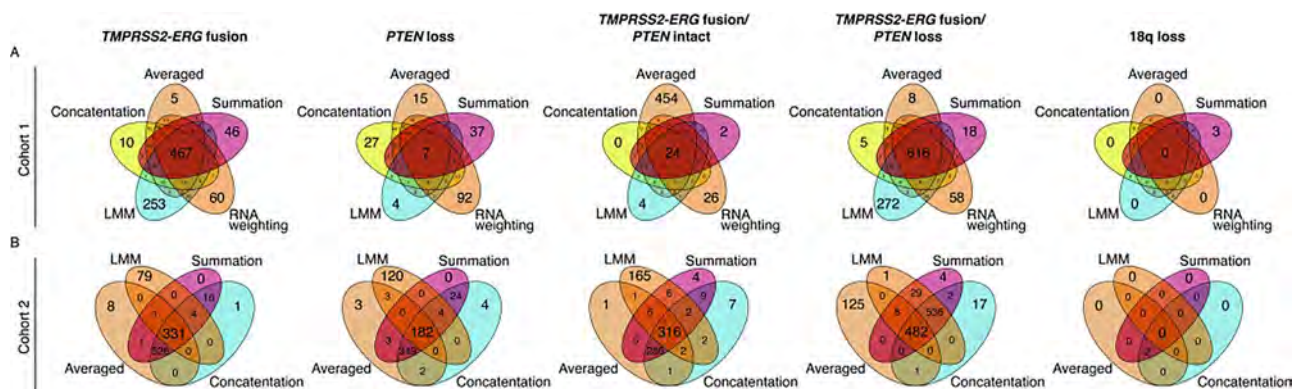


Figure 3. Set analysis of genes related to TMPRSS2-ERG fusion, PTEN loss, TMPRSS2-ERG fusion/PTEN intact, TMPRSS2-ERG fusion/PTEN loss and chr18q loss in cohorts 1 and 2. (A) Venn diagram depicting the shared DEGs between each analysis method in cohort 1. The number of DEGs common between all five methods are 467, 7, 24, 616 and 0 for TMPRSS2-ERG fusion, PTEN loss, TMPRSS2-ERG fusion/PTEN intact, TMPRSS2-ERG fusion/PTEN loss, and chr18q loss, respectively, in cohort 1. (B) Venn diagram depicting the shared DEGs between each analysis method in cohort 2. The number of DEGs common between the four methods are 331, 182, 316, 482 and 0 for TMPRSS2-ERG fusion, PTEN loss, TMPRSS2-ERG fusion/PTEN intact, TMPRSS2-ERG fusion/PTEN loss and chr18q loss, respectively, in cohort 2.

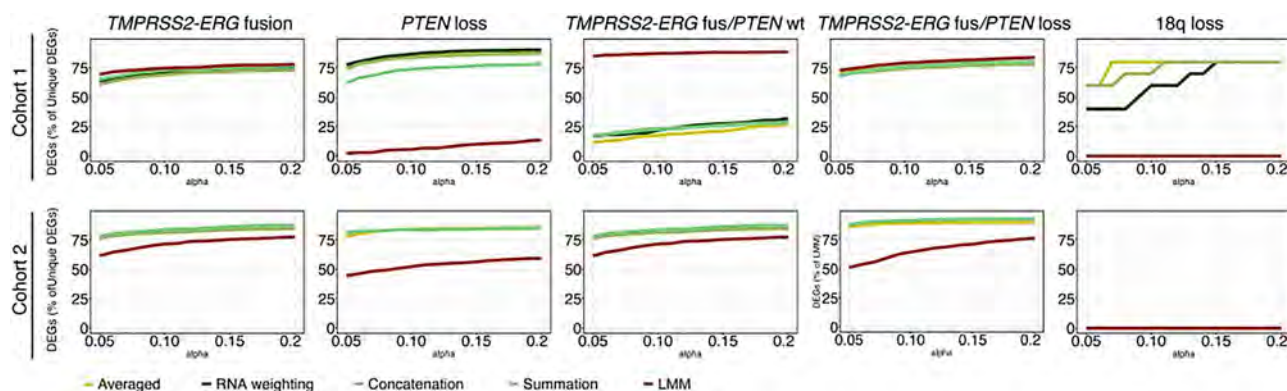


Figure 4. Sensitivity analysis of DEGs to alpha. For each genomic alteration analysis, the total number of unique DEGs from each method were pooled. The filtering threshold were set to $|\log_2 \text{fold-change}| > 1$ followed by increasing alpha to determine the percentage of significant DEGs. The increase in the alpha from 0.05 to 0.20 did not lead to a convergence $> 75\%$ of unique DEGs across genotype in cohort 1. Sensitivity curves were grouped the closest in the three TMPRSS2-ERG fusion genotypes. Averaged, concatenation and summation are consistently grouped together with increasing alpha from 0.05 to 0.20 in cohort 2. LMM approached convergence with the other methods in cohort 1 only in two genotypes (TMPRSS2-ERG fusion and TMPRSS2-ERG fusion/PTEN loss).

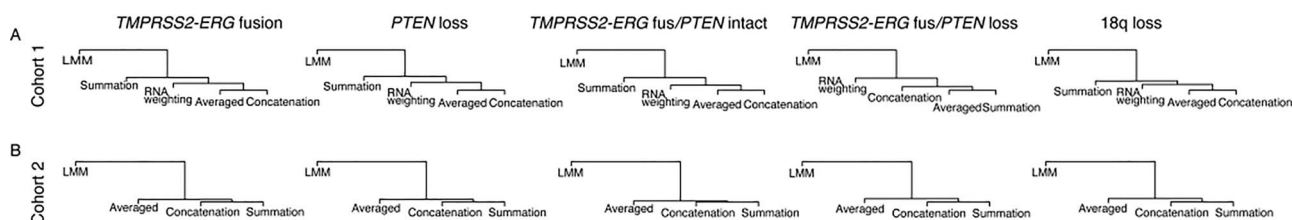


Figure 5. The relatedness of the five methods were assessed by hierarchical clustering of $\log_2$ fold-change from the five methods in cohort 1 (A) and four methods in cohort 2 (B). The type of genomic comparator is indicated above each tree.

fusion, PTEN loss and 18q loss gene expression in cohort 1 and 55–59% for cohort 2, respectively. In contrast, genomic alterations explained a median of 0% variance in both cohorts when accounted for as a fixed effect.

Comparison of DEGs to a standardized gene set

As blocking for genomic alterations reduced variance in the LMM, we still have observed that multiple methods for deriving DEGs for each genotype generally produced overlapping but distinct results. Thus, a key question is whether one approach arrives at a more accurate representation of gene expression

changes driven by TMPRSS2-ERG, PTEN or 18q mutations. To benchmark these approaches, we next derived ‘ground-truth’ gene sets by performing an integrated analysis of the prostate adenocarcinoma cancer genome atlas (TCGA-PRAD) cohort to derive a DEG list of the same five genotypes described above: (1) TMPRSS2-ERG fusion-positive versus TMPRSS2-ERG fusion-negative, (2) PTEN loss versus PTEN intact, (3) TMPRSS2-ERG fusion-positive and PTEN intact versus TMPRSS2-ERG fusion-negative and PTEN intact, (4) TMPRSS2-ERG fusion-positive and PTEN loss versus TMPRSS2-ERG fusion-negative and PTEN intact and (5) chr18q loss versus chr18q intact. For each of these gene sets, up to 150 significantly up-regulated genes in the

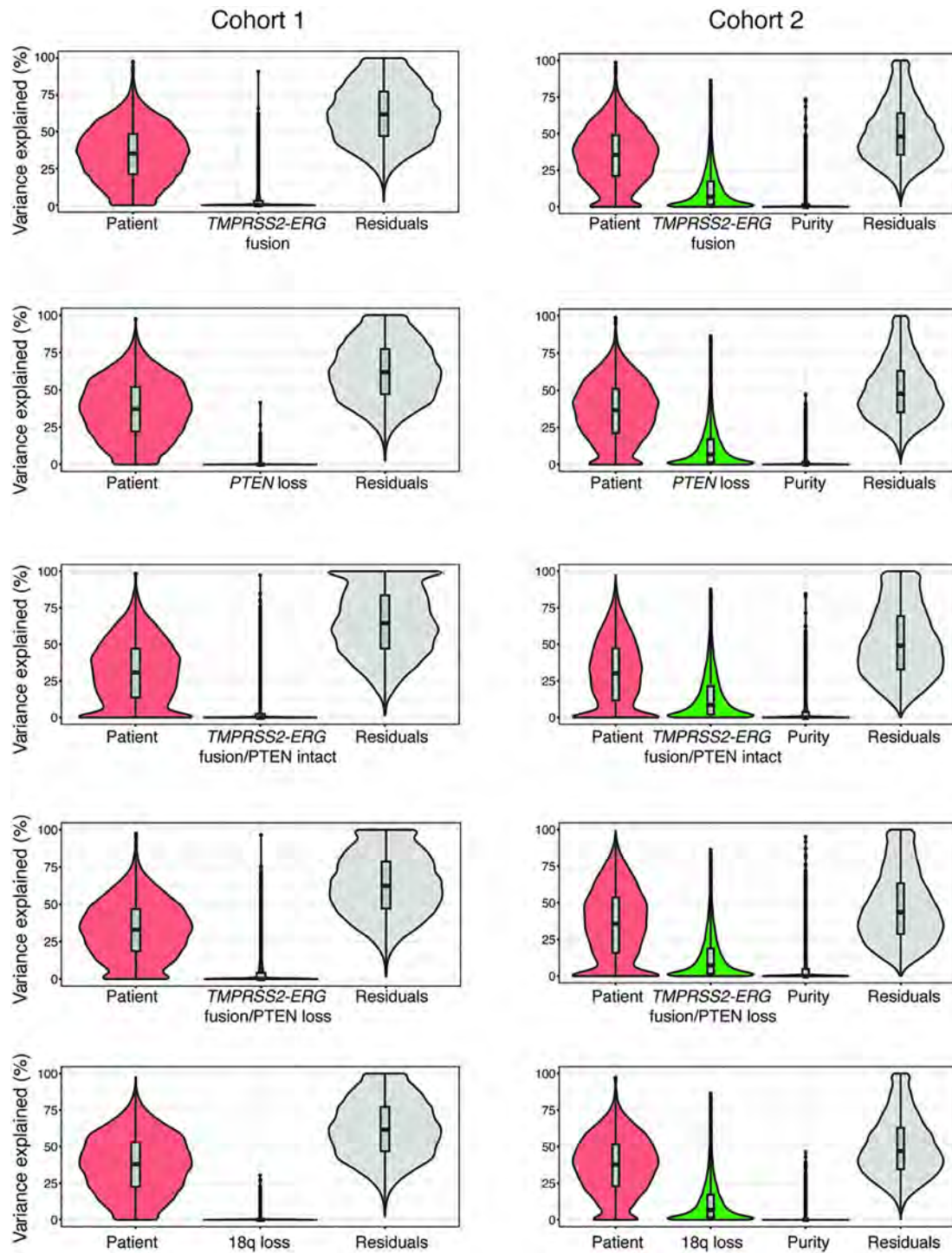


Figure 6. Decomposition of gene expression variation into patient, genotypic feature and residuals from cohort 1 (left) and cohort 2 (right). Patient, genomic features and residuals explain a median of 35%, 0% and 62% for *TMPRSS2-ERG* fusion; 37%, 0% and 62% for *PTEN* loss; 31%, 0% and 64% for *TMPRSS2-ERG* fusion/*PTEN* intact; 33%, 0% and 62% for *TMPRSS2-ERG* fusion/*PTEN* loss; and 38%, 0% and 62% for 18q loss, respectively. For cohort 2, patient, genomic features, purity and residuals can explain a median of 35%, 7%, 0% and 48% for *TMPRSS2-ERG*, 37%, 7%, 0% and 48% for *PTEN* loss; 30%, 9%, 0% and 49% for *TMPRSS2-ERG* fusion/*PTEN* intact; 36%, 7%, 0% and 44% for *TMPRSS2-ERG* fusion/*PTEN* loss; and 38%, 7%, 0% and 47% for 18q loss.

perturbed phenotype (e.g. *TMPRSS2-ERG* fusion-positive) were used to generate the gene set. Overall, *TMPRSS2-ERG* fusion calls were made in 478 cases (172 cases were positive for the fusion), *PTEN* status was determined in 427 cases (103 cases harbored *PTEN* loss-of-function mutations or copy number losses) and

18q status was called in 416 cases (with 61 cases harboring 18q losses). After determining the DEGs for each genotype, we selected the top 150 up-regulated genes for each gene set, as the average number of genes across the mSigDB Hallmark gene sets is also 150. The genes comprising these signatures

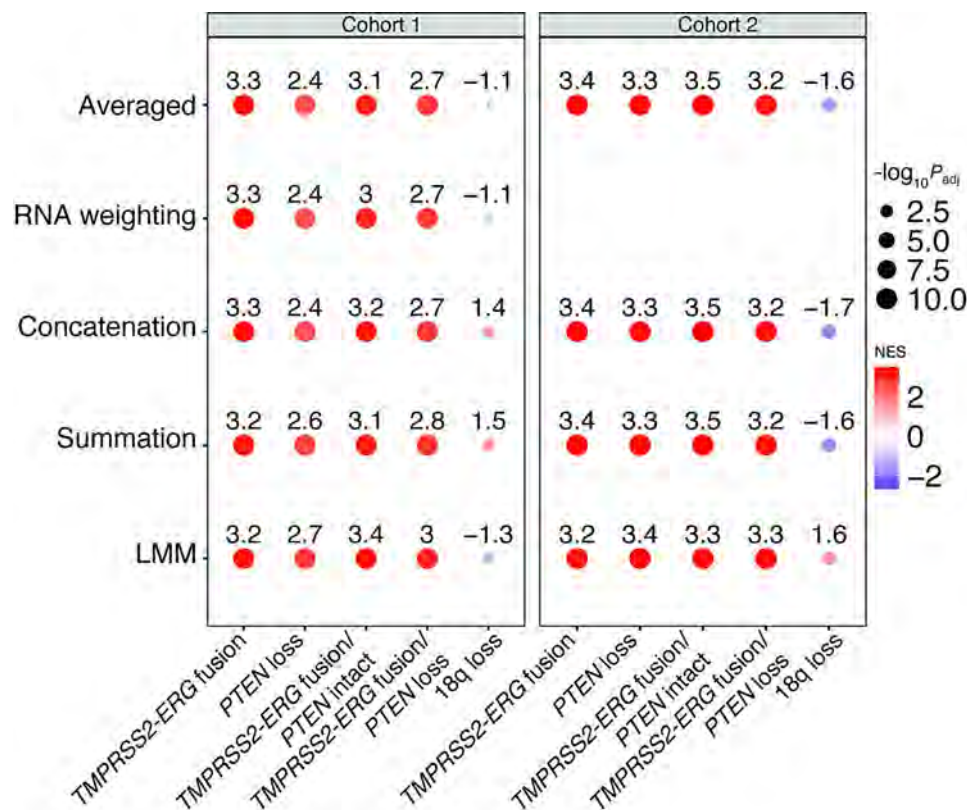


Figure 7. Bubble plot illustrating the NES, colored from low (blue) to high (red) and $-\log_{10}$ adjusted P-value (size of the dot) derived from GSEA of TMPRSS2-ERG fusion, PTEN loss, TMPRSS2-ERG fusion/PTEN intact, TMPRSS2-ERG fusion/PTEN loss, and chr18q loss using differential expression analysis outputted from counts averaging, RNA weighting, concatenation, summation and LMM.

are provided in [Supplementary Table S11](#) available online at <http://bib.oxfordjournals.org/>.

Finally, we performed gene set enrichment analysis (GSEA) to assess the sensitivity of each aggregation approach for cohorts 1 and 2 based on known alteration status to each corresponding TCGA PRAD-derived gene set. Employing normalized enrichment score (NES) as a measure of sensitivity, the five methods produced a range of NES that was highly dependent on genomic alteration. As depicted in [Figure 7](#), GSEA of TMPRSS2-ERG fusion/PTEN intact produced the highest and statistically significant NES for both cohorts, ranging from 3.1 to 3.4 (cohort 1) and 3.3 to 3.5 (cohort 2). Consistent with its ability to block for heterogeneous variables, LMM resulted in the highest statistically significant NES for three out of four alterations in cohort 1 and for two out of four alterations in cohort 2. Despite PTEN loss showing the fewest number of DEGs for both cohorts ([Table 1](#)), the NES for PTEN loss was greatest for LMM in both cohorts. As a negative control, 18q did not approach the biologically significant threshold of $NES \pm 2$ in either cohort.

Discussion

With the increasing recognition of tumor heterogeneity as a significant prognostic factor for patient outcome [3, 8, 26], experiments that account for tumor heterogeneity show promise to improve the statistical power of differential gene expression analysis. As a practical matter, the more frequently a tumor is measured, within-tumor variance for particular genes can

indicate relative importance toward salient phenotypes and genotypes. Any intra- and inter-tumoral transcriptomic variance could be assessed using statistical models, such as a LMM, which incorporate random and fixed effects for repeated measures and heterogeneity, respectively. However, the benefits to experimental designs such as these have yet to be fully explored, especially in the context of other aggregation methods that collapse multi-region samples into a single sample. Therefore, we investigated herein the impact of four methods for aggregation of RNA-seq counts and compared them to linear mixed modeling for detecting DEGs related to the important PCA genotypes of TMPRSS2-ERG fusion, PTEN loss and chr18q loss.

These three target genotypes range in frequency and relevance in PCa, with TMPRSS2-ERG fusion generally being a very early event in PCa tumorigenesis, followed by PTEN loss and 18q loss [18, 27]. This is reflected in part by the clonal or subclonal nature of these events, the frequent co-occurrence of TMPRSS2-ERG and PTEN loss in the same tumor and the potential ability to isolate pure populations of tumor cells by using laser capture microdissection based on stains detecting these events. Due to microdissection in cohort 1, the specificity of transcriptome underpinning the phenotypic differences between TMPRSS2-ERG fusion-positive and -negative tumors between cases was so great that all aggregation methods performed comparably ([Figure 5A](#)). In contrast, the loss of chromosome 18q, which is likely a passenger event, did not produce robust DEGs and GSEA NES from any methods. For PTEN loss in both cohorts, the DEGs derived by a LMM were

substantially different than all other methods (Figure 5B). The simplest explanation for this observation is that a per-patient classification of a genotype such as 'PTEN loss' demonstrates more heterogeneity across the tumor despite apparent 'spill over' from TMPRSS2-ERG fusion, as PTEN-intact and PTEN-reduced tumor cells harbored strikingly different inter-lesion and intra-lesion gene expression profiles. Additional evidence from variance decomposition supports the notion that majority of gene expression variation is not due to genomic changes but is attributable to differences between patients or other unaccounted variables.

Indeed, when we used GSEA NES as a metric for detection sensitivity (Figure 7), DEGs associated with PTEN loss benefitted most from an LMM analysis that was capable of modeling the intra-patient or intra-lesion variability as a blocking effect. In addition, the co-occurrence of relatively homogeneous TMPRSS2-ERG fusion and PTEN loss within each focus from cohort 1 created additional challenges during the PTEN loss analysis for the four aggregation methods but were significantly reduced by LMM. Thus, inclusion of the foci variability using models such as LMM significantly increased the sensitivity and specificity of an analysis to arrive at per-patient phenotypes. While we included the addition of PTEN status as a parameter for TMPRSS2-ERG status to mitigate the strong effects of TMPRSS2-ERG fusion on transcription, doing so in standard practice would require its status known a priori, and patient-level transcriptomic studies often lack genomic and histologic annotations, and thus the inclusion of such parameters would not be always practical.

Compared to cohort 1, which was comprised of LCM tumor foci, cohort 2 was intrinsically more heterogeneous within-foci. The approach used for sampling prostate tumors used in cohort 2 more closely mirrors that used in clinical settings, whereby a large region expected to represent the most aggressive region is generally selected for molecular profiling [28]. Thus, when more than one area was selected in this manner, the variance in DEG profiling the entire patient was distinctive with an LMM, whereas averaging/summing counts or FASTQ concatenation arrived at similar profiles. However, when we considered NES as a basis for this comparison, generalized to all three target genotypes, no single method outperformed the other. Nonetheless, the advantage to LMM is the use of blocking factors for fixed effects and the ability to assess within-lesion or within-patient heterogeneity against one or more salient phenotypes.

Conclusions

The aggregation of multiple transcriptomes from a single tumor to estimate per-patient phenotypes will vary in its reliability as a direct function of heterogeneity. Tumors often harbor heterogeneous alterations within the same geographic region, resulting in vastly different gene expression profiles between nearly adjacent tumor cells. Experimental designs that include tumor resampling have improved the power to detect driver genotypes and the variance of gene expression or biomarker profiles related to phenotype. Although we found that a LMM was a robust aggregation method that performed consistently well in analyses with homogeneous and heterogeneous phenotypes within individual tumors, patient-level summarization methods, including RNA-seq count averaging and summation, showed benefit for detecting gene expression changes that were associated with more homogeneous genotypes. Consequently, the method of per-patient gene expression summarization is highly context-dependent on any salient target genotypes and the tolerance of variability for formulating robust conclusions.

Key Points

- Within- and between-lesion tumor heterogeneities can exist within the same patient. Strategies to sampling the same tumor multiple times can improve detection of intratumoral phenotypic variability.
- The performance of different methods to aggregate multiple transcriptomes into per-patient summaries was highly dependent upon the phenotype of interest.
- Dominant or clonal transcriptomic signals in PCA, such as the TMPRSS2-ERG fusion, were robust to multiple aggregation methods, although subclonal alterations, such as PTEN loss, were more conducive to LMMs.
- Comparison of different aggregation methods to benchmarked gene expression profiles can identify unappreciated sources of within-patient transcriptomic heterogeneity based on defined genomic alterations.

Data availability

The data underlying this article are available in: dbGaP at <https://www.ncbi.nlm.nih.gov/gap/> and can be accessed with accession ID phs001938.v2.p1; SRA at <https://www.ncbi.nlm.nih.gov/sra> and can be accessed with accession ID PRJNA579899; and NCI GDC at <http://portal.gdc.cancer.com> and can be accessed with the project ID TCGA-PRAD.

Code availability

All codes used in the preparation of this work are available at <https://github.com/CBIIT/lgcp>.

Supplementary data

Supplementary data are available online at <https://academic.oup.com/bib>.

Data availability

The data underlying this article are available in the Database of Genotypes and Phenotypes (dbGaP) and Sequence Read Archive (SRA) at <https://www.ncbi.nlm.nih.gov/gap/> and <https://www.ncbi.nlm.nih.gov/sra>, respectively, and can be accessed with PRJNA579899 (SRA) and phs001938.v2.p1 (dbGaP).

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EZH2 inhibition activates a dsRNA-STING-interferon stress axis that potentiates response to PD-1 checkpoint blockade in prostate cancer

Katherine L. Morel^{1,22}, Anjali V. Sheahan^{1,22}, Deborah L. Burkhart¹, Sylvan C. Baca², Nadia Boufaied³, Yin Liu⁴, Xintao Qiu², Israel Cañadas⁵, Kevin Roehle⁶, Max Heckler⁶, Carla Calagua⁷, Huihui Ye⁸, Constantia Pantelidou², Phillip Galbo⁹, Sukanya Panja¹⁰, Antonina Mitrofanova^{10,11}, Scott Wilkinson¹², Nichelle C. Whitlock¹², Shana Y. Trostel¹², Anis A. Hamid², Adam S. Kibel¹³, David A. Barbie², Atish D. Choudhury², Mark M. Pomerantz², Christopher J. Sweeney², Henry W. Long², David J. Einstein¹⁴, Geoffrey I. Shapiro², Stephanie K. Dougan⁶, Adam G. Sowalsky¹², Housheng Hansen He⁴, Matthew L. Freedman², Steven P. Balk¹⁴, Massimo Loda¹⁵, David P. Labbé^{3,16}, Brian M. Olson¹⁷ and Leigh Ellis^{1,18,19,20,21} ✉

Prostate cancers are considered to be immunologically ‘cold’ tumors given the very few patients who respond to checkpoint inhibitor (CPI) therapy. Recently, enrichment of interferon-stimulated genes (ISGs) predicted a favorable response to CPI across various disease sites. The enhancer of zeste homolog-2 (EZH2) is overexpressed in prostate cancer and known to negatively regulate ISGs. In the present study, we demonstrate that EZH2 inhibition in prostate cancer models activates a double-stranded RNA-STING-ISG stress response upregulating genes involved in antigen presentation, Th1 chemokine signaling and interferon response, including programmed cell death protein 1 (PD-L1) that is dependent on STING activation. EZH2 inhibition substantially increased intratumoral trafficking of activated CD8⁺ T cells and increased M1 tumor-associated macrophages, overall reversing resistance to PD-1 CPI. Our study identifies EZH2 as a potent inhibitor of antitumor immunity and responsiveness to CPI. These data suggest EZH2 inhibition as a therapeutic direction to enhance prostate cancer response to PD-1 CPI.

Prostate cancer (PCa) is currently the most commonly diagnosed noncutaneous malignancy and the second most common cause of cancer death among men in the United States¹. Unfortunately, metastatic castration-resistant PCa (mCRPC) still remains incurable, despite recent advances in therapy options for these patients. Although CPI can generate dramatic responses in about 15–20% of patients with a number of cancer types, including melanoma, kidney and bladder cancer, this occurs in approximately 5% of PCa patients. Resistance toward CPI in PCa patients is thought to be related to low tumor immunogenicity and an immunosuppressive tumor microenvironment.

EZH2 is the methyltransferase catalytic subunit of the polycomb repressive complex 2 (PRC2) that trimethylates Lys27 of histone H3 (H3K27me3) to promote transcriptional repression². Increased expression and activity of EZH2 are important contributors to PCa initiation and progression^{3,4}. EZH2 can negatively regulate ISGs, including Th1-type chemokines, immune checkpoint molecules like PD-L1 and major histocompatibility complex (MHC) expression in multiple tumor cell types^{5,6}. More recently, it had been shown that silencing of MHC-I antigen-processing genes was mediated by coordinated transcriptional silencing by PRC2 (ref. 7). Likewise, diffuse large B-cell lymphomas harboring EZH2-activating mutations

¹Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA, USA. ²Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. ³Cancer Research Program, Research Institute of the McGill University Health Centre, Montréal, Canada. ⁴Department of Medical Biophysics, University of Toronto and Princess Margaret Cancer Center University Health Network, Toronto, Canada. ⁵Blood Cell Development and Function Program, Fox Chase Cancer Center, Philadelphia, PA, USA. ⁶Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, MA, USA. ⁷Department of Medicine, Hematology-Oncology Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA. ⁸Department of Pathology, University of California at Los Angeles, Los Angeles, CA, USA. ⁹Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY, USA. ¹⁰Department of Health Informatics, Rutgers School of Health Professionals, Newark, NJ, USA. ¹¹Rutgers Cancer Institute of New Jersey, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA. ¹²Laboratory of Genitourinary Cancer Pathogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA. ¹³Department of Urology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. ¹⁴Hematology and Oncology Division, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA. ¹⁵Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA. ¹⁶Division of Urology, Department of Surgery, McGill University, Montréal, Canada. ¹⁷Departments of Hematology and Medical Oncology and Urology, Emory University School of Medicine, Atlanta, GA, USA. ¹⁸Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. ¹⁹Cancer Program, The Broad Institute of MIT and Harvard, Cambridge, MA, USA. ²⁰Division of Medical Oncology, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA. ²¹Cedars-Sinai Samuel Oschin Comprehensive Cancer Institute, Los Angeles, CA, USA. ²²These authors contributed equally: Katherine L. Morel, Anjali V. Sheahan. ✉e-mail: Leigh.Ellis@cshs.org

exhibit low expression of MHC-I and MHC-II molecules⁶. Together, tumors with increased function of EZH2 often display immunosuppressive tumor microenvironments and immunotherapy resistance.

Dysfunction of epigenetic regulation within a cancer cell, including effects mediated by EZH2, DNA methyltransferases, histone deacetylases, BET bromodomains and lysine-specific demethylase 1 have proven to be critical mediators of acquired tumor immune escape. Subsequent inhibition of these epigenetic mechanisms results in increased tumor immunity and successful combination with CPI in preclinical cancer models^{5,8–15}. Importantly, recent data from a phase Ib/II clinical trial, ENCORE-601 (NCT02437136), illustrated the power of epigenetic therapy to restore sensitivity in a subset of melanoma patients who had progressed on an inhibitor of PD-1 (ref. ¹⁶).

Targeting epigenetic mechanisms mediated by EZH2 have not been tested for their ability to induce response to CPI in PCa. In the present study, we demonstrate that chemical and genetic inhibition of EZH2's catalytic function derepresses endogenous dsRNA, leading to a notable induction of ISGs in PCa tumor cells. Activation of this dsRNA-ISG tumor cell response by EZH2 inhibition is dependent on activation of the stimulator of interferon (IFN) genes (STING) and leads to reversal of resistance to anti-PD-1 therapy in B6-MYCCaP PCa tumors in vivo. Overall, these data provide important evidence for targeting chromatin and epigenetic regulators such as EZH2 to overcome immunotherapy resistance in PCa.

Results

Using three-dimensional (3D) tumor organoids derived from a genetically engineered mouse model of PCa (GEMM) that expresses oncogenic *cMYC*¹⁷, *Ezh2* floxed alleles¹⁸ and an inducible *Cre* recombinase driven by the prostate-specific antigen (PSA) promoter¹⁹ (EMC mouse) we demonstrated that genetic or chemical inhibition of EZH2 catalytic activity resulted in diminished organoid growth (Fig. 1a,b), accompanied by a significant decrease in DNA synthesis and H3K27me3 (Fig. 1a,b). RNA-sequencing (RNA-seq) and gene set enrichment analysis (GSEA) revealed enrichment of type I (IFN- α) and II (IFN- γ) gene signatures (Fig. 1c and Extended Data Fig. 1). Interrogation of leading-edge genes related to IFN signaling from mouse PCa organoids revealed increases in expression of Th1 chemokines (*Cxcl9*, *Cxcl10* and *Cxcl11*), antigen-presentation genes (*B2m* and *Tap1*) and IFN- γ -regulated genes (*Stat1* and *CD274/Pd-l1*). The enrichment of IFN-response genes was further corroborated when master regulator (MR) analysis using MARINA was applied to both RNA-seq datasets from 1C to identify transcription factors (TFs) driving this pattern of gene expression. Overlap of both MR lists (top 200 ranked by NES, normalized enrichment score) showed that a set of 36 TFs was common in both conditions. These included important machinery for control of ISGs including *Stat1*, *Icam1*, *Ifi16*, *Sp100*, *Trim22*, *Trim29*, *Irf1*, *Irf8* and *Irf9* (Fig. 1d and Supplementary Table 1). Specifically, *Stat1* and *Irf* proteins are shown to form transcriptional machinery that regulates ISG expression²⁰.

To determine whether loss of EZH2 catalytic activity was associated with enrichment of ISGs in human PCa, a 29-gene EZH2 repression signature was derived using differentially expressed (DE) genes after chemical inhibition of EZH2 from Fig. 1c (Supplementary Table 2) applied to independent human PCa RNA-seq datasets (Supplementary Table 3). Previously, an independent EZH2 repression signature was reported²¹ and, although both signatures were mutually exclusive, they were significantly correlated with each other (Extended Data Fig. 1). Of importance, EZH2 activity was not altered because of changes in *EZH2* gene expression (Extended Data Fig. 1). On quartile distribution of patients, differential gene expression between quartile 4 (lowest EZH2 activity) and quartile 1 (highest EZH2 activity) validated our in vitro murine results by indicating that patients with the lowest EZH2 activity were enriched for type

I/II IFN-response genes (Fig. 1e and Extended Data Fig. 2). In line with our in vitro data, low EZH2 activity in PCa patients was also associated with increased expression of Th1 chemokines (*CXCL10* and *CXCL11*), antigen-presentation genes (*B2M* and *HLA-A*) and IFN- γ -regulated genes (*STAT1* and *IRF9*) (Extended Data Fig. 2).

Recently, epigenetic targeted therapies were shown to induce ISGs by derepression of dsRNA^{8,10,11}. This mechanism, termed 'viral mimicry', involves the re-expression of dormant transposable elements after treatment with epigenetic therapies^{8,10,11}. This instructs the cancer cell to adapt and respond as if infected by an exogenous virus and mount an innate immune defense via induction of dsRNA sensor machinery and ISGs²². Treatment with EZH2 inhibitors significantly induced total intracellular levels of dsRNA in murine and human 3D PCa organoids (Fig. 2a), and in murine PCa tissue in vivo (Fig. 2b). Using immunofluorescence, human prostatectomy samples with >5% tumor PD-L1 expression (PD-L1 high) exhibited low H3K27me3 levels and increased dsRNA levels. Adversely, patient samples with <5% tumor PD-L1 positivity (PD-L1 low) demonstrated high H3K27me3 expression and low dsRNA expression (Fig. 2c,d and Supplementary Table 4). In accordance with these data we also found increased expression of innate immune receptors including STING and toll-like receptor 3 (TLR3) (Fig. 4a and Extended Data Fig. 2), and dsRNA sensors RIG-I and MDA5 (Extended Data Fig. 2) occurred in patient samples with low EZH2 activity. Also enriched in patients with low EZH2 activity were a novel subclass of endogenous retroviral sequences (ERVs) contained in genes co-regulated by STAT1 and EZH2 called stimulated prime antisense retroviral coding sequences (SPARCS). SPARCS genes housing ERVs were demonstrated to induce an innate immune response on expression (Extended Data Fig. 2)²³. We next overlaid both mouse and human IFN- α/γ DE genes from Fig. 1 and identified an overall 97-gene IFN gene signature (Extended Data Fig. 3). It is interesting that, although SPARC gene expression is shown to be induced by IFN treatment, only 1 SPARC gene (*IFI44L*) appeared in our 97-gene IFN gene signature. It was also observed that our gene set did include enrichment of *IFI44*, *IFI27* and *OASL*, which are type I IFN-stimulated 'viral mimicry' genes shown to be upregulated by DNA methyltransferase inhibition^{24,25}. Moreover, there was an enrichment of biological and molecular Gene Ontology terms, including innate immune response, dsRNA binding and Tap binding (Extended Data Fig. 3).

To further understand mechanisms underlying the observed increased expression of ISGs by EZH2 inhibition, we utilized published human PCa patient and cell-line chromatin immunoprecipitation (ChIP)-seq datasets^{26–28}. In human patient samples, our 97 ISGs did not display overall enrichment for H3K27me3, indicating that no direct repression of these genes is regulated by EZH2 catalytic activity (Fig. 3a). Instead, we found that these genes contain enrichment of H3K27ac and open chromatin regions (Fig. 3a and Extended Data Fig. 3). Likewise, we observed, in LNCaP cell lines treated with genetic or chemical inhibition of EZH2, enrichment of IFN- α/γ gene sets (Extended Data Fig. 3). Furthermore, EZH2 inhibition in LNCaP cells exhibited a dramatic increase in H3K27ac at these ISGs (Fig. 3b). As expression of these ISGs was dramatically increased after treatment with epigenetic therapies, it suggests that these genes are primed for rapid activation. As we had already observed a significant induction of tumor cell dsRNA levels after EZH2 inhibition (Fig. 2), we further analyzed H3K27me3 and H3K27ac locations in LNCaP cells. We observed a total of 302 genes that contained ERVs within their 3'-UTR (Supplementary Table 5) which, on EZH2 inhibition, resulted in direct loss of H3K27me3 and concurrent gain of H3K27ac (Fig. 3c,d). These data indicate that direct derepression of ERVs catalyzes the increased expression of ISGs after EZH2 inhibition.

