A single-copy Sleeping Beauty transposon mutagenesis screen identifies new PTEN-cooperating tumor suppressor genes

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The overwhelming number of genetic alterations identified through cancer genome sequencing requires complementary approaches to interpret their significance and interactions. Here we developed a novel whole-body insertional mutagenesis screen in mice, which was designed for the discovery of Pten-cooperating tumor suppressors. Toward this aim, we coupled mobilization of a single-copy inactivating Sleeping Beauty transposon to Pten disruption within the same genome. The analysis of 278 transposition-induced prostate, breast and skin tumors detected tissue-specific and shared data sets of known and candidate genes involved in cancer. We validated ZBTB20, CELF2, PARD3, AKAP13 and WAC, which were identified by our screens in multiple cancer types, as new tumor suppressor genes in prostate cancer. We demonstrated their synergy with PTEN in preventing invasion in vitro and confirmed their clinical relevance. Further characterization of Wac in vivo showed obligate haploinsufficiency for this gene (which encodes an autophagy-regulating factor) in a Pten-deficient context. Our study identified complex PTEN-cooperating tumor suppressor networks in different cancer types, with potential clinical implications.

Although the sequencing of genomes from cancer cells has identified multiple genetic modifications underlying the carcinogenic process1, its ability to pinpoint players that are altered by nongenetic mechanisms is limited, and discerning between driver and passenger alterations still represents a major challenge. Moreover, such a strategy is not designed to elucidate cooperation between multiple driver genes. Indeed, some genes that were previously thought to be passengers are now believed to cooperate with other cancer-related genes and have thus become drivers2.

Insertional mutagenesis in mice is a valuable method for genome-wide functional studies. Transposon-based somatic screens have identified new genes that are involved in the pathogenesis of different cancer types. Transposon mobilization in mice with known cancer-predisposing mutations has also uncovered synergistic mechanisms between networks that drive cancer progression3–13. Here we have enhanced the capabilities of transposon-based insertional mutagenesis to identify cooperating cancer driver events, by developing a system that allows both disruption of a known targeted gene and single-copy transposon mobilization to occur simultaneously in the same cell. Using this strategy we performed a mutagenesis screen in mice that was aimed at identifying loss-of-function alterations that cooperate with the inactivation of Pten, a tumor suppressor gene commonly deleted or mutated in cancer. In parallel, and for comparison with a more conventional setting, we generated another cohort of mice that harbor an additional concatemer of Sleeping Beauty transposon, which encodes an autophagy regulator, is a novel obligate haploinsufficient tumor suppressor gene.

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Received 8 December 2016; accepted 24 February 2017; published online 20 March 2017; doi:10.1038/ng.3817
RESULTS
A Sleeping Beauty–dependent inactivatable Pten allele
To identify tumor suppressors that collaborate with Pten deficiency for cancer progression, we generated mice carrying a Pten allele that was inactivatable after Sleeping Beauty (SB)-mediated transposition (PtenSBm2/+; Fig. 1a and Supplementary Fig. 1a–f). Briefly, Pten exon 5, which encodes the phosphatase domain, was flanked by two SB terminal repeats (TRs). In the absence of SB, the Pten allele functions normally, but it becomes inactivated after SB-transposase-mediated mobilization of the transposon, which can be re-inserted in the genome, potentially generating an additional loss-of-function mutation in another locus of the same cell. As demonstrated by a trapping assay in which the loss of function of the X-linked Hprt gene provides resistance to 6-thioguanine in male mouse embryonic stem (ES) cells, the mobilized Pten exon 5, which bears its natural splice acceptor, functions as a gene-disrupting element (Supplementary Fig. 2a).

PtenSBm2/+ mice were intercrossed with mice that carried the gene encoding the SB11 transposase in the Rosa26 locus (Rosa26SB11/SB11) to induce transposition, as well as a mutated Blm allele (Blmm3/m3) to favor loss of heterozygosity (LOH) of the inactivating genetic events, as Blm deficiency increases mitotic recombination4,6,15. This produced PtenSBm2/+; Rosa26SB11/+; Blmm3/m3 mice (hereafter referred to as PSB mice). Additionally, to enrich for the number of transposon-induced mutations in each cell, a concatemer with ~35 copies of an inactivating transposon (inactivating transposon 2 m; ITP2m) was established in a subset of mice (PtenSBm2/+; ITP2m; Rosa26SB11/+; Blmm3/m3; hereafter referred to as PSB mice) (Fig. 1b and Supplementary Fig. 2b). Also, ITP2m; Rosa26SB11/+; Blmm3/m3 and Rosa26SB11/+; Blmm3/m3 mice (hereafter referred to as ISB and SB mice, respectively) were generated as controls. Mice were monitored and euthanized at the onset of signs of morbidity.

Identification of Pten-cooperating tumor suppressors
PSB and PISB mice had a wide range of neoplastic lesions—including prostate, breast, skin, endometrial, intestinal and adrenal tumors with high incidence, as well as lymphomas, thyroid, lung and uterine tumors (Fig. 1c,d and data not shown). These corresponded to cancer types that are commonly observed in Pten-deficient mouse models1,6,16. Mean survival was shorter in the PISB cohort as compared to that in the PSB cohort (337 d vs 419 d) (Fig. 1e), although both groups showed similar incidences of total and malignant tumors (Fig. 1c).

These results showed a synergy between PtenSBm2 and ITP2m in accelerating tumorigenesis.

To identify transposon integrations, we performed Illumina sequencing of sheared and barcoded tumor DNA as previously described17. We sequenced 127 prostate tumors (PSB, n = 45; PISB, n = 82), 26 breast tumors (PSB, n = 12; PISB, n = 14) and 125 skin tumors (PSB, n = 43; PISB, n = 82) to yield 1,193,651 nonredundant transposon insertions with a minimal read coverage of 2 (Supplementary Tables 1–6). Subsequently, we applied statistical analyses, using Gaussian kernel convolution, separately to six data sets, which corresponded to the insertions identified in tumors from the same tissue (prostate, breast or skin) and in the same mouse cohort (PSB or PISB), to identify genomic loci hits that occurred more frequently than those predicted by chance18. We predicted that these common insertion sites (CIS) were likely to contain cancer driver genes. Because SB shows a tendency to re-integrate close to the transposon donor locus (a phenomenon known as local hopping)14,19, we excluded CIS that mapped to the donor chromosomes (chromosome 19 for PtenSBm2 and chromosome 14 for ITP2m) and compiled lists of the CIS in tumors from PSB and PISB mice (prostate, 190 and 1,170, respectively; breast, 101 and 291, respectively; skin, 287 and 665, respectively) (Table 1 and Supplementary Tables 1–7).

