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Genetic Ablation of Caveolin-1 Drives Estrogen-Hypersensitivity and the Development of DCIS-Like Mammary Lesions

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Caveolin-1 (Cav-1) loss-of-function mutations are exclusively associated with estrogen receptor-positive (ER(+)) human breast cancers. To dissect the role of Cav-1 loss-of-function in the pathogenesis of human breast cancers, we used Cav-1^{-/-} null mice as a model system. First, we demonstrated that Cav-1^{-/-} mammary epithelia overexpress two well-established ER co-activator genes, *CAPER* and *Foxa1*, in addition to ER- α . Thus, the functional loss of Cav-1 may be sufficient to confer estrogen-hypersensitivity in the mammary gland. To test this hypothesis directly, we subjected Cav-1^{-/-} mice to ovariectomy and estrogen supplementation. As predicted, Cav-1^{-/-} mammary glands were hyper-responsive to estrogen and developed dysplastic mammary lesions with adjacent stromal angiogenesis that resemble human ductal carcinoma *in situ*. Based on an extensive biomarker analysis, these Cav-1^{-/-} mammary lesions contain cells that are hyperproliferative and stain positively with nucleolar (B23/nucleophosmin) and stem/progenitor cell markers (SPRR1A and β -catenin). Genome-wide transcriptional profiling identified many estrogen-related

genes that were over-expressed in Cav-1^{-/-} mammary glands, including *CAPER*—an ER co-activator gene and putative stem/progenitor cell marker. Analysis of human breast cancer samples revealed that *CAPER* is overexpressed and undergoes a cytoplasmic-to-nuclear shift during the transition from pre-malignancy to ductal carcinoma *in situ*. Thus, Cav-1^{-/-} null mice are a new preclinical model for studying the molecular paradigm of estrogen hypersensitivity and the development of estrogen-dependent ductal carcinoma *in situ* lesions. (Am J Pathol 2009, 174:1172–1190; DOI: 10.2353/ajpath.2009.080882)

Estrogen receptor signaling is tightly linked to the pathogenesis of human breast cancers. Estrogen receptor α (ER- α) is a ligand-activated transcription factor, which on the binding of the proper nuclear co-activators, initiates

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the transcription of downstream estrogen-responsive target genes.

Epidemiological studies have shown that prolonged exposure to endogenous estrogen from early menarche and late menopause, and hormone replacement therapy, are considered risk factors for the development of breast cancer.¹ However, normal mammary epithelial cells within mature terminal duct lobular units rarely divide and are mainly ER- α negative, where only 10 to 15% of the cells express the receptor.²

In the earliest stages of mammary tumorigenesis, such as ductal hyperplasia, atypical hyperplasia, and early DCIS lesions, ER- α becomes up-regulated in luminal mammary epithelial cells.³⁻⁵ Approximately 70% of invasive breast cancers express ER- α , in all of the cells that are actively proliferating.^{3,4} These observations suggest that increased expression of ER- α is an important initiating step in the development of human breast cancers. Indeed, current therapeutic approaches for ER- α positive breast tumors include the use of estrogen receptor blockers, such as tamoxifen, or aromatase inhibitors, which prevent the conversion of androgens to estrogens.

Caveolin-1 (Cav-1) is the main structural protein of caveolae, flask-shaped invaginations of the cell membrane, which compartmentalize important signaling molecules. Cav-1 is predominantly expressed in epithelia, fibroblasts, adipocytes, type I pneumocytes, and endothelial cells.⁶ Interestingly, Cav-1 has been mapped to the D7S522 locus (7q31.1), a hot spot for deletions in many types of human cancers.^{7,8} Accordingly, several human breast cancer cell lines have reduced Cav-1 levels when compared with benign cells, and Cav-1 re-expression in these cells causes a 50% reduction in cell proliferation and an ~15-fold decrease in anchorage-independent growth.⁹ Moreover, oncogene-transformed NIH-3T3 cells expressing H-Ras (G12V), v-Abl, or Bcr-Abl, have decreased expression levels of Cav-1 and its re-expression decreases their anchorage-independent growth in soft agar.¹⁰ Recently, we have shown that Cav-1 levels are also decreased in human cancer-associated fibroblasts isolated from invasive breast tumors.¹¹ More importantly, functional replacement of Cav-1 in cancer-associated fibroblasts, via a cell-permeable Cav-1 mimetic peptide, reverted their hyperproliferative phenotype by inhibiting RB-hyperphosphorylation.¹¹

Cav-1^{-/-} null mice also show several abnormal mammary gland phenotypes. For example, Cav-1^{-/-} mammary glands develop a mild hyperplasia, accompanied by an induction of ER- α protein expression in their luminal mammary epithelia, in addition to accelerated mammary gland development during pregnancy.^{12,13} When Cav-1^{-/-} mice are crossed with cancer-prone mouse models, such as MMTV-PyMT mice, significantly larger mammary tumors develop at an earlier age.^{14,15} Similarly, when Cav-1^{-/-} mice are crossed with mice lacking the Ink4a tumor suppressor, Cav-1/Ink4a double-knockout mice develop more pronounced mammary hyperplasia, accompanied by enhanced mammary fibrosis and ductal side-branching.¹⁶ These studies strongly suggest an important role for Cav-1 as a tumor suppressor in the mammary gland.

Interestingly, when genomic DNA from human breast tumors was analyzed for the presence of Cav-1 mutations, a proline-to-leucine substitution at position 132 was discovered.^{17,18} Remarkably, this P132L mutation behaves in a dominant-negative manner, resulting in the misfolding and mislocalization of wild-type Cav-1 in cultured cells.¹² Other mutations in the Cav-1 gene have been recently reported, such as W128stop, Y118H, S136R, I141T, Y148H, and Y148S.¹⁸ Further analysis revealed that all of these Cav-1 mutations exclusively cosegregate with ER- α positive tumors.¹⁸ In fact, virtually 35% of breast cancer patients with ER- α positive tumors harbor Cav-1 mutations.¹⁸ These findings suggest a possible causative role for Cav-1 loss-of-function in the up-regulation of ER- α expression and/or estrogen-dependent cell proliferation. To directly test this hypothesis, we exposed ovariectomized Cav-1^{-/-} mice to estrogen (E2) and analyzed their mammary gland phenotypes.

Here, we demonstrate that estrogen-treatment of Cav-1^{-/-} mice results in the development of ductal carcinoma *in situ* (DCIS)-like lesions, consistent with the idea that Cav-1 loss of function conveys estrogen hypersensitivity. These Cav-1^{-/-} mammary lesions also express high levels of B23/nucleophosmin, a known marker for recurrence in ER(+) breast cancer patients undergoing tamoxifen-based anti-estrogen therapy.¹⁹⁻²¹ Conversely, expression of B23/nucleophosmin is dramatically down-regulated to nearly undetectable levels in wild-type mice treated with estrogen. Thus, there is a complete reversal of the normal effects of estrogen treatment on B23/nucleophosmin expression in Cav-1^{-/-} mammary glands.

Materials and Methods

Animals

This study was conducted according to the guidelines of the National Institute of Health and the Thomas Jefferson University Institute for Animal Studies. Cav-1^{-/-} null mice were generated, as previously described.²² All mice used in this study were in the FVB/N genetic background.^{14,15}

Antibodies and Other Reagents

Rabbit polyclonal antibodies to Cav-1, ER- α , and progesterone receptor (PR), and a mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to SPRR1A and a mouse monoclonal antibody against Foxa1 were purchased from Abcam (Cambridge, MA) and the rabbit polyclonal antibody against CAPER was purchased from Biovision (Mountain View, CA). Phospho-specific rabbit polyclonal antibodies against phospho-ERK-1/2 (Thr 202/Tyr 204) and phospho-RB (Ser 807/11) were purchased from Cell Signaling Technology (Danvers, MA). A mouse monoclonal antibody against β -catenin was purchased from BD Biosciences (San Jose, CA). A mouse monoclonal anti-

body against B23/nucleophosmin was purchased from Zymed, Inc. (San Francisco, CA). Rabbit polyclonal antibodies against ARC were purchased from Cayman Chemical (Ann Arbor, MI). The immunohistochemistry visualization kit LSAB2 and the antibody to α -smooth muscle actin were purchased from DAKO (Carpinteria, CA). Hematoxylin counterstain was purchased from Sigma-Aldrich (St. Louis, MO). Placebo (cat # SC-111) and 17- β -estradiol pellets (cat # SE-121; 7.5 mg; 60-day; slow-release) were purchased from Innovative Research of America (Sarasota, FL).

