



# Aberrantly high expression of the CUB and zona pellucida-like domain-containing protein 1 (CUZD1) in mammary epithelium leads to breast tumorigenesis

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The peptide hormone prolactin (PRL) and certain members of the epidermal growth factor (EGF) family play central roles in mammary gland development and physiology, and their dysregulation has been implicated in mammary tumorigenesis. Our recent studies have revealed that the CUB and zona pellucida-like domain-containing protein 1 (CUZD1) is a critical factor for PRL-mediated activation of the transcription factor STAT5 in mouse mammary epithelium. Of note, CUZD1 controls production of a specific subset of the EGF family growth factors and consequent activation of their receptors. Here, we found that consistent with this finding, CUZD1 overexpression in non-transformed mammary epithelial HC11 cells increases their proliferation and induces tumorigenic characteristics in these cells. When introduced orthotopically in mouse mammary glands, these cells formed adenocarcinomas, exhibiting elevated levels of STAT5 phosphorylation and activation of the EGF signaling pathway. Selective blockade of STAT5 phosphorylation by pimozone, a small-molecule inhibitor, markedly reduced the production of the EGF family growth factors and inhibited PRL-induced tumor cell proliferation *in vitro*. Pimozone administration to mice also suppressed CUZD1-driven mammary tumorigenesis *in vivo*. Analysis of human MCF7 breast cancer cells indicated that CUZD1 controls the production of the same subset of EGF family members in these cells as in the mouse. Moreover, pimozone treatment reduced the proliferation of these cancer cells. Collectively, these findings indicate that overexpression of CUZD1, a regulator of growth factor pathways controlled by PRL and STAT5, promotes mammary tumorigenesis. Blockade of the STAT5 signaling pathway downstream of CUZD1 may offer a therapeutic strategy for managing these breast tumors.

The mammary gland is a dynamic organ in that it undergoes cycles of proliferation, differentiation, and regression with every pregnancy (1, 2). Tight control of the signaling pathways orchestrating each of these steps is essential for appropriate function of the mammary epithelium (3). Expansion of the ductal epithelium through branching and development of secretory alveoli occur during pregnancy as the mammary gland prepares for lactation (4). Terminal differentiation of the mammary gland observed at lactation is marked by the expression of milk protein genes and the production of milk by the alveolar epithelial cells (5). The peptide hormone prolactin (PRL)<sup>3</sup> plays a central role in regulating mammary gland development during pregnancy and lactation (6). Binding of PRL to the prolactin receptor (PRLR) initiates a signaling cascade in which members of the Janus kinase (JAK) family, JAK1 and JAK2, and the signal transducer and activator of transcription 5 (STAT5) participate (7–13). STAT5, a transcription factor, is phosphorylated and activated by the JAKs associated with PRLR (14–19). The changes in gene expression brought about by active STAT5 are essential for proliferation and differentiation of the mammary epithelium during pregnancy and lactation (17, 20–24). In this way, PRL and STAT5 play critical roles in alveolar development and terminal differentiation of the mammary gland.

Signaling via phosphorylated STAT5 is key to the proliferation of mammary epithelial cells during alveologenesis (23, 25–27). Mitogenic genes under the control of STAT5 encode cyclin D1 and certain members of the epidermal growth factor (EGF) family, including epiregulin (EREG), which induce expansion of the mammary epithelium before alveolar differentiation (28–31). The EGF family ligands signal through their cognate ErbB receptors to promote proliferation and differentiation of the mammary epithelium during various stages of mammary gland development (32, 33). Communication

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This article contains Figs. S1 and S2 and Experimental Procedures.

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<sup>3</sup> The abbreviations used are: PRL, prolactin; PRLR, PRL receptor; ER $\alpha$ , estrogen receptor  $\alpha$ ; JAK, Janus kinase; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; EGFR, EGF receptor; H&E, hematoxylin and eosin; BrdU, bromodeoxyuridine; IHC, immunohistochemistry; qPCR, quantitative real-time PCR; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PCNA, proliferating cell nuclear antigen; RF, relative fluorescence; ICC, immunocytochemistry; DAPI, 4',6-diamidino-2-phenylindole.

between, and regulation of, the PRL/STAT5 and ErbB receptor pathways are essential for alveologenesis in preparation for lactation.

The PRLR signaling, which is critical for normal mammary gland development, is dysregulated in certain types of breast cancer (34). Aberrant PRL/STAT5 signaling induces excessive proliferation and thereby triggers unchecked growth, leading to mammary tumorigenesis (32, 35–37). Genes regulated by PRL/STAT5 influence proliferation and differentiation of breast cancer cells (31, 38). In an oncogene-induced tumor model (MMTV-PyVT), *Prl*<sup>-/-</sup> mice exhibit delayed tumor development and slower (30%) tumor progression when compared with control mice (39). In another study, local overexpression of PRL in the mouse mammary gland resulted in ubiquitous development of mammary carcinomas in aged mice and PRL-induced carcinomas displayed high levels of nuclear pSTAT5 (24, 40). Transgenic mice expressing constitutively active STAT5 exhibit hyperproliferative mammary epithelium, delayed involution, and a predisposition to mammary tumor formation (41). Collectively, these studies indicate that aberrant PRL/STAT5 signaling contributes to breast cancer.

Similarly, an increased signaling through the EGF pathway has long been implicated in the proliferation of mammary epithelial cells in breast cancer (32, 42–44). Many drugs that inhibit activation of certain ErbB receptors have been developed to treat breast cancer (45, 46). Analysis of ErbB receptor expression and activation via phosphorylation in human breast tumors provides important prognostic information and predicts responses to these targeted therapies (47). Therefore, it is conceivable that targeting a cellular factor that controls both the EGF and PRL signaling pathways would provide an effective and personalized treatment plan in breast cancer patients (48–50).

Our recent studies revealed that the CUB and zona pellucida-like domain-containing protein 1 (CUZD1) may fit such a role (51). Gene ontology analysis indicates an association of the *Cuzd1* gene product with cell division, cell cycle, and cell proliferation (52). Structurally, CUZD1 is composed of two tandem CUB domains, a ZP domain, and a putative transmembrane domain (53, 54). Presently, little information exists describing the function of these domains, but they are often found in proteins that regulate developmental processes (55, 56). Studies illustrating the presence of CUZD1 in specific tissues have provided some functional analysis. We previously reported that CUZD1, also known as estrogen-regulated gene 1 (ERG1), is an estrogen-regulated gene in the rodent reproductive tract and is important for mammary epithelial cell proliferation during pregnancy and lactation (51, 53). Additionally, expression of *Cuzd1* has been identified in the pancreas, epididymis, human ovarian cancer cells, and human embryonic stem cells (57–61). Leong *et al.* (61, 62) demonstrated the importance of CUZD1 in cell growth and proliferation of a human ovarian cancer cell line and proposed a potential role of CUZD1 in chemotherapeutic resistance. Efforts have also been made to develop serum-based assays using CUZD1 as a biomarker for ovarian cancer and pancreatic cancer; however, controversial reports support the need for additional studies (61, 63–69). Our recent work fills a gap in the body of knowledge surrounding CUZD1 by detailing

the molecular signaling pathway of CUZD1-induced proliferation in mammary epithelial cells (51). The *Cuzd1*-null mouse model provided evidence that CUZD1 is a mediator of PRL/STAT5 signaling in the mammary gland during puberty and pregnancy that controls STAT5 phosphorylation in mammary epithelial cells. Notably, CUZD1-mediated STAT5 activation drives the expression of certain EGF family growth factors, such as EREG, which acts via the ErbB1 and ErbB4 receptors. In the absence of *Cuzd1*, therefore, proliferation of the mammary epithelium during alveolar development is dramatically reduced due to the loss of signaling via these receptors. These data led us to hypothesize that the opposite may also be true; increased *Cuzd1* expression may lead to excessive proliferation of the mammary epithelium, leading to tumorigenesis.

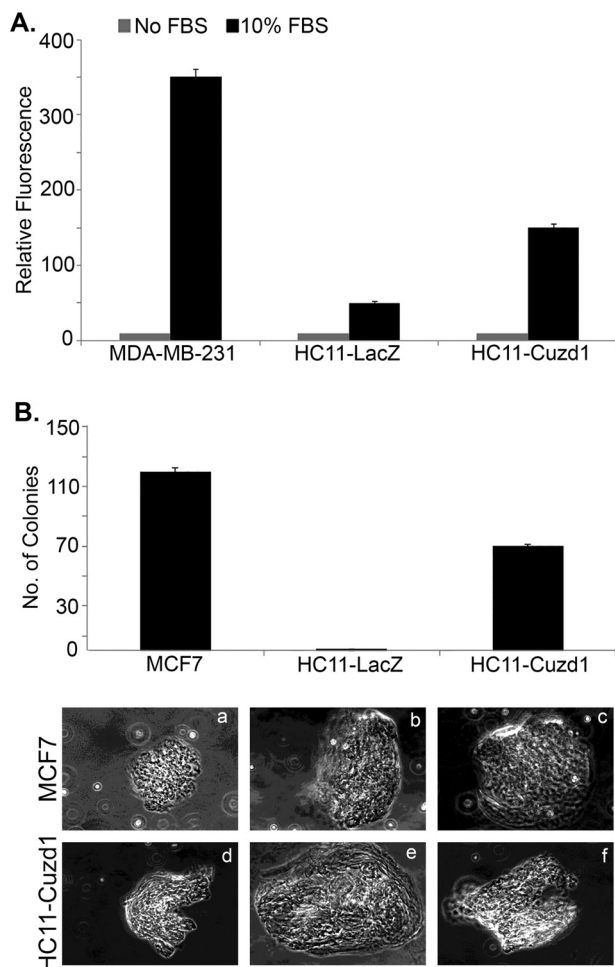
In this study, we tested the concept that overexpression of CUZD1 in mammary epithelial cells may drive constitutive activation of the STAT5 pathway and inappropriate stimulation of the EGF family growth factor pathways, leading to uncontrolled cell proliferation. We demonstrate that such dysregulation of CUZD1 and its downstream STAT5 and EGF receptor pathways indeed leads to breast carcinoma. Furthermore, we provide evidence that pimizide, a selective inhibitor of STAT5 phosphorylation, is able to suppress CUZD1/STAT5-driven mammary epithelial proliferation and tumorigenesis, presenting it as a potential therapeutic drug target in breast cancers in which the STAT5 pathway plays a major role.