We observed 717 CIS that were specific to prostate tumors, 191 that were specific to breast tumors and 329 that were specific to skin tumors, whereas 101 CIS were common to all three tumor types. Of these, seven CIS were present in all of the lists (six in total) that we generated from the prostate, breast and skin tumors from the PSB and PISB mice, which included well-known Pten-cooperating genes (Nf1, Cul3 and Trnc6b) and candidate tumor suppressor genes (Kansl1, Arhi1, Dyrk1a and Chad2) (Fig. 1f and Supplementary Table 8)12,20–22. In summary, these genome-wide screens identified comprehensive sets of tumor suppressor genes that cooperate with Pten in a global or tissue-specific manner.

Transposition model mimics human prostate tumorigenesis
We focused on prostate cancer because of its high incidence in humans and in our mice, as well as the central role of Pten in its pathogenesis. Pten+/− mice develop non-invasive prostate tumors with incomplete penetrance23, whereas complete tumor penetrance is observed after prostate-specific Pten inactivation24. The presence of concomitant genetic alterations in Pten+/− mice has also been shown to trigger prostate cancer progression25–30. We hypothesized that the combination of the PtenSBm2 allele and expression of the SB transposase in PSB and PISB mice would facilitate the coupled inactivation of Pten and cooperating tumor suppressors. Moreover, the Blm deficiency should favor LOH of both the inactivated Pten allele and the transposon-targeted loci, accelerating tumor formation and progression.

In agreement with these predictions, all male PSB (n = 49) and PISB (n = 49) mice showed prostate gland enlargement, which, in a high proportion (50% and 72%, respectively), progressed to invasive adenocarcinoma (Fig. 1c). Similarly to that seen in human prostate cancer, progression was accompanied by a decrease in Pten protein levels and a correlative increase in the proportion of actively proliferating (Ki67-positive) cells. Moreover, the contiguity of p63 staining, which is characteristic of normal epithelium and of prostate intraepithelial neoplasias (PIN), was lost in invasive adenocarcinomas (Fig. 2a,b).

In addition, Pten LOH was detected in 9/9 analyzed tumors (Fig. 2c). These data indicate that prostate tumorigenesis in our model mirrors the progression of human prostate cancer, confirming suitability of this mouse model for the molecular characterization of this malignancy.

Prostate CIS are enriched in human genes involved in cancer
Notably, 70% of the prostate CIS genes found in PSB mice overlap with those found in PISB mice (P < 2.2 × 10^-16; Fisher’s exact test) (Fig. 2d and Supplementary Table 7), indicating that the same signaling pathways support prostate cancer development in both cohorts (significant overlaps were also observed in breast (P = 3.6 × 10^-16) and skin tumors (P < 2.2 × 10^-16) by Fisher’s exact test; Supplementary Fig. 3a,d and Supplementary Table 7). To explore the biological and clinical relevance of the genes identified in the prostate tumors, we determined their enrichment in genes that are known to be involved in human cancer. CIS genes with human homologs (hCIS) in tumors from PSB (n = 160 PSB hCIS) and PISB (n = 1,047 PISB hCIS) mice, as well as those shared between both groups (hereafter referred to as PSB∩PISB hCIS; n = 115 PSB∩PISB hCIS) were analyzed independently. PSB, PISB and PSB∩PISB hCIS were significantly enriched in known and candidate genes involved in cancer (hereafter referred to as cancer genes) that are listed in the Cancer Gene Census (CGC) database (PSB, P = 5.50 × 10^-16; PISB, P < 2.2 × 10^-16; PSB∩PISB, P = 1.22 × 10^-14; by Fisher’s exact test) and in the Network of
Cancer Genes (NCG)\(^\text{31}\) (PSB, \(P = 0.005377\); PISB, \(P = 4.27 \times 10^{-7}\); PSB\(\cap\)PISB, \(P = 0.003752\); by Fisher’s exact test), respectively (Supplementary Fig. 4a,b and Supplementary Tables 9 and 10). This highlights the potential of combining human cancer sequencing with transposon-based screening for prioritizing and cross-validating candidate cancer genes.

We next asked whether hCIS were enriched in human prostate cancer genes. Genomic rearrangements and copy-number variations represent a major source of DNA alteration in prostate cancers. Given the exclusive inactivating capacity of our transposons, we compared the list of hCIS to genes that are recurrently deleted in human prostate cancers.

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Moreover, consideration of only deleted genes in the TCGA database that were also downregulated in prostate cancer led to sharply increased significance of the intersections ($P = 0.00088$, $P = 4.74 \times 10^{-10}$ and $P = 0.00086$, respectively; by Fisher's exact test), further supporting the involvement of these genes as tumor suppressor genes (Supplementary Fig. 4d,e and Supplementary Table 12).