Surgical Procedures

For the ovariectomy procedure, 5-week-old female FVB/N wild-type (wild-type; Cav-1^{+/+}) and Cav-1 knockout (KO; Cav-1^{-/-}) mice were initially anesthetized using xylazine: ketamine (5 mg/kg:50 mg/kg). A single dorsal incision, followed by ligation of the ovarian arteries and veins with a 4-0 silk suture was performed, followed by the excision of both ovaries. The incision site was subsequently closed with a 5-0 silk suture and the mice were given a subcutaneous injection of analgesic (buprenorphine, 0.1 mg/kg). Mice were allowed to recuperate for 2 weeks before being randomly assigned to either 1 or 2 replacement pellets containing 17- β -estradiol (7.5 mg/pellet; 60-day slow-release; 125 μ g/day) or placebo pellets ($n = 7$ to 10 for each group).

Implantation of slow-release pellets was performed under anesthesia, by lifting the skin on the lateral side of the neck of the mice, and by making an incision equal in diameter to that of the pellet. Then, with a pair of forceps, a horizontal pocket of about 2 cm beyond the incision site was created, and the pellet was introduced. The incision site was closed with a 5-0 silk suture.

Whole Mount Analysis of Mammary Glands

Mice were sacrificed by inhalation of CO₂ (compressed CO₂ gas cylinder) at 8 weeks following pellet implantation. Then, the fourth (inguinal) mammary glands were excised, spread onto glass slides, fixed, and stained as previously described.¹⁴ Briefly, mammary glands were fixed in Carnoy's fixative (six parts of 100% ethanol: three parts of CHCl₃: one part of glacial acetic acid) for 2 to 4 hours at room temperature. Mammary gland samples were then washed in 70% ethanol for 20 minutes and changed to decreasing amounts of ethanol and finally to distilled water. The mammary glands were stained overnight in a solution of 0.2% carmine and 0.5% aluminum potassium phosphate (Sigma-Aldrich, St. Louis, MO). The samples were then dehydrated using a graded ethanol series and left in xylene to clear the fat. Mammary gland whole mounts were stored in methyl-salicylate and photographed using an Olympus DP71 camera with the DP manager software version 3.1.1.208, using the same magnification. The degree of branching was assessed as previously described.^{23,24} Briefly, the number of primary, secondary, and tertiary branch points on stained whole mounts were counted on each whole mount and aver-

aged individually. Primary branches are defined as mammary ducts that originate from the nipple region and extend to the leading edge of the gland, secondary branches are the ones arising from the primary branches and finally the tertiary branches are the ones that initiate from the secondary ducts. Ductal thickness was determined by measuring the diameter of multiple primary branches using Image J software version 10.2.

Immunohistochemistry

Paraffin-embedded slides were dehydrated in a series of graded ethanol and were left in xylene for 10 minutes. Following the dehydration, the slides were rehydrated in a series of graded ethanol and completely rehydrated in distilled water for 10 minutes. The endogenous peroxidase activity was inhibited with 3% of hydrogen peroxide (Fisher, Hampton, NH) for 30 minutes at room temperature. The slides were blocked with 10% goat normal serum (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) for 1 hour at room temperature. The slides were then incubated with the primary antibody overnight at 4°C. The next day, the slides were washed with PBS and incubated with the biotinylated mouse or rabbit secondary antibody included in the immunohistochemistry visualization kit LSAB2 (Dako, Carpinteria, CA). The remainder of the protocol was performed according to the manufacturer's instructions. The slides were counterstained using Mayer's hematoxylin (Sigma, St-Louis, MO). The slides were dehydrated with graded alcohols, as before, and left in xylene for 10 minutes before mounting them with PermOUNT (Thermo Fisher Scientific, Hampton, NH).

Gene Profiling

Gene profiling (DNA microarray) was performed on mammary gland tissues surgically isolated from estrogen-treated Cav-1^{-/-} mice compared with their Cav-1^{+/+} counterparts (three mice per group). These studies were performed essentially as we have previously described for other cell types.²⁵ Briefly, RNA was extracted from tissues by TRIzol method (Invitrogen Corporation) according to the manufacturer's instructions. The RNA was further purified using RNeasy Micro Kit (Qiagen, Valencia, CA) and reverse transcribed using Superscript III First-Strand Synthesis System (Invitrogen Corporation) and T7-dT24 primer (Sigma Genosys). The single stranded cDNA was converted to double stranded cDNA and purified. The double stranded cDNA was used as a template to generate biotinylated cRNA using RNA Transcription Labeling Kit (Enzo, New-York, NY) and the labeled cRNA was purified. The cRNA (15 μ g) was fractionated to produce fragments of between 35 and 200 bp and hybridized to the mouse array (Affymetrix, Santa Clara, CA). The hybridization was performed in accordance with Affymetrix protocols. The arrays were scanned at 570 nm with a confocal scanner from Affymetrix.

Figure 1. Cav-1^{-/-} mammary glands overexpress ER co-activator genes CAPER and Foxa1. Cav-1^{-/-} mice are known to over-express ER- α in the luminal mammary compartment. To determine whether Cav-1^{-/-} mice express other components of the ER-transcriptional machinery, we immunostained Cav-1^{-/-} mammary glands with a panel of antibodies directed against distinct ER co-activator genes. Note that two ER co-activator genes, CAPER and Foxa1, are dramatically overexpressed in Cav-1^{-/-} mammary glands; expression of ER- α is also shown for comparison. Interestingly, CAPER is overexpressed in both the luminal epithelial cells (**white arrow**) and the mammary stromal compartment (**black arrow**). Paraffin-sections from ovariectomized wild-type and Cav-1^{-/-} mice were used to better synchronize the cells within the mammary gland.

Array Data Analysis

Analysis of the arrays was performed as previously described using the statistical package R and the limma library of the Bioconductor software package.^{25,26} Nor-

Figure 2. Experimental approach for determining the estrogen-dependent phenotypes of Cav-1^{-/-} mammary glands. **A:** Schematic diagram. Female wild-type and Cav-1^{-/-} mice were subjected to bilateral ovariectomy (OVX) at 5 weeks of age and allowed to recover for 2 weeks. Then, the mice were randomly given either estrogen or a placebo for 8 consecutive weeks (60 days) via the implantation of a slow-release pellet. Two dosages of 17- β -estradiol (E2) were evaluated in parallel using 7.5 mg (L; low; 125 μ g/day) and 15.0 mg (H; high; 250 μ g/day) pellets. At the end of the 60-day treatment period, the mice were sacrificed and the morphology of the mammary gland was assessed, by whole mount analysis and using paraffin-embedded tissue sections. In all cases, 7 to 10 mice were used for each treatment group and for each genotype. WT, Cav-1^{+/+}; KO, Cav-1^{-/-}. **B:** Whole mount analysis. Note that wild-type mice treated with placebo have few branches with narrow ducts. Following the treatment with 17- β -estradiol, the wild-type mammary glands do not seem very different, with the exception of twofold more primary branches. Note that Cav-1^{-/-} mice treated with placebo resemble the wild-type mammary gland. In striking contrast, Cav-1^{-/-} mice are markedly more responsive to 17- β -estradiol (E2) treatment, as shown by increases in ductal thickening and secondary and tertiary branching. Representative images of mammary glands from mice treated with low (L) dose E2 are shown. Images were taken with a $\times 1.5$ objective. The boxed area is shown at higher magnification to illustrate the increased branching (see **arrowhead**) observed in E2-treated Cav-1^{-/-} mammary glands. Inset magnification: $3\times$.

malization of the array was performed using a robust multiarray analysis. A fold-change of greater than 2 was generally used as a criterion for differential gene expression. Gene ontology analysis was performed using the DAVID 2007 bioinformatics resource. Gene lists for estrogen responsive genes were from the following sources.^{27–30} Gene sets for ES-cell associated transcripts, as well as Nanog, Oct4, Sox2, and Myc target genes, were previously described.³¹ The analysis was performed on three independent mammary glands for each experimental group.