As stated, multiple regulators that drive viral defense mechanisms, including STING, were upregulated in PCa patient tumors

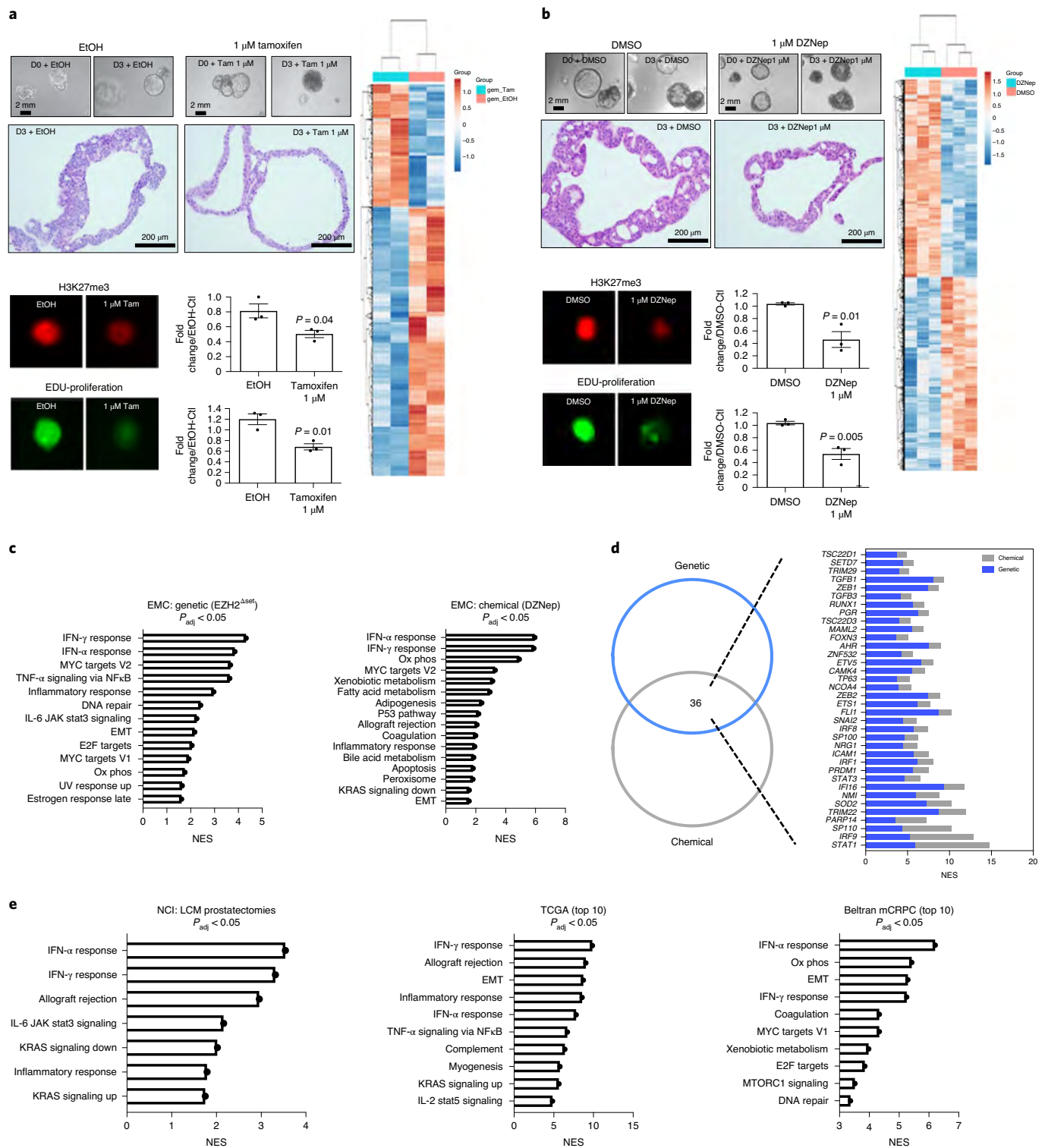


Fig. 1 | EZH2 negatively regulates type I/II ISGs in PCa. **a, b**, EZH2 catalytic activity in EMC PCa mouse organoids inhibited by genetic or chemical inhibition of EZH2 catalytic activity. Chemical and genetic EZH2 inhibition decreases H3K27me3, DNA replication and gene expression. Gene expression differentials were generated by RNA-seq. H3K27me3 and EDU (2'-deoxy-5-ethynyluridine) signal intensity were analyzed by ImageStream flow cytometry. *P* values were generated using a two-tailed, unpaired Student's *t*-test with Welch's correction. Data were generated from *n* = 3 independent experiments and displayed as mean $\pm$ s.e.m. **c**, GSEA revealing enrichment of type I/II IFN gene signatures in mouse PCa organoids after EZH2 inhibition. Ox phos, oxidative phosphorylation. **d**, Master regulation analysis of RNA-seq data from 1C (overlap of top 200 TFs ranked by NES) enriched for TFs that regulate type I/II IFN-response genes. **e**, GSEA reveals enrichment of type I/II IFN gene signatures in human PCa patients with the lowest EZH2 activity. Sample numbers used are for NCI-laser capture dissection prostatectomies (*n* = 21 samples), for the Cancer Genome Atlas (TCGA; *n* = 204 samples) and for Beltran mCRPC (*n* = 17 samples).

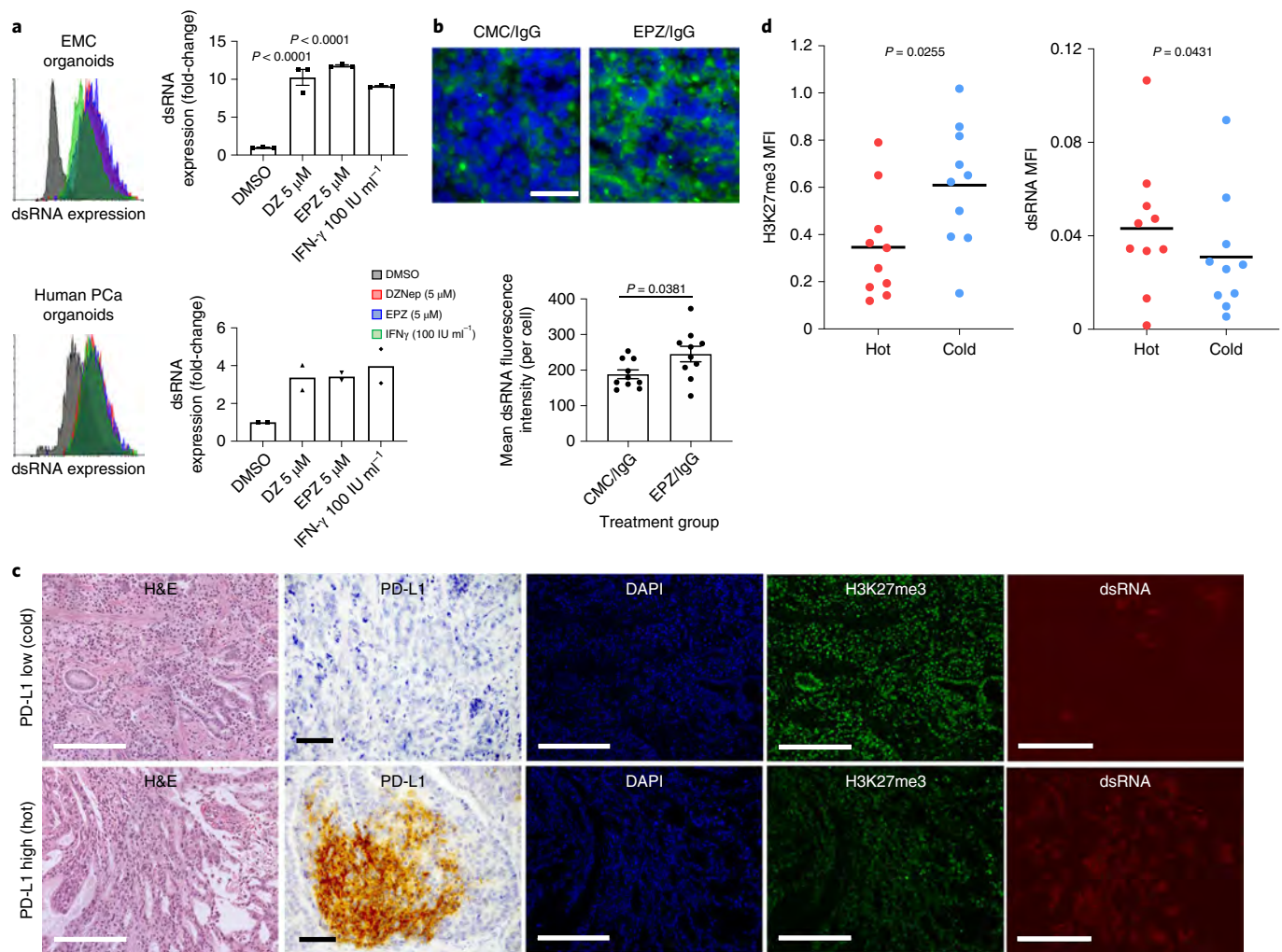


Fig. 2 | EZH2 inhibition derepresses endogenous dsRNA. a,b, Inhibition of EZH2 induces expression of dsRNA in mouse and human PCA organoids (**a**) and PCA tissue in vivo (**b**). Data for **a** were generated from $n=3$ (EMC mouse) or $n=2$ (human) independent experiments and $n=10$ independent mice for **b**. Scale bars, 25 μ m. P values for **a** were generated using a one-way ANOVA, Tukey's multiple-comparison test for **a** and a two-tailed, unpaired Student's t -test with Welch's correction for **b**, and displayed as mean $\pm$ s.e.m. **c,d**, Histopathology analysis of human prostatectomy samples indicating tumors with $>5\%$ PD-L1 tumor-positive staining (PD-L1 high, $n=10$ patients) exhibits low H3K27me3 and high dsRNA staining. Tumors with $<5\%$ tumor-positive staining (PD-L1 low, $n=10$ patients) exhibit high H3K27me3 and low dsRNA staining (**c**). **d**, Quantification of MFI for H3K27me3 and dsRNA staining performed in **c**. MFI, mean fluorescence intensity. Scale bars, 100 μ m.

with low EZH2 activity (Fig. 4a and Extended Data Fig. 2). To validate a molecular link between endogenous tumor cell dsRNA and ISG upregulation by EZH2 inhibition, we used both chemical and genetic methods to attenuate STING function in two independent murine PCA cell lines. These cell lines either overexpressed *MYC* or harbored loss of *Pten*. STING was expressed in both cell lines (Fig. 4b) and loss of STING activity did not affect the induction of dsRNA by EZH2 inhibition, but did significantly suppress the ability of EZH2 inhibition to induce MHC-I and PD-L1 expression (Fig. 4c and Extended Data Fig. 4). These results demonstrate the importance of STING for upregulation of genes critical for increasing anti-cancer immunogenicity in response to EZH2 inhibition.

We next proposed that EZH2 inhibition would sensitize PCA tumors in vivo to PD-1 CPI. Support of this proposition was indicated by human PCA samples with low EZH2 activity being significantly enriched for two gene signatures demonstrated to predict response to CPI. These are a MImm score²⁹ and a T-cell-inflamed gene signature³⁰ (Extended Data Fig. 5). Using a B6-HiMYC PCA transgenic tissue transplant model¹⁷, EZH2 inhibition (EPZ) or

PD-1 CPI did not display antitumor activity individually; however, combination treatment produced a significant therapeutic effect without extensive toxicity (Fig. 4b and Extended Data Fig. 5). EZH2 inhibition in vivo was also associated with loss of tumor H3K27me3 levels and increased tumor expression of PD-L1 (Extended Data Fig. 5 and Fig. 5b). Also noted was a reduction of PD-1 in tumor-infiltrating CD8⁺ but not CD4⁺ T cells (Extended Data Fig. 7). We further validated the ability of EZH2 inhibition to increase gene and protein expression of PD-L1 in murine and human PCA models (Fig. 5c and Extended Data Fig. 5). In addition, correlation analysis of patient PCA samples indicated that the patients with the lowest EZH2 activity had substantial enrichment of PD-L1 gene expression (Extended Data Fig. 5). As the significant induction of PD-L1 expression was dependent on STING in PCA models after treatment with EZH2 inhibitors, we sought to determine whether increased PD-L1 was functionally relevant. For this, we used an in vitro cytotoxicity assay. Using our murine PCA cell lines on a B6 background with either wild-type PD-L1 or knockout (KO) PD-L1 (Extended Data Fig. 6), we pretreated tumor

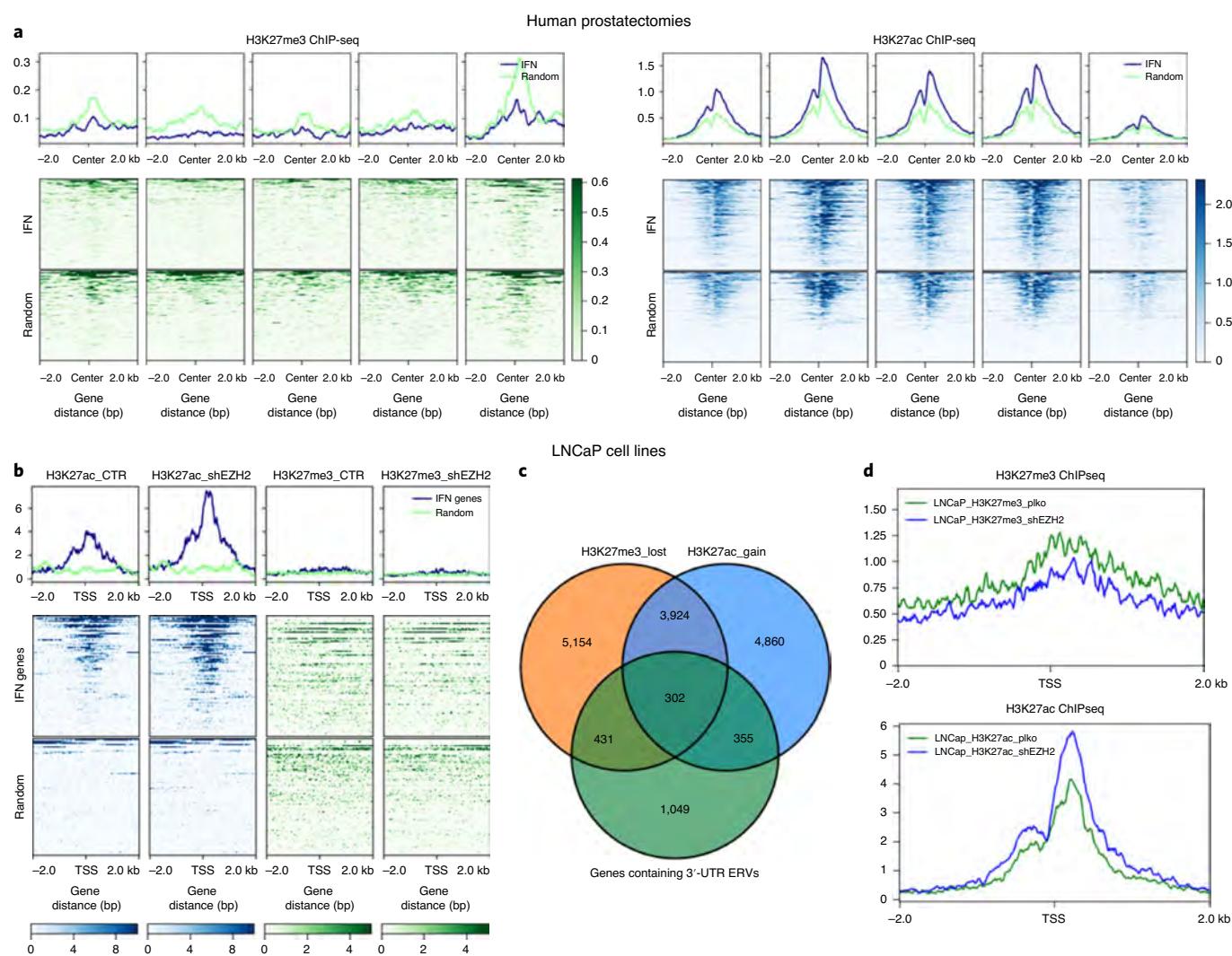


Fig. 3 | ISGs are poised for activation by EZH2 inhibition. **a**, Heatmaps of normalized H3K27me3 and H3K27ac signaling intensities of 97 ISGs from 5 independent patient prostatectomy samples. Each patient sample was analyzed once for each histone mark. The blue line represents the intensity signal of the 97 ISGs described in Extended Data Fig. 3 and the green line represents the signal intensity of randomly selected genes. **b**, LNCaP cell lines treated with nonspecific or EZH2-targeted short hairpins indicating, on EZH2 knockdown ISG display, no direct association with H3K27me3, but accumulation of increased H3K27ac. **c**, Venn analysis of LNCaP cell lines indicating a total of 302 genes that concurrently lose H3K27me3 and gain H3K27ac that also contain ERV sequences within their 3'-UTR that are upregulated after EZH2 knockdown. **d**, Average H3K27me3 and H3K27ac peak intensity associated with the promoter regions of the 302 genes identified in **c**. Data for **b–d** were generated from previously published ChIP-seq data (accession no. GSE107780). There was a single replicate for each histone ChIP and the treatment condition was provided and used to generate data.

cells with dimethylsulfoxide (DMSO) or an EZH2 inhibitor before co-incubation with murine splenocytes on an FVB background and evaluated immune cytotoxicity (Fig. 5d). Our results clearly demonstrated that inhibition of EZH2 activity resulted in a significant loss of immune-mediated cytotoxicity, which was dependent on tumor cell upregulation of PD-L1. Strikingly, immune-mediated cytotoxicity was restored in EZH2 inhibitor-treated cell lines by the addition of a PD-1 antibody, and this combination effect was also dependent on tumor PD-L1 upregulation (Fig. 5d).

Further assessment of the tumor microenvironment revealed that EZH2 inhibition and combination groups showed increased accumulation of CD4⁺ and CD8⁺ T cells (Fig. 6a). No change to intratumoral frequency of regulatory T cells (T_{reg} cells) was observed (Extended Data Fig. 7). Although both CD4⁺ and CD8⁺ T-cell trafficking was increased, only CD8⁺ T cells were significantly activated in PD-1 CPI and combination groups (Fig. 6b). With this observed increase in T-cell trafficking, we further interrogated whether EZH2

inhibition could increase Th1 chemokine expression in tumor cells. We treated both B6-MYCCaP and Pten KO murine PCa cell lines with the EZH2 inhibitors DZNep and EPZ. Overall, inhibition of EZH2 activity resulted in a drastic upregulation of Th1 chemokines interleukin (IL)-2, IL-12 p40p70, IL-12 p70 and tumor necrosis factor α (TNF- α). There was no observed upregulation of IFN- γ in cell lines by either EZH2 inhibitor (Fig. 6c and Extended Data Fig. 6). Analysis of gene expression data from patient tumor samples further validated that low EZH2 activity was most associated with increased expression of Th1 cytokines. Th2 and Th17 cytokines³¹ were less represented in tumors with low EZH2 activity (Extended Data Fig. 6). Additional evaluation of the microenvironment also showed no significant alterations in the frequency of myeloid or granulocytic myeloid-derived suppressor cells (MDSCs; Extended Data Fig. 7); however, significant increases of intratumoral M1 tumor-associated macrophages (TAMs), with concurrent loss of M2 TAMs, were observed in EPZ, PD-1 CPI and combination-treated groups

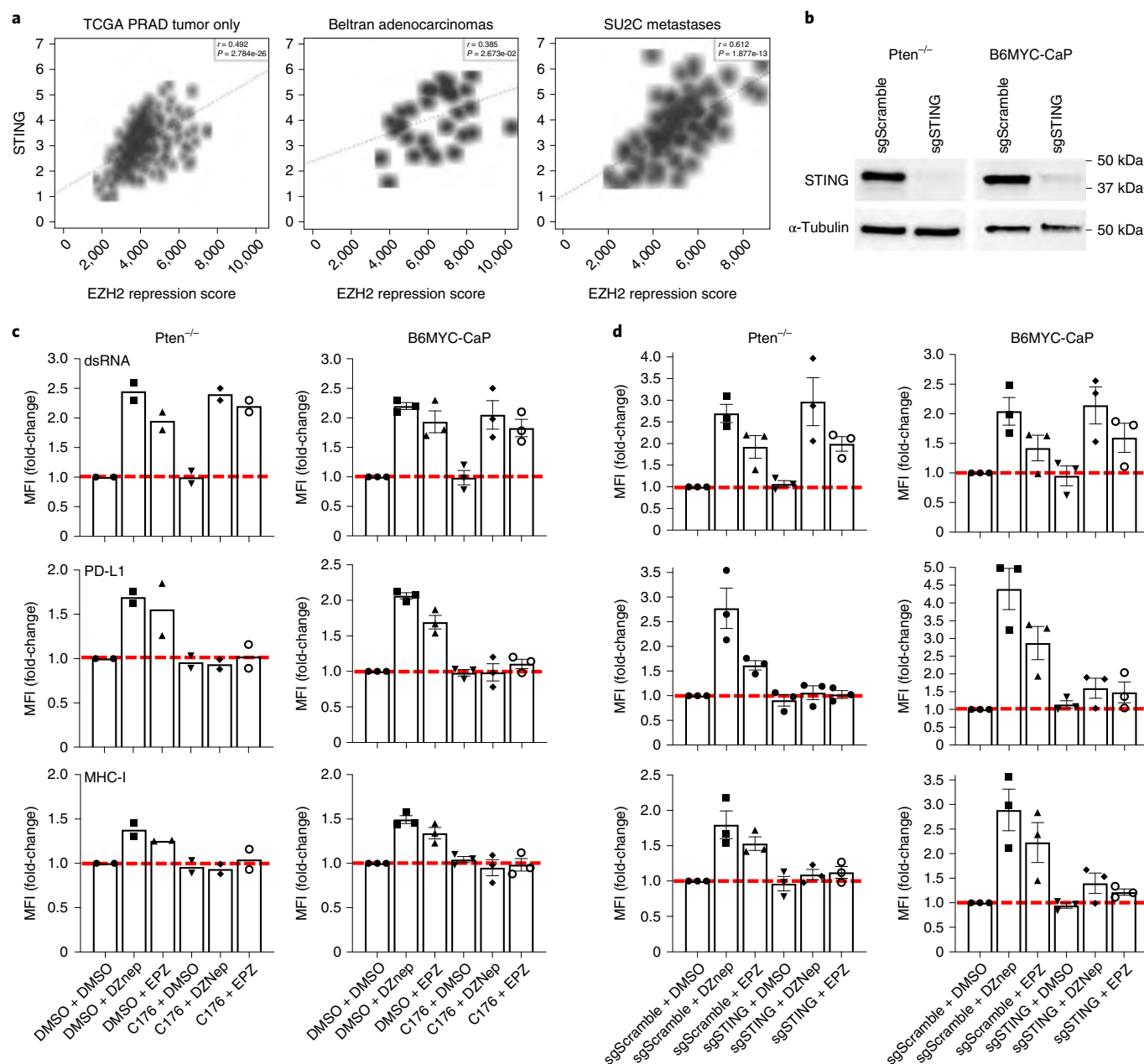


Fig. 4 | Activation of ISGs is STING dependent. **a**, Human PCa gene expression data demonstrating increased expression of the dsRNA sensor STING significantly correlated with low EZH2 catalytic activity. Sample numbers used are $n = 118$ samples for SU2C, $n = 408$ samples for TCGA and $n = 33$ samples for Beltran adenocarcinomas. **b**, Cropped western blots showing validation of STING KO in Pten^{-/-} and B6-HiMYC-CaP mouse PCa cell lines. Western blot analysis was performed once ($n = 1$ genotype/treatment condition). **c,d**, Chemical (**c**) or genetic (**d**) inhibition of STING demonstrating that EZH2 inhibitor activation of IFN-stimulated molecules, PD-L1 and MHC-I proteins, are dependent on STING activity. Data were generated using flow cytometry analysis from $n = 3$ independent experiments and displayed as mean $\pm$ s.e.m. P values can be seen in Extended Data Fig. 4 and were generated using a two-tailed, unpaired Student's t -test with Welch's correction. MFI, mean fluorescence intensity. Note that data from Pten KO cells in **c**, treated with C176 to inhibit STING, were generated from $n = 2$ independent experiments and displayed as the mean value. All values are presented as fold-change normalized to control conditions (DMSO + DMSO or sgScramble + DMSO). The red line indicates the normalized value of the control.

(Fig. 7). Overall, combination treatment provided the most significant increase in the M1:M2 TAM ratio. In concordance with our data, it was shown that inhibition of PRC1 in double-negative PCa models resulted in increased T-cell tumor infiltration and decreased immunosuppressive cells, and provided superior therapeutic benefit when combined with CPI³², implicating polycomb complexes as important mediators for immunotherapy response in PCa.

Discussion

Identifying mechanisms driving resistance toward CPIs in PCa patients remain a critical requirement. Although progress has been made describing molecular events that increase response, including patients with DNA-damage repair pathway defects^{33,34}, biallelic loss of CDK12 (ref. ³⁵), recycling of PD-L1 in patients lacking a common mutation in speckle-type POZ protein (SPOP)³⁶ and inhibition

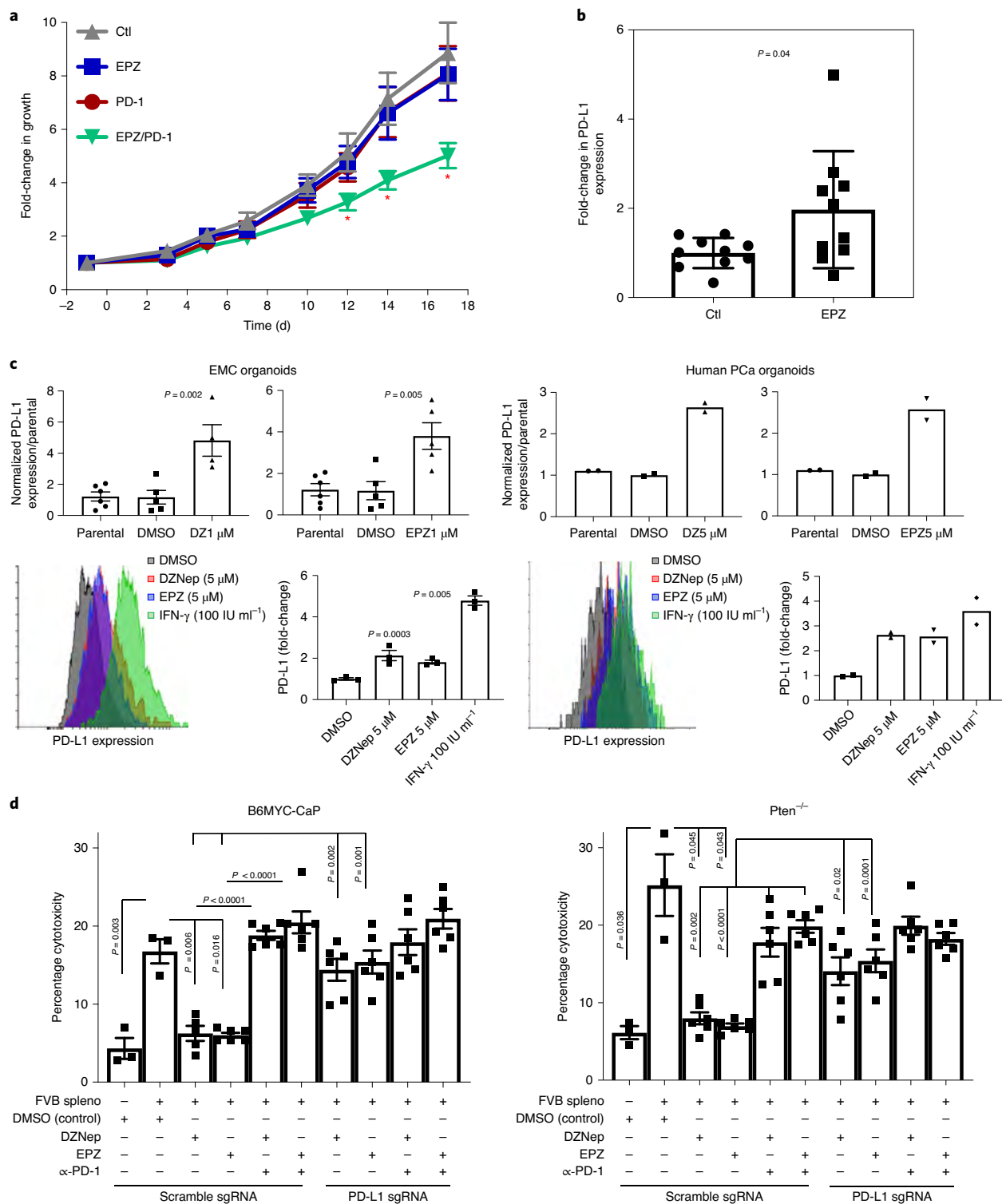


Fig. 5 | EZH2 inhibition sensitizes murine prostate tumors to PD-1 CPI and is dependent on tumor PD-L1 activation. **a**, EZH2 inhibition combines with PD-1 blockade to significantly inhibit prostate tumor progression in vivo. Each group consists of $n = 10$ mice. The P values were generated using a multiple Student's t -test. Asterisks represent: day 12, $P = 0.043$; day 14, $P = 0.017$; day 18, $P = 0.011$. **b**, EZH2 inhibition increases PD-L1 tumor expression ($n = 10$ mice/tumors per treatment group). The P values were generated using a two-tailed, unpaired Student's t -test with Welch's correction and displayed as mean $\pm$ s.e.m. Ctl, control. **c**, Mouse and human PCa organoids treated for 96 h with EZH2 inhibitors notably upregulating PD-L1 gene and protein expression. Data were generated from $n = 3$ (EMC mouse, left) or $n = 2$ (human, right) independent experiments and displayed as mean or mean $\pm$ s.e.m. The P values were generated using a one-way ANOVA, Tukey's multiple-comparison test and represent DMSO versus treatment. **d**, Upregulation of tumor PD-L1 expression functionally assessed using an in vitro cytotoxicity assay. Inhibition of immune cell cytotoxicity after EZH2 inhibition was rescued by PD-1 blockade. This rescue is dependent on tumor PD-L1 upregulation. Data were generated from $n = 6$ independent experiments and displayed as mean $\pm$ s.e.m. The P values were generated using a two-tailed, unpaired Student's t -test with Welch's correction. B6MYC-CaP and Pten $^{-/-}$ denote the murine 2D cell lines generated from the genetically engineered mouse models of PCa.

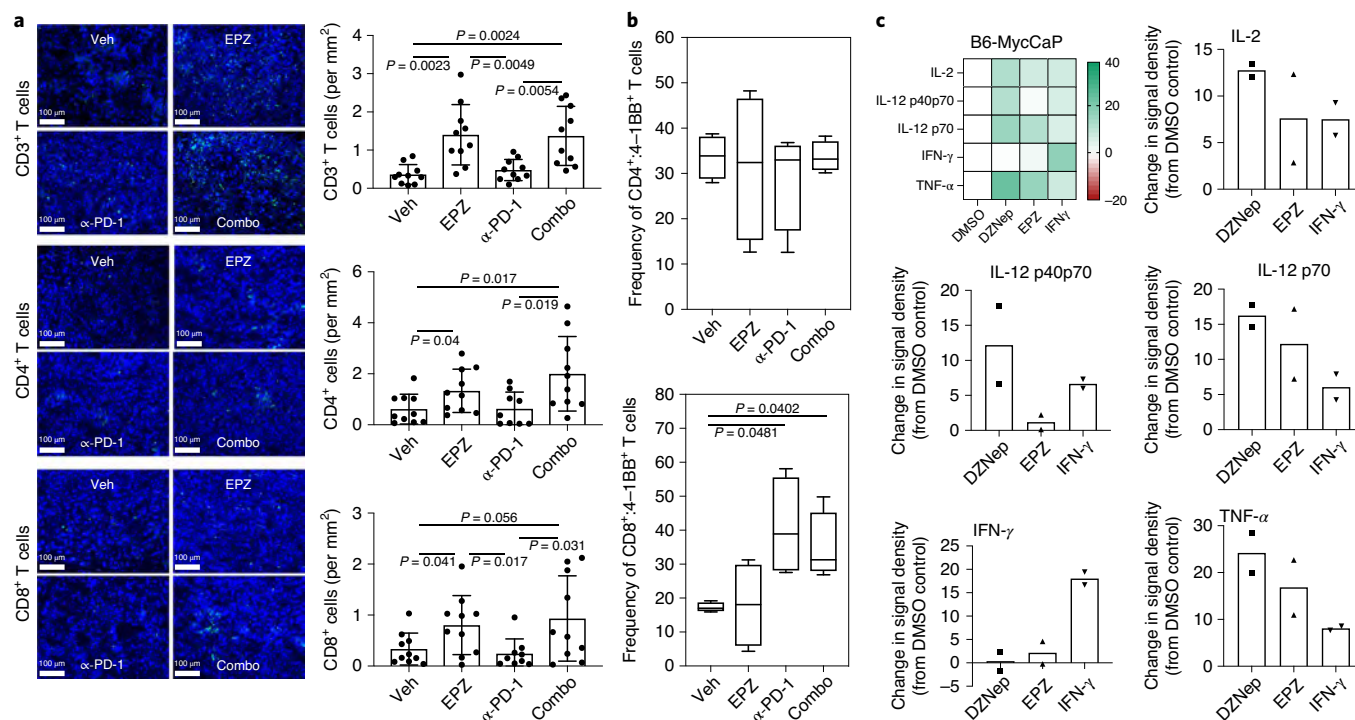


Fig. 6 | EZH2 inhibition increases T-cell infiltration and induces PCa cell Th1 chemokine expression. **a**, EZH2 inhibition alone or in combination (Combo) with PD-1 blockade significantly increasing CD3⁺, CD4⁺, CD8⁺ tumor T-cell trafficking. Scale bar, 100 μm. Each group analyzed consisted of $n = 10$ mice. Data were generated by quantification of fluorescently positive cells using an EVOS FL Auto 2 Cell Imaging System. Immunofluorescent images represent scanned tumor samples. Veh, vehicle. **b**, PD-1 blockade alone or in combination significantly increased activated CD8⁺ T cells but not CD4⁺ T cells. Each group analyzed consisted of $n = 4$ mice. Box plots are displayed as minimum to maximum distribution. All P values for **a** and **b** were generated using a two-tailed, unpaired Student's t -test with Welch's correction. **c**, Murine B6-MycCaP cells treated with EZH2 inhibitors increasing expression of Th1 chemokines. Data were generated by use of a cytokine western blot array and are displayed as the mean from $n = 2$ independent experiments.

of IL-23 (ref. ³⁷), most patients currently treated with CPI remain unresponsive.

Epigenetic regulation of immunogenicity has become one major focus to delineate mechanisms utilized by tumors to evade immune system eradication and resistance to currently available immunotherapies, specifically CPIs. It is becoming more evident that the primary mechanism mediated by epigenetic regulators is the repression of repeat element sequences (dsRNA) and immune response genes. Our work demonstrates that, in PCa models and patient samples, loss of EZH2 catalytic function results in the derepression of dsRNA and genes involved with IFN response, antigen presentation and T-cell attraction. In addition, we demonstrate that detection of increased dsRNA levels and subsequent gene response is dependent on intratumoral PD-L1 and STING activation and function. It will be important to further delineate whether activation of STING is due to dsRNA detection via crosstalk with RIG-I or whether dsRNA is being converted by reverse transcription to dsDNA. Reverse transcription of dsRNA to dsDNA may result in presentation of exclusive tumor cell antigens. This discovery could result in the production of personalized vaccine approaches that may be placed in combination strategies with epigenetic therapies such as EZH2 inhibitors.

Importantly, our work and that of others demonstrate that epigenetic targeted therapies reprogram the tumor microenvironment by a positive effect on T-cell populations. Epigenetic mechanisms, including those mediated by EZH2, have been shown to inhibit overall T-cell differentiation and tumor infiltration³⁸. Specific to EZH2, it has been demonstrated as an important regulator of differentiation in CD4⁺ T cells and T_{reg} cells^{39,40}. In T_{reg} cells, loss of

EZH2 resulted in degradation of FOXP3, allowing T_{reg} cells to be reprogrammed to a T-helper cell phenotype⁴¹. Our in vivo data did not show a change to total T_{reg} intratumoral numbers, but do induce a significant increase in CD4⁺ and CD8⁺ T cells within the tumor microenvironment. In addition, we induced downregulation of PD-1 expression in CD8⁺ T cells by EZH2 inhibition, suggesting a reversal of exhaustion that allows for their ability to be reactivated through a combination with anti-PD-1 therapy. Most exciting, in human studies, the inhibition of EZH2 in CD8⁺ T cells by CPI-1205 has resulted in increasing cytotoxic activity⁴¹.

MDSCs have also been demonstrated to be an important component of an immunosuppressive tumor microenvironment. Specific to PCa, a subset of patients with CRPC were shown to have their AR signaling activated by MDSC-secreted IL-23 (ref. ³⁷). Our data indicated that EZH2 inhibitor monotherapy or combination did not dramatically alter MDSC tumor infiltrate populations. Instead, we identified a significant effect by EZH2 inhibition and combination treatments on TAM tumor infiltrate composition. TAMs can be polarized to be tumor inhibiting (M1 TAMs) or tumor promoting (M2 TAMs). M2 TAMs have also been shown to be a major component of the immunosuppressive PCa microenvironment⁴². B6-HiMYC tumors displayed a considerable increase in M2 TAMs, which were significantly decreased after treatment, while, conversely, M1 TAMs were dramatically increased, overall supporting reprogramming toward an antitumor microenvironment.

