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TITLE: Understanding Molecular Mechanisms of Secondary Resistance in Patients with Metastatic Castrate-Resistant Prostate Cancer

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14. ABSTRACT One of the fundamental ways to slow down the progression of prostate cancer in men is to reduce the action of androgens, such as testosterone, on prostate cancer tissue. Certain drugs directly reduce androgen levels, while others, such as bicalutamide (Casodex), prevent androgen action on its receptor. However, even with these 'androgen-deprivation' therapies, a proportion of cancers escape and progress—this phenomenon is termed 'primary resistance'. To counteract this resistance, a second generation of anti-androgen therapies, namely enzalutamide (Xtandi), apalutamide (Erleada), darolutamide (Nubeqa) and abiraterone (Zytiga) has emerged over the last decade. While each drug effectively overcomes primary resistance, all tumors finally escape and progress. Such 'secondary resistance' currently poses a major clinical challenge, as patients rapidly follow a downhill course. The paucity of our understanding of mechanisms that underpin resistance has prevented us from developing new therapies. Mouse and cellular models have highlighted molecular pathways—however, not all are applicable to the prostate cancer patient. The focus of this study is therefore to understand the genetic and molecular events that lead to secondary resistance at the level of each individual patient . During the award period, we have obtained biopsy tissue from 24 patients with lethal prostate cancer using state-of-the-art radiology called molecular imaging. As we know that the cellular composition of biopsy tissue is not homogenous, we have used technologies, such as single cell gene sequencing, to measure the expression of cancer-associated genes at the level of each single cell. We have employed computational tools to understand which genes are potential drivers of the cancer and, importantly, of secondary resistance. We have identified that JAK/STAT and FGFR signaling is critical mediating resistance through a process known as lineage plasticity where cancer cells alter their identity. Furthermore, by reconstructing the cellular architecture in three dimensional cultures termed 'organoids', we have manipulated these target genes to study their effects on cancer progression. In all, we have identified and characterized novel targets that could be interrogated by new drugs to overcome secondary resistance.					
15. SUBJECT TERMS Prostate cancer, single cell RNA sequencing, human tumors, patient derived organoids, CRISPR editing, secondary resistance					
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1. INTRODUCTION

Prostate cancer is the second leading cause of cancer-related mortality in men, accounting for ~31,000 deaths in the U.S. alone during 2019. Despite the remarkable success of second-generation anti-androgen therapies, notably enzalutamide and abiraterone, secondary resistance poses an ongoing **clinical challenge**. Androgen receptor (AR) reactivation through gene amplification or mutation contributes to secondary resistance in almost 70% of patients with advanced castrate-resistant prostate cancer (CRPC). Mutations have been described in certain AR regulators, such as *FOXA1*, a pioneer transcription factor, *NCOR1/2*, a negative regulator, and *ZBTB16*, an AR-inducible gene that negatively regulates AR. Alterations in the PI3 kinase pathway that are enriched in advanced CRPC have also been shown to modulate AR signaling. Nonetheless, there is increasing concern regarding the emergence of AR-independent pathways in CRPC patients that have the poorest prognosis. One such pathway arises from 'lineage plasticity', a transdifferentiation phenomenon that, in the context of prostate adenocarcinoma, refers to the loss of typical luminal features, accounting for ~20% of secondary resistance. Notably, these tumors are enriched for *TP53* and *RB1* co-deletions that evolve clonally during transdifferentiation. An extreme form of lineage plasticity is the transformation of prostate adenocarcinoma into a neuroendocrine cancer, importantly, with AR loss. Similar biologic processes are noted in *EGFR* mutated non-small cell lung cancers and *BRAF*-mutated melanomas.

There have been attempts to recapitulate the genomic and signaling alterations in both AR-driven and AR-independent CRPCs in cell lines and mouse models. For example, a random mutagenesis screen revealed a *de novo* AR(F877L) mutation in human prostate cancer cells, LNCaP, which were under selective pressure from enzalutamide. This promiscuous mutation caused enzalutamide to convert to an AR agonist. Furthermore, an AR bypass pathway dependent on glucocorticoid receptor signaling emerged in LREX cells, a clonal line derived from parent LNCaP-AR cells following long-term enzalutamide exposure. Interestingly, CHIP-seq on LREX cells revealed overlapping AR and GR cistromes. In the context of *TP53* and *RB1* co-deletions as a cause of secondary AR-independent resistance, shRNA-mediated knock down of both genes in LNCaP and CWR22Pc-EP cells triggered the transition from a luminal to a basal epithelial phenotype with AR loss. This was, in part, mediated through a 10- to 50-fold upregulation of the epigenetic reprogramming gene *SOX2*, with androgen sensitivity being restored partially upon *SOX2* inhibition. Lineage plasticity has similarly been observed in mutant mice lacking *Tp53*, *Rb1* and *Pten*. Notably, a population of cells from these compound mutants display neuroendocrine features and increased *Sox2* and *Ezh2* expression. Our data also show that organoids derived from *Tp53^{fl/fl}/Rb1^{fl/fl}* mice lose their luminal features after ~4 weeks of lentiviral-Cre transduction, and that this transdifferentiation is accelerated with enzalutamide. Despite these functional studies, and substantial efforts at delineating genomic and transcriptomic landscapes for patients with advanced CRPC, we still lack robust drugable targets to counter the resistance to second-generation AR-directed therapies.

Here, we present a unique platform whereby we will leverage whole body molecular imaging to prospectively guide the acquisition of AR-positive and AR-negative CRPC biopsies, which will be subjected to single cell transcriptomic analyses and organoid-based functional assays. Indeed, it is now possible to determine radiographically AR expression in metastases using highly specific radiotracer probes for imaging. As expected, patients who are strongly avid for the uptake of ¹⁸F-dihydrotestosterone (F-DHT) and ⁶⁸Ga-prostate-specific membrane antigen (PSMA) are mostly AR-driven, while F-DHT- and PSMA-negative tumors are likely to be AR-independent. Our **hypothesis** is that there are unique transcriptomic signatures, genes and pathways in residual metastatic cancer cell populations in CRPC patients, and that functional studies with mouse and patient-derived organoids will identify those that are actionable. Our overall **goal** is therefore to utilize our **imaging-sequencing-organoid** platform towards two Specific Aims.

Specific Aim 1: To identify potentially actionable target genes in CRPC metastases utilizing molecular imaging to guide biopsies for single cell transcriptomic analyses.

Specific Aim 2: To determine the function of the identified target genes through CRISPR knockdown and/or doxycycline-induced overexpression in mouse and patient-derived organoid lines.

2. KEYWORDS

Prostate cancer, single cell RNA sequencing, human tumors, patient derived and murine organoids, CRISPR editing, secondary resistance

3. ACCOMPLISHMENTS

a. Research Specific Goals

	Months	MSKCC
SPECIFIC AIM 1: To identify potentially actionable target genes in CRPC metastases utilizing molecular imaging to guide biopsies for single cell transcriptomic analyses.		
Major Task 1: Collect metastatic prostate cancer biopsies for generation of single cell RNA–sequencing and patient–derived organoids.		
Subtask 1: Use advanced molecular imaging, namely ⁶⁸ Ga-PSMA and ¹⁸ F-DHT PET imaging to guide biopsies. Number of human biopsies: 24 Protocols: Biopsies and advanced imaging performed under approved IRB 12–245 and 06-107, and 16-306 and 00-095, respectively.	1–24	100%
Subtask 2: Digest tumors into a single cell suspension in preparation for single cell RNA–sequencing and organoid generation. Number of human biopsies: 24 [example HMP04 in Project Narrative] Method(s): Collagenase, trypsin, and FACS–sorted live cells are prepared. Single cell sequencing using 10X Chromium Kit. Organoids seeded in matrigel with adaptive media conditions.	1–24	100%
<i>Milestone(s) Achieved:</i> Generation on an ongoing basis of high–quality single cell RNA–sequencing data and matched patient-derived organoids.	1–24	100%
Major Task 2: Analyze single cell RNA–sequencing data using complex bioinformatic pipelines.		
Subtask 1: Process single cell RNA–sequencing data for identification of candidate genes Number of scRNA-seq files: 20 Methods: Pre-processing using SEQC, visualization of data, graph–based subpopulation clustering, trajectory inference, and identification of differentially expressed genes [refer to ‘Project Narrative’].	1–24	100%
<i>Milestone(s) Achieved:</i> Identification of candidate genes.	1–24	100%
SPECIFIC AIM 2: To determine the function of the identified target genes through CRISPR knockdown and/or doxycycline–induced overexpression in patient–derived organoid lines.		
Major Task 3: Perform CRISPR knockdown or doxycycline inducible overexpression of candidate genes in organoid models.		
Subtask 1: CRISPR knockdown of <i>HES6</i> and <i>MLLT11</i> in HMP–04, and <i>SERPINE2</i> and <i>SFRP1</i> in MSK-PCA3 organoids Lines: HMP-04 and MSK-PCA3 organoid lines.	1–3	0%

Method: Transduction of lentivirus–containing sgRNA and Cas9 at an MOI of 10 ⁵ . Confirmation of knockdown by Western blot.		
Subtask 2: CRISPR knockdown or doxycycline–inducible overexpression of newly–identified candidate genes by scRNA–sequencing. Lines: Matched patient–derived or patient–xenograft–derived organoid line. Method: CRISPR knockdown or doxycycline–inducible overexpression	4–24	100%
Milestone(s) Achieved: Successful knockdown or overexpression of candidate genes.	1–24	75%
Major Task 4: Use functional assays to study the function of candidate genes in matched patient–derived or patient–xenograft derived organoids.		
Subtask 1: Immunohistochemistry of HES6 and MLLT11 for HMP04, and SERPINE2 and SFRP1 in MSK-PCA3. Lines: Non–targeted and mutant HMP04 and MSK-PCA3 organoids. Methods: Immunohistochemistry with HES6 (Abcam #218037), MLLT11 (Abcam #109016), SERPINE2 (Abcam #154591), and SFRP1 (Abcam #4193) antibodies	1–3	0%
Subtask 2: Hematoxylin and eosin staining, immunohistochemistry for luminal, basal, and neuroendocrine markers, and immunohistochemistry of other candidate genes in patient derived organoids. Lines: Matched non–targeted and mutant organoid line. Method: Hematoxylin and eosin staining, immunohistochemistry for CK5 (basal), CK8 (luminal), and SYP and CGA (neuroendocrine), and immunohistochemistry with antibody directed to candidate gene.	4–24	100%
Subtask 3: Enzalutamide–sensitivity after knockdown or overexpression of HES6 and MLLT11 in HMP04 and SERPINE2 and SFRP1 in MSK–PCA3. Line: Non–targeted and mutant HMP04 and MSK–PCA3 organoids (~5000). Method: Viability measured in triplicate by Cell Titer-Glo.	1–24	0%
Subtask 4: Enzalutamide–sensitivity after knockdown or overexpression of newly–identified candidate genes. Line: Matched non–targeted and mutant organoids (~5000). Method: Viability measured in triplicate by Cell Titer-Glo.	1–24	100%
Subtask 5: Orthotopically engraft non–targeted or mutant HMP04 and MSK–PCA3 organoids in SCID mice and measure tumor response to enzalutamide. Lines: 24 SCID mice [6 mice for 3 triplicates of non–targeted and mutant organoids x 4 genes]. IACUC 06-07-012. Method: Organoids will be marked with GFP and orthotopically engrafted into SCID mice. Treatment with enzalutamide or vehicle will be followed by tumor volume measurements.	1–11	0%
Subtask 6: Orthotopically engraft non–targeted or mutant organoids for newly identified select candidate genes in SCID mice and measure tumor response to enzalutamide.	12–24	50%

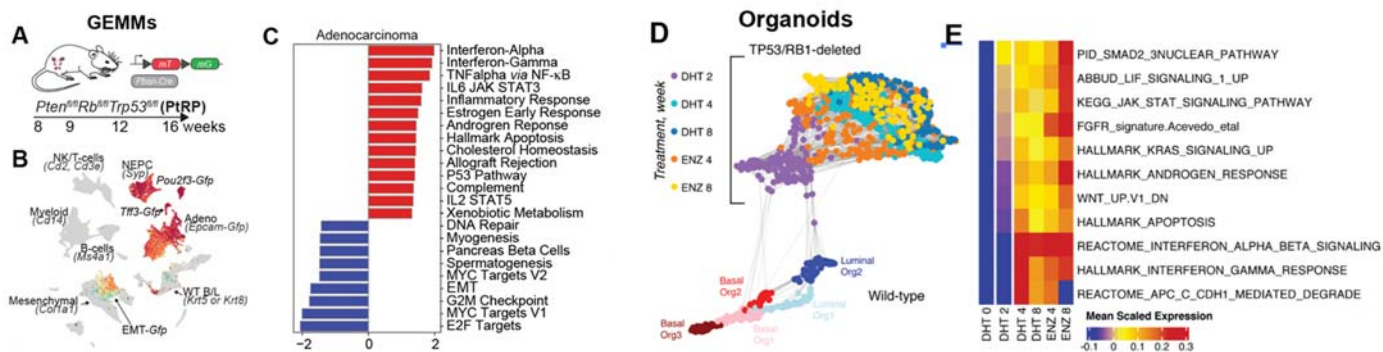


Figure 2: Upregulation of JAK/STAT and FGFR Signaling as a Function of Plasticity. To delineate the molecular programs involved in the progression of lineage plasticity after *Tp53* and *Rb1* deletion, we performed single cell RNA–sequencing (scRNA–seq) in both GEMM (*Tp53*^{fl/fl}; *Rb1*^{fl/fl}; *Pten*^{fl/fl} mice) and *Tp53/Rb1*–deleted organoids. In GEMM plastic and stem–like adenocarcinoma cells, JAK/STAT and interferon– α/γ expression programs were significantly up–regulated. **(A)** Shown is data from 9 wild type mice and 13 GEMM mice at labelled time points. **(B)** UMAP of GEMMs (N=67,622 cells) shown for all cell types based on imputed *Gfp* expression as a marker for mutant cells. **(C)** Enriched signaling pathways using GSEA in plastic adenocarcinoma cells highlighting inflammation and JAK/STAT pathways (scale -2.5 to 2, normalized enrichment score, $P < 0.05$). To determine whether a similar plasticity phenotype can be propagated *ex vivo*, we transduced *Cre*–expressing lentivirus in *Tp53*^{fl/fl}/*Rb1*^{fl/fl} murine prostate organoids. In this reductionist system, we observed a gradual loss of cystic morphology and luminal markers by 4 weeks (hyperplastic) with the gain of migratory neural protrusions by ~8–10 weeks (referred to as ‘slithering’). Organoids at 4 and 8 weeks showed a gain of mesenchymal markers, such as *Snai1*, *Zeb2*, and *Vim*. Strikingly, by performing scRNA–seq, we quantified tumoral plasticity (entropy based) and noted that JAK/STAT was the top correlated genetic program. **(D)** Shown above is a force directed layout (FDL) of *Tp53/Rb1*–deleted single cells from organoids colored by treatment (dihydrotestosterone, DHT; enzalutamide, ENZ) and timepoint (wildtype or 2–, 4–, and 8–weeks post–deletion). Wild–type cells are grouped into Basal Org 1–3 and Luminal Org 1–2 based on Phenograph clusters and canonical basal and luminal markers. **(E)** Heatmap of pathways most highly correlated with a measure of plasticity (entropy based) across timepoints. Each pathway is measured as the average Z–score of gene expression. Moreover, as our organoid media lacked serum, we reasoned that JAK/STAT maybe activated by other highly correlated ligand–receptor pairs. This analysis identified FGF1/FGFR, where the addition of the FGF1 ligand enhanced slithering in our organoid system.