## Results

### Overexpression of *Cuzd1* leads to transformation of HC11 cells

To test whether the overexpression of *Cuzd1* promotes transformation of mammary epithelial cells, we employed HC11 cells, a non-transformed mammary epithelial cell line derived from pregnant BALB/c mice. As described previously, a lentiviral expression vector harboring a full-length cDNA encoding *Cuzd1* or  $\beta$ -galactosidase (control) was integrated into HC11 cells to generate stable cell lines which constitutively express elevated levels of *Cuzd1* (HC11-Cuzd1) or  $\beta$ -galactosidase (HC11-LacZ) (51). Western blot analysis indicated that HC11-Cuzd1 cells overexpress CUZD1 about 2-fold over the HC11-LacZ control cells (51). These cells also expressed prolactin receptor and low levels of estrogen receptor  $\alpha$  and progesterone receptor. We then subjected these cells to a cell invasion assay using Boyden chambers. The HC11-Cuzd1 cells exhibited enhanced motility and were able to migrate across a barrier, whereas control HC11-LacZ cells failed to penetrate the membrane (Fig. 1A). We then subjected these cells to a soft agar assay to assess their anchorage independent growth, a well-known marker of cell transformation (70). As shown in Fig. 1B, HC11-Cuzd1 cells formed multiple large colonies when cultured in soft agar, whereas the HC11-LacZ cells remained as single cells in the agar. As a control, we used MCF7 breast cancer cells, which are known to form robust colonies on soft agar (Fig. 1B). These findings indicated that the overexpression of *Cuzd1* in HC11 mammary epithelial cells altered their growth and migratory properties, two important hallmarks of precancerous cells.

## Role of *CUZD1* in breast tumorigenesis



**Figure 1. Overexpression of *Cuzd1* leads to enhanced motility and anchorage-independent growth of HC11 cells.** *A*, overexpression of *Cuzd1* leads to enhanced motility of HC11 cells. Serum-starved MDA-MB-231 cells (positive control), HC11-LacZ, or HC11-Cuzd1 cells were placed in Boyden chambers and allowed to migrate toward 10% FBS for 72 h. The number of invading cells was quantified using CyQuant fluorescence labeling and compared with corresponding cells unexposed to the serum chemoattractant. Data are represented as relative fluorescence  $\pm$  S.E. (error bars) from at least three biological replicates ( $p = 0.04$ ). *B*, overexpression of *Cuzd1* promotes anchorage-independent growth in HC11 cells. MCF7 cells (positive control), HC11-LacZ, or HC11-Cuzd1 cells were plated in medium containing soft agar. Colonies were allowed to form for 16 days and stained with crystal violet overnight. Visible colonies ( $>0.5$  mm) were counted using a dissecting microscope. Data are represented as number of colonies ( $>0.5$  mm)  $\pm$  S.E. from at least three biological replicates ( $p = 0.0003$ ). Images show representative colonies of MCF7 (*a–c*) and HC11-Cuzd1 cells (*d–f*).

### Introduction of HC11-Cuzd1 cells into the mammary gland generates adenocarcinomas

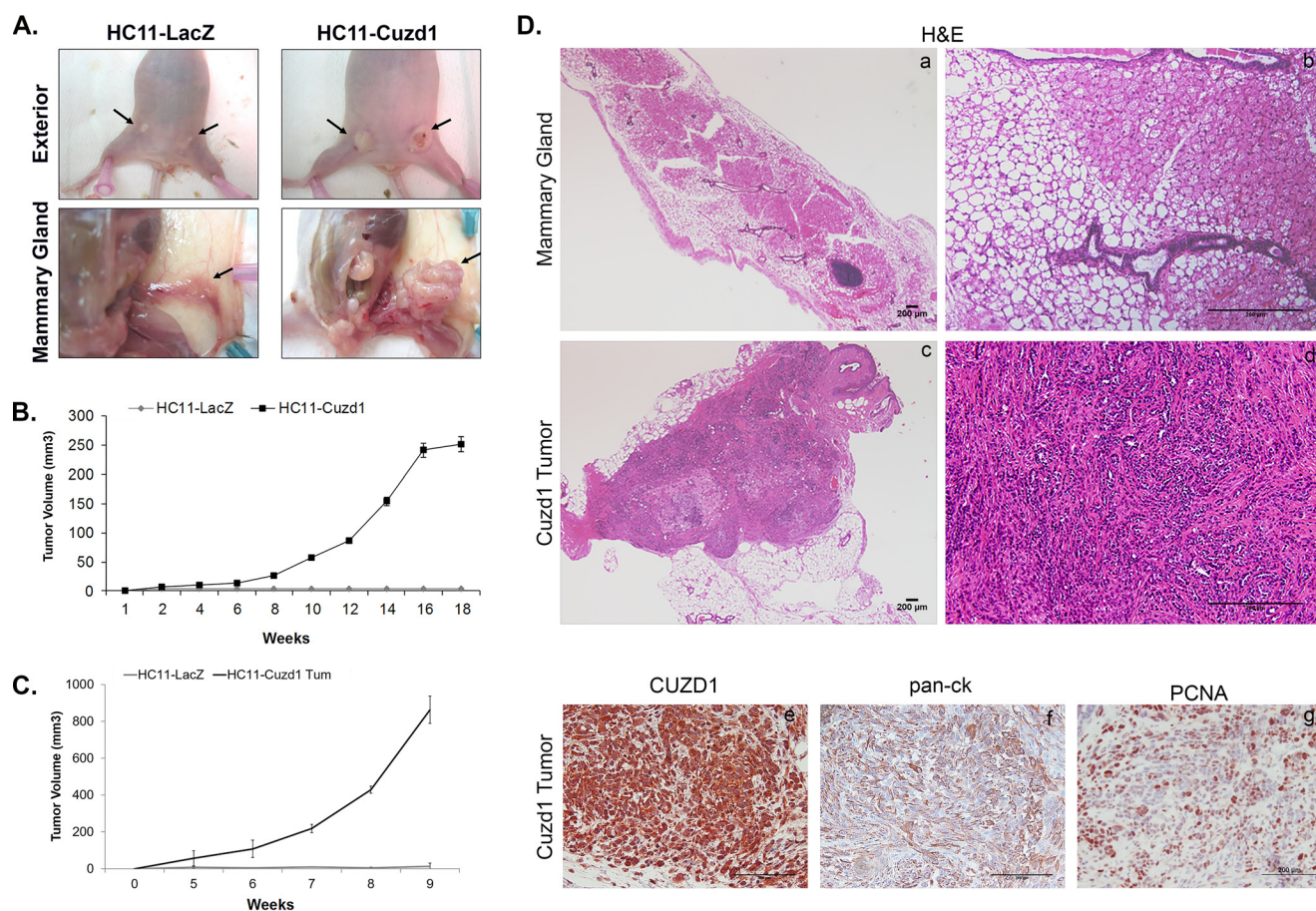
To further evaluate the tumorigenic properties of HC11-Cuzd1 cells *in vivo*, these cells were mixed with Matrigel and orthotopically injected into the mammary gland duct of nude mice through the nipple. Mice injected with HC11-LacZ cells served as a control for tumor growth. After a latency period of about 6 weeks, mice injected with HC11-Cuzd1 cells manifested palpable tumors, which continued to grow over 18 weeks (Fig. 2A). All of the mice injected with HC11-Cuzd1 cells had tumors ranging in size between 200 and 250 mm<sup>3</sup>, whereas mice injected with control HC11-LacZ cells did not form any detectable tumor (Fig. 2B). Tumor growth was measured weekly, and tumor volume was calculated.

We further examined this breast tumorigenesis process in immunologically intact BALB/c mice. HC11-Cuzd1 or HC11-LacZ cells were introduced in mammary glands of these mice as described above. Again, all BALB/c mice injected with HC11-Cuzd1 cells developed mammary tumors, whereas those receiving HC11-LacZ showed no tumor formation. Through dissection and enzymatic digestion of the mammary tumors established by injection of HC11-Cuzd1 cells in BALB/c mice, we isolated cells from these tumors (HC11-Cuzd1 Tum). These HC11-Cuzd1 Tum cells appeared to be phenotypically similar to HC11-Cuzd1 cells. When HC11-Cuzd1 Tum cells were orthotopically injected into the fourth mammary gland pair in BALB/c mice, mammary tumors developed and grew rapidly to reach a volume of about 50 mm<sup>3</sup> at 5 weeks, 200–250 mm<sup>3</sup> at 7 weeks, and eventually a volume of almost 900 mm<sup>3</sup> at 9 weeks (Fig. 2C). These tumors grew larger and more rapidly than those formed by HC11-Cuzd1 cells, which were less than 300 mm<sup>3</sup> at 18 weeks.