Our study provides vital insight into how epigenetic mechanisms mediated by EZH2 drive resistance toward CPI in PCa. Collectively, EZH2 inhibition in tumor cells induces dsRNA intracellular stress, resulting in an increased STING-ISR response, reprogramming of

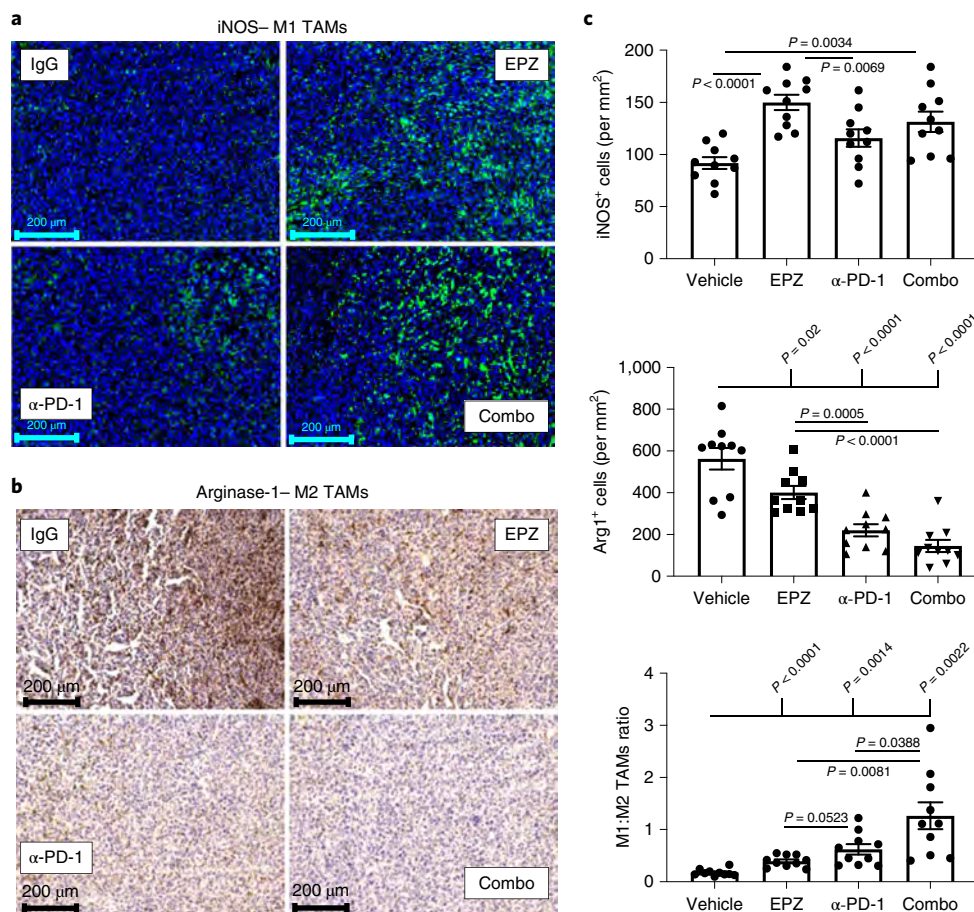


Fig. 7 | EZH2 inhibitor combination with PD-1 CPI alters the immunosuppressive tumor microenvironment. **a,b**, Example micrographs indicating alterations of M1 TAMs (**a**) and M2 TAMs (**b**) within the tumor microenvironment after treatment. Scale bar, 100 μ m. **c**, Quantification indicating that combination therapy (Combo) provides the most significant overall changes to the tumor microenvironment M1:M2 TAM ratio. The *P* values were generated using a two-tailed, unpaired Student's *t*-test with Welch's correction. Each group analyzed consisted of *n* = 10 mice. Data were generated by quantification of fluorescent or 3,3'-diaminobenzidine-positive cells using an EVOS FL Auto 2 Cell Imaging System. Images represent scanned tumor samples.

TAM infiltration, promotion of CD8⁺ T-cell activation and sensitivity to PD-1 blockade *in vivo*. These findings provide a substantial insight into PCa tumor immunity and propose the potential for patient stratification by EZH2 activity, generating the rationale to develop combinatorial use of EZH2 inhibitors with PD-1 CPI as a strategy to increase PCa response to checkpoint immunotherapy.

Methods

Further information on the research design is available in the Nature Research Reporting Summary linked to this article. Patient prostatectomy samples used for organoid generation were collected under Dana-Farber Cancer Institute protocol nos. 01-045 and 09-171 with patient consent. All remaining patient samples/data used in the present study had been collected with patient consent obtained by the respective studies.

Experimental models. The Institute of Animal Care and Use Committee at the Dana-Farber Cancer Institute approved all mouse procedures. C57BL/6N and FVB mice were obtained from Charles River Labs (strains 027 and 207, respectively). *Pten*^{flx};Pb-Cre, Pb-HiMYC, *Ezh2*^{flx} and PSA-Cre(ERT2) strains have been described previously^{17,19,43–46}. All models were validated by genotyping PCR analysis before use in subsequent studies using genomic DNA extracted from mouse ears or tails. Genotyping primers used are detailed in Supplementary Table 7. The *Ezh2*^{flx};Pb-HiMYC;PSA-Cre(ERT2)^{pos} mice (EMC mouse strain) generated in the present study were of a mixed background consisting of FVBN and C57BL/6.

Organoid models were generated using previously described methods and maintained as previously published¹⁷. Clinical samples were provided under institutional review board (IRB) approval (protocol no. 17-571; L.E.). Human

organoids were generated from two independent patient samples: (1) prostatectomy and (2) pleural effusion sample provided by A. Kibel and A. Choudhury, respectively (IRB protocol no.: 01-045; Gelb Center DFCI/HCC). Murine EMC organoids were generated from the dorsolateral prostates of 8-week-old GEMMs, whereas the *Pten*^{flx} 3D organoids were generated from end-stage prostate tumors at age 61 weeks. Mouse organoids were validated by genotyping and recombination PCRs. The primers are detailed in Supplementary Table 7.

Pten^{flx} and B6MYC-CaP murine cell lines have been previously described^{17,48}. LNCaP cells were obtained from American Type Culture Collection. PD-L1 KO models and controls were generated using pSPCas9(BB)-2A-Puro (PX459) v2.0, which was a gift from F. Zhang (Addgene plasmid no. 62988: <http://n2t.net/addgene:62988>)⁴⁹. PD-L1 KO and control cells were generated by transfecting with the PX459:sgPD-L1 or empty PX459 vector using Lipofectamine 2000 DNA Transfection Reagent (Thermo Fisher Scientific, catalog no. 11668) following the manufacturer's instructions. Cells were selected with puromycin. PD-L1 KO was validated by quantitative PCR with reverse transcription PCR (RT-qPCR) after stimulation with 100 U ml⁻¹ of IFN- γ . STING KO and control cell lines were generated as described in Pantelidou et al.⁵⁰ and used as a pooled population for all experiments. STING KO was validated by western blotting.

Mouse cytokine arrays. Mouse cell lines were treated with DZNep (5 μ M), EPZ (5 μ M), IFN- γ (100 U ml⁻¹) or DMSO control for 4 d, with medium and drugs changed on day 2. Th1-related cytokine changes were analyzed by mouse cytokine antibody array (Abcam, ab133993) according to the manufacturer's instructions.

Thapy experiments. *In vitro* assays. For all *in vitro* therapy experiments, cells were seeded at the following concentrations: two-dimensional (2D) cell lines were seeded at a concentration of 25,000 cells per well of a 24-well plate; organoids were

seeded at a concentration of 20,000 cells per 40 μ l of Matrigel disk. In both cases, each well was treated with either 1 μ M or 5 μ M DZNep or EPZ6438, DMSO or 100 U ml⁻¹ of IFN- γ control.

In vitro cytotoxicity assay. The spleens from FVB mice were mashed through a sterile 40- μ m cell strainer (Corning) that had been pre-wetted with sterile phosphate-buffered saline (PBS; Gibco). Red blood cells were lysed using a commercial ACK Lysing Buffer (Gibco). Splenocytes were stored at 2×10^6 cells ml⁻¹. Cultures were treated with 5 μ M DZNep or EPZ6438, or DMSO control for 4 d. After EZH2 inhibitor treatment, tumor cells were washed with PBS, digested to a single-cell suspension with TrypLE (Gibco) and washed with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). After washing by centrifugation, cells were resuspended in DMEM + 10% FBS, and re-plated into nonadherent, 96-well, round-bottomed plates. Cells were allowed to incubate with 10 μ g ml⁻¹ of anti-mouse PD-1 antibody or immunoglobulin (Ig)G control for 30 min at room temperature (Supplementary Table 6). After incubation, splenocytes derived from FVB mice were added at a tumor cell:splenocyte ratio of 1:10. Cells were co-cultured with splenocytes for 8 h, after which the plates were spun down and 50 μ l of supernatant was extracted for assessment of cytotoxicity. Cytotoxicity was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. Cytotoxicity was measured using a SpectraMax plate reader (Molecular Devices).

In vivo therapy experiment. Pb-HiMyc-derived tumor tissue¹⁷ was sectioned into 2-mm² tumor chunks and subcutaneously implanted into syngeneic C57BL/6N mice (Charles River Labs). Then, 4 d after the implant, mice were treated with either 250 mg kg⁻¹ of EPZ0011989 (Epizyme) or 0.5% carboxymethylcellulose by oral gavage twice daily, 200 μ g of anti-PD-1 (29F.1A12) or IgG control (2A3) by intraperitoneal injection every 3 d, started on day 5 after initiation of EZH2 inhibition therapy, or a combination (Supplementary Table 6). Tumors and mouse weight were measured three times weekly by caliper measurements. Treatment toxicities were assessed by body weight, decreased food consumption, signs of dehydration, hunching, ruffled fur appearance, inactivity or nonresponsive behavior.

Immunohistochemical and immunofluorescent staining and quantification.

In vitro samples. The 2D cell lines were seeded in a μ -Slide 8-Well chambered coverslip (ibidi, catalog no. 80826) and treated as previously described. Cells were washed with PBS (Gibco) and fixed with 4% paraformaldehyde for 15 min. After a 5-min PBS wash, cells were permeabilized by the addition of PBS containing 0.25% Triton X-100 for 15 min. After two 5-min washes with PBS, cells were incubated with a blocking solution (1% bovine serum albumin in PBS-T (PBS + 0.1% Tween-20)) for 1 h. Cells were then incubated with diluted primary antibody in blocking solution overnight at 4°C. After three 5-min PBS washes, cells were incubated with diluted secondary antibody in blocking solution for 1 h at room temperature in the dark. After three 5-min PBS washes, coverslips were imaged using an EVOS FL Auto 2 Cell Imaging System (Thermo Fisher Scientific). The antibodies are given in Supplementary Table 6.

In vivo samples. For immunohistochemistry (IHC), 4- μ m-thick, formalin-fixed, paraffin-embedded sections were prepared using standard sodium citrate antigen-retrieval methods. Primary antibodies were diluted in 1.25% horse serum/PBS and incubated overnight in a humidified chamber at 4°C overnight. IHC staining was carried out using the ImmPRESS horseradish peroxidase anti-mouse IgG (peroxidase) Polymer Detection Kit (Vector Laboratories) following the manufacturer's instructions for visualization. For immunofluorescence (IF), 4- μ m-thick sections were cut from frozen OCT blocks. Tissue sections were fixed in 2% paraformaldehyde for 20 min at room temperature and permeabilized in 0.1% Triton X-100 for 10 min, washed in PBS-T, then blocked for 1 h at room temperature with 5% goat serum + PBS-T. Sections were incubated with primary antibody in a humidified chamber at 4°C overnight, washed in PBS-T and coverslipped with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories). The antibodies used are detailed in Supplementary Table 6. For analysis, 20 representative images from each tumor were taken using an EVOS FL Auto 2 Cell Imaging System. Staining intensity was scored using analysis pipelines generated in Image J software⁵¹ (IHC staining) or CellProfiler software⁵² (IF staining).

Clinical samples. Human prostatectomy tissue collection and assessment have been previously described by Calagua et al.⁵³. Briefly, scoring was performed semiquantitatively as follows: 0 (negative or <1%), 1 (1–4%), 2 (5–24%), 3 (25–49%) and 4 ($\geq$ 50%). The antibodies are detailed in Supplementary Table 6. These tissues were stained by IF for detection of dsRNA and H3K27me3. After standard citrate buffer antigen retrieval, sections were incubated with primary antibodies overnight at 4°C, followed by incubation with a fluorescent conjugated secondary antibody for 1 h at room temperature. For analysis, 100 representative images from tumor regions were taken from each tissue sample using an EVOS FL Auto 2 Cell Imaging System. Staining intensity was quantified as mean pixel intensity per sample using analysis pipelines generated in CellProfiler software⁵².

Flow cytometry. In vitro analysis. The Click-iT EdU Alexa Fluor-488 Flow Cytometry Assay kit (Thermo Fisher Scientific) was used to measure DNA synthesis according to the manufacturer's instructions. For H3K27me3, PD-L1 and dsRNA staining, organoid disks were digested to a single-cell suspension by treatment with TrypLE (Gibco), which was in turn deactivated by resuspension in DMEM (Gibco) supplemented with 10% FBS (Sigma). Cells were washed with PBS (Gibco) by centrifugation at 500g and 4°C, and fixed with 4% paraformaldehyde for 15 min. After another PBS wash, cells were permeabilized by the addition of ice-cold methanol to a final concentration of 90% methanol. The suspension was incubated for 30 min on ice. After two washes with FACS buffer (PBS supplemented with 10% FBS), cells were resuspended in primary antibody prepared in FACS buffer (antibodies detailed in Supplementary Table 6). These cell suspensions were incubated overnight in the dark at 4°C or for 1 h at room temperature. H3K27me3 and Edu were analyzed using an Amnis ImageStream Mark II (Luminex) and dsRNA and PD-L1 with a BD LSRFortessa (BD Biosciences).

In vivo tumor analysis. Tumors were dissociated into single-cell suspensions in PBS on ice. Cells were washed with PBS (Gibco) by centrifugation at 500g and 4°C, and fixed with 4% paraformaldehyde for 15 min. After another PBS wash, cells were permeabilized by the addition of ice-cold methanol to a final concentration of 90% methanol and incubated for 30 min on ice. After two washes with FACS buffer (5% FBS/PBS), cells were resuspended in primary antibody prepared in FACS buffer. These cell suspensions were incubated at 4°C or for 1 h at room temperature, protected from light. Cells were washed twice in FACS buffer and analyzed on a BD LSRFortessa, separated into 'CD45⁻' and 'CD45⁺' events. The antibodies used are given in Supplementary Table 6.

In vivo tumor immune profiling. Tumor cell suspensions were stained using two different antibody panels: lymphocytes or myeloid, using appropriate IgG and full minus one controls, followed by analysis on an LSRII flow cytometer (BD Biosciences). Antibodies for the various immune panels are as follows: lymphocyte panel (Ghost Dye Red 780, anti-human CD8 (dump channel), anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD45 and anti-mouse PD-1); and myeloid panel (Ghost Dye Red 780, anti-human CD8, anti-mouse CD11b, anti-mouse CD45, anti-mouse Ly6C, anti-mouse Ly6G and anti-mouse I-A/I-E). After surface staining, fixation and permeabilization (BD Cytofix and BD Cytoperm), cells were stained for the following intracellular markers: lymphocyte panel (Foxp3, Ki67 or the appropriate IgG controls). After staining, cells were analyzed on an LSRII flow cytometer (BD Biosciences). Cells were gated based on singlets, size/nucleation, Ghost Dye Red 780-negative events and dump-negative events ('Live events'). Cells were then separated into CD45⁻ and CD45⁺ events, and immune populations were defined as follows: CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, CD3⁺CD4⁺Foxp3⁺ T_{reg} cells, granulocytic MDSCs (CD11b⁺MHC-II⁻Ly6C⁺Ly6G⁺) and monocytic MDSCs (CD11b⁺MHC-II⁻Ly6G⁻Ly6C^{hi}). The antibodies used are detailed in Supplementary Table 6.

RT-qPCR. The qPCRs were performed in accordance with MIQE guidelines⁵⁴. RNA was harvested using a standard TRIzol protocol according to the manufacturer's instructions. Complementary DNA was synthesized using the qScript cDNA SuperMix (Quantabio), according to the manufacturer's instructions. The SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for PCRs with the cycling conditions recommended in the manufacturer's instructions. The primers used are detailed in Supplementary Table 7.

Sequencing analysis. RNA-seq data generation. EMC mouse organoids were seeded at a concentration of 20,000 cells per 40 μ l of Matrigel disk (1 disk per well of a 24-well plate) and treated with 5 μ M DZNep, DMSO control, 1 μ M Tam or ethanol control for 3 d. RNA was harvested from samples using TRIzol (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA libraries were prepared with the TruSeq stranded messenger RNA sample preparation kits (Illumina) from 500 ng of purified total RNA, according to the manufacturer's protocol. The resultant RNA dsDNA libraries were quantified by Qubit fluorometer, Agilent TapeStation 2200 and RT-qPCR using the Kapa Biosystems library quantification kit according to the manufacturer's protocols. Uniquely indexed libraries were pooled in equimolar ratios and sequenced on a single NextSeq 500 Sequencing Platform (Illumina) run with single-end 75-bp reads. Sequencing reads were aligned to the UCSC mm9 reference genome assembly and gene counts were quantified using STAR (v.2.5.1b)⁵⁵ and normalized read counts (reads per kilobase per million) were calculated using Cuffdiff (v.2.2.1)⁵⁶.

EZH2 repression score. DE gene analysis and GSEA were first performed using RNA-seq data obtained from EMC mouse 3D organoids treated with DZNep ($n = 3$) and EMC mouse 3D organoids treated with the DMSO vehicle ($n = 3$). DZNep versus DMSO RNA-seq data were used to generate a 29-gene signature, which contained the most DE genes with human homologs. Weights were again defined as the $-\log_{10}$ of the adjusted P value multiplied by the sign of the log₂(fold-change). The EZH2 repression score was generated for each tumor sample by multiplying the log₂(transformed count data) for each of the 29 human

orthologous genes, using its established weighting and summing these 29 values for each sample.

ChIP-seq and ATAC-seq of patient samples. Fresh-frozen radical prostatectomy specimens from patients with localized PCA were obtained from the Dana-Farber Cancer Institute Gelb Center biobank, under Dana-Farber Cancer Institute/Harvard Cancer Center IRB-approved protocols (nos. 01-045, 09-171). Hematoxylin and eosin (H&E)-stained slides from each case were reviewed by a genitourinary pathologist. Areas estimated to be enriched >70% for prostate tumor tissue were isolated for analysis. ChIP-seq was performed using the protocol previously described²⁷ with antibodies to H3K27Ac (Diagenode, catalog no. C15410196) and H3K27me3 (Cell Signaling Technology, catalog no. 9733S). Libraries were sequenced using 75-bp reads on the Illumina platform. ATAC-seq (assay for transposase-accessible chromatin with high-throughput sequencing) was performed at Active Motif using fresh-frozen Gelb Center RP tumor and normal epithelium specimens. The tissue was manually dissociated, isolated nuclei were quantified using a hemocytometer and 100,000 nuclei were tagged as previously described²⁷, with some modifications⁵⁸ using the enzyme and buffer provided in the Nextera Library Prep Kit (Illumina). Tagmented DNA was then purified using the MinElute PCR purification kit (QIAGEN), amplified with ten cycles of PCR and purified using Agencourt AMPure SPRI beads (Beckman Coulter). All samples were processed through the computational pipeline developed at the DFCI Center for Functional Cancer Epigenetics (CFCE) using primarily open-source programs. Sequence tags were aligned with the Burrows-Wheeler aligner (BWA) to build hg19 of the human genome, and uniquely mapped, nonredundant reads were retained⁵⁹. These reads were used to generate binding sites with Model-Based Analysis of ChIP-seq 2 (MACS v.2.1.1.20160309), with a *q* value (false discovery rate threshold of 0.01 (ref. 60)). The ChIP-seq and ATAC-seq data are reported separately⁵⁸.

ENV sequence identification. Fastq files were downloaded from the ENA public repository (<https://www.ebi.ac.uk/ena/browser/view/PRJNA421359>). ChIP-seq fastq files were processed using GenPipes 'ChIPseq' pipeline steps 1–8 (ref. 61). In brief, raw reads are trimmed and filtered for quality using Trimmomatic and then mapped to the human reference genome (GRCh38) using the BWA to generate Bam files. Peaks were then called using MACS2 software (v.2.1.2)⁶⁰ with default setting. Generated bed files were further analyzed using R environment (v.3.6.2). The findOverlappingPeaks-function from the ChIPpeakAnno_3.20.1 package⁶² was used to identify overlapping peaks. Peaks were annotated according to genomic location and closest gene using the ChIPseeker_1.22.1 package⁶³ and the reference genome annotation package TxDb.Hsapiens.UCSC.hg38.knownGene_3.10.0. The list of peaks that lost H3K27 trimethylation on EZH2 knockdown were generated by overlapping LNCaP siCtrl H3K27me3 peaks with LNCaP siEZH2 H3K27me3 peaks. The list of peaks that gained H3K27 acetylation on EZH2 knockdown was generated by overlapping LNCaP siCtrl H3K27ac peaks with LNCaP siEZH2 H3K27ac peaks. To generate the list of genes with 3'-UTR, we followed the procedure described in Canadas et al.²³. The 5,880 3'-UTRs that have ERVs were subset to keep only unique gene symbols. We then intersected this list of 2,137 genes to the closest genes with peaks that lost methylation and gained acetylation.

Statistics and reproducibility. The sample size was based on the number of samples available for computational analysis of human samples. For mouse RNA-seq analysis, chemical and genetic inhibition of Ezh2 was performed in triplicate and duplicate, respectively. No statistical method was used to predetermine sample size and experiments were not randomized. The investigators were blinded for in vivo therapy experiments because one person conducted treatment and an independent person recorded tumor measurements. For analysis of all IHC and IF staining (clinical samples and in vivo tumor samples), the slides were coded before analysis to blind researchers to treatment groups and scored digitally to prevent potential bias. For in vitro experiments, blinding was not possible because the same researchers who treated the cells ran the analysis. However, all analysis within experiments was run at the same time in an automated manner, so prior knowledge of treatment groups had no impact on data output. There was no data exclusion from any experiment in our analysis.

For RNA-seq analysis of mouse and human datasets, DE gene analysis, sample-to-sample distance calculations and principal component analysis were conducted using the DESeq2 package in R. Raw RNA-seq count data were processed to remove genes lacking expression in >80% of samples. Low count genes, with fewer than ten total reads, were also filtered out. After variance-stabilizing transformation, a Euclidean sample distance matrix and principal component plots were generated to compare global gene expression profiles between samples. DE gene lists were then generated. Further interpretation of gene expression data was enabled using GSEA. A ranked list was generated from the DE gene output by multiplying the $-\log_{10}$ of the adjusted *P* value by the sign of the $\log_2$ (fold-change). The ranked list was then used as an input to the GSEA preranked tool to generate enrichment scores using the Hallmark, Curated and Oncogenic Signatures gene sets in the Molecular Signatures Database. Heatmaps and unsupervised hierarchical cluster analysis, using Euclidean distance measurements, were performed using the pheatmap package in R. The corr.

test and smoothScatter functions were used for Pearson's correlation analysis and to generate scatter plots. The VennDiagram package was used to compare gene lists and generate Venn diagrams. Master regulator analysis was performed using MARINA⁶⁴. Protein association network generation and Gene Ontology analyses were performed using STRING v.11 (ref. 65).

In vitro experiments were conducted in three independent experiments and displayed as mean $\pm$ s.e.m. For in vivo experiments, ten animals were used per treatment group following our previous studies. Graph preparation and statistical analyses of in vitro and in vivo experiments were performed using GraphPad Prism software. Statistical significance for assays was assessed using either a one-way analysis of variance (ANOVA), Tukey's multiple-comparison test or Welch's corrected unpaired Student's *t*-test unless otherwise stated. Specific for in vivo tumor growth curves (Fig. 4b), a multiple Student's *t*-test was used to assess therapy response. An observation with a *P* < 0.05 was considered to be statistically significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

ChIP-seq, ATAC-seq and RNA-seq data used to support the present study have been deposited in the Gene Expression Omnibus under accession nos. GSE130408, GSE107780, GSE146617 and GSE146076. Further information and requests for resources and reagents should be directed to the lead author, L.E. (Leigh.Ellis@cshs.org). Gene expression data for LNCaP cell lines treated with EZH2 inhibitor were obtained under accession no. GSE107780. Raw and normalized expression data for 550 TCGA PCA samples were obtained from the NCI Genomic Data Commons Data Portal. Some 102 samples were excluded based on pathological criteria provided by S. Tyekucheva and M. Loda, and the remaining 448 samples (40 normal samples and 408 tumor samples) were included in subsequent analyses. The NCI data were provided by A. Sowalsky. Human PCAs have been described previously⁶⁶ and were obtained from DbGaP (study accession no. phs000909). Normalized counts from the Stand Up 2 Cancer dataset were obtained from cBioPortal⁶⁷. Any code used in this manuscript will be made available upon request. Source data are provided with this paper.

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Author contributions

K.L.M., A.V.S., D.L.B. and B.M.O. performed research, analyzed data and wrote the manuscript. S.C.B., D.P.L., C.C., H.Y., P.G., S.P., A.M., A.A.H., C.J.S. and M.L. performed research. K.R., M.H., S.K.D., A.D.C., N.B., Y.L., H.H.H., A.G.S., M.M.P., M.L.F., S.W., N.C.W., S.Y.T., A.G.S., C.P., G.I.S., X.Q. and H.W.L. provided vital reagents. A.D.C., D.P.L., H.Y., M.L.F., S.C.B., C.J.S., D.J.E., S.K.D., A.G.S., S.P.B. and B.M.O. assisted with editing the final manuscript. L.E. designed experiments, analyzed data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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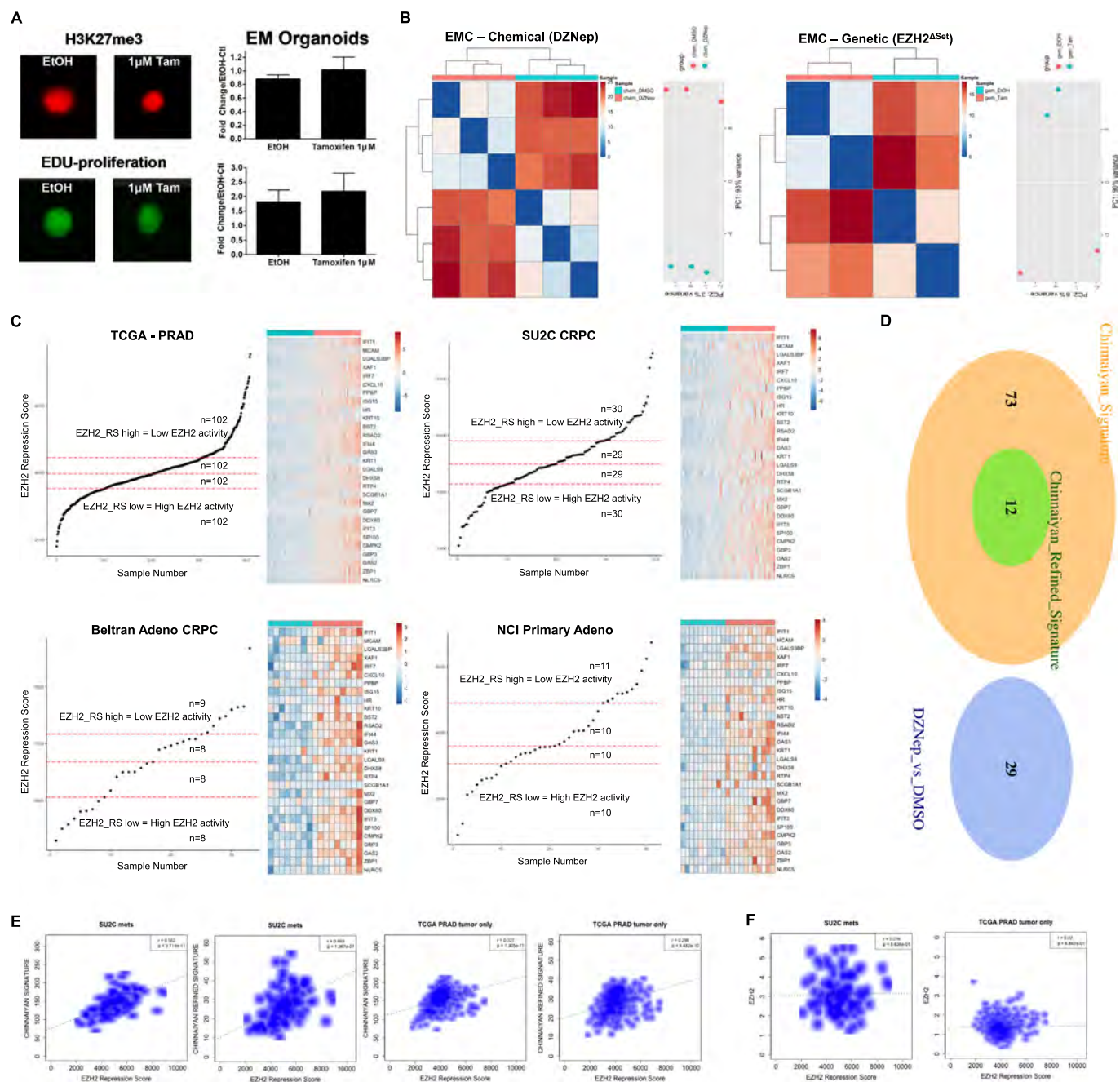
Correspondence and requests for materials should be addressed to L.E.

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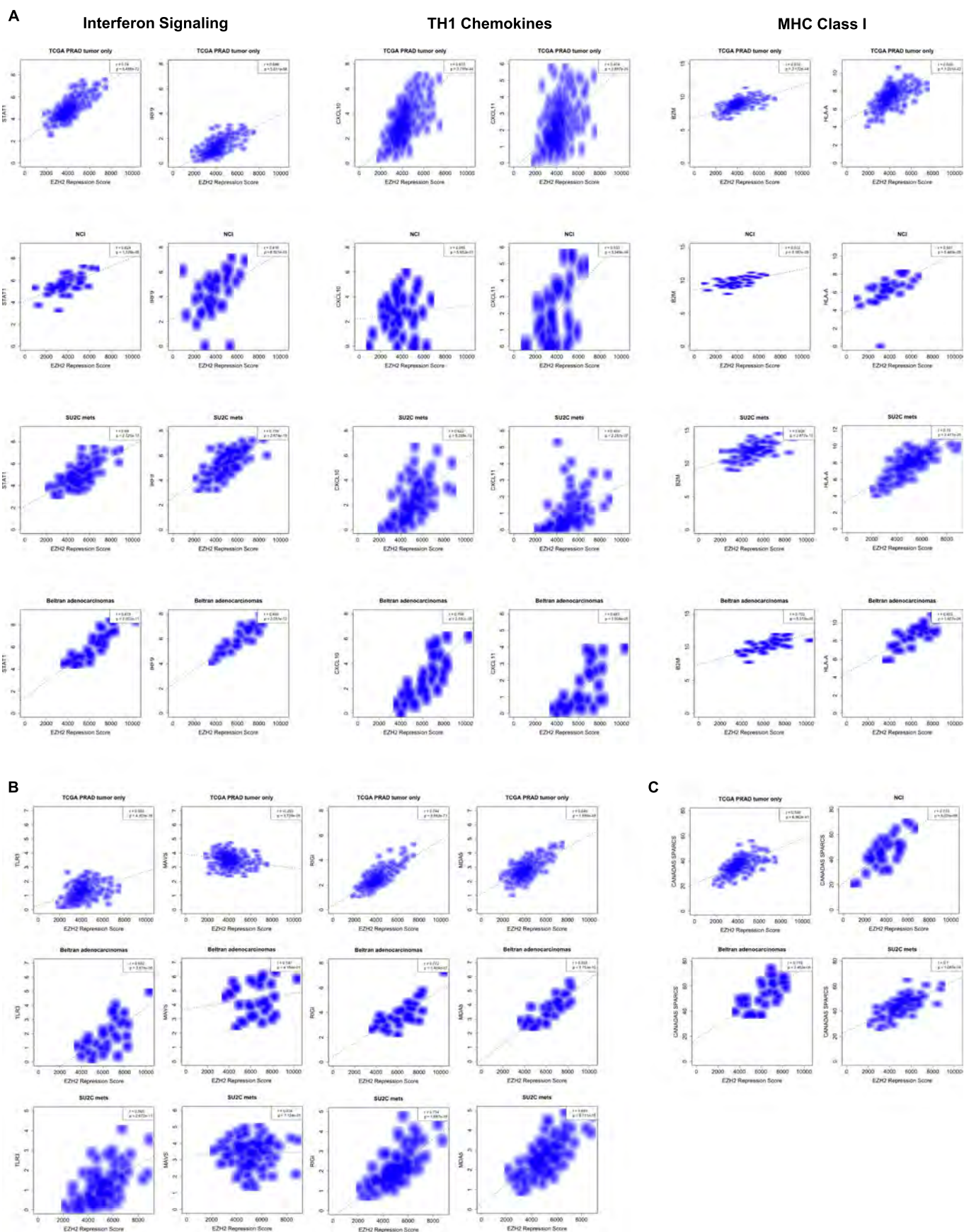
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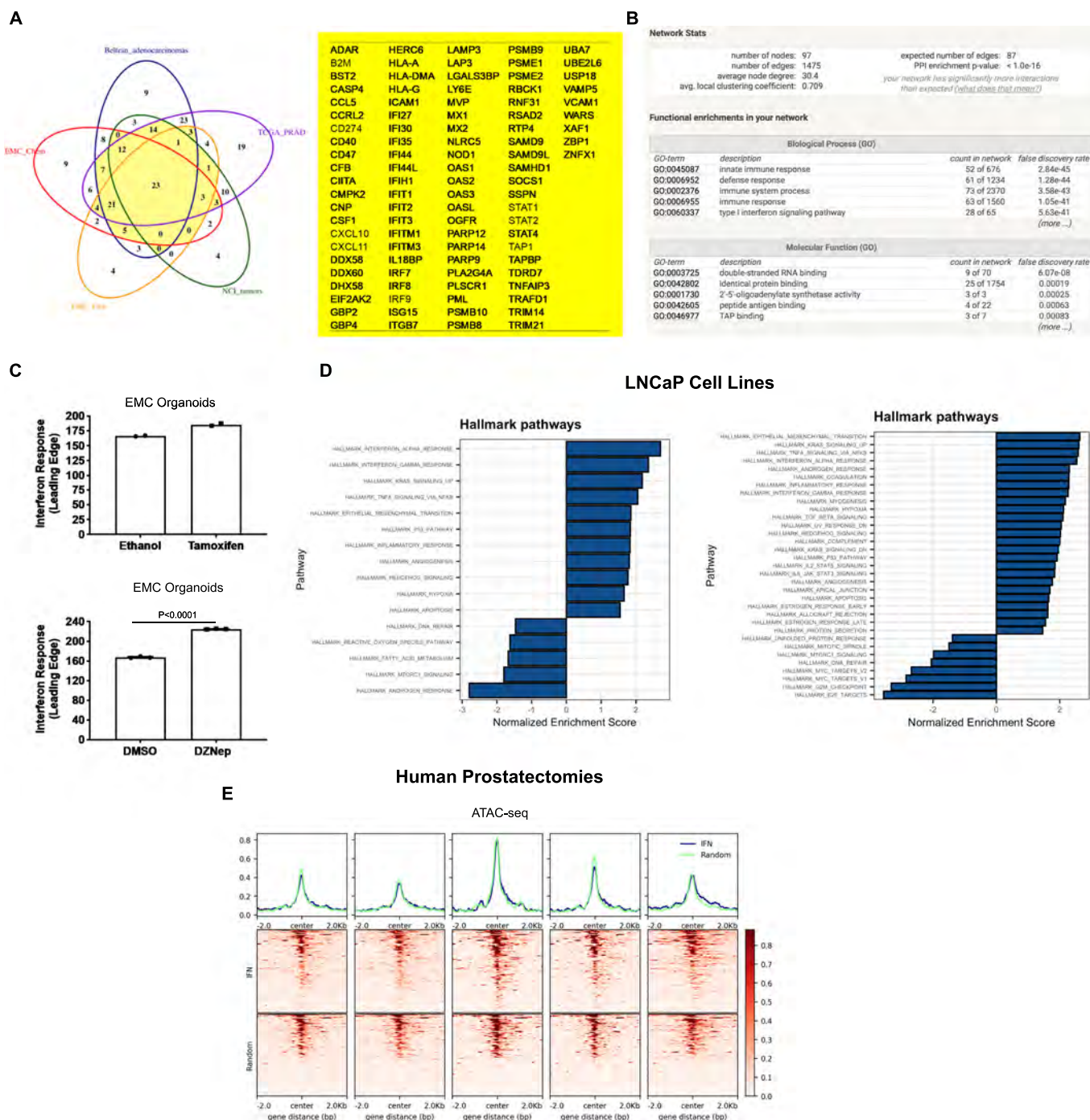


Extended Data Fig. 1 | Generation of an EZH2 Activity Gene Signature. (a) Three-dimensional PCa organoids generated from EM mice (without PSACre^{ERT2}) alleles. When treated with tamoxifen, no loss of H3K27me3 or EDU staining is indicated - demonstrating specificity of tamoxifen-PSACre^{ERT2} mediated deletion of the *Ezh2* set domain in Fig. 1. P-values were generated using a two-tailed unpaired T-test with Welch's correction. Data was generated from three (n = 3) independent experiments and displayed as mean ± SEM (b) Principal component analysis (PCA) following chemical (n = 3 independent organoid cultures per treatment group) and genetic (n = 2 independent organoid cultures per treatment group) inhibition of *Ezh2* catalytic function results in significant changes in gene expression. (c) A 29-gene signature derived from Fig. 1c (DZNep data) was used to generate signature scores for each patient within four independent human prostate cancer RNA-seq datasets. Patients were ranked highest score to lowest score and subject to quartile separation. First (blue) and fourth (red) quartiles were analyzed by supervised clustering to demonstrate expression differences within patients with lowest EZH2 activity and highest EZH2 activity. Sample numbers used for TCGA (n = 408 samples), SU2C (n = 118 samples), Beltran Adenocarcinomas (n = 33 samples), and NCI Primary Adeno (n = 41 samples). (d) Our 29-gene signature derived from demonstrates complete independence from a previously published polycomb repression signature. (e) Our 29-gene signature demonstrates significant correlation with a previously published polycomb repression signature in 2 independent human PCa gene expression datasets. (f) EZH2 activity is not determined by EZH2 mRNA expression. Pearson correlation coefficient analysis was utilized to generate data for E-F.