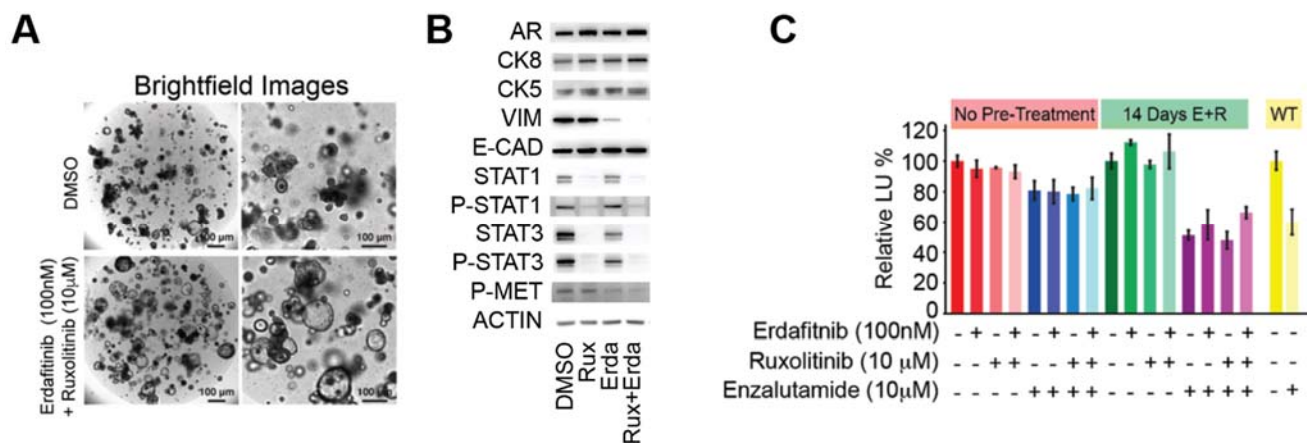


Figure 3: Reversal of Plasticity and Restoration of ARSI Sensitivity by Co–Inhibition of JAK and FGFR. As JAK/FGFR were upregulated after *Tp53/Rb1* deletion, we perturbed these pathways using a combination of genetic and pharmacologic approaches. CRISPR knockout or pharmacologic inhibition by ruxolitinib of JAK1/JAK2 reversed organoid phenotype from hyperplastic to cystic (from ~5% to 20%). Combinatorial treatment with ruxolitinib and the pan–FGFR inhibitor erdafitinib led to strikingly enhanced cystic reversion up to 60% and a dose–dependent increase in AR expression. **(A)** Shown are representative bright field micrographs and H&E staining for *Tp53/Rb1*–deleted organoids with CRISPR knockout of *Jak1/Jak2* or after 14 days exposure to DMSO or ruxolitinib (10 μ M) and/or erdafitinib (100 nM) (scale bar: 100 μ m). **(B)** Western blots showing the effect of dual inhibition with ruxolitinib and erdafitinib for 14 days. Notable is the loss of VIM, a mesenchymal marker, and increased CK8 and AR expression. We next asked whether dual inhibition

resulted in a re-sensitization to ARSIs. Of note is that ruxolitinib and/or erdafitinib did not display anti-proliferative effect; instead, there was a prominent effect in restoring cystic morphology. **(C)** Importantly, however, in the reprogrammed organoids, enzalutamide (10 μ M) reduced proliferation by 50%—this effect magnitude was strikingly comparable to the anti-proliferative effect of enzalutamide on untreated wild-type ARSI-sensitive organoids (10,000 cells *per well* in triplicate; viability by CellTiterGlo after 7 days treatment).

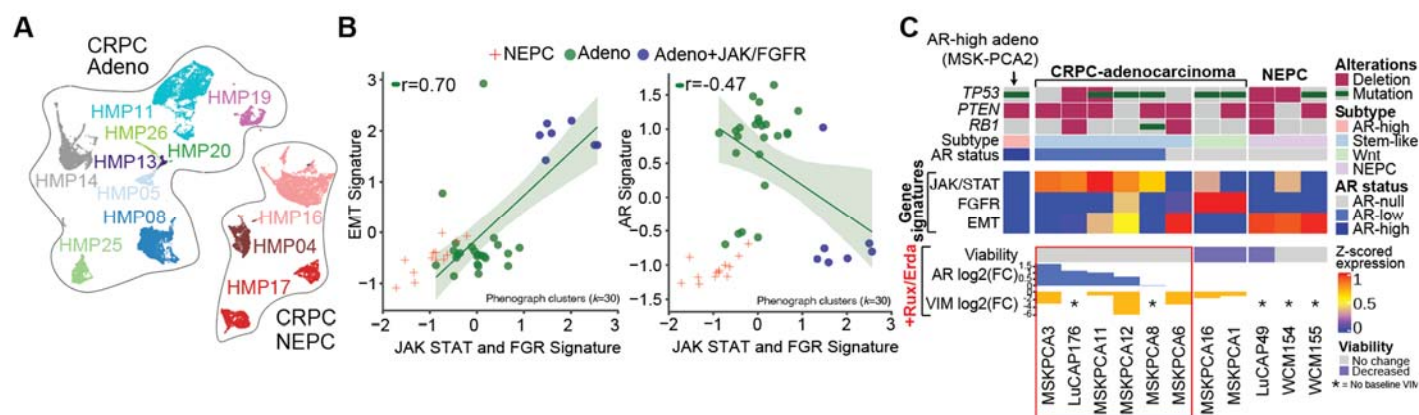


Figure 4: JAK/STAT and FGFR in Human Tumors and Tumoroids. CRPC patients underwent interventional radiology-guided biopsies using molecular imaging. **(A)** UMAPs of metastatic tumor cells (N=27,338 cells) colored by tumor ID and grouped by tumor type. **(B)** Tumor cells were annotated by PhenoGraph clusters ($k=30$) and divided into 3 groups defined by the Z-score of either JAK/STAT and FGFR or NEPC signatures. Two scatterplots of PhenoGraph clusters (points) are shown based on the Z-score of a combined JAK/STAT plus FGFR signature (x axis) vs. EMT and AR (y axis), respectively. Linear fits were calculated for adenocarcinoma clusters only; the corresponding Pearson's correlation is noted. **(C)** To functionally assess JAK/STAT reprogramming in human tumors, we treated tumoroids with erdafitinib (Erda) and ruxolitinib (Rux) and examined lineage reversion using AR (luminal) and VIM (mesenchymal) expression. Shown are baseline attributes, including *TP53*, *PTEN* and *RB1* genotypes, transcriptional and accessibility subtypes [PMID: 35617398] (14), and AR status *per* protein expression; a heatmap of baseline gene signatures in the tumoroids using bulk RNA-seq data based on mean Z-scored expression of JAK/STAT and FGFR signaling, and EMT gene sets; and functional changes in tumoroids with Rux plus Erda, including cell viability, and log2 fold change (FC) in AR and VIM protein expression. Importantly, only AR null to low tumoroids, which displayed high levels of JAK/STAT, showed an increase in AR and reduction in VIM after Rux and Erda treatment for 14 days.

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

Training:

Art of Flow Sorting, MSKCC
Mouse Training and Xenografts, MSKCC

Professional Development:

Invited Oral Presentation, Calico Labs, 2023
Poster Presentation, Genitourinary American Society of Clinical Oncology (ASCO), 2023
Invited Oral Presentation, Interdepartmental Genitourinary Retreat, MSKCC, 2023
Invited Oral Presentation, International Conference of the Genetics Society of Korea (ICGSK), 2022
Oral Podium Presentation, American Association for Cancer Research (AACR), 2022
Invited Talk, Drug Resistance and Sensitivity (DRSN Moonshot) Meeting, Cancer Moonshot Initiative, 2021
Invited Talk, Human Oncology Pathogenesis Program, MSKCC, 2020
Presentation at lab meetings every other month

d. How were the results disseminated to communities of interest?

Our manuscript was published in *Science*

1. Chan JM*, Zaidi S*, Love J, Zhao JL, Setty M, Wadosky KM, Gopalan A, Choo Z, Persad A, Choi J, LaClair J, Lawrence KE, Chaudhary O, Xu T, Masilionis I, Linkov I, Wang S, Lee C, Barlas A, Morris MJ, Mazutis L,

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

Nothing to report.

4. IMPACT

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

Our studies are highly impactful as, for the first time, as we have identified novel JAK/STAT and FGFR pathways that interact to underpin lineage plasticity and AR-insensitivity in both murine and human tumors. *In vivo* studies are ongoing which should help validate JAK/STAT and FGFR as targets for triple combinatorial therapy using currently FDA-approved agents, namely ruxolitinib, erdafitinib, and ARSI. The significance of these findings to treating resistant disease, without the need to develop a new therapeutic, could be of great benefit to our patients. Furthermore, we are continuing to grow our bank of human prostate tumors that have undergone scRNA-sequencing with an aim to elucidate meaningful targets and further inform non-tumor intrinsic mechanisms of resistance. Lastly, while we have focused on the JAK/STAT and FGFR pathway in Specific Aim 2 given its immediate therapeutic implications, however we have identified a number of other targets (*MLLT11*, *SFRP1*, *HES6*, *SERPINE2*, *ASCL1*, *NEUROD1*), which we are characterizing in ongoing studies.

b. What was the impact on other disciplines?

While these findings have clear mechanistic and therapeutic implications on prostate cancer progression and ARSI resistance, there is likely conservation of the plasticity program and its components in *EGFR*- and *KRAS*-mutant lung cancers, which also develop with *TP53* and *RB1* alterations, and, when exposed to next generation inhibitors transform in small cell lung cancer.

c. What was the impact of technology transfer?

Nothing to report

d. What was the impact on society beyond science and technology?

Our work is ultimately focused on improving patient care. We are hopeful these findings, particularly the potential for triple therapy using JAK/STAT, FGFR and AR directed agents, will translate into the clinic (ongoing trial to start at MSKCC) and benefit both patient survival and quality of life.

5. CHANGES/PROBLEMS

a. Changes in approach and reasons for change

As noted above, we discovered in our expanded cohort of human scRNA-seq data, that JAK/STAT signaling was upregulated in patient samples that underwent lineage plasticity. We also have strikingly noted a similar up-regulation of JAK/STAT (and FGFR) signaling pathway(s) in complementary models—namely, *Tp53*, *Rb1*, and/or *Pten* deleted GEMMs and organoid models. Given the consistency and therapeutic import of these observations, we expanded our focus to study this pathway and established additive effects in restoring ARSI sensitivity of co-inhibiting *Jak1/2* and *Fgfr*, both genetically and pharmacologically. For future studies, we will focus on delineating the role of other novel targets, including *MLLT11*, *SFRP1*, *HES6*, *SERPINE2*, *ASCL1*, *NEUROD1* in mediating ARSI resistance. These studies are ongoing.

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

Nothing to report

c. Changes that had a significant impact on expenditures

Nothing to report

d. **Significant changes in use of human subjects, vertebrate animals, biohazards, and/or select agents?**

Nothing to report

e. **Significant changes in use or care of human subjects**

Nothing to report

f. **Significant changes in use or care of vertebrate animals.**

Nothing to report

6. **PRODUCTS**

Publications, conference papers, and presentations:

Journal publications:

Chan JM*, **Zaidi S***, Love J, Zhao JL, Setty M, Wadosky KM, Gopalan A, Choo Z, Persad A, Choi J, LaClair J, Lawrence KE, Chaudhary O, Xu T, Masilionis I, Linkov I, Wang S, Lee C, Barlas A, Morris MJ, Mazutis L, Chaligne R, Chen Y, Goodrich DW, Karthaus WR, Pe'er D, Sawyers CL (2022) Lineage plasticity in prostate cancer depends on JAK/STAT inflammatory signaling. **Science**. doi:10.1126/science.abn0478. PMID: 35981096 (***Joint first authors**).

Other publications include:

Ryu V., Gumerova A., Korkmaz F., Kang S. S., Katsel P., Miyashita S., Kannangara H., Cullen L., Chan P., Kuo T., Padilla A., Sultana F., Wizman S. A., Kramskiy N., **Zaidi S.**, Kim S. M., New M. I., Rosen C. J., Goosens K. A., Frolinger T., Haroutunian V., Ye K., Lizneva D., Davies T., Yuen T., Zaidi, M. (2022). Brain atlas for glycoprotein hormone receptors at single-transcript level. **eLife**, 11, e79612. <https://doi.org/10.7554/eLife.79612>. PMID: 36052994. PMC9473692.

Bellone S, Roque DM, Siegel ER, Buza N, Hui P, Bonazzoli E, Guglielmi A, Zammataro L, Nagarkatti N, **Zaidi S**, Lee J, Silasi DA, Huang GS, Andikyan V, Damast S, Clark M, Azodi M, Schwartz PE, Tymon-Rosario JR, Harold JA, Mauricio D, Zeybek B, Menderes G, Altwerger G, Ratner E, Alexandrov LB, Iwasaki A, Kong Y, Song E, Dong W, Elvin JA, Choi J, Santin AD. A phase 2 evaluation of pembrolizumab for recurrent Lynch-like versus sporadic endometrial cancers with microsatellite instability. **Cancer**. (2021) Dec 7. doi: 10.1002/cncr.34025. Epub ahead of print. PMID: 34875107.

Miyahira AK, Zarif J, Coombs C, Flavell RR, Ruso JW, **Zaidi S**, Zhao D, Zhao SG, Pienta KJ, Soule HR. (2021) Prostate cancer research in the 21st century: report from the 2021 Coffey-Holden Prostate Cancer Academy Meeting. **Prostate**. (2021). Nov 3. doi: 10.1002/pros.24262. PMID: 34734426.

