

AWARD NUMBER: W81XWH-19-1-0323

TITLE: Identifying the Molecular Mechanisms of Prostate Tumorigenesis Associated with Multigenic 3p13-14 Locus Loss Using a Novel CRISPR-Organoid Platform

PRINCIPAL INVESTIGATOR: Weiran Feng

CONTRACTING ORGANIZATION: Sloan Kettering Institute for Cancer Research  
New York, NY

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Fort Detrick, Maryland 21702-5012

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# REPORT DOCUMENTATION PAGE

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<b>1. REPORT DATE</b> July 2021		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 01Jul2020 - 30Jun2021	
<b>4. TITLE AND SUBTITLE</b> Identifying the Molecular Mechanisms of Prostate Tumorigenesis  Associated with Multigenic 3p13-14 Locus Loss Using a Novel  CRISPR-Organoid Platform				<b>5a. CONTRACT NUMBER</b>	
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				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  Weiran Feng  E-Mail: fengw@mskcc.org				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Sloan Kettering Institute for Cancer Research 1275 York Avenue, New York NY 10065-6007 USA				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Human prostate cancers can feature chromosome-scale alterations, but it remains challenging to determine the causal relationship between these alterations and cancer formation. We established a mouse primary prostate organoid platform to model the large 3p13-14 locus deletion, which accounts for 15~20% of prostate cancer patients.  We performed bulk RNA- and ATAC-seq analysis to study the role of <i>Foxp1</i> , a putative tumor suppressor from this region, which revealed an inflammatory response induced by <i>Foxp1</i> loss and led us to study its role (and the role of the entire locus) in an immunocompetent environment. We developed a highly efficient approach to edit organoid genome through a single step which is compatible with downstream transplantation into immunocompetent hosts. Orthotopic transplantation revealed a synergistic effect between <i>Foxp1</i> loss and host immune system. Together, our study provides mechanistic insight into the tumor suppressor locus and serve as a paradigm for studying other frequent chromosomal alterations in prostate cancer.					
<b>15. SUBJECT TERMS</b> Prostate cancer genome, large chromosomal deletions, primary organoids, CRISPR editing, prostate cancer modeling, 3p13 locus, <i>Foxp1</i>					
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## 1. INTRODUCTION:

Chromosomal alteration is a hallmark of the prostate cancer genome. One example is the large deletion of the hexagenic 3p13-14 locus, which occurs in 15~20% of prostate cancers. However, it remains unknown how this chromosomal deletion impacts cancer formation. While our preliminary data has revealed a tumor suppressor function of *Foxp1*, which resides in this region, further study of the entire locus is limited by the lack of effective tools to model this event. In this study, we developed a primary prostate organoid platform for efficient generation of the 3p13-14 deletion and rapid cancer modeling in vivo. We utilized this platform to 1) reveal the mechanisms of tumorigenesis upon *Foxp1* loss and 2) to identify additional tumor suppressive subregions from the locus that cooperates with *Foxp1* loss. Together, our study will provide both mechanistic insight and a comprehensive view about the putative tumor suppressor locus and serve as a paradigm for studying other frequent chromosomal alterations in prostate cancer.

## 2. KEYWORDS:

Prostate cancer genome, large chromosomal deletions, primary organoids, CRISPR editing, prostate cancer modeling, 3p13 locus

## 3. ACCOMPLISHMENTS:

### What were the major goals of the project?

<b>Specific Aim 1: Uncover the tumor suppressive mechanisms of Foxp1</b>		
<b>Major Task 1: Determine the oncogenic potential of a single copy loss of Foxp1</b>	<b>Months</b>	<b>% completion</b>
Subtask 1: Prepare the <i>Foxp1</i> <sup>+/-</sup> ; <i>Pten</i> <sup>-/-</sup> organoid line for orthotopic transplantation  # mouse: 1  Method: mouse prostate organoid culture; lentiviral transduction; adenovirus infection	1-3	100
Subtask 2: Compare the oncogenic potential of <i>Pten</i> <sup>-/-</sup> , <i>Foxp1</i> <sup>+/-</sup> ; <i>Pten</i> <sup>-/-</sup> and <i>Foxp1</i> <sup>-/-</sup> ; <i>Pten</i> <sup>-/-</sup> mouse prostate organoid lines by orthotopic transplantation  # mouse: 24 (total)  Method: prostate orthotopic transplantation, bioluminance imaging, prostate histological analysis	3-12	100
<i>Milestone(s) Achieved: Elucidation of the possible effects of Foxp1 haploinsufficiency in prostate tumorigenesis</i>	12	100
<b>Major Task 2: Determine the impact of Foxp1 loss on cell fitness</b>		
Subtask 1: Examine whether an additional Foxp1 loss confers a growth advantage to Pten-null prostate organoids  Mouse prostate organoid lines (already established): <i>WT</i> , <i>Pten</i> <sup>-/-</sup> , <i>Foxp1</i> <sup>-/-</sup> and <i>Pten</i> <sup>-/-</sup> ; <i>Foxp1</i> <sup>-/-</sup>  Method: CellTiter-Glo® proliferation assay	1-6	100
Subtask 2: Examine whether an additional Foxp1 loss alleviates cellular senescence triggered by Pten loss  Method: Senescence-associated β-galactosidase assay	1-6	N/A  (no growth advantage revealed)
Subtask 3: Pro/anti-proliferative signaling marker analysis	1-6	N/A

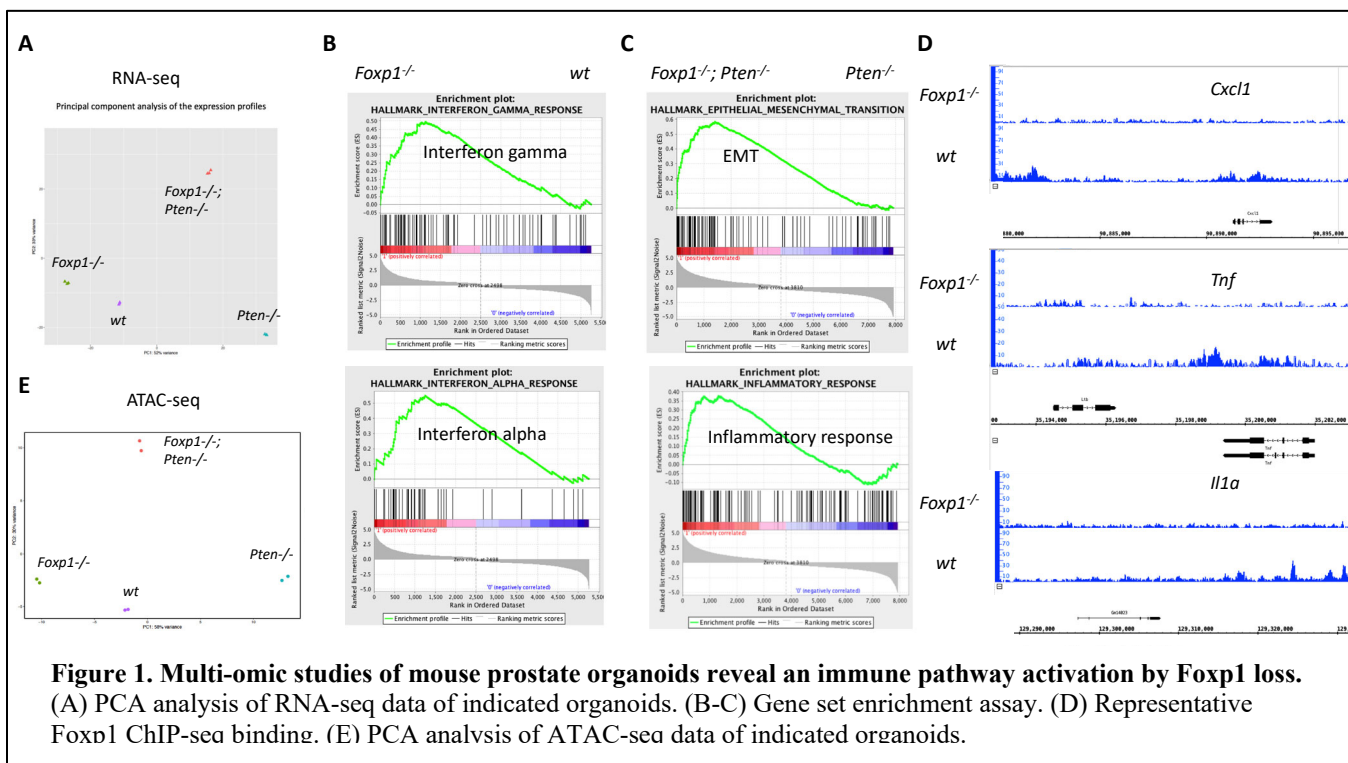
Method: Western blot		(no growth advantage revealed)
<i>Milestone(s) Achieved: Determination of a tumor suppressive role for Foxp1 and on its functional interaction with Pten at the cellular level</i>	6	100
<b>Major Task 3: Evaluate Foxp1 transcriptional regulation of Ar signaling through transcriptomic, cistromic and chromatin accessibility profiling</b>		
Subtask 1: Examine the functional interaction between Foxp1 and Pten on Ar transcription output  Mouse prostate organoid lines (already established): <i>WT</i> , <i>Pten</i> <sup>-/-</sup> , <i>Foxp1</i> <sup>-/-</sup> and <i>Pten</i> <sup>-/-</sup> ; <i>Foxp1</i> <sup>-/-</sup>  Method: RNA-seq	1-6	100
Subtask 2: Examine how Foxp1 impacts the Ar cistrome and whether this explains its effects on Ar transcription output, in the presence of absence of Pten  Method: ChIP-seq	1-9	80
Subtask 3: Measure the impacts of Foxp1 on the chromatin accessibility landscape and test whether this explains the Ar cistrome changes, in the presence of absence of Pten  Method: ATAC-seq	1-9	80
Subtask 4: Unbiased identification of novel downstream target genes and pathways regulated by Foxp1, in the presence of absence of Pten  Method: Pathway, motif and cross-reference analyses with data generated above	9-12	80
<i>Milestone(s) Achieved: Publication of a peer-reviewed manuscript on the oncogenic molecular circuits triggered by Foxp1 loss in cooperation with Pten loss</i>	12-18	80
<b>Specific Aim 2: Identify tumor suppressors within <i>Foxp1-Shq1</i> that cooperate with Foxp1</b>		
<b>Major Task 1: Identify individual prostate tumor suppressor genes that cooperate with <i>Foxp1</i></b>		
Subtask 1: Individually perturb the five candidate genes (other than <i>Foxp1</i> ) from the <i>Foxp1-Shq1</i> region in <i>Foxp1</i> <sup>-/-</sup> <i>Pten</i> <sup>-/-</sup> mouse prostate organoids  Method: Genome editing by CRISPR, western blot	1-6	0
Subtask 2: Tumor formation assay to identify individual prostate tumor suppressor genes that cooperate with <i>Foxp1</i>  # mouse: 52 (total)  Method: prostate orthotopic transplantation, bioluminescence imaging, prostate histological analysis	6-15	0
<i>Milestone(s) Achieved: Identification of bi-genic cooperation within the <i>Foxp1-Shq1</i> locus that stimulates prostate tumor formation and assessment of the relative effect of paired loss of <i>Foxp1</i> and the candidate gene in comparison to the whole <i>Foxp1-Shq1</i> locus</i>	15	0

<b>Major Task 2: Identify multigenic tumor suppressive subregions within <i>Foxp1-Shq1</i></b>		
Subtask 1: Generate a deletion series within the <i>Foxp1-Shq1</i> interval in <i>Pten</i> <sup>-/-</sup> organoids  Method: Genome editing by CRISPR	1-3	50
Subtask 2: Tumor formation assay to identify multigenic tumor suppressive subregions within <i>Foxp1-Shq1</i>  # mouse: 52 (at most)  Method: prostate orthotopic transplantation, bioluminescence imaging, prostate histological analysis	6-18	50
<i>Milestone(s) Achieved: Publication of a peer-reviewed manuscript on the cooperative events within the <i>Foxp1-Shq1</i> locus that drive prostate cancer formation together with <i>Foxp1</i> loss</i>	18-24	50

## What was accomplished under these goals?

In the the first funding period (2019-2020), our research objectives were to 1) elucidate the underlying mechanism of *Foxp1*-*Pten* functional interaction and 2) to establish genetic tools to identify the multigenic tumor suppressive subregions within *Foxp1-Shq1*.