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Low EZH2 Activity is Associated with Enrichment in IFN Signaling and dsRNA Sensory Machinery. **(a)** Genes representing IFN signaling (*STAT1*, *IRF9*), Th1 chemokines (*CXCL10*, *CXCL11*), and MHC Class I molecules (*B2M*, *HLA-A*) were shown to be enriched in PCa patients with low EZH2 activity. **(b)** Genes representing intracellular sensors of dsRNA (*TLR3*, *MAVs*, *RIG-I*, *MDA5*) were shown to be enriched in PCa patients with low EZH2 activity. **(c)** Genes from Canadas et al. (2018) described as ‘SPARCs’ regulated by STAT1 and EZH2 that house endogenous retroviral sequences important for inducing an innate immune response, were shown to be enriched in PCa patients with low EZH2 activity. Sample numbers used for TCGA (n = 408 samples), SU2C (n = 118 samples), Beltran Adenocarcinomas (n = 33 samples), and NCI Primary Adeno (n = 41 samples). All data was generated by using Pearson correlation coefficient analysis.



Extended Data Fig. 3 | EZH2 Inhibition Regulates Innate Immune Signaling in Prostate Cancer. (a) Overlay of five independent differentially expressed IFN α and IFN γ gene lists from mouse and human RNA-seq data provided a merged gene list of 97 ISGs. (b) String analysis of the generated 97 type I/II IFN gene list reveals significant enrichment of biological processes including *innate immune response*, *defense response*, and *type I interferon signaling pathway*. Moreover, molecular function terms including *double-stranded RNA binding*, *peptide antigen binding* were also significantly enriched. (c) Mouse RNA-seq data was queried to demonstrate that our 97 IFN gene signature is upregulated in response to loss of EZH2 catalytic activity. Data was generated by performing two ($n=2$) independent (genetic inhibition) or three ($n=3$) independent experiments and displayed as the mean or mean $\pm$ SEM respectively. Statistical data was generated by performing a two-tailed unpaired T-test with Welch's correction. (d) LNCaP RNA-seq data was queried to demonstrate that upon EZH2 genetic (left) or chemical (right) inhibition results in enrichment of IFN α/γ gene sets. Data was generated from previously published RNA-seq data - GSE107780. Triplicate, $n=3$ /treatment group RNA-seq experiments were available for analysis. (e) Heatmaps of normalized ATAC signal intensities of 97 ISGs from were analyzed in 5 independent patient prostatectomy samples. Each patient sample was analyzed once.

PD-L1

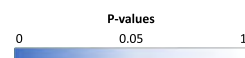
	BYMYC-CaP							Pten ^{-/-}					
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sgScramble + DMSO							sgScramble + DMSO						
sgScramble + DZNep	0.03	1					sgScramble + DZNep	0.05	1				
sgScramble + EPZ	0.05	0.11	1				sgScramble + EPZ	0.02	0.1	1			
sgSTING + DMSO	0.31	0.03	0.06	1			sgSTING + DMSO	0.5	0.04	0.01	1		
sgSTING + DZNep	0.17	0.02	0.09	0.24	1		sgSTING + DZNep	0.71	0.04	0.04	0.45	1	
sgSTING + EPZ	0.24	0.02	0.08	0.37	0.79	1	sgSTING + EPZ	0.79	0.05	0.01	0.47	0.83	1
	DMSO + DMSO	DMSO + DZNep	DMSO + EPZ	C-176 + DMSO	C-176 + DZNep	C-176 + EPZ		DMSO + DMSO	DMSO + DZNep	DMSO + EPZ	C-176 + DMSO	C-176 + DZNep	C-176 + EPZ
DMSO + DMSO	1						DMSO + DMSO	1					
DMSO + DZNep	0	1					DMSO + DZNep	0.05	1				
DMSO + EPZ	0.02	0.04	1				DMSO + EPZ	0.31	0.72	1			
C-176 + DMSO	0.7	0	0.01	1			C-176 + DMSO	0.67	0.02	0.28	1		
C-176 + DZNep	0.92	0.01	0.01	0.96	1		C-176 + DZNep	0.45	0.01	0.27	0.81	1	
C-176 + EPZ	0.25	0	0.01	0.2	0.45	1	C-176 + EPZ	0.88	0.08	0.29	0.72	0.63	1

MHC-I

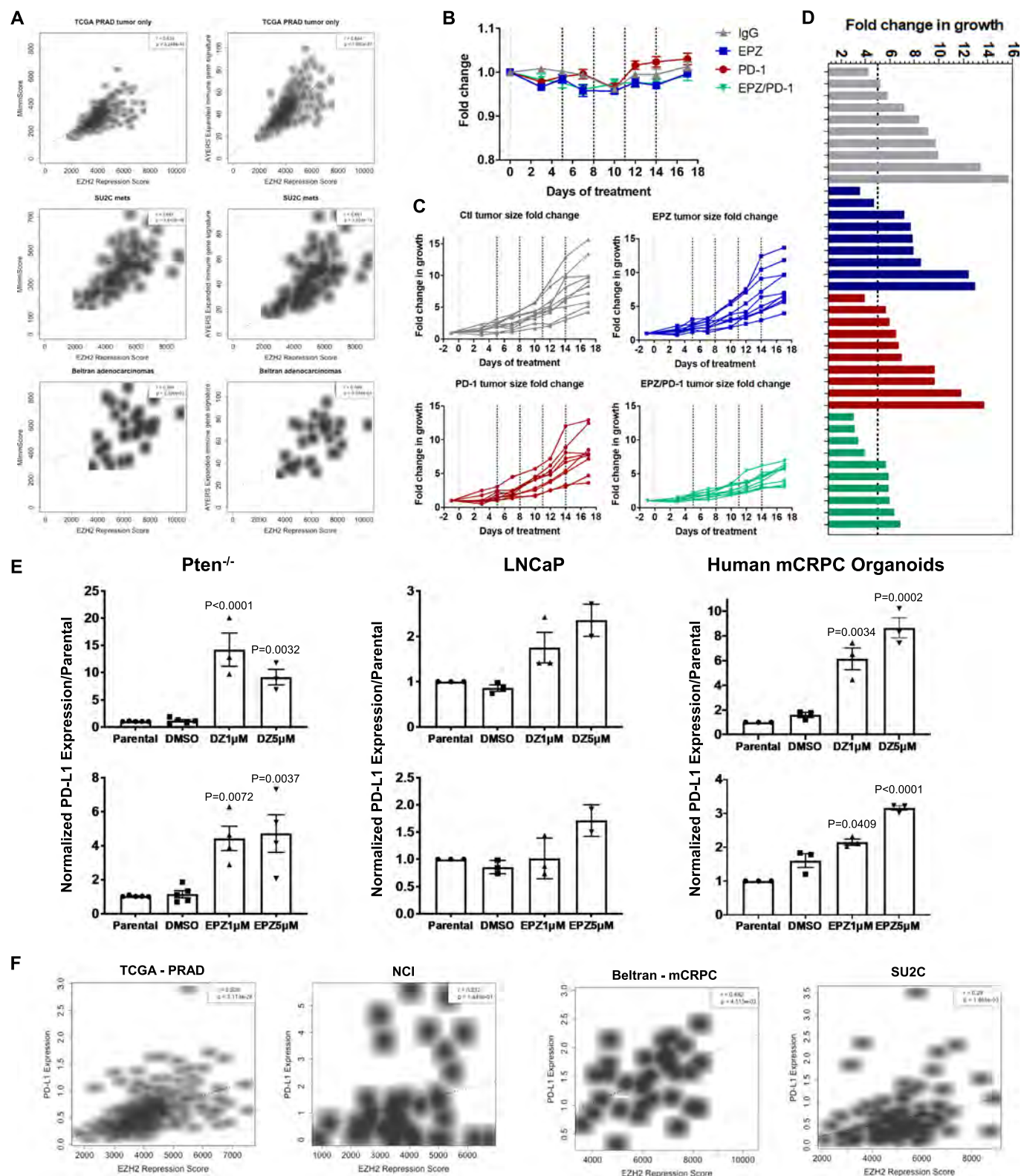
	BYMYC-CaP							Pten-/-					
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sgScramble + DMSO	1						sgScramble + DMSO	1					
sgScramble + DZNeP	0.05	1					sgScramble + DZNeP	0.06	1				
sgScramble + EPZ	0.06	0.32	1				sgScramble + EPZ	0.03	0.31	1			
sgSTING + DMSO	0.42	0.04	0.09	1			sgSTING + DMSO	0.75	0.03	0.02	1		
sgSTING + DZNeP	0.2	0.05	0.11	0.15	1		sgSTING + DZNeP	0.33	0.06	0.02	0.36	1	
sgSTING + EPZ	0.08	0.05	0.09	0.05	0.48	1	sgSTING + EPZ	0.28	0.06	0.03	0.29	0.8	1
	DMSO + DMSO	DMSO + DZNeP	DMSO + EPZ	C-176 + DMSO	C-176 + DZNeP	C-176 + EPZ		DMSO + DMSO	DMSO + DZNeP	DMSO + EPZ	C-176 + DMSO	C-176 + DZNeP	C-176 + EPZ
DMSO + DMSO	1						DMSO + DMSO	1					
DMSO + DZNeP	0.01	1					DMSO + DZNeP	0.03	1				
DMSO + EPZ	0.03	0.13	1				DMSO + EPZ	0.01	0.36	1			
C-176 + DMSO	0.34	0	0.02	1			C-176 + DMSO	0.67	0.06	0.15	1		
C-176 + DZNeP	0.64	0.01	0.03	0.42	1		C-176 + DZNeP	0.45	0.06	0.11	0.81	1	
C-176 + EPZ	0.84	0.01	0.02	0.51	0.79	1	C-176 + EPZ	0.76	0.15	0.32	0.6	0.51	1

dsRNA

	BYMYC-CaP							Pten-/-					
	sgScramble + DMSO	sgScramble + DZNeP	sgScramble + EPZ	sgScramble + DMSO	sgSTING + DZNeP	sgSTING + EPZ		sgScramble + DMSO	sgScramble + DZNeP	sgScramble + EPZ	sgSTING + DMSO	sgSTING + DZNeP	sgSTING + EPZ
sgScramble + DMSO	1						sgScramble + DMSO	1					
sgScramble + DZNeP	0.05	1					sgScramble + DZNeP	0.02	1				
sgScramble + EPZ	0.11	0.12	1				sgScramble + EPZ	0.07	0.09	1			
sgSTING + DMSO	0.8	0.02	0.07	1			sgSTING + DMSO	0.47	0.01	0.07	1		
sgSTING + DZNeP	0.07	0.81	0.14	0.04	1		sgSTING + DZNeP	0.07	0.68	0.19	0.07	1	
sgSTING + EPZ	0.14	0.26	0.62	0.11	0.24	1	sgSTING + EPZ	0.03	0.07	0.83	0.02	0.21	1
	DMSO + DMSO	DMSO + DZNeP	DMSO + EPZ	C-176 + DMSO	C-176 + DZNeP	C-176 + EPZ		DMSO + DMSO	DMSO + DZNeP	DMSO + EPZ	C-176 + DMSO	C-176 + DZNeP	C-176 + EPZ
DMSO + DMSO	1						DMSO + DMSO	1					
DMSO + DZNeP	0	1					DMSO + DZNeP	0.07	1				
DMSO + EPZ	0.04	0.28	1				DMSO + EPZ	0.1	0.14	1			
C-176 + DMSO	0.82	0	0.02	1			C-176 + DMSO	0.97	0.02	0.04	1		
C-176 + DZNeP	0.05	0.6	0.72	0.03	1		C-176 + DZNeP	0.05	0.81	0.15	0.01	1	
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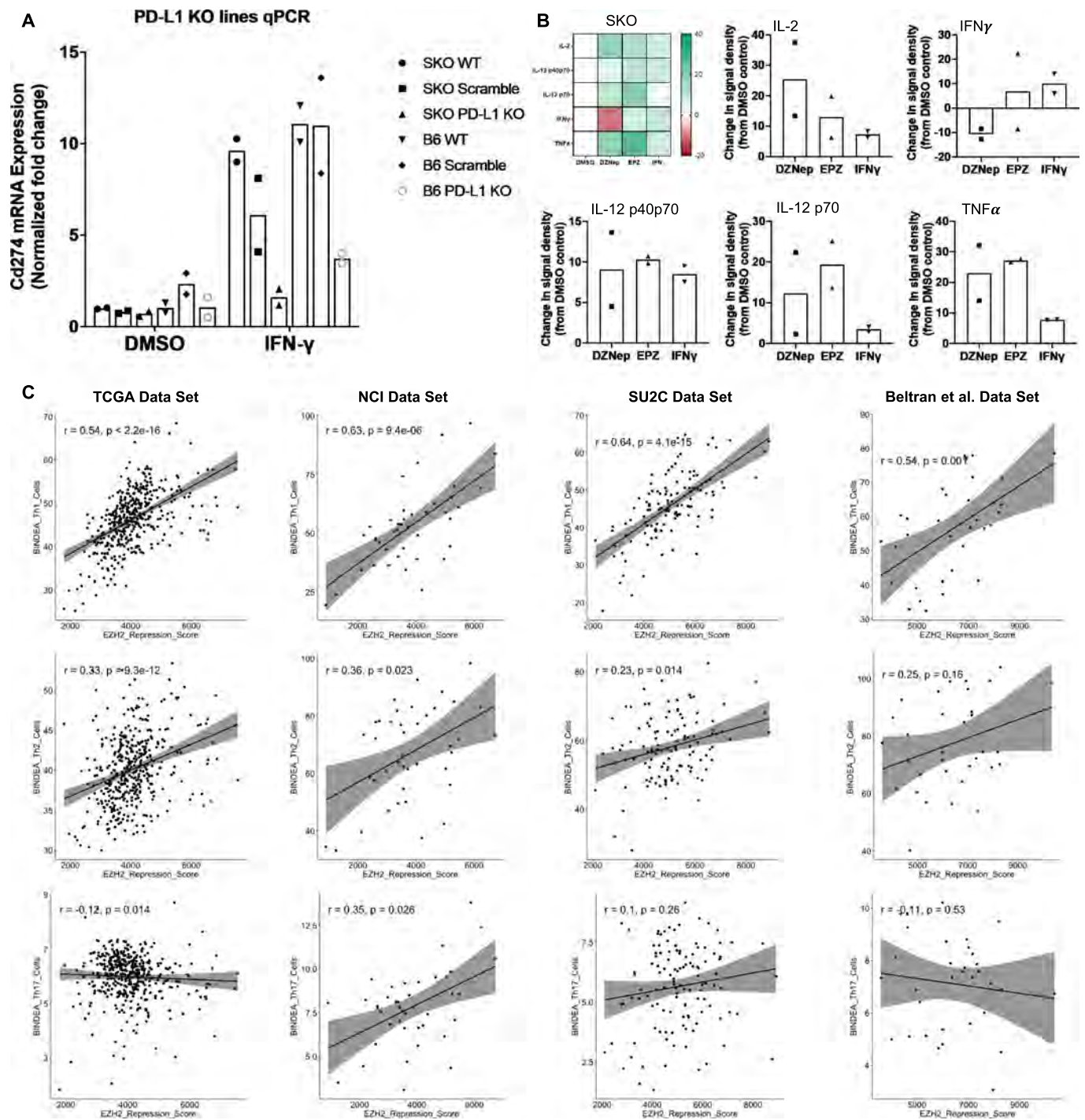
Extended Data Fig. 4 | Activation of interferon stimulated genes is STING dependent cont. Full statistical comparisons for flow cytometry analysis of PD-L1, MHC-I and dsRNA expression in B6MYC-CaP and Pten^{-/-} cells with and without STING inhibition (chemical by C-176 or genetic by sgSTING) and treated with DMSO, or EZH2 inhibitors DZNeP or EPZ. These statistical data are partnered with Fig. 4c,d and was generated by performing a two-tailed unpaired T-test with Welch's correction. Data was generated using flow cytometry analysis from three (n=3) independent experiments and displayed as mean ± SEM. P-values can be seen in Extended Data Fig. 4 and were generated using a two-tailed unpaired T-test with Welch's correction. Note: Data from Pten KO cells in Fig. 4c treated with C176 to inhibit STING was generated from two (n=2) independent experiments and displayed as the mean value.



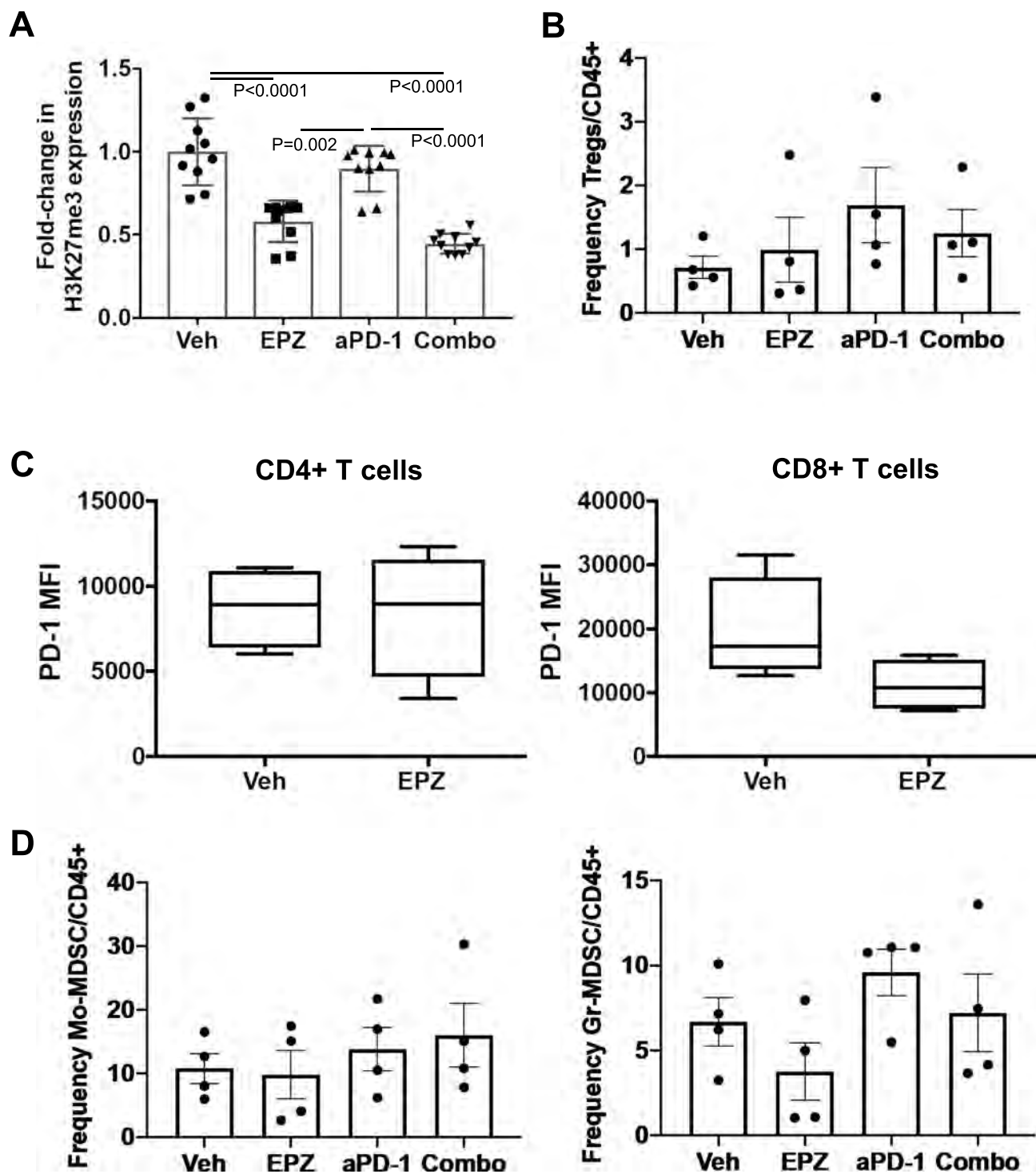
Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Low EZH2 activity is associated with increased immune gene expression related to positive response to check-point inhibition.

(a) Analysis of human RNA-seq datasets reveal immune signatures related to check-point blockade positive response are significantly enriched in PCa patients with low EZH2 activity. Sample numbers used for TCGA (n = 408 samples), SU2C (n = 118 samples), and Beltran Adenocarcinomas (n = 33 samples). (b) Normalized weights of mice (n = 10 mice/treatment group) indicate that no significant weight loss (ie: toxicity) was observed following therapy with indicated treatment cohorts. (c-d) Tumor measurements of individual tumors by waterfall or spider plots validate significant anti-tumor activity of EZH2 inhibition combined with PD-1 blockade. N = 10 mice/tumors per treatment group. (e) Mouse and human prostate cancer organoids (Pten^{-/-} and human mCRPC organoids), and human LNCaP 2D cell lines treated with indicated EZH2 inhibitors for 96 hours demonstrate upregulation of PD-L1 mRNA. Data was generated for Pten^{-/-} organoids with n = 5 independent experiments for parental and DMSO treatment groups and n = 3 independent experiments for DZ and EPZ treatment groups and displayed as the mean $\pm$ SEM. For LNCaP and human CRPC organoids, a n = 3 independent experiment was used to generate data and displayed as the mean $\pm$ SEM. Statistical data was generated by performing a two-tailed unpaired T-test with Welch's correction of DMSO verse DZ or EPZ treatment groups. (f) Human PCa gene expression data was queried to demonstrate that increased PD-L1 gene up-regulation is significantly correlated with low EZH2 activity. Sample numbers used for TCGA (n = 408 samples), NCI (n = 41 samples), SU2C (n = 118 samples), and Beltran Adenocarcinomas (n = 33 samples).



Extended Data Fig. 6 | Low EZH2 activity is associated with positive association of inflammatory immune genes. (a) B6MYC-CaP and Pten^{-/-} 2D cell lines that express Cas9 were stably infected with gRNA towards *Pd-I1* (*Cd274*). Treatment with IFN γ validates the inhibition of *Pd-I1* expression in KO cell lines. Data was generated by $n=2$ independent qRT-PCR experiments. (b) Murine Pten KO prostate cancer cells treated with EZH2 inhibitors increase expression of Th-1 cytokines. Data for A-B was generated by performing two ($n=2$) independent experiments and displayed as the mean. (c) Human prostate cancer patient correlation analysis between EZH2 repression score (X-axis) and Th1, Th2, or Th17 gene expression profiles (Y-axis). Sample numbers used for TCGA ($n=408$ samples), NCI ($n=41$ samples), SU2C ($n=118$ samples), and Beltran Adenocarcinomas ($n=33$ samples).



Extended Data Fig. 7 | Effects on the tumor microenvironment post EZH2 inhibition. (a) Representative in vivo tumor analysis indicates that EZH2 inhibition and combination significantly reduce tumor H3K27me3 expression. (b) Frequency of Foxp3+ T-reg cells was determined by flow cytometry. No significant change was observed following treatment. (c) PD-1 protein expression on CD4+ and CD8+ T-cells was analyzed by flow cytometry. Only CD8+ T-cells were observed to express lower PD-1 protein following EZH2 inhibition. Box plots are displayed as min to max distribution. (d) Frequency of Mo-MDSC and Gr-MDSC cells was determined by flow cytometry. No significant change was observed following treatment. Data was generated by analysis of (A) ten ($n=10$) independent mice or (B-D) four ($n=4$) independent mice per treatment group. Statistical data was generated by performing a two-tailed unpaired T-test with Welch's correction.

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☒ ☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☐ ☒ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

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

Data collection No software was used for data collection.

Data analysis

Flow Cytometry:

- Amnis IDEAS Analysis (vs6.0, Luminex)
- FACSDiva (vs8.0, BD Biosciences)
- FlowJo (vs10.6.1, FlowJo)

IHC/IF:

- Cell Profiler (vs 4.07, CellProfiler) - is an open-source software for measuring and analyzing cell images.
- ImageJ (vs1.51, Image J) - is an open source Java image processing program.

ChIP-seq:

MACS (vs2.1.2)

Statistics:

- Graph Pad Prism (vs8.0.1, Graph Pad) - is analysis and graphing software purpose-built for scientific research.
- R statistics (vs3.5.0, R) - is a free software environment for statistical computing and graphics.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Mouse RNA-seq dataset (Fig 1A-D, Extended Data Fig 1B and 3C) has been deposited in GEO (Gene Expression Omnibus) with the accession code GSE146076. Human ChIP-seq and ATAC-seq (Fig 3A, Extended Data Fig 4E) has been deposited in GEO (Gene Expression Omnibus) with the accession codes GSE130408 and GSE146617. Human ChIP-seq and RNA-seq (Fig 3B-D, Extended Data Fig 3D) can be accessed through GSE107780. TCGA prostate cancer samples were obtained from the National Cancer Institute Genomic Data Commons Data Portal. NCI RNA-seq data was provided by Dr. Adam Sowalsky. Beltran et al. human prostate cancer RNA-seq were obtained from DbGaP (Study Accession: phs000909).

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For in vivo studies, the sample size was selected based on previous studies treating allograft Pten-/-Rb1-/- tumors with EZH2 inhibitors (Ku et al, 2017, Science, 355(6320):78-83).
Data exclusions	There were no excluded data points.
Replication	All in vitro experiments were repeated 3 times, and data was pooled for analysis. No replicates were unsuccessful. In vivo studies were only run once.
Randomization	For in vivo studies mice were not randomized, however all mice purchased at the same time from Charles River Laboratories, were the same age and were housed in identical conditions. Mice were not assigned a treatment based on any prior. For all in vitro studies, randomization of cell lines was not possible, however all cells were treated and analyzed in identical conditions.
Blinding	For in vivo studies, mice were treated by one researcher and a separate researcher took tumor measurements at a different time to ensure the experiment was run in a blinded manner. For analysis of all immunohistochemical and immunofluorescent staining (clinical samples and in vivo tumor samples), the slides were coded prior to analysis to blind researchers to treatment groups and scored digitally to prevent potential bias. For in vitro experiments, blinding was not possible as the same researchers that treated the cells ran the analysis. However, all analysis within experiments was run at the same time in an automated manner, so prior knowledge of treatment groups had no impact on data output.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used EZH2, 5246S, Cell Signaling Technology, IHC, 1:200

Antibodies used

PD-L1, 13684S, Cell Signaling Technology, IHC, 1:400
 dsRNA, J2, Scicons, FC, IF, 1:100
 CD3, 100210, BioLegend, IF, 5 µg/mL
 CD4, 100446, BioLegend, IF, 5 µg/mL
 CD8-alpha, D4W2Z, Cell Signaling Technology, IF, 1:200
 Arginase-1, D4E3M, Cell Signaling Technology, IF, 1:50
 iNOS, D6B6S, Cell Signaling Technology, IHC, 1:200
 Tri-Methyl-Histone H3 (Lys27), 12158S, Cell Signaling Technology, FC, IHC, IF 1:100
 Cleaved Caspase 3, 9602S, Cell Signaling Technology, FC, IHC, 1:100
 Ki-67, 12075, Cell Signaling Technology, FC, 1:100
 Ki-67, 12075, Cell Signaling Technology, IHC, 1:200
 STING/TMEM173, NBP-24683, Novus Biologicals, WB, 1:1000
 MHC-I, sc-59199, Santa Cruz, FC, 1:100
 PD-L1 (mouse), 124308, BioLegend, FC, 0.25 µg/106 cells
 PD-L1 (human), 374512, BioLegend, FC, 5 µL/106 cells
 CD45, 103122, BioLegend, FC, 0.25 µg/106 cells
 CD45, 103138 BioLegend, FC, 1:100
 CD3, 100306 BioLegend, FC, 1:100
 mCD8, 100750 BioLegend, FC, 1:100
 hCD8, 301010 BioLegend, FC, 1:100
 CD4, 100548 BioLegend, FC, 1:100
 PD-1, 135228 BioLegend, FC, 1:100
 CD11b, 101263 BioLegend, FC, 1:100
 Ly6C, 128044 BioLegend, FC, 1:100
 Foxp3, 20-0191-U100, Tonbo Biosciences, FC, 1:100
 Ly6G, 80-5931-U100, Tonbo Biosciences, FC, 1:100
 4-1BB, 106106, BioLegend, FC, 1:100
 I-A/I-E, 107632, BioLegend, FC, 1:100
 anti-PD-1, BE0273, BioXCell, in vivo, 20 µg/treatment
 anti-PD-1, BE0273, BioXCell, MLR, 10 µg/mL
 IgG, BE0089, BioXCell, in vivo, 20 µg/treatment
 IgG, BE0089, BioXCell, MLR, 10 µg/mL
 Alexa Fluor 488 Goat anti-Rabbit, A-11008, Thermo Fisher, IF, FC, 1:200
 Alexa Fluor 594 Goat anti-Rabbit, A-11012, Thermo Fisher, IF, FC, 1:200

Validation

All antibodies that were used were recommended for the specific application by the manufacturer or had previously been published for the application used in this study. Matched IgG controls were used alongside target antibodies to help confirm specificity. Relevant validation/manufacturer/citation information:

EZH2, 5246S, Cell Signaling Technology
 - Peng, S., Wang, R., et al. 2019, EGFR-TKI resistance promotes immune escape in lung cancer via increased PD-L1 expression, Molecular Cancer
 - <https://www.cellsignal.com/products/primary-antibodies/ezh2-d2c9-xp-rabbit-mab/5246>
 PD-L1, 13684S, Cell Signaling Technology
 - <https://www.cellsignal.com/products/primary-anti-bodies/pd-l1-e ll 3n-xp-ra bbit-mab/13684>
 dsRNA, J2, Scicons
 - The J2 anti-dsRNA IgG2a monoclonal antibody is considered the Gold Standard in dsRNA detection.
 - <https://scicons.eu/en/antibodies/j2/>
 CD3, 100210, Biolegend
 - <https://www.biolegend.com/en-us/products/alexa-fluor-488-anti-mouse-cd3-antibody-2835>
 CD4, 100446, Biolegend
 - <https://www.biolegend.com/en-us/products/alexa-fluor-594-anti-mouse-cd4-antibody-9412>
 CD8alpha, D4W2Z, Cell Signaling Technology
 - <https://www.cellsignal.com/products/primary-antibodies/cd8a-d4w2z-xp-rabbit-mab-mouse-specific/98941>
 Arginase-1, D4E3M, Cell Signaling Technology
 - <https://www.cellsignal.com/products/primary-anti-bodies/argi-nase-1-d4e3m-xp-rabbit-mab/93668>
 iNOS, D6B6S, Cell Signaling Technology
 - <https://www.cellsignal.com/products/primary-antibodies/inos-d6b6s-rabbit-mab/13120>
 Tri-Methyl-Histone H3 (Lys27), 12158S, Cell Signaling Technology
 - <https://www.cellsignal.com/products/antibody-conj-ugates/tri-methyl-histone-h3-lys2-7-c36b-11-rabbit-mab-alexa-fluor-64-7-conj-ugate/12158>
 Cleaved Caspase 3, 9602S, Cell Signaling Technology
 - <https://www.cellsignal.com/products/antibody-conj-ugates/cleaved-caspase-3-aspl-75-d3e9-rabbit-mab-a-lexa-fl-64-7-conj-ugate/9602>
 Ki-67, 12202, Cell Signaling Technology
 - <https://www.cellsignal.com/products/primary-antibodies/ki-67-d3b5-rabbit-mab-mouse-preferred-ihc-formulated/12202> STING/
 TMEM173, NBP-24683, Novus Biologicals
 - Reinert LS, Lopusna K, Winther H et al. 2016, Sensing of HSV-1 by the cGAS-STING pathway in microglia orchestrates antiviral defence in the CNS, Nat Commun.
 - https://www.novusbio.com/products/sting-tmeme1-73-antibody_nbp2-24683
 MHC-I, sc-59199, Santa Cruz
 - <https://www.scbt.com/p/mhc-class-i-antibody-er-hr52>
 PD-L1 (mouse), 124308, Biolegend
 - <https://www.biolegend.com/en-us/products/pe-anti-mouse-cd2-7-4-b-7-h-1--pd-l1-a-ntibody-4497>
 PD-L1 (human), 374512, Biolegend
 - <https://www.biolegend.com/en-us/products/pe-anti-human-cd2-7-4-b-7-h-1--pd-l-1-antibody-15986>

CD45, 103122, Biolegend
 - <https://www.biolegend.com/en-us/products/alexa-fluor-488-anti-mouse-cd45-antibody-3100>
 CD45, 103138, Biolegend
 - <https://www.biolegend.com/en-us/products/bril-liant-violet-510-anti-mouse-cd45-anti-body-7995>
 CD3, 100306, Biolegend
 - <https://www.biolegend.com/en-us/products/fitc-anti-mouse-cd3epsilon-antibody-23>
 mCD8, 100750, Biolegend
 - <https://www.biolegend.com/en-us/products/bril-liant-violet-785-anti-mouse-cd8a-anti-body-795-7>
 CDS (human), 301010, Biolegend
 - <https://www.biolegend.com/en-us/products/pe-cy5-anti-human-cd8a-antibody-837>
 CD4, 100548, Biolegend
 - <https://www.biolegend.com/en-us/products/bril-liant-violet-605-anti-mouse-cd4-anti-body-7627>
 PD-1, 135228, Biolegend
 - <https://www.biolegend.com/it-it/products/pe-dazzle-594-anti-mouse-cd279-pd-1-anti-body-12090>
 CD11b, 101263, Biolegend
 - <https://www.biolegend.com/en-us/products/bril-liant-violet-510-anti-mouse-human-cd11b-antibody-7993> Ly6C, 128044, Biolegend
 - <https://www.biolegend.com/en-us/products/sea-orchestra-ly6c-anti-mouse-ly6c-anti-body-12513> Foxp3, 20-0191-UI00, Tonbo Biosciences
 - <https://tonbobio.com/products/apc-anti-mouse-foxp3-mf23>
 Ly6G, 80-5931-UI00, Tonbo Biosciences
 - <https://tonbobio.com/products/redfluor-710-anti-mouse-ly6g-gr-1-rb6-8c5>
 4-1BB, 106106, Biolegend
 - <https://www.biolegend.com/en-us/products/pe-anti-mouse-cd137-antibody-511-A/I-E>, 107632, Biolegend
 - <https://www.biolegend.com/en-us/products/bril-liant-violet-421-anti-mouse-ia-anti-body-714-7> anti-PD-1, BE0273, BioXCell
 - This anti-PD-1 antibody is validated specifically for in vivo therapeutic applications.
 - <https://bxccl.com/product/invivomab-anti-mouse-cd279-pd-1/>
 IgG, BE0089, BioXCell
 - <https://bxccl.com/product/rat-igg2a-isotype-control/>

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)

The Pten^{-/-} and B6MYC-CaP murine cell lines were generated by Dr. Leigh Ellis at Roswell Park Cancer Institute, as previously described (Ku et. al. 2017, Science; Ellis et. al. 2016, Prostate).

The murine 3D organoids were generated in the Ellis lab at Dana-Farber Cancer Institute. The EMC organoids were generated from the dorsolateral prostates of 8-week-old GEMMs. The Pten^{-/-} organoids were generated from end-stage GEMM prostate tumors at 61 weeks-of-age.

The human 3D organoids were generated in the Ellis laboratory at Dana-Farber Cancer Institute from 2 independent patient samples - a prostatectomy and a pleural effusion sample.

The LNCaP cell line was obtained from ATCC.

Authentication

2D and organoid cell lines generated from GEMMs were routinely genotyped by PCR to confirm identity based on Myc/Ezh2/Pten status.

PCR primers used:

MYC - Forward – AAACATGATGACTACCAAGCTTGG

MYC – Reverse – ATGATAGCATCTTGTCTTAGCTTTTCTTAATAGGG

EZH2 – Forward – CATGTGCAGCTTCTGTCTCA

EZH2 – Reverse – CACAGCCTTCTGCTCACTG

Pten – Forward – CAAGCACTCTGCGAACTGAG

Pten – Reverse – AAGTTTTGAAGGCAAGATGC

Human 3D organoids were derived from primary tissues and used directly in experiments. The LNCaP cell line was not independently authenticated as it was obtained directly from ATCC.

Mycoplasma contamination

All 2D cell lines and murine 3D organoids (including Pten^{-/-}, B6MYC-CaP and LNCaP 2D lines and EMC and Pten^{-/-} 3D organoid lines) tested negative for mycoplasma contamination using the Venor™ GeM Mycoplasma PCR Detection Kit (Sigma Aldrich). The human 3D organoids were not tested for mycoplasma contamination.