Choi J, Manzano A, Dong W, Bellone S, Bonazzoli E, Zammataro L, Yao X, Deshpande A, **Zaidi S**, Guglielmi A, Gnutti B, Nagarkatti N, Tymon-Rosario JR, Harold J, Mauricio D, Zeybek B, Menderes G, Altwerger G, Jeong K, Zhao S, Buza N, Hui P, Ravaggi A, Bignotti E, Romani C, Todeschini P, Zanotti L, Odicino F, Pecorelli S, Ardighieri L, Bilguvar K, Quick CM, Silasi DA, Huang GS, Andikyan V, Clark M, Ratner E, Azodi M, Imielinski M, Schwartz PE, Alexandrov LB, Lifton RP, Schlessinger J, Santin AD. (2021) Integrated mutational landscape analysis of uterine leiomyosarcomas. **Proceedings of the National Academy of Sciences** 118(15):e2025182118. doi: 10.1073/pnas.2025182118. PMID: 33876771; PMCID: PMC8053980.

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

Nothing to report

Other publications, conference papers, and presentations.

Invited Oral Presentation, Calico Labs, 2023

Poster Presentation, Genitourinary American Society of Clinical Oncology (ASCO), 2023

Invited Oral Presentation, Interdepartmental Genitourinary Retreat, MSKCC, 2023

Invited Oral Presentation, International Conference of the Genetics Society of Korea (ICGSK), 2022

Oral Podium Presentation, American Association for Cancer Research (AACR), 2022
Invited Talk, Drug Resistance and Sensitivity Meeting, Cancer Moonshot Initiative, 2021
Invited Talk, Human Oncology Pathogenesis Program, MSKCC, 2020
Presentation at lab meetings every other month

Website or other internet sites

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications and/or licenses

Nothing to report

Other Products

Data: N/A

Research material: N/A

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATION

What individuals have worked on the project?

Samir Zaidi (Principal Investigator, 10.8 months)

Joe Chan (Post-doctoral fellow in Dr. Pe'er laboratory, 0.96 months)

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

Please see the attached.

What other organizations were involved as partners?

Not applicable

8. SPECIAL REPORT REQUIREMENT

COLLABORATIVE AWARDS:

Not applicable

QUAD CHARTS:

Not applicable

9. APPENDICES

We have attached our recent *Science* paper describing our human single cells efforts and identification of plasticity dependence of interferon- α/γ and JAK/STAT pathways (Chan*, Zaidi* *et al.* **Science**, PMID 35981096, *Joint-first authors)

PREVIOUS/CURRENT/PENDING SUPPORT

Zaidi, Samir

PREVIOUS SUPPORT

*Title: T32 Investigational Cancer Therapeutics Training Program (ICTTP), Memorial Sloan Kettering Cancer Center (MSKCC)

*Major Goals: This is a T32 grant awarded to highly select physician trainees in hematology and medical oncology at MSKCC with the research training necessary for successful careers in the translation of cancer biology discoveries into novel molecular, immune, or cell-based therapeutics, and those experimental treatments.

*Status of Support: Completed

Project Number: 2 T32 CA009207-41

Name of PD/PI: Bajorin/Wolchok

Source of Support: NCI

Primary Place of Performance: Sloan Kettering Institute For Cancer Research

Project/Proposal Start and End Date (MM/YYYY): 08/01/1977-08/31/2024 (trainee appointment: 9/1/2019-12/31/2020)

*Total Award Amount (including Indirect Costs):

Overlap: None

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2019	12.00 calendar months
2. 2020	12.00 calendar months

*Title: Systemic Investigation of Mouse Derived Organoids Undergoing Lineage Plasticity with Human Patient-Derived Xenograft Validation

*Major Goals: This study is on mechanistic detail using organoids derived from a mouse model of prostate cancer to validate these findings, particularly the interferon signaling pathway and copy number instability, in human xenografts.

Aim 1: Investigate evolving transcription factor changes using *mouse* prostate organoids deleted for *Tp53/Rb1* using single cell ATAC- and RNA-sequencing.

Aim 2: Explore the role of interferon signaling and copy number instability in organoids from patient-derived xenografts displaying loss of *TP53/RB1*.

*Status of Support: Completed

Project Number: GC260796

Name of PD/PI: Zaidi, S

Source of Support: Conquer Cancer Foundation of the American, Society of Clinical Oncology

Primary Place of Performance: Sloan Kettering Institute For Cancer Research

Project/Proposal Start and End Date (MM/YYYY): 7/1/2021-6/30/2022

*Total Award Amount (including Indirect Costs):

Overlap: None

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	0.00 calendar
2. 2022	0.00 calendar

*No effort required by the sponsor

*Title: Understanding Molecular Mechanisms of Secondary Resistance in Patients with Metastatic Castrate Resistant Prostate Cancer

*Major Goals: This proposal will characterize novel molecular signatures in metastases from CRPC patients with an overarching goal to identify potentially actionable targets.

Aim 1: To identify potentially actionable target genes in CRPC metastases utilizing molecular imaging to guide biopsies for single cell transcriptomic analyses.

Aim 2: To determine the function of the identified target genes through CRISPR knockdown and/or doxycycline-induced overexpression in patient-derived tumor organoid lines.

*Status of Support: Completed

Project Number: PC200086

Name of PD/PI: Zaidi, S

Source of Support: Department of Defense (DOD), Prostate Cancer Research Program

Primary Place of Performance: Sloan Kettering Institute For Cancer Research Project/

Proposal Start and End Date (MM/YYYY): 1/1/2021–12/31/2022

*Total Award Amount (including Indirect Costs):

Overlap: N/A

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	10.80 calendar months
2. 2022	10.80 calendar months

CURRENT SUPPORT

*Title: Studying the Mechanisms of Lineage Plasticity in Prostate Cancer

*Major Goals: Our overall goal is to delineate, both through the use of novel human systems and previously identified functional candidates, mechanisms of lineage plasticity that are downstream of, or parallel to, TP53 and RB1.

Aim 1: Identify changes in transcriptional factor and gene programs in a wild-type human organoid model deleted for *TP53* and *RB1* over a time course

Aim 2: Characterize *Runx2* and *Barx2*, identified through single cell RNA-sequencing, in a genetically modified mouse model (GEMM) conditionally deleted for *Tp53*, *Rb1* and *Pten*.

*Status of Support: Active

Project Number: 2020YI2880

Name of PD/PI: Zaidi, S

Source of Support: Prostate Cancer Foundation (PCF)

Primary Place of Performance: Sloan Kettering Institute For Cancer Research

Project/Proposal Start and End Date (MM/YYYY): 1/4/2021–1/3/2024

*Total Award Amount (including Indirect Costs):

Overlap: None

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	0.00 calendar
2. 2022	0.00 calendar

*No effort required by the sponsor

*Title: Novel theranostic agents for neuroendocrine prostate cancer

*Major Goals: It is estimated that up to 20% of patients with advanced PCa eventually develop neuroendocrine prostate cancer (NEPC), which is typified by aggressiveness and extreme lethality. Multiple challenges must be overcome to address the needs of NEPC patients, the most critical of which include: (1) identifying the presence of NEPC in patients through an approach that is non-invasive, sensitive, and specific; and (2) developing novel treatment strategies that provide durable responses. Toward addressing these challenges, our PCF TACTICAL grant proposal explores the potential of a clinically translatable antibody targeting DLL3 as a molecularly targeted theranostic for the diagnosis and treatment of NEPC with further applications in small cell lung cancer (SCLC). Preclinical PET imaging studies with [89Zr]DFO-SC16 (Zirconium-89-labeled anti-DLL3 antibody SC16) demonstrated high specificity and sensitivity to NEPC lesions with background accumulation in AR+ and AR-null non-NEPC tumors. Therapeutic studies in NEPC tumor-bearing mice with the corresponding radiotheranostic [177Lu]-SC16 demonstrated pathologically complete responses with no tumor regrowth. We have translated

[89Zr]DFO-SC16 to the clinic, and initial imaging in NEPC patients with [89Zr]DFO-SC16 demonstrated high specificity for NEPC and an excellent safety profile. Immunohistochemical analysis of NEPC lesion samples obtained through image-guided biopsy revealed moderate to intense DLL3 expression with nominal PSMA expression. Despite the preliminary success of this trial, we are currently unable to conduct further imaging studies or initiate targeted radiotherapy studies with [177Lu]-SC16 due to the impending non-availability of the SC16 antibody. To overcome this barrier, we have initiated development of next-generation DLL3-specific antibodies for radiotheranostic applications and identified several promising lead agents.

*Status of Support: Active

Project Number: 2022 PCF TACTICAL Award

Name of PD/PI: Lewis, J

Source of Support: Prostate Cancer Foundation (formerly CaP CURE)

Primary Place of Performance: Sloan Kettering Institute For Cancer Research

Project/Proposal Start and End Date (MM/YYYY): 1/1/2023–2/31/2025

*Total Award Amount (including Indirect Costs): Overlap: None

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	0.60 calendar
2. 2024	0.60 calendar
3. 2025	0.60 calendar

*Title: NFCR GRANT APPLICATION

*Major Goals:

1. Evaluate CTC capture feasibility and validity in metastatic RCC patients treated with systemic therapies.
 - a. Evaluate the CTC detection and yield rates and correlate with radiographic tumor volumetric data.
 - b. Perform matched NGS profiling of CTC and matched tumor tissues (primary nephrectomy or metastatic site) to determine concordance and clonality estimates.
2. Determine whether dynamic changes in CTC load and profile are associated with systemic therapy response in RCC patients.
 - a. Perform baseline and on-treatment serial isolation of CTC to correlate longitudinal changes with objective response rates (via RECIST v1.1).

3. Status of Support: Active

Project Number: National Foundation For Cancer Research

Name of PD/PI: Lee, C

Source of Support: National Foundation For Cancer Research

Primary Place of Performance: Sloan Kettering Institute For Cancer Research

Project/Proposal Start and End Date (MM/YYYY): 12/1/2022 - 11/30/2023

4. Total Award Amount (including Indirect Costs):

Overlap: None

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	0.60 calendar

*Title: Perturb–Sequencing of Patient–Derived Organoids using Differentially Expressed Lineage Markers

*Major Goals: My overall goal is to functionally alter key transcriptional lineage regulators to understand how cellular states change using perturb–sequencing.

*Status of Support: Active

Project Number: Gerstner Physician Scholar

Name of PD/PI: Zaidi, S

Source of Support: Memorial Sloan Kettering Cancer Center

Primary Place of Performance: Sloan Kettering Institute For Cancer Research

Project/Proposal Start and End Date (MM/YYYY): 9/1/2022 - 8/31/2025

*Total Award Amount (including Indirect Costs):

Overlap: None

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	0.60 calendar
2. 2024	0.60 calendar
3. 2025	0.60 calendar

PENDING SUPPORT

N/A

CANCER

Lineage plasticity in prostate cancer depends on JAK/STAT inflammatory signaling

Joseph M. Chan^{1,2†}, Samir Zaidi^{3,4†}, Jillian R. Love^{3‡§}, Jimmy L. Zhao^{3‡¶}, Manu Setty^{2‡##}, Kristine M. Wadosky⁵, Anuradha Gopalan⁶, Zi-Ning Choo², Sitara Persad^{2,7}, Jungmin Choi⁸, Justin LaClair³, Kayla E. Lawrence³, Ojasvi Chaudhary², Tianhao Xu², Ignas Masilionis², Irina Linkov⁶, Shangqian Wang³, Cindy Lee³, Afsar Barlas⁹, Michael J. Morris⁴, Linas Mazutis^{2,10}, Ronan Chaligne², Yu Chen³, David W. Goodrich⁵, Wouter R. Karthaus^{3,*§}, Dana Pe'er^{2,11*}, Charles L. Sawyers^{3,11*}

Drug resistance in cancer is often linked to changes in tumor cell state or lineage, but the molecular mechanisms driving this plasticity remain unclear. Using murine organoid and genetically engineered mouse models, we investigated the causes of lineage plasticity in prostate cancer and its relationship to antiandrogen resistance. We found that plasticity initiates in an epithelial population defined by mixed luminal-basal phenotype and that it depends on increased Janus kinase (JAK) and fibroblast growth factor receptor (FGFR) activity. Organoid cultures from patients with castration-resistant disease harboring mixed-lineage cells reproduce the dependency observed in mice by up-regulating luminal gene expression upon JAK and FGFR inhibitor treatment. Single-cell analysis confirms the presence of mixed-lineage cells with increased JAK/STAT (signal transducer and activator of transcription) and FGFR signaling in a subset of patients with metastatic disease, with implications for stratifying patients for clinical trials.

Resistance to molecularly targeted therapies is often due to mutations in the drug target, but there is growing recognition of other modes of tumor escape, particularly involving next-generation inhibitors designed to circumvent target-based resistance mechanisms. For example, in epidermal growth factor receptor (EGFR)- and KRAS-mutant lung cancer and in metastatic prostate cancer, tumor cells can undergo a lineage transition from adenocarcinoma to squamous or neuroendocrine histology after treatment with the respective inhibitors (1–6). Similarly, BRAF-mutant melanomas can transition from a MITF⁺

differentiated melanocyte phenotype to more mesenchymal or neural crest stem-like cell states in response to BRAF inhibition (7–9). Transcriptomic analyses indicate that these changing cell states share features of normal developmental, regenerative, and stem cell signatures, suggesting that tumor cells co-opt these gene expression programs to escape lineage-dependent therapies (10).

The increasing prevalence of this mode of tumor escape has sparked numerous investigations into the underlying mechanism. Analysis of patient specimens, particularly at the single-cell level, has been instructive in defining the complexity of the problem, revealing notable heterogeneity within and across patients (11, 12). Because these studies typically capture a single snapshot during tumor evolution, it is difficult to discern precisely when, where, and how plasticity arises and its relationship with drug treatment and resistance.

Human prostate cancer provides an ideal model to study the role of plasticity in drug resistance because of its near universal association with prior exposure to androgen receptor (AR)-directed therapies. Prostate cancers initially present as AR⁺ invasive adenocarcinomas that express luminal lineage markers, including CK8, CK18, and kallikreins such as KLK3 (prostate specific antigen or PSA). Histopathologic criteria for diagnosis classically include a loss of TP63⁺ basal epithelial cells, underscoring the luminal-like identity of prostate cancer. Prostate cancers are uniformly sensitive to androgen deprivation therapy but eventually relapse as castration-resistant prostate cancer (CRPC).

