RapidCaP, a Novel GEM Model for Metastatic Prostate Cancer Analysis and Therapy, Reveals Myc as a Driver of Pten-Mutant Metastasis

Hyejin Cho¹, Tali Herzka¹, Wu Zheng¹, Jun Qi⁴, John E. Wilkinson⁵, James E. Bradner⁴, Brian D. Robinson², Mireia Castillo-Martin³, Carlos Cordon-Cardo³, and Lloyd C. Trotman¹

Published OnlineFirst January 20, 2014; DOI: 10.1158/2159-8290.CD-13-0346
Genetically engineered mouse (GEM) models are a pillar of functional cancer research. Here, we developed RapidCaP, a GEM modeling system that uses surgical injection for viral gene delivery to the prostate. We show that in Pten deficiency, loss of p53 suffices to trigger metastasis to distant sites at greater than 50% penetrance by four months, consistent with results from human prostate cancer genome analysis. Live bioluminescence tracking showed that endogenous primary and metastatic disease responds to castration before developing lethal castration resistance. To our surprise, the resulting lesions showed no activation of Akt but activation of the Myc oncogene. Using RapidCaP, we find that Myc drives local prostate metastasis and is critical for maintenance of metastasis, as shown by using the Brd4 inhibitor JQ1. Taken together, our data suggest that a “MYC-switch” away from AKT forms a critical and druggable event in PTEN-mutant prostate cancer metastasis and castration resistance.

**SIGNIFICANCE:** The RapidCaP system introduces fast and flexible genetics for functional analysis and therapy for endogenous metastatic prostate cancer. The approach introduces targeting of MYC as a critical strategy against PTEN-deficient lethal prostate cancer.

**ABSTRACT**

Genetically engineered mouse (GEM) models are a pillar of functional cancer research. Here, we developed RapidCaP, a GEM modeling system that uses surgical injection for viral gene delivery to the prostate. We show that in Pten deficiency, loss of p53 suffices to trigger metastasis to distant sites at greater than 50% penetrance by four months, consistent with results from human prostate cancer genome analysis. Live bioluminescence tracking showed that endogenous primary and metastatic disease responds to castration before developing lethal castration resistance. To our surprise, the resulting lesions showed no activation of Akt but activation of the Myc oncogene. Using RapidCaP, we find that Myc drives local prostate metastasis and is critical for maintenance of metastasis, as shown by using the Brd4 inhibitor JQ1. Taken together, our data suggest that a “MYC-switch” away from AKT forms a critical and druggable event in PTEN-mutant prostate cancer metastasis and castration resistance.

**SIGNIFICANCE:** The RapidCaP system introduces fast and flexible genetics for functional analysis and therapy for endogenous metastatic prostate cancer. The approach introduces targeting of MYC as a critical strategy against PTEN-deficient lethal prostate cancer.
RESULTS

Stable Transgene Delivery to Epithelial Prostate Cells by Virus Injection

To overcome the limitations of germline-based GEMMs for prostate cancer, we pursued a strategy, depicted in Fig. 1A, in which transgenes are delivered by direct injection of lentivirus (LV) into an anterior prostate gland (see Methods). Infected prostate cells are designed to express oncogenic transgenes and a marker gene, luciferase, for bioluminescence imaging to allow tracking of disease progression or the results of therapy and to guide post-mortem autopsy analysis to tissues of interest.

**Figure 1.** Stable transgene delivery to epithelial prostate cells by virus injection. A, the RapidCaP system. 1, design and production of lentivirus harboring candidate cancer genes and marker genes. 2, surgical injection of virus directly into anterior prostate gland. 3, noninvasive bioluminescence imaging to monitor disease progression and therapeutic effects. Note that adenovirus is depicted for purely aesthetic reasons. B, live imaging of LV-Luci-injected Bl6 mice and noninjected control 60 days after injection suggests persistent transgene expression. C, post-mortem autopsy imaging of LV-Luci and noninjected control mice 98 days after injection. The genitourinary (GU) tract comprises bladder (B), seminal vesicle (SV), and anterior prostate (AP). The luminescence signal is found in the (injected) right anterior prostate lobe and also in the seminal vesicle. Note that fluorescence-activated cell sorting (FACS) experiments fail to identify infected (tomato-FP) seminal vesicle cells, potentially suggesting that the seminal vesicle signal is extracellular. PCR of prostate genomic DNA reveals the luciferase transgene (300 bp) only in the LV-Luci-injected and noninjected control mice 98 days after injection. The genitourinary (GU) tract comprises bladder (B), seminal vesicle (SV), and anterior prostate (AP). D, anti-luciferase immunofluorescence on LV-Luci-injected and noninjected control prostate reveals luciferase expression in prostate luminal epithelium. Scale bar, 47 μm. E, hematoxylin and eosin (H&E) staining of LV-Luci-injected and noninjected control prostate reveals retention of normal glandular architecture. Scale bar, 100 μm.
As shown, this approach allowed successful monitoring of mice with injected prostate by live imaging (Fig. 1B), and post-mortem analysis (Fig. 1C) 60 days after injection revealed a luciferase signal only in the injected anterior prostate and adjacent seminal vesicle (see below for the discussion of the seminal vesicle signal; Fig. 2A). PCR analysis revealed the presence of the luciferase transgene in the animal with injected anterior prostate (Fig. 1C, bottom right), whereas immunofluorescence-based histology using anti-luciferase antibodies revealed expression of luciferase in the prostatic epithelium. Although infection of nonepithelial cells can by no means be excluded, the epithelial immunofluorescence signal typically clearly dominated over the stromal signal (Supplementary Fig. S1A; see also Fig. 1D). On the basis of the fluorescence-activated cell sorting (FACS) analysis with fluorescent marker transgenes, our technique infects some 0.3% of the approximately 100 million anterior prostate cells (not shown). Histologic comparison of injected and noninjected glands revealed no morphologic alterations in the injected glands, and immunohistochemistry (IHC) analysis of the PTEN pathway and the Ki-67 proliferation marker did not reveal any anomalies (Fig. 1E and Supplementary Fig. S1B). Successfully injected/injected prostates stained negative for the CD3 T-cell marker, and no signs of inflammatory responses were observed (Supplementary Fig. S1C, top and middle). These results demonstrated that viral transgene delivery and stable integration into genomic DNA in the anterior prostate epithelium is feasible with our technique.

