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A novel approach to identify driver genes involved in androgen-independent prostate cancer

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Abstract

Background: Insertional mutagenesis screens have been used with great success to identify oncogenes and tumor suppressor genes. Typically, these screens use gammaretroviruses (γ RV) or transposons as insertional mutagens. However, insertional mutations from replication-competent γ RVs or transposons that occur later during oncogenesis can produce passenger mutations that do not drive cancer progression. Here, we utilized a replication-incompetent lentiviral vector (LV) to perform an insertional mutagenesis screen to identify genes in the progression to androgen-independent prostate cancer (AIPC).

Methods: Prostate cancer cells were mutagenized with a LV to enrich for clones with a selective advantage in an androgen-deficient environment provided by a dysregulated gene(s) near the vector integration site. We performed our screen using an *in vitro* AIPC model and also an *in vivo* xenotransplant model for AIPC. Our approach identified proviral integration sites utilizing a shuttle vector that allows for rapid rescue of plasmids in *E. coli* that contain LV long terminal repeat (LTR)-chromosome junctions. This shuttle vector approach does not require PCR amplification and has several advantages over PCR-based techniques.

Results: Proviral integrations were enriched near prostate cancer susceptibility loci in cells grown in androgen-deficient medium ($p < 0.001$), and five candidate genes that influence AIPC were identified; *ATPAF1*, *GCOM1*, *MEX3D*, *PTRF*, and *TRPM4*. Additionally, we showed that RNAi knockdown of *ATPAF1* significantly reduces growth ($p < 0.05$) in androgen-deficient conditions.

Conclusions: Our approach has proven effective for use in PCa, identifying a known prostate cancer gene, *PTRF*, and also several genes not previously associated with prostate cancer. The replication-incompetent shuttle vector approach has broad potential applications for cancer gene discovery, and for interrogating diverse biological and disease processes.

Keywords: Prostate cancer, Lentiviral vector, Mutagenesis screen, Shuttle vector, Androgen-independent prostate cancer, Proto-oncogene

Background

Insertional mutagenesis screens using replicating retroviruses have identified many genes that contribute to cancer initiation and progression and have greatly improved our understanding of carcinogenesis (reviewed by Uren et al. [1]). These screens identify genomic loci which contain proviral integration sites that are identified from

different tumors, called common insertion sites (CISs). These CISs occur because integrated retroviruses dysregulate nearby genes by a variety of mechanisms, and clones with provirus insertions near dysregulated genes that provide a selective advantage become enriched [2].

To date, the majority of insertional mutagenesis screens have utilized replicating gammaretroviruses (γ RV) or transposons which have several limitations. Screens that use replicating retroviruses are limited to tissues and cell types that are permissive for replication of the virus. Because of this, the majority of screens have been performed

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in mouse hematopoietic cells or mouse mammary cells using replicating γ RV vectors. Transposons allow mutagenesis of essentially any tissue and have expanded the use of mutagenesis screens. However, a major drawback of transposon approaches is the time it takes to generate the germline transgenic or knockout lines used, and to combine multiple alleles into the same background [3]. Another limitation of transposon mutagenesis is that multiple transposition events complicate the identification of causative mutagenic events [3].

One way to greatly expand the potential of forward mutagenesis screens is to use replication-incompetent lentiviral vectors (LVs) [4]. Human immunodeficiency virus (HIV)-derived LVs that are pseudotyped with the vesicular stomatitis virus glycoprotein can efficiently transduce essentially all mammalian cell types. Replication-incompetent LVs integrate into the genome but do not replicate, and thus do not create additional insertion sites. Therefore, there are fewer potential passenger integrations than with replication-competent vectors, where driver insertional mutation events may be masked by the accumulation of bystander integrations [4,5]. Also the level of mutagenesis can be carefully controlled by adjusting the multiplicity of infection. Importantly, under the right conditions replication-competent vectors can cause cancer, which was unfortunately observed in gene therapy studies where replication-incompetent γ RV vectors caused leukemia [6,7].

Here we report a novel screen to identify genes in the progression to androgen-independent prostate cancer (AIPC) in human cells using a replication-incompetent HIV-based LV. Prostate cancer (PCa) is the second most common cause of cancer related deaths in men in the United States, and is one of the leading causes of sickness and death in men in the U.S. and Western Europe [8,9]. Despite this high prevalence, the molecular mechanisms of PCa progression still remain largely unknown, due in part to heterogeneity during tumor development [10]. PCa is initially androgen-dependent, making androgen deprivation therapy the first line of defense in combating the disease [11]. Though this treatment initially reduces tumor size, patients eventually develop AIPC, which is resistant to this primary form of therapy and is ultimately lethal [11-13]. Thus, determining the mechanisms that contribute to AIPC is critical to develop novel therapies for this advanced form of PCa. The current study was designed to identify genes involved in the progression to AIPC. The human LNCaP PCa cell line model for AIPC is well-established [11,14,15]. LNCaP cells express androgen receptor, prostate-specific antigen, and generate androgen-independence by distinct mechanisms [11,15]. Additionally, LNCaP cells readily form tumors in immunodeficient mice allowing *in vivo* studies [16,17]. A transposon-based mutagenesis screen has been

performed for PCa that specifically investigated PCa precursor lesions and genes that were involved in PCa initiation, but AIPC mechanisms were not investigated [18].

A major advantage of our approach is the use of a LV shuttle vector that allows rescue of vector LTR-chromosome junctions in bacteria as plasmids. In other retroviral and transposon-based screens, PCR is typically used to recover these proviral insertions and in turn detect dysregulated genes [1,19]. However, PCR lacks the sensitivity to detect integrations events that are rare or poorly amplified. It has previously been suggested that plasmid-based rescue of the provirus integration might eventually replace PCR methods [1]. Our study demonstrates the potential of this approach in a mutagenesis screen for AIPC.