Clinical observations and sequencing data demonstrate that CRPC has heterogeneous

phenotypes (13). Two subtypes, adenocarcinoma (CRPC-Adeno) and neuroendocrine prostate cancer (NEPC), can be broadly identified by histology and staining for luminal (AR, KLK2/3) and neuroendocrine (SYP, INSM1, CHGA/B, CD56) lineage markers. However, these markers do not capture the full spectrum of CRPC phenotypes, which include mixed luminal and neuroendocrine features, loss of canonical luminal and neuroendocrine markers (“double negative”), and gain of basal, mesenchymal, and stem-like gene expression signatures (4, 14). Phenotypic heterogeneity in CRPC is thought to be a consequence of lineage plasticity within adenocarcinomas, rather than the outgrowth of preexisting subclones with alternative lineages. This conclusion is supported by the fact that genomic alterations found in early luminal cancers, such as ERG translocations, are retained in NEPC (15). Furthermore, these diverse CRPC phenotypes cannot be clearly distinguished by tumor genotype. Although the loss of TP53 and RB1 is highly enriched in NEPC and double-negative CRPC, alterations in these tumor suppressors, as well as PTEN, are also found in CRPC-Adeno. A possible explanation is that loss of function in these genes may initiate a plastic cell state, which enables alternative lineage phenotypes to emerge during treatment with antiandrogen therapy.

Another critical question is whether anti-AR therapy, which is highly effective in inhibiting the growth of luminal epithelial tumor cells, may also accelerate the emergence of nonluminal CRPC phenotypes. The relative frequency of NEPC and double-negative CRPC cases has increased over the past decade (4), coinciding with increased clinical use of next-generation anti-AR therapeutics (such as abiraterone and enzalutamide) that inhibit AR signaling more potently than standard androgen-deprivation therapy. Although this correlation may be incidental, it begs the question of whether lineage plasticity, which is presumed to play a causal role in the development of antiandrogen resistance, is itself promoted or enhanced by anti-AR therapy.

Inflammatory signaling in adenocarcinoma cells precedes NEPC

To investigate these questions, we turned to two closely related genetically engineered mouse models (GEMMs) of prostate cancer that recapitulate the adenocarcinoma-to-NEPC transition after prostate-specific deletion of the human-relevant tumor suppressor genes *PTEN*, *RB1*, and *TP53* using CRE recombinase. Single-cell RNA sequencing (scRNA-seq) was performed on whole prostates harvested from 29 mice [9 wild type (WT); 7 *Pten*^{−/−}*Rb*^{−/−} (PtR); 13 *Pten*^{−/−}*Rb*^{−/−}*Trp53*^{−/−} (PtRP)] at relevant time points reflecting the adenocarcinoma-to-NEPC transformation (Fig. 1A and table S1) (16).

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Transcriptomes from 67,622 cells were analyzed and visualized by uniform manifold approximation and projection (UMAP), using coarse labeling to identify cell types and green fluorescent protein (*Gfp*) to mark cells that underwent recombination (Fig. 1B, fig. S1, and methods). In addition to the expected adenocarcinoma (*Epcam-Gfp*) and NEPC (*Syp*⁺) populations, we were surprised to see three additional *Gfp*⁺ clusters defined by expression of *Pou2f3* and *Delk1* (*Pou2f3-Gfp*); *Vim*, *Twist2*, and *Ncam1* (*Vim-Gfp*); and *Tff3* (*Tff3-Gfp*), respectively. *Pou2f3* encodes a transcription factor (TF) expressed by rare chemosensory cells in normal gut and lung tissue (often called tuft cells) and defines a non-neuroendocrine variant subtype of small-cell lung cancer with morphologic features distinct from ASCL1 and NEUROD1 subtypes (17, 18). *Tff3* encodes a secreted protein expressed by intestinal colunar epithelial cells involved in maintaining mucosal integrity and is a clinical biomarker for Barrett's esophagus (19).

We examined potential routes of plasticity by evaluating cell connectivity in k-nearest-neighbor graphs, visualized using force-directed layouts (FDLs) for each genotype across different time points. We noted the early appearance of *Gfp*⁺ adenocarcinoma (*Epcam-Gfp*) as well as *Tff3-Gfp* cells, followed by the expansion of *Vim-Gfp* and NEPC and *Pou2f3-Gfp* populations, with the transition to NEPC more prevalent in the PtRP genotype, indicative of the additional impact of P53 loss (Fig. 1, C and D). The presence of each of these subpopulations was confirmed by multiplex immunofluorescence (IF) and immunohistochemistry (IHC) (table S2), which documented SYP⁺ (NEPC) and VIM⁺ (VIM) cells in a background of ECAD⁺ (and PANCK⁺) epithelial cells (top row for ECAD in Fig. 1E and PANCK stain in fig. S2) as well as larger clusters of NEPC (top row) and VIM (bottom row) and foci of TFF3⁺ or POU2F3⁺ cells (bottom rows) (Fig. 1E). Although this unexpectedly diverse range of cell types, often seen within a single mouse, is of interest, we focused our analysis here on the adenocarcinoma and NEPC populations because of their established clinical importance. Deeper analysis of the *Tff3*⁺ and *Pou2f3*⁺ populations will be reported separately.

Owing to the large number of independent alleles, definitive lineage-tracing experiments cannot be easily performed in this GEMM. Consequently, we relied on various computational approaches to predict the most likely scenario by which lineage plasticity emerges. As a starting point, we reasoned that the early emergence of the adenocarcinoma population, along with its placement between WT and other mutant clusters in the k-nearest-neighbor graph, implicate it as the most likely source of plasticity (fig. S3, A to D, and methods). Therefore, we examined differentially expressed genes

(DEGs) and pathways associated with populations along this putative transition, using gene set enrichment analysis (GSEA) (Fig. 1F and tables S3 to S5). First, we noted enrichment for a stem-like (L2) luminal signature that we and others have previously reported from single-cell analysis of the normal mouse prostate (20–22) as well as enrichment for epithelial-mesenchymal transition (EMT) programs and for signatures of early developmental programs (23, 24). Most surprising was strong enrichment for a range of inflammatory response gene signatures, including *Ifn-α*, *Ifn-γ*, *Jak/Stat*, and *Tnfa* (table S4). Given the indications implicating the adenocarcinoma population as a potential source of plasticity and its clinical relevance, we used Palantir trajectory analysis (25) to consider potential routes between adenocarcinoma and NEPC (Fig. 1G and methods). We noted three major trends in TF expression: (i) those active in adenocarcinoma alone, (ii) those expressed during the putative transition to NEPC, and (iii) NEPC-specific regulators (see Fig. 1, G and H, and fig. S3, E and F, for robustness analysis and table S5). Notably, four inflammatory response TFs (Stat1, Stat2, Irf7, and Irf1), along with JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling, are specifically enriched in adenocarcinoma cells during the transition phase to NEPC (Fig. 1, G and H).

Cell-autonomous induction of plasticity in normal prostate organoids

Having documented the onset and evolution of lineage plasticity in vivo in the mouse prostate, we next asked whether plasticity is dependent on the tumor microenvironment or whether it can occur in a cell-autonomous manner. For this purpose, we turned to organoid culture because prostate epithelial cells can be grown in isolation without accessory stromal or immune cell populations from the prostate microenvironment (26). Because this system supports the growth of normal as well as malignant epithelial cells, it also provides an opportunity to examine the immediate and longer-term consequences of tumor suppressor gene deletion with precise kinetics.

Focusing initially on *Rb1* and *Trp53* deletion (to model the alterations in human NEPCs in a minimalist system), we infected *Trp53*^{loxP/loxP}; *Rb1*^{loxP/loxP} organoids with Cre-expressing lentivirus. We also leveraged a mTmG reporter cassette to identify *Trp53*^{-/-}; *Rb1*^{-/-} cells using the red-to-green color change initiated by Cre expression (movie S1). In contrast to the cystic appearance of uninfected organoids, those with *Trp53* and *Rb1* codeletion lost their basal-luminal polarity and cystic lumens by ~4 weeks (hereafter called hyperplastic), followed by the appearance of migratory cells and cellular protrusions outside the normally sharp exterior organoid borders at ~8 to 10 weeks (here-

after called “slithering,” based on similarity to the migratory phenotype originally reported for lung neuroepithelial bodies) (27) (Fig. 2, A and B; figs. S4, A to C, and S5; and movie S2). This phenotype was also seen after CRISPR-Cas9-mediated codeletion of *Trp53* and *Rb1* in WT organoids (fig. S4B) and required deletion of both tumor suppressors. No morphologic changes were observed after deletion of *Trp53* or *Rb1* alone (fig. S4B), consistent with earlier work in human prostate cancer cell lines (28).

To further characterize the changes induced by *Trp53* and *Rb1* codeletion, we performed bulk RNA-seq, Western blot analysis, and IF or IHC analysis of various lineage markers. The most dramatic findings were reduced expression of luminal lineage genes such as *Nkx3.1*, *Dpp4* (*Cd26*), *Krt18*, and *Krt8* and increased expression of mesenchymal genes such as *Snail*, *Zeb2*, and *Vim* (Fig. 2, C to E; fig. S4, C to E; and table S6). GSEA analysis of the bulk RNA-seq data confirmed enrichment of EMT (Fig. 2F and table S7) as seen in PtRP mice, which was further supported by increased protein levels of Slit3 (also in the leading edge of the EMT gene set) and the migratory slithering phenotype observed morphologically.

Most surprising, however, was strong enrichment for the same inflammatory response pathways (e.g., *Jak/Stat*) that we saw earlier in PtRP GEMMs, as well as overexpression of *Fgfr1* [log2 fold change of 5.0; false discovery rate (FDR) < 7 × 10⁻¹⁹⁹] and the ligands *Fgf1*, *Fgf2*, *Fgf7*, and *Fgf10* (all fold change >4; FDR < 5 × 10⁻⁸) (Fig. 2, E and F). We confirmed this activation biochemically, with robust pStat1 and pStat3 activation in *Trp53*^{-/-}; *Rb1*^{-/-} organoids that was reversed by 48-hour treatment with the Jak kinase inhibitor ruxolitinib (Rux) (Fig. 2, F and G, and fig. S4F). Thus, the early manifestations of plasticity seen in GEMMs can be recapitulated in normal prostate epithelial cells in culture in the absence of any accessory cells, indicative of a cell-autonomous process.

However, unlike the later NEPC phenotype observed in PtRP mice and previous reports of *Trp53*^{-/-}; *Rb1*^{-/-} GEMMs (29), we failed to detect pronounced expression of *Syp* or other neuroendocrine markers (*Chga*, *Chgb*, *Ascl1*, or *Neurod1*), even in *Trp53*^{-/-}; *Rb1*^{-/-} organoids passed for >12 months. Postulating that this might be due to the absence of critical prostate microenvironmental factors in organoid culture media, we asked if the organoid phenotype evolved further after orthotopic (OT) implantation of *Trp53*^{-/-}; *Rb1*^{-/-} organoids into *Trp53*^{+/+}; *Rb1*^{+/+} hosts. After confirming highly efficient engraftment of hyperplastic and/or slithering stage organoids in a pilot experiment (by detection of GFP⁺ cells at 4 weeks in the dorsal lobe of hormonally intact NSG mice), we scored lineage phenotype and tumorigenicity, as well as the effect of

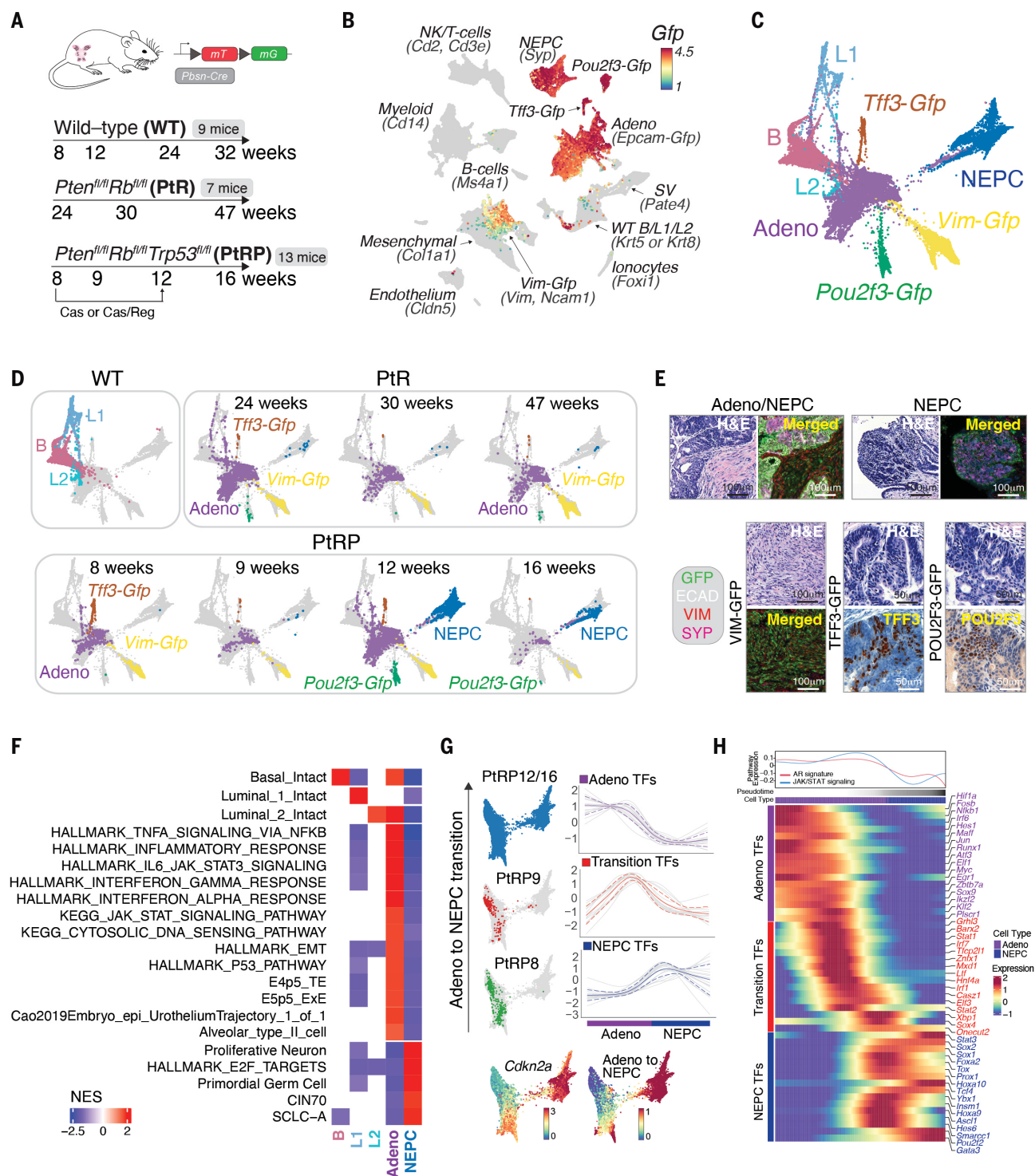


Fig. 1. Inflammatory JAK/STAT signals emerge in adenocarcinoma before transition to NEPC in GEMMs. (A) Experimental design includes WT (9 mice), PtR (7 mice, *PbCre: Rosa26^{mT/mG} Pten^{fl/fl} Rb1^{fl/fl}*), and PtRP (13 mice, *PbCre: Rosa26^{mT/mG} Pten^{fl/fl} Rb1^{fl/fl} Trp53^{fl/fl}*) at labeled time points. Time points for WT included 8 (one mouse), 12 (two mice), 24 (three mice), and 32 (three mice) weeks; for PtR included 24 (three mice), 30 (two mice), and 47 (two mice) weeks; and for PtRP included 8 (one mouse), 9 (one mouse), 12 (four mice), and 16 (one mouse) weeks relevant to the adenocarcinoma-to-neuroendocrine transition (4–6). Furthermore, six PtRP mice were castrated at 8 weeks of age for either a total of 4 weeks (three mice, “Cas”) or 2 weeks

