Genetic Ablation of the Amplified-in-Breast Cancer 1 Inhibits Spontaneous Prostate Cancer Progression in Mice

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Introduction

Prostate cancer is a major health problem as it was diagnosed in more than 230,000 American men in 2004 (1). Prostate cancer–related death is closely associated with dissemination and metastasis. Therefore, understanding prostate cancer progression before metastasis will improve therapeutic approaches. The progression of prostate cancer is believed to proceed through multiple steps, from prostatic intraepithelial neoplasia (PIN) to locally invasive carcinoma and finally to metastatic disease (2). Altered expression of oncogenes or tumor suppressor genes plays a vital role in prostate tumor initiation and progression.

The amplified-in-breast cancer 1 (AIB1, SRC-3 or NCoA3) is a transcriptional coactivator that was initially found overexpressed in breast and ovarian cancers (3–7). AIB1 is a member of the p160 steroid receptor coactivator (SRC) family that also contains SRC-1 and SRC-2 (TIF2 or GRIP1; refs. 4, 8). Biochemical and in vitro cellular experiments have shown that the SRC family members can interact with steroid receptors in a ligand-dependent manner and enhance their transcriptional activities (9). In addition, AIB1 can interact with and coactivate a group of other p300/CBP-associated transcription factors, such as E2F1, Ets, nuclear factor κB, and signal transducers and activators of transcription (10–14).

In addition to breast and ovarian cancers, AIB1 is also overexpressed in endometrial and prostate cancers (5–7, 15–17). The connection between AIB1 overexpression in steroid-promoted cancers and its coactivator activity for steroid receptors shown by in vitro studies has strongly suggested that AIB1 plays an important role in hormonal promotion of tumorigenesis (6). However, recent studies have also found AIB1 amplification and overexpression in colorectal carcinomas, esophageal squamous cell carcinomas, gastric cancers, hepatocellular carcinomas, and pancreatic cancers (18–20), suggesting that AIB1 also participates in steroid hormone–independent cancers and contributes to tumorigenesis through transcription factors that are not steroid receptors.

Transgenic mouse models have been used to dissect the role of AIB1 in cancer. Overexpression of AIB1 under the control of mouse mammary tumor virus long terminal repeat in transgenic mice leads to tumor formation in the mammary gland, lung, pituitary, and uterus (21), suggesting a sufficient role of AIB1 overexpression in tumorigenesis. To understand the physiologic role of AIB1, our laboratory has generated AIB1−/− mice. These AIB1−/− mice exhibited growth retardation and impaired insulin-like growth factor (IGF) signaling (22). Our laboratory has also shown that AIB1 deficiency suppressed v-Ha-ras–induced breast cancer initiation and progression in an ovarian hormone–independent manner, and AIB1−/− mice were resistant to chemical carcinogen–induced mammary tumorigenesis (23, 24). These results imply that AIB1 participates in the initiation and/or progression of mammary tumors.

Emerging evidence suggests that AIB1 may also play a crucial role in prostate cancer. AIB1 was shown to be recruited to the prostate-specific antigen promoter by androgen receptor (AR) in cultured cells (25) and found to be overexpressed in human prostate tumors (16). Furthermore, AIB1 overexpression in these tumors correlated positively with cell proliferation and inversely
with cell apoptosis. Conversely, knockdown of AIB1 in cultured prostate cancer cells decreased cell proliferation, delayed G1-S transition, and increased cell apoptosis (16). These findings suggest that AIB1 expression might be an important regulator for prostate tumorigenesis and for proliferation and survival of cultured prostate cancer cells. However, the tumorigenic stage-associated profile of AIB1 expression and the in vivo role of AIB1 expression in the entire process of prostate cancer initiation and progression remained to be characterized.

In this study, we have examined AIB1 expression and its developmental function in the mouse prostate and generated transgenic adenocarcinoma of the mouse prostate (TRAMP) mice with wild-type (WT) or knockout (KO) AIB1 to investigate the role of AIB1 in prostate cancer initiation and progression. We show that AIB1 is only expressed in the stromal and basal cells of normal mouse prostates and is not required for androgen-stimulated luminal epithelial proliferation and prostate regeneration. AIB1 expression is induced in the transformed epithelial cells during prostate cancer progression. Most importantly, inactivation of AIB1 inhibits prostate tumor formation and arrests prostate tumor progression into poorly differentiated adenocarcinomas. Thus, the tumorigenesis-induced AIB1 expression in the epithelial cells is essential for prostate cancer progression.

Materials and Methods

Mice. Animal protocols were approved by the Animal Care and Use Committee of Baylor College of Medicine. AIB1+/−/ mice were generated by replacing a portion of the AIB1 genomic DNA with the promoterless LacZ sequence as described (22). Because AIB1+/−/ mice with C57BL/6j strain background were lethal before birth, the AIB1 mutant colony was maintained by breeding heterozygous mice with a mixed genetic background of 50% 129SvEv and 50% C57BL/6j. All offspring were genotyped by PCR as previously described (22). The C57BL/6j TRAMP mice harboring the Probasin-SV40 Tag transgene were previously reported (26). Female C57BL/6j TRAMP mice were bred to AIB1−/−/ male mice with 50% C57BL/6j and 50% 129SvEv genetic background to obtain female AIB1+/−/TRAMP and male AIB1+/−/ TRAMP founders with a mixed genetic background of 75% C57BL/6j and 25% 129SvEv. Subsequently, these founder mice were bred to generate WT/TRAMP, AIB1+/−/TRAMP, and AIB1−/−/ TRAMP mice. This breeding strategy ensured that all experimental mice were heterogeneous to the SV40 transgene and the mixed genetic background of 75% C57BL/6j and 25% 129SvEv was randomly distributed in all three genotype groups with equal opportunities. DNA was extracted from the ear biopsy and PCR was done for genotypic analysis as described (22, 26).