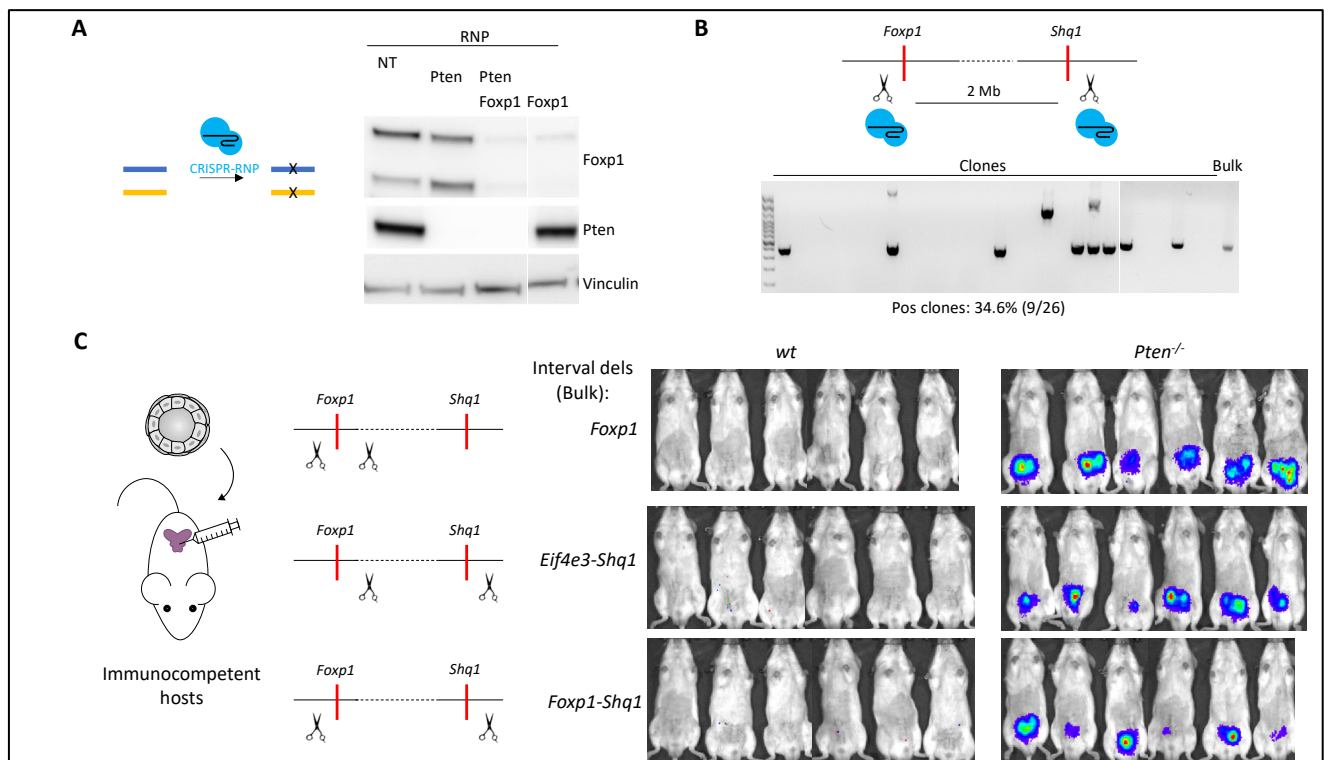
To achieve the first objective, we set out to test whether *Foxp1* loss confers growth advantage to *Pten*-null cells. However, growth analysis did not reveal significant changes (data not shown), suggesting that additional *Foxp1* loss may impact cell behaviors in a growth-independent manner. To test this possibility, we performed unbiased transcriptomic, cistromic and chromatin accessibility profiling assays using the organoids with different genetic backgrounds. Different organoids demonstrated distinct transcriptomic states (**Fig 1A**). Gene set enrichment analysis of differentially expressed genes revealed that *Foxp1* loss activated inflammatory/interferon pathways in both *wt* and *Pten*<sup>-/-</sup> backgrounds (**Fig 1B,C**). Furthermore, cistromic analysis showed that *Foxp1* is bound to either bodies or peripheries of various inflammation-related genes (**Fig 1D**). Given the putative role of *Foxp1* as a transcriptional repressor, these results converge on a model that *Foxp1* loss activates inflammatory pathways in part



by derepressing key players in the pathway. Initial chromatin accessibility analysis (ATAC-seq) also demonstrated highly separable chromatin opening profiles of different organoids, consistent with their distinct transcriptional status (**Fig 1E**). Further over-lapping analyses between the multi-omic approaches will help us identify key downstream effectors of Foxp1 that mediates the induction of immune-related pathways.

To achieve the second objective, we proposed to perform a series of chromosomal interval deletions encompassing various combinations of genes at the *Foxp1-Shq1* locus. Our initial plan was to create these deletion series via lentiviral-based CRISPR deletions before orthotopic transplantations of the engineered organoids into immune-deficient mice. However, the inflammatory/interferon pathways prompted us to investigate tumor-immune interactions as a potential explanation of the tumor suppressive role of Foxp1. Given the immunogenic effects of Cas9, Cas9 stable expression via the proposed viral-based CRISPR approaches may induce unwanted artificial immune responses. In order to set up a system that is compatible with transplantation into immunocompetent hosts, we developed a CRISPR ribonucleoprotein (CRISPR-RNP)-based organoid editing approach. By electroporation, we achieved nearly saturating knockouts of either individual *Foxp1*, *Pten* genes or the combination of the two through a single step (**Fig 2A**). Dual RNPs also created the deletion of the entire 2 Mb *Foxp1-Shq1* locus at a remarkable efficiency of > 30% when assayed at the clonal level (**Fig 2B**). Interval deletions spanning either the entire *Foxp1* gene or the *Foxp1-Shq1* region sparing *Foxp1* (*Eif4e3-Shq1*) was also generated at comparable or even higher efficiencies (up to 50%). We conclude that the large interval deletions are sufficiently represented in the bulk organoid population. Importantly, RNP editing only involves transient presence of the editing machinery. In theory, the “hit-and-run” editing avoids artificial immune responses caused by chronic presence of the editing machineries and in turn allow for the downstream tumor-immune studies. The RNP-based rapid organoid editing technique is now published (Feng, Zhen et al., 2021 PNAS).

We next sought to transplant the engineered bulk organoids into syngeneic immunocompetent hosts. Grafting signals from engineered *wt* organoids were lost by an early time point of 1 wk, indicating a grafting failure, whereas those from the *Pten*<sup>-/-</sup> correspondents persisted (**Fig 2C**). These early

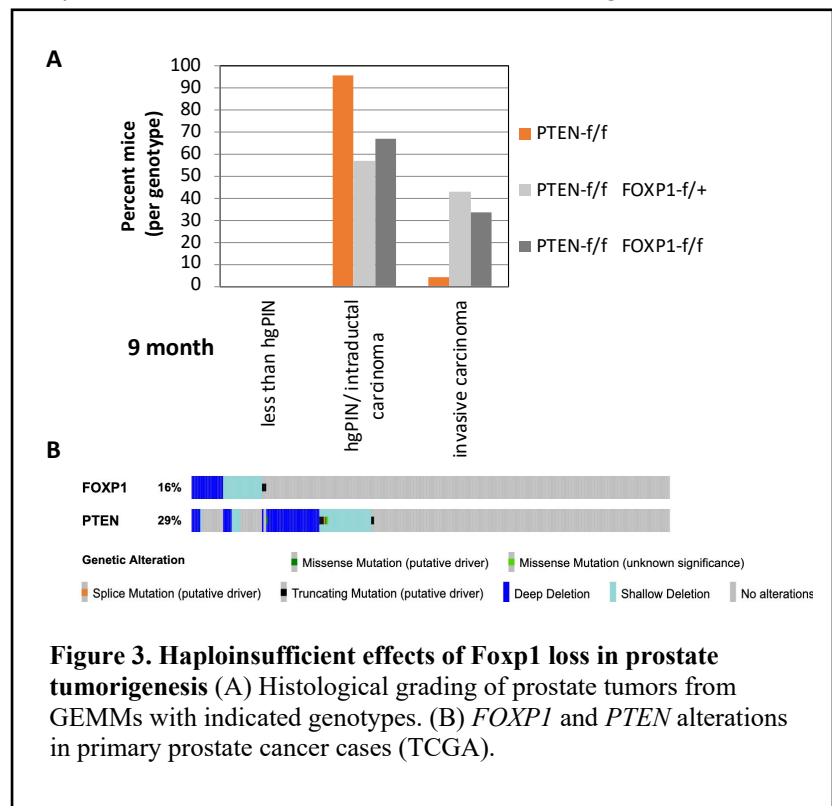


**Figure 2. Efficient CRISPR-RNP-mediated organoid editing for immunocompetent transplantations.** (A) WB after treatment with indicated RNP(s). (B) Fusion-specific PCR to detect dual-RNP-mediated *Foxp1-Shq1* deletions. (C) Bulk, luciferase-expressing *wt* prostate organoids treated with indicated RNP pairs were orthotopically transplanted to syngeneic immunocompetent hosts. Bioluminescence imaging at 1 wk post transplantation is shown on the right.