For histological analysis of mammary tumors, mice were sacrificed at 18 weeks following injection of HC11 cells. The tumors and mammary glands from mice injected with HC11-Cuzd1 and HC11-LacZ cells, respectively, were removed and examined by H&E staining (Fig. 2D, *a–d*). The mammary glands injected with HC11-LacZ cells appeared normal (Fig. 2D, *a* and *b*). The tumors that arise in mammary glands injected with HC11-Cuzd1 cells were verified by pathological examination as adenocarcinomas with lobular characteristics (Fig. 2D, *c* and *d*). When these tumors were examined by immunohistochemical staining using a CUZD1-specific antibody, we observed a robust expression of the CUZD1 protein in both cytoplasmic and nuclear compartments (Fig. 2D, *e*). The retention of CUZD1 and pan-Cytokeratin expression in the tumor indicated that the cells in the tumor mass were indeed derived from the original HC11-Cuzd1 epithelial cell line (Fig. 2D, *e* and *f*). These tumors also expressed a high level of PCNA, indicative of highly proliferative cells in the tumor tissue (Fig. 2D, *g*). We noted liver and lung lesions consistent with metastasis of primary adenocarcinoma to other organs in 10% of nude mice with HC11-Cuzd1 tumors. These results established that overexpression of *Cuzd1* produces a tumorigenic phenotype in HC11 mammary epithelial cells, which manifest in breast adenocarcinomas *in vivo*.

### *Cuzd1*-induced mammary tumorigenesis is mediated by the ErbB signaling pathway

We have previously shown that *Cuzd1* controls the phosphorylation and activation of STAT5, downstream production of a subset of EGF family ligands, and consequent phosphorylation of two key ErbB receptors, ErbB1 and ErbB4 (51). We therefore investigated, using IHC, the phosphorylation status of STAT5 in tumors from nude mice orthotopically injected with HC11-Cuzd1 cells. As expected, we detected ample expression of STAT5 in the tumor sections (Fig. 3B). Using an antibody that recognizes activating STAT5 phosphorylation (Tyr-694), we observed phosphorylated STAT5 (pSTAT5) in the tumors (Fig. 3A). We also assessed the expression levels of the EGF family ligands EREG, EPGN, and NRG1 and the activation states of the ErbB receptors in *Cuzd1*-overexpressing breast tumors. The



**Figure 2. Orthotopic injections of HC11-Cuzd1 cells form adenocarcinomas *in vivo*.** A, external images of HC11-Cuzd1 cell tumors in nude mice. HC11-LacZ or HC11-Cuzd1 cells were injected orthotopically into the nipple of the fourth mammary gland of immunocompromised female nude mice. Mice were sacrificed 18 weeks postinjection and examined for tumor growth. *Top panels*, an exterior view of the animal; *bottom panels*, mammary gland after dissection. B, tracking growth of HC11-Cuzd1 tumors in nude mice. Tumor volume was quantified weekly using digital calipers from time of injection (week 0) to time of sacrifice (week 18). Tumor volume =  $\frac{1}{2}(\text{length} \times \text{width}^2) \pm \text{S.E.}$  (error bars),  $n = 15$  in each group. C, tracking growth of HC11-Cuzd1 Tum tumors in BALB/c mice. HC11-LacZ or HC11-Cuzd1 Tum cells were injected orthotopically into the nipple of the fourth mammary gland of female BALB/c mice. Tumor volume was quantified weekly using digital calipers from time of injection (week 0) to time of sacrifice (week 9). Tumor volume =  $\frac{1}{2}(\text{length} \times \text{width}^2) \pm \text{S.E.}$ ,  $n = 15$  in each group. D, immunohistochemical analysis of HC11-Cuzd1 tumors. Mammary glands and tumors were collected from mice injected with HC11-LacZ and HC11-Cuzd1, respectively, 18 weeks postinjection. The specimens were fixed, embedded in paraffin, sectioned, and subjected to H&E staining and imaged at  $\times 5$  (a and c) and  $\times 40$  (b and d) magnification. IHC analysis was carried out with antibodies against CUZD1 (e) (red), pan-cytokeratin (f) (red), and PCNA (g) (red) and counterstained with hematoxylin (blue). Magnification,  $\times 40$ . Scale bars, 200  $\mu\text{m}$ . Data are representative images from  $n = 5$ .

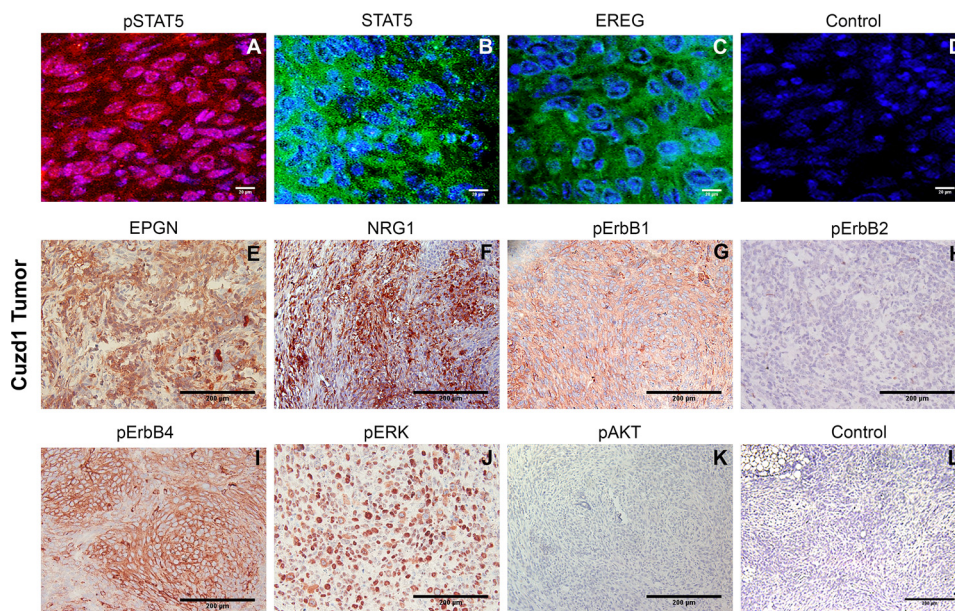
expression of high levels of EREG, EPGN, and NRG1 proteins was evident in these tumors (Fig. 3, C, E, and F). Additionally, abundant active (phosphorylated) forms of ErbB1 and ErbB4 were present in the tumors (Fig. 3, G and I). However, activated (phosphorylated) ErbB2 was undetectable in the tumor sections (Fig. 3H). These results are consistent with the hypothesis that CUZD1-driven breast tumorigenesis involves phosphorylation and activation of STAT5 and production downstream of a subset of EGF family growth factors and activation of ErbB1 and ErbB4 signaling but is not dependent on ErbB2 activation.

Activation of ErbB receptors is often accompanied by activation of downstream ERK and/or PI3K-AKT pathways. To assess ERK and AKT activation in *Cuzd1*-overexpressing tumors, sections of tumors were probed with antibodies specific for phosphorylated ERK1/2 or phosphorylated AKT1/2/3. Our results showed the presence of abundant pERK (Fig. 3J) and an absence of pAKT (Fig. 3K), indicating that ERK signaling is indeed activated and that it potentially regulates proliferation of the *Cuzd1*-driven breast tumor cells, but their proliferation is not dependent on the AKT pathway.

#### Inhibition of STAT5 phosphorylation suppresses the proliferation of HC11-Cuzd1 cells *in vitro*

Our previous studies indicate that overexpression of *Cuzd1* leads to increased STAT5 activity (51). We therefore investigated the importance of upstream PRLR signaling on CUZD1-mediated STAT5 activation. To determine whether nuclear translocation of CUZD1 as well as phosphorylation, nuclear localization, and transcriptional activity of STAT5 are dependent on PRLR activity, we used siRNA to knock down expression of *Prlr* in HC11 cells overexpressing *Cuzd1* (Fig. 4A). The phosphorylation of STAT5, as well as the nuclear translocation of pSTAT5 and CUZD1, was reduced following abatement of PRLR signaling as compared with control siRNA (Fig. 4B). Chromatin immunoprecipitation for pSTAT5 and/or FLAG-CUZD1 demonstrates that the loss of *Prlr* impairs enrichment of the STAT5/CUZD1-binding site on the *Ereg* promoter (Fig. 4C). These data indicate that intact signaling through PRL/PRLR is essential for CUZD1-mediated STAT5 activity.

## Role of *CUZD1* in breast tumorigenesis



**Figure 3. The ErbB1 and ErbB4 pathways are activated in HC11-Cuzd1 tumors *in vivo*.** Tumors were collected 18 weeks post-injection, fixed, embedded in paraffin, sectioned, and subjected to IHC analysis using antibodies against pSTAT5 (A) (red), STAT5 (B) (green), EREG (C) (green), EPGN (E) (red), NRG1 (F) (red), pErbB1 (G) (red), pErbB2 (H) (red), pErbB4 (I) (red), pERK (J) (red), and pAKT (K) (red) and counterstained with hematoxylin (blue) or DAPI (blue). Control sections were stained in the absence of a primary antibody (D and L). Magnification,  $\times 40$ . Scale bars, 20  $\mu\text{m}$  (A–D) and 200  $\mu\text{m}$  (E–L). Data are representative images from  $n = 5$ .