**Prostate-Specific Delivery of Cre Recombinase Results in Focal Pten/Trp53-Deficient Disease**

Analysis of mouse models of prostate cancer revealed that loss of Trp53 function is a critical step for disease progression in Pten pathway–mutant animals (21, 22). Thus, we next injected lentiviral Cre-luciferase double-transgenic virus (LV-Cre/Luci) into the prostates of Pten\textsuperscript{loxp/loxp}, Trp53\textsuperscript{loxp/loxp} mice. As shown in Fig. 2A, LV-Cre/Luci–injected mice typically showed a strong luciferase signal in the genitourinary region. Post-mortem analysis (69 days after injection) revealed that the injected anterior prostate and associated seminal vesicle are the luminescence source—discussed below. In contrast, 62% of lentiviral luciferase (LV-Luci) virus–injected mice demonstrated loss of signal within 78 days (not shown). PCR analysis of genomic DNA from anterior prostate revealed recombination of the tumor suppressors, Pten and Trp53, specifically in the LV-Cre/Luci–injected right anterior prostate (Fig. 2B, ΔPten, ΔTrp53). Strikingly, anterior prostate IHC analysis (see Fig. 2C) revealed the highly focal nature of disease initiation in this model; only a small region within one gland showed increased proliferation (Ki-67), loss of Pten, and strong Akt activation, whereas the vast majority of glands in the prostate retained completely normal morphology and staining for these proteins (Fig. 2C, right, pAkt, Pten). Note that increased proliferation and cell expansion were not seen in stromal cells. Indeed, after histopathologic analysis of more than 60 prostate-injected cases, the only cell type in which we observed expansion was epithelial prostate cells. Furthermore, we again observed no immunogenicity of the viral injection procedure as shown by CD3 staining (see Supplementary Fig. S1C). These data demonstrated that the RapidCaP system triggered focal disease initiation, which is thought to be a feature of the human disease-initiation process. Note that, in contrast, the probasin-driven Cre conditional knockout (cKO) systems of Pten and Trp53 or Smad4 delete their target genes in every cell of the prostate epithelium, which results in massive expansion of prostate size to several centimeters in diameter (22, 23) and is accompanied by a nonepithelial sarcoma phenotype (19).

**Deletion of Pten and Trp53 Triggers Disease Dissemination**

Analysis of the human prostate cancer genome has revealed that Pten and Trp53 are codeleted in half of all metastatic samples (3, 21). In mice, however, the complete Pten and Trp53 deletion in the prostate resulted in massive and lethal tumor growth (22, 23). To investigate disease progression in the above RapidCaP system (LV-Cre/Luci injection into Pten\textsuperscript{loxp/loxp}, Trp53\textsuperscript{loxp/loxp} prostate), mice were imaged for a period of several months. As shown in Fig. 3A, the luminescence signal was initially observed in the lower abdominal region (up to 25 days after injection). Later, however, strong signal spread was detected in the upper body and midbody (72–140 days after injection). Typically, the signal first disseminated to the neck and later to the mid-abdomen, as shown. Quantification of whole-body, primary and secondary signals (Fig. 3B) revealed how they both increased over time. Most importantly, no spreading of the luminescence signal was observed in any of the control injection cohorts (see Fig. 3C). As mentioned above, 62%, or 18 of 29 non–gene-modifying control-injected mice, instead showed a decrease of the primary luciferase signal over time (see Supplementary Fig. S2 for typical examples of a RapidCaP time course). Overall (see Fig. 3C), 50% of animals showed disease dissemination 4 months after injection. In post-mortem analysis, secondary signals were confirmed in lymph nodes (mediastinal, lumbar, and caudal), spleen, liver (shown in Fig. 3D), pancreas, and lung. Organs near the genitourinary tract (excluding bladder) were also often signal-positive. Importantly, LV-Cre/Luci–injected prostate was macroscopically indistinguishable from noninjected prostate (Fig. 3D, ni vs. inj), revealing a stark contrast to the probasin-Cre–driven Pten-Trp53 cKO model. To date, after analysis of more than 300 injected animals, those without gene alteration never showed luciferase signal spread in post-mortem analysis. Note that the seminal vesicle on the side of the injected prostate was invariably luminescence-positive from the day of injection, also in luciferase-only control injections. This argues against positive biologic selection, and in agreement, we never found phenotypic changes by IHC analysis of seminal vesicles (not shown). In fact, seminal vesicles have never shown any phenotypes in the many whole-body knockout models driven by heterozygosity of Pten (11, 15, 21). Recombination of Pten and Trp53 in the signal-positive secondary organ sites was confirmed by PCR (Fig. 3E). Histopathologic examination of prostates revealed focal invasion as shown by the frequent focal budding of epithelial cells (Fig. 3F).