Results

Efficient LV transduction results in a library of mutagenized PCa cells where clonality can be rapidly assessed by shuttle vector rescue

To identify candidate genes involved in the progression to AIPC we used a replication-incompetent LV, LV-SFFVEGFP (Figure 1A) that has a strong spleen focus-forming virus promoter known to dysregulate genes [20], and also includes a bacterial origin of replication and a kanamycin resistance gene to allow identification of integration sites by rescue of shuttle vector plasmids in *E. coli*. Our approach uses random shearing of genomic DNA to avoid restriction site bias [21] and does not require any PCR amplification so it also eliminates PCR-based bias. The LV expresses EGFP gene from the spleen focus-forming virus promoter, allowing for efficient tracking of transduced cells *in vitro* and *in vivo* (Figure 1B). The androgen-dependent human PCa cell line, LNCaP, was transduced in triplicate with LV-SFFVEGFP resulting in three independent cultures of LNCaP cells denoted shuttle vector-mutagenized (SVM) -A, -B, and -C. The transduction frequency was over 99% as assessed by EGFP expression.

The clonality of these cultures prior to selection for androgen independence was evaluated to ensure that cells used for the mutagenesis screen were polyclonal. We reasoned that a highly polyclonal pool of integrations, in essence a library, would improve the ability of our screen to identify AIPC progression genes. High-throughput sequencing of shuttle vector rescued plasmids was performed to identify provirus-chromosome junctions (Figure 1C,D). Sequences were aligned to the human genome to identify the provirus integration site in the human genome. A clonality calculation was performed to approximate the number of unique integrations present in each sample prior to selection for androgen-independence. By counting both the number of new integration sites in each survey, and accounting for the number of previously identified integration sites,

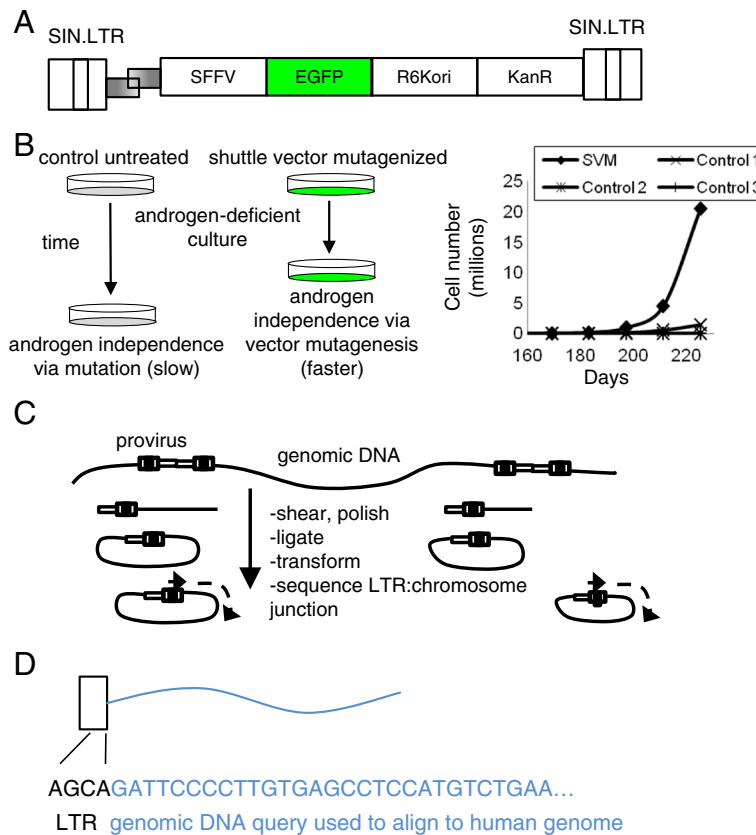


Figure 1 LV-mediated mutagenesis screen. A) Schematic of LV-SFFVEGFP vector. The strong spleen focus-forming viral (SFFV) promoter drives the expression of enhanced green fluorescent protein (EGFP). The vector includes a R6K origin of replication (R6Kori) and kanamycin resistance gene (KanR) for rescue in *E.coli*. Both long terminal repeats are self-inactivating (SIN.LTR). **B)** Experimental outline for *in vitro* LV-mediated insertional mutagenesis screen. Mutagenized sample (SVM-A) became androgen-independent before control, non-mutagenized cells. At this time genomic DNA was extracted and analyzed by our shuttle vector rescue approach. **C)** Shuttle vector rescue. Genomic DNA is sheared into smaller fragments. The ends are polished and the fragments are ligated into plasmids. Plasmids are transformed into electrocompetent *E. coli*. Using an LTR-specific primer, the junction between the provirus LTR and the chromosome DNA is sequenced. **D)** To identify the proviral integration site the chromosomal DNA at the junction is used as a query and aligned to the human genome to identify the integration site.

the approximate number of unique integration sites in a population could be calculated. A sample calculation for SVM-A is shown in Additional file 1: Figure S1. In this example, five surveys were performed and a clonality of 7.2×10^3 unique integration sites was calculated. The more surveys that are performed, the larger N will become, improving the accuracy of the clonality estimate, so this is a minimum estimate. Clonality was similar for cultures SVM-B and SVM-C. Sequencing confirmed that there was no evidence of cross contamination between independent cultures, as no identical integration sites were identified between the three mutagenized cultures. These data showed that we had a highly polyclonal starting population prior to selection for androgen independence.