Measurement of prostate response to androgen. For all surgical procedures, mice were anesthetized by i.p. injection of Avertin (2.5% in saline, 15 μL/g body weight). Mice (WT and AIB1−/−/ littersmates) were castrated via a scrotal incision at 3 or 10 weeks of age. At 14 days after castration, 10-mm-long silastic capsules (Dow 02-285) filled with testosterone propionate (Sigma) were implanted s.c. in the mice. At the time of sacrifice, all prostate lobes were collected and weighed. To explore detail structure, the prostate was incubated in 1% collagenase (Sigma) for 10 min as described (27). The numbers of distant tips and branches were counted under a stereomicroscope (27).

Tissue examination and tumor grading. Before sacrifice, each mouse was anesthetized and weighed. All major organs were inspected for evidence of primary tumors and metastases. Necropsy notes and photographs were taken for documentation. At the time of sacrifice, the lower genitourinary tract, including the bladder, seminal vesicles, and all prostate lobes, was removed en bloc and placed in a Petri dish containing PBS to prevent desiccation. The bladder was emptied and the tissues were gently blotted to remove excess PBS; then, the entire genitourinary tract was weighed. Under a dissecting microscope, the dorsal, lateral, ventral, and anterior lobes of the prostate were separated and weighed. Periaortie lymph nodes were removed and weighed.

The following specimens were collected for histology: periaortie lymph nodes, liver, lung, seminal vesicles, and anterior, dorsal, lateral, and ventral lobes. All tissue samples were fixed in 10% (v/v) phosphate-buffered formalin for 12 h, dehydrated, and embedded in paraffin. The tissues were histologically evaluated and graded by examining the H&E-stained sections under a light microscope. The prostatic tissue was identified as normal, PIN, and well, moderately, or poorly differentiated adenocarcinomas according to the grading system previously described (28).

Immunohistochemistry and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. Immunohistochemistry was done as described (24). Sections were incubated with the following primary antibodies at 4°C overnight: monoclonal anti-T antigen (BD PharMingen), monoclonal anti–E-cadherin (BD Transduction Laboratory), monoclonal anti–cytokeratin 8 (C8k; Developmental Studies Hybridoma Bank), monoclonal anti-Ki67 (BD PharMingen), polyclonal anti-AR, and monoclonal anti-p53 (Santa Cruz Biotechnology). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining was done as previously described (22). X-Gal–stained sections were further fixed in formalin for 30 min before counterstaining with nuclear fast red for 2 min.

In situ hybridization. Prostate tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5-μm thickness. Rehydrated sections were sequentially treated with 0.2 N HCl for 15 min at 70°C, 2× SSC for 15 min at 70°C, and 3 μg/ml proteinase K for 15 min at 37°C. After washing in PBS containing 100 mM/L glycine, tissues were fixed in 4% paraformaldehyde for 20 min, acetylated with acetic anhydride in 0.1 mol/L triethanolamine (pH 8) for 10 min, and washed with 5× SSC for 15 min. Prehybridization was carried out in 50% formamide, 5× SSC, 500 μg/ml salmon sperm DNA, 250 μg/ml tRNA, and 5× Denhardt’s solution for 4 h. The mouse AIB1 antisense probe and its negative control sense probe were previously described (29). The probes were made with the DIG RNA Labeling System (Roche Applied Science), treated with DNase I, and checked on an agarose gel for quality control. Tissues were incubated overnight in the hybridization solution with probes at 65°C and washed with 2× SSC and then 0.1× SSC at 65°C. The sections were incubated in 100 mL/L Tris-HCl (pH 7.5) containing 2% blocking powder (Roche Applied Science) and 150 μmol/L NaCl, and then in anti-digoxigenin-AP (Roche Applied Science) at 1:1,000 dilution for 2 h. Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mL/L Tris-HCl (pH 9.5), 100 μmol/L NaCl, and 50 μmol/L MgCl2 for 5 h at room temperature. Slides were washed in 10 mL/L Tris-HCl (pH 8) with 1 mL/L EDTA and mounted with Aquamount Solution (Lerner Labs.).

Immunoblot analysis. For harvesting prostate samples at all ages for immunoblot analysis, the entire individual prostate lobes with or without tumors were carefully dissected under stereomicroscope. For a tumor at 30 weeks, both peripheral and central areas of solid tumor tissues were collected for Western blot. Samples were immediately frozen in liquid nitrogen and stored at −80°C till tissue lysates were prepared and immunoblot analysis was done as previously described (24). Primary antibodies used for immunoblot analysis are as follows: monoclonal anti–T antigen, monoclonal anti–E-cadherin (BD Transduction Laboratory), monoclonal anti–SRC-1 (Biomedica, Foster City, CA), monoclonal anti–SRC-2 (BD PharMingen), polyclonal anti-AR (Santa Cruz Biotechnology), polyclonal anti-AIB1 (gift from Dr. R. Wu, Baylor College of Medicine, Houston, TX), and polyclonal anti–α-actin (Sigma).