### General features of PSB and PISB prostate cancer CIS genes
To identify the genes that contributed to prostate cancer progression, we focused on the curated PSB∩PISB hCIS (117 mouse genes corresponding to 115 human orthologs) (Supplementary Table 13). The analysis of their transposon integration pattern (as well as of those of breast and skin tumors) showed the presence of insertions scattered along these genes, consistent with their involvement in...
Figure 2. Characterization of Pten-inactivated prostate cancer and identification of genes that potentially drive its progression. (a) Representative images of H&E, Pten, p63 and Ki67 staining of normal tissue and of prostatic intraepithelial neoplasia (PIN) and adenocarcinoma lesions from PSB and PISB mice (n = 114). Scale bars, 50 µm. (b) Quantification of staining for the indicated proteins in normal tissue, and in the PIN and adenocarcinoma components from 114 prostate tumors from PSB and PISB mice. (c) Quantitative PCR analysis for detection and quantification of Pten alleles in tail–tumor pairs from nine PSB and PISB mice (1–9). PtenSBm2 represents the targeted Pten allele after transposon mobilization. Tails from PtenWT/WT (A), PtenSBm2/SBm2 (B), PtenSBm2/WT (C) and Pten∆SBm2/WT (D) mice were used as controls. (d) Overlap between PSB (n = 190) and PISB (n = 1,170) prostate CIS genes. P < 2.2 × 10−16 by Fisher’s exact test. (e) Distribution of PSB∩PISB human CIS genes across 127 prostate tumors. Blue boxes highlight PSB∩PISB hCIS genes containing SB insertions in each tumor. Histograms show the number of PSB∩PISB hCIS genes with insertions per each tumor. Red horizontal lines indicate the median of PSB∩PISB hCIS genes with insertions in tumors from PSB and PISB mice. Correlation between the frequency of prostate tumors from PSB and PISB mice with insertions in each PSB∩PISB hCIS gene (n = 117). Pearson correlation coefficient, P < 2.2 × 10−16 by Pearson’s correlation test. (f) Histograms of the Pearson correlation coefficients (R) between the mRNA expression of PTEN and that of PSB∩PISB hCIS genes (blue bars) or that of all genes in the genome (red bars) in human prostate tumors from The Cancer Genome Atlas (TCGA, n = 336), Taylor (n = 160) and Barbieri (n = 31) data sets. P values were calculated by the Kolmogorov–Smirnov test.
tumor suppressor functions (Supplementary Figs. 5–7). Accordingly, RNA sequencing (RNA-seq) analysis of prostate tumors from PSB and PISB mice identified chimeric transcripts of CIS genes in which transposon insertions caused disruption of their reading frames (Supplementary Table 14).

To demonstrate the significance of these CIS genes, we studied their distribution among the tumors analyzed. As shown in Figure 2e, a number of genes were hit by transposons in most neoplasms (a phenomenon also seen in breast and skin tumors; Supplementary Fig. 3b, e), with a median of 20 and 33 hits per prostate tumor in the PSB and PISB cohorts, respectively. The presence of multiple hits per tumor in the single-transposon cohort supports the co-existence of multiple tumor subclones with different insertions that cooperate with Pten disruption within a tumor. This is in line with recent human studies...
that provide evidence for polyclonality in prostate cancer\textsuperscript{32–34}. Genes that were frequently mutated in the tumors from the PSB mice were also highly mutated in the tumors from the PISB cohort ($R = 0.82, P < 2.2 \times 10^{-16}$) (Fig. 2f), demonstrating their importance for driving prostate cancer. Significant, positive correlations were also detectable in breast ($R = 0.77, P = 1.17 \times 10^{-4}$) and skin ($R = 0.88, P < 2.2 \times 10^{-16}$) tumors (Supplementary Fig. 3c,f).

To explore the potential cooperation of these genes with PTEN inactivation in driving prostate tumorigenesis, we evaluated the mRNA expression levels of PTEN and our list of PSB\textgreater PISB genes in human prostate tumors from the TCGA, Taylor\textsuperscript{35} and Barbieri\textsuperscript{36} data sets. Of note, the PSB\textgreater PISB hCIS set was highly enriched in genes whose mRNA expression levels correlated with those of PTEN, which might reflect a tendency for PSB\textgreater PISB hCIS genes to be co-regulated with PTEN (Fig. 2g). This finding suggested the validity of our screen in pinpointing genes that are involved in PTEN-related processes in prostate cancer.

**Deregulated pathways in tumors from PSB and PISB mice**

To explore the molecular and biological function of the prostate PSB\textgreater PISB CIS genes, we applied DAVID gene set enrichment analysis\textsuperscript{37} using Kyoto Encyclopedia of Genes and Genomes (KEGG), BioCarta and Gene Ontology (GO) term data sets (Fig. 3a and Supplementary Table 15) (for similar breast and skin CIS analyses, see Supplementary Tables 16 and 17). Chromatin- or histone-modifying enzymes constitute one of the most significantly enriched pathways. Histone methylase-encoding genes, such as ARID1A, or those encoding members of the MLL protein family (MLL1 and MLL5) are recurrently mutated in prostate cancer, in association with advanced disease stages\textsuperscript{36,38}. Moreover, MLL1 and MLL5 interact with the androgen receptor (AR), a key player in prostate tumorigenesis\textsuperscript{38}. Additionally, loss of the histone acetylase CREBBP cooperates with PTEN haploinsufficiency in driving prostate cancer\textsuperscript{39}.

We identified a large number of genes involved in RNA stability, splicing and transcriptional regulation. The homeodomain transcription factors encoded by MEIS1 and PBX1, which are known onco-genes for other malignancies, are downregulated at various stages of prostate cancer progression\textsuperscript{40}. Our results constitute the first in vivo indication of their tumor suppressive role in the prostate. In the case of Phx1, transposon distribution and chromic transcript data supported this view (Supplementary Fig. 5 and Supplementary Table 14). Similarly, the gene encoding the transcription factor FOXP1 has been described as an oncogene or a tumor suppressor, depending on the context\textsuperscript{41}. The distribution of insertions along Foxp1 in our screen points to a tumor suppressor role, in agreement with recent findings in human prostate cancer\textsuperscript{42} (Supplementary Fig. 5). Another notable transcription-factor-encoding gene we found in our screen and that is known to be altered in prostate cancer is ETV6. Unlike other ETS-factor-encoding genes, ETV6 is not involved in gene fusions in human prostate tumors. Instead, ETV6 is hemizygously deleted in \textasciitilde 25\% of clinically localized prostate cancer, consistent with our results\textsuperscript{43}.

Genes whose products are involved in ubiquitin-mediated proteolysis were also significantly enriched, especially those encoding E3 ubiquitin ligases. Exome sequencing of 112 prostate adenocarcinomas has previously identified SPOP, which encodes the substrate-binding subunit of the CUL3-based E3 ligase, as the gene most frequently mutated in this malignancy\textsuperscript{36}. The CUL3 CIS we observed in our screen suggests that alterations in other members of this E3 ligase complex may have similar consequences.

