INVESTIGATION OF THE ROLE OF CUZD1-STAT5 SIGNALING IN MAMMARY GLAND DEVELOPMENT AND BREAST CANCER

BY

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DISSERTATION

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ABSTRACT

In the mammary gland, genetic circuits controlled by the hormones, estrogen, progesterone and prolactin (PRL), act in concert with pathways regulated by the epidermal growth factor (EGF) family to control the growth and morphogenesis of this tissue during puberty, pregnancy and lactation. However, the precise molecular mechanisms that integrate these signaling pathways are unclear. In this study, we identified CUZD1 (CUB and zona pellucida-like domain containing protein- 1) as a novel mediator of PRL signaling in steroid hormone-primed mouse mammary gland and undertook an examination of its role in growth and differentiation of this tissue during pregnancy. CUZD1 expression is markedly induced in steroid-primed mammary epithelial cells in response to PRL treatment. *Cuzd1*-null mice exhibited a striking impairment in ductal branching and alveolar development during pregnancy, resulting in a subsequent defect in lactation. Interestingly, phosphorylation and activation of STAT5, a transcription factor that mediates PRL signaling, was absent in *Cuzd1*-null mammary tissue during pregnancy and lactation. We also noted that the expression of epiregulin (EREG), an EGF family growth factor regulated directly by STAT5, is suppressed in *Cuzd1*-null mammary gland. Protein interaction studies, using flag-tagged CUZD1 expressed in HC11 mouse mammary epithelial cells, revealed that CUZD1 associates with a multi-protein complex containing JAK1/JAK2 and STAT5. Elevated expression of CUZD1 in HC11 cells stimulated phosphorylation and nuclear translocation of STAT5. Chromatin immunoprecipitation experiments indicated that STAT5 and CUZD1 co-occupy the same regulatory region of the Ereg gene. Over-expression of CUZD1 in mammary epithelial HC11 cells induced tumorigenic characteristics, such as substrate independent growth and migration. Furthermore, HC11-Cuzd1 cells formed mammary tumors *in vivo* following orthotopic injection into nude and Balb/C mice. Mammary tumor cells derived from these animals showed elevated levels of phosphorylation and nuclear localization of STAT5 and consequent activation of the EGF signaling pathway. Blockade of JAK/STAT5 signaling through the use of a STAT5 inhibitor markedly reduced the production of the EGF family growth factors and inhibited PRL-induced tumor cell proliferation *in vitro*. It also impaired the progression of CUZD1-driven mammary tumorigenesis *in vivo*. Collectively, our findings suggest that CUZD1 plays an important role in mammary epithelial cell proliferation during mammary gland development and in tumorigenesis by facilitating JAK-STAT5 signaling and subsequent production of growth factors, such as EREG. CUZD1, therefore, emerges as a critical mediator of PRL action that controls mammary alveolar development during pregnancy and lactation and cell proliferation during tumorigenesis.

To my family and friends

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CHAPTER 1

Signaling Networks in Mammary Gland Development and Tumorigenesis

Mammary Gland Development

The mammary gland is a unique organ in that its development is not completed *in utero*, but occurs during adulthood (Fig. 1.1). In the normal mammary gland, marked ductal and alveolar development occurs during puberty, pregnancy and lactation (1–3). From birth until puberty, the mammary gland consists of a rudimentary ductal tree within the fat pad. Ductal outgrowth during puberty is marked by the formation of terminal end buds (TEBs), structurally composed of cap cells that form the outer layer around body cells (Fig. 1.2) (4,5). TEBs are sites of high cell proliferation that fill up the fat pad with a tree-like structure through elongation and branching of the ducts. Adequate proliferation of the mammary epithelium is required for ductal outgrowth, branching and the formation of alveolar structures. During pregnancy, further ductal arborization and alveolar proliferation occur. Functional differentiation of the mammary gland into a milk-producing structure is accomplished near parturition, and lactation ensues shortly after parturition (Fig 1.2) (5). At the end of lactation, involution of the gland occurs with massive cell death and remodeling of the tissue, returning it to the simple ductal structure observed in the mature virgin. Circulating steroid hormones 17β-estradiol (E) and progesterone (P), the peptide hormone prolactin (PRL), and growth factors belonging to the epidermal growth factor (EGF) family orchestrate the development, maturation and regression of the mammary gland (Fig. 1.3) (3,6–8).

Epidermal Growth Factor Signaling During Mammary Gland Development

There are ten members of the EGF family of ligands; transforming growth factor-α (TGF-α), amphiregulin (AREG), epigen (EPGN), betacellulin (BTC), heparin-binding EGF (HB-EGF), epiregulin (EREG), and four subtypes of neuregulins (NRG1-4) (9,10). TGF-α, AREG, EPGN, BTC, HB-EGF, EREG, and NRG1 are all expressed in the mammary gland at various stages of development (11). TGF-α, BTC, and HB-EGF are present in the mammary epithelium before puberty through mid-pregnancy. At puberty, AREG and EREG levels rise and remain high during early pregnancy. AREG is essential for terminal end bud proliferation and normal ductal outgrowth during puberty (12,13). During lactation, NRG1 is required for alveolar cell proliferation and differentiation (14). Members of the EGF family of ligands bind to specific receptors called ErbBs (Fig. 1.4) (15). There are four members of the ErbB family: ErbB1 (EGFR), ErbB2, ErbB3, and ErbB4 (10). The structure of ErbB receptors includes a transmembrane domain, a ligand binding domain in the extracellular region and a kinase domain in the cytoplasmic region. Upon ligand binding, these receptors form homo- or heterodimers, which leads to the activation of the kinase domain. Autophosphorylation of specific tyrosines in the tail region of each receptor triggers binding of signaling molecules within the cytoplasm, which in turn initiate downstream signaling events (16).

The differential heterodimerization of ErbB family members induced by binding of specific ligands can account for the varied effects of an activated receptor. The presence of specific ligands and increased activation of their cognate receptors can be identified in various stages of mammary gland growth and development. ErbB1 is strongly expressed in TEBs and adjacent stroma during ductal outgrowth following the onset of pubertal hormone expression (10). It is known that ErbB1 in the stroma is activated by AREG during ductal elongation, but the functional redundancy of the ErbB1 ligands results in varied developmental defects in their absence. ErbB1 expression is absolutely required for normal mammary development (17). ErbB2 in the epithelium plays a role in ductal elongation and branching, as well as cellular differentiation at parturition (18–20). ErbB3 is important for the morphology of TEBs and cell survival during mammary outgrowth (21,22). During pregnancy, ErbB4 in the mammary epithelium is required for adequate proliferation, functional differentiation of alveolar units and lactation (10,23,24). The mechanism by which ErbB4 influences alveolar differentiation of the mammary gland was previously unclear. Recent data published by Mapes et. al. indicate a novel role for CUZD1 in modulating the function of ErbB4 during alveologenesis (25). In summary, ErbB signaling is crucial during mammary development, and the removal or disruption of this signaling can have deleterious effects.

Prolactin Signaling Network During Mammary Gland Development

Circulating levels of PRL are elevated during pregnancy, and the most well-known function of PRL is to control proliferation and differentiation of the mammary epithelium in preparation for lactation. The primary site of PRL secretion is the pituitary gland; however, the presence of extrapituitary PRL synthesis is well documented and has been confirmed in the brain, decidua, mammary gland and lymphocytes (26,27). PRL signals through the prolactin receptor (PRLR) and induces a signaling cascade through the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway (Fig. 1.5) (1,28–32). Binding of PRL to PRLR induces phosphorylation of the receptor, followed by phosphorylation of JAKs. Phosphorylated JAKs recruit STATs, which are subsequently phosphorylated at critical tyrosine residues. Phosphorylated STATs then disassociate from the JAK/PRLR complex, dimerize and move to the nucleus where they act as transcription factors. Injection of PRL into mature virgin mice did not increase expression of *Stat5*, but induced phosphorylation of STAT5 in the mammary gland (33). There are two isoforms of STAT5, STAT5a and STAT5b. Although they share 95% similarity, STAT5a is the most prominent isoform active during mammary gland development (34). PRL signaling through the JAK/STAT5 pathway during late pregnancy and lactation initiates and maintains expression of the milk protein genes *Wap* and *β-casein* (32,35,36). Expression of these genes is essential for terminal differentiation of the mammary epithelium into functional milk-producing alveoli.

The development of transgenic mice has given us great insight into the importance of the PRL signaling pathway in mammary gland development. *Prl*$^{(-/-)}$ mice display normal ductal extension and arborization of the mammary epithelium during puberty, but do not undergo alveologenesis; the mammary ducts end as blunt tubes (37). As these mice are lacking proper differentiation of the mammary epithelium, they are unable to lactate. *Prlr*$^{(-/-)}$ females are also infertile due to inadequate maintenance of the corpus luteum, and although *Prlr*$^{(-/-)}$ females supplemented with progesterone were able to maintain pregnancy, the mammary epithelium did not form the alveolar buds necessary for lactation (38). *Jak1*$^{(-/-)}$ mice die within 24h of birth due to a defect in lymphopoiesis and a failure to nurse (39). *Jak2*$^{(-/-)}$ mice also die at embryonic day 12.5 because of disrupted erythropoiesis (40). Conditional *Jak2*$^{(-/-)}$ mice, created with the use of a mammary gland-specific MMTV-cre, exhibit defective proliferation and differentiation of the mammary epithelium during pregnancy and fail to lactate (41). There are two isoforms of STAT5 in the mammary gland, STAT5a and STAT5b, both of which are activated by the PRL signaling pathway (42). However, loss of STAT5a results in failed lactation due to a lack of alveologenesis with no compensation observed by STAT5b (Fig. 1.6) (43). Collectively, these data clearly outline the importance of the PRL-STAT5a signaling pathway in the development and functional differentiation of the mammary gland.

EGF Signaling and Breast Cancer

The search for targeted approaches to treat breast cancer is not a recent undertaking; we have been examining tumors for estrogen receptor status to predict patient response to tamoxifen treatment for almost 50 years (44,45). The ubiquitous use of the EGF signaling pathway by cancer cells has provoked the extensive study of this pathway and the development of many drugs targeting the ErbB receptors (46–48). Aberrant signaling through the EGF pathway has been established as a common factor in many human neoplasms, including those in the gastrointestinal tract, brain, lung, and breast (49). Overexpression of ErbB1, or the EGF ligands that signal through this receptor, results in spontaneous tumor development in mouse models and predicts large tumor size and reduced survival in breast cancer patients (48,50–52). Human trials relying only on overexpression of ErbB receptors as predictors for response to specific receptor inhibition have shown varied outcomes that are likely due to an autocrine signaling loop established by excessive EGF ligands or ErbB receptor mutations (53). A monoclonal antibody targeting HER2 (trastuzumab) has been in clinical use to treat patients with HER2-positive breast tumors since 1998, commonly in conjunction with chemotherapeutics (35,54–56). Treatment with ErbB inhibitors has also been shown to increase response to anti-estrogen treatment in hormone-resistant breast cancers (57,58). The role of ErbB4 in tumorigenesis is not as well defined, though recent studies indicate that overexpression of ErbB4 leads to tumor formation in mice (24). We provide evidence that the growth of tumors caused by

overexpression of CUZD1 is due to activated signaling through the EGF pathway via the upregulation of EGF ligands and activation of ErbB1 and ErbB4. These data propose CUZD1 as a potential drug target to decrease ErbB signaling in patients with breast tumors.

PRL and Breast Cancer

The association between the PRL signaling pathway and breast cancer has been well established in experimental models. Transgenic mice overexpressing rat PRL develop spontaneous mammary tumors within 11-15 months of age (59). PRL has also been shown to have growth stimulatory effects on mammary tumors induced by the carcinogen DMBA (60,61). Inversely, we can examine the effect of inhibition of PRL secretion by treatment with bromocriptine, which reduces serum PRL by inhibiting secretion by lactotrophs in the pituitary gland. Bromocriptine treatment reduced incidence of mammary tumorigenesis and fostered regression of existing tumors (62). Collectively, PRL plays an important role in promoting tumorigenesis as well as breast cancer progression.

The effect of PRL in breast tumorigenesis was long believed to be a phenomenon present in rodent models only. This is largely due to the observation that inhibition of pituitary PRL secretion in humans through bromocriptine treatment did not affect mammary tumor growth (63). This may be the result of the observation that human mammary cells can make their own PRL, an occurrence not present in mice. During mammary tumorigenesis, this local secretion of PRL may compensate for the lack of pituitary PRL during bromocriptine treatment (26,64). Researchers are now finding that PRL plays similar roles in the rodent and human during breast tumorigenesis, establishing this signaling pathway as an important player in breast cancer (65).

Integrated effects of PRL and EGF in mammary gland development and breast cancer

Ample evidence exists to suggest a collaboration of PRL and ErbB-mediated signaling pathways during mammary gland development and breast tumorigenesis (66–68). Upon EGF administration, STAT5 is activated to a similar degree as seen with PRL treatment (69,70). Furthermore, active ErbB4 has been shown to phosphorylate STAT5 in the mammary epithelium. *ErbB4*-null mice exhibit disrupted alveologenesis and a dramatic reduction in the expression of *Wap*, a well-known downstream gene target of STAT5 (71). Further investigation revealed STAT5 is functionally inactive due to a loss of phosphorylation in the mammary epithelium of *ErbB4*-null mice. These signaling pathways induce STAT5 activation during mid-lactation to control alveolar proliferation and differentiation as well as tumorigenesis. However, current

understanding is lacking as to how these two pathways are linked on a molecular level. Recent data from our lab positions CUZD1 as an integrator of PRL and ErbB signaling, establishing CUZD1 as a critical link between these two essential pathways in mammary gland biology (25). Furthermore, deletion of genes in this pathway that are essential for mammary gland development during pregnancy and lactation, including *Prlr*, *Jak2*, *Stat5a*, *ErbB4*, and *Cuzd1*, result in similar phenotypic defects (Fig. 1.7) (23,25,43,66,72). These data support the importance of these signaling molecules in mammary gland development and tumorigenesis.

Identification of CUZD1

Our laboratory identified CUB and zona pellucida-like domain-containing protein 1 (CUZD1) as a regulator of mammary epithelial cell proliferation, which led us to investigate the molecular pathways of CUZD1. We implemented *in vitro* and *in vivo* models to address this question. Our data indicate that CUZD1 regulates the PRL signaling pathway, influencing STAT5 phosphorylation and downstream gene expression to induce proliferation and differentiation of the mammary epithelium during pregnancy and lactation. Abatement of epithelial proliferation in the *Cuzd1*$^{(-/-)}$ mammary gland raised a very interesting question: would the overexpression of *Cuzd1* lead to an increase in proliferation and possible tumorigenesis? New data concerning the mechanism of action of CUZD1 in mammary epithelial cells allowed us to gain insight into how it is influencing tumorigenesis. Our results indicate that hyperactivation of STAT5 may be playing a role in *Cuzd1*-induced tumorigenesis. We used pimozide, a STAT5 phosphorylation inhibitor, to impede the downstream effects of *Cuzd1* overexpression and disrupt tumorigenesis.

Gene ontology analysis of *Cuzd1* associates it's gene products with cell division, cell cycle, and cell proliferation (73). It is, however, not known how CUZD1 brings about these biological processes at a molecular level. Structurally, CUZD1 is composed of two tandem CUB domains, a ZP domain and a putative transmembrane domain (74,75). Presently, little information exits describing the function of these domains, but they are often found in proteins that regulate developmental processes (76,77). Studies illustrating the presence of CUZD1 in particular tissues have provided minimal 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 (74). Additionally, expression of *Cuzd1* has been identified in the pancreas, epididymis, human ovarian cancer cells, and human embryonic stem cells (78–82). In terms of functional studies, Leong et al. 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

(82,83). 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 (82,84–90). Our recent work fills a large gap in the body of knowledge surrounding CUZD1 by detailing the molecular signaling pathway of CUZD1-induced proliferation in mammary epithelial cells and in breast tumorigenesis. This mechanistic insight into the role of CUZD1 in normal and neoplastic breast tissue is a significant contribution to the body of literature describing this molecule.

REFERENCES

1. Hennighausen L, Robinson GW. Information networks in the mammary gland . Nat Rev cell Biol. 2005 Sep;6(9):715–25.
2. Hennighausen L, Robinson GW. Signaling Pathways in Mammary Gland Development. Dev Cell. 2001;1(4):467–75.
3. Brisken C, Ataca D. Endocrine hormones and local signals during the development of the mouse mammary gland. Wiley Interdiscip Rev Dev Biol. 2015 May;4(3):181–95.
4. Humphreys RC, Krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S, et al. Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development. 1996 Dec;122:4013–22.
5. Inman JL, Robertson C, Mott JD, Bissell MJ. Mammary gland development: cell fate specification, stem cells and the microenvironment. Development. 2015 Mar 15;142(6):1028–42.
6. Brisken C, O'Malley B. Hormone Action in the Mammary Gland. Cold Spring Harb Perspect Biol. 2010 Dec 1;2(12):a003178–a003178.
7. Sternlicht MD. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis . Breast cancer Res. 2006;8(1):201.
8. Welboren WJ, Sweep FC, Span PN, Stunnenberg HG. Genomic actions of estrogen receptor alpha: what are the targets and how are they regulated? . Endocr Relat Cancer. 2009 Dec;16(4):1073–89.
9. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001 Feb 1;2(2):127–37.
10. Troyer KL, Lee DC. Regulation of mouse mammary gland development and tumorigenesis by the ERBB signaling network. J Mammary Gland Biol Neoplasia. 2001 Jan;6(1):7–21.
11. Schroeder JA, Lee DC. Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. Cell Growth Differ. 1998 Jun;2014:451–64.
12. Ciarloni L, Mallepell S, Brisken C. Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development . Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5455–60.
13. Aupperlee MD, Leipprandt JR, Bennett JM, Schwartz RC, Haslam SZ. Amphiregulin mediates progesterone-induced mammary ductal development during puberty. Breast Cancer Res. 2013 May 25;15(3):R44.
14. Forster N, Saladi SV, VanBragt M, Sfondouris ME, Jones FE, Li Z, et al. Basal Cell Signaling by p63 Controls Luminal Progenitor Function and Lactation via NRG1. Dev Cell. 2014 Jan;28(2):147–60.
15. Search G. ErbB Family Pathway Details (ErbB Family Pathway). Affymetrix eBioscience.
16. Stern DF. ErbBs in mammary development. Exp Cell Res. 2003 Mar 10;284(1):89–98.
17. Wiesen JF, Young P, Werb Z, Cunha GR. Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development . Development. 1999 Jan;126(2):335–44.

18. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling . EMBO J. 1997 Apr 1;16(7):1647–55.
19. Jackson-Fisher AJ, Bellinger G, Ramabhadran R, Morris JK, Lee K-F, Stern DF. ErbB2 is required for ductal morphogenesis of the mammary gland. Proc Natl Acad Sci U S A. 2004 Dec 7;101(49):17138–43.
20. Jones FE, Stern DF. Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation. Oncogene. 1999 Jun 15;18(23):3481–90.
21. Kim HH, Vijapurkar U, Hellyer NJ, Bravo D, Koland JG. Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein. Biochem J. 1998 Aug 15;334 (Pt 1:189–95.
22. Jackson-Fisher AJ, Bellinger G, Breindel JL, Tavassoli FA, Booth CJ, Duong JK, et al. ErbB3 is required for ductal morphogenesis in the mouse mammary gland. Breast Cancer Res. 2008 Dec 18;10(6):R96.
23. Tidcombe H, Jackson-Fisher A, Mathers K, Stern DF, Gassmann M, Golding JP. Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality . Proc Natl Acad Sci U S A. 2003 Jul 8;100(14):8281–6.
24. Wali VB, Gilmore-Hebert M, Mamillapalli R, Haskins JW, Kurppa KJ, Elenius K, et al. Overexpression of ERBB4 JM-a CYT-1 and CYT-2 isoforms in transgenic mice reveals isoform-specific roles in mammary gland development and carcinogenesis. Breast Cancer Res. 2014 Dec 17;16(6):501.
25. Mapes J, Li Q, Kannan A, Anandan L, Laws M, Lydon JP, et al. CUZD1 is a critical mediator of the JAK/STAT5 signaling pathway that controls mammary gland development during pregnancy. Hunter KW, editor. PLOS Genet. 2017 Mar 9;13(3):e1006654.
26. Ben-Jonathan N, Mershon JL, ALLEN DL, Steinmetz RW. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr Rev. 1996 Dec;17(6):639–69.
27. Nagy E, Berczi I. Hypophysectomized rats depend on residual prolactin for survival. Endocrinology. 1991 Jun;128(6):2776–84.
28. Ali S. Prolactin Receptor Regulates Stat5 Tyrosine Phosphorylation and Nuclear Translocation by Two Separate Pathways. J Biol Chem. 1998;273(13):7709–16.
29. Gao J, Hughes JP, Auperin B, Buteau H, Edery M, Zhuang H, et al. Interactions among Janus kinases and the prolactin (PRL) receptor in the regulation of a PRL response element . Mol Endocrinol. 1996 Jul;10(7):847–56.
30. DaSilva L, Howard OMZ, Rui H, Kirken RA, Farrar WL, Howard 0. M Zack, et al. Growth Signaling and JAK2 Association Mediated by Membrane-proximal Cytoplasmic Regions of Prolactin Receptors. J Biol Chem. 1994 Jul 15;2014:18267–70.
31. Vonderhaar BK. Hormonal interactions during mammary gland development. Breast Cancer Res. 2003 Oct 1;2014:34.
32. Wakao H, Gouilleux F, Groner B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J. 1994 Feb 15;2014(9):2182–91.
33. Hennighausen L, Robinson GW, Wagner KU, Liu X. Developing a mammary gland is a stat affair. J Mammary Gland Biol Neoplasia. 1997 Oct;2(4):365–72.
34. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses . Cell. 1998 May 29;93(5):841–50.
35. Burdon T, Sankaran L, Wall RJ, Spencer M, Hennighausen L. Expression of a whey acidic protein transgene during mammary development. Evidence for different mechanisms of regulation during pregnancy and lactation . J Biol Chem. 1991 Apr 15;266(11):6909–14.

36. Pittius CW, Hennighausen L, Lee E, Westphal H, Nicols E, Vitale J, et al. A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice . Proc Natl Acad Sci U S A. 1988 Aug;85(16):5874–8.
37. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, et al. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J. 1997 Dec 1;2014(23):6926–35.
38. Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, et al. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev. 1997 Jan 15;11(2):167–78.
39. Rodig SJ, Meraz MA, White JM, Lampe PA, Riley JK, Arthur CD, et al. Disruption of the Jak1 Gene Demonstrates Obligatory and Nonredundant Roles of the Jaks in Cytokine-Induced Biologic Responses. Cell. 1998;93(3):373–83.
40. Neubauer H, Cumano A, Müller M, Wu H, Huffstadt U, Pfeffer K. Jak2 Deficiency Defines an EssentialDevelopmental Checkpoint in DefinitiveHematopoiesis. Cell. 1998;93(3):397–409.
41. Wagner K-U-U, Krempler A, Triplett AA, Qi Y, George NM, Zhu J, et al. Impaired Alveologenesis and Maintenance of Secretory Mammary Epithelial Cells in Jak2 Conditional Knockout Mice. Mol Cell Biol. 2004 Jun 15;24(12):5510–20.
42. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. Proc Natl Acad Sci U S A. 1995 Sep 12;92(19):8831–5.
43. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L. Stat5a is mandatory for adult mammary gland development and lactogenesis . Genes Dev. 1997 Jan 15;11(2):179–86.
44. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: A population-based study from the California Cancer Registry. Cancer. 2007 May 1;109(9):1721–8.
45. King WJ, DeSombre ER, Jensen E V, Greene GL. Comparison of Immunocytochemical and Steroid-binding Assays for Estrogen Receptor in Human Breast Tumors. Cancer Res. 1985 Jan;45(1):293–304.
46. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level . Nat Rev cell Biol. 2006 Jul;7(7):505–16.
47. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer. 2005;5(5):341–54.
48. Atalay G, Cardoso F, Awada A, Piccart MJ. Novel therapeutic strategies targeting the epidermal growth factor receptor (EGFR) family and its downstream effectors in breast cancer. Ann Oncol Off J Eur Soc Med Oncol ESMO. 2003 Sep 1;2011(4/14/2011):1346–63.
49. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol. 1995 Jul;19(3):183–232.
50. Richard J, Sainsbury C, Needham G, Farndon J, Malcolm A, Harris A. Epidermal-Growth-Factor Receptor Status as Predictor of Early Recurrence of and Death From Breast Cancer. Lancet. 1987 Jun;329(8547):1398–402.
51. Sundvall M, Iljin K, Kilpinen S, Sara H, Kallioniemi OP, Elenius K. Role of ErbB4 in breast cancer. J Mammary Gland Biol Neoplasia. 2008 Jun 3;13(2):259–68.
52. Schroeder JA, Lee DC. Transgenie Mice Reveal Roles for TGFa and EGF Receptor in Mammary Gland Development and Neoplasia. J Mammary Gland Biol Neoplasia. 1997;2(2):119–29.
53. Arteaga CL. Epidermal Growth Factor Receptor Dependence in Human Tumors : More Than Just Expression ? Oncologist. 2002;7(suppl 4):31–9.
54. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005 Oct 20;353(16):1673–84.

55. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. N Engl J Med. 2001 Mar 15;344(11):783–92.
56. Burstein HJ. The Distinctive Nature of HER2-Positive Breast Cancers — NEJM. N Engl J Med. 2005 Oct 20;353(16):1652–4.
57. Wakeling AE, Nicholson RI, Gee JMW, Arteaga C, Osborne K, Howell A, et al. Prospects for combining hormonal and nonhormonal growth factor inhibition. In: Clinical Cancer Research. 2001. p. 4350s–4355s; discussion 4411s–4412s.
58. Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK, Osborne CK. Cross-Talk between Estrogen Receptor and Growth Factor Pathways as a Molecular Target for Overcoming Endocrine Resistance. Clin Cancer Res. 2004 Jan 1;10(1):331S–336.
59. Wennbo H, Gebre-Medhin M, Gritli-Linde A, Ohlsson C, Isaksson OG, Tornell J. Activation of the prolactin receptor but not the growth hormone receptor is important for induction of mammary tumors in transgenic mice . J Clin Invest. 1997 Dec 1;100(11):2744–51.
60. Sasso C V, Santiano FE, Lopez-Fontana CM, Pistone-Creydt V, Ezquer ME, Hapon MB, et al. Effects of Parity and Serum Prolactin Levels on the Incidence and Regression of DMBA-Induced Tumors in OFA hr/hr Rats . Biomed Res Int. 2014;2014:210424.
61. Welsch CW. Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins . Cancer Res. 1985 Aug;45(8):3415–43.
62. Welsch CW, Gribler C. Prophylaxis of spontaneously developing mammary carcinoma in C3H-HeJ female mice by suppression of prolactin . Cancer Res. 1973 Nov;33(11):2939–46.
63. Bonneterre J, Mauriac L, Weber B, Roche H, Fargeot P, Tubiana-Hulin M, et al. Tamoxifen plus bromocriptine versus tamoxifen plus placebo in advanced breast cancer: results of a double blind multicentre clinical trial . Eur J Cancer Clin Oncol. 1988 Dec;24(12):1851–3.
64. Shaw-Bruha CM, Pirrucello SJ, Shull JD. Expression of the prolactin gene in normal and neoplastic human breast tissues and human mammary cell lines: Promoter usage and alternative mRNA splicing. Breast Cancer Res Treat. 1997;44(3):243–53.
65. Harvey PW. Hypothesis Prolactin is tumorigenic to human breast: dispelling the myth that prolactin-induced mammary tumors are rodent-specific. J Appl Toxicol. 2011 Jan;32(1):1–9.
66. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and Its Receptor: Actions, Signal Transduction Pathways and Phenotypes Observed in PRL Receptor Knockout Mice: Endocrine Reviews: Vol 19, No 3. Endocr Rev. 1998 Jun;2014:225–68.
67. Merlo GR, Venesio T, Taverna D, Marte BM, Callahan R, Hynes NE. Growth suppression of normal mammary epithelial cells by wild-type p53 . Oncogene. 1994 Feb;9(2):443–53.
68. Yamauchi T, Yamauchi N, Ueki K, Sugiyama T, Waki H, Miki H, et al. Constitutive tyrosine phosphorylation of ErbB-2 via Jak2 by autocrine secretion of prolactin in human breast cancer. J Biol Chem. 2000 Oct 27;275(43):33937–44.
69. Gallego MI, Binart N, Robinson GW, Okagaki R, Coschigano KT, Perry J, et al. Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects . Dev Biol. 2001 Jan 1;229(1):163–75.
70. Vonderhaar BK. Prolactin involvement in breast cancer. Endocr Relat Cancer. 1999 Sep;2014:389–404.
71. Jones FE, Welte T, Fu XY, Stern DF. ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. J Cell Biol. 1999 Oct 4;147(1):77–87.
72. Krempler A, Qi Y, Triplett AA, Zhu J, Rui H, Wagner K-U. Generation of a conditional knockout allele for the Janus kinase 2 (Jak2) gene in mice. Genesis. 2004 Sep;2014:52–7.
73. Barrell D, Dimmer E, Huntley RP, Binns D, O'Donovan C, Apweiler R. The GOA database in 2009 - An integrated Gene Ontology Annotation resource. Nucleic Acids Res. 2009 Jan;37(SUPPL. 1):D396-403.

74. Chen D, Xu X, Zhu L-JJ, Angervo M, Li Q, Bagchi MK, et al. Cloning and Uterus/Oviduct-specific Expression of a Novel Estrogen-regulated Gene (ERG1). J Biol Chem. 1999 Nov 5;2014:32215–24.
75. Kasik JW. A cDNA cloned from pregnant mouse uterus exhibits temporo-spatial expression and predicts a novel protein. Biochem J. 1998 Mar 1;330:947–50.
76. Bork P, Beckmann G. The CUB domain. A widespread module in developmentally regulated proteins . J Mol Biol. 1993 May 20;231(2):539–45.
77. Jovine L, Darie CC, Litscher ES, Wassarman PM. Zona pellucida domain proteins . Annu Rev Biochem. 2005;74:83–114.
78. Yamazaki K, Adachi T, Sato K, Yanagisawa Y, Fukata H, Seki N, et al. Identification and characterization of novel and unknown mouse epididymis-specific genes by complementary DNA microarray technology. Biol Reprod. 2006 Sep 1;75(3):462–8.
79. Huynh H, Ng CY, Ong CK, Lim KB, Chan TW. Cloning and characterization of a novel pregnancy-induced growth inhibitor in mammary gland . Endocrinology. 2001 Aug;142(8):3607–15.
80. Imamura T, Asada M, Vogt SK, Rudnick DA, Lowe ME, Muglia LJ. Protection from Pancreatitis by the Zymogen Granule Membrane Protein Integral Membrane-associated Protein-1. J Biol Chem. 2002 Dec 27;277(52):50725–33.
81. Brink TC, Sudheer S, Janke D, Jagodzinska J, Jung M, Adjaye J. The Origins of Human Embryonic Stem Cells: A Biological Conundrum. Cells Tissues Organs. 2008;2012:9–22.
82. Leong CTC, Ng CY, Ong CK, Ng CP, Ma ZS, Nguyen TH, et al. Molecular cloning, characterization and isolation of novel spliced variants of the human ortholog of a rat estrogen-regulated membrane-associated protein, UO-44. Oncogene. 2004 Jul 22;23(33):5707–18.
83. Leong CTC, Ong CK, Tay SK, Huynh H. Silencing expression of UO-44 (CUZD1) using small interfering RNA sensitizes human ovarian cancer cells to cisplatin in vitro. Oncogene. 2007 Feb 8;26(6):870–80.
84. Hirte H, Leung F, Soosaipillai A, Kulasingam V, Diamandis EPP. CUB and zona pellucida-like domain-containing protein 1 (CUZD1): A novel serological biomarker for ovarian cancer. Clin Biochem. 2012 Aug 17;45(18):1543–6.
85. Pavlidis P, Komorowski L, Teegen B, Liaskos C, Koutsoumpas AL, Smyk DS, et al. Diagnostic and clinical significance of Crohn's disease-specific pancreatic anti-GP2 and anti-CUZD1 antibodies. Clin Chem Lab Med. 2016 Jan 1;54(2):249–56.
86. Michaels MA, Jendrek ST, Korf T, Nitzsche T, Teegen B, Komorowski L, et al. Pancreatic Autoantibodies Against CUZD1 and GP2 Are Associated with Distinct Clinical Phenotypes of Crohn's Disease. Inflamm Bowel Dis. 2015 Dec;21(12):2864–72.
87. Papp M, Sipeki N, Tornai T, Altorjay I, Norman GL, Shums Z, et al. Rediscovery of the anti-pancreatic antibodies and evaluation of their prognostic value in a prospective clinical cohort of Crohn's patients: The importance of specific target antigens [GP2 and CUZD1]. J Crohn's Colitis. 2015 Aug;9(8):659–68.
88. Liaskos C, Rigopoulou EI, Orfanidou T, Bogdanos DP, Papandreou CN. CUZD1 and anti-CUZD1 antibodies as markers of cancer and inflammatory bowel diseases. Vol. 2013, Clinical and Developmental Immunology. Hindawi Publishing Corporation; 2013. p. 968041.
89. Komorowski L, Teegen B, Probst C, Aulinger-Stöcker K, Sina C, Fellermann K, et al. Autoantibodies against exocrine pancreas in Crohn's disease are directed against two antigens: The glycoproteins CUZD1 and GP2. J Crohn's Colitis. 2013 Nov;7(10):780–90.
90. Prassas I, Brinc D, Farkona S, Leung F, Dimitromanolakis A, Chrystoja CC, et al. False biomarker discovery due to reactivity of a commercial ELISA for CUZD1 with cancer antigen CA125. Clin Chem. 2014;60(2):381–8.
91. Veltmaat J. Welcome to The Laboratory of Mammary Gland Development : Research.
92. Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng C-X, et al. Inactivation of Stat5 in Mouse Mammary Epithelium during Pregnancy Reveals Distinct Functions in Cell Proliferation, Survival, and Differentiation. Mol Cell Biol. 2004;2014(18):8037.

FIGURES

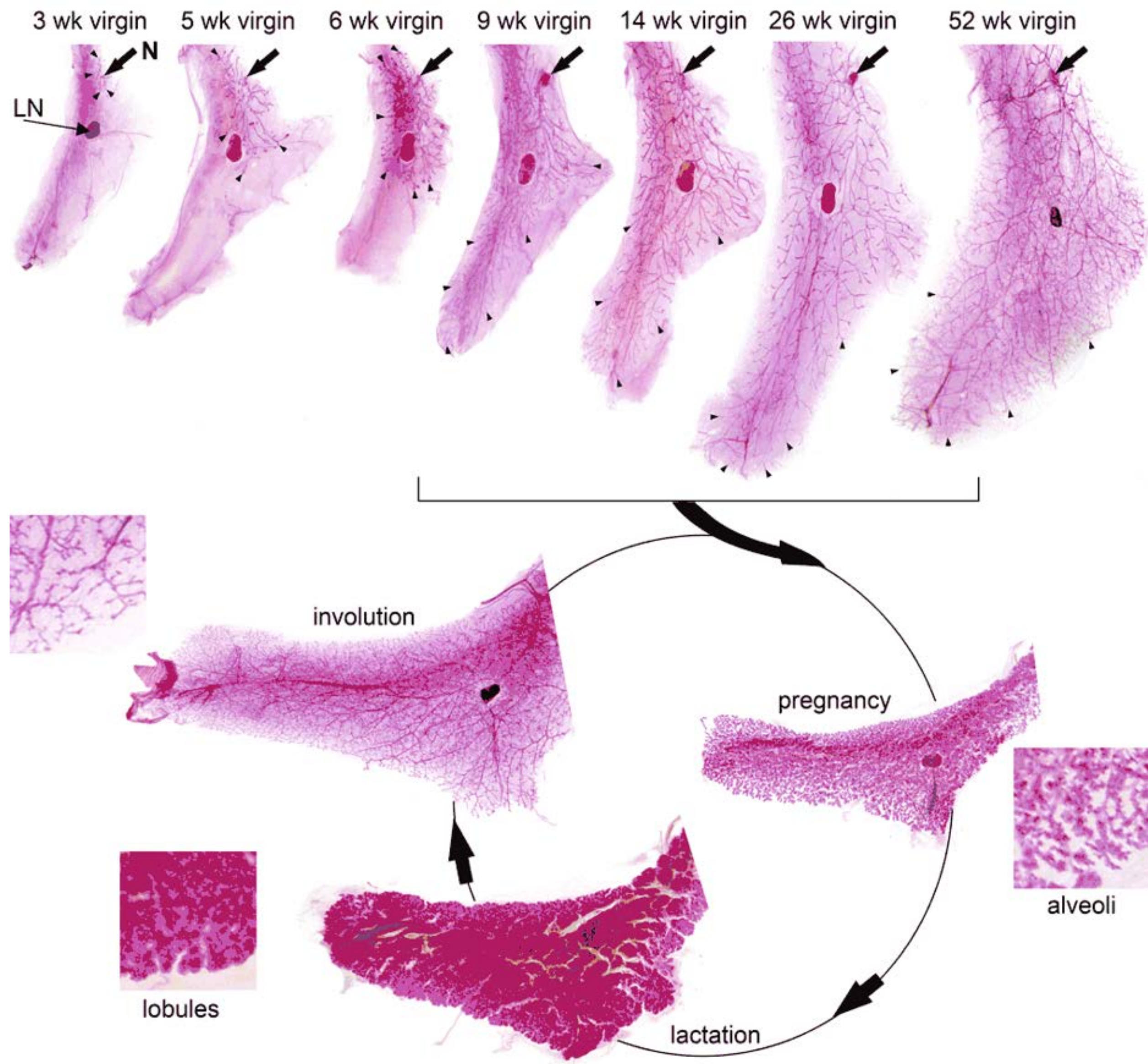

Figure 1.1. Progression and stages of mammary gland development. Changes in mammary gland morphology are shown via mammary gland whole mount staining. Original image © Copyright Jacqueline Veltmaat (91).

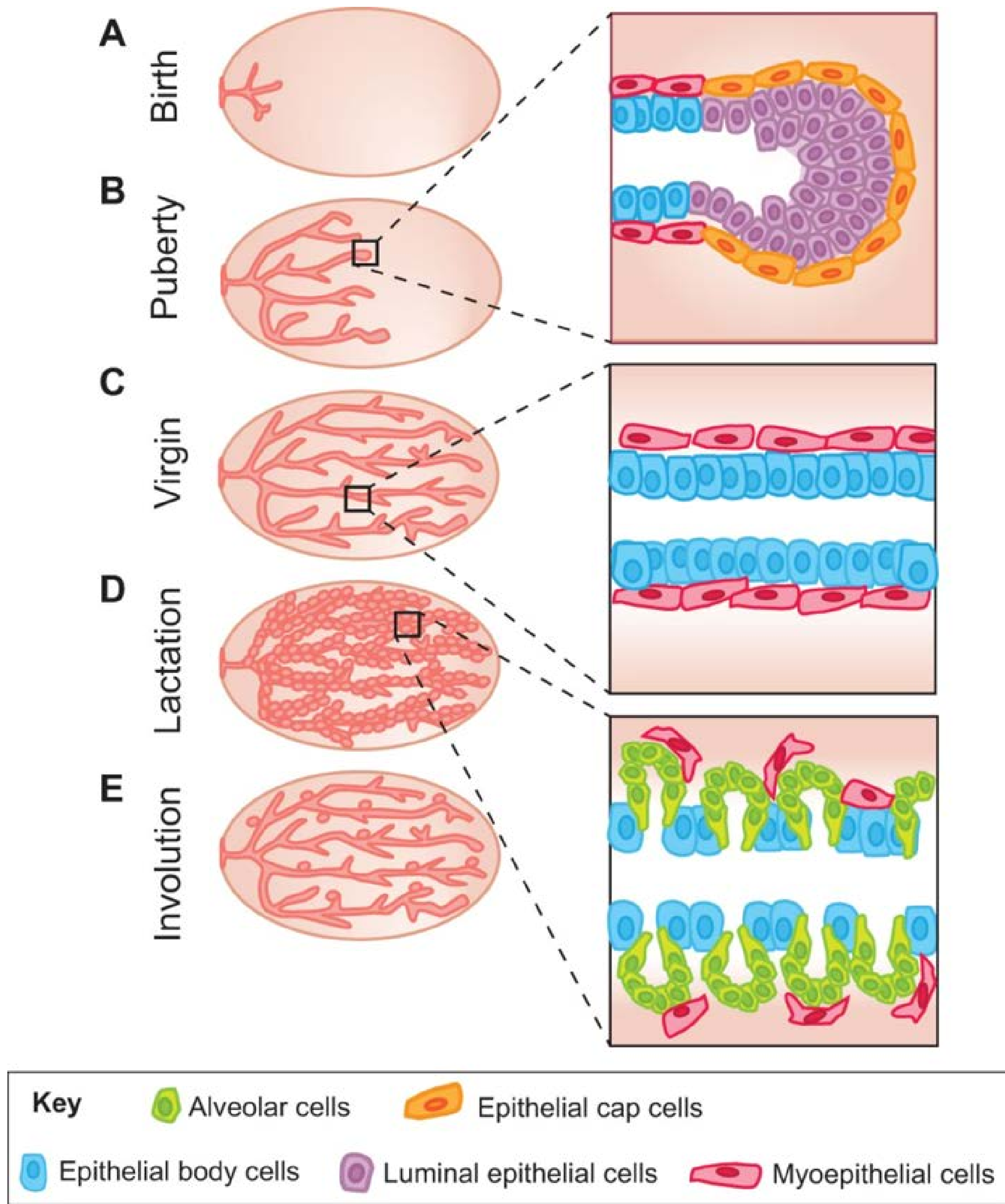

Figure 1.2. Organization of mammary epithelial cells during development. The ductal epithelium expands during puberty at sites of high proliferation called terminal end buds. Alveolar proliferation and differentiation occurs during lactation. Original image: © 2015. Published by The Company of Biologists Ltd (5).

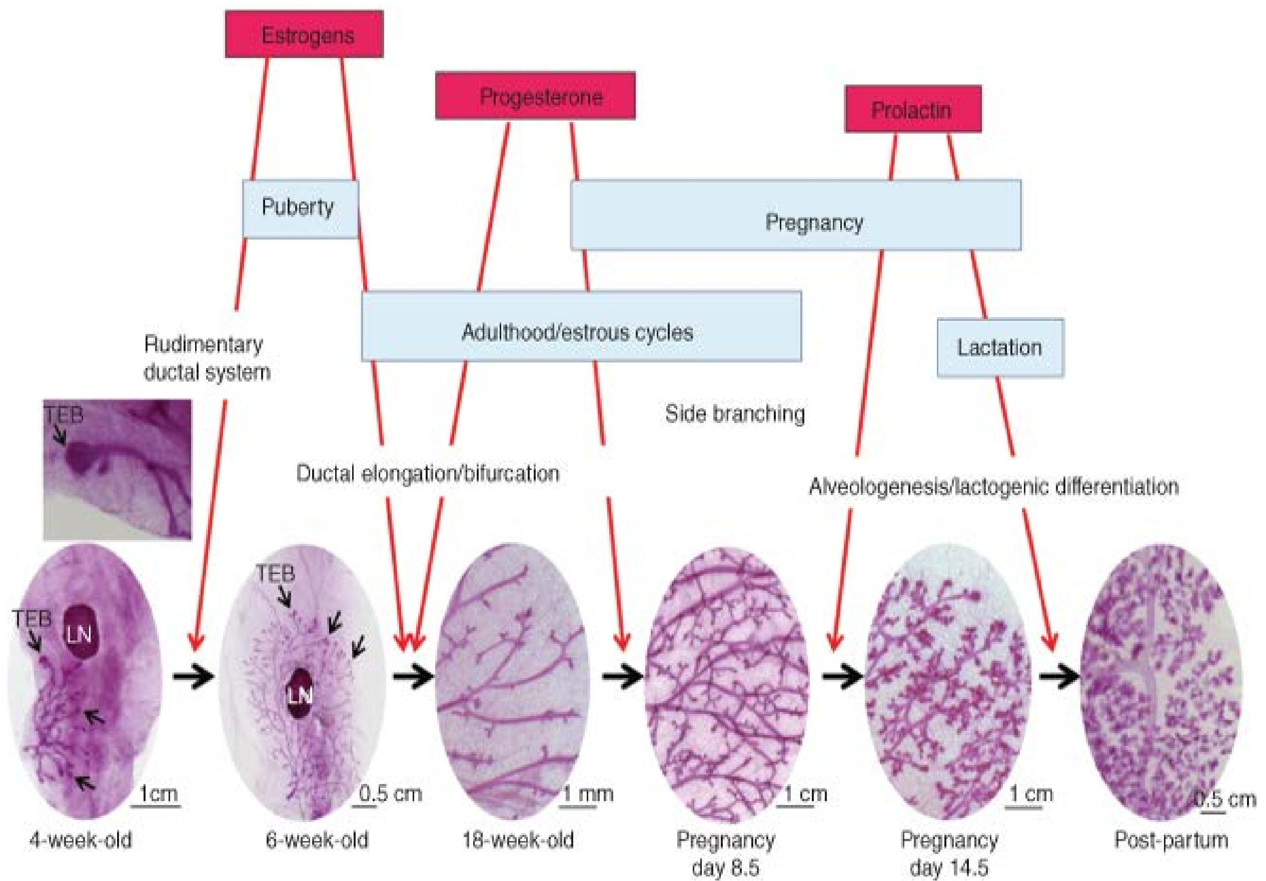

Figure 1.3. Hormonal control of mammary gland development. Elevation of specific circulating hormones control proliferation and differentiation of the mammary gland. Original image modified (3).

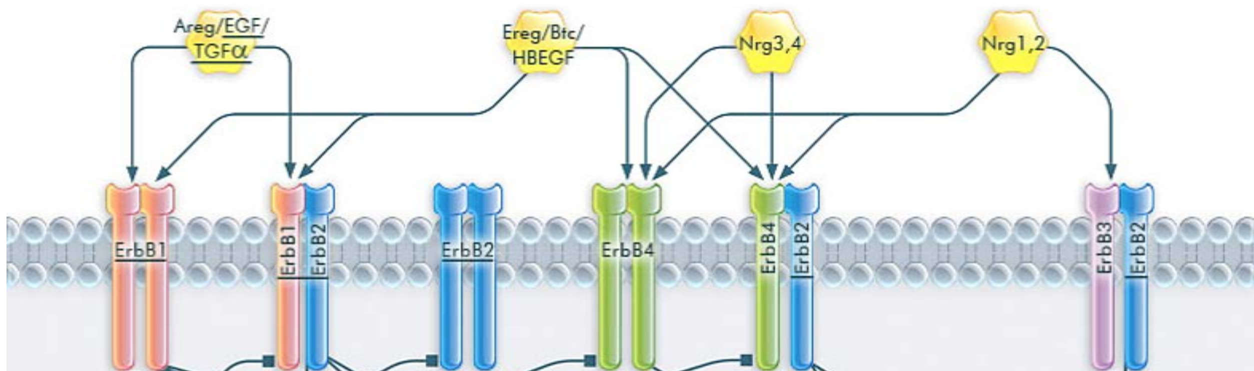

Figure 1.4. ErbB signaling. EGF family ligands bind to ErbB receptors. Original image modified (15).

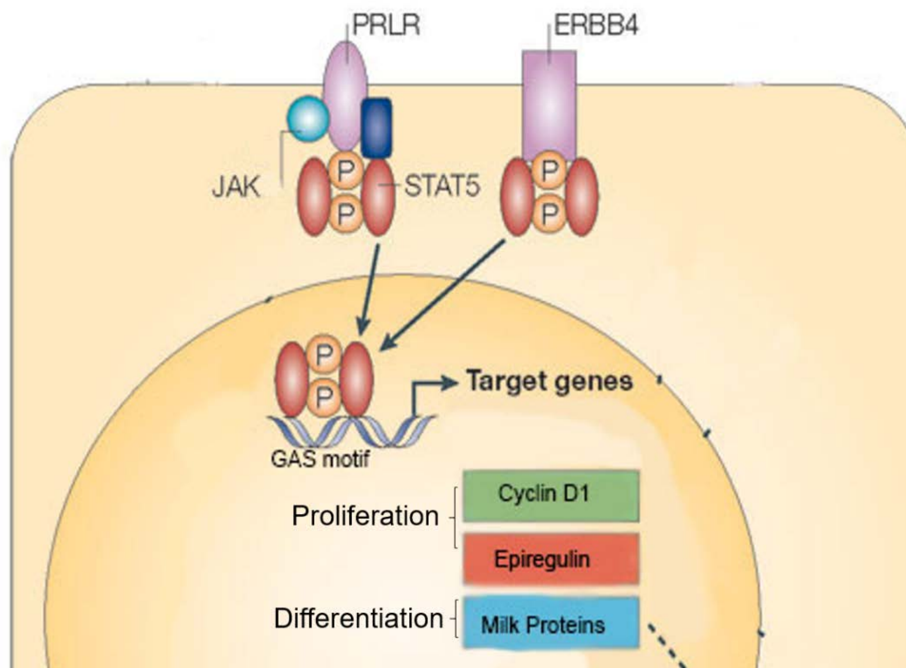

Figure 1.5. PRL signaling. PRL binds to PRLR and initiates phosphorylation of JAK1/2 and STAT5. Phosphorylated STAT5 translocates to the nucleus to bring about gene expression changes that induce proliferation or differentiation of mammary epithelial cells. ErbB4 can also phosphorylate STAT5 to induce nuclear translocation. Original image modified (1).

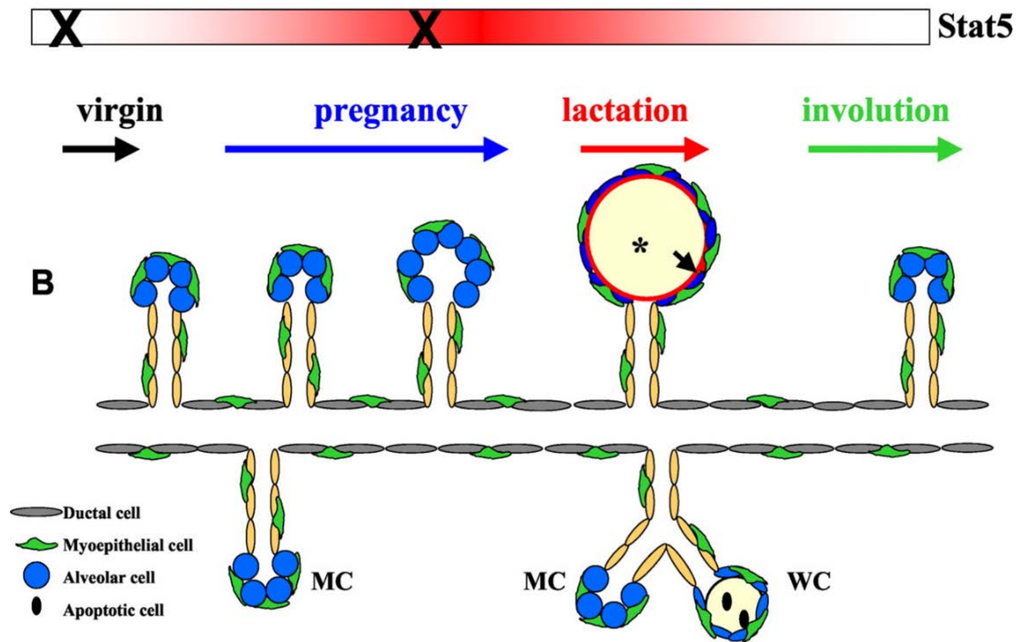

Figure 1.6. Role of STAT5 in mammary gland development. STAT5 plays an essential role in mammary gland development, primarily during pregnancy and lactation. Early loss of STAT5 results in a lack of mammary epithelial proliferation at the start of pregnancy, whereas loss of STAT5 during pregnancy arrests differentiation. Original image modified (92).

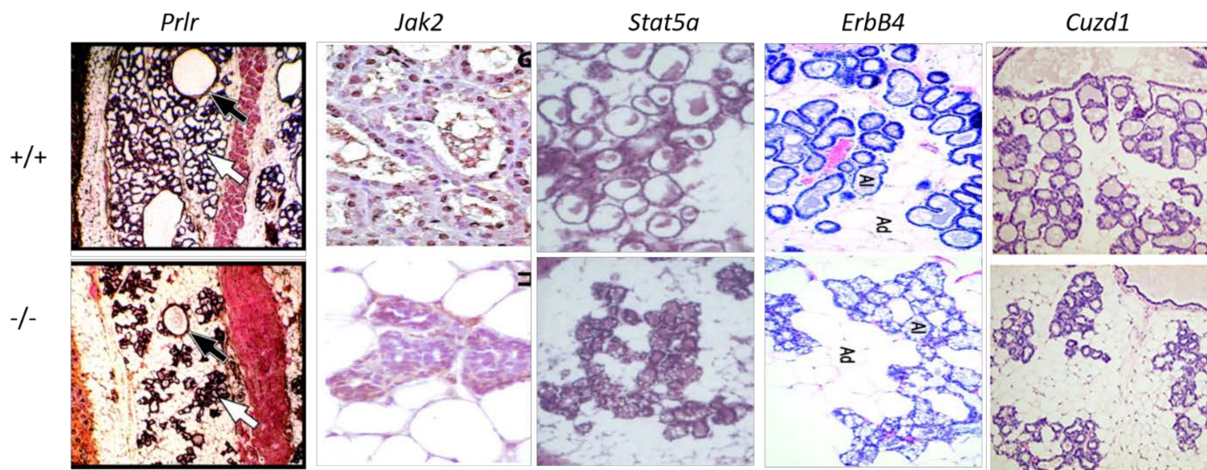

Figure 1.7. Comparison of mutant mammary glands. Mammary gland sections from *Prlr*$^{(-/-)}$, *Jak2*$^{(-/-)}$, *Stat5a*$^{(-/-)}$, *ErbB4*$^{(-/-)}$, and *Cuzd1*$^{(-/-)}$ are phenotypically similar. All mice are unable to undergo the terminal differentiation necessary for lactation. Original images modified (23,25,43,66,72).

