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Caveolin-1 Promotes Tumor Progression in an Autochthonous Mouse Model of Prostate Cancer

GENETIC ABLATION OF Cav-1 DELAYS ADVANCED PROSTATE TUMOR DEVELOPMENT IN TRAMP MICE*

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Caveolin-1 (Cav-1) is the primary structural component of caveolae and is implicated in the processes of vesicular transport, cholesterol balance, transformation, and tumorigenesis. Despite an abundance of data suggesting that Cav-1 has transformation suppressor properties both *in vitro* and *in vivo*, Cav-1 is expressed at increased levels in human prostate cancer. To investigate the role of Cav-1 in prostate cancer onset and progression, we interbred Cav-1(−/−) null mice with a TRAMP (transgenic adenocarcinoma of mouse prostate) model that spontaneously develops advanced prostate cancer and metastatic disease. We found that, although the loss of Cav-1 did not affect the appearance of minimally invasive prostate cancer, its absence significantly impeded progression to highly invasive and metastatic disease. Inactivation of one (+/−) or both (−/−) alleles of Cav-1 resulted in significant reductions in prostate tumor burden, as well as decreases in regional lymph node metastases. Moreover, further examination revealed decreased metastasis to distant organs, such as the lungs, in TRAMP/Cav-1(−/−) mice. Utilizing prostate carcinoma cell lines (C1, C2, and C3) derived from TRAMP tumors, we also showed a positive correlation between Cav-1 expression and the ability of these cells to form tumors *in vivo*. Furthermore, down-regulation of Cav-1 expression in these cells, using a small interfering RNA approach, significantly reduced their tumorigenic and metastatic potential. Mechanistically, we showed that loss or down-regulation of Cav-1 expression results in increased apoptosis, with increased prostate apoptosis response factor-4 and PTEN levels in Cav-1(−/−) null prostate tumors. Our current findings provide the first *in vivo* molecular genetic evidence that Cav-1 does indeed function as a tumor promoter during prostate carcinogenesis, rather than as a tumor suppressor.

Caveolin-1 (Cav-1)[1] is the principal structural component and marker protein of caveolae, 50–100-nm flask-shaped invaginations of the plasma membrane. Cav-1 was first identified as a tyrosine-phosphorylated substrate when avian fibroblasts were transformed with the v-Src oncogene, suggesting that Cav-1 may have a critical role in modulating cellular transformation (1). To date, three caveolin family members have been identified, with diverse tissue expression patterns in various organisms ranging from mammals and amphibians to the nematode. Such ubiquity in higher organisms insinuates the importance of the caveolin gene family for normal physiology (2). The majority of caveolin-related research has focused on caveolin-1 as the prototypical member, and the past decade has brought considerable advances in our understanding of the functions of Cav-1. Thus far, Cav-1 has been implicated in many cellular processes, from signal transduction and vesicular transport to cholesterol homeostasis and lipid transport (reviewed in Ref. 3).

Concurrently, a growing field of research is beginning to define the role of the caveolins in the development of human cancers. Identification of a novel function for Cav-1 in oncogenesis began with the demonstration that Cav-1 levels inversely correlated with soft agar growth in transformed NIH 3T3 fibroblasts (4). Subsequently, several groups have shown that recombinant expression of Cav-1 in certain human or mouse tumor-derived cell lines significantly inhibits their soft agar growth (5–8). More recently, we and others have reported that Cav-1 has *in vivo* tumor suppressor properties in certain tissues, such as the mammary gland and skin, utilizing various murine animal models (9–12). For example, using a mouse model of mammary tumorigenesis (MMTV-PyMT), we have shown that Cav-1 expression dramatically inhibits the progression of early mammary dysplastic lesions, as well as the development of primary mammary tumors and spontaneous metas-

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[1] The abbreviations used are: Cav-1, caveolin-1; TRAMP, transgenic adenocarcinoma of mouse prostate; siRNA, small interfering RNA; PCNA, proliferating cell nuclear antigen; WT, wild-type; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end label; KO, knock-out; HET, heterozygous; PIN, prostatic intraepithelial neoplasia; ERK, extracellular signal-regulated kinase; Par4, prostate apoptosis response factor-4.

tases. In terms of human cancers, mutations in the *CAV-1* gene have been identified in breast carcinomas, as well as oral squamous cell carcinomas (13, 14), and *CAV-1* gene localizes to a suspected tumor suppressor locus on chromosome 7q31.1, which is commonly deleted in a variety of human cancers (reviewed in Ref. 15). In accordance with these findings, Cav-1 protein levels are consistently down-regulated in a wide range of human cancers, including ovarian carcinomas, sarcomas, and mammary carcinomas (15).

Despite this association between Cav-1 and tumor suppression, there is accumulating evidence that Cav-1 does not function similarly in all types of human cancers. In fact, several groups have now documented that Cav-1 protein expression levels are up-regulated in other human neoplasias, including bladder, esophageal, and prostate carcinomas (16–22). These findings suggest that Cav-1 does not have transformation suppressor effects in these tissues. In fact, further clinicopathologic analyses of human bladder, esophageal, and prostate carcinomas reveal that Cav-1 up-regulation correlates positively with increased tumor grade, invasive potential, regional metastasis, and decreased overall survival (16, 17, 19, 21, 22). Therefore, these results argue that Cav-1 has tumor-*promoting* functions in certain tumor cell types, such as prostate epithelial cells. This notion has been substantiated by cell culture studies with human prostate tumor-derived cell lines. In these prostate carcinoma cell lines, Cav-1 up-regulation appears to be correlated with increased cell survival, androgen independence, and increased metastatic potential (23, 24). Taken together, these findings suggest that Cav-1 has distinct cell type-specific tumor-modulating functions, either promoting or inhibiting tumor development, depending on its cellular context.

However, it remains unknown whether Cav-1 expression can promote the development of prostate cancers *in vivo*. Previous studies have focused only on the oncogenic behavior of cultured prostate cancer-derived cell lines. Here, we employed a well established transgenic mouse model of prostate cancer, termed TRAMP (transgenic adenocarcinoma of mouse prostate), to study the potential role of Cav-1 in modulating prostate carcinogenesis *in vivo*. For this purpose, we interbred Cav-1(−/−) null mice, completely lacking Cav-1 expression, with the well characterized TRAMP mouse model (25–27). This approach allowed us to directly assess whether genetic loss of one (+/−) or both alleles (−/−) of Cav-1 alters prostate tumor development in an *in vivo* setting. Use of this autochthonous TRAMP model has many advantages, because it allows prostate tumors to develop in their native environment (*i.e.* with exposure to the proper growth factors, hormonal, immunomodulatory, and angiogenic milieu). (See the end of the "Discussion" for further elaboration on the advantages and the validity of the TRAMP model).

Here, we show that prostate tumors derived from TRAMP mice demonstrate an up-regulation of Cav-1 expression, analogous to human prostate cancers. More importantly, ablation of one or both Cav-1 alleles significantly attenuates the progression of prostate carcinoma with regard to primary tumor burden and more advanced metastatic disease. These findings are further corroborated with cell lines derived from TRAMP prostate tumors (C1, C2, and C3). Importantly, all three TRAMP cell lines express cytokeratins, E-cadherin, and the androgen receptor, confirming their epithelial and prostatic origin (28). In these cell lines, Cav-1 expression directly correlates with tumor growth. Additionally, siRNA-mediated silencing of Cav-1 gene expression in the Cav-1-expressing TRAMP C1 cell line inhibits the processes of primary tumor growth and experimental metastasis. Analysis of both (i) transgenic prostate tumors and (ii) cell line-derived tumors indicates that loss of Cav-1 results in decreased cell survival, as well as the up-regulation of pro-apoptotic markers including PTEN and Par4.

Therefore, our findings provide the first *in vivo* molecular genetic evidence that Cav-1 does indeed function as a tumor promoter during mouse prostate carcinogenesis, rather than as a tumor suppressor. As we previously reported that Cav-1 has a tumor suppressor role in the mouse mammary gland (12), our current results conclusively demonstrated that Cav-1 has clear tissue-specific functions with regard to both tumorigenesis and metastasis.

EXPERIMENTAL PROCEDURES

Materials and Expression Vectors—Mouse monoclonal antibodies to caveolin-1 (clones 2297 and 2234) and caveolin-2 were the generous gifts of Dr. Roberto Campos-Gonzalez (BD Biosciences). Antibodies and their sources were as follows: anti-Cav-1 (N-20) rabbit polyclonal antibody (from Santa Cruz Biotechnology), anti-β-actin monoclonal antibody AC-15 (Sigma), anti-β-tubulin monoclonal antibody (Sigma), anti-phospho (p)-Akt (Ser-473) polyclonal antibody (Cell Signaling, Inc.), anti-Par4 polyclonal antibody (Santa Cruz), anti-PTEN polyclonal antibody (Cell Signaling), anti-PCNA monoclonal antibody (BD Biosciences), and anti-SV-40 large T-antigen (BD Biosciences). pBABE (empty vector) and pBABE-Cav-1 constructs were as we previously described (12).