***In vitro* screen to identify genes that influence androgen-independence**

To model the clinical progression to advanced AIPC [22] *in vitro* we used a previously established method to

select for cells that become androgen-independent in culture [11,14,15]. In this model androgen-dependent LNCaP cells are cultured in charcoal/dextran-treated fetal bovine serum (CT-FBS) which is essentially devoid of androgen. This selects for those cell clones that have a proliferative advantage in an androgen-deficient environment (Figure 1B). The human LNCaP cell line has several advantages for our screen including expression of androgen receptor, androgen-dependent growth, a demonstrated ability to develop androgen-independent growth [11,14,15], and the ability to form tumors *in vivo* [16,23] to allow us to explore genes and gene pathways that mediate progression *in vivo*. Preliminary experiments showed that transfer of LNCaP cells into media supplemented with 10% CT-FBS led to a loss of cells which would have reduced the clonality of our LV-mutagenized library of insertion sites. We found that initial culture in 9.75% CT-FBS with 0.25% untreated FBS minimized cell loss. We thus cultured LV-mutagenized

and control cells in media supplemented with 9.75% CT-FBS with 0.25% untreated FBS for approximately 140 days prior to moving cultures to media supplemented with 10% CT-FBS. At this time shuttle-vector mutagenized androgen-dependent LNCaP cells were maintained in an androgen-deficient environment with CT-FBS. After 211 days, the SVM-A culture showed an increase in growth rate compared to control cultures, and was deemed androgen-independent (Figure 1B). We hypothesized that in the SVM-A culture, cells with LV proviruses near genes that influenced progression to AIPC had a selective advantage and this led to androgen-independent growth in the SVM-A culture prior to control cultures. This is expected to lead to an over-representation of cells with proviral integrants near genes that influence AIPC. Thus, analysis of provirus integration sites in these androgen-independent cells should identify dysregulated genes near vector proviruses that may mediate progression to AIPC. Genomic DNA was isolated from LV-transduced androgen-independent cells to identify candidate AIPC progression genes by shuttle vector rescue (Figure 1C).

In vivo xenotransplant approach to identify genes that influence androgen-independence

In vitro models lack the ability to identify genes involved in processes required only *in vivo* such as vascularization. We thus also performed our LV shuttle vector screen *in vivo* using a LNCaP xenograft model [16,23]. NOD.Cg-PrkdcscidIl2rgtmIwjl/SzJ (NSG) mice are severely immunocompromised, and allow for efficient engraftment of human cells [24]. Androgen-dependent control LNCaPs and mutagenized SVM-A cells prior to selection *in vitro* were injected into male NSG mice (Figure 2A). Tumors developed from the injection of both SVM-A and control LNCaP cells. Similar to androgen-dependent tumors in PCa patients, it was expected that tumor volumes would regress following androgen deprivation therapy. In patients, this is done by either a surgical or chemical castration [22]. In the *in vivo* model, this environment was created by surgical removal of the testes which are the primary source of androgens. It was expected that tumor size would decrease immediately following castration and similar to the *in vitro* model, the androgen-deficient environment would select for androgen-independent cells modeling what occurs in PCa patients [11,12]. LNCaP cells were injected in male NSG mice and formed in 6 of 7 injected mice (Figure 2B). Tumors did regress following castration and following the castration, tumor growth resumed. Tumors were allowed to grow until they reached volumes larger than the tumor size prior to castration, at which point tumors were harvested and genomic DNA was obtained for shuttle vector rescue analysis.

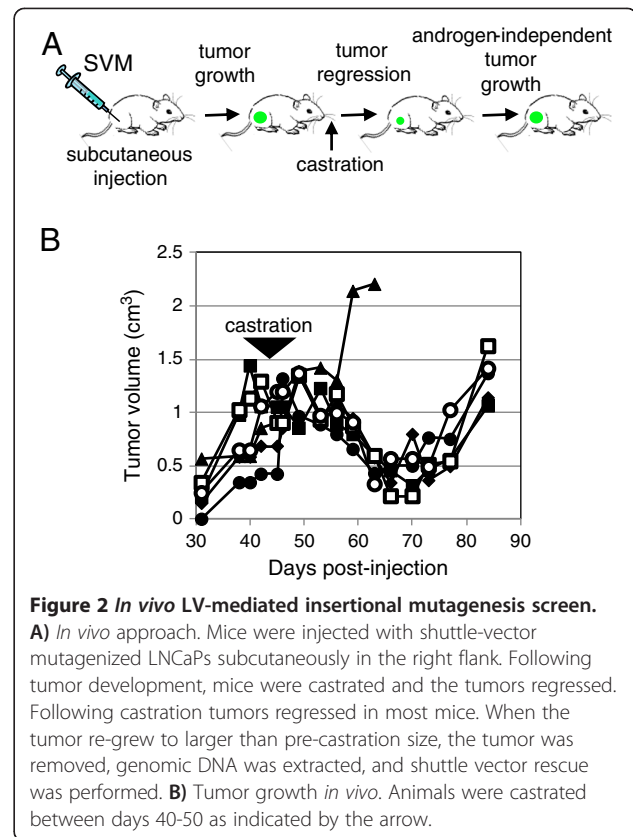


Figure 2 *In vivo* LV-mediated insertional mutagenesis screen.

A) *In vivo* approach. Mice were injected with shuttle-vector mutagenized LNCaPs subcutaneously in the right flank. Following tumor development, mice were castrated and the tumors regressed. Following castration tumors regressed in most mice. When the tumor re-grew to larger than pre-castration size, the tumor was removed, genomic DNA was extracted, and shuttle vector rescue was performed. **B)** Tumor growth *in vivo*. Animals were castrated between days 40-50 as indicated by the arrow.

Identification of genes near LV integration sites isolated from *in vitro* androgen-independent cultures and subcutaneous tumors from castrated mice

Rescue of genomic DNA from the androgen-independent culture SVM-A *in vitro* samples post-selection recovered a total of 21 unique sites (Additional file 2: Table S1). From two *in vivo* tumors analyzed post-castration we identified a total of 54 insertion sites, 27 from one tumor and 28 from the second with one site found in both tumors (Additional file 2: Table S1). All sites were single insertion sites. Custom PERL computer programs were used to analyze whether the provirus integrated within a RefSeq gene and to also provide the distance from the integration site to the nearest 3 Refseq gene transcription start sites (TSS). Only genes that had a TSS within 100 kb of the vector provirus were considered.