CHAPTER 2

CUZD1 is a Critical Mediator of the JAK/STAT5 Signaling Pathway that Controls Mammary Gland Development during Pregnancy

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ABSTRACT

In the mammary gland, genetic circuits controlled by estrogen, progesterone, and prolactin act in concert with pathways regulated by members of the epidermal growth factor family to orchestrate growth and morphogenesis during puberty, pregnancy and lactation. However, the precise mechanisms underlying the crosstalk between the hormonal and growth factor pathways remain poorly understood. We have identified the CUB and zona pellucida-like domain-containing protein 1 (CUZD1), expressed in mammary ductal and alveolar epithelium, as a novel mediator of mammary gland proliferation and differentiation during pregnancy and lactation. *Cuzd1*-null mice exhibited a striking impairment in mammary ductal branching and alveolar development during pregnancy, resulting in a subsequent defect in lactation. Gene expression profiling of mammary epithelium revealed that CUZD1 regulates the expression of a subset of the EGF family growth factors, epiregulin, neuregulin-1, and epigen, which act in an autocrine fashion to activate ErbB1 and ErbB4 receptors. Proteomic studies further revealed that CUZD1 interacts with a complex containing JAK1/JAK2 and STAT5, downstream transducers of prolactin signaling in the mammary gland. In the absence of CUZD1, STAT5 phosphorylation in the mammary epithelium during alveologenesis was abolished. Conversely, elevated expression of *Cuzd1* in mammary epithelial cells stimulated prolactin-induced phosphorylation and nuclear translocation of STAT5. Chromatin immunoprecipitation confirmed co-occupancy of phosphorylated STAT5 and CUZD1 in the regulatory regions of epiregulin, a potential regulator of epithelial proliferation, and whey acidic protein, a marker of epithelial differentiation. Collectively, these findings suggest that CUZD1 plays a critical role in prolactin-induced JAK/STAT5 signaling that controls the expression of key STAT5 target genes involved in mammary epithelial proliferation and differentiation during alveolar development.

AUTHOR SUMMARY

In the mammary gland, genetic circuits controlled by the hormones, estrogen, progesterone, and prolactin, act in concert with pathways regulated by members of the epidermal growth factor family to orchestrate growth and morphogenesis during puberty, pregnancy and lactation. We have identified CUZD1 as a novel mediator of prolactin signaling in the steroid hormone-primed mouse mammary gland during pregnancy and lactation. *Cuzd1*-null mice exhibited a striking impairment in ductal branching and alveolar development during pregnancy, resulting in a subsequent defect in lactation. Administration of prolactin failed to induce proliferation of the mammary epithelium in *Cuzd1*-null mice. Protein binding studies revealed that CUZD1 interacts with downstream transducers of prolactin signaling, JAK1/JAK2 and STAT5. Additionally, elevated expression of *Cuzd1* in mammary epithelial cells stimulated

phosphorylation and nuclear translocation of STAT5. CUZD1, therefore, is a critical mediator of prolactin that controls mammary alveolar development.

INTRODUCTION

In the mammary gland, development of an extensive ductal network during puberty and formation of lobuloalveolar units during pregnancy are critical events required for lactation. These complex developmental processes are regulated by a variety of signaling cues, including the steroid hormones 17β-estradiol (E) and progesterone (P), the peptide hormone prolactin (PRL), and the epidermal growth factor (EGF) family of growth factors (1). During pregnancy and lactation, E, P, and EGF family members act in concert with PRL to induce alveologenesis, a process in which ductal epithelial cells undergo extensive proliferation and secretory differentiation (2,3).

Circulating levels of PRL rise during pregnancy and promote proliferation and differentiation of the mammary epithelium in preparation for lactation (4-7). The prolactin receptor (PRLR) is a trans-membrane protein belonging to the cytokine receptor superfamily (8). Binding of PRL to PRLR triggers signaling events through the JAK/STAT5 pathway (9,10). Janus tyrosine kinase 1 (JAK1) and janus tyrosine kinase 2 (JAK2), associated with PRLR, are rapidly phosphorylated upon PRL binding. Signal transducer and activator of transcription 5 (STAT5), which is phosphorylated following JAK activation, undergoes dimerization and localizes to the nucleus (9,11-13). The tyrosine phosphorylation of STAT5 is essential for DNA binding and transcriptional regulation (10). Activated STAT5 binds directly to the GAS motif (TTCnnnGAA) at target genes to regulate their transcription and promote proliferation and/or differentiation of the mammary epithelium during distinct phases of mammary gland development (11). It was reported that PRL signaling through JAK2/STAT5 activates cyclin D1 transcription and nuclear accumulation in proliferating mammary epithelial cells (14). Furthermore, STAT5a has been shown to regulate transcription of other mitogenic factors, such as the EGF family member epiregulin (15,16). Terminal differentiation of the mammary gland is defined by the expression of milk protein genes in preparation for lactation. STAT5 controls the expression of several of these genes, including whey acidic protein (*Wap*) and β-casein (*Csn2*), to induce functional differentiation of the alveolar epithelial cells (11,12,17-20). These observations established that STAT5 signaling is essential for proliferation and differentiation of the mammary gland.

Ample evidence exists to suggest integrated effects of PRL and EGF receptor (ErbB) mediated signaling pathways during mammary gland development. Binding of specific EGF ligands induces differential heterodimerization of ErbB family receptors to stimulate specific intracellular signaling pathways, thereby

accounting for the varied effects of an activated receptor. Upon EGF administration, STAT5 is activated to a similar degree as seen with PRL treatment (3,20). Furthermore, active ErbB4 was shown to induce phosphorylation of STAT5 in the mammary epithelium (21). *ErbB4*$^{(-/-)}$ mice exhibit disrupted alveologenesis and a dramatic reduction in the expression of *Wap* and further investigation revealed that STAT5 phosphorylation is lost (21). These findings pointed to a possible link between signaling via EGF family receptors and STAT5 activation to control alveolar proliferation and differentiation, although the precise molecular basis of this crosstalk remains unclear.

This study reports that CUZD1 is a novel mediator of PRL and EGF signaling in mammary epithelial proliferation and differentiation during pregnancy. This protein, also known as ERG1, Itmap1, or UO-44, was originally identified in our laboratory as an E-regulated gene in the rodent uterine epithelium and later reported in other tissues (22-25). CUZD1 contains a zona-pellucida (ZP)-like domain and two tandem CUB (Complement subcomponent /C1s, Uegf, Bmp1) motifs (Fig. 2.9A). There is presently little information concerning the functional significance of these motifs, although their presence is often noted in molecules involved in developmental processes (26,27). The mouse *Cuzd1* gene shares strong sequence identity with its human ortholog, indicating functional conservation across species (25). Using a *Cuzd1*$^{(-/-)}$ mouse model and a combination of *in vivo* and *in vitro* approaches, we investigated the molecular pathways that are controlled by *Cuzd1* in the mammary gland and uncovered a novel mechanism linking CUZD1 to the PRL and EGF family growth factor signaling pathways that guide epithelial proliferation and differentiation in the mammary gland during pregnancy.

RESULTS

CUZD1 controls alveolar morphogenesis during pregnancy and lactation

We examined the expression of CUZD1 in the mammary glands of *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice at different stages of development: pubertal (5 weeks), late pregnancy (D18) and early lactation (L2). Immunofluorescence (IF) analysis of CUZD1 revealed no detectable expression in mammary tissue of *Cuzd1*$^{(-/-)}$ mice during development (Fig. 2.1, b, d, and f). CUZD1 was detected in the developing ductal epithelium of *Cuzd1*$^{(+/-)}$ mice at puberty (Fig. 2.1, a). CUZD1 immunostaining was also observed in both cytoplasmic and nuclear compartments of the ductal and alveolar epithelial cells of *Cuzd1*$^{(+/-)}$ mammary glands during alveologenesis at late pregnancy (Fig. 2.1, c). Prominent nuclear staining was seen during lactation (Fig. 2.1, e), indicating that CUZD1 may play a critical role during mammary gland development, particularly during pregnancy and lactation.

To investigate the functional role of CUZD1 in mammary gland development, we created *Cuzd1*$^{(-/-)}$ mice in which this gene is deleted from the mouse germ line by homologous recombination using mouse embryonic stem cells (Fig. 2.9B). The efficiency of gene deletion was confirmed by PCR analysis of genomic DNA (Fig. 2.9C) and northern blot analysis of *Cuzd1* mRNA expression (Fig. 2.9D). The *Cuzd1*$^{(-/-)}$ females were fertile and delivered normal size litters. However, the majority of pups from *Cuzd1*$^{(-/-)}$ dams died within 72 h of parturition and it was observed that they had insufficient milk in their stomachs. Almost all pups survived and grew normally when they were transferred to a foster dam immediately after birth. These results indicated that the *Cuzd1*$^{(-/-)}$ dams fail to produce an adequate amount of milk.

To further examine the phenotypic defects in the *Cuzd1*$^{(-/-)}$ mice, morphological analyses of whole mounts of mammary glands were performed at different stages of development. In comparison to their *Cuzd1*$^{(+/-)}$ littermates, the expansion of the epithelial tree in *Cuzd1*$^{(-/-)}$ mice was delayed at puberty (6-weeks old) (Fig. 2.2A, a and b). However, smooth muscle actin (SMA) and E-cadherin staining of *Cuzd1*$^{(-/-)}$ mammary glands at puberty indicate that there are no structural abnormalities in the cap or body cells of the terminal end buds (Fig. 2.10A, a-d) (28-29). The extent of ductal branching was modestly reduced in adult mutant females at estrous stage (10-weeks old) (Fig. 2.2A, c and d). During early pregnancy, mammary glands of mutant mice exhibited a severe deficiency in tertiary branching (Fig. 2.2A, e and f) and impaired alveolar development during late pregnancy (Fig. 2.2A, g and h) and lactation (Fig. 2.2A, i and j). Histological analysis of lactating *Cuzd1*$^{(-/-)}$ mammary glands revealed sparsely distributed alveolar units with disrupted epithelial structure in comparison to their *Cuzd1*$^{(+/-)}$ littermates (Fig. 2.2B, a-d). Collectively, these results indicated that the impairment in alveolar differentiation in *Cuzd1*$^{(-/-)}$ females during pregnancy and lactation leads to the deficiency in milk production.

Loss of *Cuzd1* impairs the ErbB signaling pathway in the mammary epithelium

The impaired alveolar development in *Cuzd1*$^{(-/-)}$ mammary glands raised the possibility that CUZD1 is involved in the control of epithelial cell proliferation. To test this possibility, we monitored the mammary epithelial proliferation in *Cuzd1*$^{(-/-)}$ mice and *Cuzd1*$^{(+/-)}$ littermates during puberty and lactation. We employed IHC analysis using an antibody against Ki67, a widely-used marker for cellular proliferation. As expected, extensive cell proliferation was observed in the mammary ductal epithelia of non-pregnant pubertal *Cuzd1*$^{(+/-)}$ mice (Fig. 2.3A, a). There was a significant reduction in the number of proliferating ductal epithelial cells in the mammary glands of pubertal *Cuzd1*$^{(-/-)}$ mice (Fig. 2.3A, b). The difference in Ki67 positive cells at puberty is quantified in Fig. 2.3A, c. When epithelial proliferation was assessed in the lactating mammary gland, we again observed a dramatic decline in epithelial proliferation in *Cuzd1*$^{(-/-)}$

mice (Fig. 2.3A, d and e). During puberty and pregnancy, *Cuzd1*$^{(-/-)}$ females maintained normal serum levels of E, P and PRL (Fig. 2.10B), indicating that tissue intrinsic factors rather than systemic hormonal disruptions caused by the loss of *Cuzd1* are responsible for this defect in mammary gland proliferation. These results demonstrated that *Cuzd1* plays a critical role in regulating side-branching and alveolar morphogenesis in female mice during pregnancy and lactation, in part by influencing pathways involved in mammary epithelial proliferation.

To identify the pathways downstream of CUZD1, a microarray analysis was performed to compare the gene expression profiles of mammary epithelial cells isolated from *Cuzd1*$^{(-/-)}$ mice and their *Cuzd1*$^{(+/-)}$ littermates on day 18 of pregnancy. This microarray identified 411 transcripts that were altered (>2-fold) in the *Cuzd1*$^{(-/-)}$ epithelium compared to the *Cuzd1*$^{(+/-)}$ epithelium (GEO Accession GSE30939). Prominent among the 377 down-regulated transcripts were the mRNAs encoding three members of the EGF family, neuregulin-1 (*Nrg1*), epiregulin (*Ereg*) and epigen (*Epgn*). Interestingly, no significant alteration was detected in the expression levels of transcripts of several other EGF-family growth factors, such as amphiregulin (*Areg*), epidermal growth factor (*Egf*), heparin binding epidermal growth factor (*Hbegf*), neurgulin-2 (*Nrg2*), neuregulin-3 (*Nrg3*) and neuregulin-4 (*Nrg4*). Gene expression changes of EGF family ligands were confirmed using real-time RT-PCR and analyzed for statistical significance (Fig. 2.3B). Furthermore, IHC analysis of EPGN and NRG1 at lactation day 2 showed a substantial decline in these EGF ligands in *Cuzd1*$^{(-/-)}$ mice (Fig. 2.3C, b and d). These data indicate that the deletion of *Cuzd1* results in reduced expression of a specific subset of EGF family ligands in the mammary epithelium during late pregnancy.

Binding of EGF ligands to ErbB receptors results in their activation via auto-phosphorylation of critical tyrosine residues, which subsequently serve as docking sites for downstream signaling molecules (30). While EREG binds to both ErbB1 and ErbB4, EPGN acts primarily via ErbB1. NRG1 binds to ErbB3 as well as ErbB4. We therefore, examined whether the observed alterations in the expression levels of *Nrg1*, *Ereg* and *Epgn* in the mammary tissue affected the ErbB receptor-mediated signaling. Mammary gland sections obtained from mice during late pregnancy were subjected to IHC, using antibodies directed against specific phosphorylated tyrosine residues critical for activation of ErbB1 (Tyr 1068), ErbB2 (Tyr 877), and ErbB4 (Tyr 1056). Abundant activating phosphorylation of ErbB1, ErbB2, and ErbB4 was observed in mammary epithelia of *Cuzd1*$^{(+/-)}$ mice, consistent with the proliferative activity seen in this tissue (Fig. 2.3D, a, c, and e). In contrast, pErbB1 and pErbB4 were markedly reduced in the *Cuzd1*$^{(-/-)}$ epithelium (Fig. 2.3D, b and d). Interestingly, phosphorylation of ErbB2 was not affected in the *Cuzd1*$^{(-/-)}$ epithelium (Fig. 2.3D, f). No alteration was observed in the total protein levels of ErbB1, ErbB2, and ErbB4 in mammary epithelia of these mice (Fig. 2.3D, insets). Collectively, these results indicated that CUZD1 is necessary f produce

the EGF family ligands, NRG1, EREG, and EPGN, which then function through ErbB receptor-mediated signaling pathways to control epithelial proliferation in the mammary gland during alveolar development.

***Cuzd1* controls the proliferation of mammary epithelial cells by modulating the ErbB signaling pathway**

We used HC11 cells, a non-transformed mammary epithelial cell line derived from pregnant mice, to examine the cell autonomous role of *Cuzd1* (31). A lentiviral expression vector harboring a full-length cDNA encoding *Cuzd1* or *LacZ* (control) was integrated into HC11 cells to generate stable cell lines which express constitutively elevated levels of *Cuzd1* (HC11-Cuzd1) or *β-galactosidase* (HC11-LacZ) (Fig. 2.11, a and b). When HC11-Cuzd1 cells were subjected to a BrdU incorporation assay, they exhibited significantly higher rates of proliferation compared to control HC11-LacZ cells (Fig. 2.4A). These data provided evidence that *Cuzd1*-dependent mechanisms indeed promote proliferation of mammary epithelial cells.

We next investigated whether CUZD1 controls the proliferation of HC11 cells by regulating the expression of the EGF growth factors. First, we examined the effects of *Cuzd1* overexpression on the expression of the EGF family members. Significantly higher levels of *Epgn, Ereg* and *Nrg1* transcripts were detected in HC11-Cuzd1 cells as compared to the HC11-LacZ cells (Fig. 2.4B). Conversely, siRNA-mediated attenuation of *Cuzd1* mRNA expression in HC11 cells led to a marked reduction in the levels of *Nrg1*, *Ereg,* and *Epgn* mRNAs without significantly altering the levels of mRNAs encoding other EGF family ligands (Fig. 2.4C). To determine which ErbB receptors play a role in CUZD1-induced cell proliferation, we performed a knock down of ErbB receptors 1-4 in HC11-Cuzd1 cells using gene-specific siRNAs (Fig. 2.12). Knock down of ErbB1, ErbB3 and ErBb4 resulted in a decrease in HC11-Cuzd1 cell proliferation as measured by a BrdU incorporation assay (Fig. 2.4D). We next wanted to determine if the loss of *Cuzd1*, and therefore the loss of specific EGF family ligands, led to a reduction in mammary epithelial cell proliferation. Using siRNA, we knocked down *Cuzd1* in HC11 mammary epithelial cells and supplemented with EPGN or NRG1 ligands. EPGN and NRG1 were both able to partially rescue proliferation of HC11 cells as compared to the ligand treated control (Fig. 2.4E). Altogether, these data strongly supported the concept that CUZD1 controls the production of specific EGF family growth factors, which act via ErbB1, ErbB3 and ErbB4 to induce mammary epithelial cell proliferation.

CUZD1-mediated STAT5 signaling is necessary for differentiation of the mammary gland

To further elucidate the molecular mechanism of CUZD1, we attempted to identify the cellular factors that interact with it. To achieve this goal, we created HC11 cells stably over-expressing recombinant FLAG epitope-tagged CUZD1 (HC11-3xFLAG-Cuzd1 cells). Soluble extracts of these cells were subjected to co-immunoprecipitation using a FLAG antibody. The immunoprecipitated proteins were recovered and submitted for mass spectrometry. The LC/MS identified peptide fragments corresponding to multiple potential interaction partners of CUZD1, including JAK1 and JAK2, protein arginine methyltransferase 5 and phosphoribosyl pyrophosphate synthetase 1.

Since JAK1/2 signaling and subsequent STAT5 phosphorylation is critical for mammary gland development, we focused on the interactions between JAK1, JAK2, and CUZD1. Co-immunoprecipitation of JAK1 and JAK2 from HC11 cell lysates was confirmed using an IP for endogenous CUZD1 and Western blot analysis (Fig. 2.5A). Interestingly, we also detected a signal for phosphorylated STAT5 in the HC11 cell immunoprecipitates (Fig. 2.5A). The presence of this complex of proteins was also confirmed using Western blot in HC11-3xFLAG-Cuzd1 cells (Fig. 2.13A). Although STAT5 was not identified as an interacting partner of CUZD1 in our proteomic analysis of the immunoprecipitate, it is conceivable that CUZD1 interacts directly with JAK1/JAK2, which exist in a larger cytosolic complex with STAT5.

In response to signaling by hormones, such as prolactin, activation of JAK1/2 leads to activation of the transcription factors STAT5a and STAT5b, which control mammary epithelial cell proliferation and differentiation during alveologenesis (7,32). Though both STAT5a and STAT5b are present in the mammary gland, STAT5a is the dominant form phosphorylated and localized to the nucleus during pregnancy and lactation (18,33). We examined the status of the activating STAT5 phosphorylation (Tyr 694) in the mammary glands of *Cuzd1*$^{(-/-)}$ mice at day 18 of pregnancy by IHC analysis. Total STAT5 protein levels were unchanged in *Cuzd1*$^{(-/-)}$ mice compared to *Cuzd1*$^{(+/-)}$ (Fig. 2.5B a and b). However, we observed a striking loss of STAT5 phosphorylation in the mammary epithelia of these mice, whereas abundant pSTAT5 was present in the mammary epithelium of *Cuzd1*$^{(+/-)}$ littermates (Fig. 2.5B, c and d, e and f). STAT5 is known to directly regulate the expression of *Wap* and *Csn2*, two milk proteins secreted by differentiated epithelial cells (8,19). We postulated that the loss of STAT5 phosphorylation impairs STAT5-dependent gene expression, leading to the observed deficiency in milk production in *Cuzd1*$^{(-/-)}$ females. To test this notion, we analyzed the gene expression levels of *Wap* and *Csn2*. The levels of *Wap* and *Csn2* transcripts were indeed markedly reduced in the mammary glands of *Cuzd1*$^{(-/-)}$ females during

lactation (Fig. 2.5C). These results formed the basis of our hypothesis that CUZD1-mediated signaling through JAK/STAT5 controls mammary epithelial cell differentiation.

To further understand the functional significance of the interaction of CUZD1 with the JAK/STAT5 pathway, we examined phosphorylation and localization of STAT5 and expression of direct transcriptional targets of pSTAT5 in HC11-Cuzd1 cells in response to PRL treatment. In this experiment, the HC11-Cuzd1 cells were treated with vehicle or PRL and STAT5 phosphorylation/localization was analyzed using immunocytochemistry. We observed that pSTAT5 immunostaining was dramatically enhanced in HC11-Cuzd1 cells (Fig. 2.5D, c and d) relative to HC11-LacZ (Fig. 2.5D, a and b) cells upon PRL treatment and, as expected, it was localized predominantly in the nucleus. PRL treatment of HC11-Cuzd1 cells did not result in a marked alteration in total STAT5 levels (Fig. 2.5D, e and f). The enhanced STAT5 phosphorylation observed in HC11-Cuzd1 cells as compared to HC11-LacZ cells was also confirmed via Western blotting (Fig. 2.13B). These data are consistent with the concept that CUZD1 promotes PRL signaling by enhancing STAT5 phosphorylation and activation.

Loss of *Cuzd1* impairs prolactin-induced lobuloalveologenesis

To investigate the role of CUZD1 in the PRL signaling pathway *in vivo*, virgin, pubertal *Cuzd1*$^{(-/-)}$ and *Cuzd1*$^{(+/+)}$ (wild type) mice were treated with E, P and PRL for 3 consecutive days to stimulate proliferation and differentiation of the mammary epithelium. To examine the gross morphological changes in the mammary epithelium following this hormonal treatment, whole mounts of mammary glands were performed. Compared to the vehicle control, *Cuzd1*$^{(+/+)}$ mice treated with E, P and PRL exhibited initiation of alveolar development (Fig. 2.6A, a and c). Conversely, *Cuzd1*$^{(-/-)}$ mice displayed a markedly reduced response to E, P and PRL treatment compared to vehicle control (Fig. 2.6A, b and d). Interestingly, we observed an elevated CUZD1 expression and nuclear localization in the mammary epithelium of *Cuzd1*$^{(+/+)}$ mice treated with E, P and PRL compared to vehicle-treated controls (Fig. 2.6B, a and c). As expected, this induction was absent in *Cuzd1*$^{(-/-)}$ mice (Fig. 2.6B, b and d).

We also observed a robust phosphorylation of STAT5 and its nuclear localization in mammary epithelia of mice treated with E, P and PRL, which was absent in *Cuzd1*$^{(-/-)}$ mice (Fig. 2.6C, a-d). Consistent with data obtained in cell lines, the expression of EREG, a direct target of STAT5, which is induced in wild-type mice upon treatment with E, P, and PRL, was absent in *Cuzd1*$^{(-/-)}$ mice (Fig. 2.6D, a and b). Overall, these data support the concept that CUZD1 is necessary for transduction of PRL signaling through the JAK/STAT pathway to induce mammary epithelial gene expression during hormone-induced alveologenesis.

CUZD1 and STAT5 co-occupy regulatory regions of target genes

CUZD1 has no nuclear localization sequence or DNA binding domain, but we observed that it was translocated to the nucleus upon stimulation with serum. We hypothesized that CUZD1 could be moving into the nucleus in association with pSTAT5. To investigate this possibility, HC11-3xFLAG-Cuzd1 cells were treated with a PRL/FBS/EGF cocktail, FBS, or a vehicle control to induce nuclear translocation of pSTAT5. Dual immunostaining was then performed to examine the cellular locations of pSTAT5 and CUZD1. In cells treated with the vehicle control, pSTAT5 and CUZD1 remained largely cytoplasmic (Fig. 2.7A, a, d, and g). Upon stimulation with FBS or PRL/FBS/EGF, pSTAT5 and CUZD1 were colocalized in the nucleus (Fig. 2.7A, b-c, e-f, and h-i). Previous studies reported that STAT5 binds directly to regulatory regions of *Ereg* and *Wap* genes to regulate their transcription (15,16). We observed that the expression of *Ereg* and *Wap* genes were up-regulated upon treatment with PRL, and their expression was further elevated in *Cuzd1*-overexpressing HC11 cells (Fig. 2.7B). Based on the protein structure of CUZD1 (Fig. 2.9A), there is no indication that CUZD1 binds to DNA, but we wanted to determine if CUZD1 and STAT5 remained in a complex when STAT5 is bound to DNA. To investigate this, we performed a ChIP re-ChIP using a STAT5-specific antibody followed by precipitation with anti-FLAG (M2) resin. Enrichment of regulatory elements in specific GAS sequences of *Ereg, Wap,* and *Csn2* indicated that CUZD1 remains bound to STAT5 in the nucleus when STAT5 is acting as a transcription factor (Fig. 2.7C). In single ChIP experiments, we confirmed STAT5 binding at in the *Ereg, Wap,* and *Csn2* GAS sequences (Fig. 2.14) as well as enrichment of these regulatory elements when we immunoprecipitated the FLAG-CUZD1 fusion protein (Fig. 2.14). The authenticity of this result was confirmed by the absence of enrichment of the *Wap, Csn2* and *Ereg* GAS sites when a FLAG ChIP was performed in HC11-Cuzd1 cells in which CUZD1 is not flag-tagged (Fig. 2.14). Collectively, our results indicated that, upon PRL-induced activation, the CUZD1-STAT5 complex translocates to the nucleus and interacts with target genes to bring about changes in gene expression that critically promote mammary epithelial cell proliferation and differentiation (Fig. 2.8).

