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A transgenic mouse model for early prostate metastasis to lymph nodes

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Abstract

The emergence of recurrent, metastatic prostate cancer following the failure of androgen-deprivation therapy represents the lethal phenotype of this disease. However, little is known regarding the genes and pathways that regulate this metastatic process, and moreover, it is unclear whether metastasis is an early or late event. The individual genetic loss of the metastasis suppressor, *SSeCKS/Gravin/AKAP12*, or *Rb*, genes that are downregulated or deleted in human prostate cancer, results in prostatic hyperplasia. Here, we show that the combined loss of *Akap12* and *Rb* results in prostatic intraepithelial neoplasia (PIN) that fails to progress to malignancy after 18 months. Strikingly, 83% of mice with PIN lesions exhibited metastases to draining lymph nodes, marked by relatively differentiated tumor cells expressing markers of basal (p63, cytokeratin 14) and luminal (cytokeratin 8 and androgen receptor) epithelial cells, although none expressed the basal marker, cytokeratin 5. The finding that PIN lesions contain increased numbers of p63/AR-positive, cytokeratin 5-negative basal cells compared to WT or *Akap12*^{−/−} prostate lobes suggests that these transitional cells may be the source of the LN metastases. Taken together, these data suggest that in the context of *Rb* loss, *Akap12* suppresses the oncogenic proliferation and early metastatic spread of basal-luminal prostate tumor cells.

Keywords

SSeCKS/Gravin/Akap12; Rb; prostate cancer; metastasis; lymph node

Introduction

Prostate cancer (CaP) is the most commonly diagnosed non-cutaneous cancer and the second leading cause of cancer mortality in U.S. men (<http://seer.cancer.gov/statfacts/html/prost.html>). During prostate carcinogenesis in humans, tumors progress from clinically undetectable precursor lesions, termed PIN (prostatic intraepithelial neoplasia), to locally invasive carcinomas often associated with metastatic disease to local lymph nodes and/or bones. Androgens act as pro-survival and proliferation factors in CaP cells such that chemical or surgical therapeutic castration to deplete serum androgen levels results in a rapid and in most cases, durable tumor regression. However, failure of androgen ablation

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therapy leads to so-called castration-recurrent (CR) CaP, marked by more aggressive, metastatic disease that progresses in the presence of very low, intracrine androgen levels (1).

Genetic comparisons between primary site and CaP metastases within the same patient have been unable to resolve whether metastatic spread is an early or late event (2–4). Comparative array-CGH analyses by Holcomb et al. (5) offer evidence of both possibilities: cases of strong relatedness between a patient's primary-site and metastatic lesions, suggesting late metastatic dissemination, versus cases in which there is more relatedness between metastases of different patients than their own primary-sites, suggesting early dissemination. Transgenic mouse CaP models have shown that at least two, and more typically, three genetic lesions are required to induce spontaneous metastasis (6). Yet, these models may be influenced by whether the oncogenic mutations are built into basal (7) or luminal prostate epithelial cells (8), or both (9). For example, building on evidence that Rb and p53 are often mutated or deleted in prostate cancer (10), a conditional Cre-loxP deletion of both Rb and p53 driven by a truncated probasin promoter (expressed in prostate and seminal vesicle epithelial cells) was produced (11). This model exhibits prostatic adenocarcinoma and lymph node metastases, whereas the loss of Rb alone causes prostatic hyperplasia (12).

SSeCKS/Gravin/AKAP12 ("Akap12") has been shown to function as a metastasis suppressor in prostate cancer, likely through its ability to scaffold key signaling and cytoskeletal proteins such as Src, protein kinase (PK)C, PKA, cyclin D and F-actin in a spatiotemporal manner (13). Akap12 re-expression suppressed macroscopic metastasis formation by inhibiting VEGF-mediated neovascularization at distal colonization sites (14). Akap12 is downregulated by Src-family kinases (15), known to be activated in CR-CaP progression (16, 17), and indeed, the forced re-expression of Akap12 suppresses Src-induced oncogenic proliferation, invasiveness and cell motility in CaP cells by directly scaffolding Src from growth factor receptor and FAK complexes to lipid rafts, without altering Src's intrinsic kinase activity (18). Consistent with its function as a tumor suppressor in the prostate, there is significant Akap12 loss in CaP progression, due to either transcriptional downregulation or allelic deletion (6q24–25.3)(19). In addition, Akap12-null mice exhibit prostate hyperplasia marked by increased levels of cell senescence (20). Akap12-null mouse embryo fibroblasts (MEF) suffer an Rb-dependent premature senescence marked by multinucleation or polyploidy, suggesting a role for Akap12 in the regulation of cytokinesis and cell aging (21). The relative ease of isolating immortalized Akap12-null MEF in which components of the Rb pathway were downregulated suggested that the combined loss of Akap12 and Rb might be sufficient for oncogenic transformation. Here, we show that male Akap12-null mice with concomitant prostate-specific Rb deletion readily formed low- and high-grade PIN lesions by eight months of age. Unexpectedly, 83% of these mice exhibit metastases in local draining lymph nodes, marked by relatively well differentiated tumor cells expressing markers of a transitional basal-luminal phenotype. Our data strongly suggest that CaP metastasis to the lymph node is an early event facilitated by the combined loss of Akap12 and Rb in the prostate.