Real-time reverse transcription-PCR. Total RNA was prepared from all prostate lobes of individual WT/TRAMP and AIB1−/−/ TRAMP mice. Real-time reverse transcription-PCR (RT-PCR) was done with 150-ng RNA using the SYBR Green method. The PCR primers for SV40 T and T mRNA were designed according to the GenBank sequence V01380. The common forward primer was 5′-ctgcataactgcgtgtaaC-3′. The reverse primer specific to SV40 T mRNA was 5′-cagccgattttgctgataC-3′. The reverse primer specific to SV40 T mRNA was 5′-ctgcataactgcgtgtaaC-3′. As an epithelial RNA loading control, the relative mRNA concentration of the keratin 18 epithelial marker was also analyzed by real-time RT-PCR as previously described (30).
Measurement of proliferation and apoptosis. Ki67 immunoreactivity and the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay were used to assess proliferation and apoptosis, respectively. At least 500 prostate cells were counted from at least three randomly selected areas from each slide (magnification, ×400). The proliferation and apoptotic indices were expressed as a percentage of cells with dark brown nuclear staining to total cells. For each time point, at least three mice of each genotype were examined.

Results

AIB1 is not expressed in the luminal epithelial cells of the normal murine prostate. As an initial step to study the role of AIB1 in the prostate, we investigated its cell type–specific expression pattern by using the phenotypically normal AIB1+/− mice harboring a knock-in LacZ reporter. This AIB1 expression reporter was inserted into the second exon of the AIB1 gene and its expression is under the control of the endogenous AIB1 promoter (22–24, 31). If expressed, this reporter produces β-galactosidase that is solely localized in the nucleus. We isolated prostate tissues from 10-week-old AIB1+/− mice and did X-Gal staining. Positively stained blue cells were located at the peripheral region of the prostatic acini and in the stromal region (Fig. 1, first image). When the basement membrane was visualized by laminin staining, the blue cells were found either in the stroma outside the basement membrane or between the basement membrane and the luminal epithelial layer (Fig. 1A, third image). The CK8-positive luminal epithelial cells were excluded from the blue color (Fig. 1A, second image) and blue cells between the basement membrane and the luminal epithelium were basal cells that specifically express p63 (Fig. 1A, fourth image). The prostate tissues from age-matched WT (AIB1+/+) mice without LacZ reporter served as negative controls and showed no X-Gal staining signals (data not shown), validating the specificity of AIB1 promoter–controlled LacZ expression in AIB1+/− prostate. These results showed that AIB1 is expressed in the stromal and basal cells but not in the luminal epithelial cells in the murine prostate. As AR is expressed in both stromal and epithelial cells of the prostate (32), these findings suggest that AIB1 is coexpressed with AR only in the stromal cells rather than the epithelial cells.

AIB1 has a subtle contribution to prostate growth and is not required for androgen-induced prostate growth. To investigate the role of AIB1 in prostate development, we examined prostatic morphogenesis in 3-, 5- and 10-week-old WT and AIB1+/− mice. We microdissected the prostate glands and counted the numbers of their distal tips and branches, which has been used as a classic index of prostate morphogenesis (27). The tip and branch numbers of anterior prostate and dorsal prostate were similar in WT and AIB1+/− prostates at all ages examined. The tip and branch numbers of ventral prostate in AIB1+/− prostates were slightly less than those in WT prostates and their differences in tip number

Figure 1. AIB1 expression and function in the murine prostate. A, AIB1 expression in the basal and stromal cells. AIB1 expression is revealed by X-Gal staining (blue color in the nuclei in first, second, and third images). No blue color is located in the nuclei of luminal epithelial (LE) cells that are positively stained for CK8 (second image). Some blue cells are located in the stromal (S) region outside of the basement membrane (BM; third image). Basement membrane is highlighted by laminin immunostaining. Other blue cells are basal cells (b) that are located between basement membrane and the luminal epithelial layer (third image) and are positively stained for p63 (fourth image). Similar results were observed in anterior, ventral, dorsal, and lateral prostate lobes. L, lumen. B, comparison of prostate growth in WT and AIB1+/− (KO) mice. The four lobes of prostate were microdissected from four or more WT and AIB1+/− mice at the indicated ages. Columns, mean number of distal tips for individual lobes; bars, SE. *, **, P < 0.001, unpaired t test. C, comparison of prostate response to androgen in adult WT and AIB1+/− (KO) mice. Ten-week-old mice (n = 6 for each time point) were castrated on day 0 and testosterone was replaced on day 21 (C+21) until day 35 (T+14). Prostate and body weights were measured at indicated time points and presented as relative prostate weight (points, mean; bars, SE). Relative weight was calculated as (prostate weight × 100) / body weight.

were statistically significant at 5 weeks and in branch number at 3 weeks. The tip and branch numbers of lateral prostate in AIB1−/− prostates were identical to those of WT prostates at 3 and 5 weeks, but were significantly less than those of WT prostates at 10 weeks (Fig. 1B; Supplementary Fig. S1A). These results suggest that AIB1 deficiency does not affect anterior prostate and dorsal prostate morphogenesis but slightly delays the growth of ventral prostate and lateral prostate.

Activation of AR in the stromal cells stimulates growth factor secretion and enhances prostate growth (33). Because AIB1 is expressed in the stromal cells, it might contribute to AR-dependent prostate growth. Therefore, we compared androgen response between AIB1−/− and WT prostates by the method previously described (34). In the first experiment, mice were castrated at the age of 3 weeks and treated with testosterone capsules for 7 or 14 days starting at the age of 5 weeks. Unexpectedly, testosterone replacement comparably increased the numbers of distal tips and branches of all prostate lobes (Supplementary Fig. S1B). In the second experiment, mice were castrated at 10 weeks, maintained for 3 weeks to allow prostate regression, and then treated with testosterone capsules for 7 or 14 days to test androgen-stimulated regeneration. Again, AIB1−/− and WT prostates comparably responded to androgen depletion and replacement. The relative weights and the numbers of distal tips and branches of anterior, ventral, dorsal, and lateral prostate were reduced significantly after castration and regenerates to the normal levels after testosterone treatment (Fig. 1C; Supplementary Fig. S1C). These results imply that loss of AIB1 function did not affect androgen or AR-dependent prostate growth, maintenance, and regeneration. Therefore, AIB1 is not an essential coactivator for AR-mediated normal prostate growth.