followed by 2 weeks of dihydrotestosterone (DHT, “Cas/Reg”) addback (three mice). (B) UMAP of GEMMs ($N = 67,622$ cells) shown for all cell types based on imputed *Gfp* expression (restricted to nonimmune cells) as a marker for mutant cells (see methods). (C) FDL of mutant *Gfp*⁺ and WT cells ($N = 28,934$) colored by cell type. WT includes luminal 1, luminal 2, and basal. Mutant cell types include adenocarcinoma, *Tff3-Gfp*, *Pou2f3-Gfp*, *Vim-Gfp*, and NEPC. (D) FDLs separated by genotype and time point and colored by cell type: WT with $N = 7435$ cells (top left); PtRP 24, 30, and 47 weeks with $N = 2565$, 662, and 1441 cells, respectively (top right); and PtRP 8, 9, 12, and 16 weeks with $N = 981$, 353, 4984, and 569 cells, respectively (bottom). (E) Hematoxylin

and eosin (H&E) and multiplex immunofluorescence (mIF) (40×) of mutant subtypes, including adenocarcinoma-to-NEPC transition, NEPC, and VIM. mIF channels include GFP (mG, green), 4',6-diamidino-2-phenylindole (DAPI) (nuclei, blue), VIM (mesenchymal, red), SYP (synaptophysin, purple), and ECAD (e-cadherin, white). Also shown are H&E and IHC of TFF3⁺ and POU2F3⁺ cells in mutant tissue. Scale bars represent 100 or 50 μ m as noted. **(F)** Heatmap of significantly enriched gene sets per cell type, restricted to WT epithelial (basal, luminal 1, luminal 2), adenocarcinoma, and neuroendocrine (NEPC) cells [FDR < 0.01, abs(NES) > 1 where NES is the normalized enrichment score; see methods]. **(G)** Dissecting the adenocarcinoma-to-NEPC transition. Shown on the left are a FDL of mutant adenocarcinoma (subsetted to adeno-B and adeno-L2) and NEPCs cells from the PtR model ($N = 16,593$ cells) colored by genotype and time. Shown at the bottom are FDLs colored by normalized $\log_2(X + 1)$ expression of *Cdkn2a* or pseudotime scaled from 0 to 1. Shown on

the right are gene trends for TF DEGs across the adenocarcinoma to NEPC branch probability that were calculated using a generalized additive model, with cubic splines across 500 equally sized bins (solid lines represent the mean; dashed lines represent the standard deviation) (25). Gene trends were grouped by PhenoGraph cluster ($k = 30$) (see methods). **(H)** TF groups include adenocarcinoma (purple), putative transition (red), and NEPC (blue). Heatmap of gene trends of select TF DEGs (PhenoGraph cluster versus the rest within PtRP; refer to methods section "Differential expression in single-cell data") from each category are ordered by the putative transition from adenocarcinoma to NEPC, with gene labels colored in the same way the aforementioned groups (scale gene trends of imputed expression, -0.5 to 1.5). The top panel shows a spline fit of the average Z-score of JAK/STAT and androgen signaling (NELSON_ANDROGEN_SIGNALING_UP) (see methods). Adeno, adenocarcinoma; B, basal; L1, luminal 1; L2, luminal 2; SV, seminal vesicles; Vim-Gfp, Vimentin-Gfp.

androgen blockade, in a larger cohort by treating half the mice with castration and daily enzalutamide (Enz) 4 weeks after OT injection. In the hormonally intact mice, engrafted cells remained Ar⁺, Syp⁺ (as in organoid culture); however, large foci of Ar⁺, Syp⁺ engrafted cells were seen in all mice in the castration with Enz group, indicating progression to a neuroendocrine-like lineage, which was a consistent finding across four independent *Trp53* and *Rb1* codeletion experiments (fig. S6, A to D). To determine if this effect of in vivo androgen blockade on lineage plasticity is also observed in culture, we returned to the organoid model to determine the effect of Enz on the slithering phenotype after *Trp53* and *Rb1* codeletion. The percentage of organoids scored as slithering increased from ~10 to >30% (~3-fold) after 7 days of Enz treatment (fig. S6E).

Collectively, the above experiments demonstrate that changes in lineage-marker expression and inflammatory response pathway activation seen in the adenocarcinoma population in PtRP mice can be recapitulated in mouse prostate organoids within weeks of *Trp53* and *Rb1* codeletion. Furthermore, anti-AR therapy (castration and/or enzalutamide) can accelerate the rate at which these changes evolve, consistent with recent data using a patient-derived xenograft model (30). To address which cells can give rise to the plasticity phenotypes described above, we took advantage of prior work showing that organoids with normal basal and luminal bilayer architecture can be generated from single luminal or basal cells (26). Basal (CD49f⁺) and luminal (CD24⁺) cells from established Rosa26-lsl-Cas9 organoids stably expressing guide RNAs targeting *Trp53* and *Rb1* were isolated by fluorescence-activated cells sorting (FACS), transduced with adenoviral Cre, seeded as single cells, expanded, and then scored after 4 weeks using the morphologic criteria for plasticity (cystic versus hyperplastic, described in Fig. 2B). About 80% of plated basal and luminal cells developed hyperplastic changes, indicating that either cell type has lineage plasticity potential in organoid culture (Fig. 2H and fig. S4, H and I).

Lineage plasticity initiates with mixing of basal-luminal identity

Our organoids constitute a tractable experimental model that initiates and elaborates lineage plasticity in a reproducible manner. To gain more molecular insight into this plasticity, we characterized emerging transcriptional cell states and gene programs by collecting scRNA-seq data, beginning 2 weeks after *Trp53* and *Rb1* codeletion (before any morphologic evidence of plasticity) as well as 4 and 8 weeks after deletion. A parallel set of cultures were treated with Enz at 2 weeks and harvested at 4 and 8 weeks (Fig. 3A and table S1) to characterize the effects of androgen blockade on plasticity (see fig. S6).

We observed great transcriptional heterogeneity both within and between each time point and condition. To systematically catalog the observed cell states, we started with the WT phenotypic landscape then followed how it remodels after perturbation. To gain a concise description of the observed phenotypic diversity, we used our SEACells algorithm (31) (see methods) to calculate metacells separately within each sample. The metacell approach (24) aggregates similar single cells into a set of distinct, highly granular cell states such that any differences between cells within a metacell are likely technical rather than biological. Aggregating counts within each metacell generates a robust (fig. S7), full transcriptomic quantification for each cell state, thus mitigating noise and sparsity in scRNA-seq data. An added benefit of metacells is the enumeration of observed cell states that are more amenable to comparison.

To establish a baseline for assessing plasticity changes in mutant organoids, we first focused on the cell states in WT organoids. PhenoGraph clustering (25) identified five distinct subpopulations, which we classified as luminal (L-Org1, L-Org2) or basal (B-Org1, B-Org2, B-Org3) based on published transcriptomes (see methods) (Fig. 3, B and C; fig. S8, A and B; and tables S8 and S9). L-Org1 cells share expression of stem-like genes (e.g., *Ly6d*, *Tacstd2*/*Trop2*) with L-Org2 cells but also express other cytokeratins (e.g., *Krt6a*, *Krt10*)

not typically associated with luminal cells. L-Org2 cells resemble stem-like (*Ly6a*/*Sca1*⁺, *Psc+*) luminal cells seen in vivo [called L2 in Karthaus *et al.* (20); LumP in Crowley *et al.* (21)] as well as canonical luminal cytokeratins (*Krt8*/*Krt18*) (20, 21). B-Org1 cells express canonical basal markers (*Krt5*, *Krt14*, *Trp63*) and B-Org2 cells express secretory proteins such as calcitonin and inhibin A, whereas B-Org3 cells are largely distinguished by expression of proliferation markers (*Mki67*, *Aurka*).

After analyzing the entire time course after *Trp53* and *Rb1* codeletion, we found no overlap between cell states in WT and mutant organoids, even at 2 weeks, when organoids were still morphologically normal. When visualized in a FDL (see methods), these metacells organize along the axis of time with a distinct gap between the WT and 2-week time point. Basal and luminal metacells identified by correlation to basal and luminal gene signatures are phenotypically well separated in the WT setting but converge together on the FDL after mutation (Fig. 3D and fig. S8, C and D; see fig. S8E for similar trends at the single-cell level). To understand these phenotypic changes, we characterized the basal and luminal features gained and lost after mutation (tables S10 to S12; see methods). Over time, mutant organoids gain a mixed phenotype, in which mutant basal metacells acquire typical luminal features (*KRT8*, *KRT18*, *PROM1*) and lose typical basal features (*KRT5*, *KRT14*). In parallel, mutant luminal metacells gain typical basal features (*KRT17*) but lose luminal markers (*PSCA*, *LY6D*) (fig. S8, F and G). At the same time, both basal and luminal mutant cells concurrently gain EMT-like features (*VIM*, *FNI*, *COL4A1/2*, *ZEB1/2*). Plasticity changes in the mutant organoid can thus be defined as a gain in mixed basal-luminal phenotype with EMT-like features. Furthermore, all of these changes are escalated by antiandrogen (Enz) treatment, consistent with the accelerated morphologic phenotypes observed in culture and in vivo.

To ensure that the observed mixed basal-luminal phenotype was not merely a loss of original basal-luminal identity or a product of

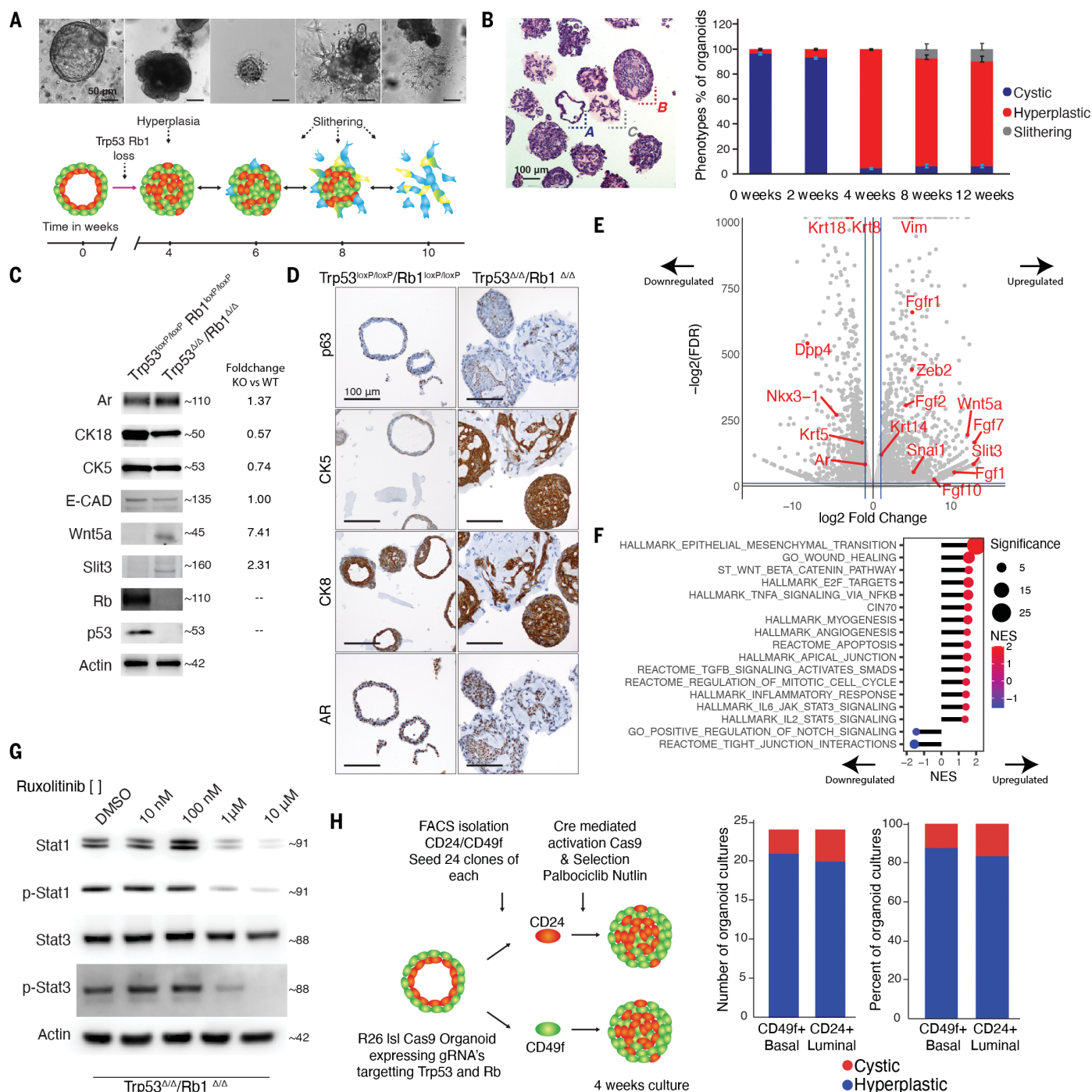


Fig. 2. Establishment of an organoid model of spontaneous lineage plasticity.