Transgenic Animal Studies—All animals were housed and maintained in a barrier facility at the Institute for Animal Studies, Albert Einstein College of Medicine (AECOM). Mice were kept on a 12-h light/dark cycle with *ad libitum* access to chow (Picolab 20, PMI Nutrition International) and water. Animal protocols used for this study were approved by the AECOM Institute for Animal Studies. Cav-1 null mice were generated as previously described (9). TRAMP mice expressing the SV40 large T-antigen, under control of the minimal rat probasin promoter, were obtained from The Jackson Laboratory (JAX) and screened for the presence of the SV40 large T-antigen by PCR, as detailed on The Jackson Laboratory website. All the mice used in this study were in the C57Bl/6 background. Cav-1(−/−) null mice were interbred with TRAMP mice to generate three male cohorts: (i) TRAMP/Cav-1(+/+) mice (WT), (ii) TRAMP/Cav-1(+/−) (heterozygous (HET)) mice, and (iii) TRAMP/Cav-1(−/−) (knock-out (KO)) mice. All TRAMP mice from this study were hemizygous for the TRAMP transgene. TRAMP transgenic mice were sacrificed at 28 weeks of age, and a full necropsy was performed on all animals, including microscopic examination of the thoracic, pelvic, and abdominal organs. Primary tumors were excised and weighed, cut into smaller portions, fixed in 10% neutral buffered formalin for 24–48 h for paraffin embedding, and 5-μm sections were cut and stained with hematoxylin and eosin for histological analysis. In cases where there was no prostate tumor evident, the prostate tissue was isolated and likewise processed for microscopic analysis. Lymph nodes and other organs were similarly processed for histological analysis. For metastasis analysis, lymph nodes and lungs were sectioned at 50-μm intervals and either stained with hematoxylin and eosin or left unstained for immunohistochemistry. Lymph nodes were photographed with a Nikon Coolscope camera and areas with metastases scored with NIH Image J software. Lung metastases were scored as the total number of metastatic foci (defined as a cluster of 10 or more cells) per lung.

Cell Culture—TRAMP cell lines (C1, C2, and C3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were previously characterized and are derived from prostate adenocarcinomas isolated and cultured from TRAMP/Cav-1(+/+) mice (28). In brief, these cell lines demonstrate androgen receptor, E-cadherin, and cytokeratin expression, confirming their prostate epithelial origin. In addition, all three cell lines no longer express detectable SV40 large T-antigen. C1 and C2 cell lines also form tumors in syngeneic (C57BL/6) or nude mice, but C1 is the only cell line that forms colonies in soft agar. C1 cells are the fastest growing *in vitro* and *in vivo*, whereas C3 cells are the slowest growing and do not form tumors *in vivo* (28).

All three cell lines were cultured in Dulbecco's modified Eagle's medium (high glucose), without sodium pyruvate, with L-glutamine, 5% fetal bovine serum, 5% Nu-Serum IV (BD Biosciences), 0.005 mg/ml bovine insulin (Sigma), 10 nM dehydroisoandrosterone (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. The target region used for siRNA-mediated caveolin-1 down-regulation was designed with the aid of the Oligoengine sequence design software (Oligoengine, Inc., Seattle, WA). A 19-nt region of caveolin-1 (nucleotide sequence GCAAGTG-

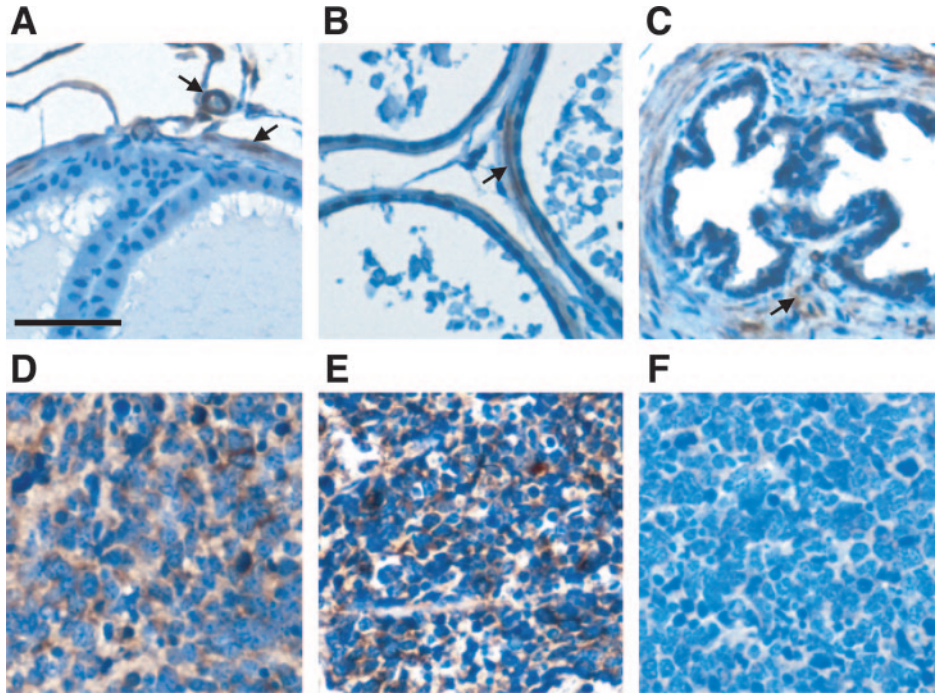


FIG. 1. **Cav-1 is up-regulated in prostate tumors derived from TRAMP mice.** Portions of normal prostate epithelial tissue from wild-type mice and advanced prostate tumors excised from TRAMP/Cav-1(+/+) or TRAMP/Cav-1(−/−) mice at 28 weeks of age were fixed in formalin and paraffin-embedded. Thereafter, 5-μm sections were cut and immunostained with a rabbit polyclonal antibody to Cav-1. Sections were counterstained with hematoxylin dye to visualize the nuclei. The anterior (*A*), lateral (*B*), dorsal (*C*), and ventral (not shown) prostate lobes in normal non-TRAMP tissue all demonstrated virtually no Cav-1 immunoreactivity in the epithelium, despite strong staining (note the *brown* color) in endothelia and smooth muscle cells (*arrows* in *panels A–C*). However, the majority of poorly differentiated prostate tumors (~80%) derived from TRAMP/Cav-1(+/+) mice display significantly more immunoreactivity directly within the cytoplasm of tumor cells (*panels D* and *E*), indicating that Cav-1 is up-regulated in advanced prostate carcinogenesis. As an important negative control, note the complete lack of staining for Cav-1 in prostate tumors derived from TRAMP/Cav-1(−/−) mice (*panel F*). All images were photographed with a Nikon Coolscope using a 20× objective and are at the same magnification. *Scale bar* = 50 μm.

TATGACGCGCAC) was chosen as a potential target and used to produce a 64-nt pair of oligos containing restriction enzyme cleavage sites and a short intervening hairpin sequence (sense: 5′-GATCCCCGCAAGTGTATGACGCGCACTTCAAGAGAGTGCGCGTCATACACTTGCTTTTTGGAAA-3′; antisense: 5′-AGCTTTTCCAAAAAGCAAGTGTATGACGCGCACTCTCTTGAAGTGCGCGTCATACACTTGCGGG-3′). These oligos were annealed and inserted into the pSUPER retroviral vector, according to the manufacturer's instructions. Production of stable cell lines by retroviral infection and puromycin selection was performed as we previously described (12).

Immunoblotting and Immunohistochemistry—Western blot analysis and immunostaining were performed essentially as we previously described in detail (9, 12).

Cell Injections—TRAMP cells were grown to confluency in 15-cm dishes, trypsinized, and counted with a hematocytometer. 2×10^6 cells in 0.1 ml of PBS were injected subcutaneously into the flanks of 2-month-old male C57Bl/6 mice, and tumors were isolated after 8 weeks. For the nude mouse experiments with stable cell lines, 5×10^5 cells in 0.1 ml of PBS were injected into the flanks of 2-month-old athymic nude male mice (Taconic Farms), and tumors were isolated after 6 weeks. Tumors were weighed and sized with calipers. Volumes were estimated using the formula $(x^2y)/2$, where x is the length and y is width. For experimental metastasis, 10^6 cells in 0.1 ml of PBS of either cell line were injected via the tail vein. Lungs were isolated 3 weeks later, fixed in formalin, and analyzed by hematoxylin and eosin staining exactly as for the transgenic study.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick-end Label (TUNEL) Staining—Briefly, 5-μm sections of WT TRAMP and KO TRAMP prostate tumors or C1/pSUPER and C1/siRNA tumors were TUNEL-stained with a kit according to the manufacturer's instructions (TACs 2 TdT *in situ* apoptosis detection kit; Trevigen, Inc., Gaithersburg, MD). TUNEL-positive nuclei were scored under a microscope using a 40× objective.

RESULTS

To determine whether Cav-1 plays an important role in prostate cancer progression, we interbred Cav-1(−/−) null mice with TRAMP mice (a mouse model of spontaneous prostate cancer) to produce three cohorts of TRAMP transgenic male mice: (i) TRAMP/Cav-1(+/+) wild-type, (ii) TRAMP/Cav-1(+/−) heterozygous, and (iii) TRAMP/Cav-1(−/−) null mice. All mice were heterozygous for the TRAMP transgene, which directs specific expression of the SV40 large T-antigen to the prostate via the rat probasin promoter. As previously reported by Greenberg and colleagues (26), TRAMP mice develop metastases to the regional lymph nodes after 28 weeks of age, with 100% penetrance and to more distant sites, such as the lung, with less frequency (26). Therefore, to study both prostate tumorigenesis and metastasis, we sacrificed mice from the three cohorts at 28 weeks of age and performed a full necropsy, including microscopic examination of the prostate. A total of 38 TRAMP animals were included in this study, including 18 Cav-1(+/+) WT, 8 Cav-1(+/−) HET, and 12 Cav-1(−/−) KO mice. TRAMP-expressing mice that died prior to the time point of sacrifice were excluded from the study. Of these, 7 of 25 (28%) WT mice died, 3 of 11 (27%) HET mice died, and 2 of 14 (14%) KO mice died. Necropsy of the WT and HET mice revealed that 100% of these mice possessed substantial tumor burden, indicating that advanced prostate cancer was the primary cause of mortality. However, the two KO mice that died did not demonstrate any significant prostate cancer development, suggesting that these mice died from other complications.