Statistical Analysis

All of the statistical analysis was performed using a Tukey-Kramer Multiple Comparison test. A *P* value of <0.05 was considered significant.

Immunohistochemistry and Automated Quantitative Analyses

Immunohistochemistry and automated quantitative *in situ* marker analysis (AQUA) analyses were performed on sections of a tissue array constructed by cutting-edge matrix-assembly³² that contained 84 invasive breast carcinomas and 20 normal breast tissues. Briefly, sections were subjected to deparaffinization, rehydration, and antigen retrieval using citrate buffer, pH 6.0 (Dako). Subsequently, tissues were blocked with peroxidase blocking reagent (Dako; Cat# S2001) and followed by 10% goat serum (Biogenex; Cat# HK-112-9K). Sections were then incubated with anti-CAPER IgG, as described above, at 1:100 dilution. Following the incubation, sections were washed 3 times with TBS containing 0.01% Tween-20 and then incubated with a mouse anti-cytokeratin antibody (Dako, Cat# AE1/AE3) for 1 hour. The CAPER antibody was detected using an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Dako, EnVision-Plus), followed by incubation with Tyramide-Cy5 (Perkin Elmer, Cat# NEL745). Cytokeratin was visualized by further incubating the sections with a mouse secondary antibody conjugated to Alexa 488 (Molecular Probes, Cat# A11034). Finally, all sections were stained with 4,6-diamidino-2-phenylindole (Vector, Cat: H1500) for nuclear

Table 1. Uterus/Body Weight Percentage Following E2 Treatment

WT uterus/body weight (%)			Cav-1 ^{-/-} uterus/body weight (%)		
Placebo	E2 7.5 mg*	E2 15 mg*	Placebo	E2 7.5 mg [†]	E2 15 mg ^{††}
0.06 +/- 0.004	0.39 +/- 0.014	0.42 +/- 0.016	0.05 +/- 0.015	0.37 +/- 0.019	0.33 +/- 0.012

E2, denotes 17- β -estradiol.

* $P < 0.001$ vs. WT placebo (Tukey-Kramer test).

[†] $P < 0.001$ vs. Cav-1 (-/-) placebo (Tukey-Kramer test).

^{††} $P < 0.001$ vs. WT E2 15 mg (Tukey-Kramer test).

visualization. Automated quantitative analysis was performed using the AQUA/PM2000 Imaging Platform (HistoRx), as described.^{33–35} Briefly, tissue array slides were scanned and images of each breast cancer tissue were captured at different channels including fluorescein isothiocyanate/Alexa 488, Cy5, or 4,6-diamidino-2-phenylindole. AQUA software was then used to identify epithelial masks based on fluorescein isothiocyanate-positive cytokeratin-expressing cells. AQUA scores for CAPER represent average signal intensities within epithelial cells.

Results

Cav-1^{-/-} Mammary Glands Overexpress ER Co-Activator Genes

Cav-1^{-/-} mice show the overexpression of ER- α in their luminal mammary epithelial cells.¹⁸ To determine whether these mice also overexpress other components of the ER-transcriptional machinery, we immuno-stained Cav-1^{-/-} mammary glands with a panel of antibodies directed against a number of distinct ER-co-activator genes. Based on this analysis, we determined that two ER co-activator genes, CAPER^{36,37} and Foxa1,³⁸ are dramatically overexpressed in Cav-1^{-/-} mammary glands (Figure 1). The expression of ER- α is shown for comparison. Note that CAPER is overexpressed in both the luminal epithelial cells and the mammary stromal compartment.

Cav-1^{-/-} Mammary Glands Display Estrogen-Hypersensitivity, with Increased Ductal Thickening, Side-Branching, and the Development of Dysplastic Mammary Lesions

Since Cav-1^{-/-} mammary glands overexpress ER- α , and two ER-co-activator genes, we speculated that they may also be functionally hypersensitive to the effects of estrogen exposure. To test this hypothesis directly, we subjected 5-week-old virgin female Cav-1^{-/-} mice to ovariectomy (OVX) and exposed them to either placebo or two different dosages of estrogen (E2). This experimental strategy is outlined in detail in Figure 2A. After 60 days, mice were sacrificed and subjected to detailed mammary gland analysis, via whole-mounts. We validated the efficacy of estrogen supplementation by independently measuring the weight of the uterus in wild-type and Cav-1^{-/-} mice (Table 1). Notably, Cav-1^{-/-} mice did not show excessive increases in uterine weight, indicating that their

estrogen-hypersensitivity phenotype (see below) may be confined to the mammary gland. Interestingly, Cav-1^{-/-} mammary glands appeared hyper-responsive to the effects of estrogen, undergoing ductal thickening and extensive side-branching, as compared with wild-type mice treated in parallel (Figure 2B). Quantitation of the estrogen-dependence of ductal thickening and branching morphogenesis is presented in Figure 3A. Note that at both lower (125 μ g/day) and higher (250 μ g/day) doses of estrogen, Cav-1^{-/-} mice show an approximately twofold increase in ductal thickening, as compared with E2-treated wild-type mice. Similarly, Cav-1^{-/-} mammary glands also show greater susceptibility to estrogen-induced branching morphogenesis, with approximately three- to four-fold increases in secondary branching and approximately a five- to seven-fold increase in tertiary branching (Figure 3B).

Interestingly, at the higher dose of estrogen, Cav-1^{-/-} mammary glands also developed abnormal mammary lesions or dysplastic foci. Mammary lesion frequency and morphological examples are shown in Figure 4. Cav-1^{-/-} mice showed a >fourfold increase in lesion frequency and an ~2.5-fold increase in lesion diameter. Development of these mammary lesions was strictly dependent on the higher-dose of estrogen and not observed in mice treated with the lower dose. Thus, we focused our efforts on understanding the nature of the high dose-dependent mammary lesions.

Estrogen-Induced Cav-1^{-/-} Mammary Lesions Morphologically Resemble Human DCIS

To understand the nature of these mammary lesions, wild-type and Cav-1^{-/-} mammary glands were surgically excised after estrogen treatment, and samples were paraffin-embedded and subjected to standard histochemical stains (H&E and Trichrome). Representative morphological examples are shown in Figure 5. Remarkably, in estrogen-treated Cav-1^{-/-} mice, these dysplastic foci clearly resemble human DCIS lesions, with complete luminal filling (Figure 5A). Trichrome staining also revealed that these lesions are often encircled or associated with small blood vessels (Figure 5B).

Virtually all of the Cav-1^{-/-} ducts showed complete luminal filling and local infiltration with small blood vessels. This pattern of vascularization has been described as “cuffing,” “hugging,” or as forming a “necklace” around DCIS lesions, and is specifically associated with high-grade DCIS lesions and has a worse prognosis.^{39–42} For additional examples of this estrogen-induced mammary stromal vascularization, see supplemental Figure S1 (available at <http://ajp.amjpathol.org>).

Figure 3. Cav-1^{-/-} mammary glands are hyper-responsive to the effects of estrogen. As outlined in Figure 2, wild-type and Cav-1^{-/-} mice were ovariectomized and subjected to estrogen supplementation. After 60 days of placebo (OVX) or E2-treatment (OVX + E2), carmine dye staining of inguinal (no. 4) mammary gland was performed. Then, quantitation of ductal thickness (A) and branching (B) was performed using NIH image J software. The number of primary, secondary and tertiary branch points in the inguinal whole mounts was assessed. Primary branches are defined as ducts that arise from the nipple and extend throughout the gland, secondary branches are ducts that arise from primary ducts, and the tertiary branches are lateral branches that arise from the secondary ducts. Results with both low (L) and higher (H) dose of E2 are shown. Note that at both lower and higher doses of estrogen, Cav-1^{-/-} mice show an approximately twofold increase in ductal thickening, as compared with E2-treated wild-type mice. Similarly, Cav-1^{-/-} mammary glands also show greater susceptibility to estrogen induced branching morphogenesis, with approximately three- to fourfold increases in secondary branching and five to sevenfold increases in tertiary branching. For (A), **P* < 0.01 when compared with wild-type treated with placebo; †*P* < 0.01 when compared with wild-type treated with E2; and ‡*P* < 0.01 when compared with placebo-treated Cav-1^{-/-} mice (*n* = 7 to 10 for each group). For (B), **P* < 0.001 when compared with wild-type treated with placebo; †*P* < 0.01 when compared with wild-type treated with E2; ‡*P* < 0.01 when compared with placebo-treated Cav-1^{-/-} mice; and §*P* < 0.01 when compared with E2 (L; low dosage) treated Cav-1^{-/-} mice (*n* = 7 to 10 for each group).