(A) At the top are representative brightfield microscopy images of the various organoid phenotypes before and 4, 6, 8, and 10 weeks after *Trp53* and *Rb1* loss. At the bottom is a schematic representation of lineage plasticity development in *Trp53* ^{Δ/Δ} ; *Rb1* ^{Δ/Δ} organoids. Scale bars represent 50 μ m. (B) On the left is a representative H&E staining of LentiCre *Trp53* ^{Δ/Δ} ; *Rb1* ^{Δ/Δ} organoids ~10 weeks after deletion with cystic (A, blue), hyperplastic (B, red), and slithering (C, silver) phenotypes. On the right is a bar graph showing the percentage of organoids with given phenotypes during the time course. The scale bar represents 100 μ m. (C) Western blot verification of DEGs identified in RNA-seq data in WT (*Trp53*^{loxP/loxP}; *Rb1*^{loxP/loxP}) organoids (left) and ~10 weeks after deletion (LentiCre *Trp53* ^{Δ/Δ} ; *Rb1* ^{Δ/Δ}) organoids (right). Proteins are as marked. Actin was used as a loading control. Fold change in protein level as determined with ImageJ is given on the right. KO.

knockout. (D) Representative IHC of basal markers (p63, Ck5), luminal marker (Ck8), and AR in WT (*Trp53*^{loxP/loxP}; *Rb1*^{loxP/loxP}) organoids (left) and ~10 weeks after deletion (LentiCre *Trp53* ^{Δ/Δ} ; *Rb1* ^{Δ/Δ}) organoids (right). Scale bars represent 100 μ m. (E) Volcano plot showing DEGs in the mutant organoid compared with WT. Blue lines indicate thresholds for significant DEGs [Bonferroni-adjusted *p* value < 0.001, abs(log2 fold change) > 1]. Red labels indicate significant DEGs of interest. (F) Lollipop plot showing significantly enriched pathways in *Trp53* and *Rb1*-deleted organoids versus WT using bulk RNA-seq based on GSEA [Benjamini-Hochberg adjusted *p* value < 0.15; abs(NES) > 1] (red indicates enriched in mutant; blue indicates enriched in WT). Lollipop size corresponds to significance, or -log2(*p* value). (G) Western blot of total Stat1 and Stat3 levels and phosphorylated (p-) Stat1 and p-Stat3 in LentiCre *Trp53* ^{Δ/Δ} ; *Rb1* ^{Δ/Δ} organoids treated with increasing doses of the JAK1/JAK2 inhibitor Rux for 48 hours. Actin was used as a loading

control. **(H)** On the left is a schematic representation of single-cell cloning experiment of basal (CD49f⁺) and luminal (CD24⁺) cells. Organoids harboring a Cre recombinase-inducible Cas9 [Rosa26 Isl-Cas9 (32)] were transduced with guide RNAs targeting *Trp53* and *Rb1* (LentiGuide Puro, guide sequences; table S23). Basal and luminal cells were sorted based on CD49f⁺ (basal) and CD24⁺ (luminal) expression, and subsequently, Cas9 was activated by transduction with adenovirus expressing Cre. About 1000 single cells were seeded and grown out. Nutlin-3 and

Palbociclib were added 3 days after Cas9 activation to select for *Trp53* and *Rb1* mutant cells. Twenty-four basal and 24 luminal clones were randomly chosen and expanded for 4 weeks. On the right is a quantification of whole-culture phenotypes (cystic or hyperplastic) of single cell-derived (CD49f⁺ or CD24⁺ FACS sorted) organoids after 4 weeks of culture. The left bar graph shows the absolute number of cultures, and the right bar graph shows the percentage of single-cell derived cultures in a cystic or hyperplastic state.

the metacell approach, we calculated basal and luminal scores for each individual cell based on basal and luminal features gained after mutation (table S12; see methods). Corresponding density plots showed that mutant luminal cells become more basal-like over time, and vice versa, resulting in a convergence between luminal and basal phenotype over time and with treatment (Fig. 3E and fig. S9A).

The apparent infidelity in basal-luminal lineage suggested that the mixed phenotype could serve as a useful proxy for quantifying plasticity. We therefore developed alternative measures of basal-luminal mixing at each time point as a proxy for plasticity (see methods). These per-sample metrics confirmed that plasticity increases over time after tumor suppressor deletion and with Enz treatment (Fig. 3F and fig. S9). As a per-cell measure that captures plasticity changes both within and across sample time points, we used a cell-type classification-based approach (25) to associate each mutant cell with a vector of classification probabilities for each WT organoid phenotype, where classification uncertainty corresponds to increased mixing and plasticity (see methods). We visualized these per-cell probabilities as a ternary plot (fig. S10, A and B). As expected, WT cells favor a single assignment to their respective phenotype, whereas mutant cells converge to maximal uncertainty at the center of the ternary plot over time. We quantified this time-dependent increase in basal-luminal mixing using entropy of cell-type probabilities, which robustly agrees with our per-sample measures of plasticity while capturing a range of plasticity within each time point and condition (fig. S10, C and D; see methods). Interestingly, Enz-treated samples display even fewer cells favoring the L-Orig2 phenotype, consistent with our transcriptional and morphological data showing that AR inhibition accelerates the loss of luminal identity (figs. S6D and S10E). Notably, we observed similar basal-luminal mixing in the PtRP mice, further validating the use of the organoid platform for interrogating plasticity phenotypes (fig. S10, F to J). Thus, several orthogonal analytical approaches consistently detect increasing plasticity over time, in both organoids and GEMMs.

Jak/Stat and Fgfr up-regulation coincides with plasticity onset and progression

To uncover the gene programs that drive plasticity after tumor suppressor gene deletion, we

leveraged our per-cell measure of plasticity to identify pathways activated in highly plastic cells. After correlating genes with our plasticity measure, we used GSEA to identify gene programs significantly associated with plasticity (fig. S11A and tables S13 and S14; see methods). Among these plasticity-associated pathways, we noted up-regulated EMT and NEPC signatures (fig. S11, B and C), even though morphological evidence of neuroendocrine transformation was not observed until these organoids were studied in vivo (fig. S6, A to D). To enrich for causal genes, we narrowed our list of candidate driver programs to pathways that meet two additional criteria: (i) activated early in the time course, because plasticity emerges before morphological changes (week 2 after *Trp53* and *Rb1* codeletion) and (ii) further enriched with Enz treatment, based on the premise that plasticity increases with therapeutic pressure (tables S15 and S16; see methods). The top resulting pathways include JAK-STAT [also leukemia inhibitory factor (LIF), interferon- γ (IFN- γ), IFN- α], fibroblast growth factor receptor (FGFR), NOTCH, and EMT (also SMAD2) (Fig. 3G and fig. S11, D to F).

Because our prostate organoids are cultured using defined, serum-free conditions that contain no ligands that might activate IFN response genes, we searched for potential mechanisms to explain the IFN response. Increased IFN signaling is often a consequence of cytoplasmic sensing of double-stranded DNA or RNA that is due to ruptured micronuclei or reactivation of endogenous retroviruses (32). However, CRISPR deletion of several genes involved in this sensing pathway [*Tmem173* (Sting), *Mavs*, *Tbk1*, *Ifih1*] had no effect on the acquisition of the hyperplastic morphology after *Trp53* and *Rb1* codeletion. We therefore explored the possibility of an autocrine loop, reasoning that if a ligand-receptor (L-R) pair activates JAK/STAT, then high abundance of the L-R should correlate with increased JAK/STAT signaling in the mutant organoid cells. A key advantage of our organoid model over the in vivo PtRP GEMM model is that it only needs to consider autocrine L-R interactions; thus, we were able to exclude cell type as a variable in L-R analysis. We evaluated a set of 74 L-R pairs known to activate JAK/STAT signaling and scored each candidate based on their correlation with a summary statistic of 33 JAK/STAT-associated genes enriched after mutation (tables S17 and S18; see methods).

The top L-R pairs identified by this analysis were LIF and its heterodimeric receptor (LIFR/IL6ST), FGF1/FGFR, IL15/IL15RA, the chemokines CCL2 and CCL5 and their cognate receptors CCR2 and CCR5, and HGF/MET (Fig. 3I and fig. S12A). To prioritize L-R pairs that cause an increase in plasticity, we applied similar criteria as for our candidate driver programs: (i) up-regulation of receptor expression early in the time course (week 2) and (ii) further up-regulation with antiandrogen treatment. FGFR and LIF (co)receptors remained as top candidate drivers after filtering, based on increased expression of FGFR1, LIFR, and IL6ST after ENZ treatment as well as early expression of FGFR1-3 and LIFR after *Trp53* and *Rb1* codeletion (Fig. 3J and fig. S12, B and C). Thus, multiple lines of evidence from PtRP GEMM mice and from *Trp53* and *Rb1*-deleted organoids implicate Jak/Stat pathway activation as an early event that precedes the acquisition of various plasticity phenotypes such as basal-luminal lineage mixing, EMT, and transition to NEPC, with FGFR and LIF prioritized as top candidates for functional interrogation based on L-R analysis.

Jak/Stat and Fgfr inhibition restores luminal lineage identity

To test these predictions at a functional level, we added recombinant FGF1 or LIF to organoids after *Trp53* and *Rb1* codeletion, reasoning that plasticity might be further enhanced by an artificial increase in ligand concentration. We also included hepatocyte growth factor (HGF), which was among the top candidates in our L-R analysis but deprioritized because of its delayed activation relative to the onset of the plasticity phenotype and thereby acted as a validation control for our prioritization schema. Recombinant FGF1, but not LIF or HGF, enhanced the magnitude of slithering (from 6 to 50% of Matrigel surface) within 3 days (fig. S13A; arrows indicate slithering), providing functional support for Fgfr signaling as a driver of plasticity.

To address the functional role of JAK/STAT signaling, we derived *Trp53*^{loxP/loxP}, *Rb1*^{loxP/loxP} organoids in which either *Jak1* or *Jak2* alone or in combination were disrupted using CRISPR-Cas9-directed single guide RNAs. We then deleted *Trp53* and *Rb1* by lentiviral Cre infection and scored the percentage of organoids with cystic, hyperplastic, or slithering phenotypes at 8 weeks, as in Fig. 2B. Disruption of

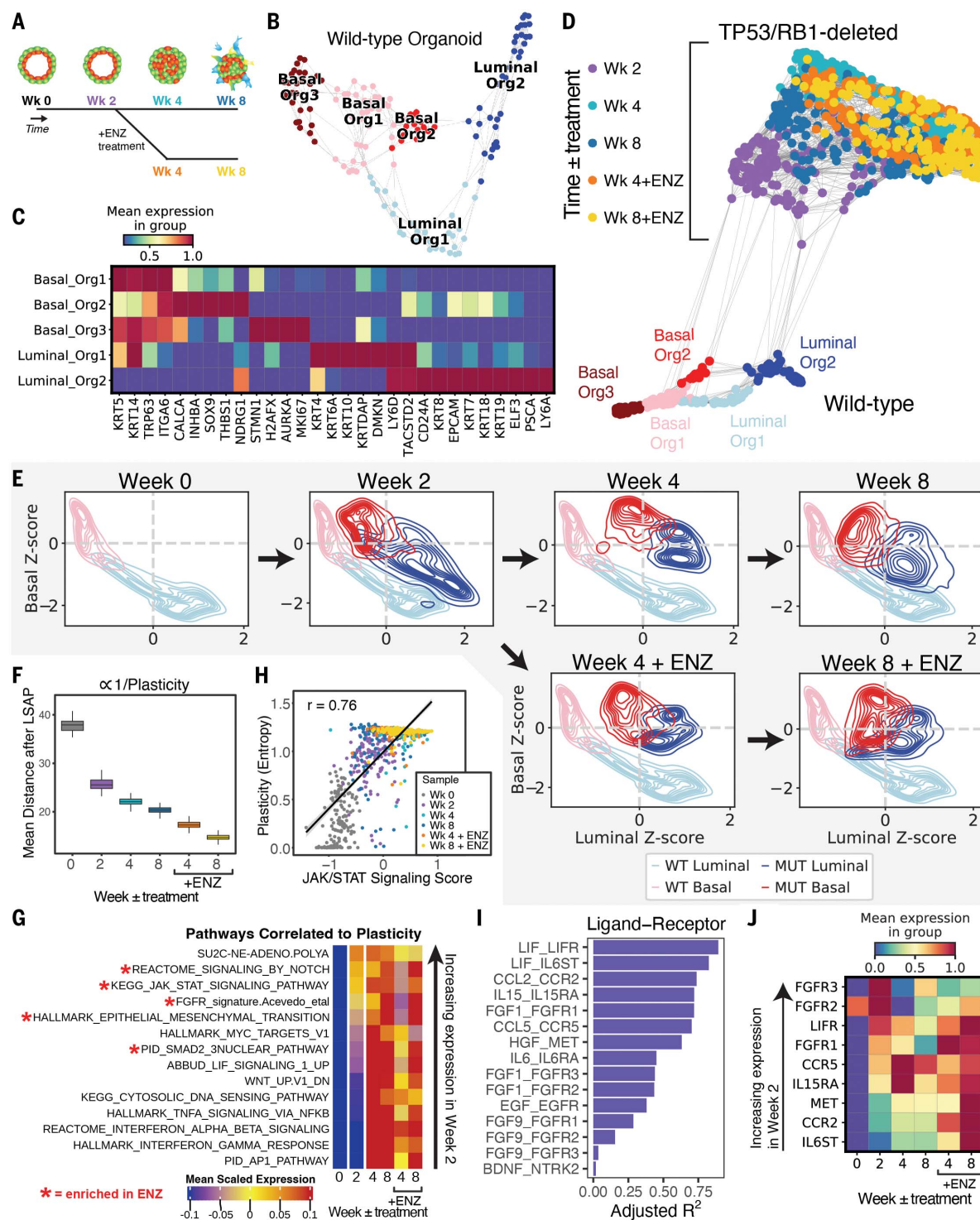


Fig. 3. Single-cell analysis of organoids reveals basal-luminal mixing, used as a proxy measure for plasticity to identify associated inflammation, JAK/STAT, and FGFR pathway activation. (A) Experimental design of organoid sequencing time course. Samples collected at weeks 0, 2, 4, and 8 are enzymatically digested and subjected to scRNA-seq. Samples from weeks 4 and 8 are collected with or without enzalutamide (ENZ) treatment. (B) FDL of WT basal and luminal SEACell metacells ($N = 142$), labeled by WT subpopulations determined by PhenoGraph clusters. Edges between metacells indicate the k -nearest neighbors ($k = 6$) (see methods). (C) Mean $\log_2(X + 1)$ expression of DEGs in each basal and luminal subpopulation in the WT organoid. (D) FDL

($k = 10$) of metacells in the prostate organoid before and after *Trp53* and *Rb1* deletion ($N = 884$), annotated by either WT subpopulations or by time and treatment after mutation. (E) Contour plots of basal and luminal cell densities at each time point, depicting a convergence of cell identities after mutation (see methods). Single cells are plotted using mean Z-scores of the set of basal genes that are gained in mutant luminal cells (y axis) and the set of luminal genes that are gained in mutant basal cells (x axis). Samples were collected at week 0 ($N = 2629$), 2 ($N = 1904$), 4 ($N = 2554$), 8 ($N = 2690$), 4+ENZ ($N = 2850$), and 8+ENZ ($N = 3050$). Gene imputation is performed using MAGIC ($k = 30$; $t = 3$) before obtaining the Z-score. See fig. S9A for a corresponding version without

gene imputation. Red, basal cell; blue, luminal cells; light colors, WT; dark colors, *Trp53* and *Rb1* mutant (MUT). (F) Mean Euclidean distance between matched basal and luminal cells, based on the linear sum assignment problem (LSAP). Mean distances within each time point are an inverse measure of plasticity, which increases with time and treatment. (G) Top pathways significantly enriched for plasticity across time points (using GSEA, Bonferroni-adjusted $p < 0.01$, NES > 1; see fig. S10C and table S14). Each pathway score is measured as the average Z-score of gene expression in each pathway among metacells. Rows (pathways) are ordered by increasing pathway score from bottom to top in the early time point at week 2. Red asterisks indicate pathways that are also enriched in ENZ-treated samples compared with untreated samples (see methods). (H) Cellular plasticity increases as a function of JAK/STAT signaling. Plasticity is measured as the average entropy of cell-type classification

probabilities per metacell. JAK/STAT score is the average Z-score of the leading-edge gene subset of KEGG_JAK_STAT_SIGNALING_PATHWAY and HALLMARK_IL6_JAK_STAT3_SIGNALING. The entropy of classification probabilities was first calculated for each single cell and then averaged per metacell for visualization. A linear fit is shown with a Pearson's correlation of 0.76. (I) Top-scoring L-R interactions known to activate JAK/STAT signaling, based on the adjusted coefficient of determination (R_{adj}^2) of the regression $JAK_STAT \sim L + R + L:R$, where JAK_STAT is the JAK/STAT signaling score and L and R represent scaled imputed ligand and receptor expression (see methods). Only L-R pairs with nonzero R_{adj}^2 are shown. (J) Mean expression (rows) by sample time point (columns) for candidate receptors that activate downstream JAK/STAT signaling in metacells. Receptor genes are ordered by increasing expression in the early time point at week 2 from bottom to top.