**Histologic Analysis Confirms Metastatic Prostate Cancer in RapidCaP**

To investigate whether the signal dissemination in RapidCaP represents prostate metastasis, histopathologic...
**Figure 2.** Prostate-specific LV-Cre/Luci delivery results in focal disease. **A,** live and post-mortem images of LV-Cre/Luci–injected $Pten^{loxP/loxP};Trp53^{loxP/loxP}$ mouse 69 days after injection. Dissection reveals similar distribution to that shown in Fig. 1C. The luminescence signal was seen in LV-Cre/Luci–injected prostate and seminal vesicle. **B,** PCR analysis confirms recombination of $Pten$ and $Trp53$ in LV-Cre/Luci–injected $Pten^{loxP/loxP};Trp53^{loxP/loxP}$ specifically in the injected right (Rt) anterior prostate (AP). **C,** IHC analysis of LV-Cre/Luci–injected $Pten^{loxP/loxP};Trp53^{loxP/loxP}$ prostate reveals a focal lesion in the luminal epithelium of one gland (H&E), which also displays increased proliferation specific to epithelial cells (Ki-67). Loss of $Pten$ expression, activation of Akt (pAkt), and increased proliferation are also found in this region (middle), which is a unique focus of disease in the cross-section of the entire anterior prostate (right). Note that surrounding stroma remains unaffected in all panels. Scale bars, 100 μm (top right) and 50 μm (all other panels). GU, genitourinary.
Figure 3. Pten/Trp53 deletion triggers disease dissemination. A, time course of a typical RapidCaP experiment (showing one LV-Cre/Luci–injected PtenloxP/loxP;Trp53loxP/loxP mouse) reveals disease spreading to distant sites starting at 72 days after injection. B, quantification of signal intensity from mouse shown in A. Total body, secondary, and primary signal measurements are shown. C, Kaplan–Meier analysis of disease dissemination reveals early onset and high penetrance, specifically in PtenloxP/loxP;Trp53loxP/loxP mice (P < 0.0001). D, live (219 days after injection) and post-mortem autopsy of an LV-Cre/Luci–injected PtenloxP/loxP;Trp53loxP/loxP mouse reveals luciferase-positive secondary organ sites (lymph nodes [LN], spleen, and liver) and a minor difference in size of prostate lobes (injected, inj. vs. noninjected, ni). Scale bar, 1 cm. E, PCR analysis reveals Pten/Trp53 recombination in the secondary tissues (weakest in kidney). Scale bar, 1 cm. F, hematoxylin and eosin (H&E) staining of an LV-Cre/Luci–injected PtenloxP/loxP;Trp53loxP/loxP prostate lesion (192 days after injection) reveals a typical budding gland with features of focal invasion. Scale bar, 50 μm.

analysis was performed on the animal shown in Fig. 4A. First, we defined markers for mutant cancer cells by analyzing the prostate. As expected, focal regions in the prostate lost Pten expression (Fig. 4B). In addition, the epithelial markers cytokeratin-8 (Ck8) and androgen receptor (Ar) were absent specifically in regions where Pten (and p53) was no longer expressed (Fig. 4B, arrows), which is in full agreement with previous results (24), and in contrast to normal prostate (Supplementary Fig. S3C, left). On inspecting the lungs of this animal (Fig. 4C and D), we found many small
Figure 4. Histogenic analysis confirms prostate cancer metastasis to lung. A, autopsy imaging of the mock-castrated RapidCaP mouse (#3) reveals disease dissemination to lung, lymph nodes, spleen, and liver at the trial endpoint (10 months after injection). B, identification of histogenic markers that define prostate cancer cells by IHC analysis of prostate in animal from A: Pten and Ck8 (black arrows; see black arrowheads for region with normal protein levels) and Pten and AR (red arrows; see red arrowheads for the region with normal protein levels). Note that “loss” of AR staining is a hallmark of Pten/p53-null prostate (see the text). Scale bar, 100 μm. C and D, IHC analysis of lung from above mouse reveals metastatic nodules that are Pten-, Ck8-, and AR-negative and positive for the prostate epithelial marker Nkx3.1. Boxes, areas of magnification. Scale bars, 100 μm (top and bottom left) and 50 μm (all other panels). E, double immunofluorescence (IF) labeling confirms double negativity for Pten and Ck8 in a metastatic lung nodule (nod) shown in C and D (yellow circles and arrowheads). Note that, in contrast, colabeling of Pten and Ck8 in adjacent normal lung epithelia (nl, white dashed circles and arrowheads) shows double-positive staining. Scale bar, 50 μm. F, anti-luciferase antibody staining and immunofluorescence analysis of lung from C to F confirms that metastatic nodules are luciferase-positive (white arrows) and Pten/Ck8-deficient (red arrowheads). Scale bar, 100 μm.
tumor nodules, some of which demonstrated tumors within intravascular spaces and others within lung parenchyma. The nodules were composed of proliferating atypical cells with large, pleomorphic, hyperchromatic nuclei and scant-to-moderate amounts of amphophilic cytoplasm. These cells were immunohistochemically characterized by loss of Pten as well as loss of Ck8 and Ar, thus matching the features of the mutant prostate epithelial cells. The metastatic nodules showed focal, faint positivity for the prostate differentiation marker Nkx3.1, further supporting prostatic origin. The loss of the low-molecular-weight Ck8 was particularly informative because the normal alveolar and bronchiolar lung epithelium displayed high expression of this cytokeratin (see also Supplementary Fig. S3C, right), whereas the metastatic nodules stood out as negative mutant epithelial islands. To confirm that the metastatic cells were truly double-negative for Pten and Ck8, double immunofluorescence labeling was performed on lungs sections. Indeed (see Fig. 4E), metastatic nodules in the lung (nod) were negative for both Pten and Ck8 (yellow circles and arrowheads), whereas normal bronchiolar epithelium (nl) showed high expression of both proteins (white, dashed circles and arrowheads). Next, we used anti-luciferase immunofluorescence to further study the metastatic nodules. Only injected prostate was stained, thus establishing our positive control for the procedure (Supplementary Fig. S3A). As shown in Supplementary Fig. S3B, lungs with metastatic nodules [hematoxylin and eosin (H&E)] showed readily detectable luciferin-positive cells and nodules (see Luci). These sites corresponded to tumor sites with loss of Pten and Ck8 staining, as shown in Fig. 4F. Importantly, Ki-67 IHC analysis revealed that metastatic nodules contained cells with a high proliferation rate (Supplementary Fig. S3D). We next confirmed that levels of the androgen-driven Nkx3.1 protein were high in prostate, the lung metastatic nodules, but markedly reduced in the castration-resistant prostate cancer (CRPC; Supplementary Fig. S3E), thus inversely correlating with the expression levels of Myc, as published previously (25). Additional cases of lung metastasis confirming the immunohistological correlations are shown in Supplementary Fig. S4. Taken together, our results are consistent with extensive prostate cancer metastasis to lung in RapidCaP.