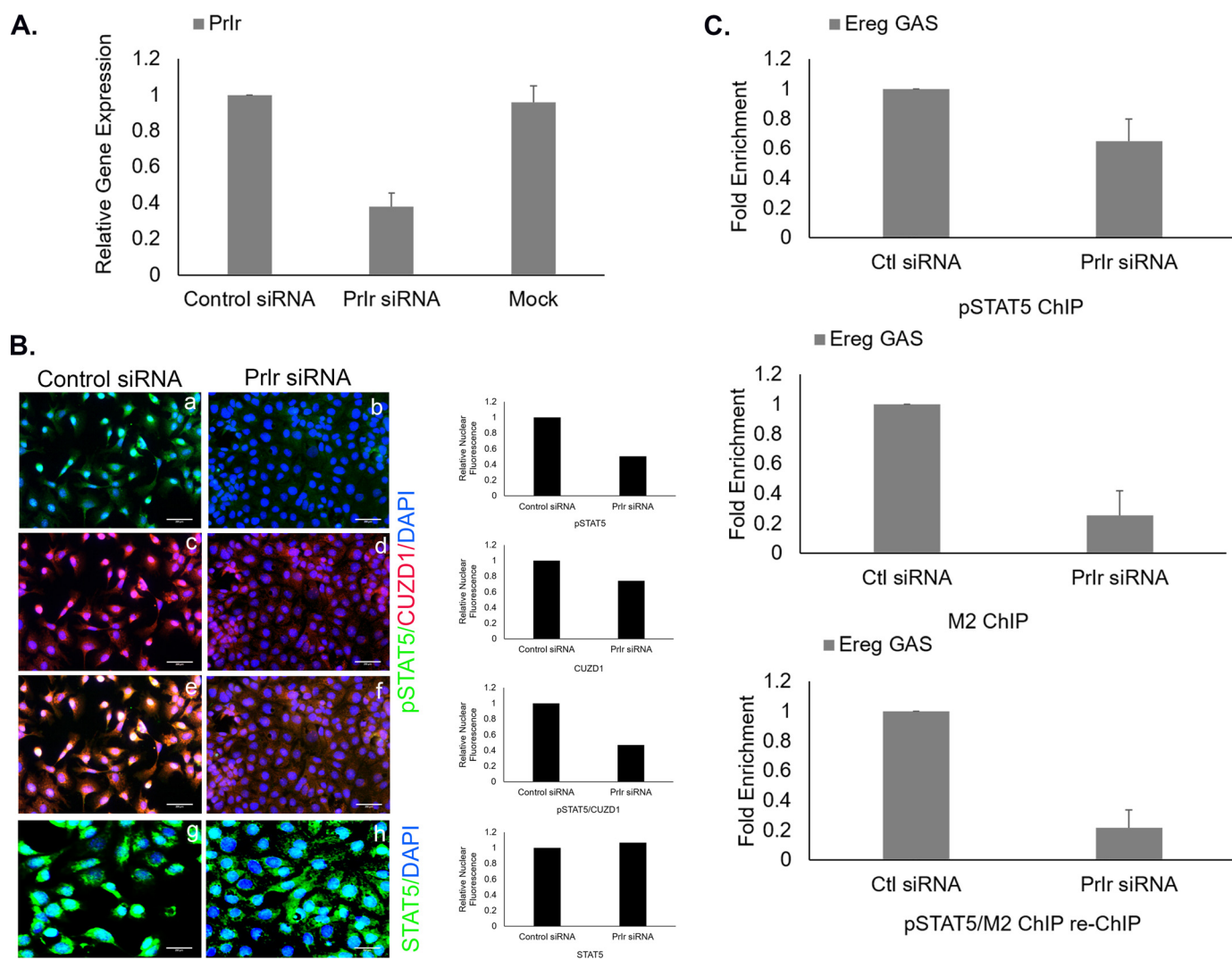
Because *CUZD1* activates the STAT5 signaling pathway to stimulate proliferation of the mammary epithelium, we hypothesized that by inhibiting STAT5, we could prevent cellular signaling downstream of *CUZD1* and suppress tumorigenesis induced by constitutive *Cuzd1* overexpression. To test this hypothesis, we treated HC11-Cuzd1 cells with PRL along with vehicle or pimoizide, a selective inhibitor of STAT5 phosphorylation. As shown by Western blotting, pimoizide treatment dramatically reduced the activating phosphorylation of STAT5 in these cells compared with the vehicle treatment (Fig. 5A). Total STAT5 protein levels were unaffected by pimoizide treatment (Fig. 5A). We further confirmed by immunocytochemistry that PRL-induced phosphorylation of STAT5 in HC11-Cuzd1 cells is attenuated by pimoizide treatment (Fig. 5B). These data established that pimoizide is an effective inhibitor of STAT5 phosphorylation in HC11-Cuzd1 cells. We previously reported that loss of STAT5 phosphorylation in *Cuzd1*-null mammary epithelium is coincident with the lack of production of a subset of EGF family ligands in this tissue (51). Consistent with this finding, we report here that inhibition of STAT5 by pimoizide also reduced the expression of *Ereg* and *Epgn* transcripts in HC11-Cuzd1 cells (Fig. 5C), confirming that inhibition of STAT5 signaling inhibits growth factor pathways critical for tumor cell proliferation. HC11-Cuzd1 cells remained over 70% viable with 24 h of pimoizide treatment compared with 90% in the vehicle control (Fig. S1) (Supplemental Experimental Procedures). A discrete shift to less than 50% viable cells is observed with 48–96 h of treatment (Fig. S1). A reduction in total cell number is detected starting at 18 h of treatment with pimoizide, indicating a reduction in proliferation before cell death occurs (Fig. S1). A BrdU incorporation assay was used to assess whether proliferation of HC11-Cuzd1 cells treated with PRL was indeed affected by pimoizide at 18 h.

As shown in Fig. 5D, significant reduction in PRL-induced proliferation of HC11-Cuzd1 cells was observed following pimoizide treatment. Collectively, these data indicate that inhibition of STAT5 phosphorylation by pimoizide reduces the proliferation of HC11-Cuzd1 cells.

### Pimoizide treatment decreases the growth of *Cuzd1*-driven mammary tumors *in vivo*

We next tested whether inhibition of STAT5 signaling by pimoizide treatment suppressed *Cuzd1*-induced mammary tumor formation *in vivo*. We orthotopically injected HC11-Cuzd1 Tum cells into the fourth mammary gland pair of BALB/c mice and treated them with oral doses of pimoizide or a vehicle control. At the end of 5 weeks of tumor growth and pimoizide or vehicle treatment, we observed a drastic reduction in tumor size in mice treated with pimoizide compared with mice treated with vehicle control (Fig. 6, A–C). Immunohistochemical analysis of STAT5 phosphorylation showed widespread pSTAT5 (Tyr-694) in vehicle-treated mammary tumors, whereas markedly diminished pSTAT5 was observed in tumors of mice treated with pimoizide (Fig. 7A, a, b, d, and e). Analysis of STAT5 staining indicated that pimoizide did not affect total STAT5 protein levels (Fig. 7A, c and f).

We also examined the levels of the EGF family growth factors EREG, EPGN, and NRG1 and monitored the activation of ErbB1 and ErbB4 in these tumors with or without pimoizide treatment (Fig. 7B). Our results showed that inhibition of STAT5 signaling by pimoizide and consequent regression of the mammary tumors are associated with suppression of the EGF signaling pathway. This is due to a decrease in production of EREG, EPGN, and NRG1 and loss of activation via phosphorylation of ErbB1 and ErbB4 in these tumors (Fig. 7B, a–e and



**Figure 4.** Signaling through PRLR is essential for CUZD1/pSTAT5 complex nuclear translocation and DNA binding. *A*, *Prlr* expression is reduced following siRNA treatment. HC11-3FCuzd1 cells were treated with 150 nM siRNA targeting *Prlr* or control siRNA for 48 h and prolactin for 6 h. mRNA was isolated, and gene expression of *Prlr* was measured. *B*, knockdown of *Prlr* reduces STAT5 phosphorylation and nuclear translocation as well as CUZD1 nuclear translocation. Cells treated with control or *Prlr* siRNA were fixed and subjected to ICC using an antibody specific for pSTAT5 (green) and CUZD1 (red) or STAT5 (green) and counterstained with DAPI (blue). Data are representative images from at least three biological replicates. Magnification,  $\times 40$ . Scale bars, 200  $\mu$ m. Immunocytochemical staining was quantified using ImageJ and expressed as relative fluorescence (RF). *C*, occupation of the *Ereg* GAS by CUZD1/STAT5 is reduced following *Prlr* knockdown. Protein-DNA complexes were precipitated using an antibody specific for pSTAT5, M2 (anti-FLAG), or a ChIP re-ChIP using pSTAT5 followed by M2. Purified DNA was subjected to qPCR using primers specific to the GAS motif of the *Ereg* promoter. Data are represented as -fold enrichment  $\pm$  S.E. (error bars) from at least three biological replicates.

*g-k*) (insets indicate total ErbB1 and ErbB4). To determine whether proliferation is reduced with pimoizide treatment, we conducted IHC using Ki67, a well-known marker of proliferation. This staining indicates that tumor cell proliferation is dramatically reduced with pimoizide treatment (Fig. 7*B, f* and *l*). Graphs accompanying immunostaining panels represent fluorescent quantification by ImageJ analysis. Collectively, these data indicate that the inhibition of STAT5 signaling by pimoizide and resulting impairment in ErbB1 and ErbB4 signaling mitigate CUZD1-induced tumorigenesis.

#### CUZD1 pathway operates in a subset of human breast cancer cells

The fact that *Cuzd1* overexpression in mammary epithelial cells leads to breast tumorigenesis in mice raised the possibility that *CUZD1* may play a role in human breast cancer. We there-