Commonly misidentified lines
(See [ICLAC](#) register)

This study did not use commonly misidentified cell lines.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals

C57BL/6N 6-week-old male mice (mus musculus, strain 027 from Charles River Laboratories)

FVB 10-12-week-old male mice (mus musculus, strain 207 from Charles River Laboratories)

Wild animals

This study did not use wild animals

Field-collected samples	This study did not use field-collected samples.
Ethics oversight	The Institute of Animal Care and Use Committee (IACUC) at Dana-Farber Cancer Institute approved all mouse procedures (Protocol number 17-003)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

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

Population characteristics	Fresh-frozen radical prostatectomy specimens from patients with localized prostate cancer for organoid generation were obtained from the Dana-Farber Cancer Institute Gelb Center biobank.
Recruitment	N/A
Ethics oversight	Samples were obtained under Dana-Farber Cancer Institute/Harvard Cancer Center IRB-approved protocols (Protocol Numbers: 01-045, 09-171). All remaining patient samples/data used in this manuscript had been collected with patient consent obtained by the respective studies.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☐ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	In vitro analysis: Organoids were digested to single cells, then were washed with PBS by centrifugation and fixed with 4% paraformaldehyde for 15 minutes. Cells were permeabilized by the addition of ice-cold methanol to a final concentration of 90% methanol. This suspension was incubated for 30 minutes on ice. Following washes with FACS buffer (PBS supplemented with 10% FBS), cells were resuspended in primary antibody or appropriate IgG control prepared in FACS buffer. Cell suspensions were incubated overnight in the dark at 4t or for 1 hour at room temperature. Cells were washed two additional times in FACS buffer and resuspended in PBS prior to cell analysis. For EdU analysis, samples were stained using the Click-iT™ EdU Cell Proliferation Kit (ThermoFisher). In vivo analysis: Tumors were mechanically dissociated and filtered into single-cell suspensions in PBS on ice. Cells were washed with PBS by centrifugation. For staining of surface targets, live cells were washed with FACS buffer (PBS supplemented with 10% FBS) and incubated with primary antibody or appropriate IgG control for 30 minutes at on ice, cells were washed and incubated with a secondary antibody where required. Cells were subsequently fixed with 4% paraformaldehyde, washed twice in FACS buffer and resuspended in PBS for analysis. For intracellular targets, cells were fixed with 4% paraformaldehyde for 15 minutes and were permeabilized by the addition of ice-cold methanol to a final concentration of 90% methanol. The cell suspension was incubated for 30 minutes on ice. Following washes with FACS buffer, cells were resuspended in primary antibody or appropriate IgG control prepared in FACS buffer and incubated overnight in the dark at 4t or for 1 hour at room temperature. Cells were washed two additional times in FACS buffer and resuspended in PBS prior to cell analysis. In vivo tumor immune profiling: Tumor cell suspensions were stained using two different antibody panels: lymphocytes or myeloid, using appropriate IgG and full minus one (FMO) controls. Antibodies for the various immune panels are as follows: lymphocyte panel (Ghost Dye™ Red 780, anti-human CD8 (dump channel), anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD45, anti-mouse PD-1); myeloid panel (Ghost Dyem Red 780, anti-human CD8, anti-mouse CD11b, anti-mouse CD45, anti-mouse Ly6C, anti-mouse Ly6G, anti-mouse I-A/I-E). After surface staining, fixation, and permeabilization (BD Cytfix and BD Cytoperm), cells were stained for the following intracellular markers: lymphocyte panel (Foxp3, Ki67, or the appropriate IgG controls).
Instrument	In vitro analysis: H3K27me3 and EdU were analyzed using an Amnis ImageStream Mark II (Luminex, Rosewell Park Cancer Institute), dsRNA and PD-L1 were analysed with a BD LSRFortessa (BD Biosciences, Dana-Farber Cancer Institute). in vivo analysis: All samples were analyzed on a BD LSRFortessa (BD Biosciences, Dana-Farber Cancer Institute). In vivo tumor immune profiling:

All samples were analysed on a LSRII flow cytometer (BD Biosciences, Emory University School of Medicine)

Software

In vitro analysis:

H3K27me3 and EdU were analyzed using IDEAS Analysis Software (Luminex), dsRNA and PD-L1 were analysed with BD FACSDiva Software (BD Biosciences) and FlowJo Software (FlowJo)

In vivo analysis:

All samples were analysed with BD FACSDiva Software (BD Biosciences) and FlowJo Software (FlowJo)

In vivo tumor immune profiling:

All samples were analysed with FlowJo Software (FlowJo)

Cell population abundance

The gating strategy selects viable and single cell population. For in vitro studies, this population made up 72-83% of the total starting populations. For in vivo studies, this population made up 39-52% of the total starting populations.

Gating strategy

In vitro analysis:

Cells were gated based on singlets, size/nucleation (viability)

In vivo analysis:

Cells were gated based on singlets, size/nucleation (viability). Cells were separated into "CD45-" and "CD45+" events.

In vivo tumor immune profiling:

Cells were gated based on singlets, size/nucleation (viability), Ghost Dye'M Red 780 negative events, and dump negative events ("Live events"). Cells were then separated into "CD45-" and "CD45+" events, and immune populations were defined as follows: CD3+CD4+ T cells, CD3+CD8+ T cells, CD3+CD4+Foxp3+ Treg, Granulocytic MDSC (CD11b+ MHCII- Ly6Clo Ly6G+), Monocytic MDSC (CD11b+ MHCII- Ly6G-).

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Sequential Prostate Magnetic Resonance Imaging in Newly Diagnosed High-risk Prostate Cancer Treated with Neoadjuvant Enzalutamide is Predictive of Therapeutic Response



Fatima Karzai¹, Stephanie M. Walker², Scott Wilkinson³, Ravi A. Madan¹, Joanna H. Shih⁴, Maria J. Merino⁵, Stephanie A. Harmon⁶, David J. VanderWeele³, Lisa M. Cordes¹, Nicole V. Carrabba³, John R. Bright³, Nicolas T. Terrigino³, Guinevere Chun¹, Marijo Bilusic¹, Anna Couvillon¹, Amy Hankin¹, Monique N. Williams¹, Rosina T. Lis⁷, Huihui Ye⁸, Peter L. Choyke², James L. Gulley¹, Adam G. Sowalsky³, Baris Turkbey², Peter A. Pinto⁹, and William L. Dahut¹

ABSTRACT

Purpose: For high-risk prostate cancer, standard treatment options include radical prostatectomy (RP) or radiotherapy plus androgen deprivation therapy (ADT). Despite definitive therapy, many patients will have disease recurrence. Imaging has the potential to better define characteristics of response and resistance. In this study, we evaluated prostate multiparametric MRI (mpMRI) before and after neoadjuvant enzalutamide plus ADT.

Patients and Methods: Men with localized intermediate- or high-risk prostate cancer underwent a baseline mpMRI and mpMRI-targeted biopsy followed by a second mpMRI after 6 months of enzalutamide and ADT prior to RP. Specimens were sectioned in the same plane as mpMRI using patient-specific 3D-printed molds to permit mpMRI-targeted biopsies to be compared with the same lesion from the RP. Specimens were analyzed for imaging and histologic correlates of response.

Results: Of 39 patients enrolled, 36 completed imaging and RP. Most patients (92%) had high-risk disease. Fifty-eight lesions were detected on baseline mpMRI, of which 40 (69%) remained measurable at 6-month follow-up imaging. Fifty-five of 59 lesions (93%) demonstrated >50% volume reduction on posttreatment mpMRI. Three of 59 lesions (5%) demonstrated growth in size at follow-up imaging, with two lesions increasing more than 3-fold in volume. On whole-mount pathology, 15 patients demonstrated minimal residual disease (MRD) of <0.05 cc or pathologic complete response. Low initial mpMRI relative tumor burden was most predictive of MRD on final pathology.

Conclusions: Low relative lesion volume at baseline mpMRI was predictive of pathologic response. A subset of patients had limited response. Selection of patients based on these metrics may improve outcomes in high-risk disease.

Introduction

Prostate cancer is the second leading cause of cancer-related death in individuals who are born biologically male in the United States, with an estimated 191,930 new cases and 33,330 deaths in 2020 (1). While radical prostatectomy or radiotherapy are potentially curative modalities for patients with localized disease, 20%–40% of patients treated with radical prostatectomy or radiotherapy will ultimately develop castration-resistant prostate cancer (2). It is imperative to evaluate new treatment strategies, particularly in individuals with high-risk disease. Androgen deprivation therapy (ADT), combined with newer generation androgen receptor (AR) axis inhibitors, such as abiraterone and enzalutamide, is widely used in metastatic castration-resistant prostate cancer (3, 4), metastatic castration-sensitive prostate cancer (5), and nonmetastatic castration-resistant prostate cancer (6). More recent studies have shown promise in reducing tumor burden and cancer stage in the neoadjuvant setting, prior to radical prostatectomy (7–10).

In particular, neoadjuvant systemic therapy has been evaluated in multiple clinical trials as a primary intervention to reduce morbidity and mortality of prostate cancer in intermediate- and high-risk patients. These studies have focused on the intensification of ADT with more potent AR axis inhibitors. A recurrent outcome measure in these trials has been the determination of the pathologic complete response (pCR) rate, which implies the complete elimination of disease, although a threshold for specifying minimal residual disease (MRD) similarly identifies exceptional responders. In an exploratory pooled analysis of three neoadjuvant studies (11), neoadjuvant

¹Genitourinary Malignancies Branch, Center for Cancer Research, NCI, NIH, Bethesda, Maryland. ²Molecular Imaging Program, NCI, NIH, Bethesda, Maryland. ³Laboratory for Genitourinary Cancer Pathogenesis, NCI, NIH, Bethesda, Maryland. ⁴Division of Cancer Treatment and Diagnosis, Biometric Research Program, NCI, NIH, Rockville, Maryland. ⁵Laboratory of Pathology, NCI, NIH, Bethesda, Maryland. ⁶Clinical Research Directorate/Clinical Monitoring Research Program, Leidos Biomedical Research, Inc., NCI Campus at Frederick, Frederick, Maryland. ⁷Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. ⁸Department of Pathology, Ronald Reagan UCLA Medical Center, Los Angeles, California. ⁹Urologic Oncology Branch, NCI, NIH, Bethesda, Maryland.

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F. Karzai and S.M. Walker contributed equally as co-first authors of this article.

P.A. Pinto and W.L. Dahut contributed equally as co-senior authors of this article.

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Corresponding Author: William L. Dahut, Center for Cancer Research, NCI, CRC 3-2571, 10 Center Drive, Bethesda, MD 20814. Phone: 301-222-3233; E-mail: dahutw@mail.nih.gov

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Translational Relevance

In high-risk localized prostate cancer, despite definitive therapy with either radical prostatectomy or radiotherapy plus androgen deprivation therapy (ADT), many patients will have recurrence of disease. Androgen receptor (AR) pathway inhibitors are increasingly being studied in men prior to prostatectomy. Studies suggest that intense ADT prior to prostatectomy may have a positive impact on time to biochemical recurrence. Incorporating imaging modalities, such as multiparametric MRI (mpMRI), to neoadjuvant AR-directed therapies may help with selection of patients who would benefit the most from this type of treatment. In this study, men with localized intermediate- or high-risk prostate cancer underwent a baseline mpMRI and mpMRI-targeted biopsy followed by a second mpMRI after 6 months of enzalutamide and ADT prior to robot-assisted radical prostatectomy. We found that low relative lesion volume at baseline mpMRI was predictive of pathologic response and may be a consideration when selecting patients for future neoadjuvant studies.

treatment prior to radical prostatectomy showed a positive impact on time to biochemical recurrence (BCR). Patients below the MRD threshold did not experience BCR in the timeframe of the meta-analysis, suggesting that complete response (CR) or MRD is a short-term surrogate outcome for BCR.

Utilizing intense ADT with enzalutamide in the neoadjuvant setting in combination with a dedicated imaging modality, such as multiparametric MRI (mpMRI), is a potentially promising strategy in high-risk localized disease. There is a critical need to incorporate newer imaging modalities to better define the initial tumor burden and subsequent response. While mpMRI has been successfully used to identify high-risk disease for sampling by biopsy (12), the role of mpMRI in the setting of neoadjuvant therapy is unclear. This feasibility trial was designed to evaluate tumors on mpMRI both before and after treatment with enzalutamide plus ADT prior to robot-assisted radical prostatectomy (RARP), and to correlate imaging findings with final pathology findings.

Patients and Methods

Study approval

This trial was approved by the Institutional Review Board of the Center for Cancer Research, NCI (Bethesda, MD; ClinicalTrials.gov identifier: NCT02430480). This study has been conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with the International Council on Harmonisation guidelines on Good Clinical Practice, all applicable

laws and regulatory requirements, and all conditions required by a regulatory authority and/or institutional review board. Written informed consent was obtained from all patients prior to performing study-related procedures in accordance with federal and institutional guidelines.

Study design

In this study, men with newly diagnosed intermediate- or high-risk localized prostate cancer were treated for 6 months with complete androgen blockade with enzalutamide, a potent AR antagonist, plus ADT with goserelin, leuprolide, or degarelix (based on discretion of the treating physician), followed by RARP. Patients underwent two mpMRIs, one at baseline and one after 6 months of treatment. Each patient underwent subsequent RARP with whole-mount histopathologic analysis to assess lesion response.

Eligible patients had pathologically confirmed intermediate- or high-risk localized prostate cancer with no prior history of treatment for prostate cancer. Intermediate-risk disease was defined as a PSA level of 10–20 ng/mL or a Gleason score of 7 or clinical stage T2b or T2c. High-risk disease was defined as PSA > 20 ng/mL at diagnosis or Gleason score of 8–10 or seminal vesicle involvement or possible extracapsular extension (on mpMRI). Patients were required to have an Eastern Cooperative Oncology Group performance status of 0–1 and baseline testosterone levels of ≥ 100 ng/dL. Patients with distant metastatic disease beyond N1 (regional) lymph nodes on conventional imaging studies (CT, MRI, or bone scan) were excluded.

This was an open-label feasibility study of enzalutamide 160 mg orally daily $\times$ 28 days for a total of six cycles plus ADT. Thirty-seven patients received goserelin 10.8 mg subcutaneously at week 0 and week 12 on-treatment. One patient had leuprolide acetate depot 22.5 mg intramuscularly $\times$ two doses. One patient received the loading dose of degarelix (240 mg subcutaneously at week 0), followed by degarelix 80 mg subcutaneously $\times$ one dose at week 4 and was transitioned to goserelin at week 8. Patients underwent two separate mpMRIs of the prostate, at baseline and after 24 weeks, followed by RARP (Fig. 1). Patients who underwent MRI-targeted combined biopsy at the NCI (Bethesda, MD) for prostate cancer diagnosis did not require an additional biopsy prior to study enrollment. If a patient had not undergone an MRI-targeted biopsy previously, a second biopsy was required.

Surgery and pathologic analysis

Initial pathology was determined by transrectal ultrasound (TRUS)-guided systematic biopsy, if there was reasonable assurance that the biopsy was from the index lesion, and/or TRUS-MRI fusion-guided biopsy prior to initiation of neoadjuvant enzalutamide plus ADT. The index lesion by mpMRI was defined as the lesion with the largest size and highest Prostate Imaging Reporting and Data Systems (PI-RADS)

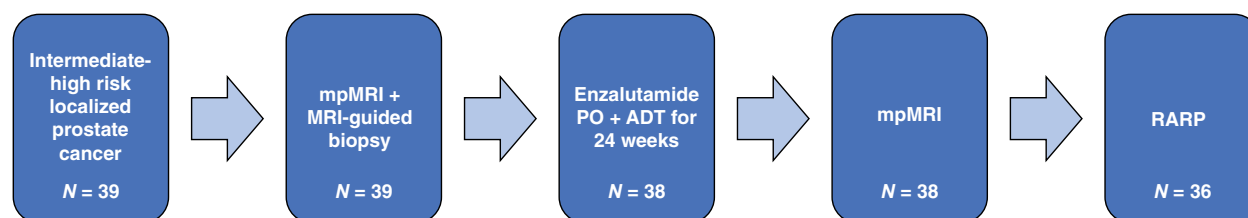


Figure 1.
Study schema.

score. All biopsy specimens were reviewed and assigned Gleason scores by a genitourinary pathologist.

RARP was performed on 36 patients after 24 weeks of complete androgen blockade and final mpMRI. A single urologist performed all, but one, RARP. All patients underwent extended lymph node dissection. Whole-mount prostate pathology slides were prepared using 3D-printed custom prostate molds to correspond with slices from each patient's final mpMRI scan (13).

All posttreatment specimens were analyzed by two nonblinded genitourinary pathologists. Postoperative pathologic staging was assigned to each patient; however, Gleason tumor grade could not be assigned to residual tumors due to treatment effect. Presence of residual tumor was noted and annotated on hematoxylin and eosin whole-mount pathology slides. For 3 patients who demonstrated substantial pathologic resistance to treatment, the NCI Laboratory of Pathology selected formalin-fixed, paraffin-embedded tissue blocks for panel-based OncoPrint Comprehensive Assay v3 (Thermo Fisher Scientific) sequencing to determine potential eligibility for ongoing clinical trials.

Image analysis of mpMRI

Prostate and lesion volumes on baseline and posttreatment mpMRIs were calculated from T2W-MRI sequences using software embedded in the PACS after manual contouring by the same radiologist (B. Turkbey). mpMRIs at baseline and after 24 weeks of neoadjuvant treatment were acquired with a 3-Tesla Scanner (Achieva; Philips Healthcare). Patients underwent imaging with integrated external (surface) phased array coil and endorectal coil. Imaging studies were interpreted by a single genitourinary radiologist with over 10 years of experience in prostate imaging. Evaluated imaging sequences included T2W-MRI, apparent diffusion coefficient (ADC) maps of diffusion-weighted imaging, high b value ($b = 2,000$ seconds/mm²), and dynamic contrast-enhanced MRI (temporal resolution = 5.6 seconds). Lesions at baseline mpMRI were assigned PI-RADS categories based on stringent interpretation of PI-RADSv2.0 guidelines (ref. 14; PI-RADSv2.1 was published near the end of this study).

All MRI visible lesions at baseline were documented and followed over the course of the study. Evidence of extraprostatic extension (EPE), seminal vesical invasion, and bulky nodal disease was noted in mpMRI reports. Each mpMRI scan was assigned an EPE grade from 0 to 3, as described previously (15): 0, no evidence of EPE; 1, large lesion-capsule contact length or capsular bulge; 2, large lesion-capsule contact length and capsular bulge; and 3, frank EPE. Only EPE grade 0 was considered "organ-confined disease." Per PI-RADSv2 guidelines, up to four MRI visible lesions per patient were targeted for biopsy and were included in lesion-level analyses, irrespective of biopsy Gleason score. Biopsy targeting was performed using a commercially available TRUS/MRI-fusion Biopsy Platform, UroNav (InVivo, Philips). Prior to biopsy, whole prostate gland MRI and MRI visible lesions were manually segmented using the DynaCad Platform (InVivo).

In addition to these qualitative mpMRI characteristics, higher order quantitative metrics were obtained from baseline mpMRI scans, including lesion volume, ADCs, and perfusion (Ktrans; calculated using a two-compartment Tofts model with standardized arterial input function). In patients with multiple contoured lesions at baseline, their volumes were added to produce a patient-level mpMRI lesion volume. To assess MRI evidence of neoadjuvant treatment response, post-treatment mpMRI scans were acquired with the same parameters; however, PI-RADS categories were not assigned because of treat-

ment effects on MRI. On both mpMRI scans, total prostate volume was measured. Relative tumor burden (RTB) volumes were calculated by dividing the combined volumes of each lesion by the prostate volume, such that RTB is expressed as the percentage of prostate volume taken by the tumor.

Residual cancer burden calculations

Residual tumor was concordantly identified by three board-certified genitourinary pathologists (M.J. Merino, R.T. Lis, and H. Ye). Pathologic analysis was performed independently from imaging analysis, and pathologists did not have access to imaging data to inform tissue analysis. All prostate specimens were completely submitted for identification of residual disease. Residual cancer burden (RCB) was measured and calculated by multiplying the number of slices through which each residual tumor extended by the largest cross-sectional width and length and block thickness (0.6 cm). Volume was further corrected by multiplying by 0.4 and the estimated tumor cellularity. MRD was considered <0.05 cc, and nonresponse (NR) was >0.05 cc. Each mpMRI visible lesion was similarly classified on the basis of the volume of the evaluable tumor remaining in the anatomic region the lesion was in. If a patient had more than one residual lesion at final pathology, the largest cross-sectional dimension of tumor was used to determine RCB for the purpose of classifying the patient as MRD or NR.

Statistics on mpMRI

Logistic regression analysis was performed to correlate clinical variables and MRI parameters, with patient response categorized as CR/MRD versus NR. Variables which were significant at the 0.05 level in the univariable analysis were considered for multivariable analysis. The performance of predictors was evaluated by the AUC with its 95% confidence interval determined by the DeLong method. The Youden index was calculated to determine the cutoffs that gave the optimal combination of sensitivity and specificity for patient response. A two-sided $P < 0.05$ was considered statistically significant for all analyses. All analyses were conducted using R 3.6.1 (R Foundation for Statistical Computing).

Results

Between June 2015 and September 2018, 39 patients with newly diagnosed localized prostate cancer were enrolled on this study and treated with enzalutamide plus ADT (see Fig. 1). The median baseline PSA was 9.56 ng/mL (1.18–985.9 ng/mL). The median grade group was 4 (Gleason score 8). Thirty-six patients had high-risk disease and 3 had intermediate-risk disease (Table 1). Three patients did not complete RARP. One patient died from a recreational drug overdose during the first month of treatment. A second patient was unable to undergo RARP due to anesthesia concerns, and 1 patient progressed on-treatment. A third patient exhibited tumor extension to the bladder while on treatment and underwent palliative transurethral resection of the prostate/transurethral resection of a bladder tumor (TURP/TURBT) due to the extent of the prostate cancer, and, thus, did not undergo RARP, so final pathology volumes were not available. He went on to receive radiotherapy to the pelvis.

Of 39 patients enrolled on-study, 3 had disease progression with enlargement of lesions on mpMRI (Fig. 2A). All three nonresponders recurred during the first year. One patient progressed during treatment (as above) and 2 progressed shortly after RARP. In addition, to date, 9 patients had recurred biochemically; 2 patients in the first year, 5 patients in the second year, and 2 patients during the third year. The

Table 1. Baseline patient characteristics for 39 patients with localized prostate cancer.

Demographics	
Median age	Year (IQR)
Race	% (N)
White	69 (27)
Black	15 (6)
Other	15 (6)
Clinical	
Median PSA (ng/mL)	# (IQR)
Median PSA density	9.56 (1.18–985.9)
Median prostate volume (cc)	0.22 (0.15–0.39)
Median MRI tumor burden (cc)	42.0 (33.0–49.0)
Risk group	3.47 (1.9–9.8)
Intermediate	% (N)
High	8 3
92 36	
Histology	
Pathologic stage	% (N)
T2	15 (6)
T3a	44 (17)
T3b	28 (11)
T4	12 (5)
N1	28 (11)
Gleason score (grade group)	% (N)
3 + 4 (2)	15.4 (6)
4 + 3 (3)	12.8 (5)
4 + 4 (4)	28.2 (11)
4 + 5; 5 + 4; 5 + 5 (5)	46.6 (17)

Note: Data are presented as *N* (IQR) or % (*N*).

Abbreviation: IQR: interquartile range.

median follow-up was 3 years. Two of the nonresponders went on to develop metastatic disease and 1 has died.

Adverse events

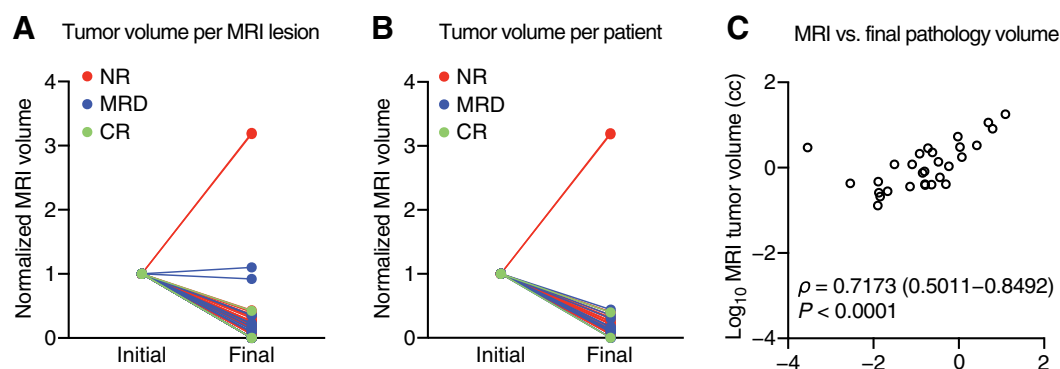
Treatment was well-tolerated, with no grade 4 events and one grade 5 event not attributable to study treatment (Supplementary Table S1). The most common adverse events were grade 1 hot flashes (34/39,

87%), grade 1 fatigue (20/39, 51%), grade 1 decrease in libido (15/39, 38%), grade 1 insomnia (12/39, 31%), and grade 1 erectile dysfunction (9/39, 23%). Grade 2 and grade 3 hypertension (18% each) was manageable with effective antihypertensive medication. No patients discontinued therapy because of toxicity. Sexual function assessment was captured through the Common Terminology Criteria for Adverse Events and not through sexual function assessment.

Response to neoadjuvant treatment on mpMRI

Within the study population, 59 lesions were detected at baseline mpMRI and 40 remained measurable at follow-up mpMRIs after 24 weeks of enzalutamide plus ADT. Fifty-five of 59 lesions (93%) demonstrated greater than 50% volume reduction on posttreatment mpMRI. Three of 59 lesions (5%) demonstrated growth in size at follow-up imaging, with two of the lesions increasing more than 3-fold in volume (**Fig. 2A**). At baseline mpMRI, all patients had measurable disease, with a median tumor burden of 3.44 cc. Median prostate volume decreased from 39.5 to 20 cc after treatment, and median PSA decreased from 9.56 ng/mL to undetectable (<0.02 ng/mL). On posttreatment MRI, 4 of 36 patients (11%) had no visible disease, and on final pathology, 2 of 36 patients (6%) were staged as a complete pathologic response (ypT0N0). Six of 36 patients (17%) had no evidence of EPE on baseline mpMRI, and this number increased to 21 patients (58%) without EPE at mpMRI after treatment. Twenty-three patients (64%) had organ-confined disease on final pathology. On baseline mpMRI, 4 patients (11%) had evidence of bladder or rectal invasion; only 1 patient had visible bladder or rectal invasion on follow-up imaging and on final pathology. **Table 2** summarizes mpMRI evidence of pathologic features before and after treatment, including EPE, SVI lymph node involvement, etc., and corresponding findings at final pathology.

Upon final pathologic analysis, nine lesions (15%) demonstrated CR, 30 lesions (52%) demonstrated MRD, and 19 lesions (33%) demonstrated NR (**Fig. 2A**). At the patient level, 2 patients (6%) demonstrated CR, 13 patients (36%) demonstrated MRD, and 22 patients (61%) demonstrated NR, including 1 patient who underwent TURP/TURBT for prostate cancer (**Fig. 2B**). Two patients with NR were determined by IHC of posttreatment tissue to have decreased AR and PSA expression in combination for positive staining for synaptophysin and Ki-67, which indicated

**Figure 2.**

Response to neoadjuvant enzalutamide plus ADT. **A**, Fifty-five of 59 lesions demonstrated >50% volume reduction. **B**, Thirty-six of 38 patients demonstrated >50% volume reduction. Lesions and patients are color coded on the basis of pathologic response to treatment at radical prostatectomy. One patient completed baseline and post-enzalutamide plus ADT mpMRI, but did not undergo RARP, thus he was included to demonstrate MRI evidence of extreme NR, but was excluded from all analyses involving pathologic response to treatment. **C**, Spearman correlation of final imaging volumes per patient with final pathologic RCB, logarithmically transformed. Values of zero were transformed to nominal values to preserve their rank within the dataset, but omitted from the graph.

Table 2. mpMRI detection of pathologic features before and after complete androgen blockage and corresponding findings on whole-mount RARP specimens ($N = 36$).

Feature	Baseline	Posttreatment	Final pathology
No visible disease	0 (0%)	4 (11%)	2 (6%)
Organ-confined disease	6 (17%)	21 (58%)	23 (64%)
Seminal vesical invasion	10 (28%)	6 (17%)	8 (22%)
Regional lymph node involvement	10 (28%)	7 (19%)	5 (14%)
Bladder/rectal invasion	4 (11%)	1 (3%)	1 (3%)

neuroendocrine differentiation (16, 17). The combined volumes of each imaging lesion per patient demonstrated a statistically significant correlation with final pathology RCBs with Spearman $\rho = 0.7173$ (Fig. 2C).

For the 3 NR patients (Supplementary Table S3) who demonstrated substantial volumes of residual disease or progression on treatment, we performed clinical cancer panel sequencing on posttreatment surgical tissue using the OncoPrint platform to assess potential drivers and determine eligibility for additional trials. Germline alterations to homologous recombination genes were observed in 2 of the 3 patients (*BRCA2* frameshift and *PALB2* nonsense), with loss-of-function alterations to *RBI* observed in all three cases. In 1 patient whose radical prostatectomy specimen demonstrated focal neuroendocrine features, biallelic inactivation of *TP53*, *PTEN*, and *RBI* was observed in conjunction with the *TPR22-ERG* fusion.

Pathology and IHC results

As enzalutamide plus ADT synergize by reducing the concentration of testosterone, dihydrotestosterone, and androgens binding to the AR, the effect of intense androgen suppression and inhibition was measured on tumors using anti-AR and anti-PSA immunostains of biopsy and surgical specimens (Fig. 3A and B). Median nuclear AR levels were substantially higher in diagnostic biopsy specimens versus residual tumor (staining indices of 0.7568 and 0.1661, respectively, $P < 0.0001$ by Welch t test; Fig. 3C). As the *KLK3* gene, encoding the PSA protein, is a primary target of AR-driven transcription, anti-PSA IHC was performed to assess AR activity *in situ*. Like AR, median cytoplasmic PSA histology scores of residual tumors were substantially lower compared with pretreatment tumor biopsies (0.1380 and 0.7062, respectively, $P < 0.0001$ by Welch t test), demonstrating reduced, but persistent AR activity (Fig. 3D).

TPR22-ERG fusion occurs in approximately 50% of prostate cancers and frequently cooccurs with the loss of *PTEN*, which, in turn, is associated with more aggressive prostate cancer (18). Therefore, ERG protein overexpression and *PTEN* reduction were evaluated using IHC in posttreatment specimens (Fig. 3E). Of 37 patients who completed treatment and underwent surgery, evaluable residual tumor was available in 32 cases, of which 14 (43.8%) harbored ERG-positive tumor cells and 21 (65.6%) demonstrated *PTEN* staining reduction or loss (Fig. 3F). Comparing pCR/MRD versus NR cases, reduction of *PTEN* showed significant differences ($P = 0.0006$, Fisher exact test) between the pCR/MRD group (two cases, 20%) versus the NR group (19 cases, 86.4%). However, the difference between groups was less with respect to ERG-positive tumor cell staining (20% vs. 54.5%, respectively; $P = 0.073$, Fisher exact test) and the combined reduction of *PTEN* with ERG-positive staining (8.3% vs. 50%, respectively; $P =$

0.0931, Fisher exact test). Therefore, reduction of *PTEN* levels is significantly associated with resistance to intense ADT driving greater residual tumor volumes.

Predicting patient-level response from baseline imaging

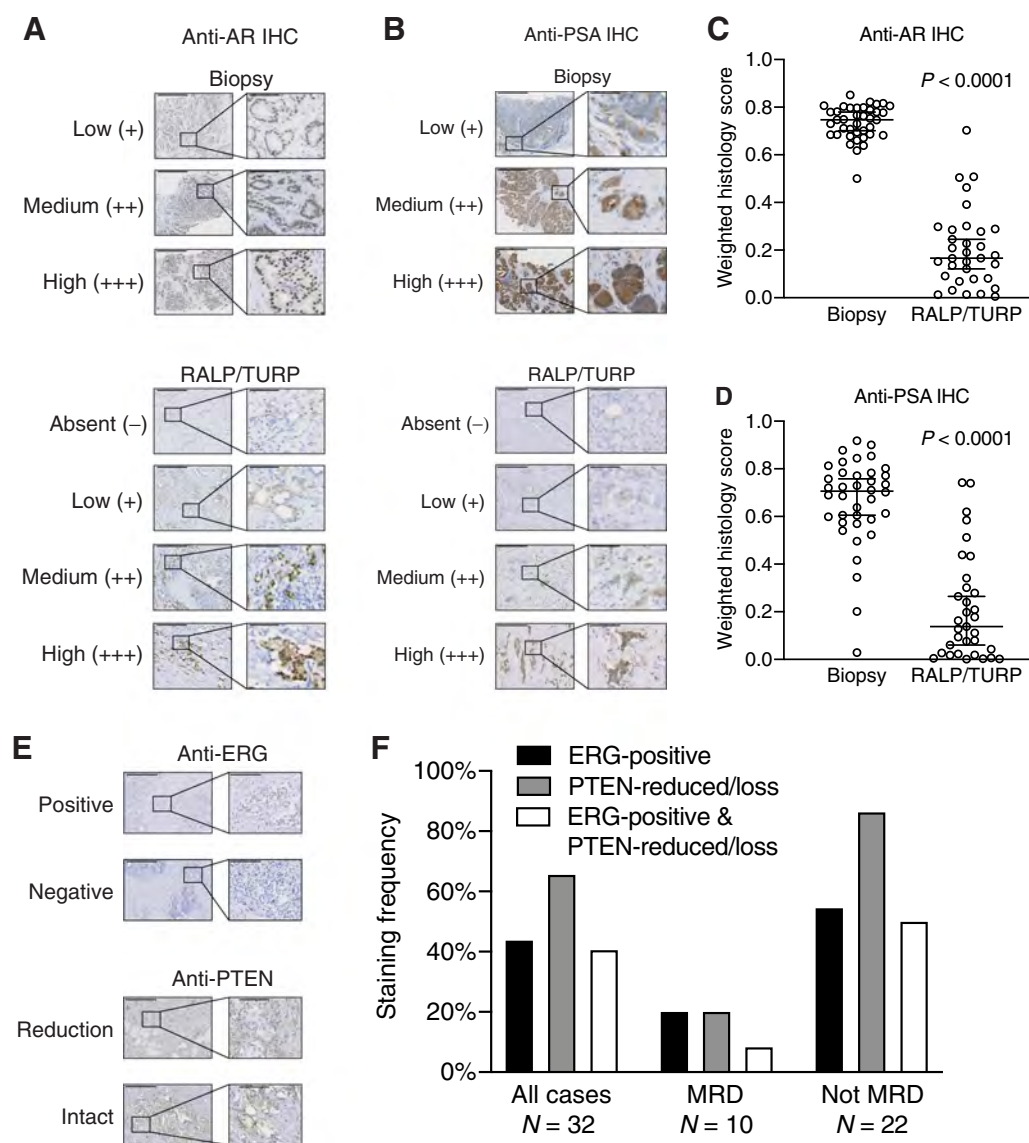
Although final imaging volumes correlated well with RCBs (see Fig. 1C), we performed extensive univariate analyses on baseline imaging features to determine whether final pathologic response could be predicted by mpMRI. Patient-level imaging characteristics associated with patient-level treatment response are listed in Supplementary Table S3. As depicted in Fig. 4A, low RTB (i.e., the combined volume of each tumor lesion divided by the total prostate volume) was the only predictor in the final multivariate logistic regression model [OR, 0.77 (0.63–0.94); $P = 0.001$]. Figure 4B shows that the AUC for RTB was 0.89 (0.79–0.99) at the optimal cutoff of 8.1% for RTB specificity (81%) and sensitivity (87%). Representative responder and nonresponder cases are shown in Fig. 4C, which were both high-risk, cT3a cases. The responder initial lesion volume was 1.56 cc, so with a baseline prostate volume of 46 cc, the RTB was 3.39%. In contrast, the nonresponder lesion volume was 7.13 cc at the start of treatment, and with a baseline prostate volume of 33 cc, the RTB was 21.6%.

Discussion

The effects of neoadjuvant therapies on prostate tumors are not uniform among patients or even in distinct cancer lesions within a patient's prostate (19). Information from mpMRI of the prostate after intense androgen deprivation can be used to assess treatment response on a per-lesion and per-patient basis, as well as for surgical planning. Predicting which patients will respond to neoadjuvant therapies based on baseline mpMRI characteristics could allow for better patient selection and overall responses to treatment in subsequent clinical trials, while patients who would not benefit from treatment would be spared from toxicities (20). mpMRI may also be able to identify patients with intrinsic resistance despite declines in PSA. This intrinsic resistance, as seen with androgen independence leading to tumor cell lineage plasticity, may play a role in resistance to AR-targeted therapy (21). Thus, perturbations in AR-regulated lineage characteristics observed phenotypically by less PSA expression and genomic loss of tumor suppressors, such as *RBI* and *TP53*, may represent aggressive prostate variants. Early identification of this high-risk population, aided by imaging, is paramount to treatment of this subset of patients.

We found that low RTB on mpMRI was the best predictor of response and was the only baseline characteristic that showed statistically significant predictive performance in multivariate analysis at both the lesion and patient levels. This was the strongest predictor of pathologic response, with an optimal cutoff of 8.1%, and may be a consideration when selecting patients for future neoadjuvant studies. Contouring of the prostate and lesions within the prostate on mpMRI to obtain a volume can be performed by radiologists with readily available software, and automated lesion segmentation software is becoming available. This makes baseline mpMRI an important prognostic tool for predicting tumor- and patient-level response to neoadjuvant intense ADT.