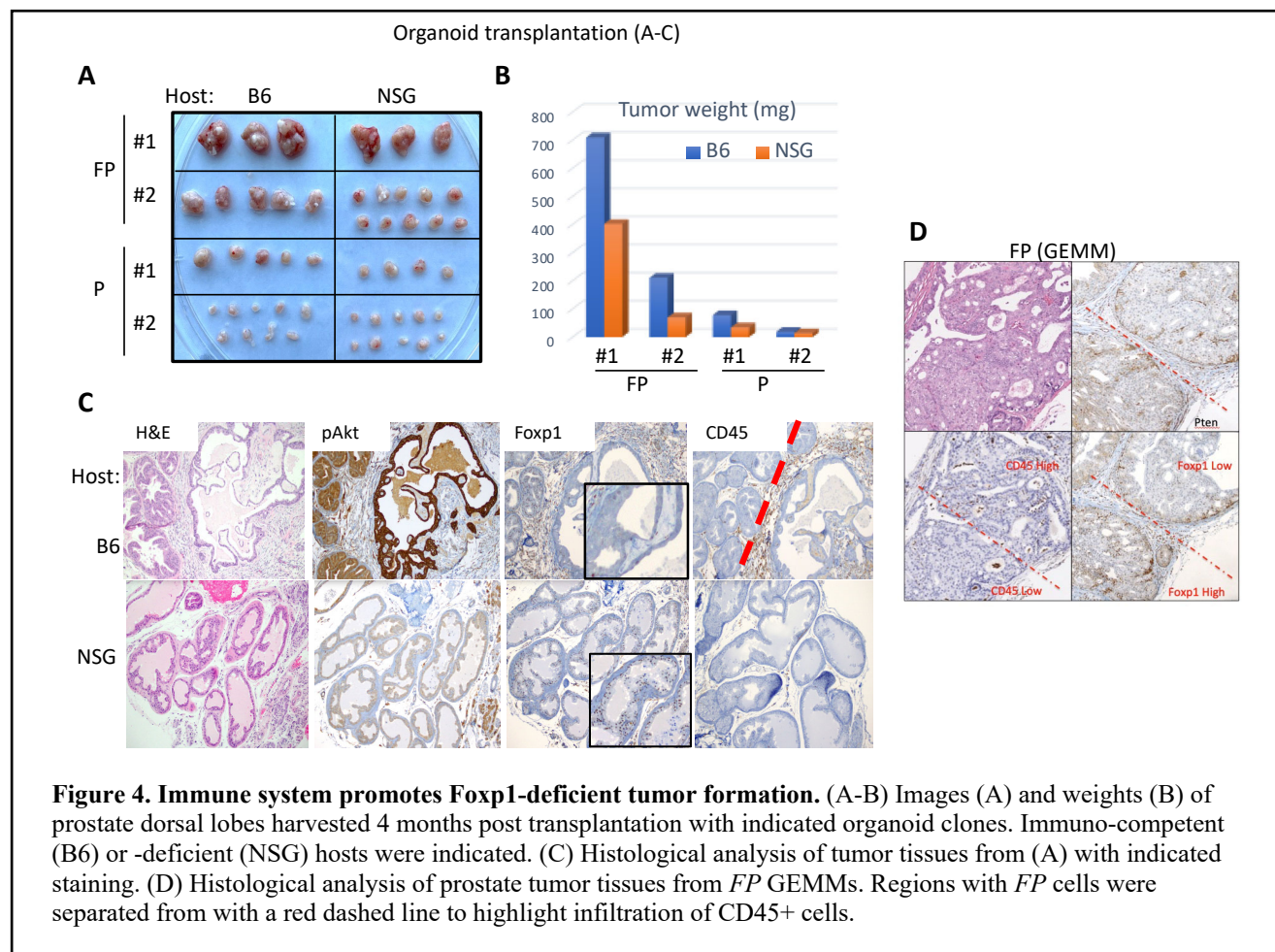
observations are consistent with a lack of tumor-prone phenotypes from *Foxp1-Shq1* loss alone in GEMMs. A follow-up comparison of these different interval deletions in *Pten*<sup>-/-</sup> transplants at a later time point will not only help us evaluate the tumor suppressive effects of these regions, but also provide additional in vivo models to interrogate potential tumor-immune responses as revealed by the above *Foxp1* studies.

In the current funding period (2020-2021), our research built on these earlier findings and focused on two directions: 1) to assess the possible effects of *Foxp1* haploinsufficiency in prostate tumorigenesis and 2) to study the impacts of immune system on tumorigenesis mediated by *Foxp1* loss.

For the first objective, we sought to find evidence in GEMMs. *Foxp1* copy number reduction, together with *Pten* loss, led to the formation of invasive carcinoma that was very rare with *Pten* loss alone (**Fig 3A**). Importantly, the cooperative effect between *Foxp1* and *Pten* was equally potent with either a single or double copy of *Foxp1* loss. These results document a haploinsufficient effect of *Foxp1* loss in prostate tumorigenesis, reminiscent of patient data where a large proportion of *FOXP1* loss is monoallelic (**Fig 3B**).



To achieve the second objective, the RNP-edited *Pten*<sup>-/-</sup> organoids with homozygous deletion of the entire *Foxp1* gene (*FP*) were transplanted orthotopically into prostates of either immuno-competent (B6) or -deficient (NSG) hosts. Both independent *FP* clones consistently displayed tumors with larger sizes and higher weights than *Pten*<sup>-/-</sup> controls. Importantly, the cooperative effects in tumor burden were strongly boosted in the presence of host immune system (**Fig 4A-B**). Indeed, histological analysis confirmed immune cell infiltration (CD45+) in tumor region with pAkt+ (indicating *Pten* loss), *Foxp1*-negative *FP* cells (**Fig 4 C**). Immune infiltration was also associated with *FP* tumors in GEMMs, further corroborating the organoid transplantation data (**Fig 4D**). These results together suggest the possibility of *FP* tumor cells interacting with immune cells to drive tumor formation. Future studies will determine which immune population is involved and further exploit the possibility of perturbing these immune cells for therapeutic purposes.



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

Training:

Mouse Genetics Workshop, MSKCC  
 Statistics for Basic Science, MSKCC

Professional development:

2019 Geoffrey Beene Retreat, Tarrytown (attended)  
 2020 13th Annual Prostate Cancer Program Retreat, Fort Lauderdale (attended)

### How were the results disseminated to communities of interest?

1. Manuscripts describing the RNP technology in rapid organoid editing and cancer modeling is published (Feng, Zhen et al., 2021 PNAS).
2. Manuscripts describing the Foxp1 biology and *Foxp1-Shq1* locus manipulation is in preparation.

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

The data obtained in the past period have brought us to an advantageous standpoint to further tackle the research questions we initially asked. The preliminary multi-omics data from the last funding period suggest a possible interaction between *Foxp1* mutant tumor cells with the immune microenvironment. Indeed, our in vivo data from this funding period confirms a role of immune system in promoting *Foxp1* mutant tumor formation. The powerful RNP-based organoid editing platform that we established generates big deletions in the bulk organoid cells through one step, which in turn enables orthotropic modeling in hosts with intact immune systems.

Moving forward, first, we will seek for an in-depth understanding of the possible roles of immune system in promoting *Foxp1* mutant tumor formation. We will tackle whether a particular immune population is functionally involved in this process and whether we can target these cells for a therapeutic opportunity.

Second, we will increase the breadth of our findings by assessing the tumor-suppressive roles of various deletion series of the *Foxp1-Shq1* locus to uncover novel tumor suppressors that cooperates with *Foxp1* loss. Based on our findings with *Foxp1*, we will leverage the edit-transplant pipeline to evaluate the tumorigenic potential of each alteration in an immunocompetent background. Together, these efforts will provide us with a both in-breadth and in-depth understanding of the multigenic 3p13 locus that is frequently lost in human prostate cancer.

#### 4. IMPACT:

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

Prostate cancer genomes commonly feature chromosomal structural alterations. Modeling these alterations, especially starting from a normal background, is key to providing insights into disease biology but remains technically challenging. For example, loss of a large, multigenic chromosomal segment at the 3p-13 locus occurs in 15~20% of human prostate cancer but how this promotes tumor formation remains unclear. During the past funding period, we have made significant progress in 1) providing insight into the possible mechanism of how loss of the *Foxp1* gene from the locus causes tumors and 2) establishing the tools to model the deletions of the entire locus in primary mouse prostate cultures. Follow-up studies in the next period will provide both a deeper understanding of cancer-forming mechanisms and a broader comprehension of the tumor-causing subregions from the whole locus. Together, this project will provide a paradigm in studying chromosome-scale alterations that frequently occur in human prostate cancer.

##### **What was the impact on other disciplines?**

An ideal cancer modeling pipeline would 1) start from a normal cell, 2) generate the desired relevant genetic alterations and 3) create cancer within the relevant tissue microenvironment. This allows for a precise interrogation of the driving genetic events, as opposed to the myriad, non-relevant passenger events. One big challenge is then to effectively create the genetic alteration from an otherwise normal background. Additional complication is conferred by unwanted host-graft immune response upon transplantation. We have addressed both challenges by creating the genetic alteration in primary cultures in an efficient and traceless manner. This in turn enables graftment into hosts with the same genetic background to minimize artificial immune responses. We anticipate that the pipeline we established can be broadly adapted for studying a spectrum of mutations across different cancer types.

##### **What was the impact on technology transfer?**

Nothing to report

##### **What was the impact on society beyond science and technology?**

Nothing to report

#### 5. CHANGES/PROBLEMS:

##### **Changes in approach and reasons for change**

To create the large deletion at the *Foxp1-Shq1* locus, we have decided to use CRISPR-RNP strategy as opposed to the viral-based strategy as we initially proposed. The primary reason is that our analysis on *Foxp1*-null organoids consistently revealed an upregulation of immune-related pathways (IFN, inflammation) regardless of *Pten* status, suggesting that an interaction with the immune system might play a role in tumor formation. Thus, a transient introduction of the editing machineries as the RNP strategy does provides the best fit for this purpose by minimizing unwanted immune responses from stable expression of potential immunogens (e.g. Cas9). Importantly, RNP editing proves to work at a much higher efficiency in both knocking out individual genes and creating large chromosomal deletions. Therefore, not only does RNP editing fit the purpose of this project alone, but serves as a superior tool for organoid genome editing in general.

Indeed, our in vivo experiments confirmed a possible role of immune system in *Foxp1* mutant tumor formation. Bases on these findings, we are planning on performing the future series of transplantation experiment in immunocompetent hosts.

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

Nothing to report

**Changes that had a significant impact on expenditures**

Nothing to report

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

Nothing to report

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

N/A

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

Nothing to report

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

Nothing to report

**6. PRODUCTS:**

**Publications, conference papers, and presentations**

**Journal publications.**

Nothing to report this period (manuscript in preparation to be submitted in next few months)

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

Nothing to report

**Other publications, conference papers, and presentations.**

Nothing to report

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

Nothing to report

**Technologies or techniques**

Development of an RNP-based approach primary organoid editing, which is highly efficient in creating large chromosomal deletions, and compatible with orthotopic transplantations in immunocompetent hosts.

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

Nothing to report

**Other Products**

Data: published manuscript

Research material: primary mouse prostate organoids carrying a variety of prostate-cancer-relevant alterations

**7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

**What individuals have worked on the project?**

Name: Weiran Feng

Project Role: Principal Investigator

Nearest person month worked: 24.00 calendar months

Contribution to Project: As the Principal Investigator of this project, Dr. Feng will oversee and coordinate all aspects of the project.

Funding Support: This award

Name: Zhen Cao, he is in the 6th year of Weill Cornell grad school

Project Role: Graduate Student

Contribution to Project: As a graduate student on this project, Mr. Cao assists Dr. Feng in the lab for work associated with this project.

Nearest person month worked: 18.00 calendar months

Funding Support: Dr. Cao is paid fully by Dr. Sawyers' (Mentor) department discretionary fund.

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

There have been no changes to Dr. Feng's active other support since the last reporting. Dr. Sawyers' updated active other support is attached with the changes noted for the last reporting

**What other organizations were involved as partners?**

Not applicable

**8. SPECIAL REPORTING REQUIREMENTS**

**COLLABORATIVE AWARDS:**

Not applicable

**QUAD CHARTS:**

Not applicable

**9. APPENDICES:**

Published manuscripts describing the RNP technology in rapid organoid editing and cancer modeling (Feng, Zhen et al., 2021 PNAS).

## Previous/Current/Pending Support

Sawyers, Charles L.