**Pten/Trp53–Deficient RapidCaP Metastasis Responds to Castration**

Androgen ablation therapy is the standard of care for advanced prostate cancer, causing widespread atrophy of prostate cells and the derived metastases. Surgical castration was conducted on mice with secondary signals, and bioluminescence imaging was continued once per week on a total of 16 animals as summarized in Supplementary Fig. S5A–SSC: 7 castrated Pten/Trp53–mutant RapidCaP animals, 7 mock-castrated Pten/Trp53–mutant RapidCaP animals, and 2 castrated LV-Luci prostate–injected normal controls. Figure 5 illustrates the typical results obtained from a group of 5 trials (Fig. 5A and B), consisting of 2 castrated animals (Fig. 5A, cast-1, cast-2; Fig. 5B, #4, #5), 2 noncastrated controls (Fig. 5B, #2, #3), one of which underwent a mock castration surgery (#3), and a nontreated wild-type (wt) mouse (#1). The castrated animals showed partial (cast-1) or complete (cast-2) therapy response within 7 weeks after castration and, importantly, both the primary and secondary signals regressed (Fig. 5A, 7 weeks; see also Supplementary Fig. S5A, graph). However, regression was followed by aggressive relapse of both primary and secondary signals, now growing much more rapidly than before castration (see Fig. 5A, 7–27 weeks; Supplementary Fig. S5A), a hallmark of human castration resistance. In contrast (see Fig. 5B and Supplementary Fig. S5B), both control animals showed steadily increasing signal intensity. Quantification (Fig. 5C and Supplementary Fig. S5C) confirmed this sharp increase in intensity for both primary and secondary disease. This resulted in a typical “hockey stick” growth behavior (compare precastration with postcastration growth) just before these animals needed to be sacrificed to prevent excessive tumor burden. Clear signs of morbidity, such as palpable tumors, low activity, hunched backs, and a rough hair coat, were observed in these animals. Figure 5D shows the quantitative analysis of regression and relapse and breakdown of the data into primary and secondary disease. This analysis revealed the kinetics of how prostate and metastatic signals in both animals responded to castration. Post-mortem analysis of animal #4, from Fig. 5B, illustrates the typical metastasis to organs including lymph nodes, liver, spleen, and pancreas (some of which were palpable). The prostate itself was massively enlarged, spanning close to 3 cm in diameter, in stark contrast to the 5- to 7-mm diameter of noncastrated Pten/Trp53–deficient RapidCaP prostate (Fig. 6A and B, bottom; see also Supplementary Fig. S6, slide overviews).

Taken together, the castration experiments confirmed that RapidCaP mice developed prostate metastatic disease, which initially responds to androgen deprivation and eventually relapses to develop CRPC. It is likely that residual disease after castration, as shown in the Cast-1 animal (Fig. 5A, Cast-1, 7 week), is due to preexisting AR-low/negative cells such as the ones shown in Fig. 4B and D, which could expand upon castration to form AR-negative CRPC tumors (see Fig. 6C). To study the molecular makeup of castration-resistant disease, we turned to IHC analysis.

**Myc Expression Can Drive Metastasis in the Absence of Akt Activation**

IHC analysis of the relapsed prostate tumor (see Fig. 6C and Supplementary Fig. S6) confirmed the consistent emergence of anaplastic cells with loss/background levels of Pten, Ar, and Ck8, consistent with mutant prostate of noncastrated mice (shown in Fig. 4B–F). However, Akt was not activated despite Pten loss (Fig. 6C and Supplementary Fig. S6C, right). We then analyzed components of the phosphoinositide 3-kinase (PI3K) pathway to understand whether feedback mechanisms (26, 27) or parallel Pdk1 kinase activation (28) was suppressing pAkt. As shown (Fig. 6D), Pdk1 was not phosphorylated in the castration-resistant tumor, in which pAkt levels were low. Furthermore, we found even lower ribosomal protein S6 phosphorylation after castration than in wt prostate (see also Supplementary Fig. S7C for IHC analysis of phospho-S6). These results suggested that PI3K signaling is low in metastasis and after castration. Instead, strong Myc staining was seen in both nuclei and cytoplasm throughout the tumor mass (Fig. 6C and Supplementary Fig. S6, Myc overview). Note that the mock-castrated Pten/Trp53–deficient RapidCaP prostate showed weak or absent
Figure 5. Metastatic signals respond to castration. A, live imaging time course of two castrated RapidCaP mice (LV-Cre/Luci–injected Pten<sup>loxP/loxP</sup>; Trp53<sup>loxP/loxP</sup> mouse). Surgical castration was performed 5 months after injection on mice harboring distant secondary disease (see Cast-1). Imaging analysis reveals differential response and recurrence of disease after castration. B, three-dimensional plot of signal intensity time course from A, including control mice 1, wt untreated mouse; 2, RapidCaP mouse; 3, mock-castrated RapidCaP mouse; 4 and 5, castrated RapidCaP mice. C, quantification of luminescence signals in castrated animals reveals a sharp increase in the rate of disease progression after relapse. "C" denotes castration. D, close-up analysis of graphs from C shows that both primary and secondary disease respond to castration, confirming their hormone dependence.
**Figure 6.** Analysis of CRPC. 

**A,** post-mortem analysis of Cast-1 animal after relapse (see Fig. 4A) shows metastasis to distant organs and massive prostate enlargement.

**B,** castration results in prostate epithelium atrophy and diffuse cytoplasmic AR staining (top). Note that Pten/Trp53 loss in RapidCap does not cause a significant increase in prostate size (bottom). Scale bar, 100 μm. 

**C,** IHC analysis of Cast-1 prostate shows loss of Akt activation, lost/low cytokeratin epithelial and basal cell markers Ck8 and Ck5, respectively. In contrast, there is strong activation of Myc in tumor cells in the therapy-resistant prostate tumor. Scale bars, 1 mm (Myc overview) and 50 μm (all other panels).

**D,** Western blot analysis of PI3K reveals no feedback (pS6) or parallel pathway (pPdk1) activation of castration-resistant tumor shown in C. 

**E,** a strong increase in Myc mRNA expression is detected in the castration-resistant tumor from C. Error bars, SD. ***, P = 0.0001. 