Cav-1^{-/-} mammary epithelial cells within these lesions also show nuclear atypia and nuclear heterogeneity (Figure 6A). Nuclei often appear pale, with prominent nucleolar staining. These nuclear changes are thought to be associated with mammary cell transformation, and are characteristic of high-grade DCIS lesions (See <http://suropathcriteria.stanford.edu/breast/dcis/>). These nuclear changes were not observed in wild-type animals treated identically. Cav-1^{-/-} mammary ductal lesions show an abnormal distribution of myoepithelial cells (Figure 6B). In wild-type ducts, the myoepithelial cells appear normal

and form a continuous layer surrounding the ducts. In contrast, in Cav-1^{-/-} ducts, the myoepithelial cells appear in a discontinuous distribution, and are often mislocalized in the center of the lesions. These changes are suggestive of a potentially invasive phenotype.

Cav-1^{-/-} Mammary Lesions are Hyperproliferative, Contain Mammary Stem/Progenitor Cells, and Show Signs of Angiogenic Stromal Activation

To gain mechanistic insight into the development of Cav-1^{-/-} mammary lesions, we next assessed their estrogen-dependent expression of a panel of well-characterized biomarkers. First, we determined the status of progesterone receptor (PR-A/B) expression by immunostaining.

Figure 7 illustrates that these mammary lesions show the dramatic up-regulation of PR immunostaining. As PR expression is estrogen-dependent and is often used as a positive marker or “reporter” of activated ER-α receptor signaling, our results are consistent with the idea that ER-signaling is highly-active in Cav-1^{-/-} mammary epithelial cells.

We also examined the expression of proliferative markers, such as PCNA, a cofactor of DNA polymerase, that is synthesized in the early G1 and S phases of the cell cycle. PCNA has been shown to be dramatically increased in terminal end buds (TEBs) by proteomic approaches.⁴³ As predicted, our results directly show the upregulation of PCNA in these estrogen-induced Cav-1^{-/-} mammary lesions (Figure 8A). Similar results were obtained with an antibody directed against phospho-RB (Ser 807/811), another established marker of cell cycle progression (Figure 8B). PCNA is a known RB/E2F target gene.⁴⁴ Cav-1^{-/-} mammary glands also show extensive mammary stromal activation of the Ras-p42/44 MAP kinase pathway. In the Cav-1^{-/-} mammary stroma, the levels of phospho-ERK-1/2 appear dramatically elevated (Figure 8C). However, no activation of ERK-1/2 was observed in the absence of estrogen, in either wild-type or Cav-1^{-/-} mammary samples. These changes are suggestive of a highly-activated mammary stroma.

In accordance with our results from phospho-ERK-1/2 immunostaining, increased mammary stromal vascularization was not strictly limited to the areas surrounding ductal lesions, but was uniformly increased throughout the mammary fat pad (Figure 9A). Mechanistically, this may be secondary to increased expression of vascular endothelial growth factor (VEGF), an angiogenic growth factor, in the Cav-1^{-/-} mammary stroma under conditions of estrogen-stimulation (Figure 9B). Notably, increased VEGF expression has been previously shown to be associated with the angiogenic “cuffing” of human DCIS,⁴⁵ as we see in Cav-1^{-/-} DCIS-like lesions (Figures 5B and 9A).

Finally, we also assessed the expression of markers that are associated with mammary stem/progenitor cells, small proline-rich repeat protein 1A (SPRR1A) and β-catenin. SPRR1A is a TEB marker that is preferentially expressed in TEBs during mammary gland development, as compared with mammary ductal cells.^{43,46–48} TEBs are thought to be enriched in mammary stem/progenitor cells. Consistent with

Figure 4. Cav-1^{-/-} mice develop abnormal mammary lesions (dysplastic foci) in response to estrogen. When the mammary whole mounts were analyzed following treatment of Cav-1^{-/-} mice with a higher (H) dose of E2 (15.0 mg slowly delivered over a 60-day period), the presence of foci was detected. Right, Representative high power images of Cav-1^{-/-} mammary lesions are shown stained with Carmine dye. In the boxed area, shown at higher magnification, **arrows** point at mammary lesions (dysplastic foci). Quantitation of mammary lesion frequency (**upper left**) and size (**lower left**) was performed using NIH image J software. Although the wild-type mice show some minor increases in mammary lesion formation, following 17- β -estradiol treatment (E2), Cav-1^{-/-} mice are markedly hyper-responsive to 17- β -estradiol treatment (E2). Cav-1^{-/-} mice show a greater than fourfold increase in lesion frequency and an ~2.5-fold increase in lesion diameter. These differences are highly statistically significant. [†] $P < 0.01$ when compared with wild-type treated with E2; [‡] $P < 0.01$ when compared with placebo-treated Cav-1^{-/-} mice. WT, Cav-1^{+/+}; KO, Cav-1^{-/-} ($n = 7$ to 10 for each group). Inset magnification: 10 $\times$.

the idea that SPRR1A is a stem/progenitor cell marker, SPRR1A has also been implicated in axonal outgrowth; it is highly expressed during axon regeneration, after nerve injury, and is associated with neuronal growth cones.⁴⁸ The Wnt/ β -catenin signaling pathway is activated in stem cells, including mammary progenitor cells.⁴⁹

Figure 10 shows the up-regulation SPRR1A and β -catenin in these estrogen-induced Cav-1^{-/-} mammary lesions (panels B and C). Importantly, we also independently validated that SPRR1A is preferentially expressed in TEBs, as compared with mammary ducts in young virgin wild-type mice (Figure 10A). In this regard, several

studies have suggested that a certain subset of mammary progenitor cells may express ER- α and PR.^{50–52}

Cav-1^{-/-} Mammary Lesions Express Elevated Levels of B23/Nucleophosmin, a Nucleolar Marker Protein and Predictor of Tamoxifen Resistance

Since Cav-1^{-/-} mammary lesions showed such striking nucleolar prominence (Figures 5 and 6), we examined the

Figure 5. Estrogen-induced Cav-1^{-/-} mammary lesions morphologically resemble DCIS, and show adjacent stromal angiogenesis. To understand the nature of these estrogen-induced mammary lesions, wild-type and Cav-1^{-/-} mammary glands were surgically excised after estrogen treatment, and samples were paraffin-embedded and subjected to standard histochemical stains. Interestingly, in estrogen-treated Cav-1^{-/-} mice, these dysplastic foci clearly resemble human DCIS lesions, with complete luminal filling. Representative images of H&E- (**A**) and Trichrome-stained (**B**) sections are shown. Trichrome staining also revealed that these lesions are often encircled or associated with small blood vessels (**arrow**). Also, note that collagen-staining (light blue) is conspicuously reduced surrounding Cav-1^{-/-} mammary lesions, suggestive of E2-induced local collagen degradation. Boxed areas are shown at higher magnification to highlight nuclear morphology and nucleolar prominence in Cav-1^{-/-} mammary lesions. Images were taken with a $\times 60$ objective. Inset magnification: 9 $\times$.