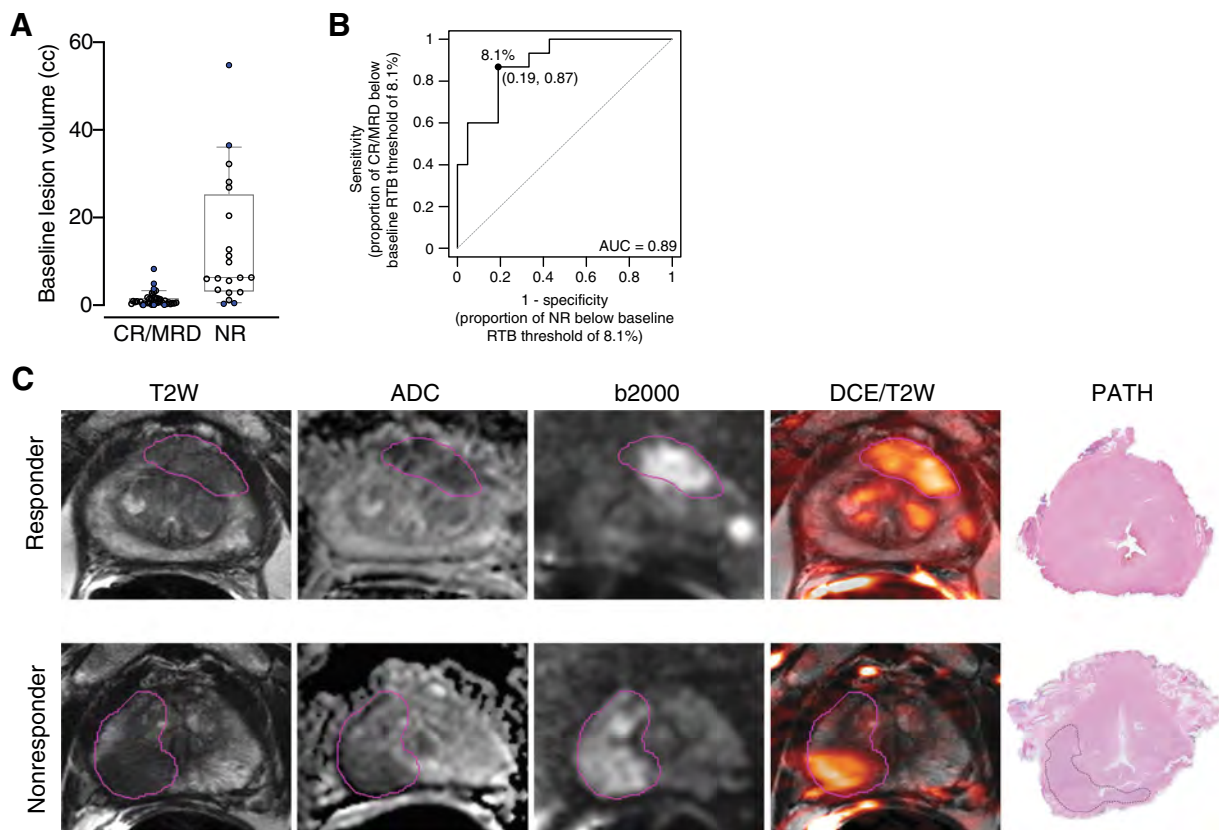
Posttreatment mpMRI revealed a significant decrease in tumor volume after therapy in an overwhelming majority of patients, although at baseline and after 6 months of treatment, it is not possible to determine the kinetics of tumor reduction during treatment. It is important to note that while 4 of 36 patients had no visible disease on mpMRI, on final pathology only 2 of 36 patients had complete pathologic response. However, studies suggest lesions undetected by

**Figure 3.**

Histologic analysis of *in situ* tissue phenotypes. Representative micrographs of low, medium, and high histology scores for anti-AR (**A**) and anti-PSA (**B**) immunostaining of biopsies (top) and absent, low, medium, and high histology scores for posttreatment surgical RALP or TURP specimens (bottom). There were no examples of absent anti-AR or PSA staining in pretreatment biopsied tumor tissue. Scale bar, 500 μ m. Inset scale bar, 100 μ m. Tissue sections immunostained with anti-AR from biopsies ($N = 39$ cases representing 82 different tissue blocks) and posttreatment surgical specimens ($N = 33$ cases representing 61 different tissue blocks; **C**) or anti-PSA from biopsies ($N = 38$ cases representing 79 different tissue blocks) and posttreatment surgical specimens ($N = 33$ cases representing 64 different tissue blocks; **D**) were quantified with Definiens to measure stain intensity. Histology scores were reported on a weighted index of 0 to 1, and multiple slides from the same individual were added before computing the index. Each circle represents a single patient. Bars represent median and 95% confidence interval. $P < 0.0001$ for both AR and PSA staining, by Welch t test. **E**, Representative micrographs of positive (overexpressed) and negative nuclear ERG immunostaining (left) and reduced or intact PTEN (right) in posttreatment tissue. Scale bar, 500 μ m. Inset scale bar, 100 μ m. Note that nuclear ERG intensity is reduced relative to endogenous ERG expression in endothelial cells on account of its expression being driven by (reduced) AR activity. **F**, Posttreatment tissue sections were immunostained with anti-ERG and anti-PTEN antibodies and the frequency of ERG-positive tumor cells (any percentage positive), or PTEN-reduced or PTEN-deficient (at least 5% of tumor cells) cells is shown. pCR/MRD versus NR for PTEN was different at $P = 0.0006$ by Fisher exact test.

mpMRI may be lower in grade and size than detected lesions, making mpMRI an important tool in risk-stratifying prostate cancer (22). Nonetheless, among all patients, the residual cancer volumes estimated by posttreatment mpMRI correlated well with final pathology at $\rho = 0.7173$, making the serial mpMRI a meaningful measure for assessing organ-wide response when comprehensive pathologic analyses are not routinely feasible.

Previous studies have demonstrated that the positive staining for the *TMPRSS2-ERG* fusion and *PTEN* loss by IHC is enriched in patients with increased volumes of residual disease (8, 23). We similarly observed that NR patients exhibited increased rates of positive ERG staining and negative *PTEN* staining than CR/MRD, although only *PTEN* reduction was statistically significant ($P = 0.0006$). In 3 patients who demonstrated minimal tumor reductions on posttreatment

**Figure 4.**

Low RTB is a statistically significant predictor of final pathologic outcome. **A**, Baseline lesion volumes in cc, based on the final pathology status for each patient. $N = 39$ for CR/MRD lesions and $N = 20$ for NR lesions. $P < 0.001$ by two-sided Mann-Whitney test. **B**, ROC curve for pretreatment RTB yielding an AUC of 0.89 and optimal threshold of 8.1%. **C**, Baseline mpMRIs of 2 different patients with high-risk localized prostate cancer and corresponding final pathology. Responder, 72-year-old with a PSA of 5.81 ng/mL had a PI-RADS 5 lesion in the left apical-mid anterior peripheral zone which revealed Gleason 4+4 = 8 cancer at targeted biopsy. Baseline RTB was 3.4% of the prostate. Final pathology revealed pCR. Nonresponder, 66-year-old with a PSA of 5.53 ng/mL, had a large PI-RADS 5 lesion in the right apical-base peripheral zone which revealed Gleason 4+5 = 9 cancer at targeted biopsy. Baseline RTB was 21.5% of the prostate. Final pathology revealed the patient was a nonresponder with RCB of 0.24 cc.

mpMRI, we performed additional genomic analysis to assess eligibility for biomarker-driven studies; in particular, loss of homologous recombination genes would indicate eligibility for treatment with olaparib. In 2 of these 3 patients, germline pathologic variants to *BRCA2* and *PALB2* were observed. Interestingly, one- or two-copy loss of *RB1* was found in all 3 patients, and biallelic inactivation of *TP53* was also observed in 2 patients. Although these findings are anecdotal, they emphasize the consequence of bypassing the AR via *RB1* loss (often in conjunction with loss of tumor suppressors or due to a defect in DNA damage response). As these genomic events are more frequently associated with the “AR low” phenotypes of localized prostate cancer (23), it aligns with our finding that the vast majority of all posttreatment specimens expressed less AR and PSA despite variable volumes of residual disease. Because AR indifference, including neuroendocrine transdifferentiation (24), can result from treatment with AR axis inhibitors, like enzalutamide and abiraterone, the use of genomic analysis to infer resistance mechanisms is needed in the neoadjuvant setting. Furthermore, earlier evaluation with imaging and molecular characteristics might better predict resistance, enabling discontinuation in patients unlikely to benefit from neoadjuvant therapy.

The results of our study reveal a significant increase in the number of patients with organ-confined disease at imaging prior to RARP. It has been suggested that patients receiving neoadjuvant ADT prior to radical prostatectomy are more likely to have negative surgical margins at radical prostatectomy (25, 26). The effect of negative margins on BCR has not been well characterized in patients who undergo neoadjuvant ADT prior to surgery, and some studies have described a lack of significant decreases in rates of BCR in this population compared with those who undergo radical prostatectomy alone (27, 28). While this study and other similar phase II studies have shown favorable imaging and pathologic responses to neoadjuvant AR antagonists plus ADT in patients with high-risk localized disease, long-term follow-up for overall survival needs to be considered (8, 29).

There are several limitations to this study. First, a small sample size of 36 patients limits the power of our statistical analyses. Second, all mpMRI scans were interpreted and contoured by a single radiologist who specializes in prostate mpMRI, which could limit reproducibility of results. However, new lesion-segmenting software may soon mitigate this problem. Third, mpMRIs were acquired using an endorectal coil, which may have influenced the results. Most mpMRIs are increasingly obtained without an endorectal coil. However, the

endorectal coil was used here to ensure the highest quality images. Fourth, although clinical grade Oncomine sequencing identified 3 patients with RB loss, only 1 of these utilized a pretreatment biopsy sample, and were not confirmed using anti-RB IHC on any of the specimens. Finally, the short-term follow-up of this study precludes using time to BCR as an outcome to assess neoadjuvant intense ADT. However, the finding that low residual tumor volumes (i.e., CR and MRD) were associated with freedom from BCR (11) implies that the 15 patients in our study who exhibited CR/MRD are less likely to experience BCR within 3 years than the 22 patients who did not.

To our knowledge, this is one of the first studies to combine serial mpMRI imaging with neoadjuvant intense ADT in prostate cancer. On the basis of our findings, imaging with mpMRI may enhance patient selection for neoadjuvant treatment. Although most (but not all) of patients with the RTB had CR/MRD, nearly every patient experienced tumor shrinkage regardless of initial tumor burden, and this finding was consistent with previous neoadjuvant intense ADT studies (8–10). Thus, patients with higher RTBs at baseline were less likely to benefit from the regimen in this study, they might benefit from either a longer duration of intense ADT or an alternative or precision-guided treatment, with such decision being guided by a combination of clinical, pathology, and imaging assessments. Neoadjuvant treatment in our patient population was tolerable, and extension of treatment beyond 6 months may be feasible.

Neoadjuvant enzalutamide with ADT prior to RARP significantly reduces tumor burden at mpMRI in most patients with localized high-risk prostate cancer. Baseline low RTB at mpMRI was the strongest predictor of pathologic response, with an optimal cutoff of 8.1%, a consideration when selecting patients for future neoadjuvant studies. In addition, imaging can enhance evaluation for high-risk molecular signatures, where combination with imaging can be used to identify patients who are unlikely to benefit from intensive neoadjuvant ADT. In this study, we found mpMRI can be used to assess treatment responses, and 3 patients with adverse pathologic features and multiple deleterious genomic alterations also experienced RCB. Deeper molecular analysis of this and other neoadjuvant studies [i.e., ClinicalTrials.gov identifier NCT03860987 involves mpMRI at mid-treatment along with 18F-DCFPyL (PSMA) imaging] will facilitate patient selection and development of appropriate endpoints for neoadjuvant therapies in prostate cancer.

Authors' Disclosures

D.J. VanderWeele reports other from Genzyme and personal fees from Clovis outside the submitted work. M. Bilusic reports employment with NCI. R.T. Lis reports personal fees from Janssen outside the submitted work. H. Ye reports personal fees from Janssen Pharmaceuticals (Johnson & Johnson) outside the submitted work. P.L. Choyke reports being an inventor of a method of MRI-ultrasound fusion biopsy that was used in the study; this invention is patented by the U.S. Government and licensed to Philips Medical Systems that has commercialized the method as "UraNav", however, this is not directly related to this study. A.G. Sowalsky reports grants from U.S. Department of Defense during the conduct of the study. B. Turkbey reports other from Cooperative

(research and development agreements with Philips and Nvidia), royalties from InVivo, and a patent for related intellectual property in field of prostate computer-aided diagnosis (NIH owned) outside the submitted work. P.A. Pinto reports other from Philips/InVivo Inc during the conduct of the study and outside the submitted work, and has a patent 8,447,384 issued and with royalties paid from Philips/InVivo Inc and a patent for 10,215,830 issued and with royalties paid from Philips/InVivo Inc. No disclosures were reported by the other authors.

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Authors' Contributions

F. Karzai: Conceptualization, data curation, formal analysis, investigation, writing-original draft, writing-review and editing. **S.M. Walker:** Data curation, formal analysis, investigation, writing-original draft, writing-review and editing. **S. Wilkinson:** Data curation, investigation, methodology. **R.A. Madan:** Conceptualization, data curation, investigation, methodology, writing-review and editing. **J.H. Shih:** Formal analysis, writing-review and editing. **M.J. Merino:** Data curation, formal analysis, investigation. **S.A. Harmon:** Formal analysis. **D.J. VanderWeele:** Data curation, formal analysis, writing-review and editing. **L.M. Cordes:** Investigation, writing-review and editing. **N.V. Carrabba:** Data curation, writing-review and editing. **J.R. Bright:** Data curation, methodology, writing-review and editing. **N.T. Terrigino:** Data curation, methodology, writing-review and editing. **G. Chun:** Investigation, writing-review and editing. **M. Bilusic:** Investigation, writing-review and editing. **A. Couvillon:** Investigation, writing-review and editing. **A. Hankin:** Investigation, writing-review and editing. **M.N. Williams:** Investigation, writing-review and editing. **R.T. Lis:** Investigation, writing-review and editing. **H. Ye:** Data curation, formal analysis, writing-review and editing. **P.L. Choyke:** Data curation, software, formal analysis, investigation, writing-review and editing. **J.L. Gulley:** Investigation, writing-review and editing. **A.G. Sowalsky:** Data curation, formal analysis, writing-review and editing. **B. Turkbey:** Data curation, software, formal analysis, writing-review and editing. **P.A. Pinto:** Data curation, investigation, writing-review and editing. **W.L. Dahut:** Conceptualization, data curation, formal analysis, investigation, writing-review and editing.

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Clinical Cancer Research

Sequential Prostate Magnetic Resonance Imaging in Newly Diagnosed High-risk Prostate Cancer Treated with Neoadjuvant Enzalutamide is Predictive of Therapeutic Response

Fatima Karzai, Stephanie M. Walker, Scott Wilkinson, et al.

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MEIS1 down-regulation by MYC mediates prostate cancer development through elevated *HOXB13* expression and AR activity

Nichelle C. Whitlock¹ · Shana Y. Trostel¹ · Scott Wilkinson¹ · Nicholas T. Terrigino¹ · S. Thomas Hennigan¹ · Ross Lake¹ · Nicole V. Carrabba¹ · Rayann Atway¹ · Elizabeth D. Walton¹ · Berkley E. Gryder² · Brian J. Capaldo¹ · Huihui Ye^{3,4} · Adam G. Sowalsky¹

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Abstract

Localized prostate cancer develops very slowly in most men, with the androgen receptor (AR) and MYC transcription factors amongst the most well-characterized drivers of prostate tumorigenesis. Canonically, MYC up-regulation in luminal prostate cancer cells functions to oppose the terminally differentiating effects of AR. However, the effects of MYC up-regulation are pleiotropic and inconsistent with a poorly proliferative phenotype. Here we show that increased MYC expression and activity are associated with the down-regulation of *MEIS1*, a HOX-family transcription factor. Using RNA-seq to profile a series of human prostate cancer specimens laser capture microdissected on the basis of MYC immunohistochemistry, MYC activity, and *MEIS1* expression were inversely correlated. Knockdown of MYC expression in prostate cancer cells increased the expression of *MEIS1* and increased the occupancy of MYC at the *MEIS1* locus. Finally, we show in laser capture microdissected human prostate cancer samples and the prostate TCGA cohort that *MEIS1* expression is inversely proportional to AR activity as well as *HOXB13*, a known interacting protein of both AR and *MEIS1*. Collectively, our data demonstrate that elevated MYC in a subset of primary prostate cancers functions in a negative role in regulating *MEIS1* expression, and that this down-regulation may contribute to MYC-driven development and progression.

Introduction

Locally advanced prostate cancers harbor a limited number of recurrently altered genes whose expression change at the earliest stages of tumor development. These include down-regulation of the tumor suppressors *NKX3-1* and *PTEN* (often

due to genomic deletion), up-regulation of *ERG* (due to fusion with *TMPRSS2*), and up-regulation of *MYC*, which often co-occurs with a single-copy gain of chromosome 8q24 [1–5]. Although up-regulation of *MYC* in most neoplastic tissues is a very early event that contributes to self-renewal and proliferation, localized prostate cancer (PCa) is not proportionally as hyperproliferative as metastatic prostate cancers which frequently harbor focal amplification of *MYC* [6, 7]. In part, the effects of the androgen receptor (AR) in terminally-differentiated luminal prostate cells are disrupted by MYC and other co-factors including FOXA1 and *HOXB13* to re-engage proliferative processes during tumorigenesis [7–11].

Recently, increased awareness that the vast majority of prostate cancers are indolent has led to increased molecular profiling of tumor biopsies prior to definitive treatment. Although MYC expression has been observed in the cancer precursor high-grade prostatic intraepithelial neoplasia, MYC expression in indolent-appearing tumor cells predicts the presence of higher-grade disease and is associated with poor differentiation [3–5]. In localized prostate cancers, up-regulated MYC has been further associated with alterations in nucleoli structure, concomitant with increased biogenesis of ribosomal

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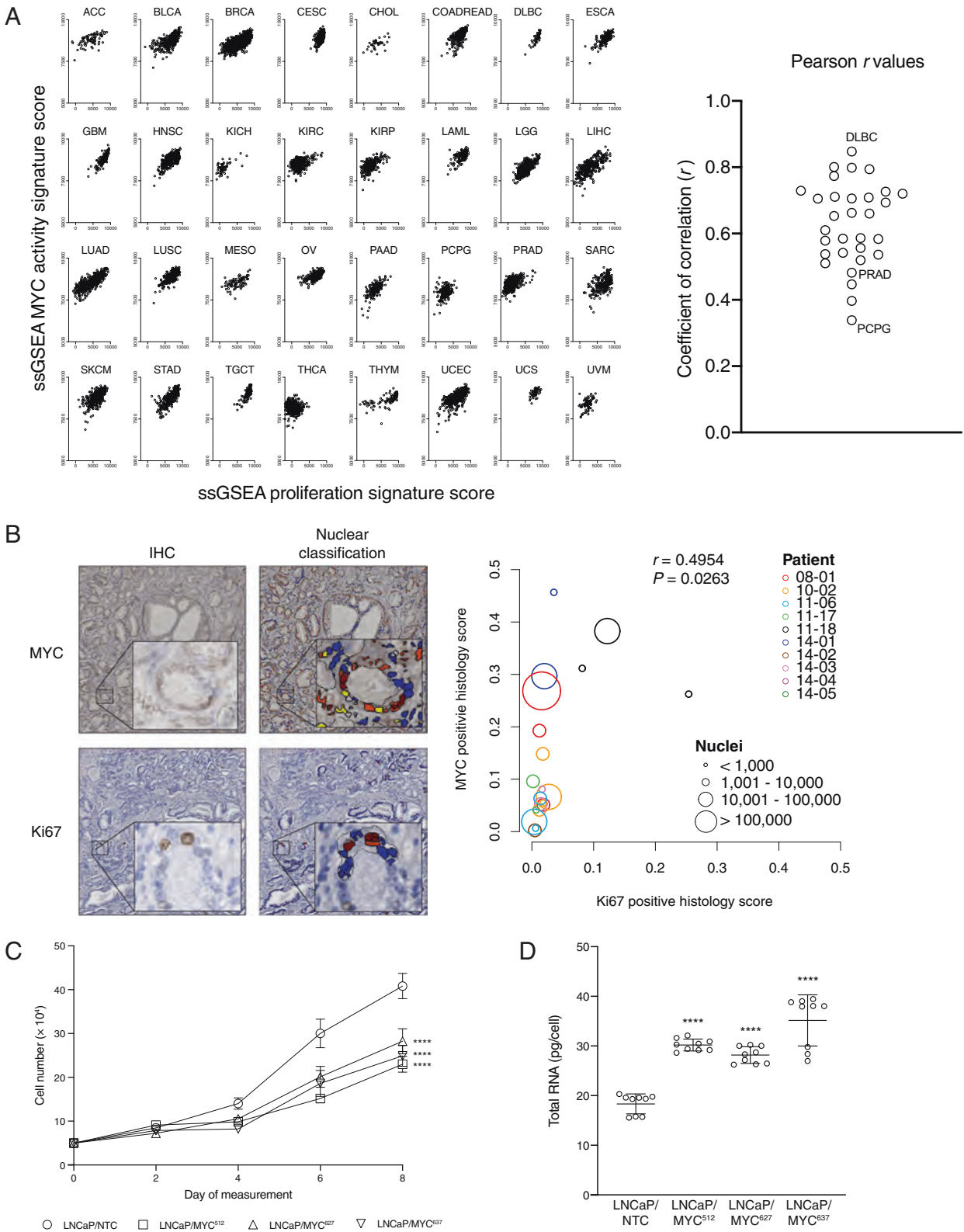
✉ Adam G. Sowalsky
adam.sowalsky@nih.gov

¹ Laboratory of Genitourinary Cancer Pathogenesis, National Cancer Institute, NIH, Bethesda, MD 20892, USA

² Genetics Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA

³ Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA 02215, USA

⁴ Present address: Department of Pathology, University of California Los Angeles, Los Angeles, CA 90095, USA



RNA, increased purine metabolism, and expression of the telomere RNA subunit *TERC* [12–14]. These effects contrast sharply with the phenotype of highly amplified MYC, which is enriched in metastatic and treatment-resistant prostate cancers,

and mirrors the role of MYC in other cancer types via effects on AKT to contribute toward cell division and survival [15–17]. Nonetheless, even in primary PCa, MYC protein expression is diffuse and heterogeneous [3].

◀ **Fig. 1 MYC and proliferation in human cancers.** **a** Left: correlation of the 54-gene ssGSEA MYC activity signature score and the 17-gene ssGSEA proliferation signature score for each tumor type from the TCGA pan-cancer cohort. All scatter plots are on the same scale. Right: plot of Pearson r coefficients of correlation from each scatter plot shown on the left. All tumor types showed a positive correlation, ranging from 0.3386 (PCPG: pheochromocytoma and paraganglioma) to 0.8473 (DLBC: diffuse large B-cell lymphoma). PRAD: prostate adenocarcinoma, $r = 0.4822$. All correlations were significant at $P < 0.001$ except THCA (thyroid carcinoma; $P = 0.5543$). **b** Left: representative IHC staining used as input for automated image analysis with Definiens Tissue Studio and the processed file used for quantification of histologic score via nuclear classification, for anti-MYC and anti-Ki67 immunostains. Right: unweighted scatter plot and Pearson correlation of the quantified intensities of anti-Ki67 vs. anti-MYC stain. Each open circle represents a contiguous quantified region, color-coded by patient. Multiple regions from the same patient indicate multiple noncontiguous regions were measured, and the size of each circle indicates the number of nuclei considered for the analysis. **c** Assessment of cell growth in vitro as a function of MYC knockdown. LNCaP cells expressing non-targeting or MYC-targeting hairpins were seeded at a density of 50,000 cells per well and counted every 2 days in triplicate using a hemocytometer by three different individuals blinded to the identities of the cultures. Data shown represents the mean $\pm$ standard error of ten independent experiments. **** $P < 0.0001$ for each cell line vs. control by two-way ANOVA. **d** RNA content as a function of MYC knockdown. Total RNA was extracted from 1×10^6 pelleted LNCaP cells expressing non-targeting or MYC-targeting hairpins. RNA concentrations were measured by Nanodrop and total RNA was calculated per cell. Data represents mean $\pm$ standard deviation of three independent experiments conducted in triplicate, with open circles representing individual replicate RNA quantity values (**** $P < 0.0001$ for each cell line vs. control by two-way ANOVA).

We sought to examine the genetic contribution of MYC to the development and progression of primary PCa in the context of dysregulated growth by using anti-MYC immunohistochemistry (IHC) and performing laser capture microdissection on populations of human prostate tumor cells with varying expression of MYC protein. We show using transcriptome profiling that increased MYC activity is inversely correlated with expression of Myeloid Ecotropic viral Insertion Site 1 (*MEIS1*), a transcription factor that interacts with and regulates the activity of HOX homeodomain transcription factors, including HOXB13 [18–20]. We determined that MYC binding to the *MEIS1* locus decreases as MYC levels increase, and that *MEIS1* expression is negatively correlated with *HOXB13* expression and AR activity.

Results

MYC activity is weakly associated with proliferation in primary prostate cancer

Although MYC orchestrates a broad range of biological functions, it has been shown in many cancers that MYC drives tumorigenesis by potentiating or stimulating cell

growth and proliferation [21]. To assess the relationship between MYC activity and proliferation across cancer types, we evaluated MYC activity and cell proliferation rate in TCGA pan-cancer cohort using ssGSEA with MYC activity and proliferation signatures [22]. As shown in Fig. 1a, signatures for MYC activity and proliferation were positively correlated in all cancers with the vast majority showing strong positive correlations ($r > 0.5$). However, prostate adenocarcinoma (PRAD) was below this cut-off as one of the weakest correlating cancer types ($r = 0.4822$, 95% C.I. 0.4112–0.5472, $P < 0.0001$). Repeating this analysis with a prostate cancer-specific gene set generated by MYC overexpression in LNCaP cells [11], PRAD correlation with proliferation increased modestly ($r = 0.5609$, 95% C.I. 0.4972–0.6186, $P < 0.0001$), but was still below the median correlation coefficient (Supplementary Fig. 1). To validate this observation, we screened a new cohort of treatment naïve Gleason score 7 prostate tumors for MYC and Ki67 expression by IHC. Although MYC was expressed in a majority of tumor cells, Ki67 expression was limited to $< 5\%$ of cells (Fig. 1b, left), consistent with median Ki67 indices from large cohorts of GS7 tumors in the 2–4% range [23, 24]. We then applied automated image analysis to quantify millions of nuclei across 20 tumor foci from ten different cases. While the MYC positive histology score ranged from 0 (low expression) to 0.46 (high expression), the Ki67 positive histology score was < 0.1 in 18 out of 20 foci (Fig. 1b, right). An unweighted Pearson correlation demonstrated that MYC IHC was weakly but positively correlated with Ki67 ($r = 0.4954$, 95% C.I. 0.06772–0.7693, $P = 0.0263$), similar to what we observed in the PRAD TCGA cohort, indicating that the transcriptional signatures applied to the larger dataset accurately recapitulated MYC and Ki67 protein abundance. Because high Ki67 expression was not associated with high MYC expression, proliferation may have been dependent upon MYC activity, but MYC expression alone was not sufficient for proliferation.

To further examine the role of MYC in prostate tumorigenesis, we knocked down MYC in LNCaP cells, which had physiologically-elevated levels of MYC. Like primary PCa, LNCaP cells express AR, are androgen sensitive, and grow slowly despite elevated MYC [25]. Using MYC-knockdown cells as a model of MYC-low PCa cells, we show in Supplementary Fig. 2 that we achieved ~50% reduction in MYC transcript and MYC protein expression. As anticipated, knockdown of MYC was associated with a modest decrease in cell number at each time point (Fig. 1c) but the decrease in cell proliferation was not proportional to the decrease in MYC expression. The relationship between MYC and proliferation in other cancer types also stipulates a role for MYC as a universal amplifier of transcription [26], alleviating constraints on cell growth and proliferation

[27]. However, we observed the opposite: the total amount of cellular RNA increased when *MYC* was knocked down in LNCaP cells (Fig. 1d). Together, these findings indicate that the cellular behavior of *MYC* in PCa contrasts with other tumor types, and that *MYC* does not act solely in a proliferative capacity.

Increased *MYC* is associated with specific up- and down-regulation of target genes

We hypothesized that analysis of tumors with different levels of *MYC* expression would identify genes that may contribute to *MYC* activity in PCa pathogenesis. Using samples of human radical prostatectomy specimens stained with anti-*MYC*, we identified concomitant regions of high- and low-*MYC* expression in the same patients. We subjected these within-patient sets of tumor foci to laser capture microdissection (Fig. 2a), controlling for PTEN and ERG status by confirming their concordance within each case. Of the 19 foci microdissected, we designated 42% *MYC*-high ($n = 8$) and 58% *MYC*-low ($n = 11$). We performed RNA-seq on these cases and derived a limited gene set of 293 up- or down-regulated genes, with only 15 genes showing expression changes of fourfold or more with adjusted P values < 0.10 (Fig. 2b and Supplementary Table 2). Consistent with our observation of increased RNA in LNCaP cells expressing *MYC* knockdown hairpins (see Fig. 1d), we observed more genes down-regulated than up-regulated in *MYC*-high vs. *MYC*-low tumor foci.

To further refine genes of potential interest, we analyzed a second dataset comprised of whole transcriptome sequencing from 499 primary tumors in the TCGA PRAD cohort. Using the mRNA expression values of genes in the *MYC* activity signature (see Fig. 1a and Supplementary Table 1), we compared the top 20 and bottom 20 ranked cases based on average median absolute deviation-modified z -score (Supplementary Fig. 3), and established a second list of differentially expressed genes (Fig. 2c and Supplementary Table 3). As these cases were compared between patients rather than within-patient, a far greater number of genes were differentially expressed (1346) with fold-change of at least 4 and adjusted P values < 0.05 .

When comparing differentially expressed genes between *MYC*-high vs. *MYC*-low tumors, *MYC* expression was consistently greater in the *MYC*-high group in the LCM primary PCa cohort (Fig. 2b and Supplementary Table 2). As *MYC* RNA and protein expression often do not correlate due to the tight posttranslational regulation of *MYC* [28, 29], the statistically insignificant increase of *MYC* expression in the *MYC*-high group was not unexpected. However, *MYC* was amongst the up-regulated genes in the *MYC* activity high group, confirming proper stratification in the TCGA PRAD cohort as well (Fig. 2c and

Supplementary Table 3). Taking an inclusive approach to combine both datasets, we identified 293 and 7390 differentially expressed genes between *MYC*-high and *MYC*-low prostate tumors in the LCM tissue and TCGA PRAD cohorts, respectively (Fig. 2d, e) that were concordantly regulated in the same direction. Of these, 221 were in common at a false discovery rate threshold of 0.10 for LCM tissue and 0.05 for TCGA PRAD (Fig. 2e and Supplementary Table 4).

MEIS1 is negatively associated with *MYC* expression and *MYC* activity

Given that we observed more genes down-regulated in *MYC*-high tumors than we would have expected if *MYC* were functioning as a genome-wide transcriptional amplifier, we hypothesized that collective analyses of gene expression might reveal coordinated down-regulation of biological processes that may contribute to *MYC*-driven PCa tumorigenesis. We therefore performed gene set enrichment analysis (GSEA) using both the LCM and TCGA cohort datasets as inputs, limiting our analyses to gene sets with differentially expressed genes shared by the LCM and TCGA cohorts. As depicted in Fig. 3a, our initial comparative analyses demonstrated significant and concordant enrichment of 762 gene sets (Supplementary Table 5). To narrow our focus further, we refined our search only to include gene sets based on ChIP-seq target discovery studies with the premise that increased *MYC* activity would misregulate a multitude of transcriptionally-driven pathways. These analyses identified 11 gene sets, a preponderance of which were associated with development and survival (Table 1).

Of note, gene sets related to NOTCH, RUNX1, HOXA9, and TNF were negatively enriched in *MYC*-high vs. *MYC*-low tumors. This suggested that one or more transcription factors exert effects in opposition to *MYC*, and may be guiding *MYC*-mediated misregulation of these pathways. In seeking a gene or common regulator between these gene sets, we identified the transcription factor *MEIS1* as an important regulator in each of the aforementioned pathways. For example, *MEIS1* has been shown to regulate genes in the NOTCH pathway [30] and sensitizes cells to TNF [31]. Moreover, *MEIS1* is essential for the expression of genes driven by the HOXA9-NUP98 fusion in acute myeloid leukemia [32–34]. Therefore, we examined whether *MEIS1* expression was associated with increased *MYC* activity as a proxy for its role in PCa development.

We observed that *MEIS1* expression was reduced in *MYC*-high cases compared with *MYC*-low tumors, for both the LCM and TCGA cohorts (Supplementary Tables 2 and 3, respectively). At a case-by-case level, *MEIS1* expression was negatively correlated with *MYC* (Fig. 3b; $r = -0.4321$, 95%

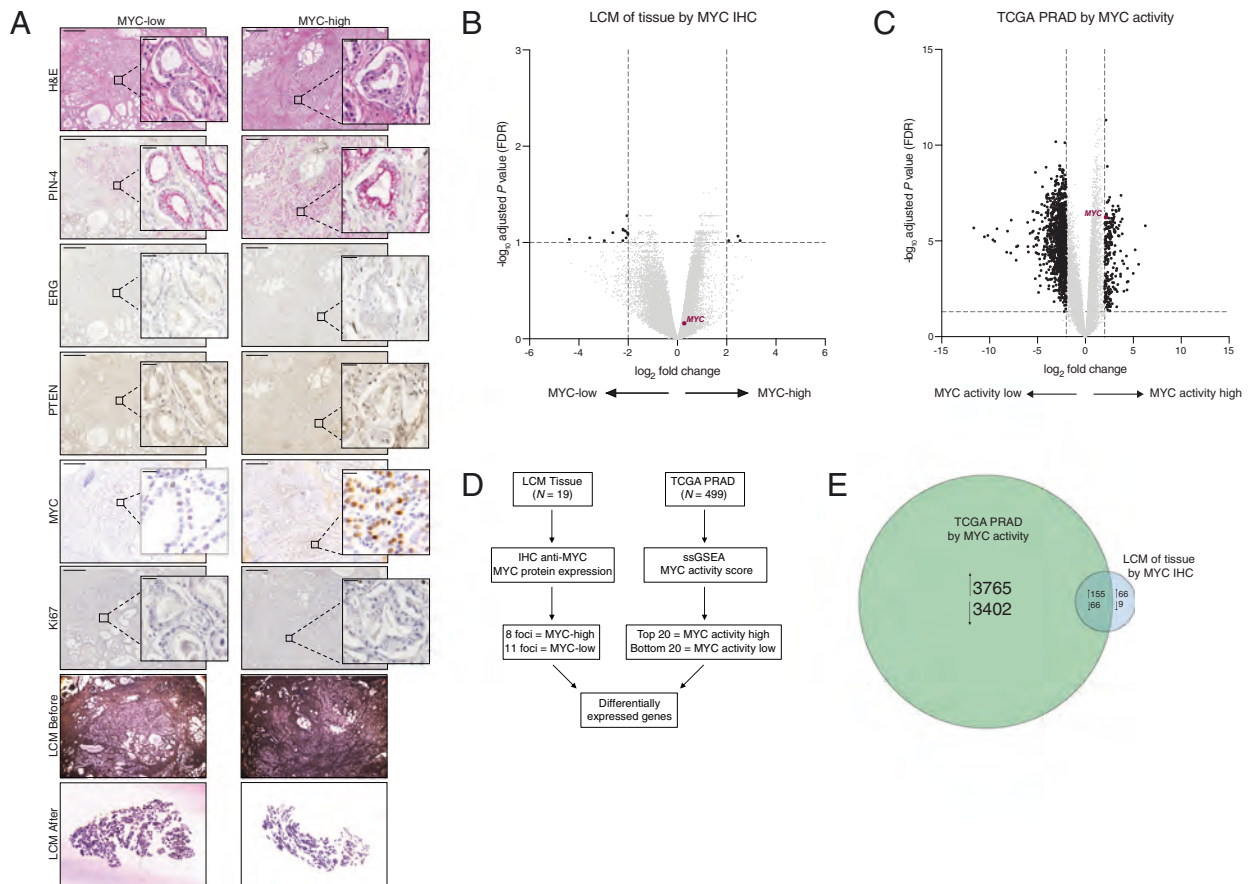


Fig. 2 Effect of MYC levels on gene expression. **a** Representative IHC of case 14-03 showing heterogeneity of MYC expression within a single patient. Serial sections were stained with H&E and PIN-4 cocktail to distinguish benign glands and high grade prostatic intraepithelial neoplasia from tumor foci; anti-ERG and anti-PTEN immunostains were performed to select regions with concordant phenotypes. Micrographs of stained tissue before and after laser capture microdissection (LCM) are also shown. Scale bar: 500 μ m; inset scale bar: 20 μ m. **b** Volcano plot depicting differentially expressed genes in MYC-high vs. MYC-low tumors in the LCM cohort, with the false discovery rate (adjusted $-\log_{10} P$ value) on the y-axis and the $\log_2$ -transformed fold-change (MYC-high vs. MYC-low) on the x-axis. Expression of *MYC* is shown in red. Black dots represent genes with

fold-change $\geq \pm 4$ and adjusted P value < 0.10 for Welch's t test. **c** Volcano plot depicting differentially expressed genes in MYC activity high vs. MYC activity low tumors in the TCGA PRAD cohort, with the false discovery rate (adjusted $-\log_{10} P$ value) on the y-axis and the $\log_2$ -transformed fold-change (MYC activity high vs. MYC activity low) on the x-axis. Expression of *MYC* is shown in red. Black dots represent genes with fold-change $\geq \pm 4$ and adjusted P value < 0.05 for Welch's t test. **d** Flowchart of the strategy used to identify lists of differentially expressed genes from the LCM and TCGA PRAD cohorts. **e** Euler diagram depicting distinct and shared differentially expressed genes between the LCM and TCGA PRAD cohort. Circles are drawn to scale.