Enrichment of vector proviruses within PCa susceptibility loci after selection for androgen-independence

We hypothesized that our approach should enrich for vector integration sites near regions known to be involved in PCa progression. The 75 integration sites (Additional file 2: Table S1) were mapped relative to previously described PCa susceptibility loci (Additional file 3: Table S2) and compared to 412 control integration sites obtained from transduced LNCaP cells prior to selection

for androgen-independence, and also to a random *in silico* generated data set of 10,000 sites (Figure 3). The percent of vector integrations within PCa susceptibility loci for LNCaP cells cultured in androgen-deficient medium was significantly higher than from the LNCaP cells prior to culture in androgen-deficient culture medium ($p < .001$) and also significantly higher than from the random sites ($p < .001$). This demonstrates a significant enrichment of vector proviruses near loci previously associated with PCa when cells are grown under androgen-deficient conditions.

Meta-analysis of expression of candidate PCa genes near vector proviruses

We used data from previously published microarray analysis of patient tumors to identify the genes in our dataset that were most likely to contribute to AIPC based on dysregulation in patient tumors. This approach has the advantage that multiple data sources may improve the power of the screen, and may improve the ability to identify genes that are clinically relevant due to their dysregulation in patient tissues. We used OncoPrint™ (Compendia Bioscience, Ann Arbor, MI) [25] to compare microarray data from 16 different studies [26-39] that evaluated gene expression in normal prostate tissue

vs. PCa tissue (prostate carcinoma or prostate adenocarcinoma) to evaluate the candidate progression genes identified near vector proviruses listed in Additional file 2: Table S1. We reasoned that candidate driver genes identified in our screen were likely to be over or under-expressed in PCa tissue and that meta-analysis of previously published microarray studies would be a powerful way to screen our candidates for driver genes likely to be potential biomarkers or therapeutic targets. To evaluate over or under-expression we used both the rank and a multiple comparisons-corrected p-value as calculated by OncoPrint™. Candidate genes with p-values $< .005$ and ranks less than 1000 in either the over- or under-expression multiple comparisons were considered as potential driver genes (Table 1). Promising candidates identified from the androgen-independent *in vitro* culture were *ATPAF1* (ATP synthase mitochondrial F1 complex assembly factor 1) and *TRPM4* (transient receptor potential cation channel, subfamily M, member 4). Promising candidates identified from the androgen-independent *in vivo* tumor were *GCOM1* (GRINL1A complex locus 1), *MEX3D* (mex-3 RNA binding family member D), and *PTRF* (polymerase I and transcript release factor). Three of these genes, *ATPAF1*, *MEX3D* and *TRPM4*, are over-expressed in PCa tissue, while two are under-expressed, *GCOM1* and *PTRF* (Figure 4 and Additional file 4: Figure S2). The locations of the five vector integrants were mapped relative to genomic loci using the University of California, Santa Cruz (UCSC) Genome Browser (Additional file 5: Figure S3 and Additional file 6: Figure S4).

Validation of genes involved in AIPC by RNAi knockdown confirms involvement of *ATPAF1* and in PCa progression

To validate the effects of *ATPAF1* and *PTRF* on androgen-independence we used LV-mediated RNAi. For *ATPAF1* and *PTRF* a set of commercially available pGIPZ LV vectors targeting different sites on each gene were first screened to find an efficient shRNA target (Additional file 7: Figure S5). The pGIPZ vector with the most effective shRNA was then used to transduce LNCaP cells. Cells were then cultured with puromycin to eliminate untransduced cells. Knockdown was confirmed by RT-PCR (Additional file 7: Figure S5C) and then puromycin-resistant cells were cultured under androgen-deficient conditions to select for androgen-independence. *PTRF* is under-expressed in PCa tissue, so *PTRF* knockdown was expected to lead to more rapid proliferation under androgen-deficient conditions. Conversely, *ATPAF1* is overexpressed in PCa tissue, so knockdown of this gene should impair proliferation under androgen-deficient conditions. After 31 days of culture in androgen-deficient medium, as expected knockdown of *PTRF* resulted in higher cell numbers than cells transduced with the control vector, however the increase

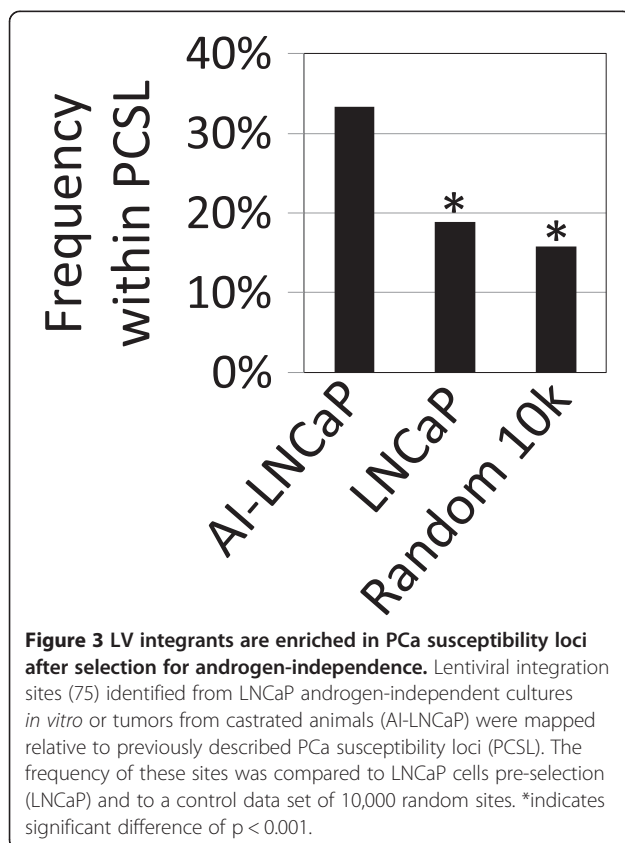


Table 1 Candidate AIPC genes

Gene	Source	In gene/distance	PCSL	Expression	p-value
<i>ATPAF1</i>	<i>In vitro</i>	yes	none	over	0.002
<i>GCOM1</i>	<i>In vivo</i>	yes	none	under	0.003
<i>MEX3D</i>	<i>In vivo</i>	32,561	19p13.3	over	9.70E-04
<i>PTRF</i>	<i>In vivo</i>	62,474	17q21-22	under	0.003
<i>TRPM4</i>	<i>In vitro</i>	yes	near 19q13.4	over	1.13E-04