### NEW ACTIVE

**5 R01 CA193837-06** (PI: Sawyers) 2/9/2021-1/31/2026 1.80 calendar  
NCI  
Role of ETS factors in specifying prostate luminal cell identity and androgen receptor dependence  
**Major Goals:** In this proposal, we investigate how mutations in ETS factors, clinically important oncogenic drivers of prostate cancer, promote luminal cell identity and regulate chromatin architecture of cancerous prostate tissues, including by co option of AR function.  
**Agency Contact:** Funmi Elesinmogun, Email: elesinmf@mail.nih.gov, Phone: (240) 276-6313  
**Overlap:** None

**3 U54 CA224079 04 S1** (PI: Sawyers/Nelson) 9/30/2020 - 8/31/2022 \*0.00 calendar  
NCI  
**Major Goals:** The MSKCC UW/Fred Hutch Prostate Cancer Drug Resistance and Sensitivity Center (Supplement)  
The overarching goal of this DRSC proposal is to evaluate translational therapies across a unique set of preclinical organoid and patient derived xenograft (PDX) models, and to catalyze the initiation of clinical studies in patients most likely to benefit based on appropriate biomarker profiles.  
**Agency Contact:** Funmi Elesinmogun, Email: elesinmf@mail.nih.gov, Phone: (240) 276-6313  
**Overlap:** None  
\*No effort required. The work for this supplement will take place at the partnering Site, Fred Hutch, under the direction of Dr. Peter Nelson.

**GC260677 (PI: Leslie)** 9/1/2020 - 8/30/2022 0.12 calendar  
Geoffrey Beene Cancer Research Center  
Epigenetic and transcriptional regulation in FOXA1 mutant prostate and breast cancer  
**Major Goals:** The goal of this project is to elucidate the molecular details of how this co-option of pioneering TF activity occurs.  
Aim 1. Characterize the epigenetic and transcriptional effects of mutant FOXA1 at a single cell level.  
Aim 2. Decipher the motif grammar of mutant FOXA1 alleles through analysis of TF cistromes.  
**Role:** Co-PI  
**Agency Contact:** Vicky Baudin, baudinv@mskcc.org  
**Overlap:** None

**SK2020-1592** (PI: Sawyers) 12/1/2020 – 11/30/2023 0.00 calendar\*  
Calico Life Sciences ( to lab)  
MSK - Calico Single Cell Collaboration  
**Major Goals:** The MSK-Calico collaboration is centered around two initial research projects. Project 1 focuses on lineage plasticity in adenocarcinomas of the lung and prostate as a mechanism of acquired drug resistance. Project 2 focuses on the dynamics of tumor microenvironment change, including response to therapy and PTPN2 treatment, in prostate, lung, liver, pancreatic, and rectal cancers. The Sawyers lab will share relevant prostate cancer models and data to support the collaboration.  
**Role:** Principal Investigator  
**Agency Contact:** Bob Cohen, Calico Principal Investigator; rlc@calicolabs.com  
**Overlap:** None  
\*No effort required by sponsor.

## INACTIVE SUPPORT

**I12 0007 (PI: Sawyers)** 1/1/2019 - 12/31/2020 0.36 calendar  
Starr Cancer Consortium  
Defining prostate cancer cells of origin through single cell profiling  
This proposal fits the RFA category of "Disease Applications" because it applies the relatively new molecular technology of single cell RNA sequencing (scRNA seq) to the study of prostate cancer.  
**Agency Contact:** Sylvie Le Blancq, Email: leblancs@mskcc.org  
**Overlap:** None

**5 R01 CA193837-05 (PI: Sawyers)** 4/1/2015-1/31/2020 1.90 calendar  
NCI  
Defining the Role of ERG in Modulating the AR Cistrome and Antiandrogen Sensitivity  
**Major Goals:** This project will shed light on the molecular mechanism by which ERG causes prostate cancer and the impact of ERG on response to therapies directed against the androgen receptor, the common form of treatment for metastatic prostate cancer.  
**Specific Aims:** Aim 1. Decipher the mechanism of ERG-mediated reprogramming of the AR cistrome  
Aim 2. Understand the role of PTEN loss in modulating the ERG transcriptome.  
Aim 3. Determine the role of ERG expression in sensitivity to AR and PI3K inhibition.  
**Agency Contact:** Funmi Elesinmogun, Email: elesinmf@mail.nih.gov, Phone: (240) 276-6313  
**Overlap:** None

## CURRENT SUPPORT

**Howard Hughes Medical Institute (PI: Sawyers)** 3/1/2008- 8/31/2025  
**Major Goals of this project:** Patient oriented research into molecularly targeted therapy of cancer  
**Agency Contact:** Edit Biro, MAS, Howard Hughes Medical Institute, 1230 York Ave, Box 269, New York, NY 10065  
**Overlap:** None

**2 R01 CA155169-09 (PI: Sawyers)** 5/1/2012-12/31/2022 1.81 calendar  
NCI/NIH  
Understanding Resistance to Next Generation Antiandrogens  
**Major Goals:** This project focuses on a novel mechanism of acquired resistance to hormone therapy in castration resistant prostate cancer (CRPC) called lineage plasticity. During the initial 5 year funding cycle, we focused primarily on two other mechanisms of resistance: mutation or amplification of the androgen receptor (AR) and bypass of AR signaling through upregulation of the closely related glucocorticoid receptor.  
**Agency Contact:** Grants Management Specialist: Rogers Gross, (240) 276-7589, rogers.gross@nih.gov  
**Overlap:** None

**1 U54 CA224079-04 (PI: Sawyers)** 9/1/2017-8/31/2022  
NCI/NIH  
The MSKCC-UW/Fred Hutch Prostate Cancer Drug Resistance and Sensitivity Center

- Admin Core (PI: Sawyers) 0.91 calendar
- Project 1 (PI: Sawyers) 0.91 calendar
- Project 2 (PI: Chen; Co-I: Sawyers) (lab) 0.46 calendar

**Major Goals:** 26,000 men die each year in the US from metastatic prostate cancer because the disease develops resistance to the primary treatment, called hormone therapy. DNA sequencing studies of tumors from these patients has revealed new strategies to treat prostate cancer with novel drugs in combination with hormone therapy. This application assembles a team of experts on this topic to work together to test these new therapies

in laboratory models of prostate cancer developed directly from patients and identify those that should be accelerated into clinical development.

**Agency Contact:** Funmi Elesinmogun, Email: elesinmf@mail.nih.gov, Phone: (240) 276-6313

**Overlap:** None

**1 P01 CA228696 02 (PI: Kantoff/ Sawyers)** 9/1/2019 - 8/31/2024\* 0.60 calendar  
NCI

The Impact of DNA Damage Repair Abnormalities in Prostate Cancer (RP3: Functional Evaluation and Interpretation of DNA Damage Response Variants in Prostate Cancer)

Alterations in genes that help repair damaged DNA are seen in 25% of men with metastatic castration resistant prostate cancer, the lethal form of prostate cancer.

**Role:** Project Leader

**Agency Contact:** Kelly Filipinski, Kelly.Filipinski@nih.gov

**Overlap:** None

\*Dr. Sawyers was replaced as Project Leader on this award as of 8/31/2021. Dr. Sawyers therefore committed no effort past 8/31/2021.

**2 P50 CA092629-19 (PI: Scher)** 9/1/2016 – 8/31/2022

National Institutes of Health

MSKCC Spore in Prostate Cancer

- RP-3 (PI: Sawyers) 0.60 calendar  
Heterogeneity in metastatic CRPC and the role of TP53 mutation as a molecular determinant
- Core F (PI: Sawyers) ( to lab) 0.60 calendar  
Administrative Core

**Major Goals of this Project:** As a public health concern, prostate cancer is the second deadliest cancer in men. The translational research projects in this program aim to use knowledge of animal and human prostate cancer biology to develop and test interventions related to the prevention, early detection, diagnosis, prognosis, and treatment of prostate cancer in men.

This allocation is split between the following: Development Research Program; Core F, Administrative Core; Project 3, Heterogeneity in metastatic CRPC and the role of TP53 mutation as a molecular determinant; and Project 4, Investigating and targeting the glucocorticoid receptor (GR) in enzalutamide- and abiraterone.

Role: Core Co-Director of Core F, Co-Director of Development Research Program, Principal Investigator of Project 3 and Project 4.

**Specific Aims of the Overall Grant:**

1. To interrogate the genomics and molecular pathways relevant to prostate cancer progression
2. To improve prognostic models for early detection of potentially lethal cancers
3. To identify and validate clinically relevant biomarkers
4. To develop novel agents and therapeutic strategies

**Agency Contact:** Renee Carruthers, Email: carruthersr@mail.nih.gov Phone: 301-631-3018 Fax: 301-451-5391

**Overlap:** None

**5 P30 CA008748-54 (PI: Thompson)** 1/1/2014-12/31/2023 0.60 calendar  
NCI to lab

Cancer Center Support Grant (Cancer Biology and Experimental Pathology Program)

The CCSG funds support MSK's research infrastructure. These shared resources facilitate the research activities of the clinical, translational and laboratory programs at the Cancer Center.

**Agency Contact:** Funmi Elesinmogun, Email: elesinmf@mail.nih.gov, Phone: (240) 276-6313

**Overlap:** None

**2 T32 CA160001-10 (PI: Sawyers)** 8/1/2016 - 7/31/2022 (NCE) 0.00 calendar  
NCI to lab)

Translational Research in Oncology Training Program

**Major Goals:** The training program for translational cancer research will provide opportunities to postdoctoral PhD trainees to learn about human oncology and pathogenesis, and work collaboratively with clinicians to advance the treatment of cancer patients. The goals are: to help basic scientists to develop a strong clinical background so that they may effectively bring discoveries from bench to bedside; and to foster interdisciplinary research and collaboration.

**Specific Aims:** 1) To provide broad and intensive translational research training for PhDs by offering enhanced opportunities to orient their research to biomedically relevant problems. 2) To prepare Trainees to successfully collaborate during their career with researchers with different backgrounds. 3) To prepare and assist Trainees to successfully transition to research independence.

**Agency Contact:** Renee Carruthers, Email: carruthersr@mail.nih.gov, Phone: 301-631-3018, Fax: 301-451-5391

**Overlap:** None

**W81XWH-20-1-028, PC190310 (PI: Sawyers)** 9/30/2020 - 9/30/2023 0.60 calendar  
Congressionally Directed Medical Research Programs

Defining Transcription Factor Networks Governing Androgen Receptor Null Prostate Cancer

**Major Goals:** Aim 1. Determine gene expression and chromatin landscape changes associated with potent AR inhibition Aim 2. Identify vulnerabilities responsible for maintenance of the AR low/null state Aim 3.

Characterize the AR low/null state in CRPC patients using single cell and organoid technologies

**Role:** Principal Investigator

**Agency Contact:** Joshua Mckean, Joshua.D.Mckean3.Civ@Mail.Mil

**Overlap:** None

**5 R25 CA057732-27 (PI: Sawyers)** 07/11/2016 - 11/30/2021 0.50 calendar  
NIH/NCI ( to lab)

Molecular Biology in Clinical Oncology Workshop


**Major Goals:** The workshop is primarily designed for oncologists in training at the fellow level. Space is also made available to a limited number of senior oncologists. The development and application of a range of cellular and molecular biological approaches to the study of gene structure and expression, cellular growth control, and malignant transformation have resulted in remarkable advances in the diagnosis, treatment, and prevention of cancer.