C.I. -0.7409 – 0.0275 , $P = 0.0323$) and MYC activity (Fig. 3c; $r = -0.5527$, 95% C.I. -0.7372 to -0.2713 , $P = 0.0001$) in the LCM and TCGA cohorts, respectively. To assess whether this association occurred in unselected populations of primary PCa, we analyzed RNA-seq data from an additional 69 microdissected tumor foci and the entire TCGA PRAD cohort ($n = 499$). Although weaker than the MYC-selected cohorts, the negative relationship between *MEIS1* and *MYC* expression was still observed (Fig. 3d, e).

MYC negatively regulates *MEIS1* expression

Recently, Bhanvadia et al., postulated that higher *MEIS1* expression conferred a less aggressive PCa phenotype [19].

Based on these results, we hypothesized that repression of *MEIS1* expression by MYC may contribute to the aggressivity of MYC-driven PCa. We engineered LNCaP cells with a non-targeting hairpin as MYC-high and LNCaP cells with three different *MYC*-targeting hairpins as MYC-low (see Supplementary Fig. 2), with variable efficiency of *MYC* knockdown to replicate the biological variability observed in patient samples. We then performed ChIP-seq against MYC in these cells to generate genome-wide site maps and ascertain chromatin occupancy at *MEIS1*. In these cells, we had observed increased overall transcriptional output (see Fig. 1d), and we controlled for this phenomenon using *Drosophila* chromatin spike-in controls.

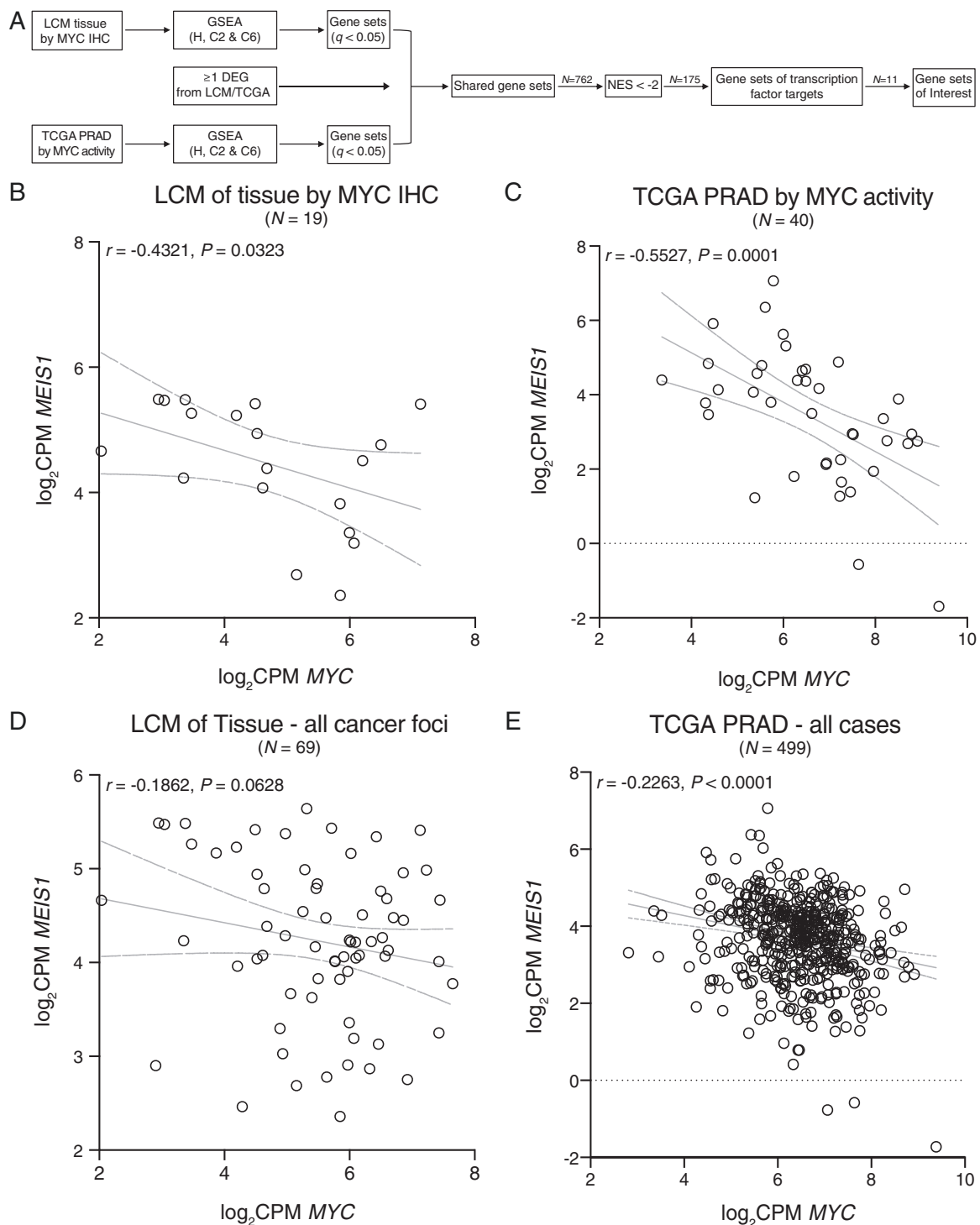


Fig. 3 Association between *MYC* and *MEIS1* expression. **a** Flow-chart of the strategy used to identify potential *MYC* target pathways based on differentially expressed genes. **b–e** Correlation of the $\log_2$ CPM expression levels between *MYC* and *MEIS1* in the initial LCM

cohort ($N = 19$, **b**), the initial TCGA PRAD cohort ($N = 40$, **c**), the entire LCM primary PCa cohort ($N = 69$, **d**), and the entire TCGA PRAD cohort ($N = 499$, **e**). The gray dotted lines represent the 95% confidence intervals for each correlation.

The quality of our *MYC* ChIP DNA was assessed using Active Motif's ChIP-IT qPCR Analysis Kit and primers targeting a *MYC* super-enhancer region prior to sequencing. As shown in Supplementary Fig. 4A, an enrichment of six-

to eight-fold was achieved for each experiment and thus submitted for subsequent ChIP-seq analysis. We identified ~18,000 and 27,000 high confidence peaks ($P < 1 \times 10^{-9}$) in LNCaP/NTC and LNCaP/shMYC consensus sites (that is,

Table 1 List of common gene sets negatively enriched in MYC-activity high vs. low comparing LCM tissue and TCGA cohorts.

MSigDB gene set	LCM tissue		TCGA	
	NES	Q value	NES	Q value
Onder CDH1 Targets 3 DN	−2.041	4.27E-03	−2.193	6.96E-03
Ono AML1 Targets DN	−2.154	2.18E-03	−2.135	6.96E-03
Klein Targets of BCR ABL1 Fusion	−2.192	1.80E-03	−2.274	6.96E-03
Hess Targets of HOXA9 and MEIS1 DN	−2.206	1.63E-03	−2.192	6.96E-03
Takeda Targets of NUP98 HOXA9 Fusion DN	−2.216	1.43E-03	−2.005	6.96E-03
Phong TNF Targets UP	−2.222	1.41E-03	−2.207	6.96E-03
Kim GLIS2 Targets UP	−2.431	1.75E-04	−2.066	6.96E-03
Hecker IFNB1 Targets	−2.497	1.50E-04	−2.086	6.96E-03
Servitja Islet HNF1A Targets UP	−2.542	8.51E-05	−2.164	6.96E-03
Vilmas NOTCH1 Targets UP	−2.575	8.93E-05	−2.342	6.96E-03
Gaurnier PSMD4 Targets	−2.768	0.00E+00	−2.311	6.96E-03

NES normalized enrichment score.

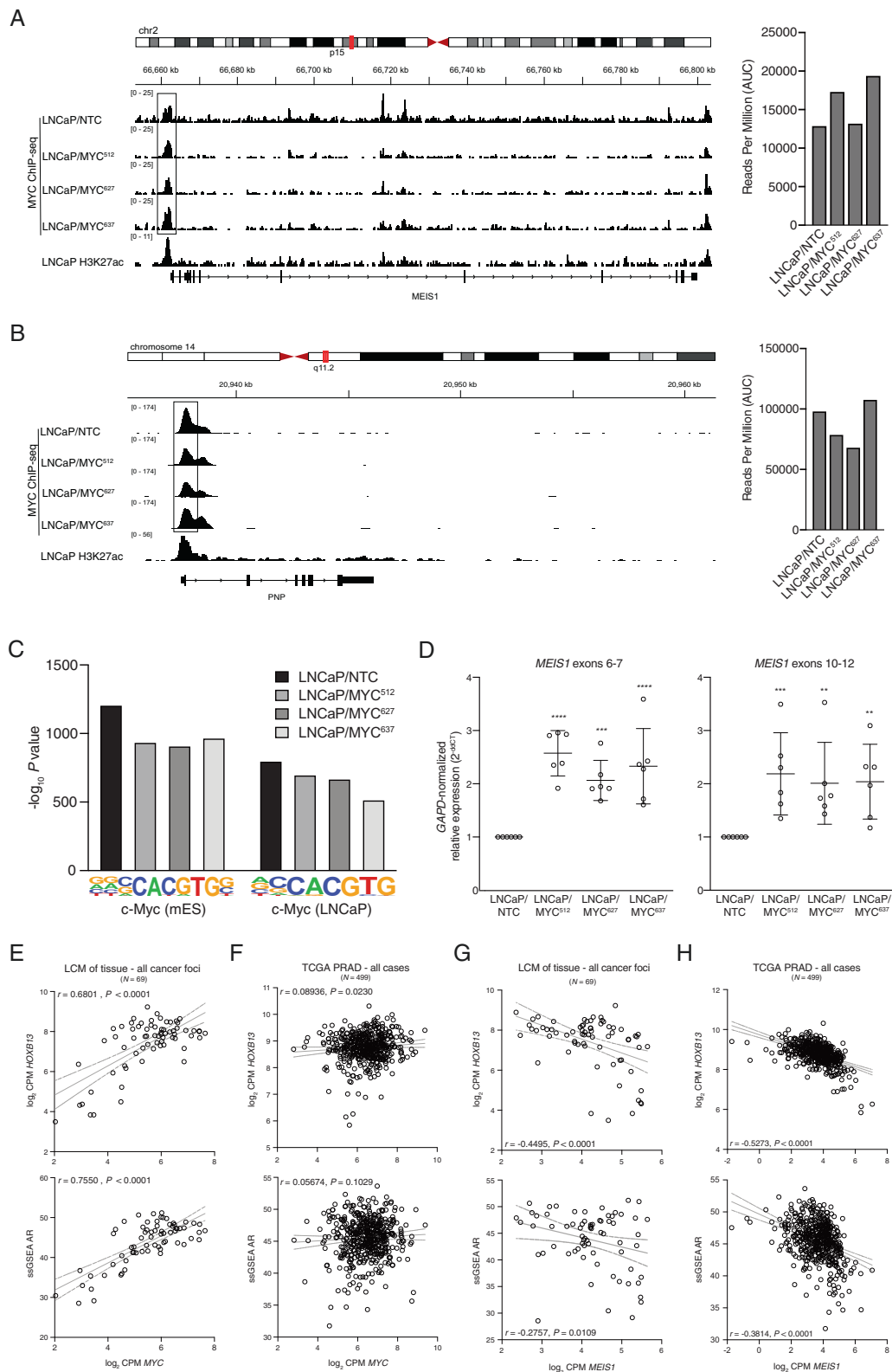
sites common in all three *MYC* knockdown lines), respectively; and of these, 98% of all peaks were shared (Supplementary Fig. 4B). These sites overlapped with those reported in multiple publicly-available MYC ChIP-seq datasets ([11, 35–37]), with the extent of overlap ranging from 22 to 90% (Supplementary Table 6). Of note, when compared with another LNCaP MYC ChIP-seq, the overlap ranged from 77 to 90%, validating the technical results of our experiment. Applying functional annotation of binding sites, the majority of MYC-bound peaks were either promoter proximal or enriched for intronic/intergenic DNA in both control and *MYC* knockdown cell lines (Supplementary Fig. 4C). This distribution was also similar to that of previous MYC ChIP-seq datasets, providing orthogonal validation of our data (Supplementary Fig. 4D).

When we examined MYC ChIP-seq peaks in the vicinity of *MEIS1*, we observed that proportional MYC recruitment to *MEIS1* was increased 1.29-fold on average, relative to global binding in each of the three *MYC* shRNA lines relative to control (Fig. 4a). In contrast to *MEIS1*, we would anticipate that genes positively regulated by MYC would demonstrate reduced recruitment of MYC at their loci when MYC levels are reduced by shRNA, counter to what we observed in Fig. 4a. When we examined binding events at the known MYC-regulated gene *PNP*, we observed 1.17-fold less MYC recruitment, on average, to the *PNP* locus (Fig. 4b). For both *MEIS1* and *PNP*, ChIP-seq for H3K27ac marks in parental LNCaP cells also identified a peak at the same genomic position, indicating that the MYC ChIP-seq was identifying chromatin of active enhancer elements. Similar results were observed for two additional MYC targets, *NOLC1* and *EIF5A* (Supplementary Fig. 5A–B) of 1.28-fold less binding at each locus. The increased occupancy of MYC at *MEIS1* was far more pronounced when peaks were normalized to *Drosophila* spike-in controls (Supplementary Fig. 5C), although this also resulted in

minimal differences between control and *MYC* knockdown for MYC binding at *PNP* (Supplementary Fig. 5D). Nonetheless, motif enrichment analysis at MYC-ChIP peaks showed decreases at canonical MYC binding sites upon *MYC* knockdown (Fig. 4c).

We then sought to determine if the observed increase in chromatin occupancy translated to altered *MEIS1* transcription by qRT-PCR. Knockdown of *MYC* resulted in increased abundance of the *MEIS1* transcript, consistent with the observed increase in MYC recruitment at *MEIS1* (Fig. 4d). Together, these results support the role of MYC in the negative regulation of *MEIS1* in primary PCa.

In PCa, *MEIS1* functions to direct transcriptional specificity and activity of *HOXB13* and act as a negative regulator of AR [20, 38]. Therefore, we next determined whether *HOXB13* expression or AR activity were altered in the context of MYC activity. Indeed, there were statistically significant positive correlations between *MYC* and *HOXB13* mRNA levels in both the entire LCM tissue cohort (Fig. 4e, top; $r = 0.6801$, 95% C.I. 0.5285–0.7897, $P < 0.0001$) and the entire TCGA PRAD cohort (Fig. 4f, top; $r = 0.0894$, 95% C.I. 0.0016–0.1758, $P = 0.0230$) although the strength of correlation in the TCGA cases was notably less than the purer LCM tissue cohort. We also observed a positive correlation between *MYC* mRNA levels and the ssGSEA scores for AR activity [39] in both the LCM cohort (Fig. 4e, bottom; $r = 0.7550$, 95% C.I. 0.6311–0.8413, $P < 0.0001$) and the TCGA cohort (Fig. 4f, bottom; $r = 0.0567$, 95% C.I. −0.0312–0.1438, $P = 0.1029$), although statistical significance was reached solely in the LCM cohort. Moreover, there was a statistically significant negative correlation between *MEIS1* and *HOXB13* expression in both the LCM cohort (Fig. 4g, top; $r = -0.4495$, 95% C.I. −0.6202 to −0.2381, $P < 0.0001$) and the TCGA cohort (Fig. 4h, top; $r = -0.5273$, 95% C.I. −0.5879 to −0.4608, $P < 0.0001$). This inverse association was also observed between *MEIS1*



expression and AR activity in the LCM (Fig. 4g, bottom; $r = -0.2757$, 95% C.I. -0.4810 – 0.0417 , $P = 0.0109$) and TCGA (Fig. 4h, bottom; $r = -0.3814$, 95% C.I. -0.4540 to

-0.3038 , $P < 0.0001$) cohorts. Given the potential impact of the *HOXB13* G84E SNP (rs138213197) on *MEIS1* interaction, expression, and function [20], we performed SNV

◀ **Fig. 4 Decreased MYC expression increased MYC occupancy at MEIS1 and MEIS1 expression.** **a** IGV depiction of MYC and H3K27ac binding events in LNCaP MYC knockdown cell lines, showing increased MYC occupancy at the MEIS1 locus in cells harboring MYC knockdown. **b** IGV depiction of MYC and H3K27ac binding events in LNCaP MYC knockdown cell lines, showing decreased MYC occupancy at the PNP locus in cells harboring MYC knockdown. Graphs to the right of (**a**, **b**) depict the area under the curve (AUC) of read counts for the peaks outlined by the boxes. **c** The confidence of identifying known MYC binding motifs as identified by HOMER are shown for the LNCaP MYC knockdown cell lines, measured by $-\log_{10} P$ value for enrichment. Q values (adjusting for false discovery) for these enrichments are zero. **d** Quantitative reverse-transcription PCR of GAPD-normalized MEIS1 transcript in LNCaP MYC knockdown cell lines relative to control, measuring across the splice boundary of exons 6–7 (left) or exons 10–12 (right). Bars and whiskers represent the mean $\pm$ standard deviation of six independent experiments conducted in triplicate, plotted individually as open circles (** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ by Student's t test). **e**, **f** Correlation of the $\log_2$ CPM expression level for MYC with the $\log_2$ CPM expression level for HOXB13 (top) and the 266-gene ssGSEA AR activity signature score (bottom) in the entire LCM cohort ($N = 69$, **e**) and the entire TCGA PRAD cohort ($N = 499$, **f**). The gray dotted lines represent the 95% confidence intervals for each correlation. **g**, **h** Correlation of the $\log_2$ CPM expression level for MEIS1 with the $\log_2$ CPM expression level for HOXB13 (top) and the 266-gene ssGSEA AR activity signature score (bottom) in the entire LCM cohort ($N = 69$, **g**) and the entire TCGA PRAD cohort ($N = 499$, **h**). The gray dotted lines represent the 95% confidence intervals for each correlation.

analysis of germline samples from the LCM and TCGA cohorts (data not shown). However, none of the LCM cases carried the G84E SNP, and only five TCGA cases which carried the SNP (CH-5748, CH-5771, G9-6370, KK-A8IG, and V1-A9OF), were not amongst the MYC-high or MYC-low group (see Fig. 3c). Taken together, our data suggest that in MYC-high tumors, PCa development is collectively mediated by increased AR activity and HOXB13 expression resulting from MEIS1 down-regulation.

Discussion

In many cancer types, the role of amplified MYC in mediating tumorigenesis has been linked to genes involved in ribosomal biogenesis, universally up-regulated transcription, proliferation, and reprogramming cells to a pluripotent state [40]. A subset of advanced prostate cancers also harbor amplified MYC, but it is distinct from the up-regulated MYC that is a hallmark of many localized prostate cancers [3, 14–16]. In the current study, we used transcriptome profiling to assess subpopulations of prostate tumors based on differential MYC protein expression and MYC activity, and we similarly compared differentially expressed genes and pathways within the larger prostate TCGA cohort based on MYC activity. Finding that increased MYC activity was inversely proportional to overall transcription, we focused

on down-regulated pathways, identifying a negative correlation between MYC activity and MEIS1 expression, with MYC directly involved in the negative regulation of MEIS1 as demonstrated by knockdown and chromatin immunoprecipitation analyses. The inverse association between MEIS1 expression and AR extended further to HOXB13 expression, indicating that in a subset of primary PCa, decreased expression of MEIS1 may be necessary for AR and HOXB13 to drive tumor development and progression.

From its discovery as an oncogene to the subsequent challenges associated with targeting MYC pharmacologically, efforts have shifted in identifying targetable MYC effector genes or other targetable co-factors that are necessary for MYC activity [41, 42]. Efforts to dissect functions of MYC have frequently relied on cancer models in which MYC levels rise up to 20-fold, in contrast to 1–2 fold physiological elevation of MYC expression in PCa [2, 6, 43]. Not surprisingly, the phenotypes associated with MYC up-regulation differ. For example, MYC expression in the activation of lymphocytes [26] or in Burkitt's lymphoma [27] is associated with a universal amplification of transcription, while we observed that in LNCaP PCa cells with up-regulated MYC, knocking down MYC by <50% with shRNA consistently increased the total amount of RNA produced per cell.

The differences in MYC function in PCa extend to the long-standing relationship between MYC and proliferation [40]. Here, we report a series of PCa tissues, serially-sectioned and stained with anti-MYC and anti-Ki67 antibodies, that show a weak proportional relationship. While up to 50% of luminal PCa cells were positive for MYC expression, proliferation measured by Ki67 affected <5% of cells. We show a similar relationship in the prostate TCGA cohort and PCa cell lines, suggesting that while proliferating cells may harbor MYC activity, MYC alone is not sufficient for proliferation in PCa. This is generally in agreement with findings that MYC expression alone is not reflective of an increased proliferative fraction of PCa cells [5].

A key finding from our study is the negative relationship between MYC activity and MEIS1 expression. In the context of MYC's role as a universal transcriptional amplifier, a target gene of MYC-mediated repression could simply be considered a technical anomaly reflecting unequal numbers of cells used for analysis [26]. However, we show that transcriptional signatures of MYC activity and MEIS1 are inversely correlated in two large independent cohorts, which is largely consistent with the finding that tumors and PCa cells with increased MEIS1 expression show decreased enrichment of MYC target gene sets by GSEA [19]. Importantly, we further demonstrate here with ChIP-seq that the effects on MEIS1 expression are due in part to increased MYC occupancy at the MEIS1 locus, such that by using

shRNA to lower the level *MYC* with biological variability observed in patient samples, it resulted in repositioning of *MYC* at specific sites. However, the intensity of *MYC* binding at *MEIS1*, as measured by the peak height in IGV, is substantially less than the peak heights of known direct targets of *MYC* such as *PNP*, *NOLC1* and *EIF5A*, and the increased expression of *MEIS1* in the context of decreased *MYC* expression precludes *MYC* as a direct transcriptional activator. Indeed, *MEIS1* is a direct target of MIZ-1 [44], which functions to increase the expression of *MEIS1* in the absence of *MYC*. Therefore, one possible explanation for our observations is that a *MYC*-regulated transcriptional repressor also sits at the *MEIS1* promoter and competes with *MYC* for direct binding to MIZ-1 or another positive regulator of *MEIS1* transcription. In such a model, *MYC* would only be bound to *MEIS1* indirectly, and the interaction between *MYC*/MIZ-1 complexes and other transcriptional regulators of *MEIS1* is of interest for future study.

Previously, Bhanvadia et al. [19] demonstrated that tumors with increased *MEIS1* are potentially less aggressive, based on studies of LAPC-4 PCa cells expressing shRNA against *MEIS1* and that tumors with lower levels of *MEIS1* were at greater risk of biochemical recurrence and metastasis. Specifically, they reported a step-wise decrease in *MEIS1* expression during PCa progression, but that knockdown of both *MEIS1* and *MEIS2* were necessary to suppress tumor development in vivo. *HOXB13*, a homeodomain transcription factor, has been shown to regulate AR activity while shRNA against *HOXB13* in LAPC4 cells inhibits their growth [18]. Based on these prior findings, as *HOXB13* physically interacts with *MEIS1* [18], tumors expressing less *MEIS1* would be expected to display greater *HOXB13* expression and AR activity. Indeed, we show an inverse relationship between *MEIS1* expression and AR activity in two independent cohorts, which is consistent with previous observations of a positive correlation between *MYC* expression and AR activity [7]. However, we did not observe a statistically significant inverse relationship between *MYC* and *MEIS2* (see Supplementary Table 2).

Our study has an important limitation in that we did not assess the relationship between *MYC* and *MEIS1* in additional human cell line or mouse model systems. Our focus on increased *MYC* activity in locoregional PCa is distinct from highly amplified *MYC* in metastatic PCa. The LNCaP model is the only commercially available PCa cell line that is genomically similar to localized PCa, derived from a regional lymph node, and is both androgen-dependent and luminal in origin. Mouse models with robustly overexpressed *MYC* and all other PCa cell lines derived from advanced metastases exhibit a high proliferative index, which does not accurately model early PCa, which is a slow-growing disease.

In summary, our analysis of *MYC*-expressing prostate tumors demonstrates an inverse relationship with *MEIS1*

expression, which in turn is negatively correlated with *HOXB13* expression and AR activity. Mechanistically, our data demonstrate that *MEIS1* is a directly repressed target of *MYC*, and via effects on *HOXB13* link *MYC* activity to AR activity. The potential clinical significance of the inverse *MYC*/*MEIS1* relationship warrants further investigation as AR-directed therapies are introduced earlier in the clinical course of disease, and *MEIS1* levels may indicate potential sensitivity to treatment.

Materials and methods

Study approval

This research was conducted in accordance with the principles of the Declaration of Helsinki. All patients provided informed consent prior to participating in tissue procurement protocols, and all samples were deidentified as per institutional policies. The collection of radical prostatectomy specimens was approved by the Dana Farber/Harvard Cancer Center Institutional Review Boards, under protocol numbers 11–250, 15–008, and 15–492. The collection of prostate biopsy specimens was approved by the National Institutes of Health Institutional Review Board, under protocol number 15-c-0124. Tissues were fixed in formalin and embedded in paraffin according to standard procedures.

Pan-cancer analysis

Gene-level normalized FPKM expression values for The Cancer Genome Atlas (TCGA) RNA-seq data were downloaded from the NCI Genomic Data Commons (<https://gdc.cancer.gov>). Cases were filtered for tumor samples within each organ type and cases with missing gene expression values were removed. Single-sample gene set enrichment analysis (ssGSEA) was performed on the GenePattern server (<https://cloud.genepattern.org>) using the ssGSEA Projection module version 9.1.1 with the following parameters: weighting exponent: 0.75; combine mode: combine.add; sample normalization method: none. For each TCGA tumor type, ssGSEA projection values were obtained for a 17-gene *MYC*-independent proliferation signature, a 54-gene *MYC* activity signature [22], and a prostate cancer-specific gene set generated by *MYC* overexpression in LNCaP cells [11]. The genes used for analysis are given in Supplementary Table 1.

Cell lines and derivatives

RNA knockdown of *MYC* expression was achieved using the SMARTvector lentiviral shRNA system (Dharmacon) and a standard second-generation packaging system. hCMV-Turbo GFP targeting vectors were *MYC* 512: V3SH11240-2251

90433 (GGTCGATGCACTCTGAGGC, targeting ORF); MYC 627: V3SH11240-226339523 (TTGATCATGCATTGAAAC, targeting 3' UTR); MYC 637: V3SH11240-226439183 (GTAGAAATACGGCTGCACC, targeting ORF); and non-targeting control: VSC11707. Briefly, 293FT cells (ThermoFisher) were reverse-transfected with a lentiviral shRNA expression vector in a 10 cm dish, using envelope (pCMV-VSV-G, #8454, Addgene) and packaging (pCMV-dR8.2 dvpr, #8455, Addgene) vectors following the ThermoFisher ViraPower protocols. Media containing lentivirus were harvested two days post-transfection, passed through a 0.45 µm filter, aliquoted and frozen. HT-1080 cells (ATCC) were used for titrating virus following the ThermoFisher ViraPower protocol.

LNCaP cells (clone FGC, catalog number CRL1740) were purchased from ATCC. Cell line authentication by STR profiling and mycoplasma testing was performed every 6 months (Laragen, Inc). LNCaP cells were seeded in 6-well plates at 1×10^5 cells per well and transduced with lentivirus at a multiplicity of infection of 1 in complete media for 24 h. Complete media were then replaced one day post-transduction, and selection with puromycin (1 µg/mL, Life Technologies) began 2 days post-transduction. Transduction was confirmed by visual confirmation of GFP fluorescence in >90% of cells, and knockdown was confirmed by qPCR and Western blotting.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 for Mac. Statistical tests used and relevant variables are indicated in the legend of each figure.

Additional methods including qPCR, western blotting, bioinformatic analyses, histology and ChIP-seq are provided in Supplementary Information.

Data availability

Human tissue RNA-seq data has been deposited into the Database of Genotypes and Phenotypes (<https://dbgap.ncbi.nlm.nih.gov/>), accession ID phs001813.v1.p1. Human tissue gene expression data has been deposited into the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>), accession ID GSE130046. ChIP-seq data has been deposited into the Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>), accession ID SRP218384 and GEO, accession ID GSE135942.

Code availability

The code used to perform all analyses is available at <https://github.com/CBIIT/lgcp>.

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Author contributions Design of the study: NCW, BEG, BJC, AGS; Tissue collection and pathology: HY; Immunohistochemistry and microdissection: NCW, SYT, SW, RL, NVC, RA, EDW; Data collection: NCW, NTT, STH, RL, NVC, RA, Library preparation and NGS: NCW, SYT; Data analysis: NCW, SYT, NTT, BEG, BJC, HY, AGS; Paper preparation: NCW, AGS.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Integrated radiologic, genomic and pathologic analysis revealed intratumoral divergent responses to neoadjuvant anti-androgen therapy

Scott Wilkinson¹, Huihui Ye², Nicholas T. Terrigino¹, Nicole Carrabba¹, Rayann Atway¹, Shana Y. Trostel¹, John R. Bright¹, S. Thomas Hennigan¹, Rosina T. Lis³, Ross Lake¹, Stephanie Harmon¹, Baris Turkbey¹, Peter A. Pinto¹, Peter L. Choyke¹, Fatima Karzai¹, David J. VanderWeele¹, Kathleen Kelly¹, William L. Dahut¹, and Adam G. Sowalsky¹

Author affiliations:

¹National Cancer Institute, NIH, Bethesda, MD, 20892

²Department of Pathology, UCLA, Los Angeles, CA, 90095

³Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, 02215

Background:

Localized prostate cancers are uniquely genetically variable and frequently multifocal, comprising both spatially separate regions and multiple independently evolving clones within each region. The multifocality of prostate cancer is a well-acknowledged problem for diagnosis, and the differential aggressiveness of potentially independent clones remains largely unknown. Further, appropriate prostate cancer staging using multiparametric MRI (mpMRI) and biopsy tissue can be confounded by sampling error. To date, there has been no understanding of whether this variability influences management decisions for localized prostate tumors. Here, we sought to identify the sensitivity and genomic profile of distinct localized tumors from a single patient following systemic intense neoadjuvant androgen deprivation therapy.

Methods:

A 66-year-old man with high risk prostate cancer was enrolled onto a Phase 2 study of neoadjuvant androgen deprivation therapy plus enzalutamide. mpMRI was performed at baseline, showing a single semi-contiguous lesion encompassing the right apical-mid peripheral zone also extending into the left distal apical peripheral zone. MR/ultrasound-fusion targeted biopsy was performed before undergoing 6 months of intense neoadjuvant androgen deprivation therapy (goserelin and enzalutamide). Biopsies of the left- and right-sided tumors showed differing histologies. A second mpMRI was performed following therapy before radical prostatectomy. Laser capture microdissection was performed to isolate pure populations of tumor cells from the pre-treatment biopsy and post-treatment surgical specimen. Whole exome sequencing was performed on microdissected tissues to identify somatic mutations and copy number alterations, which were further used to assess tumor clonal architecture and genomic/phenotypic evolution of treatment resistant tumor.

Results:

At baseline, mpMRI identified a large semi-contiguous tumor extending from the right apical-mid peripheral zone to the left distal apical zone, which was further resolved into two independent lesions sampled separately upon targeted biopsy. The biopsies showed remarkably differencing histologies, with one lesion TMPRSS2:ERG fusion positive and PTEN reduced, while the other lesion lacked the fusion and had heterogeneous PTEN staining. After six months of systemic therapy and radical prostatectomy, mpMRI and pathology showed near complete response of one tumor and substantial resistance of the other, which exhibited a large intraductal component. The resistant focus exhibited strong AR expression, reduced but present TMPRSS2:ERG expression, PTEN reduction, and low expression of potential bypass mechanisms including AR-V7 and GR.

Using point mutations as definitive clonal markers, we found that two clonally-independent tumors exhibited intrinsic heterogeneity at baseline which correlated with response or resistance. Whole exome data highlighted a divergence in the status of tumor suppressor genes implicated in prostate cancer progression both pre- and post-treatment. The pre-treatment resistant focus contained a one copy arm-level deletion of *PTEN* and a focal deletion in *TP53*, both of which were not present in the responding focus. The post-treatment intraductal resistant tumor contained an additional *PTEN* and *TP53* copy loss, rendering the intraductal resistant tumor both negative for both PTEN and p53. The responding tumor focus lacked any genomic hits to *PTEN* but showed reduced PTEN expression, suggesting alternative mechanisms of PTEN expression reduction.

Conclusions:

These data highlight a patient containing a polytumor, defined by two completely independent primary tumor systems in the same organ. Resistant intraductal tumor containing AR overexpression and reduced bypass mechanisms and dedifferentiation markers highlight a castration-resistant adenocarcinoma. Evolutionary trajectories that resulted in tumor heterogeneity in this case likely contributed to our observation that two independent prostate tumor nodules with distinct genetic alterations responded differently to neoadjuvant intense ADT.

Multiple primary prostate tumors with differential drug sensitivity

Scott Wilkinson¹, Huihui Ye², Nicholas T. Terrigino¹, Nicole Carrabba¹, Rayann Atway¹, Shana Y. Trostel¹, John R. Bright¹, S. Thomas Hennigan¹, Rosina T. Lis³, Ross Lake¹, Stephanie Harmon¹, Baris Turkbey¹, Peter A. Pinto¹, Peter L. Choyke¹, Fatima Karzai¹, David J. VanderWeele¹, Kathleen Kelly¹, William L. Dahut¹, and Adam G. Sowalsky¹

Author affiliations:

¹National Cancer Institute, NIH, Bethesda, MD, 20892

²Department of Pathology, UCLA, Los Angeles, CA, 90095

³Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, 02215

Background:

The differential aggressiveness of potentially independent prostate cancer clones remains largely unknown. Appropriate prostate cancer staging using multiparametric MRI (mpMRI) and biopsy tissue can be confounded by sampling error. To date, there has been no understanding of whether clonal variability influences management decisions for localized prostate tumors. We sought to identify the sensitivity and genomic profile of distinct localized tumors from a patient following systemic intense neoadjuvant androgen deprivation therapy (ADT).

Methods:

A 66-year-old man with high risk prostate cancer enrolled in a Phase 2 study of intense neoadjuvant ADT (goserelin + enzalutamide; inADT). Baseline mpMRI showing a single semi-contiguous lesion encompassing the right apical-mid PZ extending into the left distal apical PZ. MR/US-fusion targeted biopsy was performed before 6 months of inADT. A second mpMRI was performed before radical prostatectomy. Whole exome sequencing on microdissected tumor foci identified somatic mutations and copy number alterations, which were further used with immunohistochemistry to assess tumor clonal architecture and genomic/phenotypic evolution of treatment resistant tumor.

Results:

We found that two clonally-independent tumors exhibited intrinsic heterogeneity at baseline which correlated with response or resistance. Biopsies of distinct left- and right-sided tumors showed differing histologies. mpMRI and pathology showed near complete response of the left-sided tumor and substantial resistance of the right-sided tumor, which exhibited a large intraductal component. Histology and whole exome data highlighted a divergence in the status of PTEN and TP53, tumor suppressor genes implicated in prostate cancer progression.

Conclusions:

These data highlight that even nascent prostate cancer is heterogenous and neoadjuvant therapeutic strategies will need to consider this for clinical optimization. Evolutionary trajectories that resulted in tumor heterogeneity in this case likely contributed to our observation that two independent prostate tumor nodules with distinct genetic alterations responded differently to neoadjuvant intense ADT.

Integrated radiologic, genomic and pathologic analysis revealed intratumoral divergent responses to neoadjuvant anti-androgen therapy

Scott Wilkinson¹, Huihui Ye², Nicholas T. Terrigino¹, Nicole Carrabba¹, Rayann Atway¹, Shana Y. Trostel¹, John R. Bright¹, S. Thomas Hennigan¹, Rosina T. Lis³, Ross Lake¹, Stephanie Harmon¹, Baris Turkbey¹, Peter A. Pinto¹, Peter L. Choyke¹, Fatima Karzai¹, David J. VanderWeele¹, Kathleen Kelly¹, William L. Dahut¹, and Adam G. Sowalsky¹

Author affiliations:

¹National Cancer Institute, NIH, Bethesda, MD, 20892

²Department of Pathology, UCLA, Los Angeles, CA, 90095

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Background:

Localized prostate cancers are uniquely genetically variable, comprising both spatially separate regions and multiple independently evolving clones within each region. The multifocality of prostate cancer is a well-acknowledged problem for diagnosis, and the differential aggressiveness of potentially independent clones remains largely unknown. Further, appropriate prostate cancer staging using multiparametric MRI (mpMRI) and biopsy tissue can be confounded by sampling error. To date, there has been no understanding of whether this variability influences management decisions for localized prostate tumors. Here, we sought to identify the sensitivity and genomic profile of distinct localized tumors from a single patient following systemic intense neoadjuvant androgen deprivation therapy.

Methods:

A 66-year-old man with high risk prostate cancer was enrolled onto a Phase 2 study of neoadjuvant androgen deprivation therapy plus enzalutamide. mpMRI was performed at baseline, showing a single semi-contiguous lesion encompassing the right apical-mid peripheral zone also extending into the left distal apical peripheral zone. MR/ultrasound-fusion targeted biopsy was performed before undergoing 6 months of intense neoadjuvant androgen deprivation therapy (goserelin and enzalutamide). Biopsies of the left- and right-sided tumors showed differing histologies. A second mpMRI was performed following therapy before radical prostatectomy. Laser capture microdissection was performed to isolate pure populations of tumor cells from the pre-treatment biopsy and post-treatment surgical specimen. Whole exome sequencing was performed on microdissected tissues to identify somatic mutations and copy number alterations, which were further used to assess tumor clonal architecture and genomic/phenotypic evolution of treatment resistant tumor.