Materials and Methods

Generation of mutant mice

Akap12^{-/-};*Pb-Cre*;*Rb*^{fl/fl} mice were generated by first producing *Akap12*^{-/-};*Rb*^{fl/fl} mice from crossing C57B/L6 *Akap12*^{-/-} mice (20) with FVB/N;129 *Rb*^{fl/+} mice (11), and then intercrossing *Akap12*^{+/-};*Rb*^{fl/+} parents. *Akap12*^{-/-};*Rb*^{fl/fl} females were then mated to C57B/L6 xDBA2 *Pb-Cre* males (11), and the *Akap12*^{+/-};*Pb-Cre*^{+/-};*Rb*^{fl/+} males mated to *Akap12*^{-/-};*Rb*^{fl/fl} females over several generations to yield *Akap12*^{-/-};*Pb-Cre*;*Rb*^{fl/fl} mice. Note that the *Pb-Cre* allele was only passed through males in the intermediate

lineages because of oocyte expression of the probasin promoter (23). All experiments were carried out in compliance with NIH guidelines and under the supervision of the RPCI-IACUC.

Genotyping

Rb^{fl/fl} alleles were identified by PCR of tail-snip DNA using primers Rb18M3' (5'GGCGTGTGCCATCAATG3') and Rb19EM5' (5'CTCAAGAGCTCAGACTCATGG3'); amplification of wild-type and floxed *Rb* gene sequences resulted in 235bp and 283bp DNA fragments, respectively, whereas Cre-excised alleles resulted in 260bp PCR products using primers Rb212M5' (5'CGAAAGGAAAGTCAGGGACATTGGG3') and Rb18M3'. Wild-type and *Akap12*^{-/-} genotypes were identified as described previously (20).

Immunohistochemistry (IHC) and immunofluorescence (IFA) analyses

Tissues were fixed in 10% buffered formalin (VWR, Radnor, PA) for 24h at RT prior to embedding in paraffin, and sectioning (5 µm). Serial tissue sections each containing separated prostate lobes, seminal vesicle, lymph nodes (inguinal and pelvic), lung, liver and kidney were stained first with hematoxylin and eosin (H&E) and then stained for Ki67 (Leica Biosystems, Buffalo Grove, IL, cat. #NCL-Ki67p; dilution 1:500), TUNEL, cytokeratin (CK) 5 (Covance, Princeton, NJ, cat. #PRB-160P; 1:2000), CK8 (Covance, cat. #MMS-162P; 1:1000), CK14 (Abcam, Cambridge, MA, cat. #Ab7800; 1:100), E-cadherin (Becton Dickinson Biosciences, San Jose, Ca, cat. #610181; 1:500), androgen receptor (AR) (Upstate Biotechnology-Millipore, Billerica, MA, cat. #06-680; 1:200), vimentin (Sigma, St. Louis, MO, cat. #A5691; 1:400) or p63 (Santa Cruz Biotechnology, Santa Cruz, CA, cat. #sc-8431; 1:1000) as described previously (20) using a DAKO Autostainer Plus system. Slides were dehydrated with graded alcohols and xylenes, then coverslipped, imaged and analyzed using an Aperio digital pathology system (Spectrum software v.11.0.0.725). Double IFA staining was performed on paraffin-embedded tissues with p63 (1:1000), CK5 (1:1000), and AR (Santa Cruz, cat. #sc-816; 1:100). Secondary antibodies include 1:1000 dilutions of AlexaFluor 568- or 488-goat-anti-mouse IgG or goat-anti-rabbit IgG (Invitrogen-Molecular Probes, Grand Island, NY). Fluorescent images were acquired on a Nikon TE 2000-E inverted microscope (Melville, NY) using Metavue v.7.7.6.0 software (Molecular Devices, Sunnyvale, CA). Histological assessment was based on published guidelines (22). p63/CK5/AR IHC co-staining was quantified on an Aperio Imaging System. 200–300 total epithelial cell nuclei were counted in at least 6 independent prostate regions, and the number of p63+/CK5+, p63+/CK⁻, or p63+/AR+ cells were reported as a percentage of total epithelial cells in a lobe.