Candidate AIPC genes identified from provirus integration sites recovered from both the *in vitro* mutagenesis screen and the *in vivo* subcutaneous tumor model. Source indicates whether the gene was identified from the *in vitro* screen or the *in vivo* screen. In gene/distance indicates whether the LV provirus was within the gene transcription unit, or if outside the transcription unit indicates the distance in base pairs from the proviral integration to the transcription start site of the gene. PCSL indicates if the provirus was within or near a PCa susceptibility locus. Expression indicates if the gene was over or under-expressed in PCa tissue using the Oncomine™ database. Rank is the Oncomine™ ranking of over or under-expressed genes. p-value is the Oncomine™ p-value for over or under-expression.

did not reach statistical significance ($p = 0.25$) (Figure 5). Knockdown of *ATPAF1* resulted in significantly fewer cells in androgen-deficient medium than the control by day 31 of culture ($p < 0.05$).

Discussion

PCa remains a significant health problem and a leading cause of cancer related death in the United States and parts of Europe [8,9]. However, many of PCa's molecular mechanisms remain elusive. Though treatable in its early, androgen-dependent stages, PCa often progresses to a lethal, untreatable, androgen-independent form. Here, we report for the first time, a retroviral mutagenesis screen using a replication-incompetent LV vector to identify genes that mediate progression to AIPC. Our shuttle vector approach efficiently recovered integration sites that could be rapidly mapped to genomic loci. We observed a strong enrichment of provirus integrations in PCa susceptibility loci when integration sites were compared between transduced cells prior to culture in androgen-deficient medium and after cells had become androgen-independent. This suggests that integrated vector proviruses dysregulated nearby genes that allowed these clones to proliferate in the absence of androgen, and provided a selective advantage resulting in their enrichment in a polyclonal population. While this has been widely exploited for retrovirus mutagenesis screens, we show here for the first time that a replication-incompetent LV can be used to identify candidate genes that mediate AIPC. By performing meta-analysis of our data with patient data we identified 5 promising candidate AIPC genes from 75 unique integration sites.

High-throughput gene expression studies [40-42], comparative genome hybridization studies [43,44], and proteome studies [45-47] have previously been used to identify PCa genes. However differentiating driver genes from passenger genes using these approaches has been difficult. Retroviral mutagenesis identifies driver genes by dysregulating genes near vector proviruses that provide a selective advantage. It also models a major mechanism for PCa, the formation of double-strand breaks (DSBs) (Figure 6)

which can result in gene fusions such as TMPRSS-ERG [48,49]. These gene fusions often place constitutively active promoters next to oncogenes, resulting in activation of an oncogene. LV-based mutagenesis can model mutations caused by DSBs as we use a strong viral promoter/enhancer to dysregulate neighboring genes, analogous to the juxtaposition of strong promoters to nearby proto-oncogenes (Figure 6C).

Using a replication-incompetent LV shuttle vector has several advantages. LVs can be produced at high titer and the vesicular stomatitis virus glycoprotein used to pseudotype the LV has a broad tropism allowing for efficient transduction of essentially any target mammalian cell. Thus our approach can be used to identify genes involved in numerous oncogenic processes. For example, such a screen could be used to identify genes involved in the progression to metastatic breast cancer. Further, the approach can be used to identify genes involved in virtually any biological process that involves cells that undergo a selective pressure. A novel advantage of our approach is in the method of recovery of the provirus integration site. Previous mutagenesis screens have used PCR to detect proviral integrations but PCR is limited in its ability to identify rare or poorly amplified insertions [1]. Additionally, PCR sequence read lengths are shorter than those recovered using a shuttle vector rescue technology [21]. The longer sequence reads provided by shuttle vector allow for a more accurate localization of the provirus integration. There are some limitations to the design of our mutagenesis screen. The LV used contains self-inactivating (SIN) LTRs with modified U3 region in the 3' long-terminal-repeat (LTR) sequences and with deleted viral enhancers. The lack of transcriptionally active LTRs in SIN LVs reduces genotoxicity relative to lentiviral or gammaretroviral vectors with an intact LTR [20]. Future studies will explore the effect of retroviral vector type and design on the mutagenic potential.

Our screen identified five candidate PCa progression genes; *ATPAF1*, *GCOM1*, *MEX3D*, *PTRF*, and *TRPM4*. *TRPM4* has been associated with the development of a cancer phenotype, particularly with cell proliferation