AIB1 expression is induced in prostate epithelial cells during tumorigenesis. To investigate the kinetic expression and the contribution of AIB1 during prostate carcinogenesis, we used the TRAMP model (26). In TRAMP mice, the minimal rat probasin promoter drives the SV40 expression (T and t antigens, PB-Tag) in prostate epithelium to induce adenocarcinoma. These TRAMP mice develop poorly differentiated invasive prostate adenocarcinomas within 30 weeks (28). We generated WT/TRAMP, AIB1+/−/TRAMP, and AIB1−/−/TRAMP mouse genotypes analyzed by PCR. Top, the detected AIB1 WT (450 bp) and mutant allele (230 bp). Bottom, the detected TRAMP transgene (600 bp). F and M, breeding pair of female and male mice. B, Western blot analysis of AIB1 in prostates and tumors in WT/TRAMP and AIB1−/−/TRAMP mice with indicated ages. A significant increase of AIB1 protein is associated with the progression of prostate tumorigenesis in WT/TRAMP mice. -Actin was used as a loading control. C, analysis of AIB1 expression during prostate tumor initiation and progression. All prostate lobes were isolated from AIB1+/−/TRAMP mice with a LacZ knock-in reporter and from WT/TRAMP mice without the reporter (negative control for X-Gal staining) at indicated ages. X-Gal staining was done on cryosections. Note that AIB1 expression signals (blue) were located in the stromal cells at all ages (asterisks). AIB1 expression was not detected in the epithelium at 12 wks (arrows) but detected in some of the transformed epithelial cells in the PIN region at 18 wks. Further elevated AIB1 expression was seen in most of the epithelial or tumor cells in moderately differentiated adenocarcinoma (MDA) and poorly differentiated adenocarcinoma (PDA) at 24 and 30 wks. Arrows, epithelial cells. WDA, well-differentiated adenocarcinoma. D, in situ hybridization. In situ hybridization was done with AIB1 antisense and sense riboprobes on prostate cryosections prepared from 12-, 18-, 24-, and 30-wk-old WT/TRAMP mice. AIB1 mRNA signals (brown to purple) were specifically detected by the antisense probe but not the negative control sense probe and these signals were increased in moderately and poorly differentiated adenocarcinomas at 24 and 30 wks.
undetectable in prostates of 12-week-old WT/TRAMP mice, but became detectable in prostates of 12- to 24-week-old WT/TRAMP mice and significantly increased in prostate tumors at 30 weeks (Fig. 2B). As expected, no AIB1 protein was detected in AIB1<sup>+/−</sup>/TRAMP prostates at all time points, validating the absence of AIB1 in AIB1<sup>+/−</sup>/TRAMP mice. These results show that AIB1 protein is overproduced in mouse prostate tumors.

Next, we addressed what cell type overproduced AIB1 during prostate cancer progression in these mice. We monitored the kinetic cell type–specific AIB1 expression during prostate carcinogenesis by X-Gal staining of cryosections from the AIB1<sup>+/−</sup>/TRAMP prostates and tumors harboring the knock-in LacZ reporter (22). At the age of 12 weeks, AIB1 expression was exclusively found in stromal cells and basal cells but not in the luminal epithelial cells in all four prostate lobes (Fig. 2C for dorsal prostate; data not shown for other lobes). These results are consistent with the results observed in AIB1<sup>+/−</sup> mice (Fig. 1A), showing that AIB1 is not expressed in normal prostate luminal epithelium. Surprisingly, AIB1 expression appeared in certain transformed epithelial cells of PIN and well-differentiated adenocarcinoma at 18 weeks and became more intensive in >60% of the epithelial cells of moderately differentiated adenocarcinoma at 24 weeks (Fig. 2C). At 30 weeks, very strong AIB1 expression signals were observed in most of the tumor cells of poorly differentiated adenocarcinoma (Fig. 2C). No X-Gal staining signal was observed in WT/TRAMP control prostates and tumor tissues that did not carry the knock-in LacZ reporter (Fig. 2C). In situ hybridization using an AIB1 antisense riboprobe confirmed the significant increase of AIB1 mRNA.
expression in moderately and poorly differentiated adenocarcinomas at 24 and 30 weeks. The specificity of the in situ hybridization was verified by using the sense riboprobe as negative control, which did not detect any signal in the same set of samples (Fig. 2D).

Taken together, these results showed that AIB1 is induced in the luminal epithelial cells in early stages of prostate carcinogenesis and both the number of epithelial cells expressing AIB1 and the level of AIB1 expression in individual transformed epithelial cells are positively associated with the prostate cancer progression.

Loss of AIB1 suppresses prostate tumorigenesis and increases survival rate. To estimate the overall effect of AIB1 on oncogene-induced prostate tumorigenesis and survival, we studied more than 50 WT/TRAMP and AIB1−/−/TRAMP mice for their prostate cancer-related mortality and tumor development. Owing to the large prostate tumor and occurrence of metastasis, WT/TRAMP mice (Fig. 3A) and AIB1−/−/TRAMP mice (data not shown) died between 16.8 and 35.6 weeks. Fifty percent of WT/TRAMP mice died by the age of 26.8 weeks. In contrast, AIB1−/−/TRAMP mice died between 28.1 and 50 weeks and ~50% of them lived longer than 39 weeks (Fig. 3A). Statistical analysis showed that loss of AIB1 significantly prolonged the life span of AIB1−/−/TRAMP mice compared with that of WT/TRAMP mice (P < 0.0001, log-rank test).