RNA *in situ* hybridization of paraffin embedded tissue using DIG-labeled cRNA probes

241-bp cDNA templates for antisense or sense RNA probes were generated by PCR amplification (95°C/3 min; 37 cycles of 95°C/30 sec, 55°C/30 sec, 72°C/30 sec) of pBabeHygro-Cre plasmid DNA (gift of Eugene Kandel, RPCI) with the following primer pairs containing T7 RNA polymerase promoter sites (underlined):

Sense: forward, 5'-
TAATACGACTCACTATAGGGGCATTTCTGGGGATTGCTTA-3'

reverse, 5'-CCCGGCAAAACAGGTAGTTA-3

Anti-sense: forward, 5'-GCATTTCTGGGGATTGCTTA-3'

reverse, 5'-TAATACGACTCACTATAGGGCCCGGCAAAACAGGTAGTTA-3'

Digoxigenin-UTP-labeled antisense and sense RNA probes were generated by *in vitro* transcription using a DIG RNA Labeling Kit (Roche Applied Science, cat. #11175025910)

according to the manufacturer's instructions. The concentration of RNA probes was measured using a Thermo Scientific-Nanodrop and then adjusted with DEPC-treated water to 500 ng/ μ l. *In situ* hybridization was carried out as follows: 5 μ m sections of the formalin-fixed paraffin-embedded mouse prostate tissue was deparaffinized in three changes of xylene for 7 min each, washed twice in 100% ethanol for 5 min, followed by rehydration in a graded series of ethanols (95%, 70%, and 50%, 5 min each) and two washes in DEPC water for 3 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in DEPC water for 15 min and rinsed twice with DEPC water. The sections were then incubated at 37°C for 30 min in Tris-buffered saline (TBS: 50 mM Tris, pH 7.5, 180 mM NaCl) containing proteinase K (20 μ g/ml). After two washes in TBST (TBS plus 1% Triton X-100) for 3 min each, the sections were pre-hybridized for 1 h at 55°C in with 100 μ l hybridization buffer (Chemicon, Temecula, CA, cat. #S4040). DIG labeled sense or anti-sense probes were diluted to 1 ng/ μ l in 150 μ l of hybridization buffer, denatured at 65°C for 2 min and put on ice. Tissue sections were covered with the denatured RNA probes and incubated for 16–18 h at 55°C. Unhybridized probe was removed by washing in 2X pre-warmed SSC (300 mM NaCl, 30 mM trisodium citrate, pH 7.0) at 45°C for 10 min and RT for 5 min, and subsequently incubated for 30 min at 37°C with 250 μ l of RNase Cocktail (Invitrogen-Ambion, cat. # AM2286) diluted 1:30 in 2X SSC. The slides were washed twice at high-stringency in pre-warmed 50% formamide in 2X SSC for 30 min at 55°C, once with pre-warmed 0.08X SSC for 20 min at 55°C, and then rinsed twice with TBST. Probe hybridization was detected using a DAKO autostainer, as follows: tissue sections were blocked with Super Block (Thermo-Pierce, cat. #37535) for 1 hr, incubated for 30 min with anti-DIG-HRP conjugate (Roche, #11207733910; 1:100) in Super Block, three washes with TBST for 5 min each, and then for 15 min with biotinylated rabbit anti-sheep Ig (Vector Labs, Burlingame, CA, cat. #BA-6000; 1:1000). Signal enhancement was facilitated by 30 min incubation with ABC reagent (Vector, cat. #PK 6100), 5 min incubation with DAB substrate (Dako-Agilent, Carpinteria, CA, cat. #K3467) and counterstaining with modified Harris hematoxylin (Thermo-Richard-Allan Scientific, Waltham, MA, cat. #72704) for 20 sec. The slides were dehydrated with graded alcohols and then xylenes, coverslipped and digitally analyzed using an Aperio system.