**F,** FISH analysis reveals Myc gene amplification in disease progression from prostate to lung metastasis of the same animal. Myc gene amplification is also observed in a CRPC. The percentage of nuclei with >2 copies is indicated; scale bar, 5 μm. LN, lymph nodes; qPCR, quantitative PCR; Lt, left.
and exclusively nuclear Myc (Supplementary Fig. S3F), similar to normal prostate (not shown). Myc RNA expression sharply increased in castration-resistant cancer (Fig. 6E), and by using FISH analysis (Fig. 6F), we found that the Myc gene was amplified in 32% of CRPC nuclei. We also observed Myc amplification in metastasis (21% frequency), whereas the corresponding primary prostate showed no gene amplification. These data suggested that Myc and not phospho-Akt (pAkt) may be driving metastasis and castration-resistant cancer, in contrast to disease initiation, where we observed strong Akt activation (see pAkt; Fig. 2C). We frequently saw mixed prostate glands with pAkt high and low staining regions (Supplementary Fig. S7A), which also showed low pS6 and Ck8 staining but were positive for Myc and AR. These findings suggest that the low pAkt phenotype first emerges in the prostate and is selected for during metastasis and castration resistance, along with partial amplification of the gene and strongly increased transcription.

Next, we made full use of the RapidCaP system for the discovery and validation of a metastasis driver gene. First, we studied Akt and Myc status in prostate metastasis to lung. As shown (Fig. 7A), metastasis to lung in the mock-castrated animal showed absence of Akt activation but high Myc levels in cytoplasm and nuclei of all metastatic nodules. This situation was comparable to the IHC staining observed in the castrated animal prostate (Fig. 6C), but very different from the mock-castrated animal’s own prostate (see Supplementary Fig. S3F). Therefore, our results suggested that increased and/or mislocalized Myc may contribute specifically to the metastatic process (see Discussion). To test this hypothesis, we generated a cohort of Myc-driven RapidCaP mice.

A retroviral construct harboring Myc and luciferase was used (see Supplementary Fig. S7B and Methods) and validated in vitro before injecting into mice (not shown). Two animal genotypes were selected for injection: Pten^{loxP/loxP} (“Pten hyper”) mice with 25% reduced Pten levels (11), to test whether Myc cooperates with activation of Akt in driving metastasis, and WT mice, to test whether Myc can drive metastasis on its own. As shown in Fig. 7B (top), Pten^{loxP/loxP} mice with Myc-luciferase retrovirus (RV-Myc-Luci)-injected prostates retained their luminescence signals until they were sacrificed for analysis, and so did the WT mice (not shown). There were, however, no signals detected in live imaging beyond the lower abdominal region in both genotypes, suggesting that no strong dissemination to distant organs had occurred. Typical post-mortem imaging (Fig. 7C and Supplementary Fig. S7C, 54 days after injection) revealed luciferase positivity of the injected (right) anterior prostate lobes in both genotypes (yellow arrows), and Myc overexpression was confirmed by quantitative reverse transcription PCR (qRT-PCR; Supplementary Fig. S7D). Importantly, post-mortem visualization revealed that the majority of Myc-virus–injected animals had secondary signals (green arrows). These were limited to organs close to prostate, such as local lymph nodes and epididymal fat pads, confirming the lack of distant dissemination observed in live imaging. Statistical cohort analysis (Fig. 7B, bottom) showed significant frequencies of local disease dissemination in both the Pten-mutant and WT mice, even if a significant increase in local metastasis was observed in the mutant cohort. Thus, Myc overexpression suffices to induce local metastasis in normal prostate, but the process can be achieved through even only modest suppression of Pten.

Finally, to probe whether Myc was needed to drive growth or maintenance of the metastatic cells, we used the Myc-suppressing Brd4 inhibitor JQ1 (29–31) on mice with established Pten/Trp53-deleted metastases. We confirmed that JQ1 administration (daily at 50 mg/kg) suppressed Myc transcription in prostate tissues already after 4 days (Supplementary Fig. S8). As shown (Fig. 7D), JQ1 inhibited growth of metastatic disease within 17 days and had little effect on primary disease signal (quantified in Fig. 7E). Taken together, our data suggest that the spontaneously increased Myc expression observed in metastasis or castration resistance is a key driver and a potential drug target against Pten-mutant metastasis.

**DISCUSSION**

Virus-based in vitro gene transfer has been used to study various cancer types (32, 33). The RapidCaP system presents us with new technology for the exploration of metastatic prostate cancer and its therapy. This approach has delivered two unexpected results: First, we showed that deletion of the Pten and Trp53 tumor suppressors suffices to trigger metastatic prostate cancer at high penetrance within a few months. Second, Pten/Trp53-deficient metastasis shows no Akt activation, but rather Myc activation instead.

The first result validates our previous analysis of mouse genetics and human prostate cancer genomes, where we found that p53 is a gatekeeper for metastatic progression of PTEN-mutant prostate cancer (21). The result is corroborated by our finding that p53 is haplosufficient for preventing distant metastasis, as Pten^{loxP/loxP}+/--; Trp53^{−/+}; RapidCaP mice do not present metastases (not shown). These findings are in line with a critical role of the p53-mediated senescence response after Pten loss (22, 34, 35) and its potential in preventing metastatic prostate cancer (36). These results could not readily be obtained with the classic Pten-mutant GEMMs where metastatic disease is not, or only rarely, observed, as primary prostate cancers grow to lethal size (22, 23). In patients, this genetic setting occurs in more than half of the recently analyzed metastatic cancer genomes (3, 37, 38).

The second result demonstrates the utility of the RapidCaP approach, which allows for identification and fast validation of metastasis mechanisms. The loss of Akt activation in Pten-deficient metastatic disease and castration-resistant tumors is unexpected. This could suggest that these tissues no longer respond to PI3K pathway inhibition, despite Pten loss, a hypothesis that can be tested using RapidCaP. The metastatic Pten/Trp53-mutant disease shows clearly less pAkt signal than the primary disease-initiating counterpart (compare Fig. 6C, right, and Fig. 2C, right). Although the mechanism of pAkt suppression remains to be determined, it is apparent that Myc is overexpressed in this setting, suggesting the existence of a Myc switch in Pten-mutant metastatic disease progression. Intriguingly, in this setting, Myc is also strongly expressed in the cytoplasm, in contrast to the nuclear, weak Myc expression in normal prostate. Recently, a cytoplasmic cleavage product of Myc, termed Myc-Nick, has been shown to control muscle cell differentiation (39). Thus, it will be interesting to see if Myc-Nick is present in the metastatic or castration-resistant lesion.
Figure 7. Myc succeeds Akt activation and is required for growth of metastatic prostate cancer. 