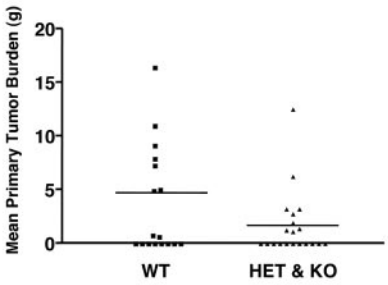
Caveolin-1 Is Up-regulated in Advanced Prostate Tumors Derived from TRAMP Mice—To determine whether Cav-1 is normally up-regulated during the process of mouse prostate carcinogenesis, we first performed immunostaining on normal (non-TRAMP) wild-type prostate tissue and prostate tumors derived from TRAMP/Cav-1(+/+) mice at 28 weeks of age. These prostate tumors were harvested from mice with a large tumor burden and metastatic disease, indicating the advanced

A

Prostate Tumor Incidence in TRAMP Mice.

Genotype	Incidence (%)	*N*
TRAMP/Cav-1 (+/+)	56	18
TRAMP/Cav-1 (-/-)	33	12

B



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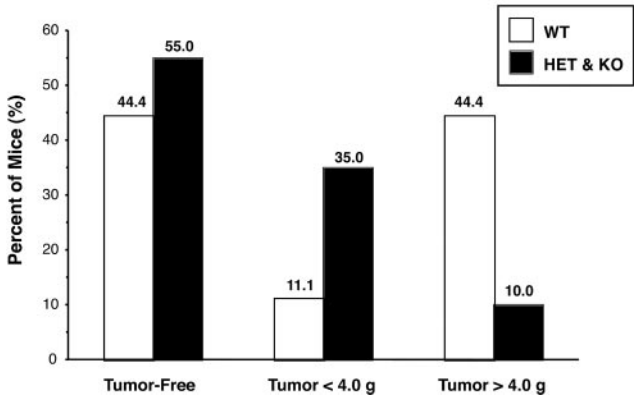


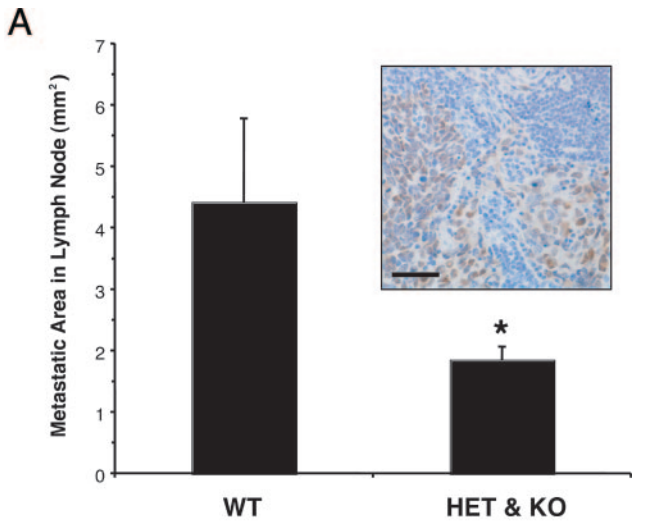
FIG. 2. **Genetic ablation of one or both Cav-1 alleles impedes prostate tumorigenesis.** *A*, incidence of prostate tumors at 28 weeks of age in male TRAMP mice. Note that 56% of TRAMP/Cav-1(+/+) (WT) mice ($n = 18$) developed tumors, as compared with only 33% of TRAMP/Cav-1(−/−) (KO) mice ($n = 12$). *B*, mean tumor wet weight/mouse (primary tumor burden) at 28 weeks of age. WT mice ($n = 18$) have prostate tumors weighing 4.72 ± 1.53 g on average, as compared with 1.28 ± 0.47 g for HET mice ($n = 8$) and 1.96 ± 1.1 g for KO mice ($n = 12$). Note that combining HET and KO cohorts ($n = 20$) results in a statistically significant ~2.8-fold decrease in mean tumor burden, with tumors weighing on average 1.69 ± 0.38 g (Student's *t* test, *p* value < 0.05). *C*, size distribution of tumors comparing WT and combined HET and KO cohorts. The percentage of mice that developed no tumor, a tumor <4 g, or a tumor >4 g is shown. Note that the majority of the WT prostate tumors are >4 g (44%, $n = 18$). In contrast, the majority of HET and KO tumors are <4 g (35%, $n = 20$).

nature of these tumors. Histological examination of these tumors also revealed that they were poorly differentiated high grade prostate carcinomas. We first examined Cav-1 expression in normal prostate epithelium of the anterior, lateral, dorsal, and ventral lobes. Virtually no immunoreactivity was observed in the epithelium of all prostate lobes in wild-type mice, despite abundant staining for Cav-1 in the endothelium, a cell-type that normally expresses high levels of Cav-1 (Fig. 1, *A–C*). On the other hand, the majority of tumors isolated from TRAMP/Cav-1(+/+) mice showed strong Cav-1 immunostaining directly within the prostate tumor cells, in addition to the endothelium (Fig. 1, *D* and *E*). As controls, immunostaining was performed with prostate tissue from Cav-1(−/−) mice (not shown) and prostate tumors from TRAMP/Cav-1(−/−) mice (Fig. 1*F*). Importantly, no Cav-1 immunostaining was detected in these Cav-1(−/−) null control tissues. These results indicate that Cav-1 is expressed at very low levels in untransformed prostate epithelium but is significantly up-regulated during the acquisition of transformed properties, ultimately leading to the development of high grade prostate tumors. These findings parallel observations made for Cav-1 in human prostate and provide further validation for using this animal model to study the role of Cav-1 in prostate tumorigenesis.

Loss of Cav-1 Significantly Impedes the Development of Primary Tumor Growth—Upon necropsy at 28 weeks of age, 56% of TRAMP/Cav-1(+/+) mice demonstrated a grossly evident prostate tumor. In comparison, the incidence of a grossly identifiable tumor in TRAMP/Cav-1(−/−) mice was only 33% (Fig. 2*A*). Next, to assess total tumor burden, prostate tumors were carefully excised and weighed. The mean tumor wet weight per mouse was 4.72 ± 1.53 g for the WT cohort and 1.96 ± 1.1 g for the KO cohort, indicating that the complete loss of Cav-1 markedly impacts prostate tumor burden in TRAMP mice. Interestingly, inactivation of one allele of *CAV-1* in HET mice was also sufficient to result in substantial reductions in total tumor burden (1.28 ± 0.47 g). As a consequence, results obtained with HET and KO cohorts were combined. Combining the HET and KO cohorts resulted in a statistically significant 2.8-fold reduction in mean primary tumor burden/mouse (1.69 ± 0.38 g; $p < 0.05$, Student's *t* test; Fig. 2*B*). Furthermore, only 10% of HET and KO mice developed large tumors (defined as >4.0 g) as compared with 44% of the WT TRAMP mice (Fig. 2*C*). Taken together, these results clearly indicate that partial or complete loss of Cav-1 is sufficient to impede prostate carcinogenesis.

Histological examination of the prostate tumors derived from WT, HET, or KO TRAMP mice revealed that all tumors were high grade, poorly differentiated carcinomas (not shown). Of those mice that did not develop visibly identifiable (gross) tumors, we isolated their prostates and evaluated them microscopically. Previous microscopic analysis of TRAMP mice at 6 months of age revealed numerous types of microscopically identifiable prostate cancer, including areas of prostatic intraepithelial neoplasia (PIN), phylloides-like lesions, and small areas of well differentiated or poorly differentiated neoplastic tissue in the dorsal, lateral, ventral, and anterior prostate (27). Histologically, we found no significant differences among the prostates of WT ($n = 8$), HET ($n = 3$), or KO ($n = 8$) TRAMP mice at 28 weeks of age. All mice developed microscopic evidence of disease, ranging from early (low grade PIN) to late (poorly differentiated) transformed tissue in all lobes to approximately the same degree (not shown). This suggests that the partial or complete loss of Cav-1 does not affect the early transformative steps in the establishment of prostate cancer, but rather delays progression to more advanced invasive disease.

Loss of Cav-1 Attenuates the Development of Regional and Distant Metastatic Disease—Because Cav-1 up-regulation has been detected in human metastatic prostate cancer and correlates positively with the Gleason score and extraprostatic extension (21, 22), we also sought to determine whether the loss of Cav-1 affected the development of metastatic disease in TRAMP mice. By 28 weeks, we detected the presence of metastases in regional pelvic lymph nodes in 100% of the TRAMP mice, regardless of *CAV-1* gene status, through a combination of hematoxylin and eosin staining and immunohistochemistry for the SV40 large T-antigen. This is consistent with previous studies in WT TRAMP mice that have reported 100% incidence of metastases to lymph nodes at this age (26). However, we found that partial or complete loss of Cav-1 resulted in substantial decreases in the mean total area occupied by these metastases in the regional lymph nodes (Fig. 3*A*). Per pelvic lymph node, TRAMP/Cav-1(+/+) mice demonstrated



B

Pulmonary Metastatic Incidence in TRAMP Mice.