**Agency Contact:** Jeannette F Korczak, korczakj@mail.nih.gov

**Overlap:** None



# Rapid interrogation of cancer cell of origin through CRISPR editing

Weiran Feng<sup>a,1</sup>, Zhen Cao<sup>a,b,1</sup>, Pei Xin Lim<sup>c</sup>, Huiyong Zhao<sup>d</sup>, Hanzhi Luo<sup>e</sup>, Ninghui Mao<sup>a</sup>, Young Sun Lee<sup>a</sup>, Aura Agudelo Rivera<sup>a</sup>, Danielle Choi<sup>a</sup>, Chao Wu<sup>f</sup>, Teng Han<sup>a</sup>, Rodrigo Romero<sup>a</sup>, Elisa de Stanchina<sup>d</sup>, Brett S. Carver<sup>a,f,g</sup>, Qiao Wang<sup>h</sup>, Maria Jasin<sup>c</sup>, and Charles L. Sawyers<sup>a,i,2</sup> 

<sup>a</sup>Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065; <sup>b</sup>Weill Cornell Graduate School of Medical Sciences, Weill Cornell Medicine, New York, NY 10021; <sup>c</sup>Developmental Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065; <sup>d</sup>Antitumor Assessment Core Facility, Memorial Sloan Kettering Cancer Center, New York, NY 10065; <sup>e</sup>Molecular Pharmacology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065; <sup>f</sup>Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY 10065; <sup>g</sup>Division of Urology, Memorial Sloan Kettering Cancer Center, New York, NY 10065; <sup>h</sup>Key Laboratory of Medical Molecular Virology of Ministry of Education/National Health Commission/Chinese Academy of Medical Sciences, School of Basic Medical Sciences, Shanghai Medical College of Fudan University, Shanghai 200032, China; and <sup>i</sup>HHMI, Memorial Sloan Kettering Cancer Center, New York, NY 10065

Contributed by Charles L. Sawyers, June 23, 2021 (sent for review June 4, 2021); reviewed by William G. Kaelin Jr. and William G. Nelson

**The increasing complexity of different cell types revealed by single-cell analysis of tissues presents challenges in efficiently elucidating their functions. Here we show, using prostate as a model tissue, that primary organoids and freshly isolated epithelial cells can be CRISPR edited ex vivo using Cas9–sgRNA (guide RNA) ribonucleoprotein complex technology, then orthotopically transferred in vivo into immunocompetent or immunodeficient mice to generate cancer models with phenotypes resembling those seen in traditional genetically engineered mouse models. Large intrachromosomal (~2 Mb) or multigenic deletions can be engineered efficiently without the need for selection, including in isolated subpopulations to address cell-of-origin questions.**

CRISPR | organoids | editing | cancer modeling

**M**odels to study the earliest stages in cancer progression must, by definition, start with normal cells to assess the consequences of a suspected oncogenic perturbation. Inbred mouse strains and genetically engineered mouse models (GEMMs) have, for decades, served as gold standards for such studies but require significant time (years) to generate, breed, and age mice. CRISPR technology has greatly accelerated the pace of generating GEMMs through delivery of sgRNAs (guide RNAs) and/or Cas9 to tissues such as lung and liver (1–3). The ability to grow primary tissues ex vivo as organoids and introduce precise genetic changes into these cultures provides an alternative platform to model genomic alterations with speed and efficiency, as reported for intestine and prostate (4–7). Here we demonstrate highly efficient (50 to 90%) editing of primary prostate epithelial organoid cultures, including multigenic or intrachromosomal (>2 Mb) deletions, through transient electroporation of Cas9–sgRNA ribonucleoprotein (cRNP) complexes. We also show cRNP-based CRISPR editing can be performed on freshly isolated prostate epithelial cells and then transplanted orthotopically into the prostates of recipient mice in a single day, enabling extremely rapid generation of in vivo cancer models in immunodeficient as well as immunocompetent settings. Finally, we show that this approach can be used to address cancer cell-of-origin questions by multigenic editing selectively in luminal versus basal epithelial cells.

## Results and Discussion

As a first step to optimize CRISPR editing in primary organoids, we compared the efficiency of a conventional virus-based approach, in which vectors expressing Cas9 and sgRNAs are stably introduced into cells (4), to the cRNP approach, in which Cas9 protein and sgRNAs are transiently electroporated into cells (8–10). Prostate organoids derived from a GEM model of ERG-positive human cancers (*R26<sup>LSL-ERG/LSL-ERG</sup>;Pten<sup>fl/fl</sup>;Pb-Cre, EPC*) (11) were used as a model in which the ERG transgene is activated via a Cre-mediated removal of the preceding Lox-STOP-Lox (LSL)

cassette. There is no endogenous Erg expression in these cells. We quantified editing efficiency by flow cytometric assessment of loss of ERG expression, an assay sensitive enough to distinguish between biallelic (*R26<sup>+/+</sup>*), heterozygous (*R26<sup>LSL-ERG/+</sup>*), or absence of ERG expression (*R26<sup>ko</sup>*), created by CRISPR knockout of the *R26<sup>+/+</sup>* allele) (Fig. 1A).

To evaluate the viral approach, we stably introduced a Cas9-expressing vector into organoids, confirmed protein expression in the bulk population (Fig. 1B), then introduced a second vector containing a sgRNA targeting ERG and a cis-linked mCherry cassette. Flow cytometric analysis confirmed loss of ERG expression in ~34% of mCherry-positive cells (Fig. 1C). Using the cRNP method, we observed a dose-dependent increase in editing

## Significance

**Modeling cancer formation requires introduction of relevant oncogenic perturbations into normal cells in a tissue/cell-type-specific manner. Genetically engineered mouse models are powerful but require significant time and cost to generate and maintain. The ability to edit primary epithelial cells ex vivo followed by orthotopic transplantation provides an alternative strategy for cancer modeling but requires efficient gene editing, typically in a multiplex fashion. Here we successfully engineer multigenic perturbations or chromosomal rearrangements in primary prostate organoids through single-step Cas9–sgRNA ribonucleoprotein electroporation. This approach can also address cell-of-origin questions by directly editing and transplanting freshly isolated subpopulations without the intermediate step of organoid culture, providing a rapid complement to traditional lineage tracing approaches.**

Author contributions: W.F. and Z.C. designed research; W.F., Z.C., P.X.L., H.Z., H.L., N.M., Y.S.L., A.A.R., D.C., C.W., T.H., and Q.W. performed research; W.F., Z.C., N.M., E.d.S., B.S.C., and M.J. contributed new reagents/analytic tools; W.F., Z.C., N.M., B.S.C., M.J., and C.L.S. analyzed data; and W.F., R.R., and C.L.S. wrote the paper.

Reviewers: W.G.K., Harvard Medical School; W.G.N., Johns Hopkins University School of Medicine

Competing interest statement: C.L.S. is on the board of directors of Novartis, is a co-founder of ORIC Pharmaceuticals, and serves on the scientific advisory boards of the following biotechnology companies: Agios, Arsenal, Beigene, Blueprint, Column Group, Foghorn, Housey Pharma, Nextech, KSQ Therapeutics, and PMV Pharma. He is a coinventor of the prostate cancer drugs enzalutamide and apalutamide, covered by US patents 7,709,517, 8,183,274, 9,126,941, 8,445,507, 8,802,689, and 9,388,159 filed by the University of California, Los Angeles.

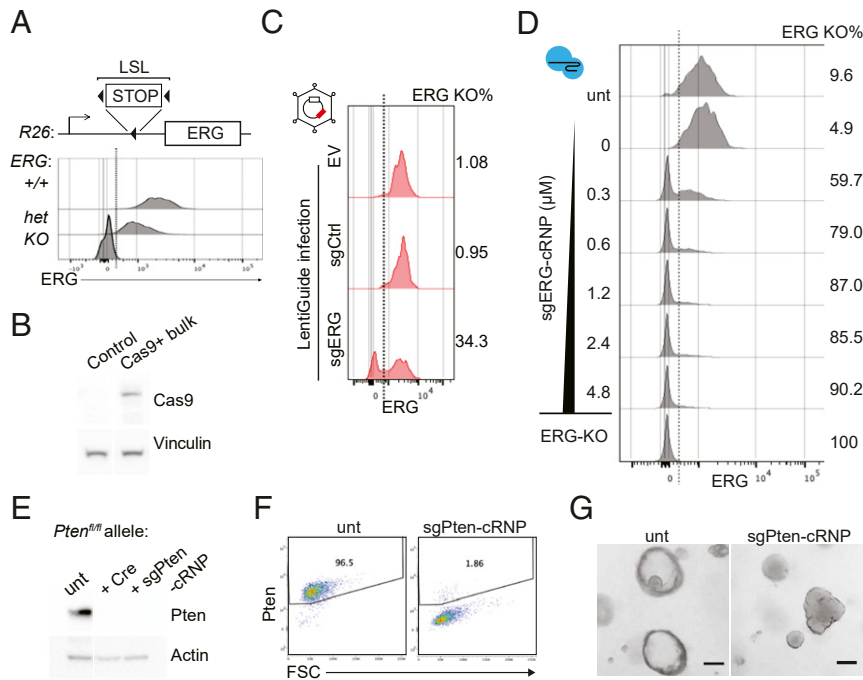
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<sup>1</sup>W.F. and Z.C. contributed equally to this work.

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2110344118/-DCSupplemental>.

Published August 5, 2021.



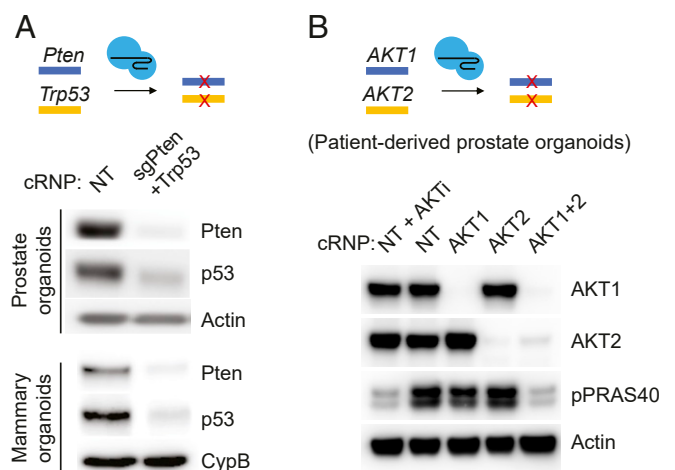
**Fig. 1.** Efficient one-step organoid population editing by cRNP. (A) (Top) Schematic describing the  $R26^{LSL-ERG}$  allele. LSL, LoxP-STOP-LoxP cassette. (Bottom) ERG detection in organoid cells by intracellular flow cytometry. (B) Cas9 protein detection in bulk EPC organoid cells after Cas9 lentiviral transduction. (C) ERG loss in mCherry-labeled sgRNA-infected  $R26^{LSL-ERG/LSL-ERG};Pten^{fl/fl};Pb-Cre$  (EPC) organoids as measured by intracellular flow cytometry. EV, empty vector control. (D) sgERG-cRNP dosage titration in EPC organoids with corresponding editing outcomes as measured by ERG intracellular flow cytometry ( $n = 1$ ). Unt, untreated. (E) Western blot comparing efficiency of editing the  $Pten^{fl/fl}$  allele by Adeno-Cre and sgPten-cRNP in  $R26^{LSL-ERG/LSL-ERG};Pten^{fl/fl}$  organoids. (F) Pten loss in wild-type (WT) organoids by cRNP as measured by intracellular flow cytometry. (G) Representative images showing a hyperplastic morphology displayed by sgPten-cRNP-treated organoids. (Scale bar, 100  $\mu$ m.)

efficiency after electroporation of sgERG-cRNP, with up to 90% of cells showing loss of ERG expression (Fig. 1D). Although it may be possible to achieve greater deletion efficiency using the viral approach by increasing the level of Cas9 expression, we were struck by the remarkable efficiency and simplicity of the cRNP method, as well as the speed, since fully edited cells were obtained within 3 d following electroporation. In addition to its simplicity, the cRNP approach offers the advantage of transient Cas9 expression and potentially reduced off-target editing as a consequence (8, 12, 13).