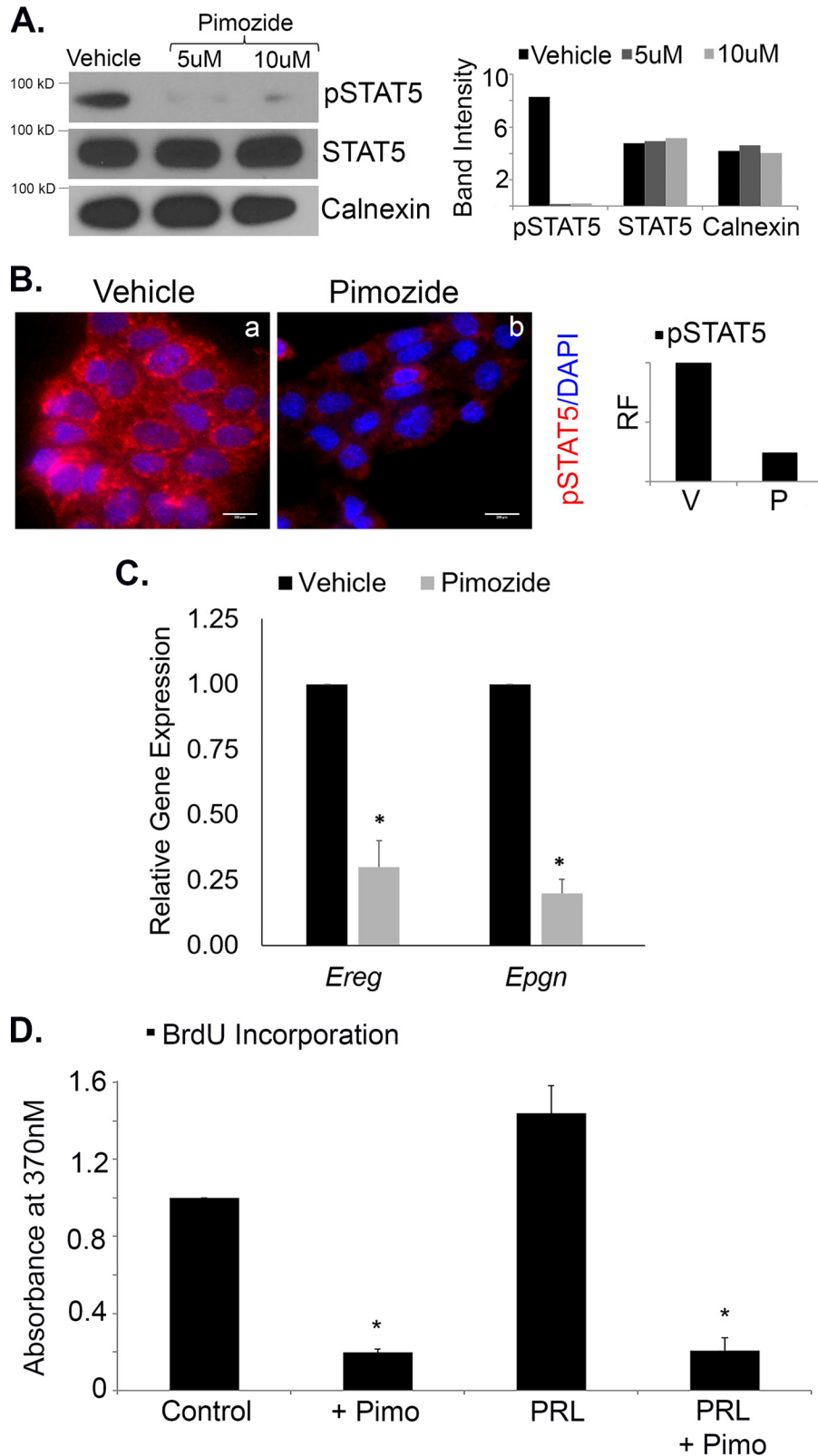
fore examined the expression of *CUZD1* transcripts in several human breast cancer cell lines. As shown in Fig. 8*A*, *CUZD1* transcripts are expressed in certain human breast cancer cell lines, including MCF7, but are undetectable in other breast cancer cell lines. Interestingly, the *CUZD1* protein was mostly cytosolic in MCF7 cells when these cells were grown in serum-free medium, but it rapidly translocated to the nucleus upon treatment with serum (Fig. 8*B*).

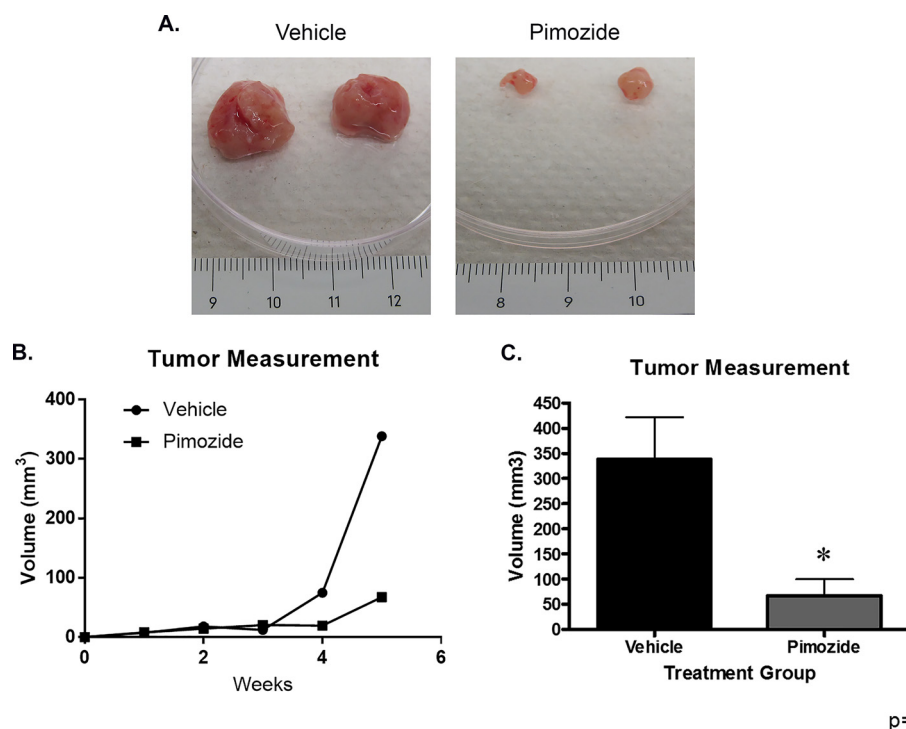
To examine the effects of *CUZD1* protein on STAT5 and ErbB signaling pathways in MCF7 cells, we created a stable cell line in which *CUZD1* is overexpressed (MCF7-Cuzd1). MCF7 cells overexpressing  $\beta$ -galactosidase were used as a control (MCF7-LacZ). MCF7-Cuzd1 cells overexpressed *Cuzd1* mRNA 20-fold compared with the MCF7-LacZ control cells (Fig. S2). Whereas STAT5 phosphorylation was evident in MCF7-LacZ cells (Fig. 8*C, a*), the level of pSTAT5 increased in

## Role of *CUZD1* in breast tumorigenesis

MCF7-Cuzd1 cells (Fig. 8C, c). Pimozide treatment strongly inhibited STAT5 phosphorylation in both MCF7-Cuzd1 and MCF7-LacZ cells (Fig. 8C, b and d). We then tested whether *CUZD1* expression in MCF7 cells is linked to the production of the EGF family growth factors by these cells. Elevated *CUZD1*

expression in MCF7-Cuzd1 cells led to increased expression of *EREG*, *EPGN*, and *NRG1* transcripts compared with their levels in MCF7-LacZ cells (Fig. 8D). Finally, we performed a BrdU incorporation assay by growing MCF7-Cuzd1 cells in the presence or absence of pimozide. Our results showed that pimozide





**Figure 6. Pimozide treatment suppresses growth of HC11-Cuzd1-Tum cell tumors *in vivo*.** *A*, gross tumor size is reduced following pimozide treatment. Representative images of tumors isolated from vehicle and pimozide-treated mice are shown. *B*, time course of tumor growth in mice treated with pimozide. Tumor volume in vehicle- and pimozide-treated mice was measured over the course of 5 weeks using digital calipers. Tumor volume =  $\frac{1}{2}(\text{length} \times \text{width}^2) \pm \text{S.E.}$  (error bars). *C*, end tumor volume is reduced with pimozide treatment. Final tumor volume in vehicle- and pimozide-treated mice was measured using digital calipers. Tumor volume =  $\frac{1}{2}(\text{length} \times \text{width}^2) \pm \text{S.E.}$ ,  $n = 15$ .

markedly inhibited the proliferation of MCF7-Cuzd1 in growth medium containing serum (Fig. 8E). Collectively, these results indicated that CUZD1-mediated activation of STAT5 signaling and downstream activation of ErbB1 and ErbB4 pathways are likely to play a critical role in controlling the proliferation of certain types of human breast cancer cells, and the use of pimozide is highly effective in countering the growth of these cells.

## Discussion

It is well documented that, during pregnancy and lactation, PRL functions through PRLR in the mammary epithelium to activate a molecular signaling cascade involving phosphorylation of PRLR and JAKs. This is followed by recruitment of STAT5 to this protein complex and subsequent activation of STAT5 through phosphorylation by JAKs. Activated STAT5 then translocates to the nucleus to regulate target gene expression (71). Our recent studies identified CUZD1 as a key cellular protein that functions as an essential regulator of STAT5 acti-