Genotype	Incidence (%)	*N*
TRAMP/Cav-1 (+/+)	50	12
TRAMP/Cav-1 (+/-)	25	8
TRAMP/Cav-1 (-/-)	22	9

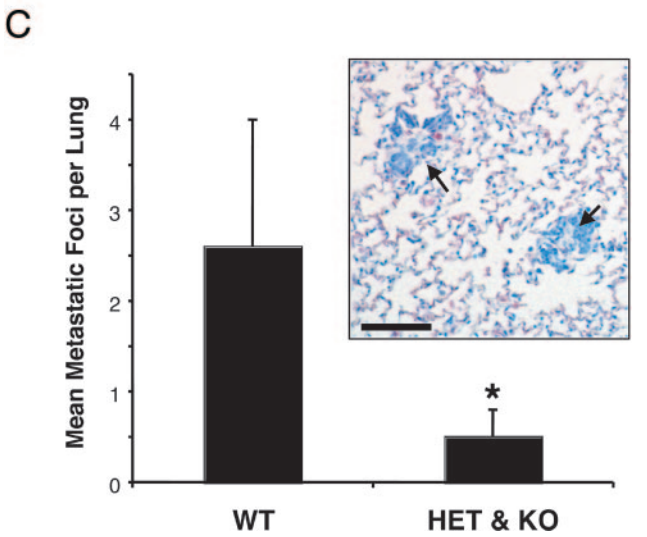


FIG. 3. **Partial or complete loss of Cav-1 significantly reduces metastatic disease.** *A*, examination of the pelvic lymph nodes from TRAMP mice. Lymph nodes were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. In combination with the hematoxylin and eosin staining, immunohistochemistry for SV40 large T-antigen (*Inset*; *scale bar* = 50 μm) was performed on serial sections to delineate areas with prostate tumor metastases. Positive areas were scored using NIH Image J software. WT TRAMP mice (n = 18) developed on average 4.41 ± 1.37 mm^2 of total metastatic area/pelvic lymph node. Interestingly, HET and KO TRAMP mice (n = 17) showed a statistically significant ~2.4-fold reduction in total metastatic area, with 1.84 ± 0.22 mm^2/node (Student's t test, p value < 0.05, *asterisk*). *B*, metastatic incidence in the lungs of TRAMP mice. Lungs were paraffin-embedded, sectioned at 50-μm intervals, and stained with hematoxylin and eosin. 50% of WT mice (n = 12) developed pulmonary metastatic foci, as compared with only 25% of HET mice (n = 8) and 22% of KO mice (n = 9). *C*, metastatic foci (operationally defined as a cluster of 10 or more cells) were scored under a microscope using a 10× objective. The mean number of pulmonary metastatic foci/WT mouse (n = 12) was 2.6 ± 1.4. HET and KO mice (n = 17) demonstrated a marked ~5.2-fold statistically significant reduction in number of metastases, with 0.5 ± 0.3 metastatic foci/mouse (Student's t test, p value < 0.05, *asterisk*). Inset depicts two metastatic foci (*arrows*) in the lungs of a WT TRAMP mouse (*scale bar* = 100 μm).

~4.41 ± 1.37 mm^2 of total metastatic area, in comparison with 1.68 ± 0.19 mm^2 for TRAMP/Cav-1(+/−) mice and 1.99 ± 0.38 mm^2 for TRAMP/Cav-1(−/−) mice. Combining the HET and KO cohorts together resulted in a statistically significant 2.4-fold decrease in metastatic area ($p < 0.05$, Student's t test).

Additionally, we also examined the lungs of TRAMP mice for the development of distant metastatic disease, which occurs with significantly less frequency than metastasis to regional lymph nodes. We found that the partial or complete loss of Cav-1 resulted in significantly lower levels of pulmonary metastatic foci; 50% (6/12) of TRAMP/Cav-1(+/+) demonstrated at least one metastasis, as compared with 25.0% (2/8) of TRAMP/Cav-1(+/−) mice and 22.2% (2/9) of TRAMP/Cav-1(−/−) mice (Fig. 3*B*). Furthermore, the mean number of pulmonary metastases in TRAMP/Cav-1(+/−) and TRAMP/Cav-1(−/−) mice was significantly reduced (5.2-fold) from 2.6 ± 1.4 foci in TRAMP/Cav-1(+/+) mice to 0.5 ± 0.3 foci in TRAMP/Cav-1(+/−) and (−/−) mice (Fig. 3*C*). Additional metastases were also detected in the liver and spleen of TRAMP/Cav-1(+/+) mice (2/18 or 11%) but not in TRAMP/Cav-1(+/−) or (−/−) mice (not shown). Taken together, these results demonstrated that TRAMP mice possessing one or both inactivated alleles of *Cav-1* are less prone to developing metastatic disease, and that wild-type Cav-1 expression contributes to extraprostatic extension, a hallmark of advanced prostate carcinoma.

SV40 large T-antigen Oncogenic Expression Does Not Alter Expression of Cav-1 in Prostate Epithelial Cells: Temporal Expression Patterns of Cav-1—An important control for this study was to assess whether the expression of the oncoprotein (SV40 large T-antigen) alters Cav-1 expression in prostate epithelium. Perhaps the differences in tumorigenesis observed in WT mice *versus* HET and KO mice were simply because of oncogene-mediated up-regulation of Cav-1, as opposed to endogenous mechanisms of Cav-1 up-regulation during prostate cell transformation. Therefore, we isolated prostate tissue from 2-month-old male wild-type and TRAMP/Cav-1(+/+) mice and performed Cav-1 and SV40 large T-antigen immunostaining. By 8–10 weeks of age, TRAMP mice had not developed advanced tumors, but *all* TRAMP mice possessed histologic evidence of both low and high grade PIN within their prostate epithelium (27). As demonstrated in Fig. 4*A*, Cav-1 is expressed at very low levels in normal prostate epithelium and there is a complete lack of SV40 large T-antigen staining (*left panels*). Likewise, Cav-1 levels are unchanged compared with wild-type in TRAMP prostate epithelium, despite an abundance of large T-antigen expression within the nuclei of prostate epithelium (Fig. 4*A*, *right panels*). Therefore, the expression of SV40 large T-antigen has no effect on the expression levels of Cav-1, suggesting that the up-regulation of Cav-1 in later stages of prostate tumor development occurs by an *endogenous* cellular process.

Interestingly, these results also indicate that up-regulation of Cav-1 during prostate tumorigenesis occurs in a temporal fashion. The TRAMP prostate epithelium shown in Fig. 4*A* is histologically consistent with low to high grade PIN, characterized by epithelial stratification and tufting, hyperchromatic and elongated nuclei, micropapillary projections, cribriform structures, and an increased number of mitotic figures. Because PIN in the TRAMP model invariably progresses to well differentiated adenocarcinoma and then to invasive, poorly differentiated adenocarcinoma, this finding demonstrated that the up-regulation of Cav-1 does not occur in these early neoplastic transitional periods. Rather, up-regulation of Cav-1 is a late occurrence, most likely established during the transformation of adenocarcinoma from a well differentiated to a poorly differentiated state.