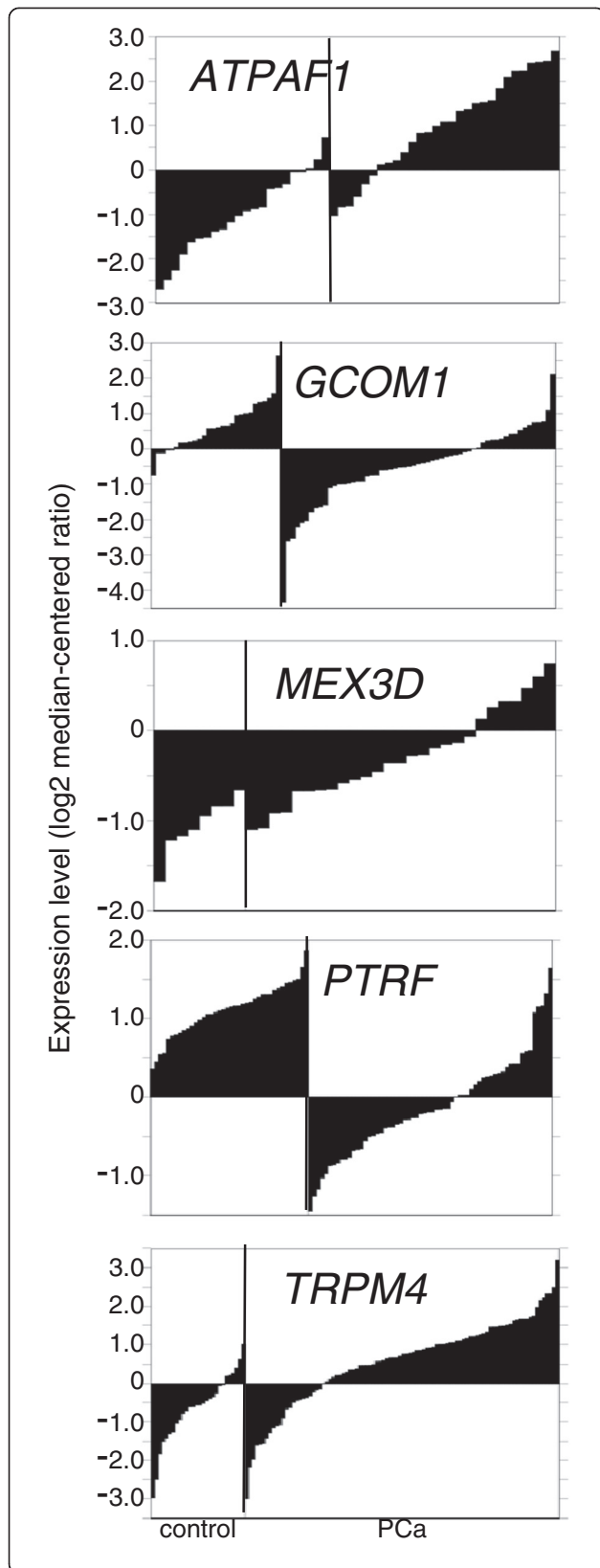


Figure 4 Differential expression in normal prostate tissue vs. PCa tissue for candidate AIPC genes. OncoPrint™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization. The OncoPrint™ database was queried using gene names for *ATPAF1*, *GCOM1*, *MEX3D*, *PTRF*, and *TRPM4* using the Cancer vs. Normal Analysis, and selecting PCa vs. Normal analysis. This provides gene expression levels in healthy prostate tissue (left side, control), and prostate carcinoma or prostate adenocarcinoma tissue samples (right side, PCa). The data is represented here as waterfall plots for each different gene.

and tumor progression [50,51], and has been specifically shown to enhance cell proliferation through up-regulation of the β -catenin signaling pathway [50,52]. *GCOM1* is a locus with a naturally occurring read-through transcription event, with one transcript encoding a fusion protein [53]. Interestingly *GCOM1* was identified as an estrogen receptor β (ER β) target gene [54]. Estrogen receptors appear to play an important role in PCa and ER β is expressed in the prostate gland [55]. ER β is the main target for phytoestrogens, and may play a role in the difference in incidence of PCa in the Western world compared to Asia where the intake of soy-based, phytoestrogen-rich food is higher [55]. *PTRF* has been associated with a number of different functions in the prostate and in cancer. In PCa, *PTRF* expression has been shown to alter the aggressiveness of the cancer [56]. *PTRF* has also been shown to be decreased significantly in LNCaP and PC3 cells and in cancer tissue [57]. *PTRF* is involved in the formation of caveolae, invaginations of the plasma membrane [58,59]. *PTRF* is also involved in localization of caveolin-1, which

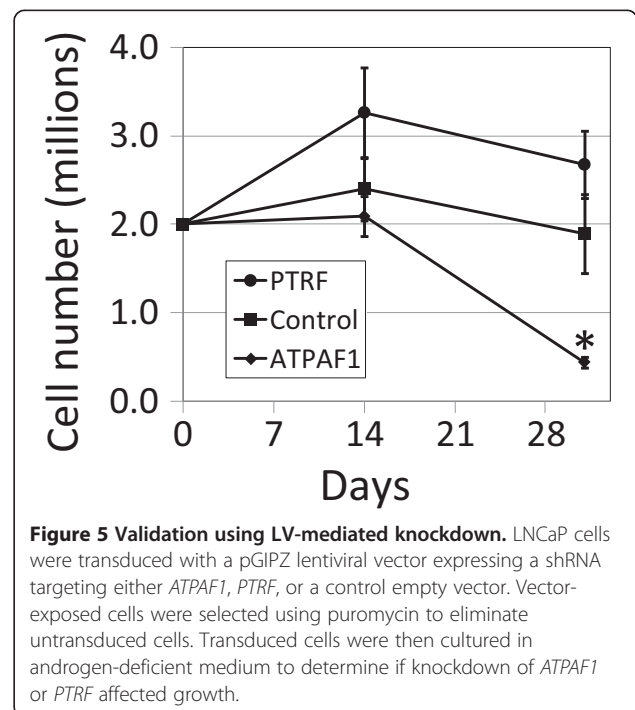


Figure 5 Validation using LV-mediated knockdown. LNCaP cells were transduced with a pGIPZ lentiviral vector expressing a shRNA targeting either *ATPAF1*, *PTRF*, or a control empty vector. Vector-exposed cells were selected using puromycin to eliminate untransduced cells. Transduced cells were then cultured in androgen-deficient medium to determine if knockdown of *ATPAF1* or *PTRF* affected growth.