Results

Combined deletion of *Akap12* and *Rb* leads to PIN within 6 months

To assess a possible cooperation between *Akap12* and *Rb* in prostate carcinogenesis, *Akap12*-null mice were crossed with *Pb-Cre;Rb^{fl/fl}* (11), resulting in *Akap12*^{-/-};*Pb-Cre;Rb^{fl/fl}* mice (hereto designated *Akap12*^{-/-};*Rb^{PE/-}*). In males, the prostate-specific composite rat probasin promoter, *Pb4*, is expressed in basal and luminal prostate epithelium in all prostatic lobes (23). Prostate-specific deletion of the *Rb* exon 19 in all lobes was confirmed by PCR (Supplementary fig. S1A) in 3 week-old mice. Importantly, no Cre-mediated recombination was detected in other organs such as the inguinal lymph nodes and kidney (Supplementary fig. S1B).

As reported previously, *Akap12*-null mice exhibit prostatic hyperplasia starting at 4 months of age (20) (Fig. 1A); similar effects were produced in *Pb-Cre;Rb^{fl/fl}* mice (11, 12). Year-old *Akap12*-null mice showed focal dysplasia in fewer than 30% of cases, and one case of low-grade prostatic intraepithelial neoplasia (LGPIN) was detected in an 18 month-old (Fig. 1B). In contrast, *Akap12*^{-/-};*Rb^{PE/-}* developed LGPIN in all of prostate lobes starting at 5 months (Fig. 1B) marked by a focal loss of columnar polarity containing cells with slightly hyperchromatic, elongated and irregular nuclei (Fig. 1A). 29% of *Akap12*^{-/-};*Rb^{PE/-}* mice also exhibited high grade (HG) PIN (Table 1) between 6–13 months of age marked by foci

highly atypical cells with severe nuclear pleomorphism and hyperchromasia (Fig. 1A). DP and LP lobes exhibited a more severe phenotype, including HGPIN, than VP or AP lobes.

In order to better classify the neoplastic phenotype of the prostate lesions in *Akap12*^{-/-}; *Rb*^{PE/-} mice according to the criteria set forth by Pienta et al. (22), DP lobes from WT, *Akap12*^{-/-}, or *Akap12*^{-/-}; *Rb*^{PE/-} mice were analyzed by immunohistochemistry (IHC) for progression biomarkers. Compared to *Akap12*^{-/-} prostates, *Akap12*^{-/-}; *Rb*^{PE/-} prostates displayed increased numbers of Ki67- and p63-positive cells, increased nuclear AR staining levels and increased cytoplasmic CK8 levels (Fig. 2, panels A, C, D and F). AR expression is E2F1-inducible, and thus, its upregulation would be expected following the loss of *Rb* (24). Total E-cadherin staining was increased although heterogeneous, and significantly, there was little of the normal cell-cell demarcation pattern still retained in the hyperplastic *Akap12*^{-/-} lesions, owing to the loss of polar epithelial organization in the PIN lesions (Fig. 2, panel B). Unexpectedly, the increased number of p63-positive cells in *Akap12*^{-/-}; *Rb*^{PE/-} lesions did not correlate with a similar increase in positivity for CK5 (Fig. 2, panel E), another basal epithelial cell marker. The relative slow growth of these PIN lesions even in the presence of increased apparent proliferation markers was likely explained by a concomitant increase in apoptosis, as measured by TUNEL assay (Supplementary figs. S2A & B). It is noteworthy that the *Akap12*^{-/-}; *Rb*^{PE/-} lesions displayed increased stromal layers as well as infiltrates of possible inflammatory cells (Fig. 1A) that were strongly positive for Ki67 and AR staining (Fig. 2, panels A and C).