As additional controls, we performed immunoblotting and

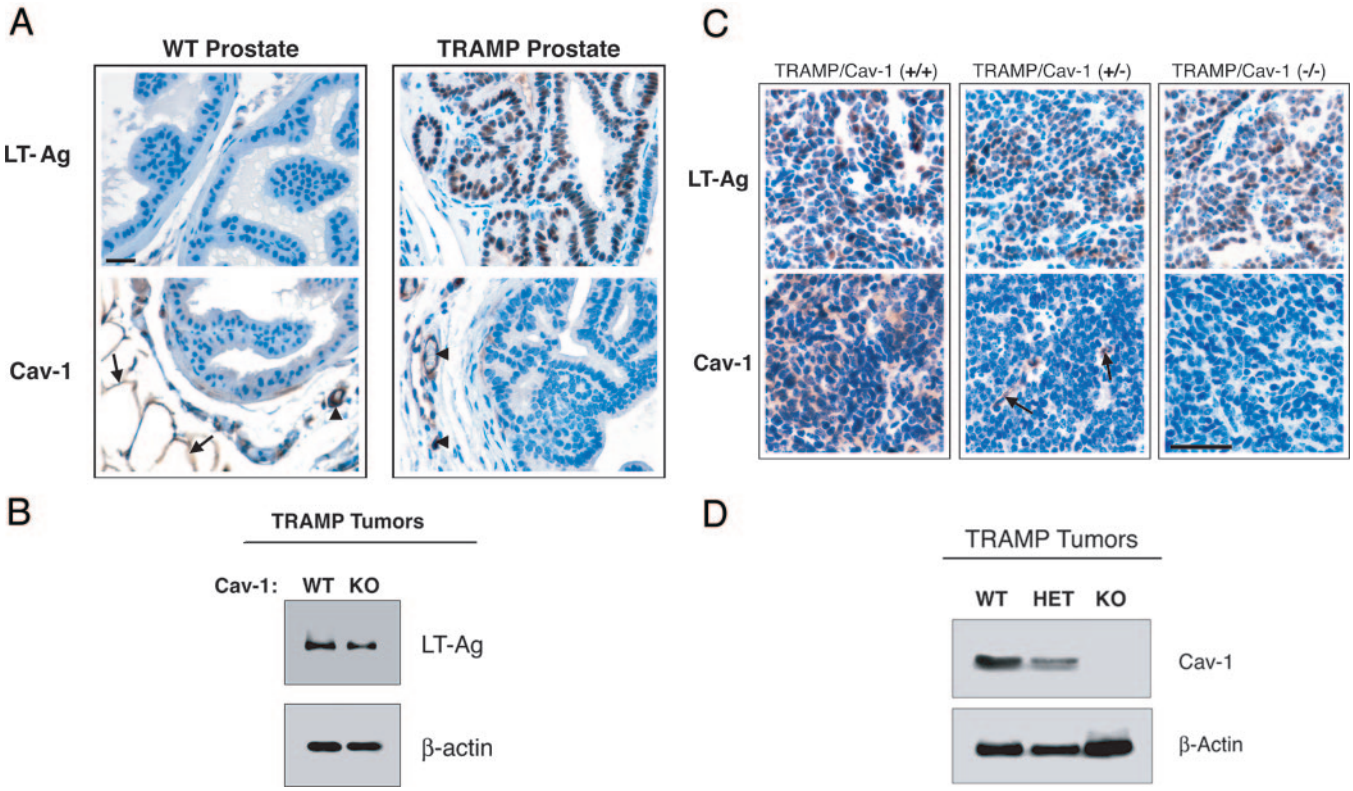


FIG. 4. **SV40 large T-antigen and Cav-1 expression levels at various stages of prostate tumor development in TRAMP mice.** *A*, immunohistochemical analysis of SV40 large T-antigen (*LT-Ag*) and Cav-1 expression in prostate tissue from 2-month-old male wild-type (*WT*) and TRAMP/Cav-1(+/+) mice. Note that the presence of LT-Ag expression in TRAMP prostate tissue (*brown* nuclear staining) does not result in up-regulation of Cav-1 expression. In both wild-type and TRAMP prostate tissue, Cav-1 is expressed at virtually undetectable levels compared with expression in poorly differentiated TRAMP tumors (see Fig. 1, *panels D* and *E*). Despite evidence of high grade PIN at this age, the lack of Cav-1 expression in TRAMP/Cav-1(+/+) prostate suggests that Cav-1 is up-regulated at a more advanced stage of prostate carcinoma development. Note the presence of positive Cav-1 staining in adipocytes (*arrows*) and capillary endothelium (*arrowheads*). All images are the same magnification (original magnification 40×). *Scale bar* = 50 μm. *B*, immunoblotting of poorly differentiated TRAMP prostate tumors at 28 weeks of age. TRAMP/Cav-1(+/+) (*WT*) and TRAMP/Cav-1(−/−) (*KO*) tumors were isolated, homogenized, separated by SDS-PAGE, and immunoblotted for SV40 large T-antigen. Two representative samples are shown. Note that levels of the SV40 transgenic oncogene are not altered because of loss of Cav-1. β-actin levels are shown as an equal loading control. *C*, immunohistochemistry of poorly differentiated TRAMP prostate tumors at 28 weeks of age. Staining for SV40 large T-antigen (*LT-Ag*) reveals that the loss of one or both Cav-1 alleles does not significantly alter the expression of this oncogene (*top panels*; *brown* nuclear staining). Interestingly, Cav-1 staining in TRAMP/Cav-1(+/−) tumors is virtually undetectable, despite positive staining in the endothelium (*arrows*). Note the positive cytoplasmic staining pattern for Cav-1 in prostate tumor cells of TRAMP/Cav-1(+/+) adenocarcinomas. All images are the same magnification (original magnification 40×). *Scale bar* = 50 μm. *D*, Cav-1 immunoblotting of poorly differentiated TRAMP prostate tumors at 28 weeks of age. Note that Cav-1 expression in TRAMP/Cav-1(+/−) tumors is reduced to a greater extent (~80–90%) than the predicted 50% reduction from inactivation of one Cav-1 allele. As expected, Cav-1 levels are absent in TRAMP/Cav-1(−/−) tumors. β-actin levels are shown as an equal loading control.

immunohistochemistry on poorly differentiated prostate tumors isolated from 28-week-old TRAMP mice. Importantly, complete loss of Cav-1 did not alter the expression of the SV40 large T-antigen, as assessed by immunoblotting (Fig. 4*B*) and immunohistochemistry (Fig. 4*C*). It has been previously demonstrated that single allelic inactivation of *Cav-1* results in an ~50% reduction in expression in various tissues (29). Therefore, we also performed SV40 large T-antigen and Cav-1 staining on prostate tumors derived from TRAMP/Cav-1(+/−) mice. Interestingly, although SV40 oncogene expression is unchanged compared with TRAMP/Cav-1(+/+) tumors, there is virtually no Cav-1 immunoreactivity detected in the prostate carcinoma cells of TRAMP/Cav-1(+/−) tumors (Fig. 4*C*). Likewise, Cav-1 immunoblotting demonstrated that these TRAMP/Cav-1(+/−) tumors show dramatic reductions in Cav-1 levels (by ~80–90%), more than the 50% reduction predicted from inactivating one Cav-1 allele (Fig. 4*D*). These findings indicated that TRAMP/Cav-1(+/−) tumors have a Cav-1 expression profile more comparable to TRAMP/Cav-1(−/−) tumors and offer an explanation as to why prostate tumorigenesis in TRAMP/Cav-1(+/−) and TRAMP/Cav-1(−/−) mice behaves similarly.

Expression of Cav-1 in TRAMP Tumor-derived Cell Lines Correlates with Primary Tumor Growth—To directly assess the role of Cav-1 in mouse prostate cancer cells with regard to tumorigenesis, we obtained three prostate carcinoma cell lines derived from WT TRAMP prostate tumors-TRAMP-C1, -C2, and -C3. Importantly, all three TRAMP cell lines express cytokeratins, E-cadherin, and the androgen receptor, confirming their epithelial and prostate origin (28).

We examined these cell lines for Cav-1 expression by immunoblotting and found that C1 demonstrated relatively high levels of Cav-1, C2 possessed moderate Cav-1 levels, whereas C3 expressed virtually no Cav-1 (Fig. 5*A*). However, Cav-2 levels were unchanged among the various cell lines. Immunocytochemistry revealed that Cav-1 localizes properly to the plasma membrane in a typical punctate staining pattern observed for Cav-1 in C1 and C2 cells (not shown). Next, we injected these cell lines into the flanks of syngeneic host male mice and observed the development of tumors. As previously reported (28), C1 and C2 cell lines form tumors, with C1 cells resulting in the largest tumors, whereas C3 cells do not form tumors, even after extended observation (Fig. 5, *B* and *C*). It is interesting to note that tumor size correlates positively with

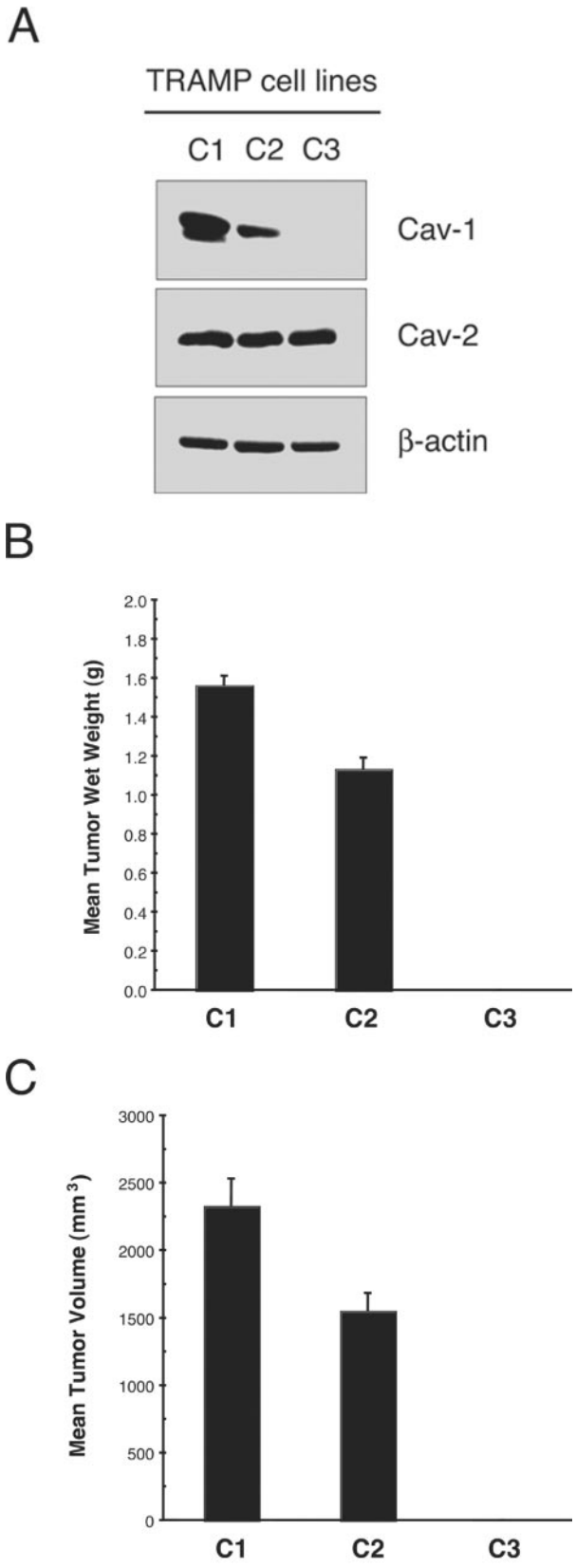


FIG. 5. **Cav-1 expression correlates with tumor growth in TRAMP cell lines.** *A*, TRAMP cell lines were grown to confluency, collected into lysis buffer, and subjected to immunoblot analysis for Cav-1 and Cav-2 expression. Note that the levels of Cav-1 are highest in C1 cells, moderate in C2 cells, and virtually absent in C3 cells. However, Cav-2 levels are equivalent in all three cell lines. β-actin levels are shown as an equal loading control. *B* and *C*, C57Bl/6 male mice were subcutaneously injected with 2×10^6 cells in 0.1 ml of PBS for each TRAMP cell line ($n = 5$ each for C1, C2, and C3). After 8 weeks, tumors were excised, weighed, and sized with calipers. Note that C1 cells formed larger tumors than C2 cells, whereas C3 cells do not form any tumors. C1 cells formed tumors weighing 1.56 ± 0.05 g and were 2330 ± 206 mm^3 in size, whereas C2 cells formed tumors weighing 1.13 ± 0.06 g and were 1550 ± 133 mm^3 in size. Differences in size and weight were statistically significant (Student's *t* test, p value < 0.05).

Cav-1 expression in these cells, suggesting that the levels of Cav-1 impact the capacity of these murine prostate carcinoma-derived cells to form tumors.