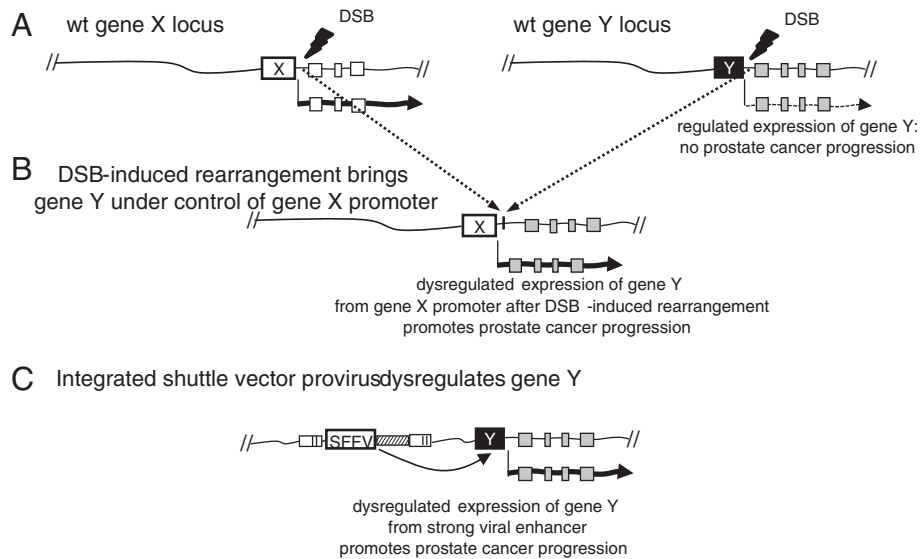


Figure 6 Shuttle vector mutagenesis models a major mechanism whereby DSB-induced rearrangements dysregulate genes. In panel **A** two loci are shown where a strong promoter expresses gene X and a weaker regulated promoter expresses gene Y. In panel **B** a DSB-induced rearrangement places gene Y under the control of the gene X promoter, dysregulating gene Y and thereby promoting PCa progression. In panel **C** an integrated shuttle vector provirus enhances expression of gene Y promoting PCa progression.

is involved in PCa severity, aggressiveness, metastasis, and androgen sensitivity [58]. Additionally, *PTRF* is under-expressed in PCa, and its expression can actually attenuate PCa disease severity and aggressiveness [58].

While these genes have been previously associated with cancer or prostate physiology, our screen was also able to identify novel candidate PCa progression genes. *MEX3D*, is a member of the Mex-3 subfamily of conserved RNA-binding proteins, which are involved in post-transcriptional regulation [60]. *MEX3D* was previously associated with chemotherapy-induced oral mucositis in acute myeloid leukemia patients [61]. *ATPAF1*, another novel target, encodes an assembly factor for the F1 component of the mitochondrial ATP synthase, which is required for assembly of the ATP synthase F₁ complex in oxidative phosphorylation [62]. *ATPAF1* is widely expressed in host tissues, but to our knowledge has never been specifically linked to cancer. However, another mitochondrial protein involved in mitochondrial oxidative phosphorylation, ATPase inhibitory factor 1 (*ATPIF1*) mediates the metabolic shift of cancer cells to a Warburg phenotype and has been identified as a promising predictive marker for clinical outcome in breast and colon cancer [63].

Our data suggests an involvement of both *PTRF* and *ATPAF1* in AIPC. *ATPAF1* knockdown resulted in significantly decreased growth relative to controls, however the increase in growth was not significant for *PTRF* knockdown. *PTRF* expression is decreased in PCa cells, including LNCaP and PC-3 [57]. We speculate that because the level of *PTRF* expression in LNCaP cells is

normally low, that any effect from knockdown by RNAi is limited. Clearly further experiments are warranted to confirm the role of *PTRF* and *ATPAF1* in AIPC, but our study is the first to our knowledge to implicate *ATPAF1* in AIPC.

Conclusions

We utilized a replication-incompetent shuttle LV as an insertional mutagen to identify genes involved in the progression to AIPC. This approach has proved effective for use in PCa, showing enrichment of proviral integrations near PCa susceptibility loci and identifying 5 potential driver genes, 1 of which was validated. Our approach has broad potential applications for cancer gene discovery, and for interrogating diverse biological and disease processes.

Methods

Cell line culture, vector production and transduction

The androgen-dependent human prostate carcinoma cell line LNCaP-FGC (ATCC CRL-1740) was cultured in RPMI-1640 supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) at 37°C in 5% CO₂. The LV shuttle vector, LV-SFFVEGFP, has self-inactivating long terminal repeats (LTRs), an internal spleen focus-forming virus promoter driving EGFP expression, and R6K γ origin of replication and a neomycin phosphotransferase gene. Vesicular stomatitis virus glycoprotein pseudotyped vector stocks were made by PEI-mediated transfection of HEK-293T cells as previously described [64]. Functional titers were determined by transduction of HT-1080

fibrosarcoma cells. Cells were cultured for 14 days post vector exposure prior to use in experiments.

Shuttle vector rescue in bacteria, identification of integration sites and clonality evaluation

Genomic DNA was isolated using the Puregene Cell & Tissue kit (Qiagen Inc., Valencia, CA) and was sheared using a Hydroshear (DigiLab Inc., Marlborough, MA). The ends of the DNA were repaired using the Terminator End Repair Kit (Lucigen Corporation, Middleton, WI). Sheared fragments were ligated using T4 DNA Ligase (New England Biolabs, Inc., Ipswich, MA) and transformed by electroporation. Shuttle vector plasmids from kanamycin-resistant colonies were sequenced using primers specific to the LV LTR. A total of 288 colonies were sequenced for the *in vitro* cultures and 96 colonies were sequenced for each tumor. The junction between the integrated provirus and the chromosome was identified and integration sites in the human genome (hg19) were determined using PERL bioinformatics programs [21] that invoke a standalone BLAT program [65]. To be considered as an integration site the alignment score had to have a canonical LTR-chromosome junction and meet additional strict criteria as previously described [21]. A control dataset of 10,000 random sites was generated as previously described [65]. The Schnabel method of multiple-census mark-recapture was used to approximate the number of unique integration sites in the entire population prior to selection for androgen independence [66,67].