Figure 6. Cav-1^{-/-} mammary lesions show nuclear changes, nucleolar prominence, mitotic figures, and an abnormal distribution of myoepithelial cells. **A:** Nuclear and nucleolar abnormalities. A higher power view of an E2-induced Cav-1^{-/-} mammary lesion is shown (Trichrome staining). Note that the mammary epithelial cells within the lesion show nuclear atypia and nuclear heterogeneity. A central mitotic figure is also present (arrow). Nuclei often appear pale, with prominent nucleolar staining. These nuclear changes are thought to be associated with mammary cell transformation. These nuclear changes were not observed in wild-type animals treated identically. **B:** Altered myoepithelial distribution. Paraffin-sections from ovariectomized and E2-treated mice were immunostained with anti- α -smooth muscle actin IgG to visualize the distribution of myoepithelial cells. Bound antibodies were detected with an HRP-conjugated secondary antibody (brown color). Samples were lightly counterstained with hematoxylin (blue color). Note that in wild-type ducts, the myoepithelial cells appear normal and form a continuous layer surrounding the ducts (Left). In contrast, in Cav-1^{-/-} ducts, the myoepithelial cells appear in a discontinuous distribution (arrow), and are often mislocalized in the center of the lesions. These changes are suggestive of a potentially invasive phenotype.

expression of B23/nucleophosmin, a well-established nuclear marker protein that is normally elevated in human breast cancers and is a predictor of recurrence in ER(+) patients treated with tamoxifen-based anti-estrogen therapy.

Figure 7. Cav-1^{-/-} mammary epithelial cells show the overexpression of PR, an estrogen-responsive target gene. The status of progesterone receptor (PR-A/B) expression was monitored by immunostaining. Note that E2-induced Cav-1^{-/-} mammary lesions show the dramatic up-regulation of PR-A/B. Since PR-expression is estrogen-dependent and is often used as a "reporter" of activated ER receptor signaling, our results are consistent with the idea that ER signaling is highly activated in Cav-1^{-/-} mammary epithelial cells. In comparison, wild-type (WT) mice (treated identically) show significantly less staining.

apy.¹⁹⁻²¹ B23/nucleophosmin expression is induced by estrogen-treatment of MCF-7 human breast cancer cells and it is constitutively elevated in tamoxifen-resistant MCF-7 cells, implicating it functionally in the development of resistance toward anti-estrogen therapy.¹⁹⁻²¹ Its expression can also serve as a surrogate marker for cell proliferation, as B23 and the nucleolus are involved in the regulation of ribosome biogenesis, and hence protein synthesis.^{53,54} Interestingly, B23 sequesters p19ARF within the nucleolus, thereby inhibiting its tumor-suppressor function.⁵⁵

Figure 11 shows that B23/nucleophosmin is clearly up-regulated in Cav-1^{-/-} mammary lesions in response to E2-treatment. Conversely, its expression is dramatically down-regulated to nearly undetectable levels in wild-type mice treated with E2. Thus, there is a complete reversal of the normal effects of estrogen-treatment on B23/nucleophosmin expression in Cav-1^{-/-} mammary glands, behaving as observed in estrogen-treated MCF-7 breast cancer cells. These findings directly support our results from genome-wide expression profiling, as several genes associated with ribosome biogenesis (*Rps9*, *Rps13*, *Rpl17*, and *Srp54*) were significantly up-regulated in the mammary glands of E2-treated Cav-1^{-/-} mice (Table 2). In fact, *Rps9* and *Rps13* are both known B23/nucleophosmin interacting proteins.⁵⁶ Since we did not observe any changes in B23/nucleophosmin transcript levels, the upregulation of *Rps9* (34.6-fold) and *Rps13* (2.0-fold) may serve to stabilize B23/nucleophosmin, thereby preventing its rapid turnover/degradation.

Figure 8. Status of proliferative markers in Cav-1^{-/-} mammary lesions. We also examined the levels of proliferative markers, such as PCNA, phospho-RB, and phospho-ERK-1/2 under conditions of estrogen-stimulation. Note that PCNA and phospho-RB (**A** and **B**, respectively) levels are clearly elevated in E2-treated Cav-1^{-/-} mammary lesions. In contrast, phospho-ERK-1/2 levels (**C**) were more highly elevated in the mammary stroma of E2-treated Cav-1^{-/-} mice. This is consistent with E2-dependent activation of the mammary stroma in Cav-1^{-/-} mice and the development of increased vascularization (stromal angiogenesis).

Upregulation of Apoptosis Repressor with Caspase Recruitment Domain, a Marker of Resistance to Apoptosis

Apoptosis repressor with caspase recruitment domain (ARC) is an inhibitor of apoptosis that confers both radi-

ation- and chemo-resistance in cultured human breast cancer cells.⁵⁷ Interestingly, ARC expression is markedly increased in the epithelium of DCIS and invasive ductal carcinoma (IDC) lesions, as well as human colon cancers.^{57,58} Thus, we examined the expression of ARC in Cav-1^{-/-} DCIS-like lesions. Figure 12 shows that after

Figure 9. Estrogen-treated Cav-1^{-/-} mice develop mammary stromal angiogenesis, and overexpress VEGF in the mammary stroma. **A:** Trichrome staining. Note that in E2-treated Cav-1^{-/-} mice, increased mammary stromal vascularization was not strictly limited to the areas surrounding ducts, but was uniformly increased throughout the mammary fat pad. To highlight this increased stromal vascularity, a boxed area is shown at higher magnification. Inset magnification: 10 $\times$. **B:** VEGF immunostaining. Interestingly, increased mammary stromal vascularization may be due to increased expression of VEGF, an angiogenic growth factor, in the Cav-1^{-/-} mammary stroma under conditions of estrogen-stimulation.

Figure 10. DCIS-like lesions in Cav-1^{-/-} mice have an increased mammary stem/progenitor cell population. **A:** Localization of SPRR1A, a TEB marker, in normal mammary glands. Paraffin sections from 6-week-old virgin female wild-type mice were immunostained with anti-SPRR1A IgG. Note that SPRR1A is preferentially expressed in TEBs, but not in mammary ducts. This is consistent with the idea that TEBs contain mammary stem/progenitor cells. An **inset**, at right, shows a higher power view of SPRR1A immunostaining. Inset magnification: 5×. **B:** Cav-1^{-/-} mammary ductal lesions show up-regulation of SPRR1A, a TEB Marker. Paraffin sections from ovariectomized and E2-treated mice were immunostained with anti-SPRR1A IgG. Note that in the Cav-1^{-/-} mammary epithelia, the levels of SPRR1A appear elevated, especially under conditions of estrogen (E2) supplementation. As SPRR1A is a TEB marker, these results suggest that there is an increase in mammary stem/progenitor cells. **C:** Cav-1^{-/-} mammary ductal lesions show the up-regulation of β-catenin. Paraffin sections from ovariectomized and E2-treated mice were immunostained with anti-β-catenin IgG. Note that in the Cav-1^{-/-} mammary epithelia, the levels of β-catenin appear highly elevated. These results suggest that the Wnt/β-catenin pathway is activated in Cav-1^{-/-} mammary epithelia, under conditions of estrogen (E2) stimulation.

E2-treatment, ARC is highly overexpressed in both the luminal epithelial cells and the mammary stromal compartment, in Cav-1^{-/-} mammary glands. As such, overexpression of ARC, an anti-apoptotic gene product,

could also contribute to the luminal filling phenotype we observe in Cav-1^{-/-} DCIS-like lesions.

Additional Mechanistic Insights from Genome-Wide Expression Profiling

To mechanistically dissect the estrogen-dependent hyperplastic phenotype of Cav-1^{-/-} mice, we surgically isolated the mammary glands from ovariectomized wild-type and Cav-1^{-/-} female mice treated with estrogen and subjected them to genome-wide transcriptional profiling (E2-treated wild-type versus E2-treated knockout). The expression levels of 250 known genes and transcripts were changed in Cav-1^{-/-} mammary glands, as compared with wild-type glands; 127 transcripts were up-regulated and 123 transcripts were down-regulated. All of these genes and transcripts changed by >twofold and achieved statistical significance ($P < 0.05$). Consistent with our morphological observations, in E2-treated Cav-1^{-/-} mammary glands, we see a number of gene changes specifically associated with estrogen receptor signaling, the upregulation of epithelial-specific markers, and pro-angiogenic factors, as well as changes associated with increased protein synthesis (ribosome biogenesis) and steroid metabolism (Table 2). Interestingly, these gene changes included the up-regulation a number of known ER co-activator genes, such as CAPER (a.k.a. Rbm39), Foxa1, and Nr4a1 (See also Figure 1). Since no changes in ER-α transcript levels were observed, we speculate that the increased levels of ER-α that we observe may be due protein stabilization effects conferred by complex formation with ER co-activator genes, thereby “priming” the mammary epithelial cells for estrogen hyper-responsiveness.