Given the efficiency of cRNP editing in the ERG model system, we extended this approach to tumor suppressor genes by first targeting *Pten* in primary mouse prostate organoids. *Pten* knockout (KO) populations were readily generated following sgPten-cRNP electroporation with >90% efficiency as measured by flow cytometry and Western blot, comparable to that observed after *Pten* deletion in  $Pten^{fl/fl}$  organoids infected with Cre-expressing adenovirus (Fig. 1E and F). In addition to loss of Pten protein expression, the edited organoids acquired known morphologic features of *Pten* loss such as a hyperplastic morphology with a filled-in lumen and loss of basal/luminal epithelial polarity (14) (Fig. 1G).

Having documented rapid, highly efficient single-gene editing in organoids (ERG, Pten), we considered the possibility of editing two genes simultaneously. We selected *Pten* and *Trp53*, which are commonly deleted in human metastatic prostate cancer and cooperatively induce prostate cancer in GEM models (15–17). Indeed, codelivery of two cRNP complexes, sgPten-cRNP and sgTrp53-cRNP, resulted in substantial ablation of both targets as measured by Western blot within 7 d following electroporation (Fig. 2A). Efficient multigenic editing is not unique to prostate, as similar results were obtained by targeting *Pten* and *Trp53* in mouse mammary organoids (Fig. 2A). We also observed robust

multigenic cRNP editing in human prostate organoids, as shown by deletion of *AKT1* and *AKT2* in an organoid line derived from a patient with metastatic prostate cancer (Fig. 2B). The near complete loss of phosphorylation of the AKT substrate PRAS40, as seen following treatment with a pan-AKT inhibitor, was observed



**Fig. 2.** Multiplexed cRNP editing in organoids across different tissues and species. (A) Western blot showing codisruption of Pten and p53 proteins in WT (C57BL/6J albino) mouse prostate organoids and mouse mammary organoids by cRNP. (B) Western blot showing codisruption of *AKT1* and *AKT2* proteins in a patient-derived prostate organoid line by cRNP. AKTi, AKT inhibitor ipatasertib treatment (500 nM for 4 h). Unt, untreated; NT, non-targeting sgRNA control.

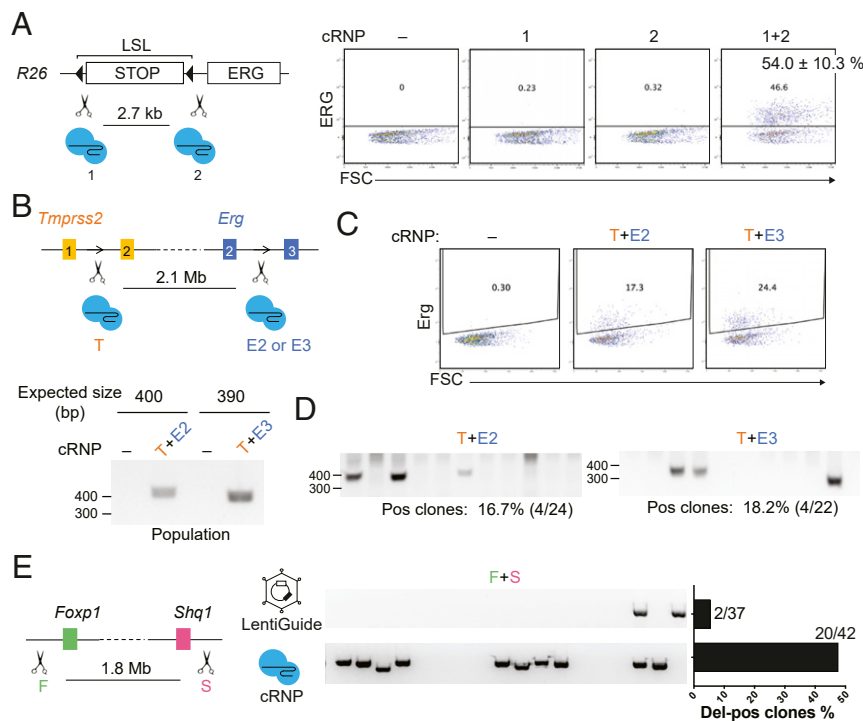
only in dual sgAKT1–cRNP/sgAKT2–cRNP electroporated organoids. In addition to confirming highly efficient multigene deletion in a human organoid model, this result demonstrates the importance of dual rather than isoform-specific AKT inhibition to block downstream signaling.

Recurrent multigenic chromosomal deletions are among the most common alterations in cancer genomes, particularly in epithelial tumors such as prostate cancer (18, 19). The high efficiency of cRNP-based gene disruption we observed in primary mouse prostate organoids led us to test whether we could efficiently engineer larger deletions. As an initial test we returned to the R26-ERG organoid model, which contains a 2.7-kb LSL cassette between the Rosa26 promoter and the ERG cDNA cassette ( $R26^{LSL-ERG/LSL-ERG}$ ), with the goal of deleting this region using cRNP (instead of Cre). Over 50% of cells electroporated with cRNPs targeting both sides of the STOP cassette displayed robust ERG expression within 3 d following electroporation, indicative of effective deletion (Fig. 3A). Importantly, ERG expression was not observed in cells electroporated with each RNP alone, ruling out large deletions created by single sgRNAs (20).

Having established that multikilobase deletions can be generated quickly and efficiently in organoids using sgRNA–cRNPs flanking the region of interest, we next asked whether we could model larger (megabase scale) deletions found in human prostate cancer. Complex translocations or deletions have been previously generated with CRISPR tools but suffer from limited efficiency, require template construct engineering, or rely on the strong selective advantage from the edited cells (e.g., tumor formation) to be successful (3, 21, 22). To address the efficiency question directly, we designed cRNPs to generate the 2.1-Mb intrachromosomal

deletion that results in aberrant expression of the ETS family transcription factor ERG in human prostate cancer (23). Using a single sgRNA targeting *TMPRSS2* intron 1 and two different sgRNAs targeting *Erg* intron 2, we detected the expected fusion products by PCR of genomic DNA from the bulk population within 3 d of electroporation (Fig. 3B and *SI Appendix*, Fig. S1A). We subsequently documented an efficiency of ~20% by flow cytometry analysis of ERG protein expression (Fig. 3C) and PCR screening of single-cell clones (Fig. 3D). These results replicate earlier work using CRISPR-directed homologous recombination to generate the *Tmprss2-Erg* translocation in mouse prostate organoids (24) but eliminate the time and effort needed to generate a genomic targeting construct containing the relevant 5' and 3' homology arms of *Tmprss2* and *Erg*.

As a second example, we chose to delete the multigenic 2.0-Mb tumor suppressor locus at human 3p13-14, found in ~15% of localized prostate cancers (18, 25). This region is of particular interest because it is almost exclusively associated with prostate cancer and lacks a canonical tumor suppressor gene within the locus. Our group previously documented that this region is, indeed, tumor suppressive in a GEM model, but this approach required a generation of a complex array of LoxP sites and extensive breeding (26). To simplify this process and simultaneously create a platform that enables rapid functional interrogation of specific genes within the locus, we tested whether a syntenic 3p13-14 deletion could be generated directly in mouse organoids, using the conventional lentiviral approach with stable Cas9 and sgRNA expression as well as cRNP, followed by screening of individual subclones. As with ERG deletion experiments discussed earlier, cRNP-based CRISPR editing was fast and efficient (20 of 42 positive clones, 48%) compared to the lentiviral Cas9/sgRNA



**Fig. 3.** cRNP generates large chromosomal deletions in mouse prostate organoids. (A) (Left) Schematic showing a paired-cRNP strategy to induce ERG expression by disrupting the preceding LSL cassette. (Right) ERG expression from the cRNP-treated  $R26^{LSL-ERG/LSL-ERG}$  organoid population was measured by intracellular flow cytometry. (B) (Top) Schematic showing *Tmprss2-Erg* fusion by paired cRNPs with different sgRNA combinations in WT organoids. (Bottom) Genomic fusion detection from the electroporated organoid populations by fusion-specific genomic PCRs. (C) ERG expression of the paired cRNP-treated WT organoid population from B was measured by intracellular flow cytometry. (D) Fusion-specific genomic PCRs of organoid clones treated with indicated cRNP pairs. DNA electrophoresis results of representative clones are shown. (E) Paired CRISPR-mediated deletion of the *Foxp1-Shq1* region (schematic shown on the Left). WT organoids were either electroporated with cRNP or infected with lentiGuide (with preengineered Cas9 expression). Fusion-specific genomic PCR of resulting organoid clones and quantification of deletion-positive clones are shown on the Right.

expression (3 of 37 positive clones, 5%); albeit higher lentiviral efficiencies might be achievable by isolation of high Cas9-expressing subclones (Fig. 3E and *SI Appendix*, Fig. S1B). Importantly, all alterations above were created without any exogenous selection. Thus, cRNP technology is a versatile and convenient tool for multigenic and long-range genome editing in organoids.

Organoids can also be used for *in vivo* cancer modeling through orthotopic injection studies, and the transient presence of Cas9 protein using the cRNP method may have advantages since it eliminates any potential risk of Cas9 immunogenicity that can occur using viral methods (27–29). To explore this, we injected the sgPten+Trp53–cRNP-edited organoids orthotopically into the prostate dorsal lobes of syngeneic recipient mice (Fig. 4A). Remarkably, 100% (six of six) B6 recipient mice developed locally invasive adenocarcinomas with median survival of ~17 wk, comparable to that observed in *Pb-Cre;Pten<sup>fl/fl</sup>;Trp53<sup>fl/fl</sup>* GEM models (16). In contrast, all mice injected orthotopically with sgPten–cRNP and/or control cRNP-edited organoids survived beyond 30 wk with no evidence of disease (Fig. 4B and C).