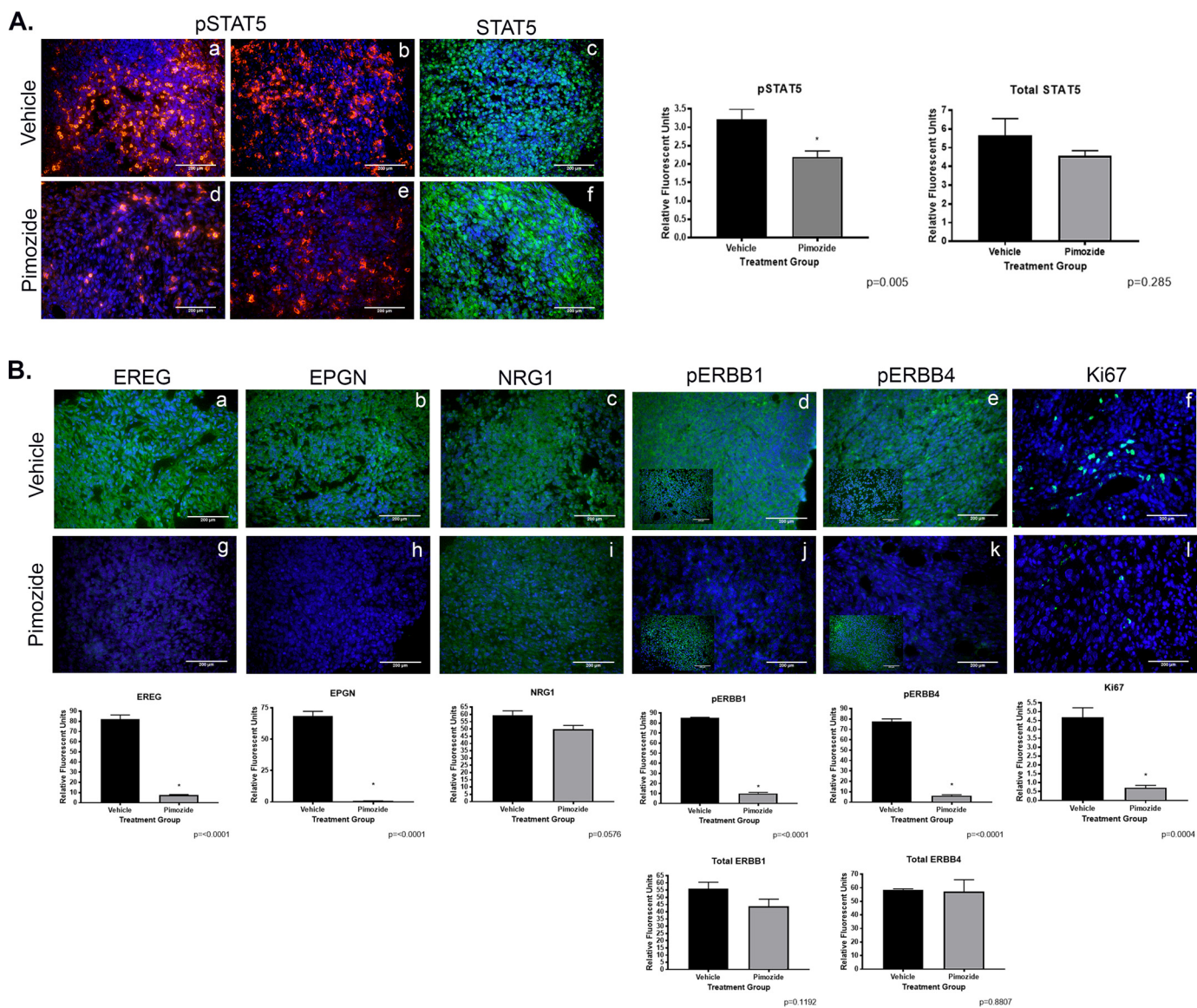
vation downstream of PRL signaling during mammary epithelial proliferation and differentiation during pregnancy and lactation (51). CUZD1 interacts with a complex containing JAK1/JAK2 and STAT5 and plays an important role in the phosphorylation and nuclear translocation of STAT5. The integral role of CUZD1 in STAT5 phosphorylation became evident with the creation of the *Cuzd1*-null mice in which the activating STAT5 phosphorylation at Tyr-694 fails to occur in the mammary epithelium, leading to a defect in its proliferation and alveolar differentiation (51). It is also of interest that the *Stat5*-null and *Cuzd1*-null mice display remarkably similar defects in mammary alveologenesis (25).

With the backdrop of the findings that CUZD1 critically influences PRL/STAT5-dependent mammary epithelial proliferation and differentiation, we examined whether dysregulation of its normal function leads to mammary tumorigenesis. Our study revealed that overexpression of CUZD1 in non-transformed mammary epithelial HC11 cells leads to precancerous transformation of these cells. Introduction of these

**Figure 5. Pimozide inhibits STAT5 phosphorylation and cell proliferation.** *A*, phosphorylation of STAT5 is reduced following treatment with pimozide. HC11-Cuzd1 cells were plated, allowed to attach overnight, and treated with PRL plus vehicle or pimozide at 5 and 10  $\mu\text{M}$  for 3 h. Cells were then lysed and subjected to Western blotting using an antibody specific for pSTAT5, STAT5, or calnexin. Band intensity was quantified using ImageJ. *B*, phosphorylation as well as nuclear localization of STAT5 is reduced following treatment with pimozide. HC11-Cuzd1 cells were plated, allowed to attach overnight, and treated with vehicle (V) or pimozide (P) at 10  $\mu\text{M}$  for 3 h. Cells were then fixed and subjected to ICC using an antibody specific for pSTAT5 (red) and counterstained with DAPI (blue). Data are representative images from at least three biological replicates. Scale bars, 200  $\mu\text{m}$ . Nuclear pSTAT5 was quantified using ImageJ and expressed as RF from at least three biological replicates. *C*, treatment with pimozide leads to a reduction in expression of specific EGF ligands. HC11-Cuzd1 cells were plated, allowed to attach overnight, and treated with vehicle or pimozide at 10  $\mu\text{M}$  for 24 h. RNA was isolated and subjected to qPCR using gene-specific primers to assess expression of *Ereg* and *Epgn*. Data are represented as relative gene expression  $\pm \text{S.E.}$  (error bars) from at least three biological replicates. *D*, treatment with pimozide reduces cell proliferation. HC11-Cuzd1 cells were plated, allowed to attach overnight, and serum-starved for 48 h. Cells were then treated with growth medium or PRL, with vehicle or pimozide for 18 h. BrdU was added 2 h before fixation, and cells were assayed for proliferation. Data are represented as absorbance at 370 nm  $\pm \text{S.E.}$  from at least three biological replicates.



## Role of CUZD1 in breast tumorigenesis

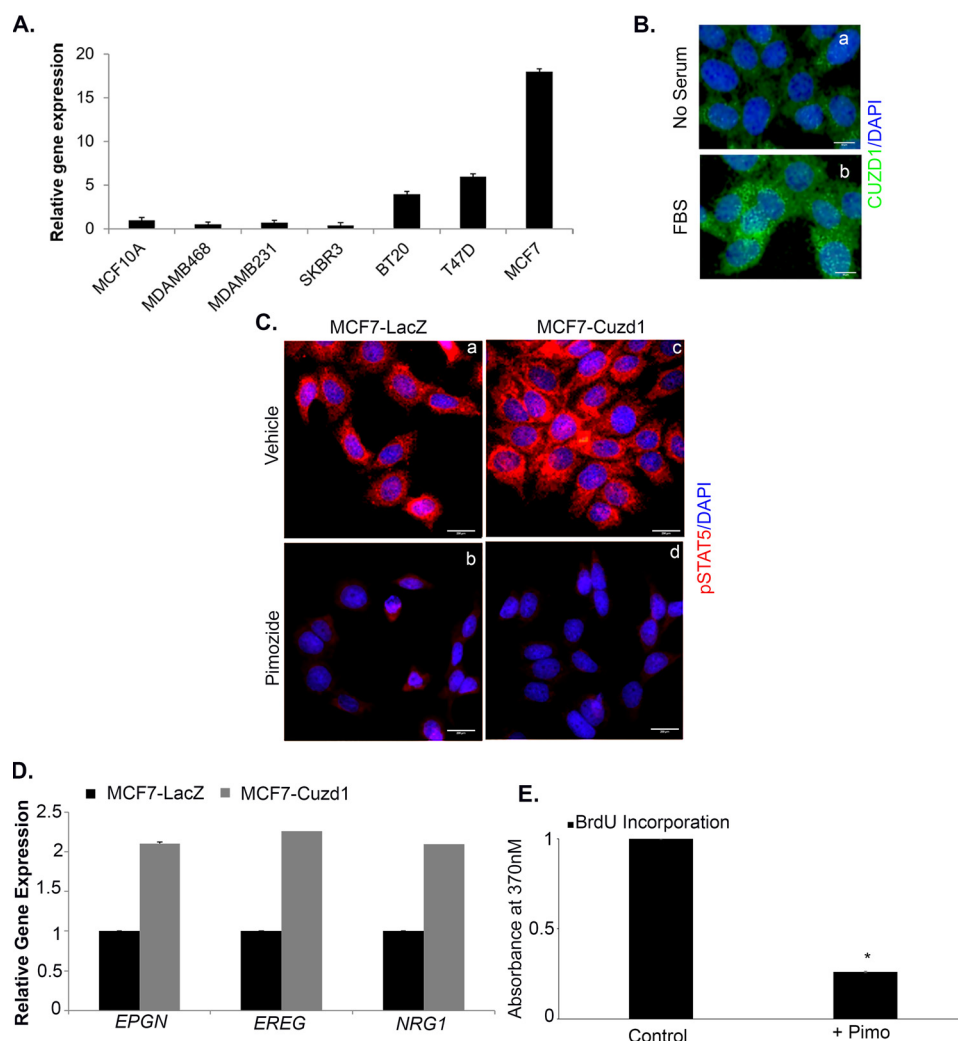


**Figure 7. Pimoizide treatment reduces STAT5 phosphorylation and blocks downstream ErbB signaling *in vivo*.** A, STAT5 phosphorylation is decreased in the tumors of mice treated with pimoizide. Vehicle-treated or pimoizide-treated tumors were collected, fixed, embedded in paraffin, sectioned, and subjected to IHC analysis with antibodies against pSTAT5 (a and b, vehicle-treated; d and e, pimoizide-treated) (red) or STAT5 (c, vehicle-treated; f, pimoizide-treated) (green) and counterstained with DAPI (blue). Magnification,  $\times 40$ . Scale bars, 200  $\mu\text{m}$ . Immunohistochemical staining was quantified using ImageJ and expressed as RF from at least three biological replicates. Data are representative images from  $n = 5$ . Signaling through the EGF pathway is altered in mice treated with pimoizide. Tumors were collected, fixed, embedded in paraffin, sectioned, and subjected to IHC analysis with antibodies against EREG (a and g) (green), EPGN (b and h) (green), NRG1 (c and i) (green), pErbB1 (d and j) (green), ErbB1 (insets in d and j) (green), pErbB4 (e and k) (green), ErbB4 (insets in e and k) (green), and Ki67 (f and l) (green) and counterstained with DAPI (blue). Magnification,  $\times 40$ . Scale bars, 200  $\mu\text{m}$ . Data are representative images from  $n = 5$ . Immunohistochemical staining was quantified using ImageJ and expressed as RF from at least three biological replicates  $\pm$  S.E. (error bars).

transformed cells in a mammary gland milieu via orthotopic injection led to the development of breast adenocarcinomas. Interestingly, tumor cells isolated from these primary tumors displayed an even more aggressive growth phenotype when transplanted to mammary glands of subsequent hosts. An important aspect of this tumorigenesis is the striking activation of STAT5 in the tumors, presumably due to constitutive overexpression of CUZD1.