DISCUSSION

E, P and PRL act in concert with the EGF family growth factors to govern mammary gland development during pregnancy and lactation (3,13). In this study, we provide evidence that CUZD1 is a novel regulator of STAT5 signaling in the steroid-primed mammary epithelium. Loss of *Cuzd1* expression in mammary epithelial cells prevented *in vivo* phosphorylation of STAT5, resulting in a severe impairment in mammary

epithelial proliferation and differentiation, which disrupts alveologenesis and prevents milk production during lactation.

A molecular link between CUZD1 and STAT5 phosphorylation has emerged from our study. Immunoprecipitation of CUZD1 from mammary epithelial cells followed by mass spectrometric and Western blot analyses revealed that CUZD1 is physically associated with several proteins, including JAK1/JAK2 and STAT5. Importantly, increased CUZD1 expression augmented PRL-induced phosphorylation as well as nuclear translocation of STAT5. The precise nature of CUZD1's association with the JAK1/JAK2/STAT5 complex and the mechanism by which it promotes STAT5 phosphorylation are presently unclear. It is conceivable that CUZD1 potentiates JAK/STAT signaling downstream of PRLR activation by acting as an adaptor protein that aids in the recruitment of STAT5 to the PRLR/JAK complex. It may also act in an accessory role in stabilizing/enhancing phosphorylation of STAT5 by JAKs. Precedence for this hypothesis is based on literature describing the roles of effector proteins that alter signaling through this complex [34]. For example, c-Src has been shown to propagate PRL initiated JAK/STAT signaling in normal mammary tissue (35). Additionally, caveolin-1 (Cav-1) has been shown to inhibit the STAT5 signaling pathway by competitively binding to the tyrosine kinase domain of JAK2, preventing interaction and subsequent activation of STAT5 (36).

Female mice lacking *Prlr*, *Jak2*, and *Stat5* are characterized by severe defects in mammary lobuloalveologenesis during pregnancy and lack of milk production during lactation (14,20,33,37-39). The *Cuzd1*$^{(-/-)}$ mice phenocopy the mammary defects observed in these mice during pregnancy and lactation, lending further support to the concept that CUZD1 is functionally linked to the components of the PRLR/JAK/STAT5 pathway during lobuloalveolar development and lactation.

The CUZD1 protein is localized in both cytoplasmic and nuclear compartments of mammary epithelial cells. When the mammary epithelial cells are grown in the absence of serum, CUZD1 is predominantly localized in the cytoplasm. Stimulation of these cells with media containing serum triggers nuclear translocation of CUZD1. This result is also recapitulated by adding a combination of PRL, EGF and serum to these cells. Since CUZD1 lacks a nuclear localization motif or a DNA binding domain, we predicted that its translocation to the nucleus is dependent on association with a transcription factor. Indeed, our results are consistent with the view that CUZD1 translocates to the nucleus in association with pSTAT5. We further demonstrated that CUZD1 is recruited along with pSTAT5 to the regulatory regions of key target genes, such as *Ereg* and *Wap*. It is plausible that EREG contributes to CUZD1-mediated epithelial proliferation

and alveolar expansion during pregnancy and lactation, as *Ereg* is a direct transcriptional target of STAT5 and has been implicated in promoting growth and survival of breast cancer cells [40-42].

Our study showed that CUZD1 controls the production of a subset of EGF family growth factors, EREG, NRG1, and EPGN, in mammary epithelium during pregnancy. Mice lacking *Nrg1* display pronounced defects in mammary alveologenesis with condensed alveoli and impaired alveolar outgrowth during pregnancy (43,44). Development of mutant mouse models showed that ErbB1, ErbB2, and ErbB3 play important roles in mammary ductal growth and fat pad penetration (45-48). *ErbB4*$^{(-/-)}$ mammary glands exhibited severe defects in alveolar proliferation and differentiation during pregnancy and lactation (21). *Cuzd1*$^{(-/-)}$ mammary glands showed impaired activation of ErbB1 and ErbB4 during pregnancy and lactation. These results are in accord with the hypothesis that the CUZD1-regulated growth factors, NRG1, EREG and EPGN, act primarily through ErbB1 and ErbB4 to exert their effects mainly during alveolar development. Consistent with this concept, there is a remarkable similarity between the mammary gland phenotypes of *ErbB4*$^{(-/-)}$ and *Cuzd1*$^{(-/-)}$ females.

In summary, our findings support a model in which CUZD1 is a downstream mediator of PRL that enhances the signaling pathway through STAT5 during proliferation and differentiation of the mammary epithelium (Fig. 2.8). CUZD1 impacts mammary epithelial proliferation and differentiation during pregnancy and lactation. It promotes production of a specific subset of the EGF-like ligands, NRG1, EREG and EPGN, which control alveolar development. These growth factors primarily function through ErbB1 and ErbB4 to regulate the proliferation and differentiation of mammary epithelial cells. Further analysis of the molecular mechanisms by which CUZD1 integrates the pathways regulated by STAT5 and the EGF family growth factors will improve our understanding of the molecular networks that underlie PRL regulation of normal mammary gland development.

MATERIALS AND METHODS

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 (IACUC). The IACUC approval number for this protocol is 16026. This approval is valid until August 22, 2019.

Gene targeting

To generate the vector for homologous recombination, about 16-kb mouse genomic DNA containing eight exons of mouse *Cuzd1* was sequenced and intron-exon boundaries were analyzed. A 4.0-kb *BamH I-Kpn I* fragment containing the 1st and 2nd exons and a 2.0-kb BamH I-EcoR I fragment containing part of 6th exon was cloned into Scrambler A and B site of pKO Scrambler NTKV-1901 targeting vector, respectively. Correct targeting resulted in deletion of gene sequence containing exons III-VI spanning the first and second CUB domains of CUZD1 protein and replaced with a neomycin resistance gene (NEO) (Fig. 2.9B). The construct was linearized and electroporated into embryonic stem (ES) cells. ES clones were selected by G418 and screened by Southern blot analysis employing a 335-bp 5'-end probe, a 390-bp 3'-end probe and a 450-bp internal probe respectively. The ES clone with appropriate homologous recombination was selected for blastocyst injection and chimaeras were generated with heterozygous ES cell lines. Heterozygous male mice were backcrossed to wildtype C57BL/6 female to generate the *Cuzd1*-null mice with pure genetic background. Progeny were genotyped by using PCR assay that identified both mutant and wild-type alleles and Southern blotting analysis with 5'-end probe (Fig. 2.9B).

Cell line and Cell Culture

HC11 cells were grown in RPMI-1640 supplemented with 5% (v/v) fetal bovine serum, 1x Penicillin-Streptomycin, 10 ng/ml EGF and 5 μg/ml insulin. In certain experiments, 2% charcoal-stripped calf serum was used.

Hormone Treatment

HC11 cells were treated with 10nm E, 10μm P and/or 50μm PRL. Ovariectomized mice were treated with vehicle controls (oil or saline), 1ng E and 1mg P (subcutaneous) and/or 50μg/g bw of PRL (intraperitoneal).

Whole mount analysis of mammary gland morphology

The inguinal mammary glands were dissected out, spread onto a glass slide and fixed in a 1:3 mixture of glacial acetic acid/100% ethanol. After hydration, slides were stained as described previously (50). Following mounting, images were captured using bright field dissecting microscope.

Immunochemistry

Paraffin-embedded mammary tissues were sectioned and subjected to IHC as described previously (49). Rabbit polyclonal antibodies against a peptide antigen containing amino acids SSPNYPKPHPEL of mouse CUZD1 were generated in our laboratory. IHC was performed on mammary tissue sections, using primary antibodies and bound primary antibodies were detected with either horseradish peroxidase (HRP)- or fluorescent label-conjugated secondary antibodies. Sections were counterstained with hematoxylin or dapi and mounted.

Cells were fixed in a 3.7% formalin solution at room temperature for 15 min followed by washing with PBS. The cells were permeabilized by 0.25% Triton X-100 in PBS for 10 min, and nonspecific binding of antibodies was blocked with 5% donkey serum for 1 h at room temperature. Cells were incubated with primary antibodies overnight at 4°C. Labeling was visualized with fluorescent label-conjugated secondary antibodies and slides were mounted in Prolong GOLD and cured for 24 h before imaging. The images of immunohistochemical staining were captured by using a Leica DM2500 light microscope fitted with a Qimaging Retiga 2000R camera (Qimaging). Immunofluorescence imaging was performed on a Leica 700 confocal microscope. These images were minimally processed on ADOBE Photoshop version 8.

Isolation of mammary epithelial cells and DNA microarray analysis

Pooled inguinal mammary glands from three mice (*Cuzd1*$^{(+/-)}$ or *Cuzd1*$^{(-/-)}$) were minced into small pieces and incubated with DMEM: F12 containing 100 U/ml hyaluronidase and 1.5 mg/ml collagenase at 37^{0}C for 2 h accompanied by shaking at 110 RPM. Following neutralization of enzyme activity with 5% FBS, the homogeneous cell mixture was centrifuged and the cell pellet was washed several times with PBS. Purified epithelial cells were frozen in liquid nitrogen and stored at –80^{0}C.

Total RNA was prepared from these cells and hybridized to Affymetrix mouse arrays (GeneChipMouse Genome 430 2.0 array) containing probes that represented ~14,000 known genes. They were processed and

analyzed according to Affymetrix protocol. Although the microarray analysis was performed using pooled mammary glands, we further confirmed gene expression changes, using RNA samples isolated from independent batches of epithelial cells isolated from *Cuzd1*$^{(+/-)}$ or *Cuzd1*$^{(-/-)}$ glands and analyzing the expression of selected genes by real-time PCR followed by statistical analyses. As shown in Fig. 2.3B, several transcripts corresponding to the EGF family ligands were indeed differentially expressed in a manner similar to that predicted by the microarray analysis. The microarray data were deposited in the publicly available GEO database with GEO Accession GSE30939.

Quantitative real-time PCR (qPCR) analysis

For qPCR, total RNA was extracted from purified mammary epithelium or cultured HC11 cells using Trizol RNA purification kit, according to manufacturer's instructions and subjected to qPCR using gene specific primers. Primer sequences are provided in Table 1. Relative mRNA levels were plotted after normalization to the loading control 36B4. The error bars represent the relative gene expression ± the standard error from three or more independent trials. Data were analyzed using a student's t-test and * indicate p-values < 0.05.

siRNA treatment

HC11 cells were transfected with siRNA against *Cuzd1* or control siRNA (non-targeting), using Lipofectamine-RNAimax reagent following manufacturer's protocol. Briefly, lipofectamine was mixed with siRNA, and allowed to form siRNA-liposome complexes, which were then added to HC11 cells at 60% confluency. After 24 h, the transfection was repeated again. Cells were harvested 48 h after the second transfection, total RNA was isolated and analyzed by qPCR using gene-specific primers.

Immunoprecipitation

HC11-3xFLAG-Cuzd1 cells were cultured with FBS, EGF and PRL for 6h, lysed and samples were precleared before immunoprecipitation (IP). The IP was done using anti-FLAG M2 or a mouse IgG control resin (according to manufacturer's directions) and the captured proteins were eluted using 3xFLAG peptide. Samples were boiled in SDS buffer and analyzed by standard Western blotting.

Mass Spectrometry

Directly following IP, protein samples were submitted to the Mass Spectrometry Laboratory at the University of Illinois at Urbana-Champaign. Liquid chromatography (LC)/mass spectrometry (MS) proteomic data were analyzed using Mascot (Matrix Science) and results were sorted by protein score.

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-STAT5 antibody (Santa Cruz, sc-835) were used overnight at 4^0C to immunoprecipitate flag-CUZD1 and STAT5, respectively. Normal mouse IgG (Santa Cruz, sc-2027) immunoprecipitation served as a negative control. Protein/DNA complexes were eluted, crosslinks were reversed and purified DNA was analyzed for enrichment in sequences of interest using qPCR.

Reagents

pKO Scrambler NTKV-1901 targeting vector was purchased from Stratagene (La Jolla, CA). G418, carmine, formalin, hyaluronidase and 1.5 mg/ml collagenase, insulin, p3XFLAG-CMV-10 Expression Vector, progesterone and 17β-estradiol, mouse IgG-agarose, ANTI-FLAG M2 affinity resin, 3xFLAG peptide were purchased from Sigma-Aldrich (St. Louis, MO). JAK1, JAK2, EREG, Ki67, pErbB1 (Tyr 1068), pErbB2 (Tyr 877), pErbB4 (Tyr 1056), pSTAT5 (Tyr694) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX). The NRG1 antibody was purchased from Thermo Scientific (Waltham, MA). Total STAT5, EPGN, ErbB1, ErbB2 and ErbB4 antibodies were acquired from Cell Signaling Technology (Beverley, MA). Horseradish peroxidase (HRP)–conjugated goat anti-mouse or goat anti-rabbit secondary antibodies, plenti6.3/V5 TOPO Trizol RNA purification kit, RPMI-1640, blasticidin, Prolong GOLD antifade reagent with 4′,6-diamidino-2-phenylindole, Cuzd1 siRNA and non-targeting siRNA and Lipofectamine-RNAimax were purchased from Life Technologies (Carlsbad, CA). Cyanine 3 or Dylight 488-conjugated anti-mouse IgG or antirabbit IgG were obtained from The Jackson Laboratory (Bar Harbor, ME). Fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). Prolactin was acquired from the National Hormone and Peptide Program (Torrance, CA).

Table 1. Primer Sequences.

Gene symbol	Gene accession	Forward primer	Reverse primer
Cuzd1	NM_008411	GCAGCAGGTGTGAAACTGAA	GATTCAAACACAGGCACGAA
Wap	NM_011709	AACATTGGTCTTCCGAAAGC	AGGGTTATCACTGGCACTGG
Csn2	NM_009972	TCCTCTCTTGTCCTCCACTA	TGTAGCATGATCCAAAGGTGA
krt19	NM_008471	CTGCTGTCTGGCAATGAGAA	CGAGGCACTCAAGGAAGAAC
36B4	NM_007475.3	CATCACCACGAAAATCTCCA	TTGTCAAACACCTGCTGGAT
Epgn	NM_053087	CGAAGAAGCAGAGGTGATCC	AATGGCTTGCTTCAGCTCAT
Ereg	NM_007950	CTACACTGGTCTGCGATGTGA	TCCAGCGGTTATGATGAGAAAC
Areg	NM_009704	AGATGTCTTCAGGGAGTG	GGTATTTGTGGTTCGTTATC
Egf	NM_010113	TTCTCACAAGGAAAGAGCATCTC	GTCCTGTCCCGTTAAGGAAAAC
Btc	NM_007568	TGAAAACCCACTTCTCTCGGT	TGCTGGAGGTAAAACAGGTCC
Nrg1	NM_178591	TCAGCAAGTTAGGAAACGACAG	ACATAAGGTCTTTCAGTTGAGGC
Nrg2	NM_001167891	ACGGATTCTTCGGACAGAGAT	CACAGGACACTTTGCTTAGGAT
Nrg3	NM_001190187	TAGGCTCCGTCAAGGAGTACG	GGGGACGTGGTAGAAGTGG
Nrg4	NM_032002	CACGCTGCGAAGAGGTTTTTC	CGCGATGGTAAGAGTGAGGA
Hbegf	NM_010415	AGATACCTGCAGGAGTTCCG	GTCATAACCTCCTCTCCTGT
Wap GAS		CATCTCTTCCTGCCCATGAC	TCGGGCATACATTGAAAAGG
Csn2 GAS		GTCCTCTCACTTGGCTGGAG	GTGGAGGACAAGAGAGGAGGT
Ereg GAS		GCGAATTGCATCCTGTGAGT	ACCCCCTCACATTTTGGAGA

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REFERENCES

1. Hennighausen L, Robinson GW. Signaling Pathways in Mammary Gland Development Developmental Cell 2001;1(4):467-475.
2. Brisken C, O'Malley B. Hormone Action in the Mammary Gland Cold Spring Harbor Perspectives in Biology 2010;2(12). PMID: 20739412
3. Gallego MI, Binart N, Robinson GW, Okagaki R, Coschigano KT, Perry J, et al. Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects Dev Biol 2001 Jan 1;229(1):163-175.
4. Vomachka AJ, Pratt SL, Lockefeer JA, Horseman ND. Prolactin gene-disruption arrests mammary gland development and retards T-antigen-induced tumor growth Oncogene 2000 Feb 21;19(8):1077-1084.
5. Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, et al. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse Genes Dev 1997 Jan 15;11(2):167-178.
6. Naylor MJ, Lockefeer JA, Horseman ND, Ormandy CJ. Prolactin regulates mammary epithelial cell proliferation via autocrine/paracrine mechanism Endocrine 2003 Feb-Mar;20(1-2):111-114.
7. Hennighausen L, Robinson GW, Wagner K-, Liu X. Prolactin Signaling in Mammary Gland Development J Biol Chem 1997;272(12):7567-7569.
8. Bazan JF. A novel family of growth factor receptors: A common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor ß-chain Biochem Biophys Res Commun 1989;164(2):788-795.
9. Rui H, Kirken RA, Farrar WL. Activation of receptor-associated tyrosine kinase JAK2 by prolactin J Biol Chem 1994 Feb 18;269(7):5364-5368.
10. Wakao H, Gouilleux F, Groner B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J 1994 May 1;13(9):2182-2191.
11. Ali S. Prolactin Receptor Regulates Stat5 Tyrosine Phosphorylation and Nuclear Translocation by Two Separate Pathways J Biol Chem 1998;273(13):7709-7716.
12. Gao J, Hughes JP, Auperin B, Buteau H, Edery M, Zhuang H, et al. Interactions among Janus kinases and the prolactin (PRL) receptor in the regulation of a PRL response element Mol Endocrinol 1996 Jul;10(7):847-856.
13. Vonderhaar B. Hormonal interactions during mammary gland development. Breast Cancer Research 2003;5:1-1.
14. Sakamoto K, Creamer BA, Triplett AA, Wagner K. The Janus Kinase 2 Is Required for Expression and Nuclear Accumulation of Cyclin D1 in Proliferating Mammary Epithelial Cells. Molecular Endocrinology 2007;21(8). PMID: 17519353
15. Sato T, Tran TH, Peck AR, Liu C, Ertel A, Lin J, et al. Global profiling of prolactin-modulated transcripts in breast cancer in vivo. Mol Cancer 2013 Jun 12;12:59-4598-12-59.
16. Zhu BM, Kang K, Yu JH, Chen W, Smith HE, Lee D, et al. Genome-wide analyses reveal the extent of opportunistic STAT5 binding that does not yield transcriptional activation of neighboring genes Nucleic Acids Res 2012 May;40(10):4461-4472.
17. Li S, Rosen JM. Nuclear factor I and mammary gland factor (STAT5) play a critical role in regulating rat whey acidic protein gene expression in transgenic mice Mol Cell Biol 1995 Apr;15(4):2063-2070.
18. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue Proc Natl Acad Sci U S A 1995 Sep 12;92(19):8831-8835.
19. Schmitt-Ney M, Doppler W, Ball RK, Groner B. Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. Mol Cell Biol 1991 Jul;11(7):3745-3755.

20. Vonderhaar BK. Prolactin involvement in breast cancer. Endocr Relat Cancer 1999 Sep;6(3):389-404.
21. Jones FE, Welte T, Fu XY, Stern DF. ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. J Cell Biol 1999 Oct 4;147(1):77-88.
22. Chen D, Xu X, Zhu LJ, Angervo M, Li Q, Bagchi MK, et al. Cloning and uterus/oviduct-specific expression of a novel estrogen-regulated gene (ERG1). J Biol Chem 1999 Nov 5;274(45):32215-32224.
23. Huynh H, Ng CY, Ong CK, Lim KB, Chan TW. Cloning and characterization of a novel pregnancy-induced growth inhibitor in mammary gland Endocrinology 2001 Aug;142(8):3607-3615.
24. Imamura T, Asada M, Vogt SK, Rudnick DA, Lowe ME, Muglia LJ. Protection from pancreatitis by the zymogen granule membrane protein integral membrane-associated protein-1 J Biol Chem 2002 Dec 27;277(52):50725-50733.
25. Leong CT, Ong CK, Tay SK, Huynh H. Silencing expression of UO-44 (CUZD1) using small interfering RNA sensitizes human ovarian cancer cells to cisplatin in vitro Oncogene 2007 Feb 8;26(6):870-880.
26. Bork P, Beckmann G. The CUB domain. A widespread module in developmentally regulated proteins J Mol Biol 1993 May 20;231(2):539-545.
27. Jovine L, Darie CC, Litscher ES, Wassarman PM. Zona pellucida domain proteins Annu Rev Biochem 2005;74:83-114.
28. Williams JM, Daniel CW. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Developmental biology. 1983 Jun 30;97(2):274-90.
29. Silberstein GB. Postnatal mammary gland morphogenesis. Microscopy research and technique. 2001 Jan 15;52(2):155-62.
30. Troyer KL, Lee DC. Regulation of mouse mammary gland development and tumorigenesis by the ERBB signaling network. J Mammary Gland Biol Neoplasia 2001;6(1):7-21.
31. Danielson KG, Knepper JE, Kittrell FS, Butel JS, Medina D, Durban EM. Clonal populations of the mouse mammary cell line, COMMA-D, which retain capability of morphogenesis in vivo In Vitro Cell Dev Biol 1989 Jun;25(6):535-543.
32. Santos SJ, Haslam SZ, Conrad SE. Signal transducer and activator of transcription 5a mediates mammary ductal branching and proliferation in the nulliparous mouse Endocrinology 2010 Jun;151(6):2876-2885.
33. Nevalainen MT, Xie J, Bubendorf L, Wagner K, Rui H. Basal activation of transcription factor signal transducer and activator of transcription (Stat5) in nonpregnant mouse and human breast epithelium. Molecular endocrinology 2002;16(5):1108-1124.
34. Rawlings JS. The JAK/STAT signaling pathway J Cell Sci 2004;117(8):1281-1283. PMID: 15020666
35. García-Martínez JM, Calcabrini A, González L, Martín-Forero E, Agulló-Ortuño MT, Simon V, et al. A non-catalytic function of the Src family tyrosine kinases controls prolactin-induced Jak2 signaling Cell Signal 2010;22(3):415-426.
36. Park DS, Lee H, Frank PG, Razani B, Nguyen AV, Parlow AF, et al. Caveolin-1-deficient mice show accelerated mammary gland development during pregnancy, premature lactation, and hyperactivation of the Jak-2/STAT5a signaling cascade. Mol Biol Cell 2002 Oct;13(10):3416-3430.
37. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, et al. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene EMBO J 1997 Dec 1;16(23):6926-6935.
38. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L. Stat5a is mandatory for adult mammary gland development and lactogenesis Genes Dev 1997 Jan 15;11(2):179-186.
39. Parganas E. Jak2 Is Essential for Signaling through a Variety of Cytokine Receptors Cell 1998;93(3):385-395.
40. Farooqui M, Bohrer LR, Brady NJ, Chuntova P, Kemp SE, Wardwell CT, et al. Epiregulin contributes to breast tumorigenesis through regulating matrix metalloproteinase 1 and promoting cell survival Mol Cancer 2015 Jul 29;14:138-015-0408-z.
41. Riese DJ, Cullum RL. Epiregulin: roles in normal physiology and cancer. Seminars in cell & developmental biology 2014 Apr 30 (Vol. 28, pp. 49-56). Academic Press.

42. Komurasaki T, Toyoda H, Uchida D, Morimoto S. Epiregulin binds to epidermal growth factor receptor and ErbB-4 and induces tyrosine phosphorylation of epidermal growth factor receptor, ErbB-2, ErbB-3 and ErbB-4 Oncogene 1997 Dec 4;15(23):2841-2848.
43. Jones FE, Jerry DJ, Guarino BC, Andrews GC, Stern DF. Heregulin induces in vivo proliferation and differentiation of mammary epithelium into secretory lobuloalveoli Cell Growth Differ 1996 Aug;7(8):1031-1038.
44. Li L, Cleary S, Mandarano MA, Long W, Birchmeier C, Jones FE. The breast proto-oncogene, HRGalpha regulates epithelial proliferation and lobuloalveolar development in the mouse mammary gland Oncogene 2002 Jul 25;21(32):4900-4907.
45. Jackson-Fisher AJ, Bellinger G, Breindel JL, Tavassoli FA, Booth CJ, Duong JK, et al. ErbB3 is required for ductal morphogenesis in the mouse mammary gland. Breast Cancer Res 2008;10(6):R96.
46. Jackson-Fisher AJ, Bellinger G, Ramabhadran R, Morris JK, Lee KF, Stern DF. ErbB2 is required for ductal morphogenesis of the mammary gland Proc Natl Acad Sci U S A 2004 Dec 7;101(49):17138-17143.
47. Wiesen JF, Young P, Werb Z, Cunha GR. Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development 1999 Jan;126(2):335-344.
48. Andrechek ER, White D, Muller WJ. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. Oncogene. 2005 Jan 27;24(5):932-7.
49. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA,Jr, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities Genes Dev 1995 Sep 15;9(18):2266-2278.
50. Li Q, Cheon YP, Kannan A, Shanker S, Bagchi IC, Bagchi MK. A novel pathway involving progesterone receptor, 12/15-lipoxygenase-derived eicosanoids, and peroxisome proliferator-activated receptor gamma regulates implantation in mice J Biol Chem 2004 Mar 19;279(12):11570-11581.