**A.** IHC analysis of pAkt (Ser473) in metastatic lung nodules of LV-Cre/Luci-injected Pten<sup>loxP/loxP</sup>;Trp53<sup>loxP/loxP</sup> mice reveals absence of pAkt activation. In contrast, Myc levels are strongly increased, especially in the cytoplasm (compared with prostate; Supplementary Fig. S3F). Note the apparent further Myc increase in castration-resistant disease (Fig. 6C) compared with this mock-castrated metastasis. Scale bar, 50 μm.

**B.** Top, typical live imaging of RV-Myc-Luci-injected Pten<sup>hy/+</sup> mice shows lower abdominal signals and absence of distant metastasis (in contrast to LV-Cre/Luci-injected Pten<sup>loxP/loxP</sup>;Trp53<sup>loxP/loxP</sup> mice; Fig. 3A). Note that Pten<sup>hy/+</sup> mice express only 75% Pten throughout their body. Bottom, Kaplan-Meier analysis of disease spread in indicated RapidCaP models reveals that Myc expression can drive local dissemination of prostate cancer and cooperate with Pten suppression. Log-rank (Mantel-Cox) testing shows that wt, Pten<sup>hy/+</sup>, as well as negative control study arms, are significantly different (P = 0.004).

**C.** Autopsy analysis of RV-Myc-Luci-injected mice reveals local, mostly lower abdominal disease dissemination from prostate (yellow arrows) to secondary sites (green arrows), including local lymph nodes. Also see Supplementary Fig. S7C.

**D.** LV-Cre/Luci, Pten<sup>loxP/loxP</sup>;Trp53<sup>loxP/loxP</sup> mice show lower abdominal signals and absence of distant metastasis (in contrast to LV-Cre/Luci-injected Pten<sup>loxP/loxP</sup>;Trp53<sup>loxP/loxP</sup> mice; Fig. 3A). Note that Pten<sup>hy/+</sup> mice express only 75% Pten throughout their body. Bottom, Kaplan-Meier analysis of disease spread in indicated RapidCaP models reveals that Myc expression can drive local dissemination of prostate cancer and cooperate with Pten suppression. Log-rank (Mantel-Cox) testing shows that wt, Pten<sup>hy/+</sup>, as well as negative control study arms, are significantly different (P = 0.004). C, autops analysis of RV-Myc-Luci-injected mice reveals local, mostly lower abdominal disease dissemination from prostate (yellow arrows) to secondary sites (green arrows), including local lymph nodes. Also see Supplementary Fig. S7C. D, treatment of metastasis in LV-Cre/Luci-injected Pten<sup>loxP/loxP</sup>;Trp53<sup>loxP/loxP</sup> mice using the Myc-antagonizing Brd4 inhibitor JQ1 [at 50 mg/kg/d] reveals regression of metastatic disease compared with the disease progression in DMSO-treated mice. E, quantification of disease regression from D shows significant effect on metastasis (*, P = 0.026) but not primary disease. Error bars, SD. N.S., not significant; DMSO, dimethyl sulfoxide.
The MYC gene is frequently amplified, especially in metastatic human prostate cancer, as part of the signature chromosome 8p amplification (3) and has previously been functionally validated as a driver of prostate cancer (14, 40). It is known to cooperate with PI3K pathway activation in mouse models of mainly primary disease (41, 42; reviewed in ref. 40). The nonepithelial (CK8-deficient) histology of Pten/Trp53-mutant metastasis remains to be validated in PTEN/TP53-mutant human prostate cancer, which represents between 54% and 65% of metastatic prostate cancer cases (3, 37, 38). The RapidCap system, however, revealed a specific role for Myc as a druggable driver of Pten-mutant metastasis. These results suggest that a quantitative and qualitative change in Myc expression succeeds Akt activation in driving prostate metastasis. Furthermore, we have seen that Pten/Trp53-mutant metastasis is still androgen-dependent, and that disease relapse is characterized by the absence of pAkt and high Myc levels. It remains to be determined whether and how Myc function suppresses Akt activity and how these two cancer pathways can best be targeted coordinately to prevent disease relapse.

Thanks to its versatility, accelerated time frame, and amenability to genome sequencing, the RapidCap system is very well positioned to explore these questions, and thus may help end death and suffering from today’s incurable prostate cancer.

METHODS

Mice

Pten+/loxP, Trp53+/loxP, Pten+/+ transgenic and C57BL/6 or 129SV/ C57BL/6 wild-type mice were used in this study. All protocols for mouse experiments were in accordance with the institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC). Pten+/loxP, Trp53+/loxP transgenic mice were generated by crossing Pten+/loxP with Trp53+/loxP (43). For genotyping, tail DNA was subjected to PCR analysis with the following primers. For Pten+/loxP, primer 1 (5′-TGTTTTGACCAATTAAAGTAGGCTGTG-3′) and primer 2 (5′-AAAAAGTTCCCTGGTGATTTTGGT-3′) were used. For Trp53+/loxP, primer 1 (5′-CAGAAAAAGGGTTAAAAACCGAG-3′) and primer 2 (5′-AGGCCATAGGAGCCAGAGA-3′) were used.

Virul Constructs

Lentiviral constructs—the Luc.Cre lentiviral plasmid (Tyler Jacks, Addgene plasmid 20985), pMD2.G, and psPAX2—were purchased. The luciferase lentiviral plasmid was generated by eliminating CreNLS from the above construct. The retroviral Myc-luciferase construct was a kind gift from Dr. Scott W. Lowe (Memorial Sloan-Kettering Cancer Center).