Basal cell proliferation in *Akap12*^{-/-}; *Rb*^{PE/-} PIN lesions

A hallmark of many transgenic mouse models of PIN or prostatic adenocarcinoma is a concomitant loss of basal cells amidst proliferating epithelial cells expressing luminal markers such as CK8 or 18 (22). However, there is evidence that prostate cancer can arise from basal epithelial cells that transition to a luminal phenotype including induction of AR and CK8 expression (7, 9). We followed up on the observation of increased numbers of p63-positive cells in the *Akap12*^{-/-}; *Rb*^{PE/-} PIN lesions by first showing that all four lobes displayed significantly increased numbers of basal cells, especially in the DP and AP, compared to WT or *Akap12*^{-/-} mice (Fig. 3A). Moreover, the normally flat cell morphology of a basal cell layer is replaced by apolar cells with enlarged, atypically-shaped nuclei containing segmented chromatin (Fig. 3B). Double staining for p63 and CK5 showed that WT and *Akap12*^{-/-} basal cells had fully concordant co-expression of both markers, whereas there was a 35% decrease p63-positive cells in the *Akap12*^{-/-}; *Rb*^{PE/-} lesions co-staining with CK5 compared to controls (Fig. 3C, *left panels*; Fig. 3D; Supplementary fig. S3). Additionally, whereas nuclear androgen receptor (AR) staining was mainly in WT or *Akap12*^{-/-} luminal cells (Fig. 3C, *right panels*; Supplementary fig. S3), many *Akap12*^{-/-}; *Rb*^{PE/-} cells displayed combined p63- and AR-positivity. Taken together, these data suggest that *Akap12*^{-/-}; *Rb*^{PE/-} PIN lesions contain both proliferating luminal as well as transitional basal-luminal epithelial cells.

Loss of *Akap12* and *Rb* results in significant prostate metastasis to the lymph node

None of the *Akap12*^{-/-}; *Rb*^{PE/-} mice aged to 24 months showed evidence of adenocarcinoma, indicating that the combined loss of *Akap12* and *Rb* was only partially oncogenic in regards to the primary prostate site. However, consistent with *Akap12* functioning as a metastasis suppressor in prostate cancer cell lines (14, 18, 25), and with the increased levels of *AKAP12* promoter hypermethylation in human prostate cancer lesions with Gleason grades 8–10 (26), 83% of the *Akap12*^{-/-}; *Rb*^{PE/-} mice that had PIN lesions also displayed metastases in their pelvic or inguinal lymph nodes (LN) (Fig. 4A; Table 2). In contrast, none of the WT or *Akap12*^{-/-} mice had such lesions. Moreover, this frequency of metastasis is significantly higher than in mice with combined deletions of *Rb* and p53 in the

prostate (83% vs. 68% $\pm$ 8)(11). All mice with LN metastases had foci of primary site HG-PIN, many of which showed clear evidence of localized invasiveness, an example of which is shown in Supplementary fig. S4A. Notably, macroscopic metastases were not detected in other peripheral organs or non-pelvic LN, and moreover, the pelvic LN lesions (Fig. 4A) were detected as early as one month following the earliest appearance of PIN lesions (Fig. 1B). We cannot rule out the possibility that other sites contain micrometastases. However, to address whether the increased metastasis in *Akap12*^{-/-};*Rb*^{PE/-} mice correlates with changes in circulating tumor cells (CTC), peripheral blood samples obtained by cardiac puncture from *Akap12*^{-/-};*Rb*^{PE/-}, *Akap12*^{-/-} or *Rb*^{PE/-} mice (6 mice/group) were analyzed by sensitive PCR for CTCs, either for the floxed form of *Rb* or for the *neo*^R cassette (in *Akap12*^{-/-} mice). Although this assay could readily detect 1-CTC in 10⁵–10⁶ buffy coat cells (Supplementary fig. S5A), no increases in CTCs were found in *Akap12*^{-/-};*Rb*^{PE/-} compared to *Akap12*^{-/-} mice (Supplementary fig. S5B). This strongly suggests that the increased LN metastasis in the *Akap12*^{-/-};*Rb*^{PE/-} mice results from increased localized trafficking, possibly through the known ability of SSeCKS/Akap12 to regulate chemotaxis (25, 27). Another characteristic of the metastases is that these lesions are primarily within the LN sinusoids, with little or no evidence of invasion to the lymphoid follicles (Fig. 4B). This suggests that the disseminating cells are a subset of primary-site PIN cells that have gained trafficking potential yet have failed to re-differentiate to establish a mature metastatic focus.