More surprisingly, although all of the 20 WT/TRAMP mice examined at the time of necropsy developed large prostate tumors, there was no sign of prostate tumor in 14 of 16 AIB1−/−/TRAMP mice examined at the time of necropsy (Fig. 4A). The other 2 AIB1−/−/TRAMP mice examined at 30 weeks of age developed small prostate tumors. Some of the prostate tumor–free AIB1−/−/TRAMP mice developed other types of tumors, including tumors in throat (3 mice), kidneys (4 mice), seminal vesicle (3 mice), and liver (1 mouse). These mice died in coincidence with these tumors.

Figure 4. Inactivation of AIB1 arrests prostate cancer progression and maintains E-cadherin expression without affecting the SV40 transgene expression. A, AIB1 deficiency inhibits prostate tumor formation. Necropsy was done to examine the genitourinary tract of 30-wk-old WT/TRAMP and AIB1−/−/TRAMP mice. Left, representative photographs of typical prostate tumors in WT/TRAMP mice. One of the genitourinary tracts is also shown as an enlarged image. T, tumor; SV, seminal vesicle. B, H&E-stained histologic sections of the prostates prepared from WT/TRAMP and AIB1−/−/TRAMP mice with indicated ages. Images were taken at ×400 magnification. See text for differences of tumor progression in these mice. H, hyperplasia. C, immunohistochemical analysis of E-cadherin during prostate tumor progression in WT/TRAMP and AIB1−/−/TRAMP mice. Sections from dorsal lobes were immunostained with an antibody against E-cadherin. Images were taken at ×400 magnification. Brown color, E-cadherin immunoreactivity. Sections were counterstained with hematoxylin. Note that E-cadherin was detected in AIB1−/−/TRAMP prostates at all ages, but in WT/TRAMP prostates E-cadherin was restricted in certain areas at 24 wks and undetectable in poorly differentiated adenocarcinoma at 30 wks. D, immunohistochemistry analysis of SV40 T antigen expression in WT/TRAMP and AIB1−/−/TRAMP prostates and tumors. Sections from dorsal lobes were reacted with T antigen antibody. Images were taken at ×400 magnification. Slides were counterstained with hematoxylin. Brown color, T antigen immunoreactivity. Note that the T antigen immunoreactivities are similar in individual epithelial and tumor cells in both WT/TRAMP and AIB1−/−/TRAMP mice, whereas the number of T antigen–positive cells increases significantly as prostate tumor progresses in WT/TRAMP mice.
Because aging mice also develop spontaneous tumors, we did not pursue these nonprostate tumors in this study. In addition, other AIB1+/−/TRAMP mice exhibited normal genitourinary tract, but their bodies were lean and they died with unknown reasons. These results indicate that AIB1 deficiency strongly suppresses prostate tumorigenesis.

To monitor prostate tumor growth in mice, body weight and the weight of genitourinary were measured as a function of age and cancer progression (28). The average body weight of AIB1−/−/TRAMP mice was lighter than that of age-matched WT/TRAmp mice (Fig. 3B), which was expected because AIB1−/−/ non-TRAMP mice were smaller than WT mice (22). There was no significant difference in the ratio of kidney weight to body weight between WT/TRAmp and AIB1−/−/TRAMP mice although their body weights were different (Fig. 3B), suggesting that relative organ weight can be used to monitor hyperplastic changes in these mice. The net genitourinary weight in WT/TRAmp mice was significantly greater than that of AIB1−/−/TRAmp littermates after 24 weeks of age (P < 0.05). The increase in relative genitourinary weight as a function of time in each cohort followed the same trend as the increase in net genitourinary weight (Fig. 3B). However, the relative and net genitourinary weights in AIB1−/−/TRAmp mice remained constant at all examined ages (Fig. 3B). A closer measurement revealed that the relative weights of individual prostate lobes showed a gradual increase from the age of 8 weeks to 30 weeks (Fig. 3B). These results showed that WT/TRAmp prostate tumors grow rapidly but AIB1−/−/TRAmp prostate only exhibit a small hyperplastic growth.

In addition, metastatic lesions in the seminal vesicle, periaortic lymph nodes, lung, and liver were observed in WT/TRAmp mice but not in AIB1−/−/TRAmp mice, including the two 30-week-old AIB1−/−/TRAmp mice with prostate tumors. Following the development of metastasis in WT/TRAmp mice, the relative weights of periaortic lymph nodes were significantly increased after the age of 24 weeks. In contrast, the relative weights of AIB1−/−/TRAmp lymph nodes remained constant from the age of 8 to 30 weeks (Fig. 3B). Histologic examination of lymph nodes also confirmed the same finding. These results suggest that loss of AIB1 inhibits both prostate tumor formation and metastasis.