Pro-angiogenic factors that were up-regulated in Cav-1^{-/-} mammary glands included two angiogenin gene family members (*Ang4* and *Ang5*) (Table 2). Interestingly, angiogenin expression is known to be up-regulated in human DCIS lesions and full-blown IDC and is strictly associated with ER-positivity and higher tumor grade.⁵⁹ Several luminal epithelial markers (cyokeratins 8/18/19 and prolactin receptor), known to be up-regulated in ER (+) breast cancers,^{60,61} were significantly up-regulated in Cav-1^{-/-} mammary lesions (Table 2).

We also analyzed an extended gene list (with transcripts achieving >1.5-fold alterations; $P < 0.05$) for changes in the levels of known estrogen-responsive genes. Supplemental Table S1 (available at <http://ajp.amjpathol.org>) shows that the transcriptional expression of ~55 known estrogen-regulated genes was changed in the appropriate direction in Cav-1^{-/-} mammary glands. This more extensive list includes Gata3, another ER-co-activator gene.⁶² In accordance with our observations that Cav-1^{-/-} mammary lesions stain positively with stem/progenitor markers, a number of ES-cell associated genes are transcriptionally up-regulated in Cav-1^{-/-} mammary glands, including Nanog, Oct4, Sox2, and Myc target genes (Table 3). Several of these ES-cell associated genes, as well as other up-regulated transcripts, are associated with malignancy and are highlighted in Tables

Figure 11. Cav-1^{-/-} mammary lesions express elevated levels of B23/nucleophosmin, a nucleolar marker protein and predictor of tamoxifen resistance. Paraffin sections from ovariectomized and E2-treated mice were immunostained with anti-B23/nucleophosmin IgG (brown color). Then, sections were lightly counterstained with hematoxylin (blue color). B23/nucleophosmin is a well-established nucleolar marker protein that is normally elevated in human breast cancers and is a predictor of recurrence in ER(+) patients treated with tamoxifen. **A:** A comparison between E2-treated mice (WT versus Cav-1^{-/-}) is shown. Images were taken with a $\times 100$ objective. Inset magnification: $3.5\times$. **B:** A comparison of all four experimental groups is shown. Images were taken with a $\times 40\times$ objective. In **(A)** and **(B)**, boxed areas are shown at higher magnification. Note that B23/nucleophosmin is up-regulated in Cav-1^{-/-} mammary lesions in response to E2 treatment. Conversely, its expression is dramatically down-regulated to undetectable levels in wild-type mice treated with E2. Thus, there is a complete reversal of the normal effects of estrogen-treatment on B23/nucleophosmin expression in Cav-1^{-/-} mammary glands. Inset magnification: $10\times$.

4 and 5. For example, Cav-1^{-/-} mammary glands show the upregulation of CD133 (a.k.a., Prom1), a well-established cancer stem cell marker that is often used to purify tumor-initiating cells.

Interestingly, based on this analysis, we identified CAPER (a.k.a., Rbm39) as a stem-cell associated gene, as it is the shared transcriptional target of three iPS (induced-pluripotency) genes, namely Nanog, Sox2, and c-Myc. As CAPER is both an ER co-activator gene and an ES cell associated gene, up-regulation of CAPER expression could account for both (i) the increased estrogen-sensitivity and (ii) the expansion of the mammary stem cell compartment, in Cav-1^{-/-} mammary glands.

Overexpression of CAPER in Human Breast Cancers

Given the association of CAPER overexpression with estrogen-hypersensitivity in Cav-1^{-/-} mice, we speculated that CAPER may also be relevant for the pathogenesis of human breast cancers. To this end, we examined the

expression of CAPER using a human breast cancer tissue microarray, consisting of 84 IDCs and 20 normal breast tissue samples. Quantitative analysis of CAPER expression was achieved by using the PM2000 AQUA automated quantitative analysis system (HistoRx, Inc). Figure 13 shows that CAPER protein expression is significantly elevated in all three tumor grades (well, moderately, and poorly differentiated). However, CAPER is most highly over-expressed in moderately differentiated breast cancers.

We also assessed the expression and distribution of CAPER by immunohistochemical staining of paraffin-embedded tissue sections. Figure 14 shows that CAPER is undetectable (not shown) or expressed at relatively low levels in normal breast tissue and assumes a cytoplasmic distribution. In contrast, CAPER is expressed at significantly higher levels in DCIS and IDC samples, where it assumes a predominantly nuclear distribution (Figure 14). Thus, our results directly establish that there is a cytoplasmic-to-nuclear shift in the distribution of CAPER during the transition from pre-malignancy to malignancy.

Table 2. A Selection of Genes Upregulated in Cav-1^{-/-} Mammary Glands in Response to Estrogen Exposure

Symbol	Gene name	Fold-up-regulation in Cav-1 KO
Estrogen receptor (ER) signaling/Regulation		
<i>Foxa1</i>	forkhead box A1	3.3
<i>Nr4a1</i>	nuclear receptor subfamily 4, group A, member 1	2.0
<i>Rbm39</i>	RNA binding motif protein 39/CAPER/EST-C79248	9.6
Epithelial specific markers		
<i>Cldn7</i>	claudin 7	2.0
<i>Cldn8</i>	claudin 8	3.3
<i>Ivl</i>	involucrin	2.8
<i>Krt18</i>	keratin 18	2.3
<i>Krt19</i>	keratin 19	3.1
<i>Krt4</i>	keratin 4	2.7
<i>Krt6a</i>	keratin 6A	2.2
<i>Krt7</i>	keratin 7	2.4
<i>Krt8</i>	keratin 8	2.4
Pregnancy and Lactation		
<i>Muc5b</i>	mucin 5, subtype B, tracheobronchial	2.1
<i>Prlr</i>	prolactin receptor	2.7
<i>Wfdc2</i>	WAP four-disulfide core domain 2	2.4
Angiogenic Factors		
<i>Ang4</i>	angiogenin, ribonuclease A family, member 4	2.1
<i>Ang5</i>	angiogenin, ribonuclease A family, member 5	6.6
<i>Fgb</i>	fibrinogen, B beta polypeptide	3.4
<i>Id1</i>	inhibitor of DNA binding 1	2.1
<i>Thbs1</i>	thrombospondin 1	5.3
<i>Tfpi</i>	tissue factor pathway inhibitor	2.1
Proteases		
<i>Adamts4</i>	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4	2.2
<i>Ctsr</i>	cathepsin R	4.1
<i>Tmprss11a</i>	transmembrane protease, serine 11a	4.8
Ribosomal proteins/Protein synthesis		
<i>Rpl17</i>	ribosomal protein L17	5.4
<i>Rps13</i>	ribosomal protein S13	2.0
<i>Rps9</i>	ribosomal protein S9	34.6
<i>Srp54</i>	signal recognition particle 54	2.8
Nuclear mRNA splicing		
<i>Cmkl1</i>	Crn, crooked neck-like 1 (Drosophila)	2.6
<i>Rbm39</i>	RNA binding motif protein 39/CAPER/EST-C79248	9.6
<i>Txn14</i>	thioredoxin-like 4	2.4
Cholesterol/Steroid metabolism		
<i>Hsd17b12</i>	hydroxysteroid (17-beta) dehydrogenase 12	2.5
<i>Osbpl7</i>	oxysterol binding protein-like 7	2.3
Solute carrier family members		
<i>Slc12a2</i>	solute carrier family 12, member 2	2.4
<i>Slc14a1</i>	solute carrier family 14 (urea transporter), member 1	2.7
<i>Slc2a3</i>	solute carrier family 2 (facilitated glucose transporter), member 3	2.6
<i>Slc20a1</i>	solute carrier family 20, member 1	2.1
<i>Slc25a12</i>	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	4.4
<i>Slc5a5</i>	solute carrier family 5 (sodium iodide symporter), member 5	3.0
<i>Slc7a2</i>	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	2.7

All of these genes and transcripts changed by ≥ 2.0 -fold and achieved statistical significance ($P < 0.05$).

Since CAPER functions as an ER co-activator gene at the level of the nucleus, the nuclear distribution of CAPER in cancer cells may reflect its constitutive activation. Notably, CAPER was also localized to the nucleolus in DCIS and IDC samples.