FIGURES

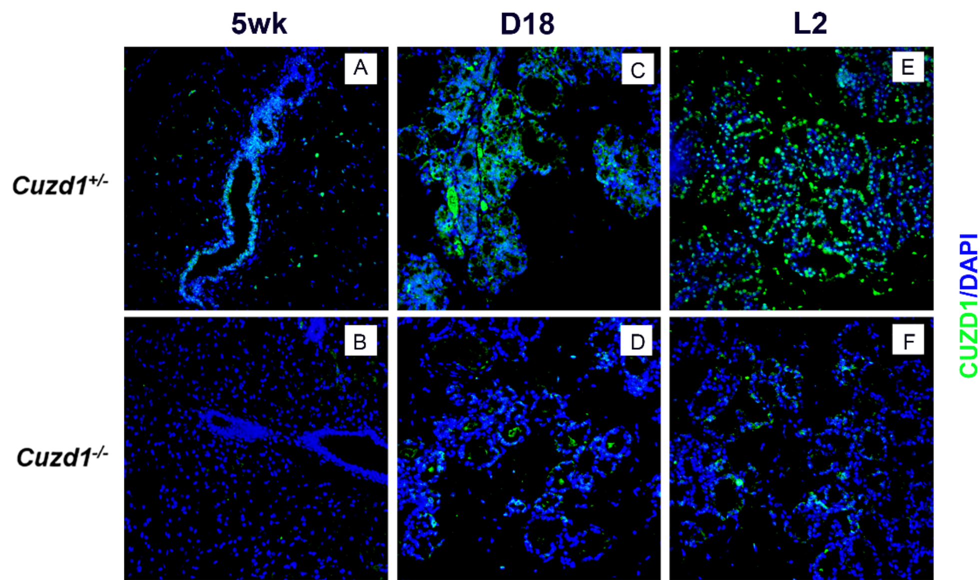


Figure 2.1. Analysis of the spatio-temporal expression of CUZD1 in the mammary glands during development. Mammary sections were obtained from *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice at puberty (5 weeks, a and b), late pregnancy (Day 18, c and d) and lactation day 2 (L2, e and f) and subjected to immunofluorescence, using rabbit polyclonal antibodies against mouse CUZD1. Magnification 20x. (Courtesy of Quanxi Li and Athilakshmi Kannan)

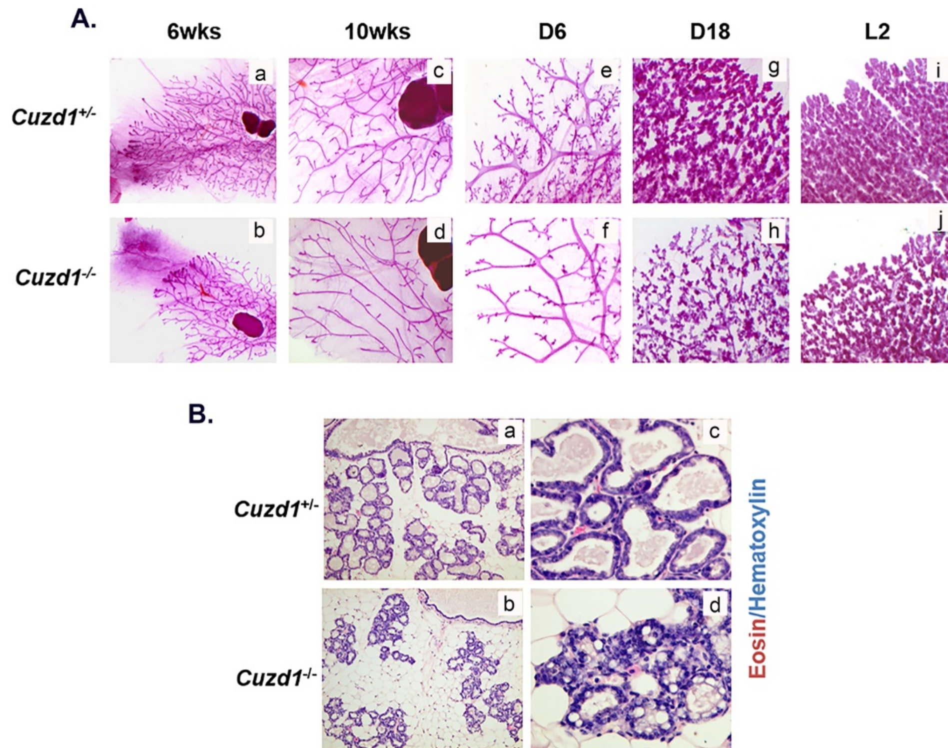


Figure 2.2. Phenotypic analysis of Cuzd1$^{(-/-)}$ mice. A. Analysis of *Cuzd1*$^{(-/-)}$ mammary gland morphology. Whole mount analysis of no. 4 inguinal mammary glands of virgin *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice at 6 weeks of age (a and b) and 10 weeks of age (c and d), pregnancy day 6 (e and f), pregnancy day 18 (g and h) and lactation day 2 (i and j). Magnification 4x. (Courtesy of Quanxi Li and Athilakshmi Kannan) **B. Histological analysis of *Cuzd1*$^{(-/-)}$ mammary gland during lactation.** Mammary gland sections of lactating (day 2) *Cuzd1*$^{(+/-)}$ (a and c) or *Cuzd1*$^{(-/-)}$ (b and d) mice were subjected to H&E analysis. Magnification 20x (a and b) and 40x (c and d). (Courtesy of Quanxi Li and Athilakshmi Kannan)

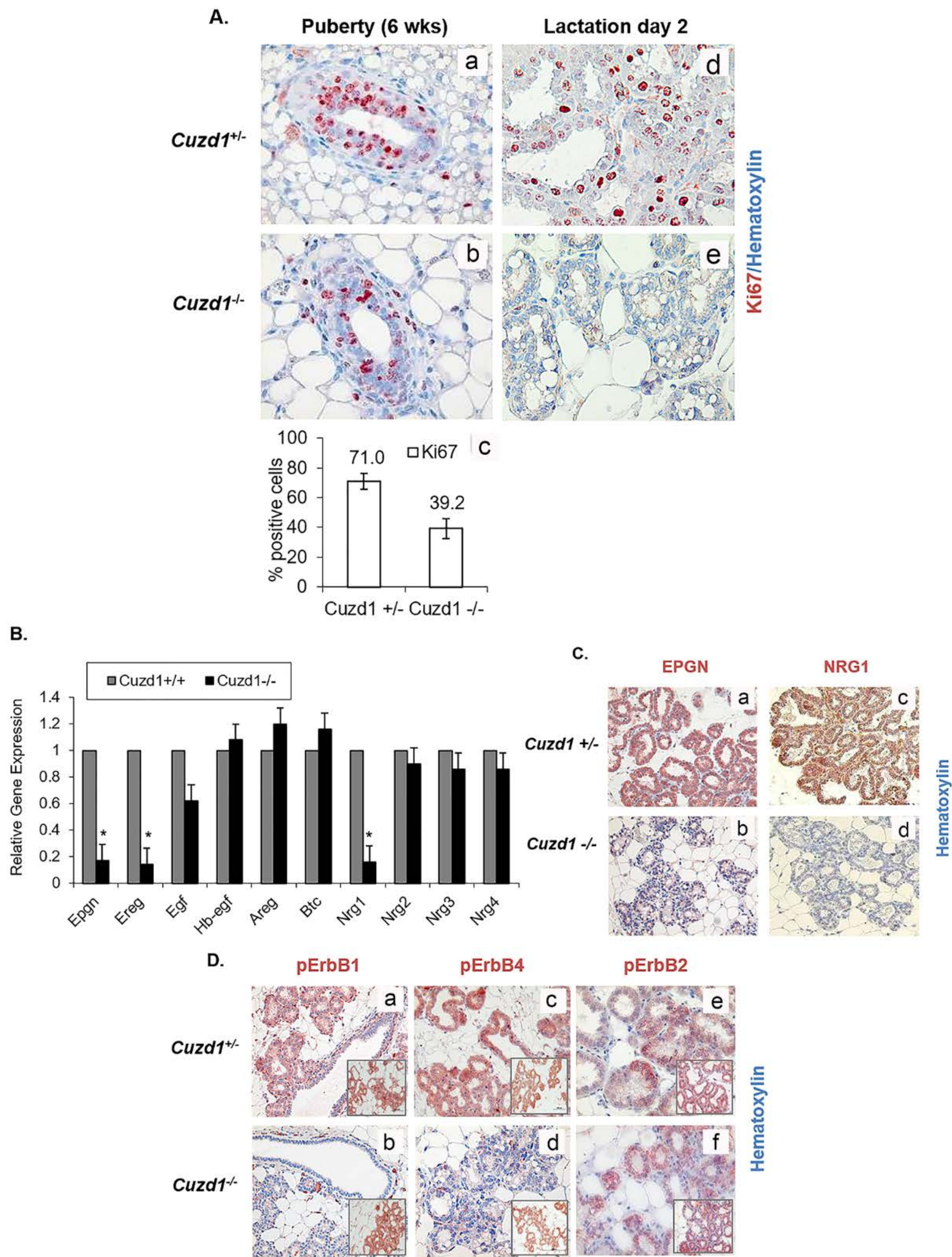


Figure 2.3. Developmental defects in *Cuzd1*$^{(-/-)}$ mammary glands are due to an impairment in EGF signaling. A. Analysis of mammary epithelial cell proliferation during development. Mammary gland sections of pubertal (6 weeks, a and b) and lactating (day 2, d and e) *Cuzd1*$^{(+/-)}$ or *Cuzd1*$^{(-/-)}$ mice were subjected to IHC analysis using an antibody against Ki67. Magnification 20x. The number of Ki67-positive cells during puberty in *Cuzd1*$^{(-/-)}$ mammary tissue (a) was estimated by ImageJ software and compared with those in *Cuzd1*$^{(+/-)}$ tissue (b). Data (c) expressed as average ± SEM of ≥3 biological replicates. (Courtesy of Quanxi Li and Athilakshmi Kannan)

(Figure 2.3 con't.) B. Expression of EGF family ligands in the *Cuzd1*$^{(-/-)}$ mammary gland. Total RNA was isolated from purified mammary epithelial cells of *Cuzd1*$^{(-/-)}$ mice and *Cuzd1*$^{(+/-)}$ littermates at day 18 of pregnancy. qPCR was performed to analyze expression levels of *Epgn*, *Ereg*, *Egf*, *Hbegf*, *Areg*, *Btc*, *Nrg1*, *Nrg2*, *Nrg3* and *Nrg4* mRNAs. Data are represented as relative gene expression ± SEM from ≥3 biological replicates. (Courtesy of Quanxi Li) **C. Expression of EPGN and NRG1 proteins.** Mammary tissue sections obtained from *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice on lactation day 2 were subjected to IHC using antibodies specific for EPGN (a and b), NRG1 (c and d). Magnification 10x. (Courtesy of Lavanya Anandan) **D. Activation of ErbB receptors in the *Cuzd1*$^{(-/-)}$ mammary gland.** Mammary tissue sections obtained from *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice on day 18 of pregnancy were subjected to IHC using antibodies specific for pErbB1 (a and b), pErbB4 (c and d) and pErbB2 (e and f). Insets show total ErbB levels. Magnification 10x. (Courtesy of Lavanya Anandan)

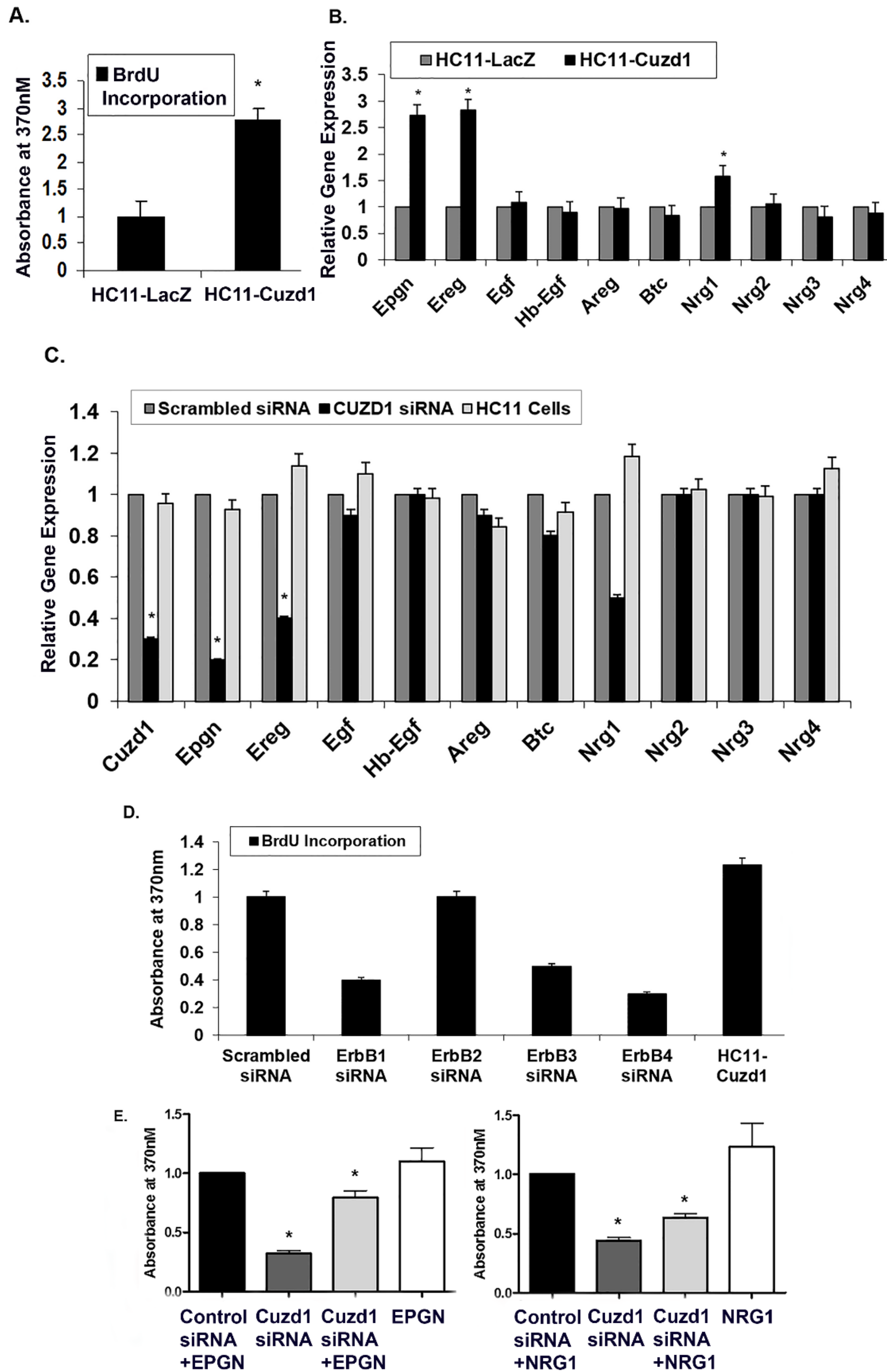


Figure 2.4. *Cuzd1* controls the expression of a subset of EGF family ligands in mammary epithelial cells. A. *Cuzd1* overexpressing cells exhibit increased proliferation. HC11-Cuzd1 and HC11-LacZ cells were cultured under serum-free conditions for 48 h and 10% FBS was added along with BrdU 24 h prior to cell harvest. BrdU incorporation was measured using an ELISA-based assay. Data are expressed as Absorbance at 370nm ± SEM from ≥3 biological replicates. (Courtesy of Lavanya Anandan)

(Figure 2.4 con't.) B. Expression of EGF family ligands in HC11-Cuzd1 and HC11-LacZ cells. HC11-Cuzd1 and HC11-LacZ cells were cultured for 48 h. qPCR was performed to analyze relative expression levels of *Epgn*, *Ereg*, *Egf*, *Hbegf*, *Areg*, *Btc*, *Nrg1*, *Nrg2*, *Nrg3* and *Nrg4* mRNAs. Data are represented as relative gene expression ± SEM from ≥3 biological replicates. (Courtesy of Lavanya Anandan) **C. Expression of EGF family ligands in *Cuzd1-silenced* HC11 cells.** HC11 cells were transfected with siRNA (100nM) targeted against *Cuzd1* or scrambled siRNA (control). Total RNA was prepared from HC11 cells 48 h after transfection and subjected to qPCR using gene-specific primers to assess the expression of *Epgn*, *Ereg*, *Egf*, *Hbegf*, *Areg*, *Btc*, *Nrg1*, *Nrg2*, *Nrg3* and *Nrg4* mRNAs. Data are represented as relative gene expression ± SEM from ≥3 biological replicates. **D. Proliferation of HC11-Cuzd1 cells upon *ErbB* perturbation.** HC11-Cuzd1 cells were transfected with siRNA (50nM) targeted against *ErbB1, ErbB2, ErbB3, ErbB4* or non-targeting siRNA (control). 48 h post transfection, the siRNA transfection mixture was removed and replaced with fresh growth medium and BrdU was administered 24 h prior to cell harvest. BrdU incorporation was measured using an ELISA-based assay. Data are expressed as Absorbance at 370nm ± SEM from ≥3 biological replicates. (Courtesy of Lavanya Anandan) **E. Proliferation of HC11 cells with *Cuzd1* knockdown and ligand supplementation.** HC11 cells were transfected with siRNA (100nM) targeted against *Cuzd1* or a non-targeting siRNA (control). 48h post-transfection, HC11 cells were supplemented with EPGN, NRG1, or a vehicle control and BrdU was added. BrdU incorporation was measured after 24h using an ELISA-based BrdU assay and resulting color reaction was measured using a plate reader at 370nm. Data are expressed as average Absorbance at 370nm ± SEM from ≥3 biological replicates.

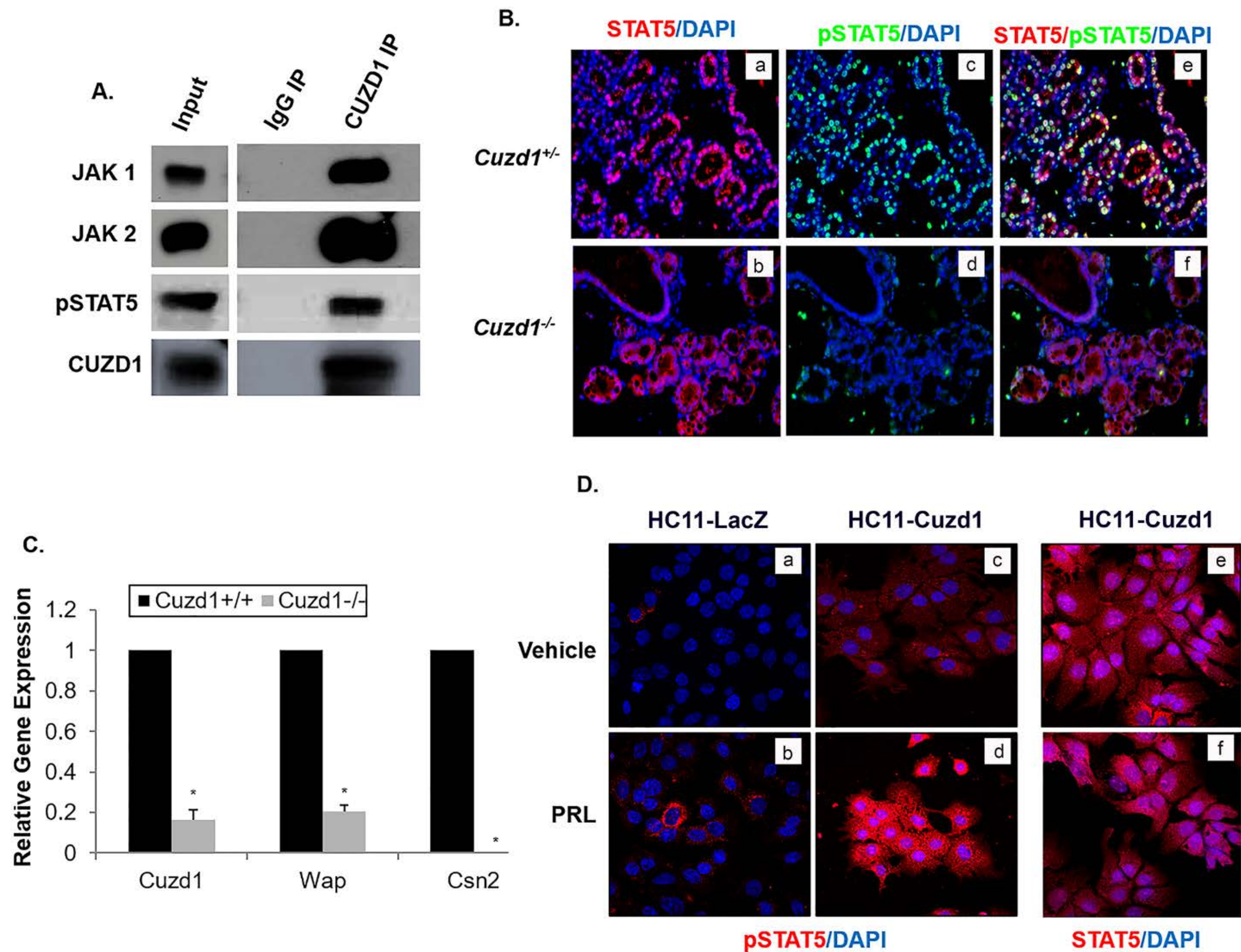


Figure 2.5. CUZD1-mediated STAT5 signaling is necessary for PRL-induced proliferation and differentiation of the mammary gland. A. CUZD1 associates with JAK1/2 and STAT5. HC11 cells were cultured for 48 h in serum-free media and then exposed to 10% FBS for 24 h. Cells were lysed and samples were immunoprecipitated with an IgG (control) or CUZD1 antibody. CUZD1 and the associated proteins were confirmed by Western blot analysis. Blots were probed with CUZD1, JAK1, JAK2 and pSTAT5 antibodies. **B. Activation STAT5 in the *Cuzd1*$^{(-/-)}$ mammary gland.** Mammary tissue sections obtained from *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice on day 18 of pregnancy were subjected to IHC using an antibody specific for pSTAT5 and total STAT5. Magnification 10x. **C. Expression of milk protein genes in the *Cuzd1*$^{(-/-)}$ mammary gland.** RNA was isolated from mammary glands of lactating day 2 mammary glands from *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice and analyzed using primers specific for *Cuzd1, Wap,* and *Csn2*. Data are represented as relative gene expression ± SEM from ≥3 biological replicates. **D. Analysis of pSTAT5 in HC11-LacZ and HC11-Cuzd1 cells.** HC11-LacZ and HC11-Cuzd1 cells were cultured for 48 h in serum-free media and then exposed to a vehicle control (a, c, and e) or PRL (b, d, and f) for 24 h. Cells were fixed and subjected to ICC using an antibody specific for pSTAT5 (a, b, c, and d) or total STAT5 (e and f). Magnification 40x.

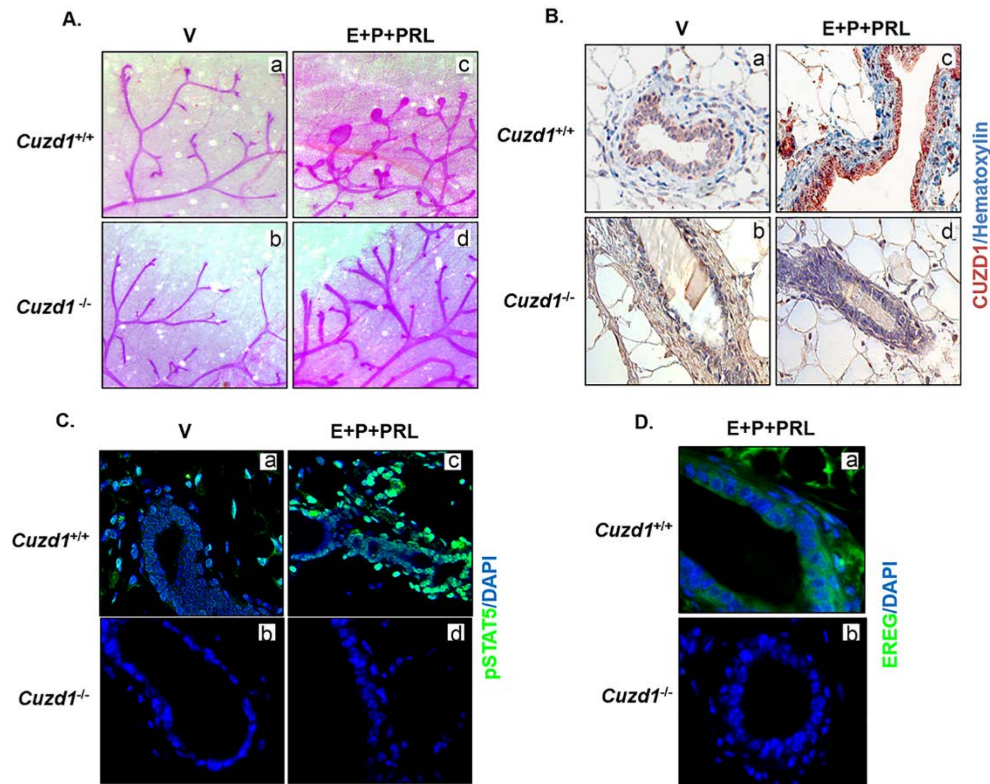
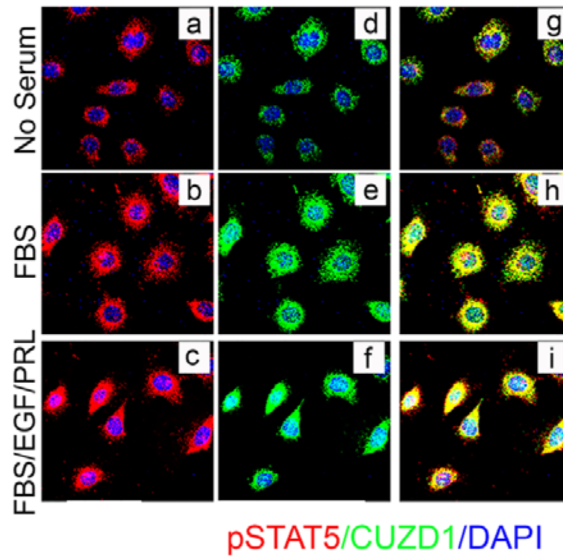
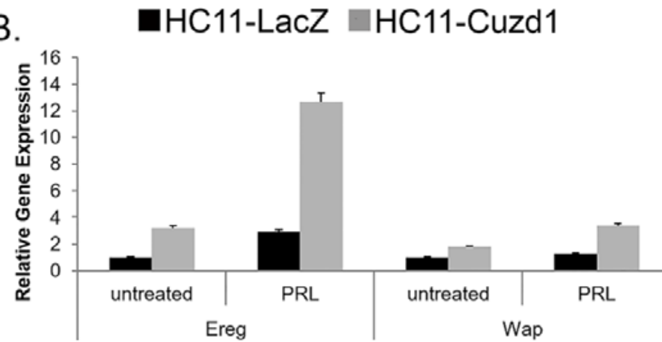


Figure 2.6. *Cuzd1*$^{(-/-)}$ mammary glands do not undergo alveologenesis in response to hormone treatment. A. *Cuzd1*$^{(-/-)}$ mammary gland morphology in response to hormonal treatment. *Cuzd1*$^{(+/+)}$ and *Cuzd1*$^{(-/-)}$ mice (n=5) were treated with a vehicle control (a and b) or E+P+PRL (c and d) for 3d. Whole mount analysis of no. 4 inguinal mammary glands of *Cuzd1*$^{(+/+)}$ and *Cuzd1*$^{(-/-)}$ mice after 3d of hormone treatment. Magnification 6.3x. **B. Analysis of CUZD1 expression.** Mammary tissue sections obtained from *Cuzd1*$^{(+/+)}$ (a and c) and *Cuzd1*$^{(-/-)}$ (b and d) mice were subjected to IHC using an antibody specific for CUZD1. **C. Activation of STAT5.** Mammary tissue sections obtained from *Cuzd1*$^{(+/+)}$ (a and c) and *Cuzd1*$^{(-/-)}$ (b and d) mice were subjected to IHC using an antibody specific for pSTAT5. **D. Analysis of EREG expression.** Mammary tissue sections obtained from *Cuzd1*$^{(+/+)}$ (a) and *Cuzd1*$^{(-/-)}$ (b) mice were subjected to IHC using an antibody specific for EREG. Magnification 20x.