We previously demonstrated that CUZD1 is also a critical regulator of a subset of EGF family growth factors, EREG, EPGN, and NRG1, which act primarily through their tyrosine kinase receptors, ErbB1 and ErbB4, to exert effects mainly during alveolar development (72). The ErbB receptors are activated

at all stages of mammary development and contribute to normal breast functions (32, 44, 72). A large body of evidence suggests that these receptors control cell proliferation, differentiation, angiogenesis, invasion, and survival of mammary cells, and their aberrant activation plays a key role in giving rise to malignant phenotypes (44, 46, 73). This raised the possibility that Cuzd1-mediated tumorigenesis may be driven through an overactive ErbB signaling network. Consistent with this prediction, the EGF family ligands EREG, EPGN, and NRG1 are robustly expressed, and activated forms of their receptors ErbB1 and ErbB4 are prominently present in the Cuzd1-overexpressing cells and adenocarcinomas. Interestingly, ErbB2, the receptor associated with shortest overall survival rates for



**Figure 8. CUZD1 signaling in the human breast cancer MCF7 cells.** A, MCF7 cells express high levels of *Cuzd1* mRNA. Human cancer cell lines were cultured in growth medium, and mRNA isolated from these cells was examined for *Cuzd1* expression. Data are represented as relative gene expression  $\pm$  S.E. (error bars) from at least three biological replicates. B, CUZD1 localization is influenced by FBS. MCF7 cells were plated, allowed to attach overnight, and serum-starved for 48 h. Cells were treated with no serum (a) or FBS (b) for 6 h. Following fixation, cells were subjected to IHC analysis with an antibody specific for CUZD1 (green) and counterstained with DAPI (blue). Data are representative images from at least three biological replicates. Scale bars, 200  $\mu$ m. C, phosphorylation of STAT5 is reduced following treatment with pimozide. HC11-LacZ and HC11-Cuzd1 cells were plated, allowed to attach overnight, and treated with a vehicle control (a and c) or a pimozide at 10  $\mu$ M (b and d) for 3 h. Cells were then fixed and subjected to ICC using an antibody specific for pSTAT5 (red) and counterstained with DAPI (blue). Data are representative images from at least three biological replicates. Scale bars, 200  $\mu$ m. D, specific EGF family ligands are up-regulated in MCF7 cells that overexpress *CUZD1*. RNA was isolated from MCF7-Cuzd1 cells and subjected to qPCR using gene-specific primers to assess expression of EREG, EPGN, and NRG1. Data are represented as relative gene expression  $\pm$  S.E. from at least three biological replicates. E, treatment with pimozide reduces cell proliferation. MCF7-Cuzd1 cells were plated, allowed to attach overnight, and serum-starved for 48 h. Cells were then treated with growth medium, with vehicle or pimozide, for 18 h. BrdU was added 2 h before fixation, and cells were assayed for proliferation. Data are represented as absorbance at 370 nm  $\pm$  S.E. from at least three biological replicates.

breast cancer and a primary target for developing therapeutics, is not active in these tumors.

During normal mammary gland development, PRL, acting via downstream STAT5 signaling, directs proliferation and extension of the ductal system. We and others have shown that the gene encoding EREG, which has been implicated in promoting growth and survival of breast cancer cells, is a direct transcriptional target of STAT5 (31, 51). Constitutive action of pSTAT5 and resulting stimulation in the production of a subset of EGF-like growth factors may therefore contribute to tumorigenesis through persistent stimulation of mammary epithelial proliferation. Drugs, such as bromocriptine, inhibit transcription of pituitary PRL, but due to the local synthesis of PRL in the mammary gland driving tumor proliferation, therapeutic inter-

vention needs to occur further downstream in the signaling pathway (74–76). Researchers have been targeting multiple steps along the PRL signaling pathway, from competitive PRLR antagonists to pharmacologic inhibition of PRL signal transducers (77). These data support a growing body of research emphasizing the need for clinical targets of PRL/STAT5 signaling in the treatment of breast cancer. Interruption of the PRL signaling pathway through inhibition of STAT5 could be an effective treatment for PRL/STAT5-driven tumors.

Development of the *Cuzd1*-dependent breast cancer model in our laboratory presents a unique opportunity to study the effects of a STAT5 inhibitor in treating breast cancers expressing a constitutively high level of pSTAT5. In this study, we repurposed a previously Food and Drug Administration-ap-

## Role of *CUZD1* in breast tumorigenesis

proved antipsychotic drug, pimozide, which is used to treat mental disorders such as Tourette syndrome and schizophrenia. Studies demonstrated that pimozide reduces viability and proliferation of breast and non-small cell lung carcinoma cells and was comparably less cytotoxic to non-cancer cells (78, 79). Although the mechanism of STAT5 inhibition by pimozide is yet to be elucidated, it selectively inhibits STAT5 phosphorylation and transcription of STAT5 target genes (80, 81). In our study, inhibition of STAT5 phosphorylation through the use of pimozide reduced the production of the EGF family members *Ereg*, *Epgn*, and *Nrg1*. Consequently, activation of ErbB1 and ErbB4 receptors was also reduced, resulting in the suppression of proliferation in *Cuzd1*-overexpressing cells. Most importantly, treatment of mice with pimozide was able to significantly inhibit breast tumorigenesis *in vivo*. Although this treatment did not prevent tumorigenesis, an increase in dosage concentration/frequency or combination with other therapies may significantly increase the effectiveness of this drug.

The *Cuzd1* gene is highly conserved between mice and humans (61). The linkage between *CUZD1* and mammary tumorigenesis in the mouse raised the possibility that it might be involved in human breast cancers. Screening of a broad panel of breast cancer cell lines for *CUZD1* expression using qPCR revealed that *CUZD1* is undetectable in ER $\alpha$ -negative tumor cells but present in ER $\alpha$ -positive tumor cells, including the well-characterized MCF7 cells. It is pertinent to mention here that *Cuzd1* (formerly known as ERG1) was originally identified by our laboratory as an estrogen-regulated gene in the uterus and was later found to be induced in breast epithelium of ovariectomized mice in response to estrogen (53). It is therefore possible that ER $\alpha$  regulates *CUZD1* expression in certain human breast cancer cells. Previous studies reported that the PRLR and ER $\alpha$  act synergistically to exert their mitogenic effects on breast cancer cells (48). It would be important to study the mechanism of this cross-regulation.

The expression of *CUZD1* in several human breast cancer cell lines raised the possibility that dysregulation of *CUZD1* may play a role in human breast tumorigenesis. We analyzed publicly available data on the cBioPortal for Cancer Genomics database to examine whether *CUZD1* expression is altered in human breast cancer (82, 83). Interestingly, data from tumors in Breast Invasive Carcinoma (The Cancer Genome Atlas, Provisional) indicate that *CUZD1* is altered in 1% of patients (10 of 960). Although this represents a small portion of this study, it is worth noting that *HER2*, *BRCA1*, and *BRCA2* were altered in 14, 4, and 5% of patients, respectively. All breast cancer samples that had alterations in *CUZD1* displayed *CUZD1* amplification and were classified as stage IIA through IIIB. Classification of tumors based on co-occurrence of genes identified by gene expression profiles, as opposed to tumor stage, can provide the necessary information to predict treatment response and clinical outcome (84–86). A subset of tumors with a *CUZD1* amplification also showed amplification of *STAT5A*, *PRL*, *PRLR*, and/or *EGFR*. There was significant co-occurrence in alterations in *CUZD1* and *STAT5A* ( $p = 0.026$ ) and *CUZD1* and *PRL* ( $p = 0.024$ ). Additionally, breast cancer samples with alterations in this gene set also had increased EGFR phosphorylation at tyrosine 1068 ( $p = 0.0112$ ). Collectively, these data indicate

that not only is *CUZD1* amplified in these breast cancer samples; the components of its signaling pathway are also amplified or increased. Although these data provide some insights regarding the relevance of *CUZD1* amplification in human breast cancer, they do not provide a complete picture of the functional amplification of *CUZD1* and *STAT5A* signaling because a large portion of these activities are conveyed through post-translational modifications on these factors. Nonetheless, based on our findings, *CUZD1* has emerged as a novel target for designing effective treatments for certain types of breast cancers.

## Experimental procedures

### Animals

Mice were maintained in the designated animal care facility at the University of Illinois, according to institutional guidelines for the care and use of laboratory animals. All experimental procedures involving mice were conducted in accordance with National Institutes of Health standards for the use and care of mice. The animal protocol describing these procedures was approved by the University of Illinois Institutional Animal Care and Use Committee.

### Cell lines and cell culture

The HC11 cell line is a non-transformed mammary epithelial cell line derived from pregnant BALB/c mice (87). These cells were grown in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum, 5  $\mu$ g/ml insulin, and 10 ng/ml EGF at 37 °C and 5% CO<sub>2</sub>. HC11-LacZ, HC11-Cuzd1, MCF7-LacZ, and MCF7-Cuzd1 cell lines were developed as described by Mapes *et al.* (51). To create the HC11-Cuzd1 Tum cell line, HC11-Cuzd1 cells were orthotopically injected into BALB/c mice and allowed to form tumors. Upon collection, tumors were minced into 1-mm pieces using scissors, and fragments were digested in Hanks' balanced salt solution (HBSS) with 6 g/liter dispase and 0.5 g/liter collagenase at 37 °C for 1 h with constant agitation. Enzymes were neutralized, and the cell suspension was passed through a 100- $\mu$ m mesh, followed by two washes with Hanks' balanced salt solution. Cells were plated on collagen-treated plates in DMEM supplemented with 10% FBS, penicillin-streptomycin, and amphotericin B. After 48 h in culture, stable *Cuzd1*-overexpressing cells were selected using blasticidin for 10 days.