Inactivation of AIB1 impedes prostate cancer progression. Morphologically, prostate cancer progression consists of multiple stages from PIN to well-differentiated, moderately differentiated, and poorly differentiated carcinomas. To determine how AIB1 deficiency affected prostate cancer initiation and progression, we did histopathologic examination. As evidenced by epithelial hyperplasia and the presence of an intact basal cell layer, PIN was developed in almost all dorsal, lateral, and ventral prostate lobes in both WT/TRAmp and AIB1−/−/TRAmp mice from 8 to 12 weeks of age (Fig. 4B and data not shown). At 18 weeks, high-grade PIN and well-differentiated adenocarcinoma were observed in dorsal, lateral, and ventral prostate lobes in WT/TRAmp mice (n = 5) as evidenced by an increase in small glands, but only PIN was observed in dorsal, lateral, and ventral prostate lobes in AIB1−/−/TRAmp mice (n = 5). By 24 weeks, three of six WT/TRAmp mice developed high-grade well-differentiated or moderately differentiated adenocarcinoma and had invasive foci. Stromal thickening and active cell proliferation through the basement membranes of small glands were seen at these stages. The other three mice developed poorly differentiated adenocarcinoma characteristic of anaplastic sheats of tumor cells. Interestingly, all AIB1−/−/TRAmp mice (n = 5) only developed high-grade PIN in dorsal, lateral, and ventral prostate lobes (Fig. 4B and data not shown). By 30 weeks, all of WT/TRAmp mice (n = 6) developed poorly differentiated adenocarcinoma. In contrast, none of AIB1−/−/TRAmp mice (n = 5) had poorly differentiated adenocarcinoma and they only formed early-stage well-differentiated adenocarcinoma (Fig. 4B and data not shown). These histopathologic results are consistent with the morphologic observation showing that most AIB1−/−/TRAmp mice did not develop visible prostate tumors. Taken together, these findings suggest that inactivation of AIB1 arrests prostate cancer progression at the stage of well-differentiated adenocarcinoma.

The above notion is further supported by the expression of epithelial markers. As prostate carcinomas progress, the epithelial cells seem to lose epithelial differentiation and acquire more mesenchymal morphology and metastatic potential (28). In clinical prostate cancer, well-differentiated tumors typically retain expression of E-cadherin whereas poorly differentiated tumors show reduced or abnormal expression (35, 36). Similar to clinical cancer, abundant E-cadherin was detected in PIN and well-differentiated adenocarcinoma lesions in all prostate lobes of 18- and 24-week-old WT/TRAmp mice, but E-cadherin was significantly reduced or absent in poorly differentiated adenocarcinoma in 30-week-old WT/TRAmp mice (Fig. 4C and data not shown). However, E-cadherin was steadily detected in all prostate lobes, PIN, and well-differentiated adenocarcinoma in AIB1−/−/TRAmp mice at all ages (Fig. 4C and data not shown). Western blot analysis of tissue lysates prepared from all prostate lobes confirmed that E-cadherin was drastically reduced in the WT/TRAmp prostate tumors but not reduced in the AIB1−/−/TRAmp prostate tumors or tumors of 30-week-old mice (Fig. 5A). Similar results were also observed for another epithelial marker, CK8. In normal prostate, CK8 is expressed exclusively in the luminal epithelia (28, 37). In the poorly differentiated carcinomas of 30-week-old WT/TRAmp mice, CK8 staining was weak or absent. Interestingly, CK8 was still expressed in the prostes or tumors of 30-week-old AIB1−/−/TRAmp mice (Supplementary Fig. S2). These results show that the epithelial property is maintained in AIB1−/−/TRAmp prostatic epithelia and tumors and thereby further support the conclusion that AIB1 deficiency impedes prostate tumor progression at the well-differentiated adenocarcinoma stage.

Effects of AIB1 deficiency on the expression of SV40 T antigen, AR, SRC-1, and SRC-2. To follow the PB-Tag transgene expression, sections representing various stages of cancer progression were immunostained with an antibody specific for SV40 T antigen. As expected for the negative control, T antigen was undetectable in the prostates of WT and AIB1−/−/ mice (data not shown). In WT/TRAmp and AIB1−/−/TRAmp mice, the T antigen immunoreactivity was comparable in the prostatic epithelial cells as early as 8 weeks of age. The T antigen was continuously expressed in prostatic epithelium and tumor cells at all examined ages (18–30 weeks) in both types of mice. The T antigen immunoreactivity was similar in individual epithelial cells of dorsal, lateral, and ventral prostate lobes in WT/TRAmp and AIB1−/−/TRAmp mice through all ages except that the T antigen expression was largely reduced in some of the poorly differentiated adenocarcinoma cells in 30-week-old WT/TRAmp mice (Fig. 4D and data not shown). To quantify the amount of SV40 t and T mRNA expression, we did real-time RT-PCR analysis using RNA samples prepared from all prostate lobes of 18-week-old WT/TRAmp and AIB1−/−/TRAmp mice. Because the PB-Tag transgene was specifically expressed in the prostate epithelial cells, the relative expression levels of SV40 mRNAs were normalized to the
levels of keratin 18 mRNA that is also specifically expressed in the epithelial cells. Our measurements revealed that the SV40 t mRNA was expressed ~2-fold higher than the SV40 T mRNA in both WT/ TRAMP and AIB1+/−/TRAMP prostate tissues. Furthermore, the expression levels of both mRNA pieces in WT/TRAMP prostate samples were the same as those in AIB1+/−/TRAMP prostate tissues (Fig. 5D). The relative expression levels of AIB1 were variable among different mice, the average amounts of AR, SRC-1, and SRC-2 were not significantly different between WT/ TRAMP and AIB1+/−/TRAMP mice (Fig. 5D). These results suggest that AIB1 deficiency has no obvious effects on the expression of AR, SRC-1, and SRC-2 in the prostate.