Retrovirus Productions and Infections

Retrovirus was produced by calcium phosphate transfection; 6 × 10^9 phenoix cells were plated in 10-cm plates 6 to 12 hours before transfection with 15 μg of target construct and 5 μg of ecdyotropic helper plasmid. Fresh media was added 12 hours after transfection and viral supernatant was collected four times, 24, 36, 48, and 60 hours after transfection. Viral supernatant was filtered through a 0.45-μm filter, then concentrated by ultracentrifugation (2 hours at 20,000 × g). Before the viral injection to prostate, an in vitro infection test for each batch of viruses was conducted.

Lentivirus Productions, Infections, and Injections

Lentiviruses were produced by calcium phosphate transfection. 293FT cells were plated for transfection density 8 × 10^5 cells per 10-cm plate. Target plasmid (10 μg) was combined with helper constructs, 8.5 μg of pMD2.G and 3.5 μg of psPAX2, for transfection. For retroviruses, ecdyotropic phoenix cells were plated in 10-cm dishes 6 to 12 hours before transfection with 15 μg of target construct and 5 μg of ecdyotropic helper plasmid. Both viruses were harvested 24, 36, 48, and 60 hours after transfection and centrifuged (4,500 rpm, 15 minutes) before filtering through 0.45-μm pore cellulose acetate filters. Viral supernatant was concentrated by ultracentrifugation (2 hours at 20,000 × g), then an in vitro infection test for each batch of viruses was conducted in advance.

Cell Lines

293FT and ecdyotropic Phoenix cells were a kind gift from Dr. Scott W. Lowe. They were authenticated for propagation of lentivirus and retrovirus, respectively.

Intraprostate Injection

After exposure to anesthesia (isoflurane, 2%), the lower half of the abdomen was shaved and the mouse was placed in a surgery hood. The mouse was constantly exposed to isoflurane via a nose cone for the entire duration of the 10-minute surgery. The shaved region was cleaned with betadine, followed by sterile PBS three times. A 0.5-inch incision in both the skin and peritoneum was made along the lower abdominal midline to allow the right anterior prostate to be positioned for injection on a sterile support. Typically, 30 μL of concentrated virus was injected into the right anterior prostate. The incision was then sutured and the skin was stapled shut using two to three stainless steel EZ Clip wound closures. After animals were observed for complete recovery from anesthesia, they were warmed under a heating lamp to regain the ability to maintain sternal recumbence and given DietGel.

Surgical Castration

An anesthetized and surgically prepared animal was placed in dorsal recumbency. Both testes were then pushed down into the scrotal sacs by gently applying pressure to the abdomen. A 1- to 2-cm ventral midline incision was made in the scrotum and the skin was retracted to expose the tunica. The tunica was pierced and the testes were pushed out one at a time. The testes were then raised to expose the underlying blood vessels and tubules. The fat pad, which adheres to testis, was then grasped with blunt forceps to locate the vas deferens with the prominent blood vessel running along it. The testis was dissected away from the fat pad and removed. The fat pad was then pushed back into the scrotal sac. All deferential vessels and ducts were replaced back into the tunica. Skin incisions were closed with stainless steel wound closures and removed 7 to 10 days after operation.

Bioluminescence Imaging

In vitro, in vivo, and ex vivo bioluminescence imaging was performed using an Xenogen IVIS Spectrum imager, which uses a highly sensitive, cooled CCD camera mounted in a light-tight camera box. For in vitro imaging, mouse embryonic fibroblasts (MEF) (1 × 10^3 cells/well) were seeded in a 12-well plate and infected with serial dilutions of virus. Twenty-four hours after infection, the medium was replaced with fresh medium and infection efficiency was measured by bioluminescence imaging 5 days after infection. For in vitro imaging, luciferin (oraluciferin, potassium salt; Gold Biotechnology) was added to each well at 150 mg/mL of final concentration in PBS and photons were collected for 3 minutes. For in vivo imaging, animals received intraperitoneal injections of 200 μg/kg luciferin 5 minutes before imaging. The animals were then anesthetized using 2% isoflurane and placed onto the warmed stage inside the camera box. The animals received continuous exposure to 2% isoflurane to sustain sedation for 3 minutes of imaging. For quantification, regions of interest were measured with standardized rectangular regions covering the mouse trunk and extremities.
The measured signal was quantified as photons per second (ph/s) using the Living Image software v.4.2 (Xenogen). Background bioluminescence in im was in the range of 3 to 6 × 10^4 ph/s. For corrected intracellular redox reactions, animals were intraperitoneally injected with 50 mg/kg of carrier containing 5% DMSO. At the indicated time points, mice were intraperitoneally injected with freshly diluted JQ1 (50 mg/kg) or a 5% DMSO saline solution. Mice were sacrificed and their entire bodies were administered to the analysis of the indicated control reverse transcription reaction.

**Histology and IHC**

Tissues were fixed in 10% buffered formalin for 24 hours, followed by gentle wash and transfer to PBS. Tissues were fixed in 10% buffered formalin for 24 hours, followed by gentle wash and transfer to PBS. Then, paraffin-embedded tissues were sectioned 6 µm thick, placed on charged glass slides, and stained with H&E, or the appropriate immunohistochemical stain. Antigen retrieval was performed by incubating the slides in 0.01 mol/L citric acid buffer (pH 6.0) at 95°C for 15 minutes. Slides were then allowed to cool to room temperature for 20 minutes in a citric acid buffer. After the slides were washed with deionized water, they were transferred to PBS (pH 7.4) for 5 minutes. The following detection and visualization procedures were performed according to the manufacturer’s protocol. Slides were counterstained in Mayer’s hematoxylin, dehydrated, cleared, and cover-slipped. Negative control slides were run without primary antibodies and signals were detected with enhanced chemiluminescence. For Western Blot Analysis, animals were humanely euthanized, and tissues were used (using the UltravioVoX Imaging System). Tissue samples were measured using the Living Image software v.4.2 (Xenogen). Background bioluminescence was in the range of 3 to 6 × 10^4 ph/s. For correct intracellular redox reactions, animals were intraperitoneally injected with 50 mg/kg of carrier containing 5% DMSO. At the indicated time points, mice were sacrificed and their entire bodies were administered to the analysis of the indicated control reverse transcription reaction.