Targeted Down-regulation of Cav-1 in the TRAMP-C1 Cell Line Diminishes Primary Tumor Growth and Experimental Metastasis—To directly address the role of Cav-1 in tumorigenesis in these cells, we down-regulated Cav-1 in C1 cells using an siRNA approach. We also chose to overexpress Cav-1 in C3 cells by a recombinant approach to see whether the forced expression of Cav-1 would be sufficient to induce tumor growth. We created stable cell lines through a retroviral approach using the pSUPER retroviral vector for the siRNA-mediated down-regulation of Cav-1 and the pBABE retroviral vector for recombinant Cav-1 overexpression. Initial characterization of the stable cell lines was performed by immunoblotting (Fig. 6*A*). Note that Cav-1 levels are markedly down-regulated in C1/siRNA cells, as compared with C1 parental or C1/pSUPER (empty vector) cells. When these cell lines were injected into the flanks of athymic nude male mice, we found a dramatic decrease in the weight (3.8-fold) and volume (4.6-fold) of tumors derived from C1/siRNA cells (Fig. 6*B*). However, overexpression of Cav-1 in C3 cells was not sufficient to drive tumor formation. These findings indicated that, although Cav-1 is not sufficient to induce tumors, high Cav-1 expression levels can promote the development of larger tumors by providing a selective advantage for neoplastic growth. Conversely, down-regulation of Cav-1 in TRAMP-C1 cells attenuates the ability of prostate carcinoma cells to form tumors.

We also assessed whether the targeted down-regulation of Cav-1 in C1 cells affected the ability of these cells to metastasize. Equal numbers of C1/pSUPER and C1/siRNA cells were injected directly into the bloodstream of athymic male mice through the tail vein (termed "experimental metastasis"). This technique allows the direct assessment of whether the loss of Cav-1 affects the ability of these prostate tumor cells to exit the bloodstream and form metastatic tumors in an ectopic site. After 3 weeks, the lungs were excised, fixed in formalin, and processed for histological analysis. Lungs derived from mice injected with C1/siRNA cells demonstrated an average of 7.6 ± 1.6 metastatic foci/lung, a dramatic reduction (~5.1-fold) in the number of pulmonary metastatic foci compared with lungs isolated from mice injected with C1/pSUPER cells, which developed an average of 38.8 ± 10.3 foci/lung (Fig. 6*C*). Additionally, pulmonary metastatic foci arising from C1/pSUPER cells were significantly enlarged compared with C1/siRNA foci (Fig. 6*D*). These results corroborate the findings from the transgenic mouse study and indicate that down-regulation of Cav-1 in prostate carcinoma-derived cells attenuates both the processes of tumorigenesis and metastasis. Moreover, the cellular studies provide direct evidence that the decreases in prostate tumorigenesis and metastasis in HET and KO TRAMP mice are cell-autonomous effects because of the intrinsic loss of Cav-1 expression in prostate carcinoma cells.

Loss of Cav-1 Results in Increased Levels of Apoptosis in TRAMP Tumors—To begin to identify how the loss of Cav-1 results in diminished prostate tumorigenesis and metastasis, we examined the processes of proliferation and apoptosis in the TRAMP-C1 cell lines, C1-derived tumors, and TRAMP prostate tumors. We have previously established that Cav-1 has a negative regulatory role in cellular proliferation in certain cell

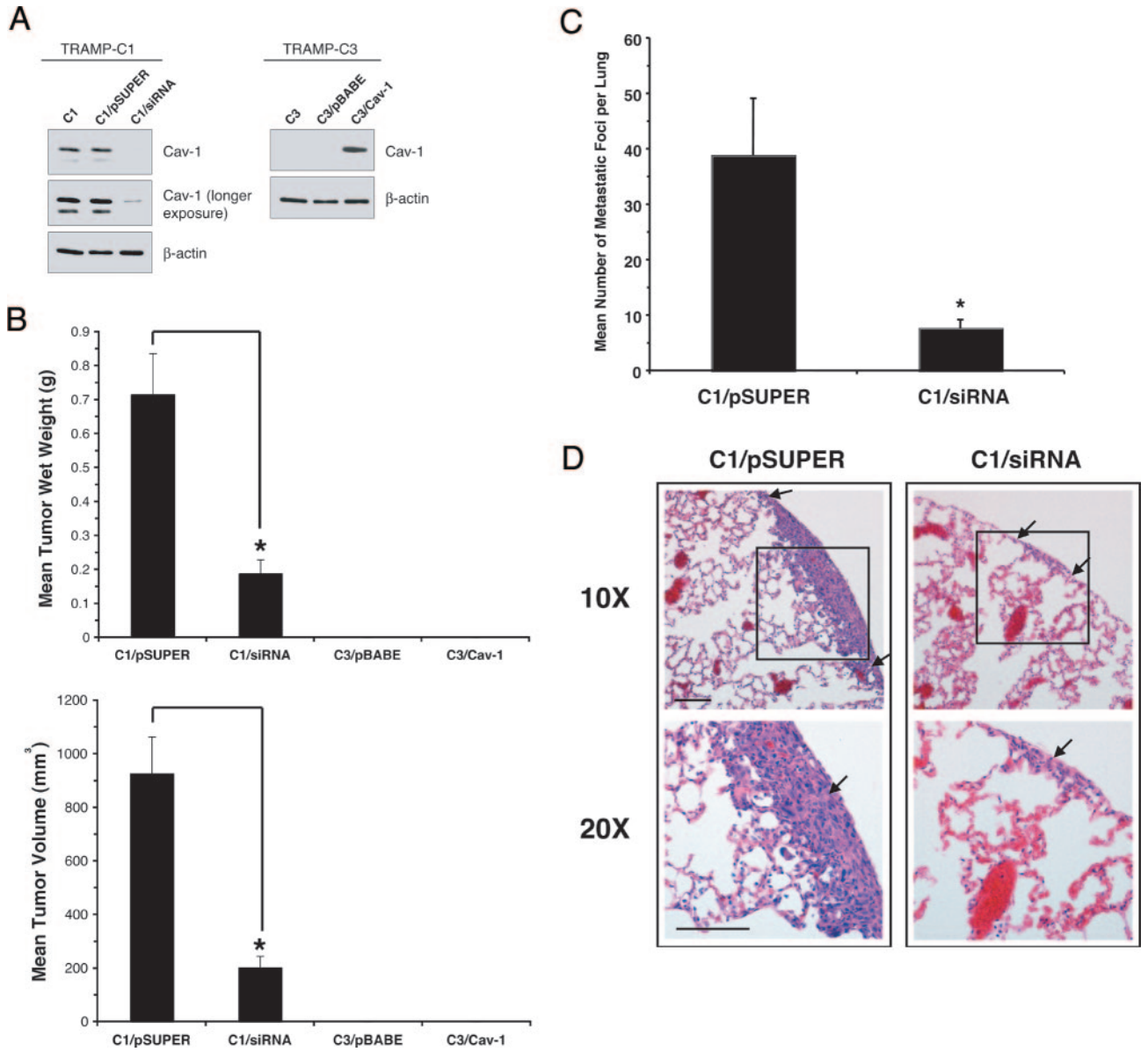


FIG. 6. **siRNA-mediated down-regulation of Cav-1 in TRAMP-C1 cells attenuates both tumorigenesis and experimental metastasis.** *A*, creation of stable TRAMP cell lines lacking or overexpressing Cav-1. TRAMP C1 cells were retrovirally infected with pSUPER (empty vector) and pSUPER-Cav-1-siRNA to down-regulate Cav-1 levels. In contrast, TRAMP C3 cells were retrovirally infected with pBABE (empty vector) and pBABE-Cav-1 to overexpress Cav-1. Thereafter, cells were selected with puromycin for 1 week to generate stable "pools" of transduced cells. Finally, cells were grown to confluency, collected into lysis buffer, and subjected to immunoblotting for Cav-1 levels. Note that Cav-1 levels are markedly down-regulated using an siRNA approach in C1 cells, as compared with parental (*C1*) or empty vector (C1/pSUPER) controls. Conversely, C3/Cav-1 cells overexpress recombinant murine Cav-1, as compared with parental (*C3*) or empty vector (C3/pBABE) control cells. β-actin levels are shown as an equal loading control. *B*, male nude mice were subcutaneously injected with 5×10^5 cells in 0.1 ml of PBS of either C1/pSUPER, C1/siRNA, C3/pBABE, or C3/Cav-1 cells. After 6 weeks, tumors were excised, weighed, and sized with calipers. Note that both C3 cell lines did not form tumors. Interestingly, C1/siRNA cells formed dramatically smaller tumors (0.19 ± 0.04 g; 203 ± 42 mm^3; $n = 14$) with a 3.8-fold reduction in weight and 4.6-fold reduction in volume as compared with C1/pSUPER cells (0.72 ± 0.12 g; 927 ± 137 mm^3; $n = 14$). Differences in size and weight were highly statistically significant (Student's t test, p value < 0.0002, *asterisk*). Data represent the average of several independent experiments, all with virtually identical results. *C*, male nude mice were injected through the tail vein with 10^6 cells of either the C1/pSUPER or C1/siRNA cell lines in 0.1 ml of PBS. Lungs were isolated after 3 weeks, processed for hematoxylin and eosin analysis, and examined under a microscope for the presence of metastatic foci (defined as a cluster of 10 or more cells). Mice ($n = 5$) injected with C1/pSUPER cells developed an average of 38.8 ± 10.3 metastatic foci/lung. In contrast, mice injected ($n = 5$) with C1/siRNA cells demonstrated a 5.1-fold reduction in metastatic foci (7.6 ± 1.6 foci; Student's t test, $p < 0.05$, *asterisk*). *D*, pulmonary metastatic foci (*arrows*) derived from C1/pSUPER cells (*left panels*) are significantly larger than C1/siRNA foci (*right panels*). Photographs were taken from 5-μm sections of the lungs stained with hematoxylin and eosin (original magnifications 10 and 20×; *scale bars* = 100 μm). *Arrows* in the *top panels* (10×) delineate the boundaries of metastatic foci. *Arrows* in the *bottom panels* (20×) indicate the position of metastatic foci.