**Inactivation of AIB1 reduces cell proliferation.** Prostate tumor progression usually involves alteration of cell proliferation and apoptosis (38–41). To determine whether loss of AIB1 function significantly decreases the proliferation indices in ventral, dorsal, and lateral prostate, we used immunohistochemistry with an antibody against Ki67, a S-phase marker, to detect proliferative cells and TUNEL assay to detect apoptotic cells. These assays were carried out at various ages in coordination with AIB1 expression analysis. The percentages of Ki67-positive cells in ventral, dorsal, and lateral prostate were high in the prostate epithelia of 8-week-old WT/TRAMP and AIB1+/−/TRAMP mice because of their rapid growth at puberty (Fig. 6I). In WT/TRAMP mice, proliferation indices in ventral, dorsal, and lateral prostate decreased at 12 weeks as the prostate matured, but significantly increased during the progression of prostate cancer from 18 to 24 weeks. However, in AIB1+/−/TRAMP mice, the proliferation indices were significantly lower in ventral and lateral prostate at 12 weeks and in ventral, dorsal, and lateral prostate at 18 and 24 weeks compared with age-matched WT/TRAMP mice. At 30 weeks, the proliferation indices remained high in WT/TRAMP tumors whereas large tumors made it impossible to isolate individual prostate lobes from these mice for proliferation assay. The proliferation indices remained unchanged in 30-week-old AIB1+/−/TRAMP mice compared with younger AIB1+/−/TRAMP mice (Fig. 6A). The proliferation rate in the anterior prostate lobe showed little changes because the PB-Tag transgene had little expression in the anterior prostate lobe due to a limited PB promoter activity. Interestingly, the apoptotic cell indices were as low as 1% to 2% in all lobes of both WT/TRAMP and AIB1+/−/ TRAMP prostate and maintained at similar levels at all examined ages, except that high apoptotic indices were observed in the central area of large tumors with extensive cell death in 30-week-old WT/TRAMP mice (Supplementary Fig. S3). In summary, these results show that loss of AIB1 function significantly decreases prostate epithelial and tumor cell proliferation induced by SV40-triggered tumorigenesis, but it has little effect on cell apoptosis.

**Discussion**

AIB1 has garnered extensive attention because of its over-expression and possible roles in human breast, esophageal, gastric, ovarian, pancreatic, and prostate cancers (3, 4, 6, 42). In certain prostate tumors, AIB1 was found to be overexpressed and its overexpression was associated with the disease recurrence (17, 43). Conversely, the knockdown of AIB1 was shown to inhibit proliferation and promote apoptosis of cultured prostate cancer cells both positive and negative to AR (16). Although these studies suggest that AIB1 might play an important role in prostate cancer,
the physiologic function of AIB1 in the androgen-regulated prostate development, the AIB1 expression profile associated with prostate cancer progression stages, and the in vivo role of AIB1 in prostate cancer initiation and progression were not established. This study shows that AIB1 is expressed in the stromal and basal cells rather than luminal epithelial cells in the normal murine prostate. Genetic deletion of AIB1 only slightly affects the prostate morphogenesis during puberty but has no obvious effects on the prostate regression following androgen withdrawal and the prostate regeneration after androgen replacement. These findings suggest that AIB1 is not an essential coactivator for AR in the normal prostate; this notion is further supported by the fact that the SV40 transgene expression driven by the androgen-responsive probasin promoter is identical in individual prostatic epithelial cells of WT/TRAMP and AIB1−/−/TRAMP mice. More importantly, this study further shows that in the prostate of WT/TRAMP mice, AIB1 expression is progressively induced in the SV40 antigen–transformed epithelial cells, which appear initially in a small cell population at the late PIN stage, subsequently in most of the transformed epithelial cells from well-differentiated to moderately differentiated adenocarcinoma stages, and finally in almost all of the tumor cells at the poorly differentiated adenocarcinoma stage (Fig. 6C). Inactivation of AIB1 in the prostate of AIB1−/−/TRAMP mice significantly extends their life span, greatly reduces the epithelial proliferation and tumor burden, maintains the expression of epithelial markers such as E-cadherin and CK8, and results in a blockade of prostate cancer progression at the well-differentiated adenocarcinoma stage (Fig. 6C). These results indicate that AIB1 expression in the prostate epithelial cells induced by the SV40 antigen–triggered cellular transformation is essential for prostate tumor growth and progression. Therefore, AIB1 may be a biomarker for prostate cancer progression and a possible therapeutic molecular target for prostate cancer treatment. The AIB1+−/− mouse line harboring the knock-in LacZ indicator for the endogenous AIB1 promoter activity is a sensitive and powerful tool to indicate temporal and spatial patterns of AIB1 expression. It has been faithfully used to detect AIB1 expression in the brain hippocampus, mammary epithelial cells, vascular smooth muscle and endothelial cells, uterine smooth muscle cells, and oocytes (22–24, 31). This mouse line is particularly useful in the case encountered in this study where AIB1 expression is low and all available AIB1 antibodies failed to reliably detect AIB1 protein in the mouse prostate by immunohistochemistry. The AIB1 expression patterns indicated by the LacZ reporter in the mouse prostate and tumors are consistent with the results of in situ hybridization in the same sets of tissues and with the results observed in the human prostate and prostate tumors. In the "normal" human prostatic epithelium, AIB1 expression is also undetectable or at very low levels (16). In human prostate
carcinomas and cancer cells, AIB1 is overproduced (17, 43). Importantly, this study provided new molecular genetic evidence showing that AIB1 expression is induced after the initiation of prostate carcinogenesis and during a period from severe PIN to well-differentiated carcinoma stages.