Jak1 or *Jak2* alone blunted up-regulation of pStat1 and pStat3 but only modestly affected development of the plasticity phenotype [~ 6 to $\sim 15\%$ increase in cystic organoids ($p = 0.01$, *Jak1* guides; $p = 0.09$, *Jak2* guides)], with most organoids progressing to the hyperplastic morphology (fig. S13, B and C). By contrast, organoids with combined *Jak1/Jak2* disruption (which more effectively blocked Stat1/3 phosphorylation) had substantial preservation of cystic morphology (> 3 -fold increase; fig. S13, B and C). We confirmed this effect pharmacologically by treatment with the dual *Jak1/2* kinase inhibitor Rux, which restored cystic morphology (from ~ 5 to $\sim 30\%$) in a dose-dependent manner (Fig. 4A and fig. S13, D and E). As further evidence of reversion to a more luminal lineage, these morphological changes were accompanied by a modest reduction in *Vim*⁺ cells and an increase in AR expression (Fig. 4, C and D), commensurate with the reduction of pStat1 and pStat3 levels back to baseline.

Having demonstrated that perturbation of either FGFR or JAK/STAT signaling can affect the plasticity phenotypes in our organoid model, we used the clinical grade FGFR inhibitor erdafitinib (Erda) to examine the functional consequences of pharmacological FGFR inhibition on plasticity, alone and in combination with JAK inhibition. Although treatment with Erda also reduced the percentage of organoids with the slithering phenotype, we observed a notable ~ 12 -fold increase (5 to 60%) in cystic morphology when fully plastic organoids were cotreated with Erda and Rux (Fig. 4, A and B).

Consistent with the morphologic change, we noted > 3 -fold reduction in cells expressing the mesenchymal lineage marker *Vim* by IHC (Fig. 4C), which was confirmed by Western blot and increased levels (1.5-fold) of the luminal markers *Ck8* and *AR* (Fig. 4D and fig. S13F). Importantly, these changes in morphology and lineage-marker expression occurred in the absence of any effects on organoid proliferation (Fig. 4E and fig. S13G), suggesting that the primary consequence of combined *Jak/Fgfr* inhibition is on lineage reprogramming rather than selective outgrowth of rare organoids that had retained cystic morphology

after *Trp53* and *Rb1* codeletion. The restoration of cystic luminal organoids after combined *Fgfr* with *Jak* kinase inhibition led us to ask if sensitivity to antiandrogen therapy is also restored. Indeed, Enz sensitivity was restored to previously Enz-resistant organoids ($\sim 50\%$ decrease in proliferation, comparable to WT organoids), but only after cystic morphology was restored by 14 days of Rux with Erda pretreatment (Fig. 4E and fig. S13H).

JAK/STAT and FGFR activation in human CRPC

To determine if the transcriptional programs linked to plasticity in our murine prostate models are used in human prostate cancer, we analyzed scRNA-seq data from 12 CRPC patient samples representing both CRPC-adeno and transformed NEPC histologies (fig. S14 and table S19) that were obtained by radiologically guided biopsies of metastatic index lesions visualized by metabolic imaging. As expected, these samples were enriched for genomic alterations in *TP53*, *RB1*, and *PTEN* or in other alterations linked to these pathways such as *CDKN2A/CDKN2B* (*RB1*) and *AKT1* (*PTEN*) (13, 33, 34) (Fig. 5A). Transcriptomes from 51,094 cells were analyzed (see methods), and the tumor cells ($n = 27,338$) revealed notable heterogeneity across samples with a clear distinction based on CRPC-adeno and NEPC histology (Fig. 5B and fig. S15, A to C). Cells were scored for JAK/STAT, FGFR, AR, EMT, and NEPC signatures (see methods), derived from published GEMM and organoid plasticity models (table S18), and a mean value was determined for each PhenoGraph cluster [k -nearest neighbors ($k = 30$)]. Scatterplots of these clusters revealed a strong positive correlation between JAK/STAT with FGFR signaling and EMT [correlation coefficient (r) = 0.70], but a negative correlation with AR signaling ($r = -0.47$) (Fig. 5D). One particular CRPC adenocarcinoma tumor (HMP13) distinctively displayed intratumoral heterogeneity, harboring a subpopulation marked by JAK/STAT and FGFR up-regulation, EMT, and down-regulation of AR signaling (fig. S15D and table S20). Mapping of TFs in this sample across pseudotime (25) revealed a similar activation of transcrip-

tional regulators, namely STAT2/3, IRF1, and IRF7 (fig. S15, E and F, for robustness analysis). Overall, clusters with JAK/STAT with FGFR- and EMT-high programs are found in 5 out of 9 (or 56%) of CRPC adenocarcinoma samples (Fig. 5E; defined by having at least > 20 cells in a cluster). These results are highly consistent with our findings from the GEMM and organoid models where JAK/STAT and FGFR are strongly correlated with EMT programs and lower AR signaling but absent in NEPC cells.

To address whether the JAK/STAT and FGFR activation we observed in human CRPC samples is functionally linked to lineage plasticity phenotypes such as EMT and NEPC, we turned to a panel of human prostate cancer organoids derived from patients with CRPC, all of which have been characterized by whole-exome sequencing and bulk RNA-seq and represent the CRPC-adeno and NEPC histology phenotypes described earlier. As expected, these patient-derived organoids (PDOs) were enriched for alterations in *TP53*, *RB1*, and *PTEN*. For the CRPC-adeno subgroup, we have previously shown that those with high AR expression (e.g., MSKPCa2) express luminal lineage markers and remain sensitive to Enz (35). We therefore focused on CRPC-adeno organoids with low or absent AR expression, as well as PDOs with NEPC histology, to determine if Rux with Erda treatment might restore expression of luminal genes as we saw in *Trp53* and *Rb1*-deficient mouse organoids. Transcriptomic analysis revealed increased JAK/STAT activation and EMT signatures in five PDOs (MSKPCa3, 8, 11, and 12 and LuCap176), all of which have low AR expression and therefore reflect the JAK/STAT-high, mixed AR-low subgroup seen in the patient biopsies (Fig. 5F, fig. S16A, and table S21). Unlike our genetically defined mouse organoid model, the human PDOs contain a complex array of genomic alterations beyond the tumor suppressor gene annotated in Fig. 5F and have growth properties that, in general, are much slower (4- to 7-day doubling times) than mouse organoids (1 to 2 days). In addition, individual PDOs often have heterogeneous expression of lineage markers, exemplified by variations in the level and percentage of cells

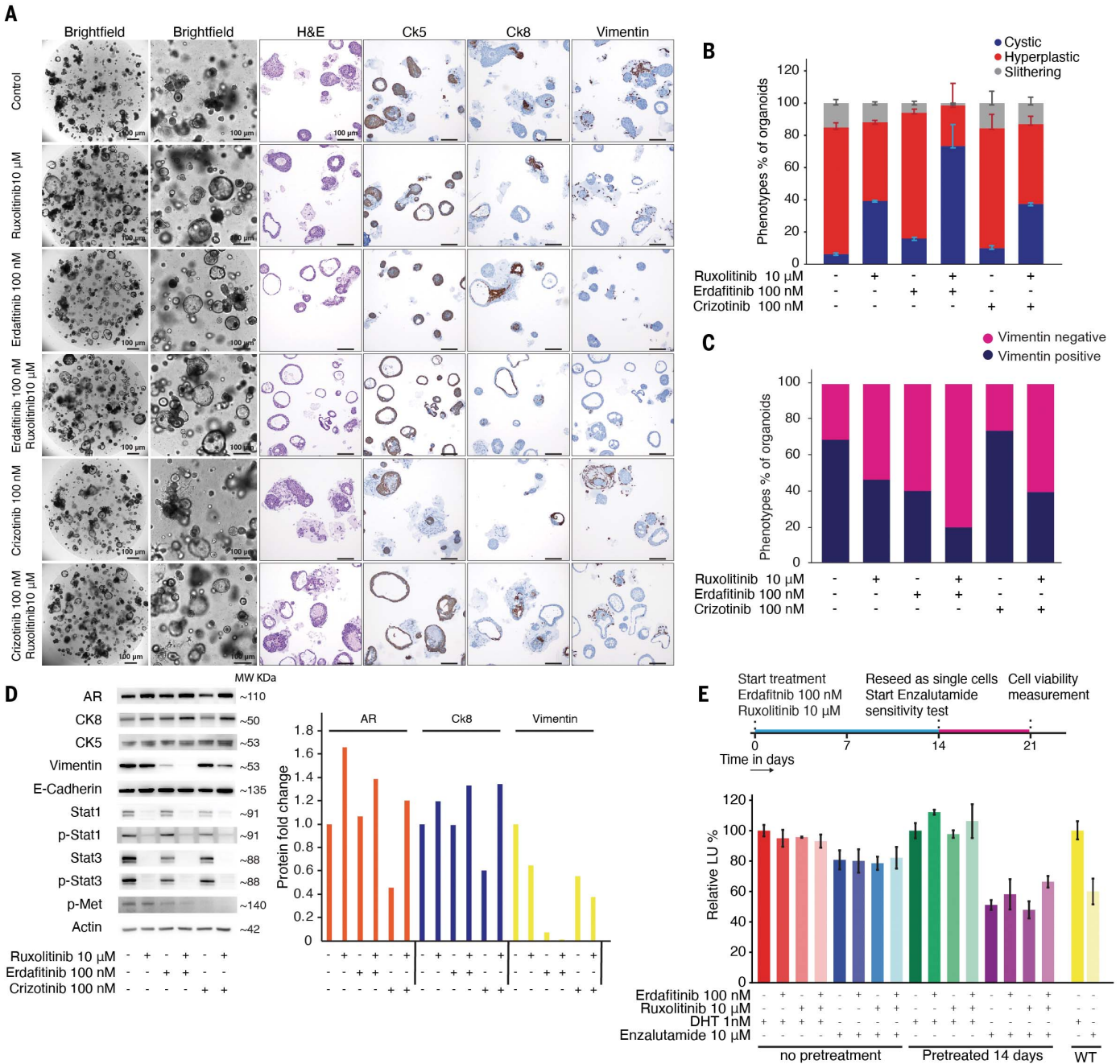


Fig. 4. Pharmacological inhibition of JAK/STAT signaling and FGFR signaling resensitizes prostate cancer organoids to androgen receptor signaling inhibitors. (A) Representative brightfield microscopy images, H&E staining, and IHC of CK8, Ck5, and vimentin of *Trp53^{Δ/Δ};Rb1^{Δ/Δ}* organoids treated for 14 days with the indicated drugs. Scale bars represent 100 μ m. (B) Quantification of phenotypes cystic (blue), hyperplastic (red), and slithering (silver) of *Trp53^{Δ/Δ};Rb1^{Δ/Δ}* organoids treated for 14 days with the indicated drugs; see methods for exact medium composition. (C) Quantification of vimentin positivity of organoids in (A). The total number of organoids that were positive for the marker were quantified and normalized. (D) On the left is a Western blot of lineage markers and JAK/STAT signaling components in *Trp53^{Δ/Δ};Rb1^{Δ/Δ}* organoids treated for 14 days with the indicated drugs; see methods for exact medium composition. Proteins were probed as indicated. Actin was used as a loading control. Shown on the right is the protein fold change for AR, Ck8, and

vimentin as determined by ImageJ analysis in *Trp53^{Δ/Δ};Rb1^{Δ/Δ}* organoids treated for 14 days with the indicated drugs. (E) At the top is a schematic overview of the resensitization drug experiments. Organoids are treated with Erda 100 nM and Rux 10 μ M for 14 days in low-epidermal growth factor (EGF) organoid medium (ENR+A83+DHT), and control organoids are cultured in low-EGF organoid medium for 14 days. Subsequently, organoids (10,000 cells per well, triplicate) are reseeded in organoid culture medium without EGF (NR+A83) that contains an AR agonist (DHT 1 nM) or antagonist (enzalutamide 10 μ M). Viability is shown at the bottom, which was measured by CellTiterGlo after 7 days of enzalutamide treatment of *Trp53^{Δ/Δ};Rb1^{Δ/Δ}* organoids that were treated for 14 days with the indicated drugs. Results were normalized to control-culture conditions. See methods for exact medium composition. The bar graph represents the relative viability to control, as measured by Cell Titer Glo Light Units (LU). Data are presented as mean $\pm$ SD.