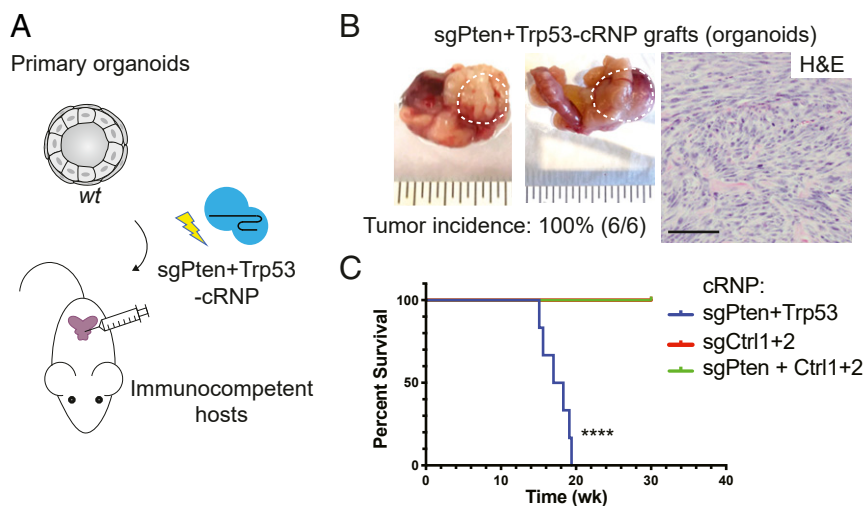
The high efficiency of cRNP-based editing in organoids led us to ask whether this technology could be applied directly to prostate epithelial cells freshly isolated from a donor mouse and then reintroduced as an orthograft into a recipient mouse, thereby avoiding the need for organoid culture. In addition to speed, this approach could potentially be used to address cancer cell-of-origin questions by comparing the outcome of edited cells in different subpopulations isolated by flow cytometry. Toward that end, we sorted freshly isolated mouse prostate epithelial cells into basal (Cd49f-high) and luminal (Cd24-high) subpopulations (30), electroporated each with sgPten–cRNP and sgTrp53–cRNP as described earlier for organoids, and separately injected each subpopulation orthotopically into the dorsal prostate lobes of recipient mice (Fig. 5A). Substantially reduced p53 and Pten protein expression was confirmed in these postedited primary cells (Fig. 5B). At the 17-wk endpoint (selected based on the organoid orthograft data described earlier; Fig. 4C), two of six mice engrafted with cRNP-edited luminal cells developed large prostate tumors (one at 14 wk due to early death), whereas no tumors were observed in four mice engrafted with basal cells (Fig. 5C). Immunohistochemical analysis confirmed the presence of glandular orthografted cells in the dorsal lobes of all mice, based on phospho-Akt staining (indicative

of *Pten* loss) and absence of p53 staining (Fig. 5D), as well as PCR analysis of the sgRNA-targeted region from bulk prostate tissue (*SI Appendix*, Fig. S2). Although additional studies are required to define the efficiency, these results document the feasibility of same-day cRNP editing of freshly isolated prostate epithelial cells for cancer modeling. The fact that tumors were observed only in mice engrafted with cRNP-edited luminal cells is consistent with prior evidence from GEM models implicating luminal cells as the cell of origin for prostate cancers initiated by *Pten* loss (31, 32).

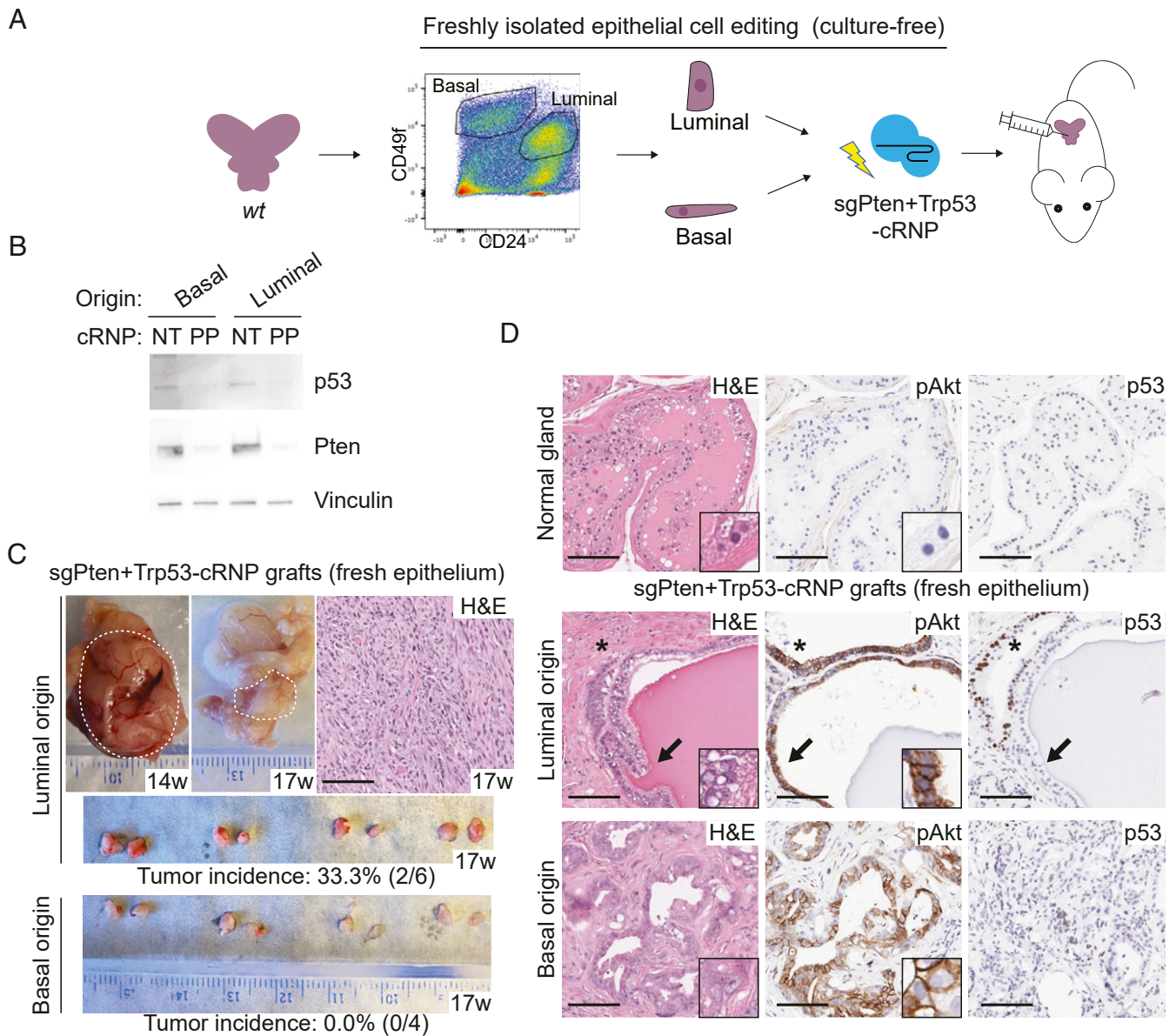
In summary, we show that cRNP-based CRISPR editing offers several advantages for cancer modeling: high editing efficiency, ability to generate single/multiple perturbations and large chromosomal deletions in a single step, and ability to edit freshly isolated epithelial cells, in bulk or after sorting into luminal or basal subpopulations, followed by same day orthotopic transfer for tumorigenicity studies *in vivo*. In addition, by eliminating the risk of an immune response against Cas9, the cRNP method is a convenient strategy to study tumor–microenvironment interactions in an immunocompetent setting without having to resort to models with germline Cas9 expression. The ability to edit freshly isolated prostate epithelial cells after separation into luminal and basal subpopulations is particularly exciting because it provides a platform to address cancer cell-of-origin questions without having to generate new mouse strains expressing tissue- or cell-type-specific recombinases. This application is particularly compelling in light of the increased complexity of cell types in tissues revealed by single-cell analysis, evidenced recently by the identification of multiple luminal subtypes in the normal prostate (33–35).

Although canonical lineage tracing experiments remain important to shed light on cell-of-origin questions in the context of a native microenvironment, our approach allows for screening through various cell types for more rapid assessment of their tumorigenic potentials. It should be noted that this method provides insight in cell of origin only in the context of mouse tissues. Validation of human relevance is always warranted. It will also be important to extend the studies reported here in prostate and breast cells to other tissues, and our laboratory has ongoing studies in primary renal and intestinal organoid cultures.

In conclusion, the speed, convenience, and scalability of the cRNP-editing approach described here provides an opportunity to generate an expansive array of cancer models covering a



**Fig. 4.** Organoid editing by cRNP enables cancer modeling in immunocompetent hosts. (A and B) Orthotopic transplantation of sgPten+Trp53–cRNP organoids (from Fig. 2A) into syngeneic immunocompetent mice (schematic shown in A). (B) Representative images of the urogenital systems at harvest (Left) and histological analysis (Right) of a prostate tumor harvested at 15 wk posttransplantation. Dashed circles highlight the prostate tumor regions. (Scale bar, 100 μm.) (C) Survival analysis of mice with orthotopic transplants of indicated prostate organoids. \*\*\*\* $P < 0.0001$  (log-rank Mantel–Cox test). Ctrl, control sgRNAs targeting intergenic regions.



**Fig. 5.** Rapid cancer modeling with cRNP-edited freshly isolated prostate epithelial cells. (A) Workflow of prostate cancer modeling by cRNP editing of freshly isolated prostate epithelial cells followed by orthotopic transplantation. (B) Western blot showing codisruption of Pten and p53 proteins in freshly isolated prostate epithelial cells. Cells from indicated lineage origin were analyzed 7 d post-cRNP treatment. NT, nontargeting sgRNA control. PP, sgPten+Trp53. (C) Images showing prostate tumor development from mice engrafted with cRNP-edited luminal or basal cells. The dorsal prostate lobes of NSG mice were transplanted with sgPten+Trp53-cRNP-treated luminal or basal cells and harvested at indicated weeks (w) posttransplantation. The urogenital systems or dorsal lobes at harvest are displayed, with an H&E staining showing tumor histology. Dashed circles highlight the prostate tumor regions. (Scale bar, 200  $\mu$ m.) (D) Histological analysis confirming the engraftment of edited cells in host prostates. The 17-wk endpoint prostate tissues carrying the sgPten+Trp53-cRNP-treated orthografts from C were stained as indicated, with high power magnifications shown in *Inset*. The lineage origins of the grafted cells are as indicated. A normal gland from the host dorsal lobe is shown in *Top*. Arrows highlight a gland with a pAkt-positive and p53-negative staining, indicative of *Pten Trp53* double knockout. Asterisk, a pAkt/p53 double positive gland, indicative of *Pten* single knockout. (Scale bar, 200  $\mu$ m.)

broader number of complex genomic alterations linked with human cancers. These models can serve as a platform to address ongoing questions in cancer biology and to evaluate novel precision oncology medicines and immunotherapies in a genetically defined setting.

### Materials and Methods

**Constructs.** To create a lentiGuide-mCherry backbone, the sgRNA scaffold of LRG (Addgene plasmid #65656) (36) was first replaced with an enhanced scaffold (37) between *BsmBI/EcoRI* sites. The GFP sequence was then replaced with mCherry using *BamHI* and *MluI*. sgRNA sequences were cloned into either lentiGuide-mCherry (sgCtrl and sgERG) or LRG (F and S) using *BsmBI*.

**GEM Models and Organoid Lines.** Mouse prostate organoid lines *EPC* and wild type (WT) (*R26<sup>LSL-ERG/LSL-ERG</sup>;Pten<sup>fl/fl</sup>* or *Pten<sup>fl/fl</sup>*) were derived from corresponding GEM models (11). *EPC* and WT (*R26<sup>LSL-ERG/LSL-ERG</sup>;Pten<sup>fl/fl</sup>*) have been previously described (38). The WT line was derived from a C57BL/6 albino mouse at the age of 3 mo. For Cas9 expression, organoid cells were transduced with lentiCas9-blast (Addgene plasmid #52962) (39) that was 30 $\times$  concentrated with Lenti-X Concentrator (Takara Bio 631231) and bulk selected with 10  $\mu$ g/mL blasticidin for at least one additional week after all control cells died. Selection was not maintained afterward. To generate *R26<sup>LSL-ERG/+</sup>;Pten<sup>-/-</sup>* organoids, bulk *R26<sup>LSL-ERG/LSL-ERG</sup>;Pten<sup>fl/fl</sup>* populations were transduced with adeno-Cre (Vector Biolabs), sorted for GFP expression, and cultured as a pool of clones with the expected genotype. To generate

the ERG-KO control used in Fig. 1C, EPC cells were electroporated with lentiGuide-mCherry-sgERG1/2 plasmids, followed by a mCherry<sup>+</sup>GFP<sup>-</sup> sorting and a second GFP-negative sorting (ERG expression is linked with ires-GFP). In all cases, ERG and PTEN status was confirmed at the protein level by intracellular flow cytometry and/or Western blot. For ERG genotyping PCR, oligos 1 and 2 were used to detect the LSL allele (164 bp). Oligos 3 and 4 were used to detect the + allele (379 bp). *Pten* genotyping primers have been previously described (40).