Because the SV40 transgene is expressed virtually in all epithelial cells of the dorsal, lateral, and ventral prostate lobes as early as 8 weeks of age and AIB1 is induced in a subpopulation of transformed prostatic epithelial cells as late as 18 weeks of age, the induction of AIB1 expression is not an immediate consequence of SV40 expression. Instead, it might be a consequence of progressive activation of cell growth signaling pathways responsible for prostate cancer progression. Although it is unclear what is the direct factor that switches on AIB1 expression in the transformed epithelium, recent studies have shown that AIB1 is a coactivator of E2F1, and E2F1 overactivation can stimulate AIB1 overexpression (12, 44, 45), which forms a positive feedback regulatory loop to maintain AIB1 overexpression and to enhance cell proliferation (45). Therefore, the SV40-induced cellular transformation may result in E2F1 overactivation and cause AIB1 overexpression in the transformed prostate epithelial cells.

The fact that 2 of 16 (12.5%) examined AIB1−/−/TRAMP mice still developed prostate tumors indicates that AIB1 deficiency does not arrest all prostate cancer progression induced by the SV40 antigen. Our data showed that SV40 antigen was expressed in most epithelial cells starting at 8 weeks, but only some of the epithelial cells became transformed and grew into tumors at a later stage. This observation suggests that the SV40 antigens alone are not sufficient to induce prostate cancer, and SV40 antigen–induced accumulation of genetic and/or epigenetic alterations is responsible for the development of prostate cancers. Because individual tumors might be induced by different signaling pathways activated by different combinations of genetic/epigenetic changes, the molecular mechanisms leading to the development of SV40-induced prostate tumors should include AIB1-dependent and AIB1-independent pathways. AIB1 deficiency should inhibit AIB1-dependent pathways and tumor progression, but would not block AIB1-independent pathways and tumor progression. This notion is further supported by multiple lines of evidence showing that AIB1 is amplified and overexpressed in some but not all of the human breast and prostate tumors (6, 16).

Our results showed that removal of AIB1 prevents SV40-transformed prostate epithelial cells from overproliferation and arrests prostate tumor development and progression in most AIB1−/−/TRAMP mice. This dramatic effect of AIB1 deficiency should be attributed to the involvement of AIB1 in multiple regulatory pathways. First, because previous studies using biochemical analysis and manipulated cell lines have shown that overexpressed AIB1 could enhance AR and Akt activities and promote cell proliferation (18, 19), one could think of the possibility that the oncogenesis-induced AIB1 expression in the transformed epithelial cells might serve as a coactivator for AR to promote cell proliferation and carcinogenesis. However, this possibility is dampened by the results showing that AIB1 expressed in the basal and stromal cells was not required for androgen-stimulated prostate growth and regeneration and AIB1 expressed in the transformed prostate epithelial cells of WT/ TAMP mice did not alter the expression level of the SV40 transgene directed by the androgen-responsive probasin promoter. Second, our previous studies have shown that AIB1 deficiency inhibits overactivation of IGF-I signaling pathways during mammary tumorigenesis by preventing IGF-I, IRS-1, IRS-2, and cyclin D1 overexpression and Akt overactivation (23, 24). Other studies have also shown that overexpression of AIB1 in transgenic mice significantly stimulates IGF-I signaling pathways and results in tumors in the mammary gland, lung, pituitary, and uterus (21). Therefore, AIB1 deficiency may suppress the overactivation of IGF-I signaling pathways induced by the prostate epithelial transformation and inhibit prostate cancer progression. Third, AIB1 has been found to increase cell size and survival capability of prostate cancer cells in culture (16, 43). Finally, AIB1 can interact with and coactivate a group of transcription factors including activator protein, Ets factors, nuclear factor-κB, and E2F1, known to be important for cell proliferation, survival, and carcinogenesis (11, 12, 14, 44, 46–48). Taken together, the blockade of prostate cancer progression by AIB1 deficiency should be a combined result from the effects of AIB1 deficiency on multiple signaling pathways. Additional investigation of the major pathways and target genes regulated by AIB1 should provide new insight into the understanding of molecular mechanisms and gene networks that govern prostate cancer progression.

Our analysis did not find appreciable change of cell apoptosis during prostate cancer progression in WT/TRAMP and AIB1−/−/ TRAMP mice, which was different from the results observed in the cultured prostate cancer cells where AIB1 depletion caused extensive apoptosis (16). Other studies showing a delay in tumor progression in TRAMP mice also showed an elevation of apoptosis (38–41). These differences may be explained by the essential role of AIB1 in prostate cancer progression. In other words, once the transformed prostate epithelial cells depend on AIB1 expression to progress to an advanced malignant stage, these tumor cells may become more dependent on AIB1 to survive. Depletion of AIB1 in malignant cancer cells such as the cultured prostate cancer cell lines would reduce their viability and result in apoptosis (16). In our in vivo model, the genetic ablation of AIB1 arrests prostate tumor progression at the moderately differentiated adenocarcinoma stage. Because AIB1 is never expressed in the AIB1−/−/TRAMP prostate epithelial cells, the partially transformed AIB1−/−/TRAMP epithelial cells still express epithelial cell markers and should be in an AIB1-independent status for survival. Thus, no obvious cell apoptosis would happen.

In summary, we have explored the role of AIB1 in prostate cancer progression using the bitransgenic mouse models. We show here that AIB1 protein is indeed induced by prostate oncogenesis and this induction of AIB1 overproduction is required for prostate cancer progression. Our results provide the first molecular genetic evidence that AIB1 regulates a gene expression program that promotes the prostate cancer progression from carcinoma in situ to invasive carcinoma. Future studies designed to understand the complex molecular mechanisms and to characterize AIB1–regulated genes responsible for promotion of prostate cancer progression should further advance our knowledge to understand and control human prostate cancer.

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References

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