The LN metastases contained tumor cells with highly epithelioid morphologies, lacking mesenchymal morphologies typical of aggressiveness. Moreover, they expressed both basal and luminal epithelial markers: strong staining for p63 (96% of mice with positive staining), CK8 and 14 (78% and 67% positivity, respectively), E-cadherin (82% positivity) and vimentin (60% positivity) but not for CK5 (0% positivity)(Fig. 4B; Supplementary fig. S6). It is noteworthy that the same CK5 antibody stained basal epithelial cells in prostate lobes (Fig. 2E) on the same slide as the LN samples, ruling out the possibility of a technical artifact. Staining for nuclear AR and E-cadherin was heterogeneous amongst cells within LN lesions (Fig. 4B), although there was a significant increase in TUNEL-positive apoptotic cells compared to controls LNs (Supplementary fig. 2C). Fewer than 5% of the LN lesions homogeneously expressed the neuroendocrine marker, synaptophysin (Supplementary fig. S4B), and taken with the expression of cytokeratins in the remaining 95%, this indicates that the vast majority were likely adenocarcinoma precursors. We excluded the possibility that the LN lesions were CD68-positive histiocytes (Supplementary fig. S6), i.e.- cells that might be recruited in cases of chronic inflammation.

Similar to the mild oncogenic penetrance of the primary PIN lesions, the LN metastases did not grow aggressively although the inguinal LNs of 10 month-old *Akap12*^{-/-};*Rb*^{PE/-} mice typically weighed 3–4 times more than *Akap12*^{-/-} controls. Notably, foci could be detected within *Akap12*^{-/-};*Rb*^{PE/-} LNs that contained cells with severe nuclear pleomorphism and abnormal nucleus to cytoplasm ratios (Fig. 4B, inset arrow) typical of HGPIN or carcinoma. PCR analysis of DNA isolated from laser microdissected LN or prostate tumor tissue indicated that the LN metastases were of prostatic origin in that they, but not tail DNA, encode Cre-mediated deletions of the *Rb*^{fl/fl} locus (Fig. 4C). Importantly, LN DNA from 3 week-old *Akap12*^{-/-};*Rb*^{PE/-} newborns were negative for the *Rb*^{fl/fl} locus deletion (Supplementary fig. S1B), strongly suggesting that the *Rb*-deleted cells in the adult LN originated from prostates.

In order to strengthen the notion that that most, if not all, the LN lesion cells were of prostate origin, we performed RNA *in situ* hybridization using single-stranded Cre probes. The Cre probe showed no hybridization signals in primary VP lobes or LN from WT, *Rb*^{PE/-} or *Akap12*^{-/-} mice (Fig. 4D; Supplementary fig. S7), whereas it induced strong

cytoplasmic signals in both the PIN and LN lesions from *Akap12*^{-/-};*Rb*^{PE/-} mice (Fig. 4D). Moreover, young *Akap12*^{-/-};*Rb*^{PE/-} mice (<4 mo.), which show no evidence primary-site PIN disease (Fig. 1B), showed no Cre expression in LN (Supplementary fig. S7). These data strongly suggest that the loss of Akap12 can potentiate an early metastatic colonization of *Rb*-negative prostate cells to local LNs, although oncogenic progression in the prostate and LN is limited to non-malignant HGPIN.

DISCUSSION

Building on our observation that the loss of Akap12 induces *Rb*-dependent senescence associated with the hyperplasia of luminal prostate epithelial cells (21), we now show that the crossing a prostate-specific *Rb* loss into an Akap12-null background induces PIN lesions that do not readily progress to adenocarcinomas. Indeed, whereas spontaneous death was rare even in 16–20 month-old *Akap12*^{-/-};*Rb*^{PE/-} mice, the median survival for the combined prostate-specific loss of p53 and *Rb* was only 200 days (11). Although the prostate lesions in *Akap12*^{-/-};*Rb*^{PE/-} mice display increased markers of proliferation, such as Ki67, it is likely that a concomitant increase in apoptosis in all four prostate lobes (as measured by TUNEL staining) antagonizes further progression to malignancy. This suggests that the combined loss of *Rb* and Akap12 fails to fully defeat all senescence pathways. Examples of parallel, tertiary senescence control mediators include p21, which mediates senescence of PC-3 CaP cells following activation of the androgen receptor (28), or SMAD4, which facilitates CaP malignancy in the background of PTEN loss (29).