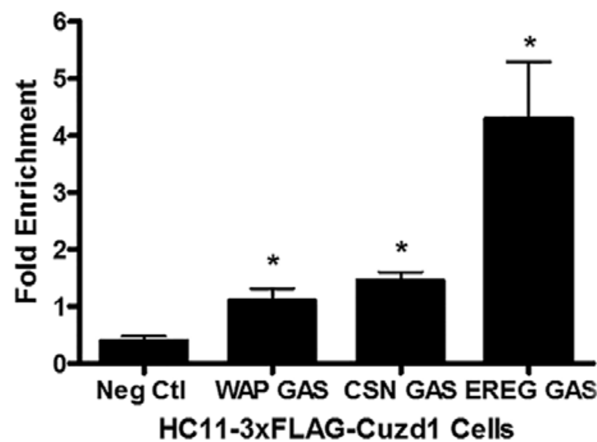


Figure 2.7. CUZD1 and STAT5 translocate to the nucleus and modulate target gene expression. A. CUZD1 translocates to the nucleus in response to culture serum. HC11-Cuzd1 cells were cultured for 48 h in serum-free media and followed by media supplemented with FBS only or a cocktail of FBS/PRL/EGF for additional 24 h. Cells were subjected to ICC using an antibody specific for CUZD1 and pSTAT5. Magnification 40X.

(Figure 2.7 con't) B. Transcription of STAT5 target genes is activated in response to PRL. HC11-Cuzd1 cells were cultured for 48 h in serum-free media and treated with PRL for 24 h. Cells were subjected to qPCR to assess the relative levels of mRNA expression for STAT5 regulated-target genes**,** *Ereg* and *Wap*. Data are represented as relative gene expression ± SEM from ≥3 biological replicates. **C. STAT5 remains in a complex with CUZD1 when bound to DNA regulatory elements.** HC11-3xFLAG-Cuzd1 cells were cultured with FBS, EGF and PRL for 6h. Protein/DNA complexes were precipitated using an antibody for STAT5 followed by FLAG (anti-M2), and subjected to qPCR using primers to GAS motifs of *Wap*, *Csn2*, and *Ereg* promoters, respectively. Data are represented as fold enrichment ± SEM from ≥3 biological replicates.

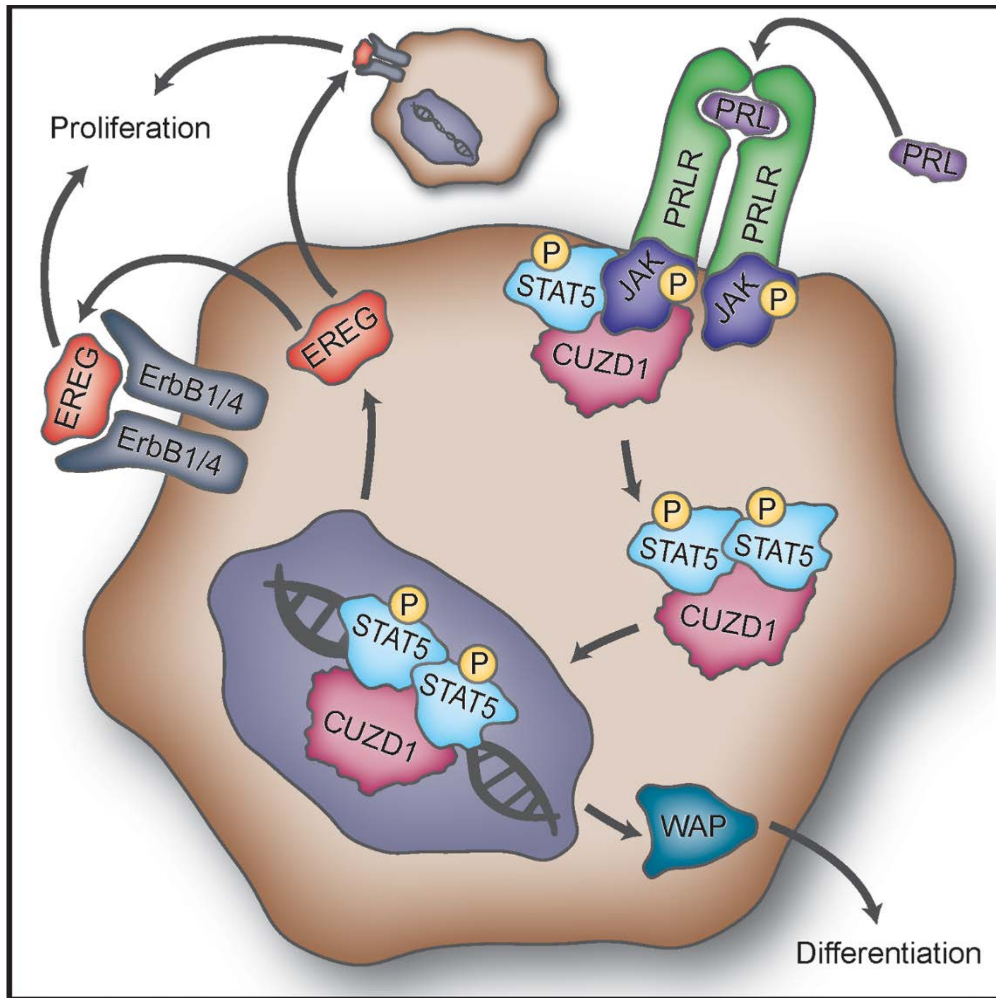


Figure 2.8. Proposed mechanism of action of CUZD1 in the mammary gland epithelium.
Binding of PRL to PRLR induces activation of JAK1/2. CUZD1 forms a complex with JAKs and potentiates activation of STAT5 downstream of PRLR. Activated STAT5 and CUZD1 translocate to the nucleus where STAT5 regulates transcription of target genes, such as *Ereg* and *Wap*. EREG acts in a paracrine and/or autocrine manner through ErbB1 and/or ErbB4 to induce mammary epithelial proliferation. The expression of WAP, a milk protein, marks the terminal differentiation of the mammary epithelium. (Image Courtesy of Jason Neff)

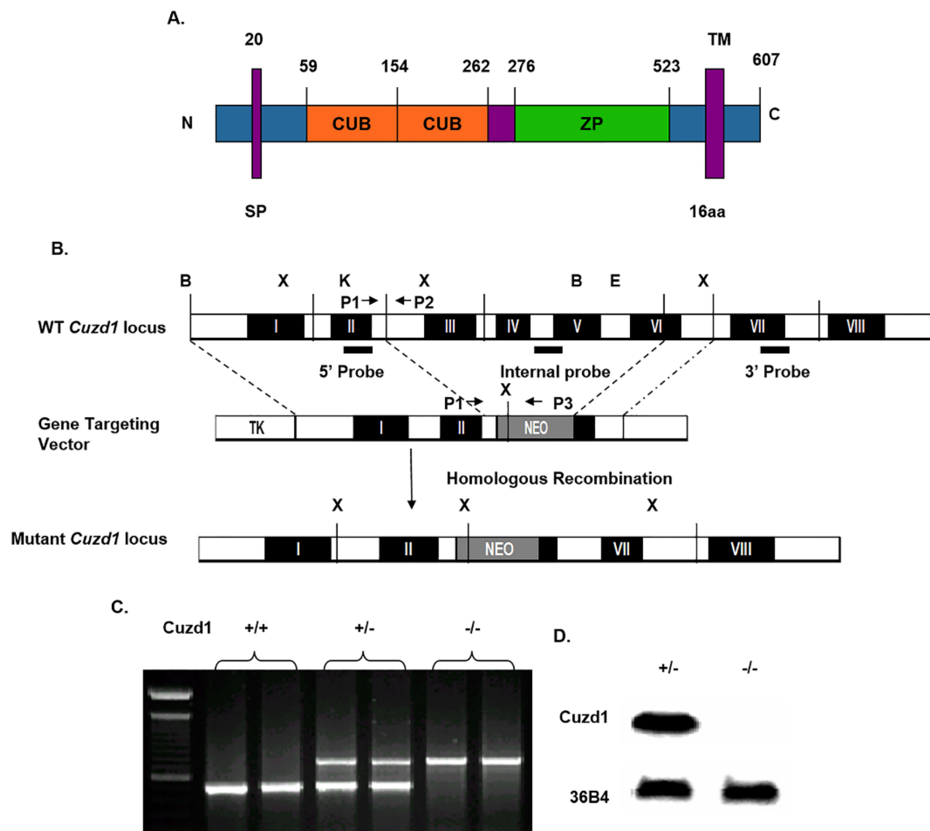


Figure 2.9. Targeting strategy for the *Cuzd1* locus. A. Protein structure of CUZD1. CUZD1 contains two tandem CUB (Complement subcomponent /C1s, Uegf, Bmp1) motifs and a zona-pellucida (ZP)-like domain. (Courtesy of Quanxi Li) **B. Map of *Cuzd1* target**. The genomic organization of the wild-type *Cuzd1* allele is shown with black boxes representing exons and white boxes representing introns. In the targeting vector, the neomycin (NEO) resistance gene is included to provide clone selection. Homologous recombination results in replacement of exons III-VI by the NEO resistance gene. P1, P2, P3 represent the locations of primers used for genotyping PCR to identify wild-type or null genomic mutation. A subset of restriction enzyme sites is shown for relative orientation and targeting vector construction: B, BamHI; E, EcoRI; X, XhoI. (Courtesy of Quanxi Li) **C. Genotyping of *Cuzd1*-null mice.** Genotyping was performed by PCR using tail genomic DNA as template and P1 and P2 or P1 and P3 as primers. The 513 bp and 782 bp DNA fragments arose from wild-type and mutant loci, respectively. (Courtesy of Quanxi Li) **D. Measurement of *Cuzd1* mRNA.** Total RNA was isolated from pregnant (day 1) uteri of heterozygous *Cuzd1*$^{(+/-)}$ and homozygous *Cuzd1*$^{(-/-)}$ mice. The RNA was subjected to Northern blotting, using P^{32}-labled probes specific for *Cuzd1* and internal control gene, 36B4. (+/+), (+/-), and (-/-) represent genomic DNA of wild-type, heterozygous and homozygous mice, respectively. (Courtesy of Quanxi Li)

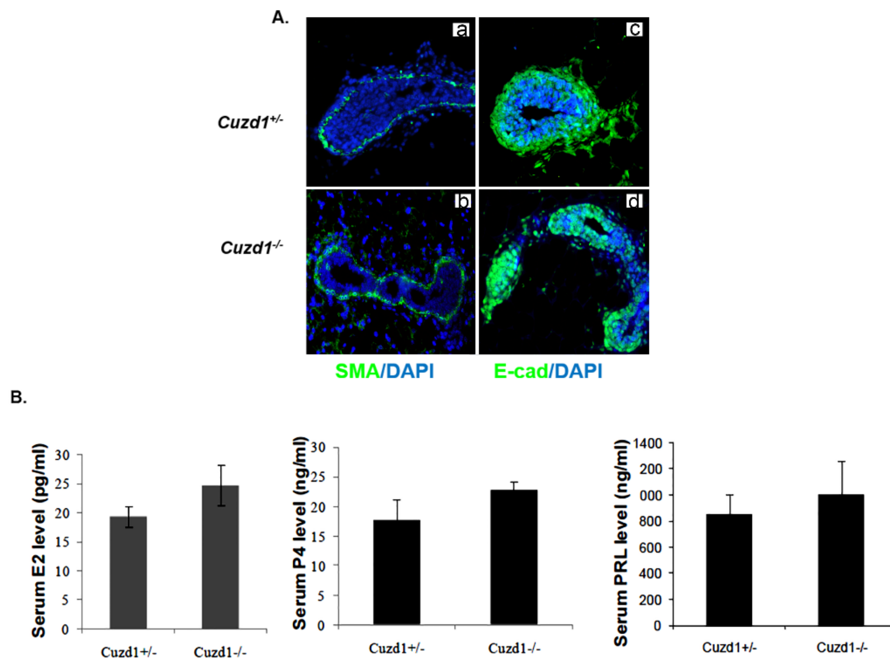


Figure 2.10. Phenotypic analysis of *Cuzd1*$^{(-/-)}$ mice. (A) Expression of SMA and E-cadherin in terminal end buds. Mammary tissue sections obtained from *Cuzd1*$^{(+/-)}$ and *Cuzd1*$^{(-/-)}$ mice were subjected to IHC using an antibody specific for SMA (a and b) and E-cadherin (c and d). **(B) Hormone level measurements.** Blood samples were collected from heterozygous *Cuzd1*$^{(+/-)}$ and homozygous *Cuzd1*$^{(-/-)}$ mice on day 18 of pregnancy. The measurements of E, P and PRL levels were performed as described in Experimental Procedures. Bars represent average values ± SEM from five animals of each genotype. (Courtesy of Quanxi Li)

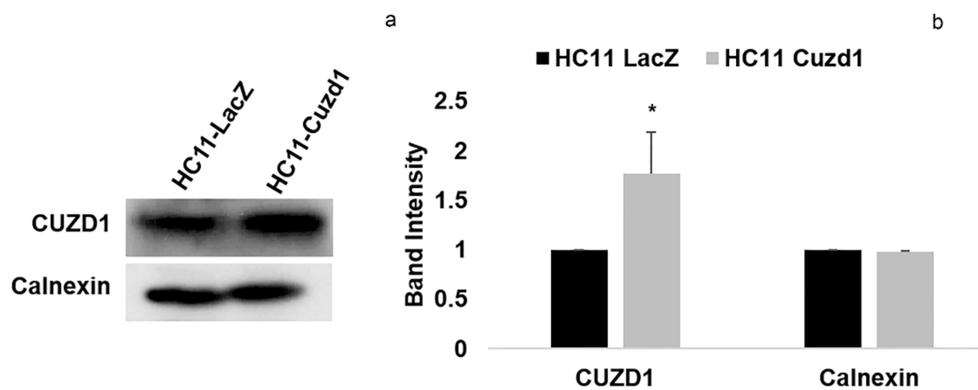


Figure 2.11. Overexpression of Cuzd1 in HC11-Cuzd1 cells. HC11 cells were transduced with lentivirus harboring *Cuzd1* or *LacZ* cDNA to create stable cells overexpressing *Cuzd1* (HC11-Cuzd1) or *LacZ* (HC11-LacZ), respectively. HC11-LacZ and HC11-Cuzd1 cells were lysed and total protein extracts were analyzed using Western blot. Blots were probed with an antibody specific for CUZD1. Calnexin was used as a loading control (a). The density of the bands was quantified using ImageJ and are expressed as Band Intensity (b).

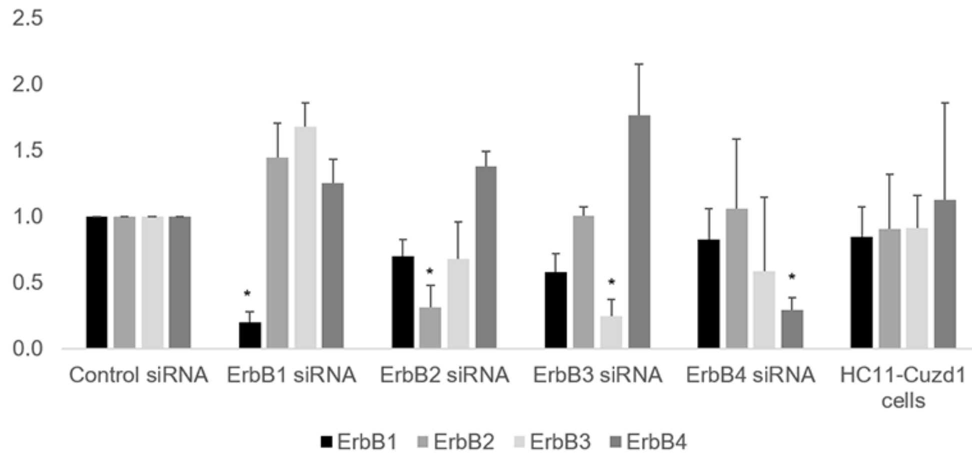


Figure 2.12. Confirmation of individual ErbB knockdown. HC11-Cuzd1 cells were transfected with siRNA (50nM) targeted against *ErbB1, ErbB2, ErbB3, ErbB4* or scrambled siRNA (control). Total RNA was isolated from these cells and subjected to real-time PCR using specific primers to validate *ErbB 1-4* mRNA expression. Data are represented as relative gene expression ± SEM.

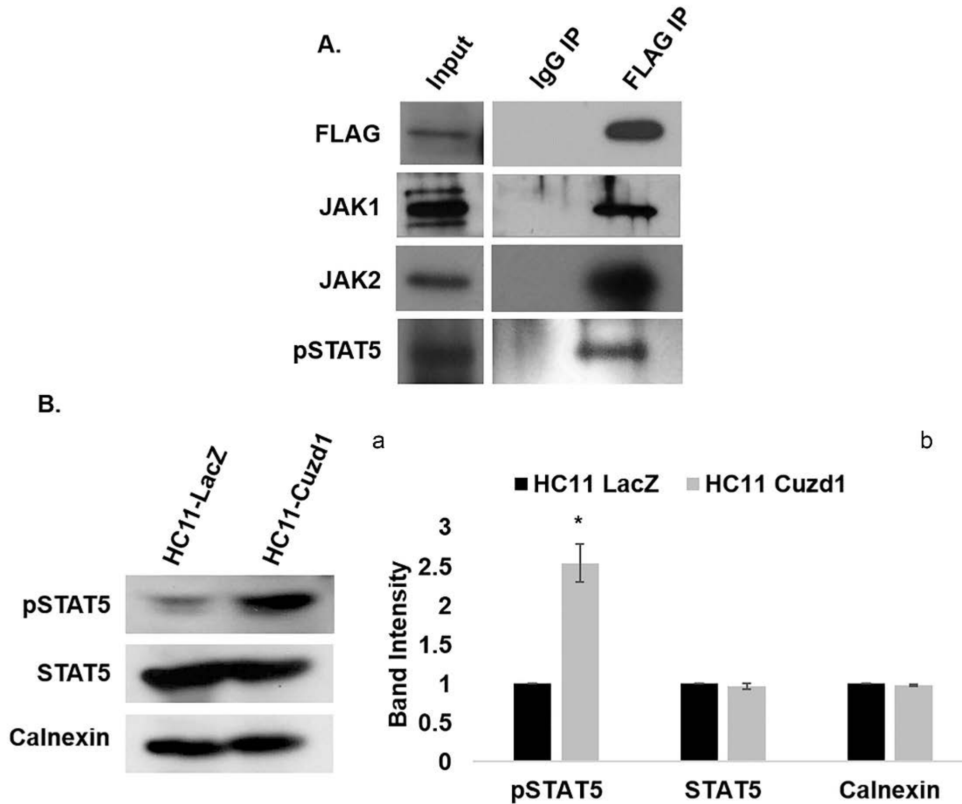


Figure 2.13. (A) Confirmation of CUZD1 protein complex in HC11-3xFLAG-Cuzd1 cells. HC11-3xFLAG-Cuzd1 cells were cultured for 48 h in serum-free media and then treated with FBS/PRL/EGF for 6 h. Cells were lysed and samples were immunoprecipitated with an IgG (control) or M2 (anti-FLAG) antibody. 3xFLAG-CUZD1 and the associated proteins were confirmed by Western blot analysis. Blots were probed with FLAG, JAK1, JAK2 and pSTAT5 antibodies. **(B). Alteration in STAT5 phosphorylation in *Cuzd1* overexpressing cells.** HC11-LacZ and HC11-Cuzd1 cells were lysed and total protein extracts were analyzed using Western blot. Blots were probed with antibodies specific to STAT5 and pSTAT5. Calnexin was used as a loading control (a). The density of the bands was quantified using ImageJ and are expressed as Band Intensity (b).

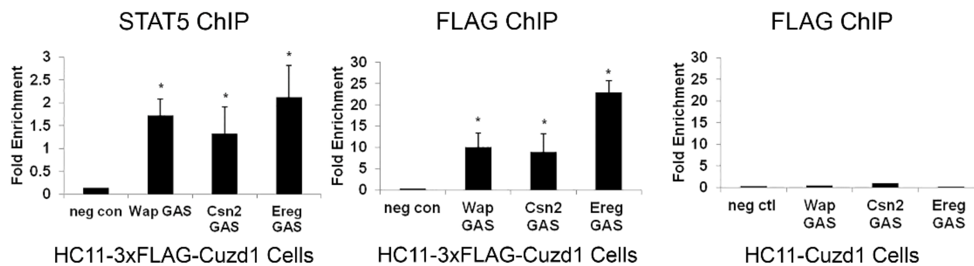


Figure 2.14. Confirmation ChIP with individual antibodies. HC11-3xFLAG-Cuzd1 and HC11-Cuzd1 cells were cultured with FBS/EGF/PRL for 6h. Protein/DNA complexes were precipitated using an antibody for STAT5 or FLAG, and subjected to qPCR using primers specific to GAS motifs of *Wap*, *Csn2*, and *Ereg* promoters, respectively. Data are represented as relative gene expression ± SEM.

CHAPTER 3

Aberrantly High Expression of the STAT5 Signaling Regulator CUZD1 in Mammary Epithelium Leads to Breast Tumorigenesis

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Running title: Role of CUZD1 in breast tumorigenesis

Keywords: breast cancer, cell proliferation, prolactin, STAT transcription factor, tumor cell biology

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ABSTRACT

The peptide hormone prolactin (PRL) and certain members of the epidermal growth factor (EGF) family play central roles in normal mammary gland development and physiology, and their dysregulation has been implicated in mammary tumorigenesis. Our recent studies revealed that the CUB and zona pellucida-like domain-containing protein 1 (CUZD1) is a critical factor for PRL-mediated activation of STAT5 in the mammary epithelium, controlling production of a subset of the EGF family growth factors and consequent activation of their receptors on mammary epithelial cells. Consistent with this finding, overexpression of CUZD1 in non-transformed mammary epithelial HC11 cells increased their proliferation and induced tumorigenic characteristics in these cells. When introduced orthotopically in mouse mammary glands, these cells formed adenocarcinomas *in vivo*. These tumors showed elevated levels of STAT5 phosphorylation and activation of the EGF signaling pathway. Blockade of STAT5 signaling by pimozide, a selective STAT5 inhibitor, markedly reduced the production of the EGF family growth factors and inhibited PRL-induced tumor cell proliferation *in vitro*. It also impaired the progression of CUZD1-driven mammary tumorigenesis *in vivo*. Analysis of human MCF7 breast cancer cells indicated that CUZD1 controls the production of the same subset of the EGF family members in these cells. Treatment with pimozide, which blocks STAT5 activation in these cancer cells, inhibited their proliferation. Collectively, these findings indicated that dysregulation of CUZD1, a regulator of growth pathways controlled by PRL and STAT5, promotes mammary tumorigenesis. Blockade of STAT5 pathway downstream of CUZD1 offers a novel therapeutic strategy for certain types of breast tumors.

INTRODUCTION

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) plays an integral role in regulating mammary gland development during pregnancy and lactation (6). Binding of PRL to the prolactin receptor (PRLR) activates a signaling cascade which includes members of the Janus Kinase (JAK) family and the Signal Transducer and Activator of Transcription 5 (STAT5) (7–13). STAT5, a transcription factor, is phosphorylated and activated by JAKs associated with PRLR (14–19). The changes in gene expression

brought about by 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

Active signaling through phosphorylated STAT5 is key to the proliferation of mammary epithelial cells during alveologenesis (23,25–27). Mitogenic genes under the control of STAT5 include Cyclin D1 and certain members of the epidermal growth factor (EGF) family, such as epiregulin (*Ereg*), which induce expansion of the mammary epithelium prior to 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 between, and regulation of, the PRL/STAT5 and ErbB 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 cell cycle progression, proliferation and differentiation in breast cancer cells (31,38). In an oncogene-induced tumor model (MMTV-PyVT), *Prl*$^{-/-}$ mice exhibit delayed tumor development and slower (30%) tumor progression when compared to control (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 a hyperproliferative mammary epithelium, delayed involution and a predisposition to mammary tumor formation (41). Collectively, these studies demonstrated that aberrant PRL/STAT5 signaling contributes to breast cancer.

Similarly, an increase in signaling through the EGF pathway has long been implicated in promoting the proliferation of mammary epithelial cells in breast cancer (32,42–44). Many drugs focused on inhibiting activation of ErbB receptors have been developed to target this pathway and treat breast cancer (45,46). Analysis of ErbB receptor expression and activation via phosphorylation in human breast tumors provides important prognostic information and predict 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) fits such a role (51). The *Cuzd1*-null mouse model provided evidence that CUZD1 is a key regulator of alveolar development and lactation. It is a mediator of PRL/STAT5 signaling in the mammary gland during puberty and pregnancy. CUZD1 controls STAT5 phosphorylation in mammary epithelial cells, and pSTAT5 performs the dual role of promoting proliferation and differentiation of the mammary epithelium. 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 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 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 pimozide, a selective inhibitor of STAT5, 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 mice. As described previously, a lentiviral expression vector harboring a full-length cDNA encoding *Cuzd1* or *β-galactosidase* (control) was integrated into HC11 cells to generate stable cell lines which constitutively express elevated levels of *Cuzd1* (HC11-Cuzd1) or *β-galactosidase* (HC11-LacZ) (51). Western blot analysis indicated that HC11-Cuzd1 cells overexpress CUZD1 about two fold over the HC11-LacZ control cells (51). These cells also expressed prolactin receptor and low levels of estrogen receptor α and progesterone receptor. We then subjected these cells to a cell invasion assay using Boyden chambers as described in the Materials and Methods. The HC11-Cuzd1 cells exhibited enhanced motility and were able to migrate across a barrier while control HC11-LacZ cells failed to penetrate the membrane (Fig. 3.1, A). We then subjected these cells to a soft agar assay to assess their anchorage independent growth, a well-

known marker of cell transformation (52). As shown in Fig. 3.1B, HC11-Cuzd1 cells formed large colonies when cultured in media containing soft agar, whereas the HC11-LacZ cells remained as single cells in the agar suspension. As a control, we used MCF7 breast cancer cells, which are known to form robust colonies on soft agar (Fig. 3.1, B). These findings indicated that the overexpression of *Cuzd1* in HC11 mammary epithelial cells altered their growth and migratory properties, two important hallmarks of pre-cancerous cells.

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 six weeks, mice injected with HC11-Cuzd1 cells manifested palpable tumors, which continued to grow over 18 weeks (Fig. 3.2, A). All of the mice injected with HC11-Cuzd1 cells had tumors ranging in size between 200-250mm^3, while mice injected with control HC11-Lacz cells did not form any detectable tumor (Fig. 3.2, B). Tumor growth was measured weekly and tumor volume was calculated as described in Materials and Methods.