Alterations in intracellular membrane trafficking, intercellular communication or cytoskeleton organization also emerged as important molecular networks that can compromise cell polarity. PTEN has essential roles in cellular polarization, in part by recruitment to the endocytic vesicles\textsuperscript{44,45}. Thus, the PTEN-interacting polarity protein PARD3, a tumor suppressor in different tumor types but unexplored in prostate cancer, emerged as a notable candidate.

Finally, our screens identified alterations of central signaling networks in prostate cancer, such as androgen receptor (AR) signaling (Nrip1, Y11, Mll1, Mll5 and Foxp1), RAS and mitogen-activated protein kinase (MAPK) signaling (Rasa1, Nf1 and Erbb2ip), and phosphatidylinositol-3 kinase (PI3K)–AKT serine/threonine kinase 1 (AKT) signaling (Pik3r1, Magi3 and Pten-competitive endogenous RNAs (ceRNAs)). In fact, a large number of putative Pten ceRNAs, including the validated ceRNAs Cnot6l and TRDc6\textsuperscript{46,47}, were present among our CIS. In the polyclonal tumors from PSB and PISB mice, disruption of Pten ceRNAs would further decrease Pten function in those subclones without Pten LOH. In a previous SB screen focusing on melanoma, 33 Pten ceRNAs were identified\textsuperscript{48}. We found a significant overlap between these and our CIS genes (PSB, $P = 6.58 \times 10^{-10}$; PISB, $P < 2.2 \times 10^{-16}$; PSB\textgreater PISB, $P = 3.63 \times 10^{-11}$; by Fisher’s exact test) (Supplementary Fig. 8a and Supplementary Table 18). Moreover, our CIS genes were significantly enriched among the PTEN ceRNAs predicted by the ceFINDER algorithm\textsuperscript{49} (PSB, $P = 4.51 \times 10^{-12}$; PISB, $P < 2.2 \times 10^{-16}$; PSB\textgreater PISB, $P = 2.44 \times 10^{-10}$; by Fisher’s exact test) (Supplementary Fig. 8b and Supplementary Table 19).

**Co-silencing of PTEN and candidate genes drives invasion**

To examine the role of the identified genes in the evolution of cancer, we used small interfering RNAs (siRNAs) to silence the expression of candidate genes in two immortalized but nontransformed human prostate cell lines, named BPH-1 and RWPE-1. In contrast to cancer cells, immortalized cells more closely resemble the primary cells they derive from and, therefore, do not contain cancer-driving genetic alterations. Thus, we tested the effect of inhibiting the expression of our CIS genes, either alone or in combination with PTEN silencing, on their invasive ability in vitro.

We selected five of the twenty genes that were most frequently hit by transposons in our screens, which were not annotated in the Cancer Gene Census and for which a role in prostate cancer had not been described so far (Fig. 3b and Table 1). These genes were ZBTT20 (zinc finger and BTB domain containing 20), CELF2 (CUGBP, Elav-like family member 2), AKAP13 (A-kinase anchor protein 13), PARD3 (Par-3 family cell polarity regulator) and WAC (WW-domain-containing adaptor with coiled coil). Co-silencing the expression of PTEN together with that of each of the candidate genes in BPH-1 and RWPE-1 cells sharply increased the invasiveness of the cell lines (Fig. 4a, Supplementary Fig. 9 and Supplementary Table 20). This synergistic effect is consistent with there being a cooperative role of these genes with PTEN in preventing malignant progression.

**Oncogenic pathways disrupted by co-silencing**

To examine the molecular basis of this phenotype, we performed RNA-seq transcriptomic profiling of BPH-1 and RWPE-1 cell lines after silencing of the expression of the candidate genes, either alone or with co-silencing of PTEN expression (Supplementary Fig. 10 and Supplementary Tables 21–25). Principal component analysis showed that the first component was determined by the cell line, whereas the second component discriminated single-gene knockdown from co-silencing conditions (Fig. 4b). This suggested the rewiring of common molecular pathways irrespective of the gene being co-silenced with PTEN.