Results:

After six months of systemic therapy and radical prostatectomy, mpMRI and pathology showed near complete response of one tumor and substantial resistance of the other tumor, which exhibited a large intraductal component. Histopathological staining and whole exome data highlighted a divergence in the status of tumor suppressor genes implicated in prostate cancer progression, with the non-responding tumor at baseline exhibiting additional alterations absent from the pre-treatment tumor that responded to therapy. Using point mutations as definitive clonal markers, we found that two clonally-independent tumors exhibited intrinsic heterogeneity at baseline which correlated with response or resistance.

Conclusions:

These data highlight that even nascent prostate cancer is heterogenous and neoadjuvant therapeutic strategies will need to consider this for clinical optimization. Evolutionary trajectories that resulted in tumor heterogeneity in this case likely contributed to our observation that two independent prostate tumor nodules with distinct genetic alterations responded differently to neoadjuvant intense ADT.

Conflicts of Interest:

Huihui Ye: Janssen (consulting)

Funding Acknowledgments:

This work was supported by the Intramural Research Program of the NIH, National Cancer Institute, the Prostate Cancer Foundation, and Department of Defense Prostate Cancer Research Program (W81XWH-19-1-0712 to S.W., W81XWH-16-1-0433 to A.G.S.).

SCOTT WILKINSON

37 Convent Drive, Room 1056, Bethesda, MD 20892

scott.wilkinson@nih.gov

EDUCATION

National Cancer Institute, National Institutes of Health, Bethesda, MD
CRTA Postdoctoral Fellow

October 2016 – Present

Emory University, Atlanta, GA

PhD in Biological and Biomedical Sciences, Cancer Biology

June 2011 – August 2016

Berry College, Mt Berry, GA

BS in Biology

December 2009

RESEARCH SUPPORT

- **CCR Excellence in Postdoctoral Research Transition Award** 2021-
National Cancer Institute, \$200,000/year
- **Early Investigator Research Award** 2019-2021
W81XWH-19-1-0712, Department of Defense, \$200,000
- **Young Investigator Award** 2018-2021
18YOUN28, Prostate Cancer Foundation, \$225,000* (NCI is an ineligible recipient of PCF funds)
- **Ruth L. Kirschstein National Research Service Award** 2015-2016
1F31CA200383, National Institutes of Health, \$86,696
- **Graduate Research Fellowship Program Recipient** 2011-2014
National Science Foundation, \$128,500

RESEARCH EXPERIENCE

Postdoctoral Research, National Institutes of Health, Bethesda, MD

October 2016 – Present

CTRA Fellow, Dr. Adam Sowalsky lab

My postdoctoral work investigates the genetic features underlying response to anti-androgen therapy in patients with localized aggressive prostate cancer. Although androgen deprivation therapies (ADT) have been utilized in the clinic for metastatic prostate cancer, the genetic features that would predict response or non-response to this therapy is as of yet not understood. We were successful in identifying genetic features within baseline tumors of exceptional response to ADT, as well as features that would predict intrinsic resistance to this therapy. I was able to utilize my graduate training in cancer cell biology to initiate functional and mechanistic studies of these genetic predictors of response to therapy, with the ultimate goal of identifying novel targets to sensitize aggressive prostate cancers to hormone therapy.

- I served as a major contributor to three successfully funded Department of Defense Prostate Cancer Research Program awards to study ctDNA in pre-surgical and post-surgical/pre-biochemical recurrence patients and to examine intrinsic resistance to neoadjuvant ADT. I conducted specific aims under the research plan, and supervised/mentored 3 postbaccalaureate fellows and 1 summer student under the project.
- I applied for, and received, research grants from the Prostate Cancer Foundation's Young Investigator Award and the DOD Early Investigator Research Award based on my preliminary work and proposed future studies in this lab.
- Served as a reviewer for Scientific Reports, as well as peer-reviewed Prostate Cancer Foundation Young Investigator Awards, NCI Graduate Student Research Program applications, and ASCB microsymposia abstracts.
- I presented my work at several national research conferences, including the American Society for Cell Biology, American Association for Cancer Research, and AACR Advances in Prostate Cancer Research.

Thesis Research, Emory University, Atlanta, GA

March 2012 – August 2016

PhD Candidate, Dr. Adam Marcus lab

The tumor suppressor LKB1 is the third most commonly mutated gene in non-small cell lung adenocarcinoma. Identifying the consequences of LKB1 mutation during lung cancer invasion and metastasis is critical to understanding the mechanism of localized tumor invasion. My graduate work focused on identifying functional consequences of domain mutation in LKB1. I identified LKB1 farnesylation as critical for driving cellular polarization and directional persistence in 2D and 3D invasion, as well as promoting its actin association during cell motility. I also highlighted the LKB1 kinase activity in regulating cell membrane dynamics and leading-edge focal adhesion deposition and maturation during cell motility.

- I applied for and received an NSF predoctoral Graduate Research Fellowship to cover my first three years of my degree, focusing on the cell biology of lung cancer during cell motility.

- Received an NIH F31 pre-doctoral fellowship for my final two years describing the role of LKB1 in regulating cell polarization and invasion in a 3D microenvironment.
- Presented my research at several local and national conferences, including presentations at ASCB, AACR Tumor Metastasis, the Winship Cancer Institute Research Symposium, and Emory's graduate student symposium.
- Served as a mentor to 2 graduate students in the lab, while also developing and teaching courses on cancer cell biology to undergraduate and high school students.

Rickettsial Zoonoses Branch, CDC, Atlanta, GA

February 2010 – June 2011

Biologist (ORISE Fellow)

- I created an ecological niche model of Rickettsial pathogens throughout the Congo basin in sub-Saharan Africa to track previous Rickettsial outbreaks and predict future outbreaks based on climate and environmental conditions.
- I traveled to Arizona three times to participate in community awareness and Epi-Aid efforts to treat for and prevent *R. rickettsii* infection in epidemic regions of rural eastern Arizona.

Rickettsial Zoonoses Branch, CDC, Atlanta, GA

June 2009 – February 2010

Guest Researcher

- I developed an ELISA to detect antibodies to *R. rickettsii* in canines, which has been deployed to eastern Arizona for an *R. rickettsii* epidemic where canines are serving as reservoir hosts for ticks harboring rickettsial disease.

Berry College, Mount Berry, GA

August 2008 – December 2009

Undergraduate Research Assistant, Dr. Chris Mowry lab

- My undergraduate research examined dietary habits of coyotes (*Canis latrans*) in Yellowstone National Park to identify the impact of reintroduction of the grey wolf (*C. lupus*) on the local ecosystem.

PUBLICATIONS

J. R. Bright, R. T. Lis, A. T. Ku, N. T. Terrigino, S. Y. Trostel, N. V. Carrabba, S. A. Harmon, B. Turkbey, **S. Wilkinson**, A. G. Sowalsky. (2021). Prostate-specific membrane antigen is a biomarker for residual disease following neoadjuvant intense androgen deprivation therapy in prostate cancer. Submitted to *Journal of Urology*.

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C. Calagua, M. Ficial, C. S. Jansen, T. Hirz, L. Del Balzo, **S. Wilkinson**, R. Lake, A. T. Ku, O. Voznesensky, D. B. Sykes, P. J. Saylor, H. Ye, H. Kissick, S. Signoretti, A. G. Sowalsky, S. P. Balk, D. J. Einstein. (2021). A Subset of Localized Prostate Cancer Displays an Immunogenic Phenotype Associated with Losses of Key Tumor Suppressor Genes. *Clinical Cancer Research*. DOI: 10.1158/1078-0432.CCR-21-0121.

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S. Wilkinson, H. Ye, F. Karzai, S. Harmon, N. T. Terrigino, D. VanderWeele, J. Bright, R. Atway, S. Trostel, N. Carrabba, N. Whitlock, S. Walker, R. Lis, H. Sater, B. Capaldo, R. Madan, J. Gulley, G. Chun, M. Merino, P. Pinto, D. Salles, H. Kaur, T. Lotan, D. Venson, P. Choyke, B. Turkbey, W. L. Dahut, A. G. Sowalsky. (2021). Nascent prostate cancer heterogeneity drives evolution and resistance to intense hormonal therapy. *European Urology*. S0302-2838(21)00207-4. DOI: 10.1016/j.eururo.2021.03.009.

K. L. Morel, A. V. Sheahan, D. L. Burkhart, S. C. Baca, N. Boufaied, Y. Liu, X. Qiu, I. Cañadas, K. Roehle, M. Heckler, C. Calagua, H. Ye, C. Pantelidou, P. Galbo, S. Panja, A. Mitrofanova, **S. Wilkinson**, N. C. Whitlock, S. Y. Trostel, A. A. Hamid, A. S. Kibel, D. A. Barbie, A. D. Choudhury, M. M. Pomerantz, C. J. Sweeney, H. W. Long, D. J. Einstein, G. I. Shapiro, S. K. Dougan, A. G. Sowalsky, H. Hansen He, M. L. Freedman, S. P. Balk, M. Loda, D. P. Labbé, B. M. Olson, L. Ellis. (2021). EZH2 inhibition activates a dsRNA–STING–interferon stress axis that potentiates response to PD-1 checkpoint blockade in prostate cancer. *Nature Cancer*. 2(4):444-456. DOI: 10.1038/s43018-021-00185-w.

F. Karzai*, S. M. Walker*, **S. Wilkinson**, R. A. Madan, J. H. Shih, M. J. Merino, S. Harmon, D. VanderWeele, L. M. Cordes, N. V. Carrabba, J. R. Bright, N. T. Terrigino, G. Chun, M. Bilusic, A. Couvillon, A. Hankin, M. N. Williams, R. T.

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*These authors contributed equally to this work.

N.C. Whitlock, S.Y. Trostel, **S. Wilkinson**, N.T. Terrigino, S.T. Hennigan, R. Lake, N.V. Carrabba, R. Atway, E.D. Walton, B.E. Gryder, B.J. Capaldo, H. Ye, A.G. Sowalsky. (2020). *MEIS1* down-regulation by MYC mediates prostate cancer development through elevated *HOOXB13* expression and AR activity. *Oncogene*. DOI: 10.1038/s41388-020-01389-7

C. S. Jansen, N. Prokhnevskaya, V. A. Master, M. Sanda, J. W. Carlisle, M. A. Bilen, M. A. Cardenas, **S. Wilkinson**, R. Lake, A. G. Sowalsky, R. Valanparambil, W. Hudson, D. McGuire, K. R. Melnick, A. Khan, K. S. Kim, Y. M. Chang, A. Kim, C. Filson, M. Alemozaffar, A. Osunkoya, P. Mullane, C. Ellis, R. Akondy, S. J. Im, A. O. Kamphorst, A. Reyes, Y. Liu, H. Kissick. (2019). TCF1+ stem-like CD8 T cells in human tumors are required to generate anti-tumor effector T cells. *Nature*. 576: 465-470.

S. T. Hennigan*, S. Y. Trostel*, N. T. Terrigino, O. S. Voznesensky, R. J. Schaeffer, N. C. Whitlock, **S. Wilkinson**, N. V. Carrabba, R. Atway, S. Shema, R. Lake, A. R. Sweet, D. J. Einstein, F. Karzai, J. L. Gulley, P. Chang, G. L. Bubley, S. P. Balk, H. Ye, A. G. Sowalsky. (2019). Low abundance of circulating tumor DNA in localized prostate cancer. *Journal of Clinical Oncology Precision Oncology*. DOI: 10.1200/PO.19.00176.

S. Wilkinson, Y. Hou, J. T. Zoine, J. Saltz, L. A. D. Cooper, A. I. Marcus. (2017). Coordinated cell motility is regulated by a combination of LKB1 farnesylation and kinase activity. *Scientific Reports*. 7:40929.

J. Konen*, **S. Wilkinson***, B. Lee, W. Zhou, Y. Jiang, A. I. Marcus. LKB1 kinase-dependent and -independent defects disrupts polarity and adhesion signaling to drive collagen remodeling during invasion. *Molecular Biology of the Cell*. 2016. 27(7): 1069-84.

**Selected for Highlights Article, Cover Image for Journal

B. Lee, J. Konen, **S. Wilkinson**, A. I. Marcus, Y. Jiang. (2016). Local alignment vectors reveal cancer cell-induced ECM fiber remodeling dynamics. *Scientific Reports*. 7:39498.

F. Liu, R. Jin, X. Liu, H. Huang, **S. Wilkinson**, D. Zhong, F. R. Khuri, H. Fu, A. I. Marcus, Y. He and W. Zhou. LKB1 promotes cell survival by modulating TIF-IA-mediated pre-ribosomal RNA synthesis under uridine depleted conditions. *Oncotarget*. 2016. 7(3): 2519-31.

EDITORIALS AND REVIEWS

S. Wilkinson, H. Ye, R. T. Lis, A. G. Sowalsky. (2021). Reply to Alessia Cimagli, Liang Cheng, Antonio Lopez-Beltran, Marina Scarpelli, and Rodolfo Montironi's Letter to the Editor re: Scott Wilkinson, Huihui Ye, Fatima Karzai, et al. Nascent Prostate Cancer Heterogeneity Drives Evolution and Resistance to Intense Hormonal Therapy. *European Urology*. DOI: 10.1016/j.eururo.2021.06.002.

S. Wilkinson, A. G. Sowalsky. Battling the two-headed dragon of prostate cancer targeted therapy. (2020). *Molecular and Cellular Oncology*. DOI: 10.1080/23723556.2020.1745037.

SCIENTIFIC SERVICE

• Reviewer, Urology	2021-Present
• Organizer: Understanding Imposter Fears and Other Distortions Forum, ASCB	December 2021
• Member: Honorary Awards Taskforce, ASCB	2020-2021
• Member: Nominating Committee, ASCB	2020
• Member: Governance Committee, ASCB	2018-2020
• Organizer: Managing Up to Maximize Mentoring Forum, ASCB	December 2020
• Organizer: Self-Empowerment for Trainees Forum, ASCB	December 2019
• Moderator: Microtubule Motors & Transport Microsymposium, ASCB	December 2019
• Moderator: New Perspectives: Frontiers of Microscopy Microsymposium, ASCB	December 2019
• Reviewer, Prostate Cancer Foundation Young Investigator Awards	2019-Present
• Reviewer, NCI Graduate Student Recruiting Program	2019

• Reviewer, <i>Scientific Reports</i>	2018 – Present
• Moderator: Cell Cycle, Cell Division, Cell Death Microsymposium, <i>ASCB</i>	December 2018
• Moderator: Cytoskeleton, Motility, and Cell Mechanics Microsymposium, <i>ASCB</i>	December 2018
• Moderator: Cancer Cell Biology Microsymposium, <i>ASCB</i>	December 2017
• Moderator: Cell Adhesion and Motility Microsymposium, <i>ASCB</i>	December 2017
• Moderator: Bioengineering and Signaling Microsymposium, <i>ASCB</i>	December 2017
• Moderator: Cytoskeletal Molecular Dynamics Microsymposium, <i>ASCB</i>	December 2016
• Moderator: Development and Invasion Microsymposium, <i>ASCB</i>	December 2016
• Moderator: Signaling and Bioengineering Microsymposium, <i>ASCB</i>	December 2016
• Moderator: Cell Motility and Migration Microsymposium, <i>ASCB</i>	December 2015
• Moderator: Actin Cytoskeleton Dynamics Microsymposium, <i>ASCB</i>	December 2015

AWARDS & HONORS

• Fellows Award for Research Excellence (FARE), NIH	2020
• Early Investigator Research Award, Department of Defense	2019-2021
• Young Investigator Award, Prostate Cancer Foundation	2018-2021
• Cover Image, Molecular Biology of the Cell	April 2016
• First Author <i>Highlights</i> Article, Molecular Biology of the Cell	April 2016
• Cancer Close-Up Imaging Award, National Cancer Institute	April 2016
• Runner-Up, DSAC Image Competition, Emory University	January 2016
• Graduate Student Travel Award, American Society for Cell Biology	December 2015
• Graduate Career Teaching Award, Emory University	September 2015
• William and Catherine Rice Endowed Research Award, Emory University	September 2015
• Honorable Mention, Ford Foundation Dissertation Fellowship	April 2015
• 2nd Place, Division Student Activity Council Symposium, Emory University	January 2015
• Center for Faculty Development and Excellence Mini-Grant, Emory University	November 2014
• Runner-Up in Winship Scientific Research Symposium, Emory University	October 2014
• Emory-HHMI Curriculum Development Fellowship, Emory University	June-August 2014
• COMPASS Outreach Award, American Society for Cell Biology	May 2014
• 1st Place in Division Student Activity Council Research Symposium	January 2014
• GDBBS Annual Award for Excellence in Student Teaching, Emory University	September 2013
• ORISE Fellow, Centers for Disease Control and Prevention	2010-2011

NATIONAL MEMBERSHIPS

Society for Basic Urologic Research	2018-Present
American Association for Cancer Research	2015-Present
American Society for Cell Biology	2013-Present
American Society for Rickettsiology	2010-2011

COMMUNITY OUTREACH AND LEADERSHIP

• Co-Chair: COMPASS leadership committee, ASCB	2019-2022
• Panelist: Politicians Don't Bite, ASCB Annual Meeting	December 2016
• Member: Public Policy Committee, ASCB	2016-2019, 2021-2022
• USA Science and Engineering Festival, ASCB Exhibitor	2016, 2018
• COMPASS Hill Day, ASCB	April 2016
• Co-Chair: Outreach Subcommittee, COMPASS, ASCB	2016-2018
• AACR Early Career Hill Day, AACR	February 2016
• Member: Education Committee, ASCB	2015-2016
• Member: Committee for Postdocs and Students (COMPASS), ASCB	2015-Present
• Imagining the Future: Atlanta Science Festival	March 2015
• Hill Day, American Society for Biochemistry and Molecular Biology	April 2014
• Students for Science, Educator	2012-2016

FORMAL TEACHING, AND MENTORING

• NIH Postbaccalaureate Mentor Program, Mentor to Jack Bright	2019-2021
• NIH Postbaccalaureate Mentor Program, Mentor to Nicholas Terrigino	2018-2021
• NIH Postbaccalaureate Mentor Program, Mentor to Nicole Carrabba	2017-2019

- **NIH Summer Internship in Biomedical Research**, *Mentor to Rayann Atway* 2017, 2019
- **Winship Summer Scholars Program**, *Mentor to Stuti Rao* 2015
- **Bio 285: The Non-Fiction of Cancer**, *Instructor, Emory University* Spring 2015
- **Mentor to Emily Summerbell, Jaquelyn Zoine, PhD Rotation Students** 2014-2016
- **Cancer: The Biology Behind the Disease**, *Instructor, Emory Pre-College Program* 2014-2016
- **Cancer: The Biology Behind the Disease**, *Course Developer* Spring 2014
- **Emory Pipeline Program**, Senior Curriculum Instructor 2013-2014
- **Infectious Disease Institute**, *Mentor to Lucy Baker, Emory Pre-College Program* 2016
- **Bio 250: Cell Biology**, *Teaching Assistant, Emory University* Fall 2012

OUTREACH WRITING

- **Promote Public Awareness of Science with a COMPASS Outreach Grant**
[ASCB Post](#), January 20, 2017
- **How to Inspire Future Generations of Scientists Without Quitting Our Day Jobs**
[ASCB Compass Points](#), August 7, 2015
- **Exploring “Alternative” Careers: Are universities doing enough to help?**
[ASCB Compass Points](#), June 26, 2015
- **The Importance of Elections in Scientific Societies (including ASCB)**
[ASCB Compass Points](#), April 10, 2015

ABSTRACTS

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2020). Multiple primary prostate tumors exhibit divergent responses to intense neoadjuvant androgen deprivation therapy. 2020 AACR Prostate Cancer meeting. Denver, CO.

*Conference was canceled due to COVID-19 pandemic.

C. S. Jansen, N. Prokhnevskaya, M. Cardenas, V. Master, J. W. Carlisle, M. A. Bilen, **S. Wilkinson**, R. Lake, A. G. Sowalsky, A. O. Osunkoya, P. Mullane, C. Ellis, A. Reyes, Y. Liu, H. Kissick. (2020). CD8 T cell infiltration predicts progression in renal cell carcinoma and stratifies low and high-risk patients with stage III disease. 2020 SESAUA Meeting, New Orleans, LA.

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2020). Multiple primary tumors with differential drug sensitivity. 2020 GU ASCO Symposium. San Francisco, CA.

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2019). Multiple primary tumors with differential drug sensitivity. 2019 ASCB Annual Meeting. Washington, DC.

C. S. Jansen, N. Prokhnevskaya, V. Master, J. W. Carlisle, M. A. Bilen, M. Cardenas, **S. Wilkinson**, R. Lake, A. G. Sowalsky, A. O. Osunkoya, P. Mullane, C. Ellis, A. Reyes, Y. Liu, H. Kissick. (2019). CD8 T cell infiltration into renal tumors required a supportive antigen presenting niche and is independent of PD-L1 status. Society for Immunotherapy in Cancer Annual Meeting. National Harbor, MD.

S. Wilkinson, H. Ye, N. T. Terrigino, N. Carrabba, R. Atway, S. Y. Trostel, J. R. Bright, S. T. Hennigan, R. T. Lis, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2019). Integrated radiologic, genomic and pathologic analysis revealed intratumoral divergent responses to neoadjuvant anti-androgen therapy. 26th Annual Prostate Cancer Foundation Scientific Retreat. Carlsbad, CA.

S. Harmon, **S. Wilkinson**, H. Ye, F. Karzai, N. V. Carrabba, R. Atway, N. Terrigino, J. Bright, R. Lake, D. VanderWeele, P. Choyke, P. Pinto, W. L. Dahut, A. G. Sowalsky, B. Turkbey. (2019). Baseline multiparametric MRI characteristics of exceptional pathologic response to neoadjuvant enzalutamide for high-risk, localized prostate cancer. 2019 RSNA Annual Meeting. Chicago, IL.

S. Wilkinson, H. Ye, N. Carrabba, R. Atway, S. Y. Trostel, T. Hennigan, R. Lake, S. Harmon, G. Chun, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2019). Combining genetic and histopathologic features to predict response to anti-androgen therapy in aggressive prostate cancer. 2019 AACR Annual Meeting. Atlanta, GA.

N. T. Terrigino, S. T. Hennigan, S. Trostel, **S. Wilkinson**, H. Ye, A. G. Sowalsky. (2019). Development of an allele-specific assay for detecting circulating tumor DNA prostate cancer. 2019 AACR Annual Meeting. Atlanta, GA.

F. Karzai, R. A. Madan, D. J. VanderWeele, G. Chun, M. Bilusic, L. M. Cordes, N. Carrabba, **S. Wilkinson**, H. Ye, S. Harmon, A. G. Sowalsky, B. Turkbey, P. A. Pinto, P. L. Choyke, J. L. Gulley, W. L. Dahut. (2019). A study of intense neoadjuvant testosterone lowering therapy with goserelin and enzalutamide (Enza) in high-risk prostate cancer (PC) with multiparametric MRI (mpMRI). 2019 GU ASCO Annual Meeting, San Francisco, CA.

A. G. Sowalsky, **S. Wilkinson**, H. Ye, S. Harmon, B. Turkbey, N. Carrabba, R. Atway, G. Chun, D. J. VanderWeele, F. Karzai, R. A. Madan, P. A. Pinto, P. L. Choyke, W. L. Dahut. (2019). Molecular and imaging correlates of exceptional pathologic response to neoadjuvant ADT plus enzalutamide. 2019 GU ASCO Annual Meeting. San Francisco, CA.

S. Wilkinson, H. Ye, N. Carrabba, R. Atway, S. Y. Trostel, S. T. Hennigan, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, W. L. Dahut, K. Kelly, D. J. VanderWeele, A. G. Sowalsky. (2018). Genetic features predictive of response to anti-androgen therapies in aggressive prostate cancer. 2018 ASCB Annual Meeting. San Diego, CA.

S. Wilkinson, H. Ye, N. Carrabba, R. Atway, S. Y. Trostel, S. T. Hennigan, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2018). Genetic features predictive of response to anti-androgen therapies in aggressive prostate cancer. 2018 SBUR Annual Meeting. Palm Springs, CA.

A. Sheahan, K. Morel, D. Burkhardt, N. Boufaied, S. Panja, C. Calagua, Y. Huang, H. Ye, S. Trostel, N. Whitlock, **S. Wilkinson**, A. G. Sowalsky, A. Kibel, M. Loda, C. Sweeney, A. Mitrofanova, S. Dougan, D. Labbe, B. Olson, L. Ellis. (2018). Therapeutic targeting of Ezh2 enhances PD-1 blockage by induction of interferon gamma response. 2018 SBUR Annual Meeting. Palm Springs, CA.

S. Wilkinson, H. Ye, N. Carrabba, R. Atway, S. Y. Trostel, S. T. Hennigan, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, D. J. VanderWeele, K. Kelly, W. L. Dahut, A. G. Sowalsky. (2018). Genetic features predictive of response to anti-androgen therapies in aggressive prostate cancer. 25th Annual Prostate Cancer Foundation Scientific Retreat. Carlsbad, CA.

D. J. VanderWeele, B. Turkbey, F. Karzai, S. Harmon, A. G. Sowalsky, H. Ye, **S. Wilkinson**, G. Chun, S. Gold, P. A. Pinto, P. L. Choyke, W. L. Dahut. (2018). Neoadjuvant androgen deprivation therapy and enzalutamide: imaging and pathological responses. 2018 ASCO Annual Meeting. Chicago, IL.

N. Carrabba, **S. Wilkinson**, H. Ye, S. Harmon, B. Turkbey, P. A. Pinto, F. Karzai, W. L. Dahut, D. J. VanderWeele, P. L. Choyke, A. G. Sowalsky. (2018). Genetic and molecular mechanisms in high risk prostate cancer. 18th Annual CCR-FYI Colloquium. Bethesda, MD.

A. G. Sowalsky, **S. Wilkinson**, H. Ye, D. J. VanderWeele, B. Turkbey, S. T. Hennigan, R. Atway, S. Y. Trostel, R. Lake, F. Karzai, S. Harmon, G. Chun, K. Kelly, P. A. Pinto, P. L. Choyke, W. L. Dahut. (2018). Molecular and histopathologic correlates of imaging and biological responses to neoadjuvant GnRH agonist plus enzalutamide for high risk prostate cancer. 2018 GU ASCO Meeting. San Francisco, CA.

D. J. VanderWeele, B. Turkbey, F. Karzai, S. Harmon, A. G. Sowalsky, H. Ye, **S. Wilkinson**, P. A. Pinto, P. L. Choyke, W. L. Dahut. (2018). Neoadjuvant enzalutamide and androgen deprivation therapy for high risk prostate cancer: early results from a feasibility trial. 2018 GU ASCO Meeting. San Francisco, CA.

S. Wilkinson, H. Ye, R. Atway, S. Y. Trostel, N. C. Whitlock, S. T. Hennigan, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, W. L. Dahut, K. Kelly, D. J. VanderWeele, A. G. Sowalsky. (2017). Genomic correlates of imaging response in men receiving intense neoadjuvant androgen deprivation therapy. 2017 ASCB Annual Meeting. Philadelphia, PA.

S. Wilkinson, H. Ye, R. Atway, S. Y. Trostel, N. C. Whitlock, S. T. Hennigan, R. Lake, S. Harmon, B. Turkbey, P. A. Pinto, P. L. Choyke, F. Karzai, W. L. Dahut, K. Kelly, D. J. VanderWeele, A. G. Sowalsky. (2017). Genomic correlates of imaging response in men receiving intense neoadjuvant androgen deprivation therapy. AACR's Prostate Cancer: Advances in Basic, Translational, and Clinical Research. Dec 2017. Orlando, FL.

N. C. Whitlock, R. Atway, **S. Wilkinson**, Q. Yang, H. Ye, A. G. Sowalsky. (2017). Genetic contribution of MYC to the development of primary prostate cancer. AACR's Prostate Cancer: Advances in Basic, Translational, and Clinical Research. Dec 2017. Orlando, FL.

S. Wilkinson, A. I. Marcus. (2016). Cellular polarization and motility during invasion is driven by a combination of LKB1 farnesylation and kinase activity. *Mol. Bio. Cell* 27, pg(311).

- R. Commander, Y. Hou, J. Konen, **S. Wilkinson**, M. Gilbert-Ross, L. Cooper, A. I. Marcus. (2016). Defining the invasive biology of clinically relevant LKB1 mutations in lung cancer. *Mol. Bio. Cell* 27, pg (312).
- R. Jin, F. Liu, X. Liu, H. Huang, **S. Wilkinson**, D. Zhong, F. Khuri, H. Fu, A. I. Marcus, Y. He, W. Zhou. (2016). The regulation of pre-ribosomal RNA synthesis by LKB1. Proceedings: AACR 107th Annual Meeting. New Orleans, LA.
- S. Wilkinson**, J. Konen, A. I. Marcus. (2016). LKB1 kinase-dependent and -independent defects disrupt polarity and adhesion signaling to create a uniquely invasive amoeboid cancer cell. 13th Annual Division Student Advisory Council Student Research Symposium. 14 Jan 2016. Atlanta, GA.
- E. Summerbell, **S. Wilkinson**, A. I. Marcus. (2016). Farnesylated LKB1 associates with actin to promote stress fiber formation in mesenchymal cells. 13th Annual Division Student Advisory Council Student Research Symposium. 14 Jan 2016. Atlanta, GA.
- S. Wilkinson**, J. Konen, A. I. Marcus. (2015). LKB1 kinase-dependent and -independent defects disrupt polarity and adhesion signaling to create a uniquely invasive amoeboid cancer cell. *Mol. Bio. Cell* 26, pg(1116).
- E. Summerbell, **S. Wilkinson**, A. I. Marcus. (2015). Farnesylated LKB1 associates with actin to promote stress fiber formation in mesenchymal cells. *Mol. Bio. Cell* 26, pg(1515).
- S. Wilkinson**, J. Konen, A. I. Marcus. (2015). LKB1 kinase-dependent and -independent defects disrupt polarity and adhesion signaling to drive collagen remodeling during invasion. AACR Tumor Metastasis Conference. Austin, TX.
- Y. Hou, **S. Wilkinson**, D. Brat, A. I. Marcus, L. Cooper. (2015). Spatiotemporal Visualization of Cell Membrane Dynamics and Protein Colocalization Reveals Correlation between Membrane Dynamics and Metastatic Invasion. The 6th ACM Conference on Bioinformatics, Computational Biology, and Health Informatics. 9 Sept 2015. Atlanta, GA.
- J. Konen*, **S. Wilkinson***, A. I. Marcus. (2015). Better together: A dual function of LKB1 in navigating the 3-D microenvironment. 12th Annual Division Student Advisory Council Student Research Symposium. 15 Jan 2015. Atlanta, GA.
- *These authors contributed equally to this work.
- S. Wilkinson**, A. I. Marcus. (2015). Farnesylation regulates a molecular switch between distinct tumor suppressive functions of LKB1. 12th Annual Division Student Advisory Council Student Research Symposium. 15 Jan 2015. Atlanta, GA.
- S. Wilkinson**, A. I. Marcus. (2014). Farnesylated LKB1 regulates cell polarization and invasion through a 3D collagen microenvironment in a kinase-independent manner. *Mol. Bio. Cell* 25, pg(2055).
- S. Wilkinson***, J. Konen*, A. I. Marcus. (2014). Twice as nice: the dual tumor suppressive function of LKB1 in regulating cell polarization and invasion. Winship Scientific Research and Academic Development Symposium. 23 Oct 2014. Atlanta, GA.
- * These authors contributed equally to this work.
- S. Wilkinson**, A. I. Marcus. Farnesylated LKB1 associates with actin to regulate cell polarity and motility. Emory University STEM Research and Career Symposium. 27 March 2014. Atlanta, GA.
- S. Wilkinson**, A. I. Marcus. LKB1 regulates cell polarity and motility in a farnesylation-dependent manner. 11th Annual Division Student Advisory Council Student Research Symposium. 10 Jan 2014. Atlanta, GA.
- S. Wilkinson**, A. I. Marcus (2013). Post-translational modification of LKB1 regulates its actin association in a kinase-independent manner. *Mol Bio Cell* 24(suppl), Abstract No. 1167.
- R. Ryan Lash, **Scott Wilkinson**, Marguerite Madden, William L. Nicholson. Transforming analog rickettsial disease data into a digital geospatial database. 2011 ESRI Health GIS Conference. 9 Sept 2011. Arlington, VA.
- Scott C Wilkinson**, R Ryan Lash, William L Nicholson. Creation of a legacy database on Rickettsial pathogens and diseases found in Africa. 2010. 24th Annual Meeting of the American Society for Rickettsiology. 2010 August 1-4. Stevenson, WA.
- S. Wilkinson**, A. J. Lepore, J. Isenhower, C. Mowry. (2009). Determination of prey eaten by coyotes in Yellowstone National Park. Symposium on Student Scholarship. Berry College. Mount Berry, GA.

PRESENTATIONS

Scott Wilkinson. LKB1: Controlling the clutch that drives invasion. Elkin Seminar Series. Emory University. 16 Nov 2015. Atlanta, GA.

Scott Wilkinson. Twice as nice: the dual tumor suppressive functions of LKB1 in regulating cell polarization and invasion. Tuesday Morning Seminar Series. Berry College. 7 April 2015. Mount Berry, GA.

Scott Wilkinson. Dual functions of LKB1 in regulating cell polarity during invasion. Integrated Cellular Imaging Data Club. Emory University. 2 April 2015. Atlanta, GA.

Scott Wilkinson. Dual functions of LKB1 in regulating cell polarization and invasion. Elkin Seminar Series. Emory University. 16 Feb 2015. Atlanta, GA.

Scott Wilkinson. Farnesylated LKB1 associates with actin to regulate cell polarity. Elkin Seminar Series. Emory University. 31 Jan 2014. Atlanta, GA.

Scott Wilkinson. LKB1 Farnesylation Regulates its Actin Association. Elkin Seminar Series. Emory University. 22 Feb 2013. Atlanta, GA.

Scott Wilkinson. Possible Interactions Between Bcl-2 Family Proteins. Elkin Seminar Series. Emory University. 2 March 2012. Atlanta, GA.

Scott Wilkinson. Targeting Vimentin Overexpression in Metastatic Cancer. Elkin Seminar Series. Emory University. 2 Dec 2011. Atlanta, GA.

Scott Wilkinson. Characterization of the BAI1 Promoter Region and Regulatory Elements in Mouse and Human DNA. Elkin Seminar Series. Emory University. 2 Sept 2011. Atlanta, GA.

Scott Wilkinson. Recent CDC Investigations into Rocky Mountain Spotted Fever in Arizona. Tuesday Morning Seminar Series. Berry College. 19 Oct 2010. Mount Berry, GA

“Predicting Response to Intense Neoadjuvant Androgen Deprivation Therapy through Spatial Genomics and Imaging Heterogeneity”

PC180854

W81XWH-19-1-0712

PI: Dr. Scott Wilkinson

Org: The Geneva Foundation

Award Amount: \$239,600.00

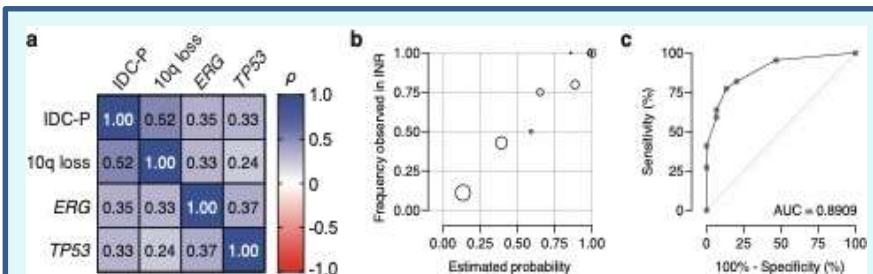


Study/Product Aim(s)

- 1) Assess genetic features of exceptional response and resistance to neoadjuvant anti-androgen therapies
- 2) Correlate mpMRI features to multiplex histopathological analysis of pre- and post-treatment tissue

Approach

We performed correlative studies of prostate cancer biopsies prior to undergoing neoadjuvant intense androgen deprivation therapy. Using residual cancer burden as an output, we then performed genomic analyses of samples stratified based on response status to identify features that correlate with treatment response.



Mapping multiple prostate biopsies to identify multiple unique lesions/patient prior to performing whole exome sequencing allows us to identify genomic features that predict response to anti-androgen therapy. Presence of IDC-P, chromosome 10q loss, nuclear ERG staining, and mutation to TP53 are predictive of incomplete/non-response to anti-androgen therapy.

Accomplishment: We completed the assessment of genetic features of therapeutic response, developing a model that combines genomic and histologic features that predicts treatment response.

Timeline and Cost

Activities	CY	FY1	FY2		
Assess genetic features of exceptional response and resistance to neoadjuvant anti-androgen therapies					
Correlate mpMRI features to multiplex analysis of pre-and post-treatment tissue					
Estimated Budget (\$K)		\$195,658	\$42,531		

Updated: 1/27/2022

Goals/Milestones (Example)

CY19 Goal –

- ☒ Biopsy Tumor Mapping and DNA Analysis

CY20 Goals –

- ☒ Mapping Radical Prostatectomy to corresponding biopsy and IHC
- ☒ Complete correlation of mpMRI to multiplex pathology

Comments/Challenges/Issues/Concerns

- If timelines change, comment here.
- If off by more than one quarter in spending, comment here.

Budget Expenditure to Date

Actual Expenditure: \$238,189.17