Discussion

Here, we provide *in vivo* evidence that one of the normal functions of Cav-1 expression is to suppress estrogen hypersensitivity in the mammary gland by negatively reg-

ulating the expression of ER- α and ER co-activator genes (summarized schematically in Figure 15). First, we show that Cav-1^{-/-} mammary glands over-express two well-established ER co-activator genes, CAPER and Foxa1, in addition to ER- α . Second, we demonstrate using ovariectomy and estrogen supplementation that Cav-1^{-/-} mammary glands are functionally hyper-responsive to the effects of estrogen. In this regard, loss of Cav-1 leads to ductal thickening, increased side-branching, and the development of DCIS-like mammary lesions, accompanied

Figure 12. Up-regulation of ARC, a marker of resistance to apoptosis. Paraffin sections from ovariectomized and E2-treated mice were immunostained with anti-ARC IgG. Note that ARC staining in E2-treated Cav-1^{-/-} mice was dramatically increased, and was not strictly limited to epithelial ductal areas, but was also uniformly increased throughout the mammary fat pad.

by stromal angiogenesis. Morphologically, they fulfill the pathological criteria for high-grade DCIS lesions, with characteristic alterations in nuclear and nucleolar morphology. Cav-1^{-/-} lesions also show an abnormal distribution of associated myo-epithelial cells, implying a potentially invasive phenotype. These DCIS-like lesions are PR(+) and PCNA(+), providing an indication the cells are undergoing estrogen-dependent hyperproliferation. Fur-

Table 3. ES Cell Related Genes that are Upregulated in Cav-1^{-/-} Mammary Glands

ES Cell Expressed Genes (4 genes)
<i>Gpr64, Hspa8, Krt8, Prom1</i>
Nanog Targets (10 genes)
<i>Hsd17b12, Id1, Krt18, Nedd4l, Rbm39*, Rpl17, Rps13, Scg5, Spag9, Tmem87a</i>
Oct4 Targets (1 gene)
<i>Spag9</i>
Sox2 Targets (5 genes)
<i>Hsd17b12, Id1, Nedd4l, Rbm39*, Spag9</i>
Myc Targets (8 genes)
<i>Atm, Hspa8, Pdk2, Rbm39*, Rps13, Slc12a2, Srp54, St6galnac4</i>
Proliferative/Cycling Genes (2 genes)
<i>Atm, Hspa8</i>

Underlined genes were up-regulated >5-fold.
 *Rbm39 = CAPER (Co-Activator of AP-1 and ER).

thermore, these Cav-1^{-/-} lesions stain positively with stem/progenitor cell markers, such as β -catenin, and SPRR1A. These lesions also show elevated levels of B23/nucleophosmin, an established nucleolar marker protein and predictor of tamoxifen resistance in human breast cancer patients. Third, using genome-wide transcriptional profiling, we identify a number of estrogen-related and estrogen-responsive genes that are significantly overexpressed in Cav-1^{-/-} mammary glands, including CAPER, *Foxa1*, and *Gata3*—which all function as transcriptional co-activators of ER- α . Finally, we directly show that CAPER is overexpressed in human breast cancers, and undergoes a cytoplasmic-to-nuclear shift in DCIS

Figure 13. Analysis of CAPER expression in human normal and cancerous breast tissue by AQUA. **A:** We examined the expression of CAPER using a human breast cancer tissue microarray, consisting of 84 IDCs and 20 normal breast tissue samples. Note that CAPER is up-regulated selectively in human breast cancer samples. Representative image are shown. CAPER (red); CK (cytokeratin; green); nuclei (blue). **B, C:** Quantitative analysis of CAPER expression was achieved by using the PM2000 AQUA automated quantitative analysis system (HistoRx, Inc). Note that CAPER protein expression is significantly elevated in all three tumor grades (well, moderately, and poorly differentiated). However, CAPER is most highly expressed in moderately differentiated breast cancers. 1, well; 2, moderate; and 3, poorly differentiated. Two different representations of CAPER expression are shown. **P* < 0.01.

Table 4. A Selection of Genes Up-Regulated in Cav-1^{-/-} Mammary Glands Associated with Malignancy

Symbol	Gene Name	Fold-up-regulation in Cav-1 KO
<i>Afp</i>	alpha fetoprotein	6.3
<i>Arg1</i>	arginase 1, liver	3.2
<i>Cd276</i>	CD276 antigen; B7-H3	2.7
<i>Gipc2</i>	GIPC PDZ domain containing family, member 2	2.1
<i>Hsd17b12</i>	hydroxysteroid (17-beta) dehydrogenase 12	2.5
<i>Id1</i>	inhibitor of DNA binding 1	2.1
<i>Indo</i>	indoleamine-pyrrole 2,3 dioxygenase	4.5
<i>Pldn</i>	palladin	5.2
<i>Prom1</i>	prominin 1; CD133	2.4
<i>Scg5</i>	secretogranin V	2.9
<i>Spag9</i>	sperm associated antigen 9	2.4
<i>Thbs1</i>	thrombospondin 1	5.3

and IDC. Nucleolar staining was also observed. Surprisingly, this is the first demonstration that CAPER overexpression is associated with DCIS lesions and human breast cancer pathogenesis.

CAPER functions as a nuclear co-activator of the AP-1 transcription factor and a co-activator of nuclear receptors, including ER- α , ER- β , and PR.^{36,37} It was cloned based on its interaction with another nuclear co-activator gene, namely ASC-2.³⁶ CAPER is identical to Rbm39/HCC1, which was first identified as a factor that facilitates pre-mRNA processing/splicing.^{36,63,64} Thus, CAPER may functionally couple transcription with pre-mRNA splicing. In this regard, it has been shown that CAPER can mediate hormone dependent alternative splicing of genes, such as VEGF.³⁷ Given that CAPER can function as a co-activator for both ER- α and AP-1, its expression may confer both estrogen-dependent and estrogen-independent increases in cell proliferation. This duality of function could potentially contribute to estrogen-independent growth that arises in response to tamoxifen resistance. Further studies are necessary to investigate if CAPER expression confers tamoxifen resistance. In accordance with the idea that Cav-1^{-/-} mammary lesions resemble human DCIS lesions, a number of human breast cancer biomarkers are transcriptionally up-regulated in Cav-1^{-/-} DCIS-like lesions, including Foxa1 and Gata3. Foxa1 is winged-

helix transcription factor of the forkhead family. It is highly overexpressed in luminal A type breast cancers, and is strictly associated with ER- α positivity.⁶⁵ Foxa1 functions as a pioneer factor that enhances the binding of ER- α to its target genes. In fact, Foxa1 expression is required to mediate the transcriptional effects of ER- α .³⁸

Gata3 expression is required for normal mammary gland development, and experimental evidence suggests that it may specify the fate of the luminal cell population.^{66,67} Gata3 is also required for estrogen-dependent cell cycle progression in mammary epithelial cells.^{66,67} In this regard, Gata3 is a marker for TEBs that are enriched in mammary stem cells.⁴⁷ In addition, both Foxa1 and Gata3 are often co-expressed with ER- α in human breast cancers.⁶⁸⁻⁷⁰

Although a supraphysiological dose of estrogen was used to accelerate DCIS lesion formation, the Cav-1^{-/-} mouse model appears to be highly relevant to the human disease, as demonstrated by the upregulation and/or overexpression of several factors known to be involved in human breast cancer pathogenesis (such as ER- α , PR, Foxa1, claudins 7/8, keratin 19, and the prolactin receptor).

Also, we identified *Cables1* as a gene that is transcriptionally down-regulated in Cav-1^{-/-} mammary glands.