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 while 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-Cuzd 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 4th mammary gland pair in BALB/c mice, mammary tumors developed and grew rapidly to reach a volume of about 50mm^3 at five weeks, 200-250mm^3 at seven weeks, and eventually reaching a volume of almost 900mm^3 at 9 weeks (Fig. 3.2, C). These tumors grew larger more rapidly than those formed by HC11-Cuzd1 cells, which were less than 300mm^3 at eighteen weeks.

For histological analysis of mammary tumors, mice were sacrificed at eighteen weeks following injection of HC11 cells into nude mice and 9 weeks following injection of HC11-Cuzd1 cells into nude mice. 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. 3.3, A-D). The mammary glands appeared normal (Fig.

3.3B). The tumors were verified by pathological examination as adenocarcinomas with lobular characteristics (Fig. 3.3, D). When the tumors were examined by immunohistochemical staining using a CUZD1-specific antibody, we observed a robust expression of CUZD1 in both cytoplasmic and nuclear compartments (Fig. 3.3, 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. 3.3, E and F). These tumors also expressed a high level of PCNA, indicative of highly proliferative cells in the tumor tissue (Fig. 3.3, G). In one out of five animals with breast tumors, we noted liver and lung lesions consistent with metastasis of primary adenocarcinoma to other organs. 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. Ample STAT5 phosphorylation was detected along with total STAT5 in the tumor sections (Fig. 3.4, A and C). 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 expression of high levels of EREG, EPGN and NRG1 proteins was evident in these tumors (Fig. 3.4, D, F, and G). Additionally, abundant active (phosphorylated) forms of ErbB1 and ErbB4 were present in the tumors (Fig. 3.4, H and J). However, activated (phosphorylated) ErbB2 was undetectable in the tumor sections (Fig. 3.4, I). 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 and an absence of pAKT, indicating that ERK signaling is indeed activated and it potentially regulates proliferation of the Cuzd1-driven breast tumor cells, but their proliferation is not dependent on the AKT pathway (Fig. 3.4, J and K).

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

Since 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 pimozide, a selective inhibitor of STAT5 phosphorylation. As shown by western blotting, pimozide treatment dramatically reduced the activating phosphorylation of STAT5 in these cells compared to the vehicle treatment (Fig. 3.5A). Total STAT5 protein levels were unaffected by pimozide treatment (Fig. 3.5A). We further confirmed by immunocytochemistry that PRL-induced phosphorylation of STAT5 in HC11-Cuzd1 cells is attenuated by pimozide treatment (Fig. 3.5B). These data established that pimozide 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 pimozide reduced the expression of *Ereg*, *Epgn* and *Nrg1* transcripts in HC11-Cuzd1 cells (Fig. 3.5C), confirming that inhibition of STATS signaling inhibits growth factor pathways critical for tumor cell proliferation. A BrdU incorporation assay was used to assess whether proliferation of HC11-Cuzd1 cells treated with PRL was indeed affected by pimozide. As shown in Fig. 3.5D, significant reduction in PRL-induced proliferation of HC11-Cuzd1 cells was observed following pimozide treatment.

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

We next tested whether inhibition of STAT5 signaling by pimozide 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 pimozide or a vehicle control. At the end of five weeks of tumor growth, and pimozide or vehicle treatment, we observed a drastic reduction in tumor size in mice treated with pimozide compared to mice treated with vehicle control (Fig. 3.6, A and B). Immunohistochemical analysis of STAT5 phosphorylation showed widespread STAT5 phosphorylation in vehicle-treated mammary tumors, whereas markedly diminished pSTAT5 was observed in tumors of mice treated with pimozide (Fig. 3.7A, a, b, d, and e). Analysis of STAT5 staining indicated that pimozide did not affect total STAT5 protein levels (Fig. 3.7A, panels c and f).

Using immunohistochemistry, 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 pimozide treatment (Fig. 3.7, B). Our results showed that inhibition of STAT5 signaling by pimozide, 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. 3.7B, a-e and g-k, insets total ErbB1 and ErbB4). To determine if proliferation is reduced with pimozide treatment, we conducted IHC using a well-known marker of proliferation, Ki67. This staining indicates that tumor cell proliferation is dramatically reduced with pimozide treatment (Fig. 3.7B, f and l). Collectively, these data support our view that the inhibition of STAT5 signaling by pimozide, and resulting impairment in proliferation 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, therefore, examined the expression of *CUZD1* transcripts in several human breast cancer cell lines. As shown in Fig. 3.8A, *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 media but it rapidly translocated to the nucleus upon treatment with serum (Fig. 3.7, 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 *β-galactosidase* were used as a control (MCF7-LacZ). While STAT5 phosphorylation was evident in MCF7-LacZ cells (Fig. 3.8C, a), the level of pSTAT5 increased in MCF7-Cuzd1 cells (Fig. 3.8C, c). Pimozide treatment strongly inhibited STAT5 phosphorylation in both MCF7-Cuzd1 and MCF7-LacZ cells (Fig. 3.8C, 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 to their levels in MCF7-LacZ cells (Fig. 3.8D). Finally, we performed a BrdU incorporation assay by growing MCF7-Cuzd1 cells in the presence or absence of pimozide. Our results showed that pimozide markedly inhibited the proliferation of MCF7-Cuzd1 in growth media containing serum (Fig. 3.8E). 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 (53). Our recent studies identified CUZD1 as a key cellular protein that functions as an essential regulator of STAT5 activation 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 pre-cancerous transformation of these cells. Introduction of these 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 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 (54). The ErbB receptors are activated at all stages of mammary development and contribute to normal breast functions (32,44,54). However, a large body of evidence suggests that aberrant activation of the ErbB receptors plays a key role in giving rise to malignant phenotypes, including cell proliferation, differentiation, angiogenesis, and invasion and survival

(44,46,55). 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 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). Constitutive expression 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 intervention needs to occur further downstream in the signaling pathway (56–58). Researchers have been targeting multiple steps along the PRL signaling pathway, from competitive PRLR antagonists to pharmacologic inhibition of PRL signal transducers (59). 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 re-purposed a previously FDA approved antipsychotic drug, pimozide, which is used to treat mental disorders such as schizophrenia, psychosis and Tourette syndrome (60,61). *In vitro* 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 (62,63). Although the mechanism of STAT5 inhibition by pimozide is yet to be elucidated, it selectively inhibits STAT5 phosphorylation and transcription of STAT5 target genes (64,65). In our study, inhibition of STAT5 phosphorylation through the use of pimozide reduced the production of the EGF family members *Ereg*, *Epgn*, and *Nrg1*, activation of ErbB1 and ErbB4 receptors, and resulted 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 the mouse and the human (66). 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 ERα-negative tumor cells, but present in ERα-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 E (67). It is therefore possible that ERα regulates *CUZD1* expression in certain human breast cancer cells. Previous studies reported that the PRLR and ERα 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 (68,69). 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(70–72). 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 indicated 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 since 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.

MATERIALS AND METHODS

Animals

Mice were maintained in the designated animal care facility at the University of Illinois, per 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 (IACUC).

Cell Lines and Cell Culture

The HC11 cell line is a non-transformed mammary epithelial cell line derived from pregnant BALB/c mice(73). These cells were grown in RPMI-1640 supplemented with 5% (v/v) fetal bovine serum, 5 µg/ml insulin and 10ng/ml EGF at 37°C and 5% CO_2. HC11-LacZ, HC11-Cuzd1, MCF7-LacZ, and MCF7 -Cuzd1 cell lines were developed as described in Mapes and Li *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 1mm pieces using scissors and fragments were digested in HBSS with 6g/L dispase and 0.5g/L collagenase at 37°C for 1 h with constant agitation. Enzymes were neutralized and the cell suspension was passed through a 100µm mesh, followed by two washes with HBSS. 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 50µm PRL followed by immunocytochemistry or RNA analysis. The STAT5 inhibitor pimozide was used at 10µM (unless otherwise designated) along-side a vehicle control (DMSO) prior to immunocytochemistry, western blotting, proliferation assays, and RNA analysis.

Boyden Chamber Cell Migration Assay

Boyden-chambers (Millipore) were placed in 24-well dishes containing chemoattract media (RPMI containing 10% FBS). Serum-starved cells HC11-LacZ or –Cuzd1 cells (1 x 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 CyQuant (Millipore) fluorometric assay per 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 (1×10^4) or MCF7 cells (control) were seeded in six-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 a 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 5×10^3 cells/well in 96-well plates and cultured overnight in full growth medium. Following 48h of serum starvation, cells were treated with selected compounds in addition to vehicle or pimozide and allowed to grow for 18h. BrdU was added and incorporation was measured after 2h using an ELISA-based BrdU assay. Resulting color reaction was measured using a plate reader at 370nm. The relative levels of BrdU incorporation from three independent measurements are shown (Mean ± SEM).

Quantitative real-time PCR (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 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 SD was calculated from individual Ct values from 3-4 replicates of a sample. Normalized mean Ct was computed as ΔCt by subtracting mean Ct of 36B4 from Ct of a target gene for control sample. ΔΔCt was then calculated as a difference in ΔCt 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 ± the standard error from three or more independent trials.

Orthotopic Intraductal Injection of Cells into the Mammary Gland

HC11-Cuzd1 or HC11-Cuzd1 Tum cells (1×10^6) were suspended in Matrigel and orthotopically injected into the nipple of the fourth abdominal mammary gland of nude or BALB/c mice. Equal numbers of mice were orthotopically injected with HC11-LacZ cells during each tumor study. Tumor length and width were measured using digital calipers and tumor volume was calculated (tumor volume=1/2(length $\times$ width^2).

Pimozide Treatment

Female BALB/c mice were orally treated with 5mg/kg body weight pimozide or a vehicle control (DMSO), suspended in corn oil, once a day for three days prior to orthotopic injection. The treatment regimen of pimozide included three days of treatment followed by three days of rest, which cycled until the end of the five weeks. Tumor volume was measured throughout the course of the five-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(74). Rabbit polyclonal antibodies against a peptide antigen containing amino acids SSPNYPKPHPEL of mouse CUZD1 were generated in our laboratory. 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 (HRP)–conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. Sections were counterstained with hematoxylin and mounted. For immunofluorescence staining, fluorescence-conjugated ant-mouse IgG or anti-rabbit IgG were used as secondary antibody and slides were mounted in Prolong GOLD and cured for 24 hours 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 ant

mouse IgG or anti-rabbit IgG were used as secondary antibody and slides were mounted in Prolong GOLD and cured for 24 hours before imaging.

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.

Statistical analysis

Statistical analysis was performed by the Student *t*-Test. Statistically significant differences ($P<0.05$) are indicated by *.

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CONFLICT OF INTEREST

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

Study concept and design: JM, LA, QL, CC, IB, MB. Acquisition of data: JM, LA, QL. Analysis and interpretation of data: JM, LA, QL, CC, IB, MB. Drafting of manuscript: JM, LA, MB. Critical revision: JM, CC, MB.

REFERENCES

1. Hennighausen L, Robinson GW. Signaling Pathways in Mammary Gland Development. Dev Cell. 2001;1(4):467–75.
2. Hennighausen L, Robinson GW. Information networks in the mammary gland . Nat Rev cell Biol. 2005 Sep;6(9):715–25.
3. Brisken C, O'Malley B. Hormone Action in the Mammary Gland. Cold Spring Harb Perspect Biol. 2010 Dec 1;2(12):a003178–a003178.
4. Watson CJ, Khaled WT. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. Development. 2008 Mar;135(6):995–1003.
5. Rillema JA. Development of the mammary gland and lactation. Trends Endocrinol Metab. 1994;5(4):149–54.
6. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, et al. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J. 1997 Dec 1;2014(23):6926–35.
7. Gao J, Hughes JP, Auperin B, Buteau H, Edery M, Zhuang H, et al. Interactions among Janus kinases and the prolactin (PRL) receptor in the regulation of a PRL response element . Mol Endocrinol. 1996 Jul;10(7):847–56.
8. Ali S. Prolactin Receptor Regulates Stat5 Tyrosine Phosphorylation and Nuclear Translocation by Two Separate Pathways. J Biol Chem. 1998;273(13):7709–16.
9. Hennighausen L, Robinson GW, Wagner K-U, Liu X. Prolactin Signaling in Mammary Gland Development. J Biol Chem. 1997;272(12):7567–9.
10. Clevenger C V, Plank TL. Prolactin as an autocrine/paracrine factor in breast tissue . J Mammary Gland Biol Neoplasia. 1997 Jan;2(1):59–68.
11. Brisken C, Kaur S, Chavarria TE, Binart N, Sutherland RL, Weinberg RA, et al. Prolactin controls mammary gland development via direct and indirect mechanisms. Dev Biol. 1999 Jun;210(1):96–106.
12. DaSilva L, Howard OMZ, Rui H, Kirken RA, Farrar WL, Howard 0. M Zack, et al. Growth Signaling and JAK2 Association Mediated by Membrane-proximal Cytoplasmic Regions of Prolactin Receptors. J Biol Chem. 1994 Jul 15;2014:18267–70.
13. Rodig SJ, Meraz MA, White JM, Lampe PA, Riley JK, Arthur CD, et al. Disruption of the Jak1 Gene Demonstrates Obligatory and Nonredundant Roles of the Jaks in Cytokine-Induced Biologic Responses. Cell. 1998;93(3):373–83.
14. Wagner K-UU, Schmidt JW. The two faces of Janus kinases and their respective STATs in mammary gland development and cancer. J Carcinog. 2011;10(1):32.
15. Wagner K-U, Rui H. Jak2/Stat5 Signaling in Mammogenesis, Breast Cancer Initiation and Progression. J Mammary Gland Biol Neoplasia. 2008 Mar 29;13(1):93–103.
16. Nevalainen MT, Xie J, Bubendorf L, Wagner K-U, Rui H. Basal Activation of Transcription Factor Signal Transducer and Activator of Transcription (Stat5) in Nonpregnant Mouse and Human Breast Epithelium. Mol Endocrinol. 2002 May;2014(3/5/2014):1108–24.
17. Hennighausen L, Robinson GW. Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. Genes Dev. 2008;22(6):711–21.
18. Ihle JN, Kerr IM. Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet [Internet]. 1995;11(2):69–74. Available from: http://dx.doi.org/10.1016/s0168-9525(00)89000-9
19. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges . Gene. 2002 Feb 20;285(1–2):1–24.
20. Schmitt-Ney M, Doppler W, Ball RK, Groner B. Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. Mol Cell Biol. 1991 Jul;2014(3/2/2014):3745–55.

21. Yamaji D, Kang K, Robinson GW, Hennighausen L. Sequential activation of genetic programs in mouse mammary epithelium during pregnancy depends on STAT5A/B concentration. Nucleic Acids Res. 2013 Feb 1;41(3):1622–36.
22. Fuh G, Wells JA. Prolactin receptor antagonists that inhibit the growth of breast cancer cell lines . J Biol Chem. 1995 Jun 2;270(22):13133–7.
23. Liu X, Robinson GW, Hennighausen L. Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation . Mol Endocrinol. 1996 Dec;10(12):1496–506.
24. Arendt LM, Rugowski, Debra E Grafwallner-Huseth, Tara A Garcia-Barchino MJ, Rui H, Schuler LA. Prolactin-induced mouse mammary carcinomas model estrogen resistant luminal breast cancer. Breast Cancer Res. 2011 Jan 28;13(1):R11.
25. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L. Stat5a is mandatory for adult mammary gland development and lactogenesis . Genes Dev. 1997 Jan 15;11(2):179–86.
26. Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng C-X, et al. Inactivation of Stat5 in Mouse Mammary Epithelium during Pregnancy Reveals Distinct Functions in Cell Proliferation, Survival, and Differentiation. Mol Cell Biol. 2004;2014(18):8037.
27. Clarkson RWE, Boland MP, Kritikou EA, Lee JM, Freeman TC, Tiffen PG, et al. The Genes Induced by Signal Transducer and Activators of Transcription (STAT)3 and STAT5 in Mammary Epithelial Cells Define the Roles of these STATs in Mammary Development: Mol Endocrinol. 2006 Mar;2014(3/5/2014):675–85.
28. Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, et al. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell. 1995;82(4):621–30.
29. Creamer BA, Sakamoto K, Schmidt JW, Triplett AA, Moriggl R, Wagner KU. Stat5 Promotes Survival of Mammary Epithelial Cells through Transcriptional Activation of a Distinct Promoter in Akt1. Mol Cell Biol. 30(12):2957–70.
30. Zhu BM, Kang K, Yu JH, Chen W, Smith HE, Lee D, et al. Genome-wide analyses reveal the extent of opportunistic STAT5 binding that does not yield transcriptional activation of neighboring genes. Nucleic Acids Res. 2012 May;40(10):4461–72.
31. Sato T, Tran TH, Peck AR, Liu C, Ertel A, Lin J, et al. Global profiling of prolactin-modulated transcripts in breast cancer in vivo. Mol Cancer. 2013 Jun 12;12:59.
32. Troyer KL, Lee DC. Regulation of mouse mammary gland development and tumorigenesis by the ERBB signaling network. J Mammary Gland Biol Neoplasia. 2001 Jan;6(1):7–21.
33. Stern DF. ErbBs in mammary development. Exp Cell Res. 2003 Mar 10;284(1):89–98.
34. Clevenger C V., Gadd SL, Zheng J. New mechanisms for PRLr action in breast cancer. Vol. 20, Trends in Endocrinology and Metabolism. 2009. p. 223–9.
35. Brisken C, Hess K, Jeitziner R. Progesterone and Overlooked Endocrine Pathways in Breast Cancer Pathogenesis. Endocrinology. 2015 Aug 4;en20151392.
36. Hynes NE, Watson CJ. Mammary Gland Growth Factors: Roles in Normal Development and in Cancer. Cold Spring Harb Perspect Biol. 2010;2(8):a003186–a003186.
37. Visvader JE, Lindeman GJ. Transcriptional regulators in mammary gland development and cancer. Int J Biochem Cell Biol. 2003;35(7):1034–51.
38. Iavnilovitch E, Cardiff RD, Groner B, Barash I. Deregulation of Stat5 expression and activation causes mammary tumors in transgenic mice. Int J cancerJournal Int du cancer. 2004 Nov 20;112(4):607–19.
39. Vomachka AJ, Pratt SL, Lockefeer JA, Horseman ND. Prolactin gene-disruption arrests mammary gland development and retards T-antigen-induced tumor growth . Oncogene. 2000 Feb 21;19(8):1077–84.
40. Rose-Hellekant TA, Arendt LM, Schroeder MD, Gilchrist K, Sandgren EP, Schuler LA. Prolactin induces ERalpha-positive and ERalpha-negative mammary cancer in transgenic mice. Oncogene. 2003 Jul 24;22(30):4664–74.

41. Iavnilovitch E, Groner B, Barash I. Overexpression and forced activation of stat5 in mammary gland of transgenic mice promotes cellular proliferation, enhances differentiation, and delays postlactational apoptosis . Mol Cancer Res. 2002 Nov;1(1):32–47.
42. Richard J, Sainsbury C, Needham G, Farndon J, Malcolm A, Harris A. Epidermal-Growth-Factor Receptor Status as Predictor of Early Recurrence of and Death From Breast Cancer. Lancet. 1987 Jun;329(8547):1398–402.
43. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol. 1995 Jul;19(3):183–232.
44. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level . Nat Rev cell Biol. 2006 Jul;7(7):505–16.
45. Atalay G, Cardoso F, Awada A, Piccart MJ. Novel therapeutic strategies targeting the epidermal growth factor receptor (EGFR) family and its downstream effectors in breast cancer. Ann Oncol Off J Eur Soc Med Oncol ESMO. 2003 Sep 1;2011(4/14/2011):1346–63.
46. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer. 2005;5(5):341–54.
47. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat. 2012 Nov 17;136(2):331–45.
48. Carver KC, Arendt LM, Schuler LA. Complex prolactin crosstalk in breast cancer: new therapeutic implications. Mol Cell Endocrinol. 2009 Aug 13;307(1–2):1–7.
49. Scotti ML, Langenheim JF, Tomblyn S, Springs AEB, Chen WY. Additive effects of a prolactin receptor antagonist, G129R, and herceptin on inhibition of HER2-overexpressing breast cancer cells. Breast Cancer Res Treat. 2008 Sep 23;111(2):241–50.
50. Howell SJ, Anderson E, Hunter T, Farnie G, Clarke RB. Prolactin receptor antagonism reduces the clonogenic capacity of breast cancer cells and potentiates doxorubicin and paclitaxel cytotoxicity. Breast Cancer Res. 2008 Aug 5;10(4):R68.
51. Mapes J, Li Q, Kannan A, Anandan L, Laws M, Lydon JP, et al. CUZD1 is a critical mediator of the JAK/STAT5 signaling pathway that controls mammary gland development during pregnancy. Hunter KW, editor. PLOS Genet. 2017 Mar 9;13(3):e1006654.
52. Macpherson I, Montagnier L. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology. 1964 Jun;23(2):291–4.
53. Sakamoto K, Creamer BA, Triplett AA, Wagner K-U. The Janus Kinase 2 Is Required for Expression and Nuclear Accumulation of Cyclin D1 in Proliferating Mammary Epithelial Cells. Mol Endocrinol. 2007;21(8).
54. Schroeder JA, Lee DC. Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. Cell Growth Differ. 1998 Jun;2014:451–64.
55. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001 Feb 1;2(2):127–37.
56. Ben-Jonathan N, Mershon JL, ALLEN DL, Steinmetz RW. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr Rev. 1996 Dec;17(6):639–69.
57. Shaw-Bruha CM, Pirrucello SJ, Shull JD. Expression of the prolactin gene in normal and neoplastic human breast tissues and human mammary cell lines: Promoter usage and alternative mRNA splicing. Breast Cancer Res Treat. 1997;44(3):243–53.
58. Clevenger C V, Furth PA, Hankinson SE, Schuler LA. The Role of Prolactin in Mammary Carcinoma. Endocr Rev. 2003 Feb;2013(1):1–27.
59. Clevenger C V, Zheng J, Jablonski EM, Galbaugh TL, Fang F. From bench to bedside: future potential for the translation of prolactin inhibitors as breast cancer therapeutics . J Mammary Gland Biol Neoplasia. 2008 Mar;13(1):147–56.
60. Mothi M, Sampson S, Meghana : Mothi, Stephanie : Sampson. Pimozide for schizophrenia or related psychoses. Mothi M, editor. Cochrane Database Syst Rev. 2013 Nov 5;(11):CD001949.

61. Sallee FR, Nesbitt L, Jackson C, Sine L, Sethuraman G. Relative efficacy of haloperidol and pimozide in children and adolescents with Tourette's disorder. Am J Psychiatry. 1997 Aug;154(8):1057–62.
62. Strobl JS, Kirkwood KL, Lantz TK, Lewine MA, Peterson VA, Worley JF, et al. Inhibition of human breast cancer cell proliferation in tissue culture by the neuroleptic agents pimozide and thioridazine. Cancer Res. 1990 Sep 1;50(17):5399–405.
63. Wiklund ED, Catts VS, Catts S V., Ng TF, Whitaker NJ, Brown AJ, et al. Cytotoxic effects of antipsychotic drugs implicate cholesterol homeostasis as a novel chemotherapeutic target. Int J cancerJournal Int du cancer. 2010 Jan 1;126(1):28–40.
64. Bar-Natan M, Nelson EA, Walker SR, Kuang Y, Distel RJ, Frank DA. Dual inhibition of Jak2 and STAT5 enhances killing of myeloproliferative neoplasia cells. Leukemia. 2012 Jun 2;26(6):1407–10.
65. Nelson EA, Walker SR, Weisberg E, Bar-Natan M, Barrett R, Gashin LB, et al. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors . Blood. 2011 Mar 24;117(12):3421–9.
66. Leong CTC, Ng CY, Ong CK, Ng CP, Ma ZS, Nguyen TH, et al. Molecular cloning, characterization and isolation of novel spliced variants of the human ortholog of a rat estrogen-regulated membrane-associated protein, UO-44. Oncogene. 2004 Jul 22;23(33):5707–18.
67. Chen D, Xu X, Zhu L-JJ, Angervo M, Li Q, Bagchi MK, et al. Cloning and Uterus/Oviduct-specific Expression of a Novel Estrogen-regulated Gene (ERG1). J Biol Chem. 1999 Nov 5;2014:32215–24.
68. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013 Apr 2;6(269):pl1.
69. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio Cancer Genomics Portal: An open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012 May;2(5):401–4.
70. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002 Jan 31;415(6871):530–6.
71. Cui Q. A network of cancer genes with co-occurring and anti-co-occurring mutations. Rogers S, editor. PLoS One. 2010 Oct 4;5(10):e13180.
72. Maskery SM, Zhang Y, Jordan RM, Hu H, Hooke JA, Shriver CD, et al. Co-occurrence analysis for discovery of novel breast cancer pathology patterns. IEEE Trans Inf Technol Biomed. 2006 Jul;10(3):497–503.
73. Danielson KG, Knepper JE, Kittrell FS, Butel JS, Medina D, Durban EM. Clonal populations of the mouse mammary cell line, COMMA-D, which retain capability of morphogenesis in vivo . In Vitro Cell Dev Biol. 1989 Jun;25(6):535–43.
74. Li Q, Cheon YP, Kannan A, Shanker S, Bagchi IC, Bagchi MK. A novel pathway involving progesterone receptor, 12/15-lipoxygenase-derived eicosanoids, and peroxisome proliferator-activated receptor gamma regulates implantation in mice . J Biol Chem. 2004 Mar 19;279(12):11570–81.

FIGURES

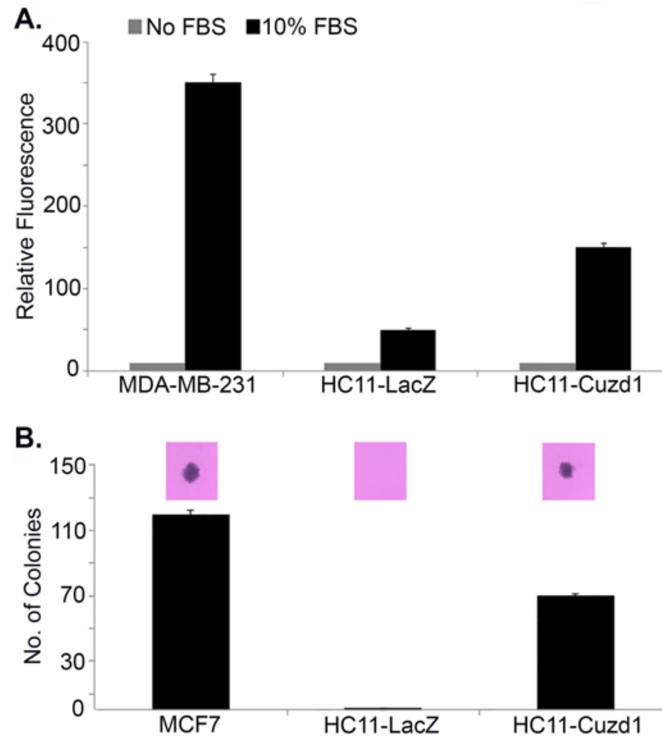

Figure 3.1. Overexpression of *Cuzd1* leads to enhanced motility and anchorage-independent growth of HC11 cells. **A. Overexpression of *Cuzd1* leads to enhanced motility of HC11 cells.** Serum starved MDA-MB-231 cells (positive control), HC11-LacZ, or HC11-Cuzd1 cells were placed in Boyden chambers and allowed to migrate toward 10% FBS for 72 h. The number of invading cells was quantified using CyQuant fluorescence labeling and compared to corresponding cells unexposed to the serum chemoattractant. Data are represented as Relative Fluorescence ± SEM from ≥3 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 media containing soft agar. Colonies were allowed to form for 16 days and stained with crystal violet overnight. Visible colonies (>0.5mm) were counted using a dissecting microscope. Data are represented as Number of Colonies (>0.5mm) ± SEM from ≥3 biological replicates. Images above bar show representative colony sizes from each treatment group (p=0.0003).