Given the lack of malignant progression of the primary prostate lesions in *Akap12*^{-/-};*Rb*^{PE/-} mice, the high incidence of LN metastasis was notable and suggests that prostate cancer metastasis to the LN can be an early event. The LN lesions, all of which derived from mice with focal HG-PIN, contained cells with epithelioid, non-aggressive morphologies and expressed both basal and luminal prostate epithelial markers, such as CK14, p63, CK8, E-cadherin and AR, but not the basal marker, CK5. This correlated in the primary-site PIN lesions with a significant increase in total p63⁺ cells yet a relative decrease in coincident CK5 co-staining. The p63⁺CK5⁻ cells in the primary PIN lesion lack typical basal cell morphologies and polarization, and the increase in this population correlates with increased nuclear AR expression. Although further experiments are required, it is interesting to speculate that the LN metastases arise from basal cell populations that are transitioning to more luminal phenotypes. Interestingly, over 50% of human prostate cancer cases with minimal primary site disease show evidence of PSA-positivity in the bone marrow (2) or disseminated tumor cells in local lymph nodes (30), strongly suggesting early metastatic dissemination. Given that the majority of initial CaP metastases in the clinical setting are found in local draining LN and bones

In regards to the CK5-negative lesions in our model's PIN and LN lesions, although human CK5-positive basal prostate epithelial cells can serve as progenitors of luminal prostate cancers (7, 31), and CK5/18-positive transitional cells have been described in human CaP (32), small populations of CK5-negative basal cells have been described (33) which our genetic alterations may have selected. Moreover, CK14-expressing prostatic basal cells can serve as CaP progenitors in transgenic models irrespective of their ability to express CK5 (9).

Previous data from our group indicated that Akap12 can suppress metastatic motility, chemotaxis and invasiveness by inhibiting PKC-mediated pathways through direct scaffolding of PKC isoforms (25) and by physically scaffolding Src away from FAK adhesion complexes, thereby attenuating MEK/ERK pathways controlling oncogenic motility (18). Thus, the ability of Akap12 to attenuate Src-induced oncogenic pathways (18,

25, 34, 35) likely contributes to its metastasis-suppressing activity, especially given the renewed focus on the role of Src-family kinases in mediating several parameters of metastatic and castration-recurrent prostate cancer (36, 37).

Our findings are consistent with a role for Akap12 in suppressing metastatic progression such that loss of Akap12 in specific tissues, such as the prostate epithelium, results in a metastasis-prone condition (Fig. 4E). Indeed, of the 37 cases of metastatic CaP from Taylor et al. (38) analyzed in the *cbio* website (<http://www.cbioportal.org/public-portal/>), the 11 cases which displayed AKAP12 downregulation correlated with a more rapid appearance of metastases compared to the 26 cases that showed no changes in AKAP12 expression levels (Fig. 4F). Taken together with our findings, these data argue strongly for a role for AKAP12 in suppressing specific parameters of metastatic growth of prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The combined loss of Akap12 and Rb results in PIN lesions

A. H&E staining of formalin-fixed paraffin sections of prostatic lobes from 8 month-old WT, *Akap12*^{-/-}, and *Akap12*^{-/-}; *Rb*^{PE-/-} mice. The hyperplasia of luminal epithelial cells found in *Akap12*^{-/-} mice (yellow arrowhead) was replaced by PIN (arrow) in *Akap12*^{-/-}; *Rb*^{PE-/-} mice. Insets show nuclear atypia with prominent nucleoli. **B.** Graphic analysis of PIN formation in WT, *Akap12*^{-/-}, and *Akap12*^{-/-}; *Rb*^{PE-/-} mice over time, based on mouse numbers (n) per genotype.

Fig. 2. IHC analysis of *Akap12*^{-/-};*Rb*^{PE-/-} PIN lesions

AP lobes from 8 month-old WT, *Akap12*^{-/-}, and *Akap12*^{-/-};*Rb*^{PE-/-} mice were stained for Ki-67 (A), E-cadherin (B), AR (C), p63 (D), CK5 (E) or CK8 (F). Cell-cell junctional E-cadherin staining in Panel B (arrow) is contrasted with cytoplasmic staining (arrowhead). A focus of increased nuclear AR staining in *Akap12*^{-/-};*Rb*^{PE-/-} vs. WT is shown in Panel C (insets; arrows).