Oligo 1: CATTCTGCACGCTTCAAAG

Oligo 2: GCCCAGTCATAGCCGAATAG

Oligo 3: GCGGTTGAGACAACTCTTC

Oligo 4: CTCTGTCTTAGCCAGGTGTGGCC

**Mouse Organoid Derivation and Culture.** Mouse prostates were isolated as described previously (14). Briefly, prostates were harvested and subsequently digested with collagenase type II (Gibco) for 2 h at 37 °C, followed by TrypLE (Gibco) digestion at 37 °C until a single-cell suspension was obtained. Digestions were supplemented with Y-27632 (10 μM) to inhibit anoikis. Cells were filtered before seeding for organoid culture. Organoid culture was maintained under standard conditions as described previously (14). Organoids were continuously grown in three-dimensional (3D) culture, with the exception of single-clone isolation and EPC culture, when the organoids were maintained as adherent culture. For morphology experiments, organoids were cultured in the absence of EGF before image acquisition under a brightfield microscope (ECHO). Mammary tumors from c-Myc-overexpressing mice (41) were dissected and cultured in the form of organoids as previously described (42).

**Patient-Derived Prostate Organoids.** Patient-derived organoids (MSK-Pca12) were generated and cultured as previously described (43). The tissues were deidentified prior to use. The 2 × 10<sup>5</sup> cells of MSK-Pca12 were engrafted subcutaneously into 6- to 8-wk-old CB17-SCID (severe combined immunodeficiency disease) male mice (Taconic). Mice were castrated when the tumor reached ~200 mm<sup>3</sup> and treated with ipatasertib (50 mg/kg) for 4 wk. Resistant tumors were collagenase/trypsin-digested into single cells and cultured in standard human organoid culture with ipatasertib (500 nM). AKT inhibitor ipatasertib was provided by Genentech under an MTA (material transfer agreement) and was dissolved in 0.5% methylcellulose and 0.2% Tween-80 for in vivo study. Acquisition of human tissue for prostate cancer organoids was performed under MSKCC IRB (Institutional Review Board of Memorial Sloan Kettering Cancer Center)-approved protocols 06-107 and 12-001.

**Preparation of Freshly Isolated Prostate Epithelial Cells for Gene Editing.** Prostates from six C57BL/6 mice (The Jackson Laboratory) were pooled together, dissociated into single cells, and stained for 1 h on ice with CD49f-PE (1:200; BD, 555736) and CD24-Alexa Fluor 647 (1:200; BioLegend, 101818). Sorted basal and luminal cells were centrifuged and directly used for electroporation and transplantation into four or six recipient NSG (NOD scid gamma) mice, respectively.

**Electroporation.** Nucleofection was performed using a basic kit for primary mammalian epithelial cells (Lonza, VPI-1005) or kit R (Lonza, VVCA-1001) for prostate organoids or a home-made nucleofection solution (150 mM KH<sub>2</sub>PO<sub>4</sub>, 24 mM NaHCO<sub>3</sub>, 3.7 mM glucose, 7 mM ATP (adenosine triphosphate)-disodium salt, 12 mM MgCl<sub>2</sub>, pH 7.4) for mouse mammary organoids. The 0.5~1 × 10<sup>6</sup> dissociated organoid cells or 350 k of freshly isolated basal/luminal cells were resuspended with nucleofection buffer, cRNP complexes, and electroporation enhancer (IDT 1075915, 1:1 molar ratio to cRNP) in a total volume of 100 μL. The cell suspension was transferred to a nucleofection cuvette and nucleofected using Lonza Amaxa Nucleofector II (program T-030 for prostate cells, X-005 for mammary cells). Cells were centrifuged and either seeded for culture (organoids) or transplanted into mouse prostates (primary lineage cells).

**Genome Editing and Editing Outcome Verification.** For cRNP editing, Cas9 was first mixed with sgRNA (IDT) for 20 min to form the cRNP complex before nucleofection. A total of 1.2 μM cRNP was used per individual sgRNA. Editing outcome was analyzed within a week. For virus-based editing, Cas9-expressing organoids were transduced with sgRNA-expressing lentiviruses. Fusion-specific PCR was performed to detect *Tmprss2-Erg* fusion or *Foxp1-Shq1* deletion: oligos 5 and 6 for CRISPRs T and E2, oligos 5 and 7 for CRISPRs T and E3, and oligos 8 and 9 for CRISPRs F and S. PCR products were subcloned using a TA Cloning™ kit (Thermo Fisher K200001) and confirmed by Sanger sequencing.

To detect indels from sgTrp53 and sgPten editing from mouse tissue, genomic DNA was recovered from formalin-fixed paraffin-embedded slides and PCRs were performed using oligos 10 and 11 (for sg53) or oligos 12 and 13 (for sgPten). Indel frequency estimation was performed by Sanger peak decomposition using TIDE (44).

Oligo 5: GTGGGAGTGCACAGAGATTGAG

Oligo 6: CTCAGGCTAATCCAGCCGTTCC

Oligo 7: ATTGTGCATCCACTCGAAGAG

Oligo 8: TAACGGGCTTAACAACCCCT

Oligo 9: CCAGGCCCAAACCATTGAAA

Oligo 10: CTGATCGTTACTCGGCTGTGC

Oligo 11: TTCCACCCGGATAAGATGCTG

Oligo 12: GGTGGTATGATAGAAAGGGTGG

Oligo 13: GATAGGCTTAAAGAACAACCTTAAC

sgRNA target sequences:

Ctrl (targeting intron): TCGTCGAGTTGGTCTACC

Ctrl-1 (targeting a nongenic region): TCGCGCTCGCGGGTCCACAG

Ctrl-2 (targeting a nongenic region): GTAGCGGTCTGGCTAGAACC

NT-1 (nontargeting, mouse) (39): CTTACGCTTGGACCGATA

NT-2 (mouse) (39): ACCCAGTATGTACTCGGGA

NT-1 (human) (39): ACGGAGGCTAAGCGTCGCAA

NT-2 (human) (39): CGCTCCGCGCCGTTCAA

ERG-1: TAACTCTGCGCTCGTCC

ERG-2: CTGCCGTAGTTCATCCCAA

ERG-3: TGCCGTAGTTCATCCCAA

Pten-1: ACCGCCAAATTTAACTGCAG

Pten-2: TGTGCATATTTATTCATCC

Pten-3: TCACCTGGATTACAGACCCG

Trp53: AAGTCACAGCATGACGG

AKT1-1: TGTGCCGCAAAGGCTCTCA

AKT1-2: TCACGTTGGTCCACATCTG

AKT2-1: TCTCGTCTGGAGAATCCACG

AKT2-2: CATCGAGAGGACCTTCCACG

LSL-1: TTCTAGGAATTTACCGGGT

LSL-2: GTTACTAGTGGATCCGAGCT

T (*Tmprss2* intron 1): ATCGTCGAGTTGGTCTACC

E2 (*Erg* intron 2): CACCACCATCCGATGACGA

E3 (*Erg* intron 2): ATTAGACCGACATTTACCG

F (*Foxp1* vicinity): GACCAAACCGGGTGATACC

S (*Shq1* vicinity): GTAGCGGTCTGGCTAGAACC

**Intracellular Flow Cytometry.** Intracellular flow cytometry was performed using a Fixation/Permeabilization Solution Kit (BD 554714). Dissociated organoid cells were fixed and permeabilized with the fixation/permeabilization buffer for 30 min on ice, followed by incubation with primary antibodies for at least 1 h at room temperature and secondary antibodies (for nonconjugated primary antibodies) for 30 min at room temperature with washes between the steps before analysis by flow cytometry (BD Fortessa). Primary antibodies used were ERG (1:500; Abcam ab92513), ERG-Alexa Fluor 647 (1:200; Abcam ab196149), ERG-Alexa Fluor 488 (1:200; Abcam ab196374), and Pten (1:500; Abcam ab170941). Secondary antibody was goat anti-rabbit Alexa Fluor 647 (1:1,000; Thermo Fisher Scientific).

**In Vivo Experiments and Histology.** All animal work was done in compliance with the guidelines of Research Animal Resource Center of Memorial Sloan Kettering Cancer Center (Institutional Animal Care and Use Committee):

06-07-012). For orthotopic transplantation of organoid cells, cells were transduced with Lenti-luciferase-P2A-Neo (Addgene plasmid #105621) (45) and selected with 500  $\mu\text{g}/\text{mL}$  G418. Dissociated organoid cells were resuspended in 40  $\mu\text{L}$  of 50% Matrigel (Corning) and 50% growth medium before injection into prostate dorsal lobes of syngeneic C57BL/6 albino mice (The Jackson Laboratory) ( $3 \times 10^6$  cells/injection, six mice per group). For orthotopic transplantation of freshly isolated prostate lineage cells, electroporated cells were resuspended in 20  $\mu\text{L}$  of 50% Matrigel (Corning) and 50% growth medium before injection into prostate dorsal lobes of immunodeficient NSG mice (The Jackson Laboratory) (90,000 cells/injection, four to six mice per group). Prostates were collected at autopsy, fixed using 4% paraformaldehyde, dehydrated with 70% ethanol, paraffin embedded, and sectioned on glass slides. Hematoxylin/eosin (H&E) and immunohistochemistry staining were carried out by the MSKCC Molecular Cytology Core or HistoWiz, Inc. The following antibodies were used for staining: pAkt-S473 (1:100; CST4060) and p53 (0.05  $\mu\text{g}/\text{mL}$ ; CST 2524).

**Western Blot.** Boiled whole cell lysates were run on a precast Tris-acetate (Thermo Fisher Scientific) or Mini-PROTEAN TGX protein gel (Bio-Rad) and

then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated overnight at 4  $^{\circ}\text{C}$  with primary antibodies against Cas9 (1:1,000; Active Motif 61577), Pten (1:1,000; Cell Signaling Technology 9188), ERG (1:1,000; Abcam ab92513), p53 (1:1,000; Cell Signaling Technology 2524), Apc (1:1,000; Millipore MABC202), AKT1 (1:2,000, Cell Signaling Technology 4060L), AKT2 (1:1,000, Cell Signaling Technology 5239S), pPRAS40-Thr246 (1:1,000, Cell Signaling Technology 2997S), Actin-HRP (horseradish peroxidase) (1:10,000; Abcam ab49900), Cyclophilin B (1:10000; Cell Signaling Technology 43603S) and Vinculin (1:10,000; Cell Signaling Technology 13901). Signal was visualized with secondary HRP-conjugated antibodies and chemiluminescent detection.

**Data Availability.** All study data are included in the article and/or *SI Appendix*.

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