types, including transformed fibroblasts and mammary epithelial cells (12, 30). Therefore, we performed cell proliferation assays and thymidine-incorporation experiments on the TRAMP-C1 cell line in culture. We found that the down-regulation of Cav-1 in C1/siRNA cells resulted in a slight proliferative advantage for growth in culture, in accordance with previous results showing that loss of Cav-1 exerts a proliferative advantage (not shown). Despite this, immunohistochemistry for proliferating cell nuclear antigen (PCNA), a well characterized marker for cellular proliferation, revealed no significant differences in the number of PCNA-positive cells in WT or KO TRAMP prostate tumors (Fig. 7*A*). PCNA staining performed

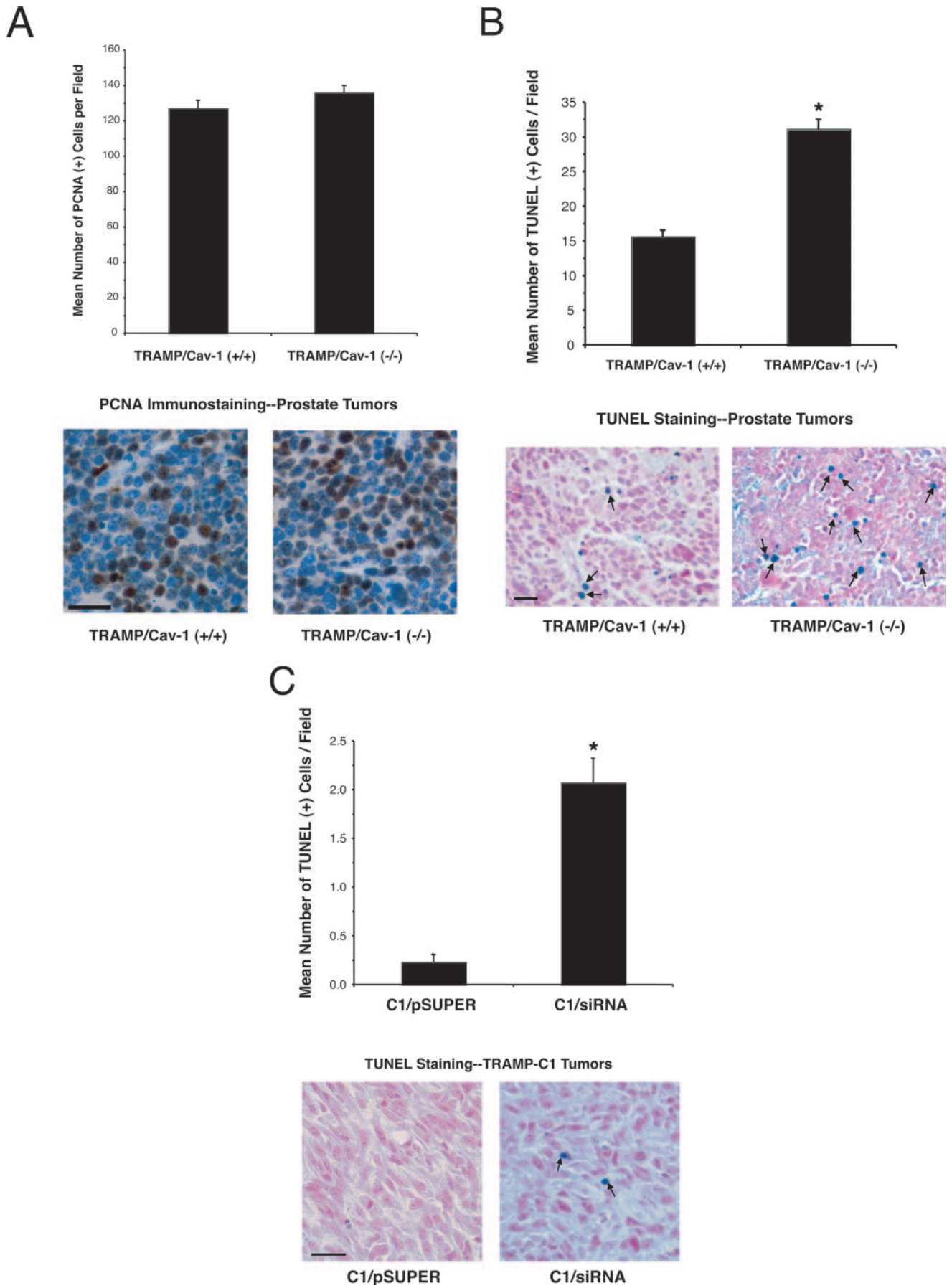


FIG. 7. **Loss of Cav-1 expression renders prostate tumor cells more susceptible to apoptosis.** *A*, PCNA immunohistochemistry of prostate tumors from WT and KO TRAMP mice reveals no significant differences in the proliferative index of these tumors. A total of 30 fields (40×) were counted/genotype. Representative PCNA photomicrographs of prostate tumors from WT and KO TRAMP mice are shown. *Scale bar* = 25 μm.

on tumors derived from C1/pSUPER and C1/siRNA cells also revealed no significant differences (not shown). We also examined the expression of cyclin D1 and phospho-ERK-1/2 in TRAMP WT and KO tumors and found no differences in the levels of these pro-proliferative molecules (not shown).

It has been established that, in prostate cancers, resistance to apoptotic stimuli plays an especially critical role during the process of tumorigenesis (31). Furthermore, a role for Cav-1 in promoting prostate cancer cell survival has been previously established in cell culture systems (24, 32). Therefore, we performed TUNEL staining to measure DNA strand breakage, a hallmark of apoptosis, in TRAMP prostate tumors and tumors derived from TRAMP-C1 cells. We found that loss of Cav-1 resulted in substantial increases in the apoptotic index of KO TRAMP tumors, as compared with WT TRAMP tumors (Fig. 7*B*). TRAMP/Cav-1(+/+) prostate tumors demonstrated an average of 15.6 ± 0.9 TUNEL-positive cells/field, as compared with 31.1 ± 1.4 TUNEL-positive cells/field in TRAMP/Cav-1(−/−) tumors. This ~2.0-fold increase was highly significant and suggests that the presence of Cav-1 in prostate carcinoma cells has an anti-apoptotic role. To examine whether the anti-apoptotic function of Cav-1 is indeed a cell-autonomous effect, we also performed TUNEL staining on tumors derived from TRAMP-C1 prostate tumor cells. Once again, we found that the loss of Cav-1 resulted in highly significant increases (9.0-fold) in the degree of apoptosis in tumors derived from C1/siRNA cells (Fig. 7*C*). Taken together, these findings clearly indicate an anti-apoptotic role for Cav-1 in prostate carcinoma cells during prostate tumorigenesis.

TRAMP/Cav-1(−/−) Null Tumors Demonstrate Increased Levels of Two Pro-apoptotic Markers, Namely PTEN and Par4—Because previous studies have implicated Cav-1 in promoting cell survival in prostate cancer cells by potentiating Akt activation (32), we assessed TRAMP tumors for Akt-activation using a phosphospecific antibody that detects active phosphorylated Akt at serine 473. Surprisingly, we did not observe significant differences in the levels of phospho (p)-Akt among the tumors (Fig. 8*A*, *top panels*). However, we observed marked increases in the levels of prostate apoptosis response factor-4 (Par4) in KO TRAMP tumors (Fig. 8*A*, *middle panels*). Par4 is a prostate apoptosis-specific marker that both induces and sensitizes prostate cancer cells to apoptosis when overexpressed (33). Moreover, the well characterized lipid phosphatase tumor suppressor, namely PTEN, demonstrated reduced levels in WT TRAMP tumors (Figs. 8*A*, *bottom panels*, and *B*). PTEN is frequently deleted or inactivated in primary human prostate cancer, and its deletion in mice results in metastatic prostate cancer (34). Interestingly, it has been previously shown that Cav-1 co-immunoprecipitates with PTEN, suggesting that these two proteins interact *in vivo* (21). Taken together, the increased Par4 and PTEN expression observed in TRAMP tumors lacking Cav-1 expression provides further molecular evidence that these Cav-1 KO tumors are indeed more susceptible to apoptosis and, therefore, are less prone to tumor and metastasis development.