Pathway enrichment analysis of transcriptional changes associated with co-silencing (versus those associated with PTEN silencing alone),
Genetic inhibition of tumor suppressor genes drives prostate cancer progression through canonical signaling pathways. (a) Effect of ZBTB20, CELF2, PARD3, AKAP13 and WAC silencing, either alone or in combination with PTEN silencing, on the invasiveness of BPH-1 (top) and RWPE-1 (bottom) immortalized human prostate cell lines. Cell invasion rate is expressed as a percentage relative to the mean values of the control invasion rates, which were set as 100%. Each circle represents an individual technical replicate. Data are the mean ± s.e.m for each condition. *P < 0.05, **P < 0.01 and ***P < 0.001 by two-tailed Student’s t-test. (b) Principal component analysis of the RNA-seq expression profiles of RWPE-1 and BPH-1 cell lines after silencing of the five candidate tumor suppressor genes in a, either alone or in combination with Pten silencing. RNA expression profiles from control siRNA (siCtr)-treated or Pten-specific siRNA (siPten)-treated cells were also included in the analysis. Component one was determined by the status of single-gene silencing versus co-silencing with Pten, regardless of the single candidate gene being co-silenced (n = 2 per condition). (c) Heat map depicting a subset of shared deregulated pathways associated with co-silencing of each of the candidate genes, as compared to that with Pten silencing only (n = 2 per condition). Selected pathways shared by at least four of the co-silencing conditions and with FDR q-values < 0.025 are represented.
Figure 5 Clinical significance of validated genes in human prostate cancer. (a) Expression of ZBTB20, CELF2, PARD3, AKAP13 and WAC mRNAs in primary prostate cancers (n = 336) versus that in benign tissue samples (n = 65) available from TCGA. (b) mRNA levels of the indicated genes in metastatic samples (n = 29), as compared to that in primary tumors (n = 131) and benign tissues (n = 19) available from the Taylor data set. In the box plots in a, b, boxes display the 25th to 75th percentiles, lines represent the medians, and whiskers represent 1.5× the interquartile range. *P < 0.05, **P < 0.01 and ***P < 0.001 by two-tailed Student’s t-test. (c, d) Scatter plots showing positive correlations between PTEN and ZBTB20, CELF2, PARD3 or WAC gene expression in primary prostate tumors from the TCGA data set (c) and in primary and metastatic tumors from the Taylor data set (d) (in d, correlation is also detected for AKAP13). R, Pearson correlation coefficient. P values were calculated by Pearson’s correlation test. Normalized RNA-seq by expectation maximization (RSEM) and log2 mRNA expression values are shown for the TCGA (a, c) and Taylor (b, d) data sets, respectively. (e) Kaplan–Meier analysis for recurrence-free survival of the 25% of patients with the lowest mRNA expression levels of each of the five genes indicated versus the survival of the remaining 75% of patients. Analyses were performed using the Taylor data set and the open web interface ‘Project Betastasis’ (http://www.betastasis.com). P values were obtained by the log-rank test.
showed common themes that were shared by the candidates (Fig. 4c and Supplementary Tables 26–35). Among the upregulated genes, enrichment was detected for pathways promoting cancer progression: epithelial–mesenchymal transition, MYC-dependent transcriptional activation, MET–RAS–MAPK signaling and PI3K–AKT–MTOR signaling. Accordingly, western blot analysis showed that, as compared to those by PTEN silencing alone, co-silencing of each of the candidates induced increased levels of phosphorylated AKT (p-AKT) and/or p-MTOR, without further reduction of PTEN levels (Supplementary Fig. 11). The downregulated genes were enriched in pathways including mitotic spindle formation, Rho GTPases, DNA damage response, and transforming growth factor (TGF)-β and AR signaling. PTEN deletion in human and mouse prostate tumors has been previously described to decrease the AR transcriptional output, as the PI3K and AR oncogenic pathways cross-regulate each other by reciprocal feedback. Several of these pathways (AR, RAS–MAPK and PI3K–AKT) were also over-represented in the PSB∩PISB CIS from prostate tumors.

Clinical relevance of validated genes

We then analyzed the mRNA expression of these genes in prostate tumors from the TCGA data set. ZBTB20, CELF2, PARD3, AKAP13 and WAC mRNA levels were significantly reduced in primary prostate cancer samples \( n = 336 \) compared with benign tissues \( n = 65 \) (Fig. 5a). To investigate more advanced stages of the disease, we analyzed the Taylor data set, which includes primary \( n = 131 \) and metastatic \( n = 29 \) samples. We observed a further reduction of mRNA expression of these genes after progression to metastasis (Fig. 5b). Additionally, a positive correlation was detected between expression of these genes and tumor progression (Fig. 5c). This was stronger and extensive to AKAP13 when more advanced tumors were included in the analysis (Fig. 5d), supporting the idea of cooperation between PTEN and these genes in preventing cancer progression. With the exception of that for CELF2, the correlation was clearer for log2(PTEN expression levels) > 8.5. Although gene-expression-independent regulatory mechanisms might operate, this observation could also reflect that the contribution of ZBTB20, PARD3, AKAP13 and WAC in preventing cancer progression was stronger when PTEN function was perturbed but not lost. In addition to these observations, recurrence-free survival of patients with tumors that expressed low levels of these genes was notably reduced (Fig. 5e). This was also true for ZBTB20, AKAP13 and WAC when the analysis was restricted to primary tumors (Supplementary Fig. 12). Taken together, these results highlight the clinical relevance of the PTEN-cooperating tumor suppressor genes we identified.

Wac: a new obligate haploinsufficient prostate cancer gene

To explore the in vivo effects of Wac disruption on prostate tumorigenesis in mice, we generated a mouse model in which there was prostate-specific homozygous inactivation of Pten (Pten\(^{-}/-\)) combined with prostate-specific heterozygous (Wac\(^{+/+}\)) or homozygous (Wac\(^{-/-}\)) deletion of Wac. Tumor development in these mice disclosed complex interconnections between the Wac and Pten deficiencies. Relative to the size of tumors in the prostate-specific Pten\(^{-}/-\) mice, prostate-specific Pten\(^{-}/-\), Wac\(^{+/+}\) mice developed larger tumors, whereas prostate-specific Pten\(^{-}/-\), Wac\(^{-/-}\) mice were protected from tumor progression (Fig. 6a). This showed a phenomenon of obligate haploinsufficiency, by which, in the presence of a Pten deficiency, partial Wac inactivation potentiated tumor growth, whereas complete Wac inactivation precluded it.

The tumor-promoting effect of partial Wac inactivation was more evident at earlier cancer development stages—it led to significantly increased tumor sizes in 4-month-old Pten\(^{-}/-\), Wac\(^{+/-}\) mice, whereas there was only a tendency toward larger tumors in mice 5 months later (Fig. 6a,b). This is consistent with the proposed role of Wac as an activator of autophagy, a process known to exert dual effects on cancer, suppressing tumor initiation and promoting the growth of established cancers.

DISCUSSION

PTEN, the second most frequently mutated or deleted gene in human cancer, is a key controller of signaling nodes in multiple tumor types. On the basis of a novel strategy that couples targeted gene inactivation to single-copy transposon mobilization, we performed a genome-wide survey for genes that cooperate with Pten in suppressing tumorigenesis in mice. Our model recapitulated the progressive histological, immunohistochemical and genetic alterations of human prostate cancer, confirming its suitability for the molecular characterization of this malignancy. Our studies identified comprehensive landscapes of tissue-specific and global Pten-collaborating genes, pathways and biological processes, including RNA metabolism, chromatin remodeling or ubiquitin-mediated proteolysis. Previous work has proven the utility of transposon-based somatic mutagenesis to identify and validate candidate cancer genes. Our strategy meets these objectives and opens new applications of somatic transposition by introducing relevant features to the field.