**RNA Extraction and qRT-PCR**

Total RNA was extracted from prostate or lymph nodes using the RQIol reagent (Life Technologies) according to the manufacturer’s instructions. RNA (2 µg) was used for first-strand synthesis and cDNA produced using random primers and SuperScript II (Invitrogen). RNA expression was measured by real-time qRT-PCR, using the Roche LightCycler 480 (Roche Applied Science) based on the SYBR Green method. Each assay was done in triplicate and the expression of each gene was calculated relative to expression of β-actin cDNA. Quantification was based on a standard curve obtained by serial dilution of the indicated control reverse transcription reaction.

**DNA FISH**

To detect cMyc, the probe was made using a nick-translated kit (Abbott Molecular; 0700-001) as per the manufacturer’s protocol. The paraffin-embedded tissue sections were treated with 5% pepsin for 20 minutes at 37°C after deparafl euminization. Tissues were permeabilized with 0.5% TX-100/PBS for 5 minutes and RNAase A was added for 1 hour at room temperature. For hybridization, cellular DNA was denatured in 70% deionized formamide/2× saline-sodium citrate (SSC) for 7 minutes at 80°C. Hybridization of oligo probes was performed in 50% deionized formamide, 10% dextran sulfate, 2× SSC, yeast tRNA, salmon sperm DNA, mouse C3H DNA, and 50 ng nick-translated probe in a humidified chamber overnight at 37°C. Tissues were washed with 50% deionized formamide/2× SSC at 37°C and then 15 minutes in 2× SSC and 1× SSC, respectively. Tissues were mounted and images obtained using a Zeiss Axio Cambridge Illumination Microscope. For quantification, the percentage of cells with Myc copies >2 was counted for more than 200 cells per tissue.

**Pten Analysis to Confirm Injection**

PCR analysis of Cre-mediated recombination in Pten^hematopoietic^ transgenic mice was performed on genomic DNA extracted from Cre virus–injected or noninjected prostate. For Pten recombination, primer 2 (5’-AAAGTTCCTCAGTGTATGTTG-3’) and primer 3 (5’-CCCCCAAGTGAATTGTTAGCTGT-3’) (ref. 11) were used. For Thr53 recombination, primer 2 (5’-CAACACAAACAGGC TAAACCAG-3’) and primer 3 (5’-GAAGCAAGAAGGAGGGGACC-3’) (ref. 14) were used. For detecting luciferase, primer 1 (5’-GAGGGTC CATCTGCAAGGT-3’) and primer 2 (5’-CACACAGTGGCATCTT TGA-3’) were used.

**Analysis of Tissue Expression**

Histological and immunohistochemical analysis of Pten expression was performed on tissue sections using the Ventana Discovery XT (Ventana Medical Systems) as previously described (21), or sections were first incubated with biotinylated secondary antibody for 30 minutes at 37°C after deparafl euminization. Tissues were washed with 50% deionized formamide, 10% dextran sulfate, 2× SSC, yeast tRNA, salmon sperm DNA, mouse C3H DNA, and 50 ng nick-translated probe in a humidified chamber overnight at 37°C. Tissues were washed with 50% deionized formamide, 2× SSC at 37°C and then 15 minutes in 2× SSC and 1× SSC, respectively. Tissues were mounted and images obtained using a Zeiss Axio Cambridge Illumination Microscope. For quantification, the percentage of cells with Myc copies >2 was counted for more than 200 cells per tissue.
Disclosure of Potential Conflicts of Interest

J.E. Bradner has ownership interest (including patents) in Tensha Therapeutics and is a consultant/advisory board member of the same. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: H. Cho, T. Herzka, L.C. Trotman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Cho, T. Herzka, W. Zheng, J.E. Wilkinson, M. Castillo-Martin, C. Cordon-Cardo, L.C. Trotman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Cho, T. Herzka, J.E. Wilkinson, J.E. Bradner, B.D. Robinson, C. Cordon-Cardo, L.C. Trotman
Writing, review, and/or revision of the manuscript: H. Cho, T. Herzka, B.D. Robinson, M. Castillo-Martin, C. Cordon-Cardo, L.C. Trotman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Cho, T. Herzka, W. Zheng, J. Qi, L.C. Trotman
Study supervision: C. Cordon-Cardo, L.C. Trotman

Acknowledgments

The authors thank Scott W. Lowe, Alea Mills, Leemor Joshua-Tor, Scott Powers, Chris Vakoc, Junwei Shi, Charles Bieberich, and the members of the Trotman laboratory for valuable discussion and help with experimental protocols and reagents; the Cold Spring Harbor Laboratory (CSHL) Animal Resources team, Lisa Bianco, Lotus Altholz, Jodi Coblinksi, and Michael Cahn, for help with RapidCap modeling, maintenance and trials; Angoul Nourjanova, Rasa Puzis, Denise Hoppe, K. Manova, and A. Barlas for help with histopathologic procedures; Scott W. Lowe, Nilgun Tasdemir, and Luke Dowe for help with reagents; and D. Tsang for help with the article.

Grant Support

This work was supported by grants to L.C. Trotman from the NIH (CA137050), the Department of the Army (W81XWH-09-1-0557), the STARR foundation, the Robertson Research Fund of CSHL, and by support from the CSHL CTD Cancer Target Discovery and Development Network Grant (U01CA168409, to S. Powers, Principal Investigator).

Received July 8, 2013; revised January 7, 2014; accepted January 8, 2014; published OnlineFirst January 20, 2014.

REFERENCES

Myc in Pten-Mutant Prostate Metastasis


RapidCaP, a Novel GEM Model for Metastatic Prostate Cancer Analysis and Therapy, Reveals Myc as a Driver of \textit{Pten}-Mutant Metastasis


Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-13-0346

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2014/01/20/2159-8290.CD-13-0346.DC1

Cited articles
This article cites 44 articles, 10 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/4/3/318.full#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/4/3/318.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.