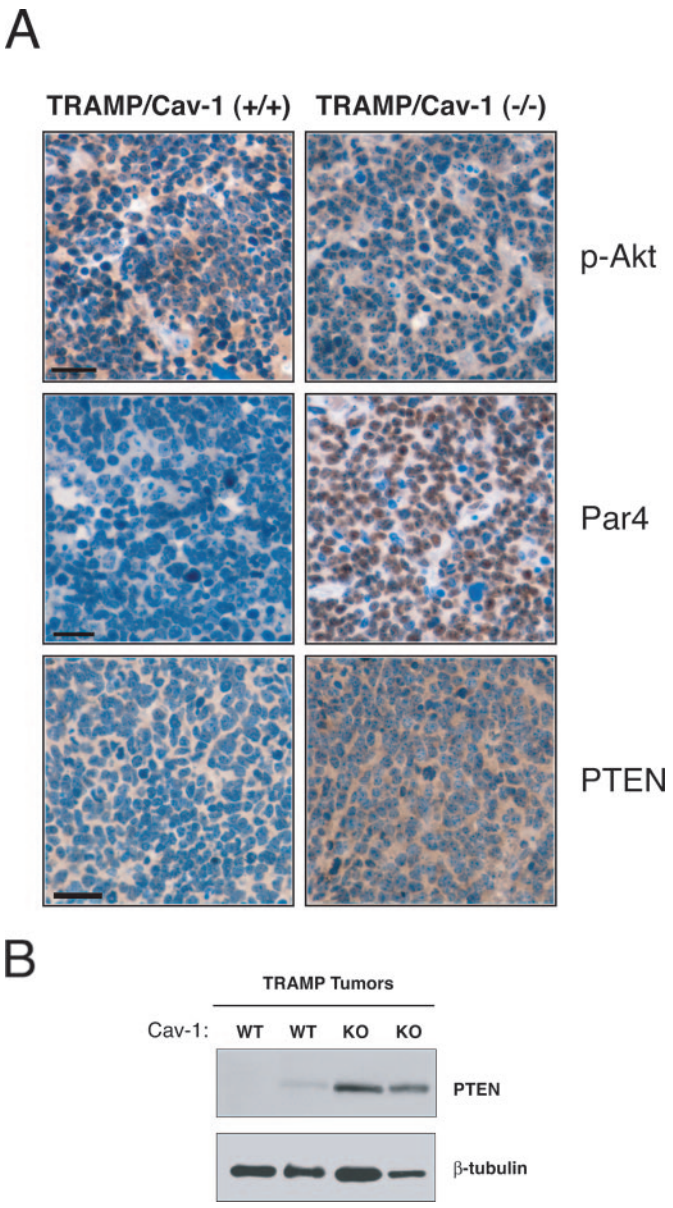


FIG. 8. **Loss of Cav-1 results in the up-regulation of two pro-apoptotic markers (PTEN and Par4) in TRAMP prostate tumors.** *A*, immunohistochemical localization of phospho (p)-Akt (Ser-473), Par4, and PTEN in WT and KO TRAMP tumors at 28 weeks of age. Representative photomicrographs are shown. Note that phospho-Akt cytoplasmic levels do not differ significantly between WT and KO tumors. In contrast, there is a striking increase in nuclear Par4 staining in KO tumors. Additionally, KO tumors also demonstrated higher PTEN levels. Finally, cytoplasmic PTEN staining is also increased in KO tumors. *Scale bars* = 25 μm. *B*, immunoblotting for PTEN levels in WT and KO TRAMP tumors. Note that PTEN levels are clearly up-regulated in KO tumors. β-tubulin is shown as an equal loading control.

DISCUSSION

One of the primary goals of cancer research is to identify genetic factors that contribute to tumorigenesis and to elucidate the mechanisms by which a given gene product can either inhibit or potentiate tumor development. In so doing, specific proteins can be selected for their potential as markers for

B, TUNEL staining of WT and KO TRAMP tumors at 28 weeks of age. TRAMP/Cav-1(+/+) tumors demonstrated an average of 15.6 ± 0.9 TUNEL-positive cells/40× field as compared with TRAMP/Cav-1(−/−) tumors, which show an average of 31.1 ± 1.4 apoptotic cells/40× field (a total of 75 fields were counted/genotype). This 2.0-fold difference is extremely significant ($p < 0.00001$, Student's *t* test, *asterisk*). A representative photomicrograph of TUNEL-stained prostate tumors from WT TRAMP (*left*) and KO TRAMP (*right*) mice is also shown. Note the increase in TUNEL-positive *blue* nuclei (*arrows*) in KO TRAMP prostate tumors. *Scale bar* = 25 μm. *C*, TUNEL staining of flank tumors derived from C1/pSUPER and C1/siRNA cells. C1/pSUPER-derived tumors demonstrated on average 0.23 ± 0.08 TUNEL-positive cells/40× field compared with 2.07 ± 0.25 cells/40× field (total of 30 fields counted/cell line). This 9.0-fold decrease is extremely significant ($p < 0.00001$), Student's *t* test, *asterisk*). A representative photomicrograph of TUNEL stained prostate tumors from C1/pSUPER (*left*) and C1/siRNA (*right*) cells is shown. Note the increase in TUNEL-positive *blue* nuclei (*arrows*) in C1/siRNA tumors. *Scale bar* = 25 μm.

cancer progression or as targets for therapeutic intervention. The Cav-1 gene has garnered attention because of its altered expression patterns in human cancers, e.g. (i) its down-regulation in ovarian carcinomas, sarcomas, and mammary carcinomas and (ii) its up-regulation in bladder, esophageal, and prostate carcinomas.

Prostate cancer is the second leading cause of cancer-related deaths in males in the United States. As such, identifying genes that serve as markers, predictors of outcome, or targets for pharmacological therapy is critical in establishing diagnoses and alleviating morbidity and mortality. Interestingly, there is an established relationship between Cav-1 protein levels and prostate cancer progression. Increased Cav-1 expression appears to correlate with higher prostate carcinoma grade and a negative outcome in clinical studies (21, 22). These studies have demonstrated important clinical correlations, but molecular genetic proof of a direct causal link between Cav-1 expression and prostate tumorigenesis is lacking. Full recapitulation of prostate tumor development using a transgenic mouse model enables investigators to directly test whether such relationships are indeed causal in nature.

Here, our current results provided the first molecular genetic evidence that Cav-1 promotes prostate tumorigenesis *in vivo*. Initial characterization of prostates derived from wild-type and TRAMP mice revealed that Cav-1 is expressed at virtually undetectable levels in wild-type and early transformed (PIN) prostate epithelium. However, Cav-1 was up-regulated in the later stages of prostate adenocarcinoma development, because poorly differentiated TRAMP tumors demonstrated significant Cav-1 expression. More importantly, we showed that loss of Cav-1 attenuates prostate cancer development by significantly reducing both primary tumor burden and metastatic disease in a transgenic prostate cancer model. Either partial or complete loss of Cav-1 is sufficient to reduce both of these indices of advanced neoplastic development. The observation that TRAMP/Cav-1(+/−) prostate tumors demonstrate markedly reduced Cav-1 levels (by ~80–90%) offers at least a partial explanation for why prostate tumorigenesis in TRAMP HET mice proceeds similarly to TRAMP KO mice.

Furthermore, we have established that these results are because of the *intrinsic* loss of Cav-1 within prostate carcinoma cells, as targeted down-regulation of Cav-1 in a mouse prostate carcinoma epithelial cell line (TRAMP-C1 cells) dramatically reduces tumor burden and experimental metastasis. We have also provided direct evidence to support previous cell culture studies that Cav-1 has pro-survival roles in prostate cancer, as both TRAMP prostate tumors and TRAMP-C1 cell line tumors lacking Cav-1 expression demonstrated increased rates of apoptosis. Taken together, these results delineate a direct causal relationship between Cav-1 expression and the development of more advanced prostate carcinomas, with regard to total primary tumor burden and metastatic disease. Our findings also validated the use of Cav-1 as a marker for prostate cancer progression and identified Cav-1 as a potential target for chemotherapeutic or gene therapy-based interventions.

Interestingly, these results directly contrast with our previous studies in mammary tissue, demonstrating that loss of Cav-1 accelerates mammary tumorigenesis and metastasis through a different transgenic model approach (9, 12). Recently, another group independently demonstrated that Cav-1 expression inhibits mammary tumor growth and metastasis (11). In these mammary carcinoma studies, Cav-1 appears to have a growth-inhibitory role, because genetic ablation of Cav-1 results in cyclin D1 overexpression and ERK-1/2 hyperactivation. In contrast, no differences were detected in ERK-1/2 activation or cyclin D1 expression between WT and Cav-1 KO TRAMP prostate tumors (not shown). Taken together, these data directly indicate that Cav-1 has tissue or cell type-specific roles with regard to tumorigenesis (*i.e.* growth-inhibitory in transformed mammary epithelium *versus* anti-apoptotic in transformed prostate epithelium). These results, taken in combination with other studies, provide clear evidence that Cav-1 up-regulation serves to provide a selective advantage to transformed cells in certain human cancers (*i.e.* prostate), whereas Cav-1 down-regulation may also promote cancer development in a completely different group of cancers (*i.e.* mammary gland). Further analysis of the molecular pathways responsible for this observed dichotomy should yield interesting insights into how Cav-1 normally functions as a "tumor suppressor" or "tumor promoter" in different cell- and tissue-specific contexts.

Previously, there was some concern regarding the validity of the TRAMP animal model for studying prostate carcinogenesis. However, this has now been resolved in favor of the TRAMP model. It was argued that a large proportion of prostate tumors arising in TRAMP mice are derived from neuroendocrine precursor cells and not from the prostate epithelium. This hypothesis was based on the presence of several neuroendocrine markers, such as synaptophysin, in advanced TRAMP prostate tumors. However, Greenberg and colleagues (27) have now provided direct evidence that this is not the case. They have demonstrated that the neuroendocrine markers present in poorly differentiated TRAMP tumors arise stochastically, as neuroendocrine markers are almost completely absent in PIN and low grade prostatic carcinoma TRAMP lesions. Additionally, the emergence of neuroendocrine markers does not directly indicate that transformed epithelial cells have "*trans*-differentiated" into neuroendocrine cells, as Greenberg and colleagues also show that the majority of these cells also co-express the androgen receptor, a protein not normally expressed by cells of neuroendocrine origin. Therefore, the presence of neuroendocrine markers more likely indicates the emergence of a distinct "neuroendocrine-like" phenotype.

Finally, because neuroendocrine markers are present in focal areas of nearly all clinical human prostatic adenocarcinomas, this acquired neuroendocrine phenotype further parallels the progression of the human prostatic disease and actually validates the use of the TRAMP model for studying prostate carcinoma progression (27). Similarly, we showed here that Cav-1 protein expression levels are indeed elevated in TRAMP prostate tumors, consistent with the up-regulation of Cav-1 in human prostate cancers (21, 22). Thus, our current results with Cav-1 further highlight the utility of the TRAMP model in studying the evolution of prostate cancers *in vivo*.

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