First, the single-copy transposon limits the number of insertions to one per cell, which is aimed at reducing the number of passenger insertions and increasing the specificity of the screen. To our knowledge, this is the first time a single-copy transposon has been shown to generate functionally relevant CIS in a somatic mutagenesis screen. Second, our approach achieves transposon-dependent targeted gene disruption, which favors the identification of insertions that cooperate with the engineered mutation (in our case, Pten inactivation). Transposons have been successfully used previously, together with Cre-dependent activation or inactivation of known oncogenes and tumor suppressors, including Pten\(^{3,6,8,11–13}\). However, using a Cre-inactivated loxp-flanked (floxed) Pten allele does not guarantee perfect coupling of Pten inactivation and transposon mobilization.
In our model, by contrast, transposition happens only in cells that have undergone \textit{Pten} inactivation, avoiding the development of transposon-induced tumors with intact \textit{Pten}. Third, our screen exclusively involves inactivating transposition. Although this precludes the identification of potential oncogenes, it facilitates interpretation of the roles of uncharacterized candidate genes.

Detailed analysis of prostate cancer CIS showed enrichment in known (CGC) and putative (NCG) cancer genes. All of the genes had insertions that were evenly distributed along their loci, which is the pattern expected for tumor suppressors. Consistently, they overlapped significantly with genes that show, concurrently, homozygous deletions and downregulation of their expression in TCGA human prostate cancer samples. In addition, they were enriched in genes whose expression levels positively correlated with those of \textit{PTEN} in human prostate cancer, supporting the specificity of the screen in the identification of \textit{PTEN}-cooperating genes. We prioritized our characterization and validation efforts on five of the genes that ranked among the 20 most frequently hit prostate cancer CIS and that were not previously described as cancer drivers. We confirmed the effect of genetically inhibiting \textit{ZBTB20, CELF2, PARD3, AKAP13} and \textit{WAC} expression in \textit{PTEN}-deficient contexts for the enhancement of invasion and the unleashing of canonical cancer-promoting pathways, and we provided evidence of their clinical relevance in human prostate cancer. Finally, we characterized \textit{Wac} as a new obligate haploinsufficient tumor suppressor \textit{in vivo}.

The obligate haploinsufficiency of \textit{Wac} agrees with its proposed role as an autophagy activator\textsuperscript{50,51}. Co-silencing of \textit{Wac} and \textit{PTEN} resulted in the downregulation of pathways directly related to autophagy, such as KEGG\_LYSOSOME and KEGG\_PEROXISOME (false discovery rate (FDR) \textit{q}-values = 0.000) (Supplementary Table 35). Heterozygous disruption of \textit{Becn1}, a gene encoding a Wac-interacting protein essential for autophagy, increases the frequency of spontaneous malignancies and accelerates hepatitis B virus (HBV)-induced hepatocellular carcinogenesis\textsuperscript{55}. However, liver tumors arising from \textit{Becn1} allele loss do not undergo LOH, suggesting obligate haploinsufficiency\textsuperscript{49}. In addition, autophagy abrogation by complete loss of \textit{Atg7} delays progression of \textit{Pten}-deficient prostate tumors\textsuperscript{56}. Our results parallel these observations, as heterozygous \textit{Wac} deletion promoted prostate tumor progression, whereas its complete loss constrained it. Autophagy exerts dual effects on cancer, suppressing initiation but promoting growth of established cancers\textsuperscript{49,52}. Accordingly, Wac inactivation led to significantly larger tumors in 4-month-old \textit{Pten}\textsuperscript{−/−}\textit{Wac}\textsuperscript{−/−} mice but only a tendency for lesions with increased size was observed 5 months later (Fig. 6a,b). These findings, which are likely to underlie the modest decrease of \textit{WAC} mRNA levels that accompanies human prostate cancer progression, are relevant for basic and translational oncology. Moreover, the dependence of \textit{Pten}-deficient tumors on \textit{Wac} function opens up potential opportunities for drug development.

Our approach recapitulates the sporadic nature of human tumorigenesis, in which mutations in relevant cancer genes occur randomly in individual cells from any tissue surrounding by healthy cells. Beyond prostate cancer, mice in our cohorts developed several tumor types for which \textit{Pten} relevance is well documented. Exploration of the potential of the novel putative tumor suppressors in preventing such cancers is warranted. Hopefully, our results will provide a basis for the development of therapeutic strategies inspired by the tumor suppressor networks identified through this screen.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank the staff members of the Research Support Facility at the Wellcome Trust Sanger Institute, the Laboratory of Molecular Medicine at IMOMA, and those at the Transgenic Animal Unit, the Molecular Histopathology Unit, the Department of Biochemistry and Molecular Biology and the Biobank of the Principality of Asturias at UOAPA, for excellent technical assistance. This work was supported by grants from the Wellcome Trust (grant no. 098051; A.B.), the Ministerio de Economía y Competitividad–Spain (grant no. SAF2014-52413; C.L.-O.) and the German Research Society (grant no. SFB1243; R.R.), as well as by funding from the Fundación María Cristina Masaveu Peterson (J.C.), the Fundación Centro Médico de Asturias (J.C.), the Fundación Bancaria Caja de Ahorros de Asturias/Liberbank (J.C. and A.A.), FEBS (J.d.l.R. and J.C.), CIBERONC (Plan Feder) (C.L.-O.), the Progeria Research Foundation (C.L.-O.), the EDP Foundation (C.L.-O.) and the German Cancer Consortium (R.R.). G.S.V. is funded by a Wellcome Trust Senior Fellowship in Clinical Science (WT095663MA). J.d.l.R. is a recipient of a FEBS Long-Term Fellowship and was a recipient of a fellowship from the Fundación María Cristina Masaveu Peterson during part of this work. J.C. was a recipient of a FEBS Long-Term Fellowship in the initial phases of this work.