***In vitro* androgen-independent culture**

Control and LV-SFFVEGFP transduced LNCaP cells were maintained in RPMI-1640 supplemented with 9.75% charcoal/dextran-treated FBS (CT-FBS) and 0.25% FBS (Atlanta Biologicals, Lawrenceville, GA), before being moved to and maintained in RPMI-1640 supplemented with 10% CT-FBS. Cells were counted using a Cellometer Auto T4 (Nexcelom Bioscience Inc., Lawrence, MA) and replated approximately every 2-3 weeks.

***In vivo* model of PCa**

All protocols involving the use of animals were approved by the Washington State University Institutional Animal Care and Use Committee and institutional guidelines for the humane use of animals in research were followed. Male 4-8 week old NSG mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). LV-mutagenized or control LNCaP cells were inoculated via subcutaneous injection. Between $1-5 \times 10^6$ cells were suspended in 100 μ L of RPMI-1640 plus 5% FBS plus 100 μ L Matrigel (BD Biosciences, Bedford, MA) and injected via a 25 gauge needle into the right flank. Tumors were visually monitored or measured once to twice weekly

and their volumes were calculated using the formula $L \times W \times H \times 0.5236$ [17].

To select for androgen-independent tumor growth *in vivo*, mice were castrated via the scrotal approach. Mice were anesthetized with isoflurane or ketamine and xylazine (80mg/kg and 6mg/kg, respectively). When tumors reached volumes larger than the tumor size prior to castration, mice were sacrificed, tumor tissue was harvested. Genomic DNA was obtained from tumor tissue using the Puregene Cell & Tissue kit (Qiagen Inc., Valencia, CA).

Validation of the effect of target genes on AIPC using LV-mediated RNAi

pGIPZ lentiviral shRNA vector sets of 3-6 shRNA vectors were obtained from Thermo Fisher Scientific (Waltham, MA) for the selected target genes. Gene knockdown was evaluated using the psiCHECK[™]-2 system (Promega Corporation, Madison, WI) using a synthesized sequence (GenScript USA, Inc., Piscataway, NJ) containing target sites for each target gene shRNA cloned into the psiCHECK[™]-2 vector. psiCHECK[™]-2 vectors containing these target fragments were co-transfected with pGIPZ shRNA vector sets for each specific target gene. The efficiency of the knockdown for each shRNA for each gene was determined with the Dual-Luciferase[®] Reporter Assay System (Promega Corporation, Madison, WI). The shRNA which demonstrated the most efficient knockdown of luciferase activity for each target gene was selected. Vesicular stomatitis virus glycoprotein pseudotyped vector stocks were made by PEI-mediated transfection of HEK-293T cells as described above. LNCaP cells for *in vitro* validation were transduced at a multiplicity of infection of 5, selected in 2.5 μ g/mL puromycin, and expanded. RT-PCR was used to quantitate the expression levels of these genes in the pGIPZ transduced LNCaP cells. Total RNA from LNCaP cells transduced with a LV carrying a shRNA sequence against the candidate genes or vector with no shRNA (EV) was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from the total RNA using Transcription First Strand Synthesis Kit (Roche Diagnostics, Indianapolis, IN). cDNA was used as a template to amplify gene specific products using primers *ATPAF1* (forward: 5'-GCCAACCAAGTTCAGCTCTT-3' and reverse 5'-GGTTCTGGGCACATTTTCAGT-3'), *PTRF* (forward: 5'-GAAGAGCTGATCAAGTCGGACC-3' and reverse: 5'-GCTTCACTTCATCCTGGTAGATCA-3') and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward: 5'-ATCCCATCACCATCTTCCAG-3' and reverse: 5'-CCATCACGCCACAGTTTCC-3'). Transduced puromycin-resistant LNCaP cells were cultured in androgen-deficient medium and cell number determined as described above.

Statistical analysis

To assess the enrichment of proviruses in PCa susceptibility loci after selection for androgen-independence, a χ^2 test of the frequency of integration sites was used. To assess the effects of shRNA knockdown of *PTRF* and *ATPAF1* on androgen-independence a Student's t-test was used.

Additional files

Additional file 1: Figure S1. Determining the clonality of LV integrants.

Additional file 2: Table S1. Proviral integration sites identified from both in vitro and in vivo androgen-independent samples. The in vitro androgen-independent culture SVM-A led to the identification of 21 unique integration sites. The in vivo androgen-independent subcutaneous (s.c.) tumors led to the identification of 54 unique integration sites. In total, 75 unique integration sites were identified.

Additional file 3: Table S2. PCa susceptibility loci.

Additional file 4: Figure S2. Oncomine meta-analysis of expression in prostate cancer tissue of genes near vector proviruses.

Additional file 5: Figure S3. Localization of vector proviruses to the human genome.

Additional file 6: Figure S4. Proviral integration sites displayed using the UCSC genome browser. The positions of the proviral integration site is indicated by the vertical line on images generated by the University of California, Santa Cruz genome browser.

Additional file 7: Figure S5. Screening of pGIPZ knockdown vectors for ATPAF1 and PTRF and confirmation of knockdown by RT-PCR.

Abbreviations

γ RV: Gammaretrovirus; LV: Lentiviral vector; AIPC: Androgen-independent prostate cancer; PCa: Prostate cancer; SFFV: Spleen focus-forming viral promoter; EGFP: Enhanced green fluorescent protein; LTR: Long terminal repeat; SVM: Shuttle vector mutagenized; PCSL: Prostate cancer susceptibility loci.

Competing interests

The authors indicated no potential competing interests.

Authors' contributions

ENS carried out the *in vitro* and *in vivo* screens, performed validation, analyzed data and drafted the manuscript. VB performed validation. AN performed the RT-PCR analysis for the validation. DTR carried out vector construction. LT generated viral vector preparations and mutagenized the LNCaP cells. GGM assisted with the study design and edited the manuscript. GDT conceived the study, drafted the manuscript and analyzed data. All authors read and approved the final manuscript.

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