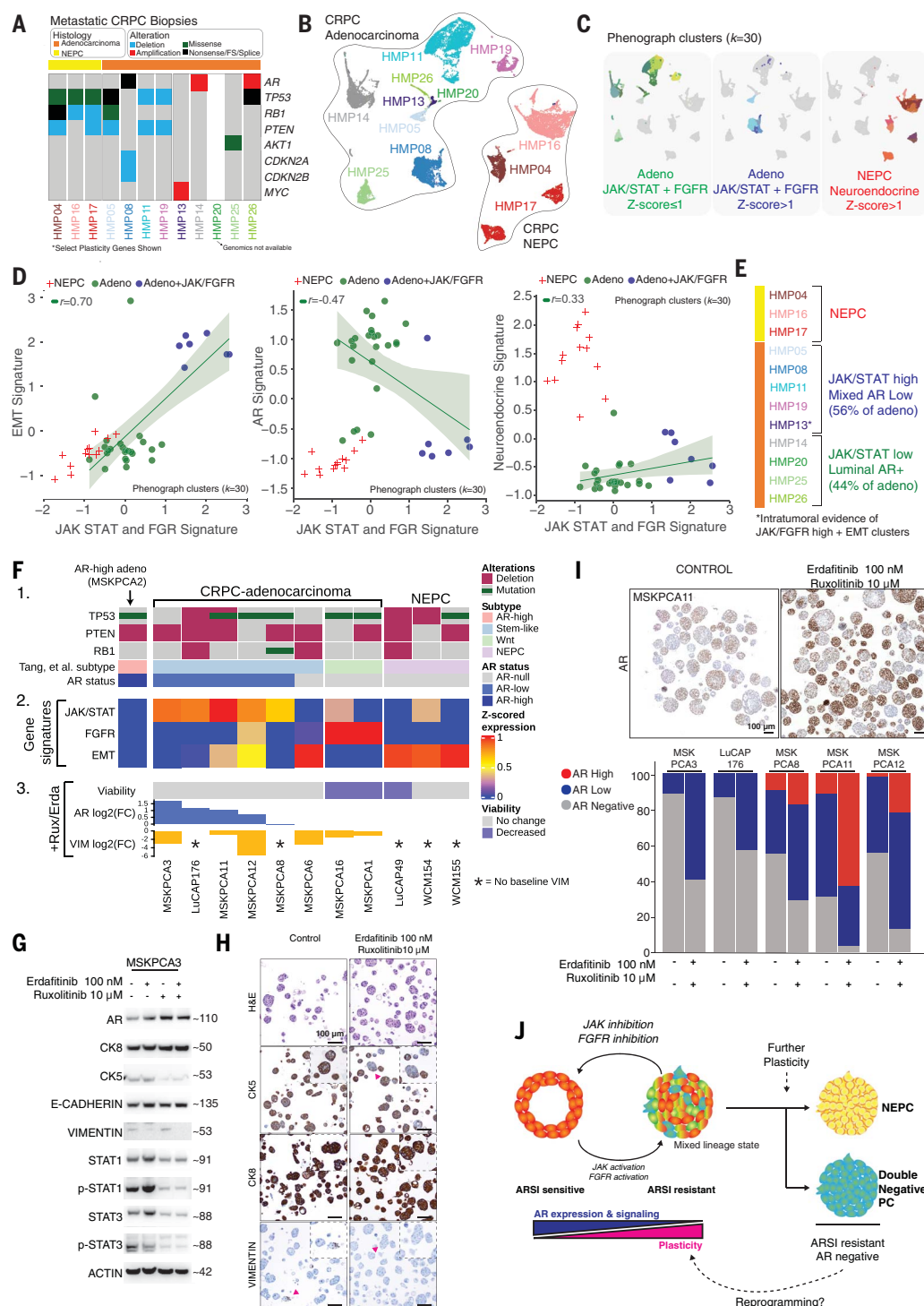


Fig. 5. Activation of JAK/STAT and FGFR signaling arises in CRPC patient biopsies and PDOs. (A) Treatment-refractory metastatic CRPC patients underwent interventional radiology-guided biopsies. High-risk index lesions were guided by advanced molecular imaging. Mutational oncogenes were labeled by sample ID (HMP, human metastatic prostate), histology (orange, adenocarcinoma; yellow, neuroendocrine prostate cancer), and mutational status (blue, deletion; red, amplification; green, missense; black, stop or frameshift). (B) UMAPs of tumor cells from patient-derived metastatic CRPC (N = 27,338 cells) colored by tumor ID and grouped by tumor type. (C) UMAPs of tumor cells from patient-derived metastatic CRPC, annotated by PhenoGraph clusters (k = 30), divided into three groups defined by the Z-score of either JAK/STAT

and FGFR or NEPC signatures: (i) AR-high and JAK/STAT+FGFR-low adenocarcinoma (JAK and FGFR signature, Z-score ≤ 1, green), (ii) AR-low and JAK/STAT+FGFR-high adenocarcinoma (JAK and FGFR signature, Z-score > 1, blue), and (iii) NEPC (neuroendocrine signature, Z-score > 1) (see methods). (D) Three scatterplots of PhenoGraph clusters (points) based on the Z-score of a combined JAK/STAT+FGFR signature (x axis) versus EMT, AR, and NEPC signatures (y axis), respectively. Linear fits were calculated for adenocarcinoma clusters only; the corresponding Pearson's correlation is noted. Clusters annotated by groups defined in (C): NEPC (red), AR-high and JAK/FGFR-low adeno (green), and AR-low and JAK/FGFR-high adeno (blue). (E) Proposed classification of metastatic CRPC samples based on JAK/STAT+FGFR and NEPC

signatures. HMP13 is assigned to the JAK/STAT+FGFR-high group, because this sample harbors a well-demarcated JAK/FGFR and EMT-high subpopulation, described in fig. S14D. **(F)** Schema of baseline attributes and functional change in human organoids with null to low AR expression after treatment with combined Rux and Erda, with hold-out sample MSKPCA2 used as reference level for the AR-high subtype. Shown are (1) baseline attributes, including an oncoprint of the *TP53*, *RBI*, and *PTEN* genotypes, transcriptional subtypes based on Tang *et al.* (36), and low versus null AR protein expression; (2) a heatmap of baseline gene signatures in the human organoids using publicly available bulk RNA-seq based on mean Z-scored expression of JAK/STAT signaling, FGFR signaling, and EMT gene sets (see methods); and (3) functional changes in human organoids with null to low AR expression after treatment with Rux and Erda, including cell viability, and log2 fold change (FC) in AR and VIM protein expression. **(G)** Western blot of lineage markers and JAK/STAT signaling components in MSKPCA3 organoid after 14 days of treatment with Erda 100 nM, Rux 10 μ M, or with Erda 100 nM and Rux 10 μ M combination. Proteins were probed as indicated. Actin was used as a loading control. **(H)** Representative histology and

IHC (CK8, CK5, and vimentin) of MSKPCA3 organoids after 14 days of treatment with Erda (100 nM) and Rux (10 μ M) combination in full organoid medium (ENRFFPN, A83-01, nicotinamide, DHT). Red arrowheads indicate organoids of interest with changed protein expression. See methods for exact medium composition. Scale bars represent 100 μ m. **(I)** Shown at the top is a representative staining of AR in control-treated MSKPCA11 organoids and those treated with Erda 100 nM and Rux 10 μ M. Shown at the bottom is a bar graph of AR IHC staining patterns in PDOs MSKPCA3, LuCAP176, MSKPCA8, MSKPCA11, and MSKPCA12. Organoids were control-treated or treated with Erda 100 nM and Rux 10 μ M. AR staining was classified as negative (gray), low intensity (blue), and high intensity (red). **(J)** Proposed model system of lineage plasticity. JAK/STAT signaling activation and FGFR signaling activation leads to an AR-low, androgen receptor signaling inhibitor (ARSI)-insensitive state that can be reprogrammed back to an ARSI-sensitive state. Potentially, the JAK/STAT-high, AR-low, ARSI-insensitive state is a cellular state preceding AR⁺, ARSI-insensitive CRPC. Mechanisms leading to AR⁺ CRPC are unknown.

with AR expression when examined on a cell-by-cell basis (Fig. 5, G to I).

Despite these differences between the mouse and human organoid models, we observed increased AR expression after 14 days of Rux with Erda treatment in four of the five AR-low and JAK/STAT-high PDOs. This change in AR expression was accompanied by increased expression of the luminal cytokeratin CK8 and decreased expression of the mesenchymal marker (VIM) (Fig. 5, F to I, and figs. S16B and S17). As with the *Trp53* and *Rb1*-deleted mouse organoid, Rux with Erda treatment did not affect the proliferation rate of the AR-low and JAK/STAT-high PDOs (fig. S18, A and B). One important distinction from the mouse system, however, is that a substantial percentage of cells within each of these Rux-with-Erda-sensitive organoids remained AR⁺ (Fig. 5I). Whether this is a consequence of the greater genomic complexity of the human versus mouse models and/or the need for longer periods of drug treatment to reprogram human organoids fully (owing to their slower growth rate) requires further study.

In addition to the luminal reprogramming phenotype seen in the AR-low, JAK/STAT-high organoids, we noted a distinct phenotype of substantial growth inhibition in two AR⁺ PDOs with CRPC-adenocarcinoma histology (MSKPCA1 and 16) (fig. S18, A and B). These PDOs are noteworthy for signatures of highly activated FGFR signaling but modest or absent JAK/STAT activation and likely reflect a dependency on FGFR for proliferation. Indeed, evidence of FGFR-dependent CRPC has been previously reported in the context of AR⁺, SYP⁺ (double negative) prostate cancer (4). Growth of one of the three NEPC PDOs (LuCaP49) was also notably inhibited by Rux with Erda despite low FGFR and JAK/STAT activation states across all three lines. The mechanism underlying this sensitivity requires further investigation.

Finally, we mapped the Rux-with-Erda sensitivity profiles to four subtypes of human CRPC

(AR⁺, Stem-like, Wnt, and NEPC) recently reported based on integrated chromatin accessibility and transcriptome analysis (36). The four PDOs that display luminal reprogramming all fall within the stem-like subtype [also defined by increased JAK/STAT activation in Tang *et al.* (36)], whereas the two CRPC-adenocarcinoma PDOs with growth inhibition fall with the Wnt subtype (Fig. 5F), providing evidence of specific and exploitable therapeutic vulnerabilities for molecularly distinct subtypes of human CRPC.

Discussion

Across multiple cancers, marked changes in tumor cell state are implicated in the resistance to targeted therapies. This lineage plasticity is particularly relevant in the context of next-generation drugs designed to overcome conventional resistance that arises from drug-target mutations. However, lineage transformation remains poorly characterized, and we even lack a basic quantitative description of phenotypic plasticity. To address this mechanism of resistance therapeutically, it is critical to understand when, where, and how cell-state changes arise—ideally, by profiling tumor cells as resistance arises. We performed scRNA-seq time-course experiments on prostate cancer GEMMs and organoids, enabling an in-depth study of the adenocarcinoma-to-NEPC transition upon deletion of tumor suppressor gene combinations relevant to human CRPC (*Trp53*, *Rb1*, *Pten*). Our analysis identified an adenocarcinoma population with mixed luminal and basal identity that emerges within 2 weeks of tumor suppressor loss and progresses to EMT or NEPC phenotypes. We developed approaches to quantify plasticity, showing that it arises in a cell-autonomous fashion in both normal luminal and basal cells and increases as migratory behavior and EMT-like transcriptional signatures are acquired. However, progression to an NEPC phenotype only occurs in vivo, presumably reflecting a need for stromal or immune cell signals that are missing from organoid culture.

Prior work (4, 15, 16, 28) established that the onset of lineage plasticity is accompanied by resistance to AR pathway inhibition, which we hypothesize to be a consequence of acquiring previously uncharacterized AR-independent cell states, including basal or mesenchymal lineages. Importantly, we also observed the converse—plasticity is initially driven by loss of tumor suppressor genes, and subsequently accelerated by AR pathway inhibition. The fact that lineage plasticity, which is initially responsible for drug resistance, is exacerbated by further drug treatment has important medical implications.

We searched for molecular drivers of plasticity by identifying therapy-induced gene programs associated with early acquisition of our newly quantifiable high-plasticity state and discovered an unexpected, robust signature of JAK/STAT activation in both organoids and GEMMs. Other manifestations of plasticity such as EMT and NEPC likely evolve later, based on the temporal sequence observed in organoids as well as absence of JAK/STAT activation in the NEPC component of GEMM tumors and human NEPC samples. This observation suggests an early “critical period” after the onset of plasticity, during which therapies might prevent, delay, or even reverse plasticity. Supporting this hypothesis, JAK (and FGFR) inhibitors restored expression of luminal markers and decreased mesenchymal markers in mouse organoids and human PDOs, but only in those with increased JAK/STAT activation. These changes evolved over several weeks of treatment in the absence of growth inhibition, consistent with a lineage reprogramming mechanism akin to differentiation therapy used in subclasses of acute myeloid leukemia (37, 38).

By searching for autocrine loops associated with JAK/STAT activation, we discovered that FGFR signaling increases with the onset of plasticity in organoids, GEMMs, and human CRPC samples. Using a highly selective pan-FGFR inhibitor (Erda), we found (i) potent

luminal lineage reprogramming in combination with JAK inhibition (Rux) in mouse organoids and PDOs, perhaps due to cross-talk between these pathways and reprogramming transcription factors or chromatin modifiers; and (ii) consistently reduced mesenchymal gene (VIM) expression in organoids after FGFR inhibition, independent of JAK/STAT activation. This is consistent with FGFR's role in tumors with an EMT phenotype, as proposed in AR⁺, SYP⁺ prostate cancer and in breast cancer (39). Importantly, the primary consequence of FGFR inhibition in PDOs with low JAK/STAT activation is growth inhibition, suggesting a dependency on FGFR for survival, which is distinct from the lineage reprogramming phenotype in PDOs with high JAK/STAT activation. Collectively, these findings suggest a therapeutic strategy by which CRPC patients with JAK/STAT-high, AR-low signaling [defined as stem-like by Tang *et al.* (36)] would be candidates for combined JAK and FGFR inhibition in order to reprogram tumor cells to an AR-high luminal state and restore sensitivity to AR-targeted therapy. Those with increased FGFR signaling and no JAK/STAT activation [defined as Wnt subtype by Tang *et al.* (36)] would also be candidates for FGFR inhibition to exploit FGFR dependency for survival. Therapeutic timing is critical, as shown by cell-state heterogeneity within a single CRPC patient (or GEMM tumor) and absence of JAK/STAT activation in NEPC. These findings provide a rationale for molecular diagnostic assays that could aid patient selection.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

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MDAR Reproducibility Checklist

Movies S1 and S2

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Lineage plasticity in prostate cancer depends on JAK/STAT inflammatory signaling

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Understanding prostate cancer plasticity

Cellular plasticity is the ability of cells to alter their identity and acquire new biological properties. In the context of cancer, plasticity may explain how certain tumors evolve and become resistant to therapy. Chan *et al.* investigated the molecular mechanisms underlying lineage plasticity in late-stage prostate cancer. Using a number of model systems, including human organoid cultures, activation of Janus kinase-signal transducer and activator of transcription (JAK/STAT) and fibroblast growth factor receptor (FGFR) signaling was found to drive prostate tumor plasticity. Pharmacological targeting of the JAK and FGFR pathways promoted lineage reprogramming in biopsy samples from castration-resistant prostate cancer patients. These findings provide insights that may help to curb prostate cancer progression and resensitize resistant tumors to effective therapies. —PNK

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