Fig. 3. *Akap12*^{-/-};*Rb*^{PE-/-} PIN lesions display hyperplasia of p63-positive, CK5-negative basal epithelial cells

A. Increased numbers of p63+ cells in *Akap12*^{-/-};*Rb*^{PE-/-} vs. WT or *Akap12*^{-/-} prostate lobes. p63+ cells counted in 6 independent low-magnification fields (at least 40 cells/field) were normalized to total numbers of epithelial cells. Error bars, S.E.; *, p<0.05; **, p<0.01. **B.** IHC staining for p63 in *Akap12*^{-/-};*Rb*^{PE-/-} vs. WT AP lobes (8 month-old) showing an increase in basal cells displaying a loss of normal basal cell morphology *Akap12*^{-/-};*Rb*^{PE-/-} mice. **C.** IFA co-staining for p63/CK5 or p63/AR in *Akap12*^{-/-};*Rb*^{PE-/-} vs. WT or *Akap12*^{-/-} AP lobes. *Arrowheads*, p63⁺CK5⁻ basal cells in PIN lesions; *arrows*, p63⁺AR⁺ basal cells. Scale bar, 20 μ m. **D.** Six independent microscopic fields of VP lobes in Panel **B** co-stained for p63 and CK5 (at least 25 cells/field) were quantified for p63+ cells that co-stain for CK5. Error bars, S.E.; *, p<0.01.

Fig. 4. Lymph node metastases in *Akap12*^{-/-};Rb^{PE-/-} mice

A. Incidence of LN metastasis in WT, *Akap12*^{-/-}, and *Akap12*^{-/-};Rb^{PE-/-} mice based on numbers of mice (n) per genotype. **B.** Characterization of cell markers by IHC. Pelvic LNs from 13 month-old *Akap12*^{-/-};Rb^{PE-/-} mice were analyzed for basal (p63, CK5 and CK14) or luminal (AR, E-cadherin) epithelial cells markers, as well for a marker of mesenchymal transition (vimentin). Note the lack of CK5 staining. Arrow, examples of HG-PIN-like cells with pleiomorphic nuclei and abnormal nuclear:cytoplasmic ratios. **C.** PCR-based confirmation of the prostatic origin of the LN tumor. PIN or LN metastasis lesions were isolated using laser capture microscopy (RPCI Pathology Resource Network- Carl Morrison, Director), and DNA isolated using a Qiagen QIAamp DNA Micro Kit was subjected to PCR using primers specific for the deleted Rb^{fl/fl} allele (Rb212M5', Rb18M3'; Supplementary fig. S1A) and then analyzed by agarose gel electrophoresis. DNA isolated from the tail snip of the same *Akap12*^{-/-};Rb^{PE-/-} mouse (PCR-positive for the undeleted Rb^{fl/fl} allele) was used as a negative control. **D.** RNA *in situ* hybridization for Cre expression in primary prostate (DP) vs. LN in 9 month-old WT, *Akap12*^{-/-} or *Akap12*^{-/-};Rb^{PE-/-} mice. Scale bars, 100 μ m. **E.** Oncomine analysis (<http://www.oncomine.org>) of Lapointe prostate cancer expression data (39) showing AKAP12 downregulation in human prostate cancer metastasis compared to levels in primary prostate cancer ("1° CaP") or normal prostate tissue. **F.** Kaplan-Meier plot analysis (<http://www.cbiportal.org/public-portal/>) of metastasis occurrence vs. time to onset in 37 prostate cancer metastasis cases from Taylor et al. (38) in which 11 cases (29.7%) displayed AKAP12 downregulation compared to levels in primary CaP lesions, versus 26 cases with no change in AKAP12 levels.

Table 1

Summary of prostatic epithelial defects in *Akap12*^{-/-};*Rb*^{PE/-} mice.

Genotype ^a	Normal	Hyperplasia	LGPIN	HGPIN
Wild-type	10/11 (91%)	1/11 (9%)	0/11	0/11
<i>Akap12</i> ^{-/-}	0/8	8/8 (100%)	1/8 (13%) ^b	0/8
<i>Akap12</i> ^{-/-} ; <i>Rb</i> ^{PE/-}	0/21	21/21(100%)	20/21 (95%)	6/21 (29%)

^a 6–13 months-old;

^b 18 month-old

Table 2

Frequency of LN metastases

Genotype ^a	LN metastases ^b
Wild-type	0/11
<i>Akap12</i> ^{-/-}	0/8
<i>Akap12</i> ^{-/-} ; <i>Rb</i> ^{PE-/-}	15/18 (83%)

^a 6–13 months-old^b Metastases were identified minimally by H&E staining and by IHC for p63, AR or E-cadherin.