HC11 cells were treated with 50  $\mu$ M PRL, followed by immunocytochemistry or RNA analysis. The STAT5 inhibitor pimozide was used at 10  $\mu$ M (unless otherwise designated) alongside a vehicle control (DMSO) before immunocytochemistry, Western blotting, proliferation assays, and RNA analysis.

### Boyden chamber cell migration assay

Boyden chambers (Millipore) were placed in 24-well dishes containing a chemoattractant (RPMI containing 10% FBS). Serum-starved HC11-LacZ or -Cuzd1 cells ( $1 \times 10^5$  cells/well) were added to the upper compartment and allowed to incubate for 72 h at 37 °C. Cells that migrated across into the lower chamber of the membrane were quantified by a CyQuant (Millipore) fluorometric assay according to the manufacturer's

instructions. Mean values were taken from three individual chambers for each of the three biological replicates.

#### **Anchorage-independent growth in soft agar**

HC11-LacZ or HC11-Cuzd1 cells ( $1 \times 10^4$ ) or MCF7 cells (control) were seeded in 6-well plates with a bottom layer of 0.48% Bacto agar in DMEM and a top layer of 0.36% Bacto agar in DMEM. Fresh DMEM containing 10% FBS was added to the top layer of the soft agar. The culture medium was changed twice per week. After 16 days, colonies were stained with 0.005% crystal violet. Visible colonies ( $>0.5$  mm in diameter) were counted from representative views from three biological replicates, and the average number of colonies per well was determined.

#### **Cell proliferation using BrdU incorporation**

Cells were plated at a density of  $5 \times 10^3$  cells/well in 96-well plates and cultured overnight in full growth medium. Following 48 h of serum starvation, cells were treated with selected compounds in addition to vehicle or pimozone and allowed to grow for 18 h. BrdU was added, and incorporation was measured after 2 h using an ELISA-based BrdU assay. The resulting color reaction was measured using a plate reader at 370 nm. The relative levels of BrdU incorporation from three independent measurements are shown (mean  $\pm$  S.E.).

#### **qPCR analysis**

For qPCR, total RNA was extracted from purified mammary epithelium or cultured HC11 cells using a TRIzol RNA purification kit. Reverse transcription was performed using the cDNA synthesis kit (Stratagene) following the manufacturer's instructions. cDNA was amplified by quantitative real-time PCR analysis using gene-specific primers and SYBR Green supermix (Applied Biosciences). For a given sample, threshold cycle (*Ct*) and S.D. were calculated from individual *Ct* values from 3–4 replicates of a sample. Normalized mean *Ct* was computed as  $\Delta Ct$  by subtracting mean *Ct* of 36B4 from *Ct* of a target gene for the control sample.  $\Delta\Delta Ct$  was then calculated as a difference in  $\Delta Ct$  values between control and experimental groups. -Fold change in gene expression was then computed as  $2^{-\Delta\Delta Ct}$ . Relative mRNA levels were plotted after normalization to the loading control 36B4. The error bars represent the relative gene expression  $\pm$  the S.E. from three or more independent trials.

#### **Orthotopic intraductal injection of cells into the mammary gland**

HC11-LacZ, HC11-Cuzd1 or HC11-Cuzd1 Tum cells ( $1 \times 10^6$ ) were suspended in Matrigel and orthotopically injected into the nipple of the fourth abdominal mammary gland of nude or BALB/c mice. During each tumor study, these experiments were repeated three times with five mice per group when tracking tumor growth and eight mice per group during pimozone treatment. Tumor length and width were measured using digital calipers, and tumor volume was calculated (tumor volume =  $\frac{1}{2}(\text{length} \times \text{width}^2)$ ).

#### **Pimozone treatment**

Female BALB/c mice were orally treated with 5 mg/kg body weight pimozone or a vehicle control (DMSO), suspended in corn oil, once per day for 3 days before orthotopic injection. The treatment regimen of pimozone included 3 days of treatment followed by 3 days of rest, which cycled until the end of the 5 weeks. Tumor volume was measured throughout the course of the 5-week treatment. The primary mammary tumors were harvested from sacrificed animals and fixed in 4% paraformaldehyde for subsequent H&E staining or immunohistochemistry. Individual tumor studies were terminated at the recommendation of the University of Illinois Division of Animal Resources veterinary staff due to high tumor burden.

#### **Immunostaining**

Paraffin-embedded mammary tissues were sectioned and subjected to IHC as described previously (88). IHC was performed on tissue sections using primary antibodies, and bound primary antibodies were detected with either immunoperoxidase or immunofluorescence secondary antibodies. For immunoperoxidase staining, horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were used as secondary antibody. Sections were counterstained with hematoxylin and mounted. For immunofluorescence staining, fluorescence-conjugated anti-mouse IgG or anti-rabbit IgG were used as secondary antibody, and slides were mounted in Prolong GOLD and cured for 24 h before imaging.

For immunocytochemistry, cells were fixed in a 3% formalin solution at room temperature for 10 min, followed by washing with PBS for 10 min. The cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min, and nonspecific binding of antibodies was blocked with 10% donkey serum for 1 h at room temperature. Cells were incubated with primary antibodies overnight at 4 °C. Fluorescence-conjugated anti-mouse IgG or anti-rabbit IgG were used as secondary antibody, and slides were mounted in Prolong GOLD and cured for 24 h before imaging.

Rabbit polyclonal antibodies against a peptide antigen containing amino acids SSPNYKPKPHEL of mouse CUZD1 were generated in our laboratory and used in IHC at 1:200 dilution. Other antibodies used in this study include pan-Cytokeratin (Santa Cruz Biotechnology, Inc., catalog no. sc-8018, IHC 1:350); PCNA (Santa Cruz Biotechnology, catalog no. sc-56, IHC 1:500); pSTAT5 (Tyr-694/Tyr-699) (Santa Cruz Biotechnology, catalog no. sc-11761, IHC 1:50, Western blot 1:500); STAT5 from Santa Cruz Biotechnology, catalog no. sc-11761, IHC 1:50, Western blot 1:1000); EREG (Santa Cruz Biotechnology, catalog no. sc-376284, IHC 1:50); EPGN (R&D Systems, catalog no. AF1127SP, IHC 1:100); NRG1 from NeoMarkers (catalog no. AP-9003, IHC 1:100); pEGFR (Tyr-1068) (Santa Cruz Biotechnology, catalog no. sc-377547, IHC 1:500); pErbB2 (Cell Signaling Technology, catalog no. 2241S, IHC 1:100); pErbB4 (Tyr-1056) (Santa Cruz Biotechnology, catalog no. sc-33040, IHC 1:100); ErbB1 (Santa Cruz Biotechnology, catalog no. sc-03, IHC 1:100); ErbB4 (Santa Cruz Biotechnology, catalog no. sc-283, IHC 1:100); Ki67 (BD Pharmingen, catalog no. 550609, IHC 1:100); pAKT1/2/3 (Santa Cruz Biotechnol-

## Role of CUZD1 in breast tumorigenesis

ogy, catalog no. sc-33437R, IHC 1:100); pERK1/2 (Santa Cruz Biotechnology, IHC 1:100); phospho-Akt1/2/3 from Santa Cruz Biotechnology, catalog no. sc-33437R, IHC 1:100); and pSTAT5 (Tyr-694) (Cell Signaling Technology, catalog no. 9351, lot 7).

### Image capture and processing of immunostaining

Images of immunohistochemical staining were captured by using a Leica DM2500 light microscope fitted with a Qimaging Retiga 2000R camera (Qimaging) or a Leica 700 confocal microscope. These images were directly documented from the scope with minimal processing to adjust the tonal range and color balance in ADOBE Photoshop version 8. ImageJ was used to quantify immunofluorescence staining.

### siRNA treatment

HC11 cells were transfected with siRNA against *Prlr* or control siRNA (non-targeting), using siLentFect reagent following the manufacturer's protocol. Briefly, siLentFect was mixed with 150 nM siRNA and allowed to form siRNA-liposome complexes, which were then added to cells at 60% confluence. Cells were harvested 48 h after transfection and 6 h after PRL treatment. Total RNA was isolated and analyzed by qPCR using gene-specific primers.

### Chromatin immunoprecipitation

ChIP assays were performed using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions with minor modifications. Anti-FLAG M2 affinity gel (Sigma, A2220) and anti-pSTAT5 antibody (Cell Signaling Technology, antibody 9351, lot 7) were used individually overnight at 4 °C to immunoprecipitate FLAG-CUZD1 and pSTAT5, respectively. Normal mouse IgG (Santa Cruz, sc-2027) immunoprecipitation served as a negative control. The ChIP re-ChIP was conducted by immunoprecipitating with pSTAT5 followed by M2. Protein-DNA complexes were eluted, cross-links were reversed, and purified DNA was analyzed for enrichment in sequences of interest using qPCR.

### Statistical analysis

Statistical analysis was performed by Student's *t* test. Statistically significant differences ( $p < 0.05$ ) are indicated by an asterisk.

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**Author contributions**—J.M., L.A., Q.L., A.N., I.C.B., and M.K.B. conceptualization; J.M. and L.A. data curation; J.M., L.A., Q.L., A.N., C.V.C., I.C.B., and M.K.B. formal analysis; J.M., L.A., Q.L., and A.N. validation; J.M., L.A., Q.L., and A.N. investigation; J.M., L.A., Q.L., and M. K. B. visualization; J.M., L.A., Q.L., A.N., C.V.C., I. C. B., and M. K. B. methodology; J.M., L.A., C.V.C., I.C.B., and M.K.B. writing-original draft; J.M., I.C.B., and M.K.B. writing-review and editing; I.C.B. and M.K.B. supervision; I.C.B. and M.K.B. funding acquisition; I.C.B. and M.K.B. project administration.

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## Role of CUZD1 in breast tumorigenesis

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