AUTHOR CONTRIBUTIONS

J.d.l.R., R.R., C.L.-O., A.B. and J.C. designed the study; J.d.l.R., J.W., R.R. and J.C. generated mouse alleles and cohorts, and performed experiments; J.d.l.R., J.W., I.R., Q.L., M.A.I., G.S.V., R.R. and J.C. performed mouse necropsies; A.A., M.S.F.-G., M.T.F.-G. and G.H. performed histopathological analysis; J.d.l.R., M.J.F. and H.P. did bibliometrics analyses; J.d.l.R., C.L.-O. and J.C. interpreted results; M.T.F., S.R.D.O., I.N., E.M., A.S. and R.E. contributed to some of the experiments; R.R., C.L.-O., A.B. and J.C. supervised the study; and J.d.l.R. and J.C. wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mouse strains. All mouse experiments were conducted in accordance with the guidelines of the Animal Scientific Procedures Act of 1986 and the Committee on Animal Experimentation of the University of Oviedo. Rosa26SB11 transposase knock-in, Blm mutant (Blm<sup>m3</sup>), prostate-specific PB-cre<sup>+</sup> deleter and Fp<sup>+</sup> deleter mice were previously described. To generate PTSA3 and PTSA4, two mice were obtained from the Wellcome Trust Sanger Institute (International Knockout Mouse Consortium). The generation of Pten<sup>Shm2</sup> and ITP2m<sup>+</sup> mice is described in the following sections. To generate the experimental and control mice used in the transposon screens, Pten<sup>Shm2</sup>, ITP2m and Blm<sup>m3</sup> mice were successively interbred to generate Pten<sup>Shm2</sup> and ITP2m; Blm<sup>m3</sup> triple-transgenic mice. At the same time, Rosa26SB11 mice were crossed with Blm<sup>m3</sup> mice, and their double-heterozygous descendants were crossed with each other to produce Rosa26SB11; Blm<sup>m3</sup> mice. Finally, Pten<sup>Shm2</sup>; ITP2m; Blm<sup>m3</sup> mice were crossed to Rosa26SB11; Blm<sup>m3</sup> mice to obtain Pten<sup>Shm2</sup>; ITP2m; Blm<sup>m3</sup> (PSB), Pten<sup>Shm2</sup>; ITP2m; Rosa26SB11; Blm<sup>m3</sup> (PISB), ITP2m; Rosa26SB11; Blm<sup>m3</sup> (ISB) and Rosa26SB11; Blm<sup>m3</sup> (SB) mice. To generate mice with a Wac conditional-knockout allele (referred to as Wac<sup>m2a(EUCOMM)/Wtsi</sup> mice), Wac<sup>m2a(EUCOMM)/Wtsi</sup> knockout-first mice were initially crossed to Fp<sup>+</sup> transgenic mice to remove the FRT-flanked disrupting cassette. Subsequently, the offspring carrying the tm2z allele was outcrossed to wild-type C57BL/6 mice, and those animals with the tm2z allele and negative for Fp<sup>+</sup> were selected and interbred. To generate a prostate-specific deletion of Pten and Wac, Pten<sup>Shm2</sup>; Wac<sup>m2a(EUCOMM)/Wtsi</sup> knockout-first mice were initially crossed to Fp<sup>+</sup> transgenic mice to remove the FRT-flanked disrupting cassette. Subsequently, the offspring carrying the tm2z allele was outcrossed to wild-type C57BL/6 mice, and those animals with the tm2z allele and negative for Fp<sup>+</sup> were selected and interbred. To generate the experimental and control mice used in the transposon screens, TRs were cloned into pBlueScript, and the following genetic elements were introduced in-between them: an adenosine splice acceptor (AV-SA), five bidirectional SV40 polyadenylation signals (pA) and a splice acceptor from exon 2 of the mouse engravILLED gene (En2). ITP2m transposons were cut out of pBlueScript and prepared for pronuclear injection, using standard techniques as described in Rad et al.<sup>37</sup>

Histopathological analysis. Mice were monitored for tumors at least twice a week, and they were euthanized before tumor masses compromised their well-being or at the onset of other signs of morbidity. For DNA extraction, tumors were snap-frozen in liquid nitrogen. For histological studies, tumors were fixed in 4% formaldehyde, paraffin-embedded, sectioned and stained with hematoxylin and eosin for morphological examination. Tumors were harvested, and sections were stained with hematoxylin and eosin for morphological analysis. Tissue samples were examined by four experienced histopathologists (A.A., M.S.F.-G., M.T.F.-G. and G.J.H.) who were blinded to the mouse genotype. Samples were excluded if the post-necropsy genotyping did not confirm the initial genotyping. Tissue samples were examined by four experienced histopathologists (A.A., M.S.F.-G., M.T.F.-G. and G.J.H.) who were blinded to the mouse genotype.

Immunohistochemistry. Tissue microarrays that contained 114 formalin-fixed paraffin-embedded mouse tumor sections (cut at 5 µm) from PSB or PISB mice were used for immunohistochemical detection of Pten, p63 and Ki67 on a DAKO Autostainer. After deparaffinization, heat-induced antigen retrieval was performed. Subsequently, primary antibody incubation was carried out using the following antibodies: mouse monoclonal anti-Pten (M3627, Agilent Technologies, 1:200), mouse monoclonal anti-p63 (1B662, Agilent Technologies, ready-to-use) and rabbit monoclonal anti-Ki67 (K1681C01, DCS Innovativ Diagnostik-Systeme, 1:150), respectively. Sections were then incubated with anti-mouse (for Pten and p63) or anti-rabbit (for Ki67) secondary antibodies from Agilent Technologies for 30 min at room temperature and then stained with the chromogen 3-3′-diaminobenzidine (DAB; Dabo). Finally, they were counterstained for 10 min with Dako Hematoxilin. Ki67 and Pten staining intensities were graded as negative (−), mild (+), moderate (+++) and intense (++++) in the PIN, adenocarcinoma and normal tissue components of each tumor.

Splinkerette PCR and Illumina sequencing. These were done as previously described.<sup>37</sup>

Identification of common integration sites (CIS). Transposon insertions were mapped to the mouse genome using the SSAHA2 algorithm. Query sequences were filtered to contain splinkerette primer sequences that were located in the transposon inverted terminal repeats (ITRs). Redundant sequences from the same tumor and merging to the same genomic location were ‘collapsed’ to a single integration. To identify those regions in the genome that were hit by transposons significantly more frequently than expected by chance (referred to as CIS), nonredundant insertions were analyzed by using a Gaussian kernel convolution-based framework<sup>18</sup> for different kernel window sizes (from 10 kb to 100 kb, in 10 kb steps, plus an extra 200-kb window). CIS predicted across multiple scales and overlapping in their genomic locations were clustered together, and only the ones obtained using the smallest windows were...
reported. SfiI, a known artifactual CIS frequently found in transposon screens, was filtered out from the definitive CIS lists. Curated lists were obtained after removal of predicted genes.