Table 5. Malignancy-Related Genes Up-Regulated in Cav-1^{-/-} Mammary Glands

Symbol	Associated malignancy
<i>Afp</i>	An estrogen-inducible estradiol-binding protein, associated with liver cancer. ⁸¹⁻⁸³
<i>Arg1</i>	Arginase levels are increased in human breast cancers. ⁸⁴⁻⁸⁷
<i>CD276</i>	A novel biomarker for aggressive human prostate cancers. ^{88,89}
<i>Gipc2</i>	Levels are elevated in human gastric cancers. ⁹⁰
<i>Hsd17b12</i>	A transcriptional target of Nanog and Sox2, which is overexpressed in human breast cancers. ⁹¹
<i>Id1</i>	A transcriptional target of Nanog and Sox2, which is associated with tumor angiogenesis and breast cancer metastasis. ^{92,93}
<i>Indo</i>	Elevated and facilitates immune escape in breast cancers. ⁹⁴
<i>Pldn</i>	Up-regulated in human pancreatic cancers, and is associated with the organization of the actin cytoskeleton and increased cell motility. Palladin is also mutated in familial pancreatic cancers. ⁹⁵⁻⁹⁷
<i>Prom1</i>	CD133 is a cancer stem cell marker often used to isolate tumor initiating cells. ⁹⁸
<i>Scg5</i>	A transcriptional target of Nanog and SNPs in Scg5 are associated with increased risk for the development of human colon cancers. ⁹⁹
<i>Spag9</i>	A transcriptional target of Nanog, Oct4, and Sox2—three iPS genes. It is overexpressed in testicular and ovarian cancers. ¹⁰⁰
<i>Thbs1</i>	Increased in human breast cancer and contributes towards increased angiogenesis and metastatic capacity. ^{101,102}

Figure 14. Changes in the expression and distribution of CAPER during human breast cancer onset and progression. Paraffin sections from normal DCIS, and well-differentiated invasive ductal carcinoma (IDC-WD) were immunostained with anti-CAPER IgG. Note that CAPER is expressed at relatively low levels in normal breast tissue and assumes a cytoplasmic distribution. In contrast, CAPER is expressed at significantly higher levels in DCIS and IDC samples, where it assumes a predominantly nuclear distribution. Representative high power images are shown. At least 3 to 5 samples were stained for each group and similar results were obtained. Boxed areas are shown to the far right at higher magnification to highlight the nuclear and nucleolar localization of CAPER in breast cancer lesions. Inset magnification: 5 $\times$.

Cables1 is a cyclin-dependent kinase-interacting protein that normally inhibits cell cycle progression.⁷¹ Interestingly, Cables1 is normally down-regulated in the endometrium in response to estrogen, and loss of Cables1 expression has been implicated in the pathogenesis of human colon, lung, ovarian, and endometrial cancers.^{72,73} Cables1 deficient mice develop endometrial hyperplasia and carcinoma *in situ* at a young age.^{72,73} Thus, Cables1 may represent a new biomarker that should be evaluated in the context of human breast cancers.

There is an extensive body of literature showing that nucleolar prominence is a “hallmark” of transformed or

malignant cells, and is associated with a poor prognosis for breast cancer patients.^{53,54} In this regard, it is important to note that Cav-1^{-/-} mammary lesions showed striking nucleolar prominence (See Figure 6A) and express elevated levels of B23/nucleophosmin, a nucleolar marker protein (See Figure 11). The nucleolus is the organelle where the biogenesis of ribosomes occurs.⁵³ In fact, we observed the up-regulation of several genes associated with ribosome biogenesis (Rps9, Rps13, Rpl17, and Srp54) (Table 2) in Cav-1^{-/-} mammary glands. This also fits well with the observation that three other up-regulated genes (CAPER, Crnk11, and Txnl4; Table 2) are involved in the regulation of nuclear mRNA splicing. Interestingly, CAPER also showed both nuclear and nucleolar localization patterns in DCIS and IDC breast cancers samples.

Based on our current studies, ribosomal protein S9 (Rps9) may be an interesting candidate biomarker. Genome-wide transcriptional profiling revealed that *Rps9* is the single most transcriptionally up-regulated gene in E2-treated Cav-1^{-/-} mammary glands (34.6-fold increased). However, little is known about Rps9 and it has never been evaluated as a cancer biomarker. Interestingly, a recent report suggests that Rps9 forms a complex with B23/nucleophosmin, a nucleolar marker protein that is involved in genomic stability and ribosome biogenesis. Functionally, knockdown of Rps9 expression using an siRNA-approach decreased protein synthesis and led to cell cycle arrest.⁵⁶ Thus, expression of Rps9 is normally required for maintaining cells in the proliferative state.⁵⁶ Notably, B23/nucleophosmin—an Rps9-interacting protein—is normally up-regulated in response to estrogen, and is associated with estrogen-independence and metastasis in breast cancer patients.^{19–21} Rps13, another member of the B23/Rps9 complex, is also up-regulated in Cav-1^{-/-} mammary glands (Table 2). *Rps13* is both a Nanog and Myc target gene (Table 3). Thus, there is an emerging relationship between the nucleolus, ribosome biogenesis, stem cells, and cancer pathogenesis. In direct support of this notion, nucleostemin, another distinct nucleolar marker protein is (i) a stem cell marker; (ii) a

Figure 15. Diagram summarizing the role of Cav-1 loss of function in estrogen-induced mammary epithelial cell proliferation. Our current findings on the role of Cav-1 loss of function in mediating estrogen hypersensitivity are illustrated schematically. Briefly, we show here that ablation of a single gene is sufficient to confer estrogen-hypersensitivity and DCIS lesion formation in Cav-1^{-/-} mammary glands, as well as adjacent stromal angiogenesis. Mechanistically, we demonstrate that genetic ablation of Cav-1 causes the overexpression of both ER- α and ER- co-activator genes (such as CAPER, Foxa1, and Gata3).

cancer biomarker; and (iii) an interacting partner for B23/nucleophosmin.^{74,75}

We also provide novel evidence that the biogenesis of DCIS-like lesions may be a “developmental disorder” related to aberrant expansion of the mammary stem/progenitor cell compartment, as we see the up-regulation of a series of TEB markers (SPRR1A, PCNA, and Gata3)^{43, 46–48} in these estrogen-induced Cav-1^{-/-} lesions. In further support of this notion, Clarke, Bundred, and colleagues have demonstrated that human DCIS lesions also show an increase in mammary stem/progenitor cells.⁷⁶ Consistent with this hypothesis, the onset of these dysplastic lesions coincides with a decrease in tertiary branching (See Figure 3A), suggesting that DCIS may developmentally arise from abnormal branching. We previously observed that isolated primary cultures of Cav-1^{-/-} mammary epithelial cells grown in 3D Matrigel cultures show defects in lumen formation, resulting in almost complete luminal filling, and that this *ex vivo* phenotype is exacerbated to the addition of exogenous estrogen.^{18,77,78} Thus, it is likely that the DCIS-like *in vivo* phenotype that we observed here can be attributed to the cell autonomous behavior of Cav-1^{-/-} mammary epithelial cells. However, we have previously shown that the Cav-1^{-/-} mammary stroma dramatically enhances the growth of transplanted tumors, leading to up to ~twofold increases in tumor size, as compared the same tumor cells grown in a Cav-1 (+/+) wild-type mammary fat pad.⁷⁹ Thus, a loss of Cav-1 in the mammary stroma may also exacerbate the formation of the Cav-1^{-/-} mammary dysplastic lesions that we observe, by providing a pro-tumorigenic and/or pro-angiogenic microenvironment.

In direct support of this hypothesis, here we observe that after estrogen-treatment, the Cav-1^{-/-} mammary stroma shows dramatic estrogen-dependent increases in vascularization and VEGF expression, as well as Ras-p42/44 MAP kinase activation (See Figures 8 and 9). Furthermore, we have recently demonstrated that isolated Cav-1^{-/-} mammary stromal fibroblasts share many characteristics with human breast cancer-associated fibroblasts, including the capacity to secrete increased levels of VEGF *in vitro*.⁸⁰ As such, this phenomenon undoubtedly deserves further study.

In summary, we show here that Cav-1^{-/-} mice are a new animal model for studying the molecular basis of estrogen-hypersensitivity and the development of estrogen-dependent DCIS lesions. In future studies, we will screen human DCIS lesions for the presence of the Cav-1 mutation(s), as we believe patients harboring these mutations may go on to develop invasive breast cancer. In addition, crossing Cav-1^{-/-} mice with other mouse models lacking specific candidate genes will allow us and others to genetically dissect the down-stream signaling elements that are required for mediating estrogen-hypersensitivity and the biogenesis of DCIS lesions. Thus, Cav-1^{-/-} mice will provide a novel genetically tractable model for understanding the complex signaling networks that govern estrogen-dependent breast cancer initiation.

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