RNA-seq analysis. RNA-seq libraries were constructed using the Illumina TruSeq Stranded RNA protocol with oligo-dT pulldown and sequenced on an Illumina HiSeq2500 by 75-bp paired-end sequencing. For RNA-seq transcriptional profiling of the BPH-1 and RWPE-1 cell lines, analysis was performed using TopHat\textsuperscript{60}, version 2.0.13. Read counts were obtained using HTSeq\textsuperscript{61} version 0.6.1 and differential expression analysis was performed using the DESeq2 software package\textsuperscript{62} version 1.14.1. RNA-seq analysis of transposon–CIS RNA chimeric transcripts was done as previously described by Temiz et al.\textsuperscript{63} Sequencing reads were aligned to the mouse reference genome GRCm38, with exon 5 of \textit{Pten} masked at the locus and the transposon sequence containing \textit{Pten} exon 5 added to the reference as a separate sequence. Alignment to this modified reference genome was performed using GSNAP\textsuperscript{64} version 2015-11-20, and fusions with \textit{Pten} exon 5 were identified using our own software implementation in the scripting language Python. Python scripts used for the fusion analysis are available upon request.

Pathway enrichment analysis. Pathway enrichment analysis was performed by DAVID\textsuperscript{65} (using KEGG, BioCarta and GO term data sets) and the GSEA\textsuperscript{66}Pre-ranked module from GSEA v3.0 (using hallmark and canonical pathways data sets\textsuperscript{66}). Results obtained with DAVID were visualized by the Cytoscape Enrichment Map plugin\textsuperscript{66}.

Gene-silencing and invasion assays. For gene-silencing experiments, BPH-1 and RWPE-1 immortalized human prostate cells, obtained from the American Type Culture Collection (ATCC) and previously checked to exclude mycoplasma contamination, were transfected with 10 nM final concentration of siRNA oligonucleotides (purchased from Life Technologies; Silencer Select Pre-Designed and Validated siRNAs) using Lipofectamine RNAi Max (Life Technologies) as per the manufacturer’s instructions. Two days later, their invasive potential was evaluated using 24-well Matrigel-coated invasion chambers with an 8-mm pore size (BD Biosciences). For BPH-1, 6 × 10\textsuperscript{5} cells were allowed to invade for 72 h using 15% FBS as a chemoattractant, whereas for RWPE-1 cells, 8 × 10\textsuperscript{5} cells were seeded and allowed to invade for 48 h, using bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) as chemoattractants. Cells that reached the lower surface were stained with crystal violet and counted under the microscope.

SDS–PAGE and western blot. Cultured cells were homogenized in SDS lysis buffer containing 100 mM Tris–HCl pH 7.4, 2% SDS, 50 mM EDTA pH 8, protease inhibitor cocktail (P8340, Sigma) and phosphatase inhibitor cocktails (P5726 and P0044, Sigma). Protein concentration was evaluated with the bicinchoninic acid assay (Pierce BCA Protein Assay Kit). Equal amounts of proteins (10 µg) were loaded onto 4–20% precast polyacrylamide gels (BioRad). After electrophoresis, gels were electrotransferred onto PVDF membranes (BioRad), blocked with 5% nonfat dry milk in TBS-T buffer (20 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature and incubated overnight at 4 °C with various primary antibodies: rabbit monoclonal anti-Pten (9188, Cell Signaling, 1:1,000), rabbit monoclonal anti-akt (4060, Cell Signaling, 1:1,000), rabbit monoclonal anti-AKT (4691, Cell Signaling, 1:1,000), rabbit monoclonal anti-phospho-akt (2971, Cell Signaling, 1:1,000), rabbit monoclonal anti-mTOR (2983, Cell Signaling, 1:1,000) or mouse monoclonal anti-GAPDH (G8795, Sigma, 1:10,000). Finally, we incubated the blots with goat anti-rabbit or horse anti-mouse horseradish-peroxidase-conjugated secondary antibodies (Cell Signaling) diluted 1:3,000 in 2.5% nonfat dry milk in TBS-T, washed them and developed the immunoreactive bands with Clarity Western ECL (Bio-Rad).

Quantitative RT–PCR. For qRT–PCR, cells were collected 72 h after transfection, and total RNA was extracted using the RNeasy Plus Mini kit (Qiagen). cDNA was synthesized with the Thermoscript RT–PCR System (Invitrogen). qPCR was carried out in triplicate for each sample using 20 ng of cDNA per reaction, TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 µL of TaqMan Gene Expression Assay probes (Life Technologies) for ZBTB20, CELF2, PARD3, AKAP13, WAC and GAPDH (which was used as an internal control for the amount of template cDNA) (Supplementary Fig. 13).

Statistical analyses. We used Microsoft Excel, GraphPad Prism or R version 3.2.0 (The R Project for Statistical Computing,\textsuperscript{67}http://www.r-project.org/) software for calculations. Specific statistical tests, number of samples and data representation used in each analysis are indicated along the main text or in the figure legends. Data were checked to meet the assumptions of each test. The sizes of the animal cohorts were estimated on the basis of previous transposon-based somatic cancer screens and after preliminary observations of near-100% penetration of prostate cancer in PSB and PISB male mice.

Data availability statement. The RNA-seq data from mouse tumors for the identification of fusion transcripts and from the transcriptomic profiling of BPH-1 and RWPE-1 cell lines, which support the findings of this study, have been deposited in ArrayExpress with the accession codes E-ERAD-610 and E-ERAD-432, respectively. All other data supporting the conclusions can be found within the manuscript and its supplementary files.

Code availability. Python scripts used for the fusion transcript analysis are available upon request.

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