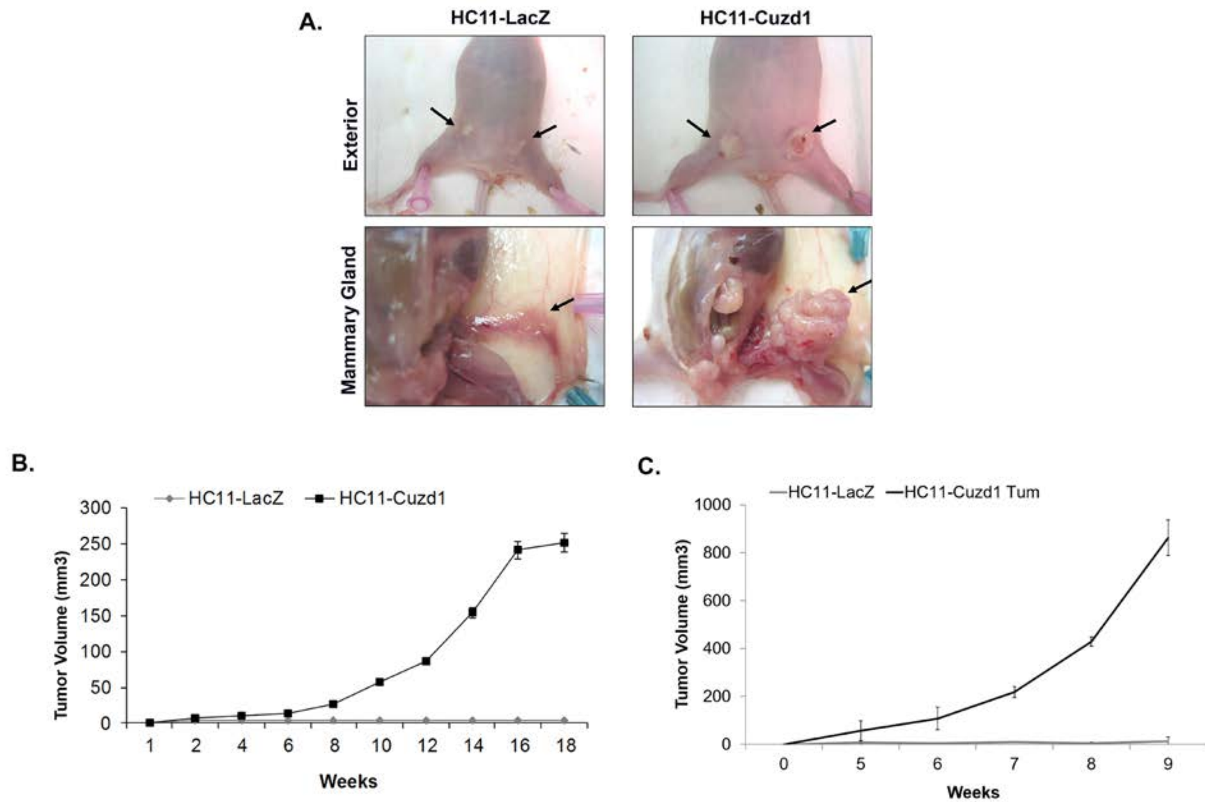

Figure 3.2. Orthotopic injections of HC11-Cuzd1 cells form mammary tumors. A. External images of HC11-Cuzd1 cell tumors in nude mice. HC11-LacZ or HC11-Cuzd1 cells were injected orthotopically into the nipple of the 4th mammary gland of immunocompromised female nude mice. Mice were sacrificed 18 weeks post injection and examined for tumor growth. Top panels represent an exterior view of the animal and bottom panels show the 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=1/2(length $\times$ width^2) $\pm$ SEM, n=5 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 4th 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=1/2(length $\times$ width^2) $\pm$ SEM, n=3 in each group.

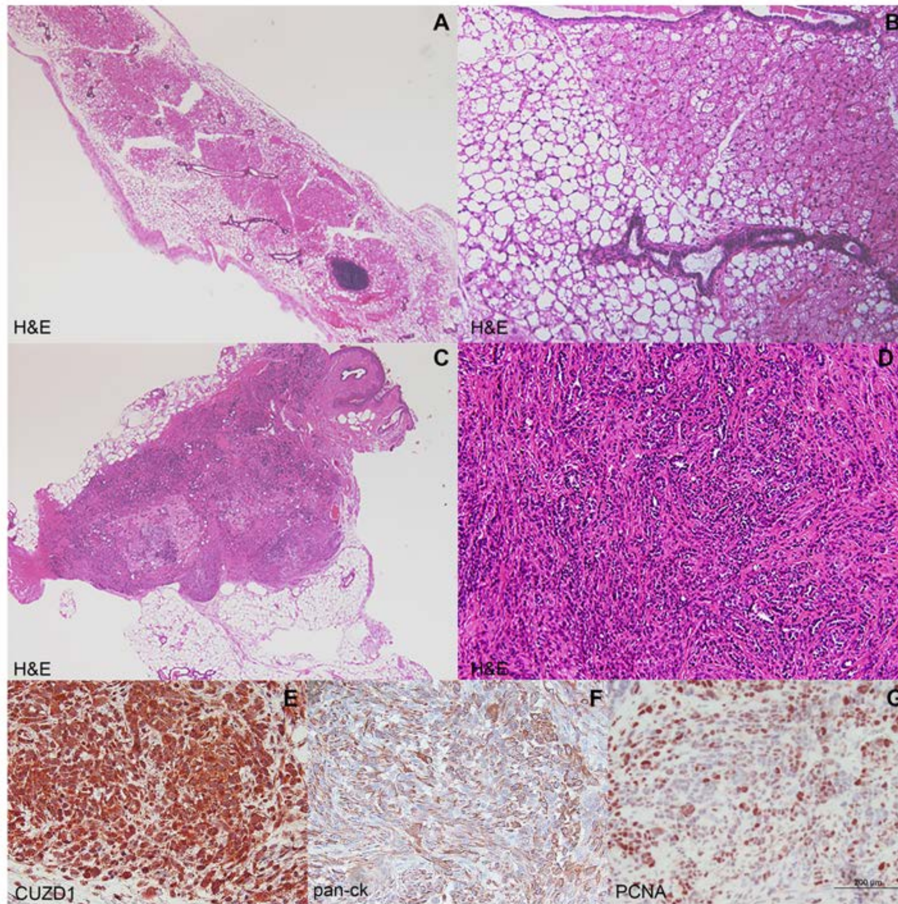

Figure 3.3. HC11-Cuzd1 cells form adenocarcinomas *in vivo*. Mammary glands and tumors were collected from mice injected with HC11-LacZ and HC11-Cuzd1, respectively, 18 weeks post-injection. The specimens were fixed, embedded in paraffin, sectioned, and subjected to H&E staining and imaged at 5x (A and C) and 40x (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, 40x. Data are representative images from n=5.

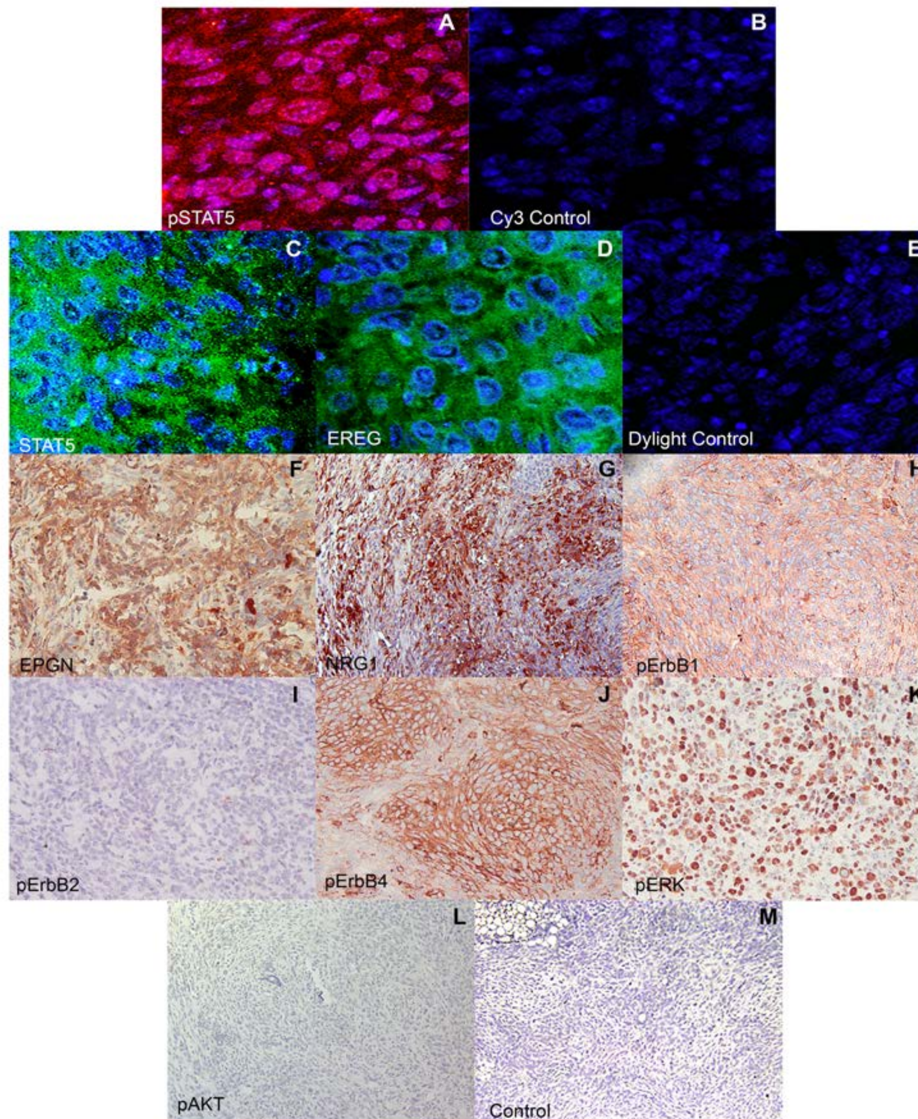

Figure 3.4. The ErbB1 and ErbB4 pathways are activated in HC11-Cuzd1 tumors *in vivo*. Tumors were collected 18 weeks post-injection, fixed, embedded in paraffin, sectioned, and subjected to IHC analysis was carried out with antibodies against pSTAT5 (A) (red), STAT5 (C) (green) EREG (D) (green), EPGN (F) (red), NRG1 (G) (red), pErbB1 (H) (red), pErbB2 (I) (red), pErbB4 (J) (red), pERK (K) (red), and pAKT (L) (red) and counterstained with hematoxylin (blue) or DAPI (blue). Control sections were stained in the absence of a primary antibody (B, E, and M). Magnification, 40x. Data are representative images from n=5.

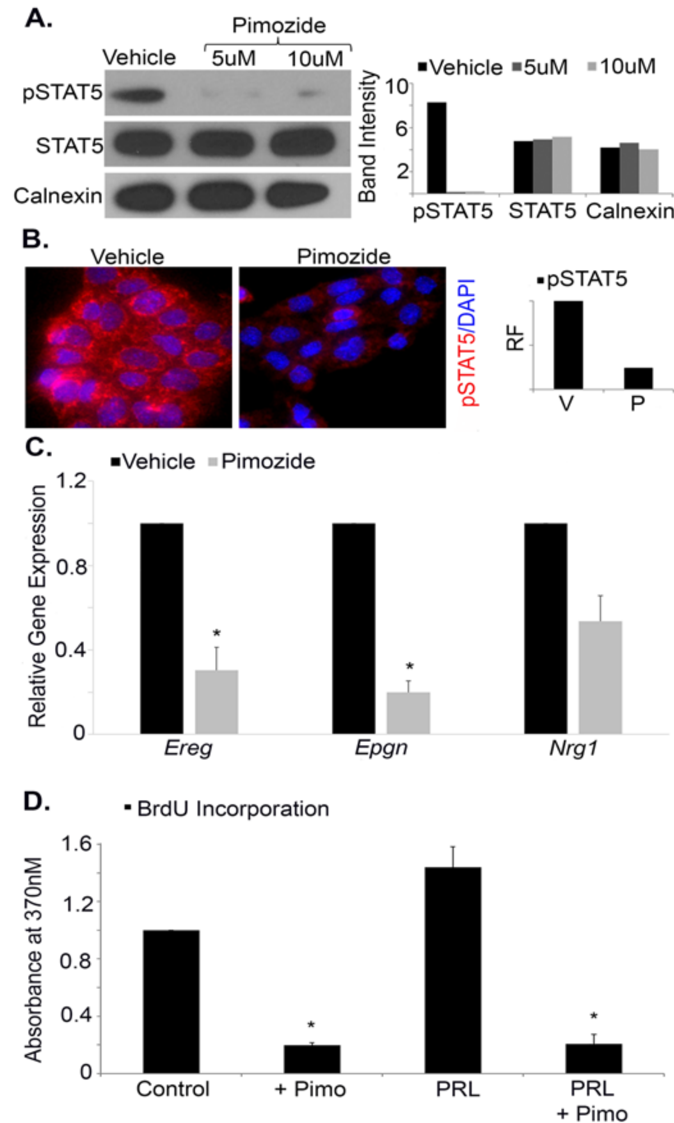

Figure 3.5. Pimozide inhibits STAT5 phosphorylation and cell proliferation. A. Phosphorylation of STAT5 is reduced following treatment with pimozide. HC11-Cuzd1 cells were plated, allowed to attach overnight and treated with PRL plus vehicle or pimozide at 5, 8 and 10uM for 3 h. Cells were then lysed 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 10uM 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 ≥3 biological replicates. Nuclear pSTAT5 was quantified using ImageJ and expressed as Relative Fluorescence (RF) from ≥3 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 10uM for 24 h. RNA was isolated and subjected to qPCR using gene specific primers to assess expression of EREG, EPGN and NREG1. Data are represented as Relative Gene Expression ± SEM from ≥3 biological replicates.

(Figure 3.5 con't.) 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 media 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 370nM ± SEM from ≥3 biological replicates.

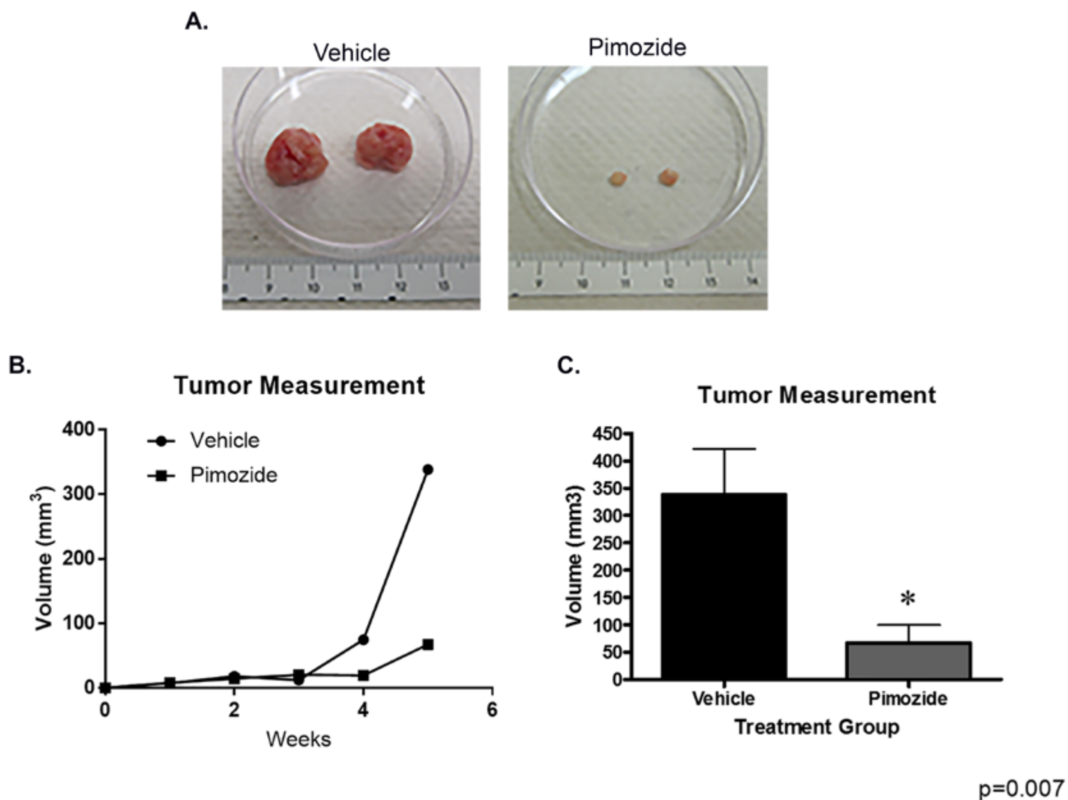

Figure 3.6. Pimozide treatment suppresses growth of HC11-Cuzd1-Tum cell tumors *in vivo*. A. Gross tumor size is reduced following Pimozide treatment. Representative images of tumors isolated from vehicle and pimozide treated mice. **B. Growth of tumors is reduced in mice treated with Pimozide.** Tumor volume in vehicle and Pimozide treated mice was measured over the course of five weeks using digital calipers. Tumor volume=1/2(length × width^2) ± SEM, n=5. **C. End tumor volume is decreased with pimozide treatment.** Final tumor volume in vehicle and Pimozide treated mice was measured using digital calipers. Tumor volume=1/2(length × width^2) ± SEM, n=5.

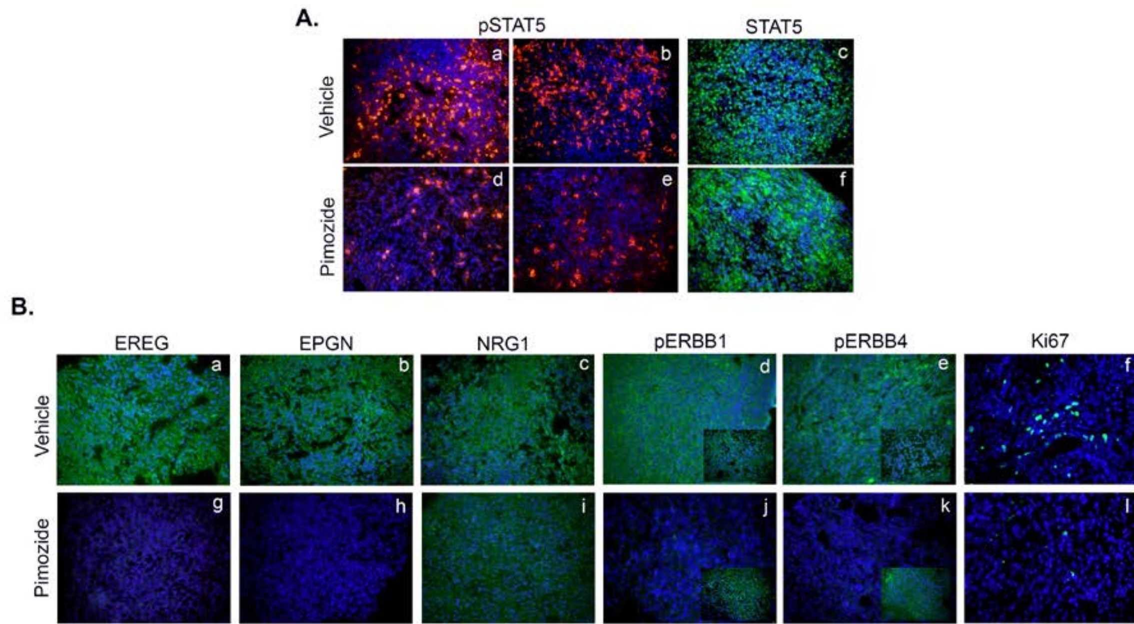

Figure 3.7. Pimozide treatment reduces STAT5 phosphorylation and blocks downstream ErbB signaling *in vivo*. A. STAT5 phosphorylation is decreased in the tumors of mice treated with pimozide. Tumors were collected, fixed, embedded in paraffin, sectioned, and subjected to IHC analysis with antibodies against pSTAT5 (a, b, d, and e) (red) or STAT5 (c and f) (green) and counterstained with DAPI (blue). Data are representative images from n=5. **B. Signaling through the EGF pathway is altered in mice treated with pimozide.** 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 on d and j) (green), pErbB4 (e and k) (green), and ErbB4 (insets on e and k) (green), Ki67 (f and l) (green), and counterstained with DAPI (blue). Data are representative images from n=5.

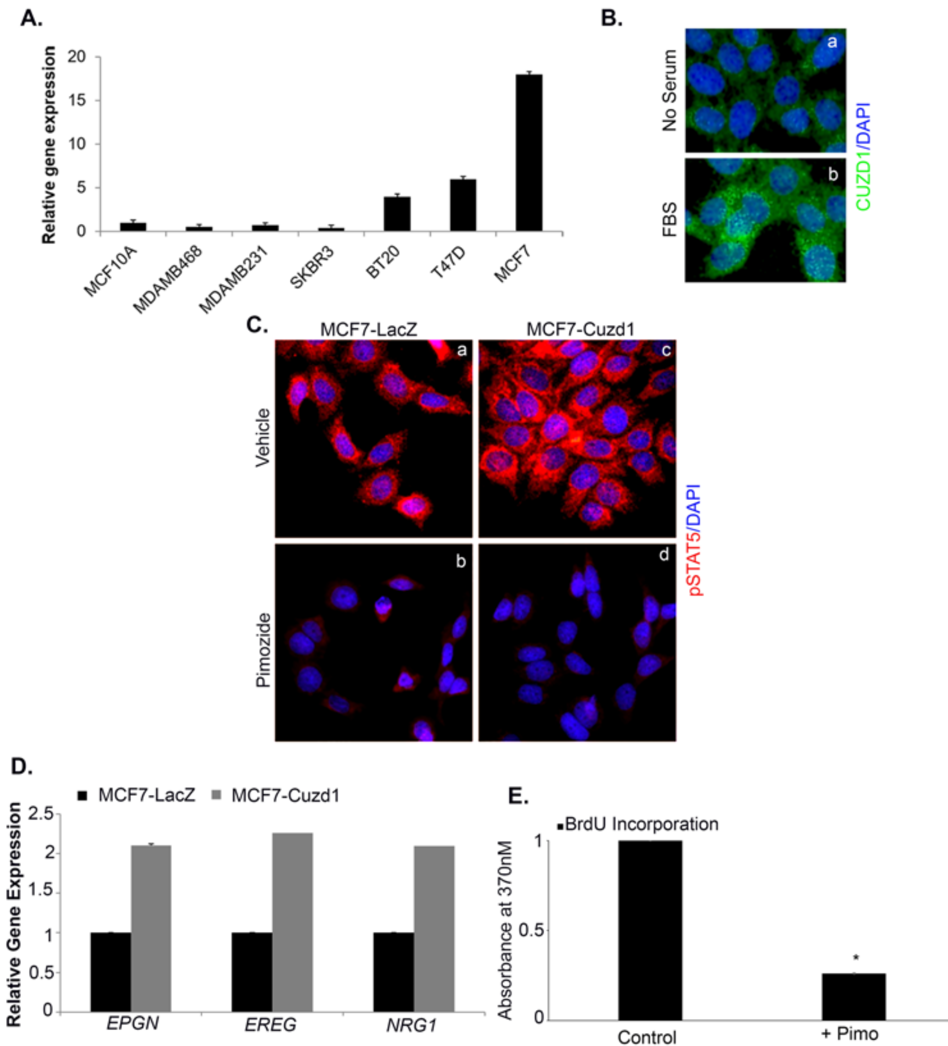

Figure 3.8. CUZD1 signaling in the human breast cancer MCF7 cells. A. MCF7 cells express high levels of *Cuzd1* mRNA. Human cancer cell lines were cultured in growth medium and mRNA isolated from these cells was examined for Cuzd1 expression. Data are represented as Relative Gene Expression ± SEM from ≥3 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 IF with an antibody specific for CUZD1 (green) and counterstained with DAPI (blue). Data are representative images from ≥3 biological replicates. **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 10uM (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 ≥3 biological replicates**. 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 ± SEM from ≥3 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 media, 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 370nM ± SEM from ≥3 biological replicates.

APPENDIX A

CUZD1 in Mammary Gland Biology: Perspective and Future Studies

In the studies described in Chapters 2 and 3, we have examined the role of CUZD1 in mammary epithelial cells during mammary gland development and tumorigenesis. We have shown that CUZD1 induces proliferation by promoting activation of STAT5 and the EGF family signaling pathway. Using a knockout mouse model, we demonstrated the importance of CUZD1 in the mammary epithelium during pregnancy and lactation. Mice lacking CUZD1 exhibit impaired STAT5 activation, fail to undergo alveologenesis, and are unable to lactate. A critical component of the phenotype of *Cuzd1*-null mice is a severe reduction in proliferation of the mammary epithelium, likely due to a loss of a subset of the EGF family ligands. This impairment in cell proliferation in the absence of CUZD1 prompted us to investigate its role in mammary tumorigenesis. In fact, overexpression of *Cuzd1* resulted in excessive mammary epithelial proliferation and generation of tumor *in vivo*. Analysis of CUZD1-overexpressing tumors revealed that STAT5, ErbB1, and ErbB4 signaling pathways are activated, and potentially play a role in the proliferation of these tumors. Collectively, CUZD1 emerged as a critical mediator of STAT5 and EGF family signaling during mammary gland development and tumorigenesis. In this final section, we will discuss some of the unresolved questions regarding the mechanism of CUZD1 and ongoing work.

Regulation of *Cuzd1* Expression in Mammary Gland: Evidence for a Role of Estrogen

Although our studies revealed the signal transduction pathways regulated by CUZD1, the mechanisms that regulate *Cuzd1* gene expression during mammary gland development remain unresolved. CUZD1 was initially discovered as an E-regulated gene in the rodent reproductive tract (1). We also investigated regulation of CUZD1 by E in mouse mammary epithelium and in human breast cancer cells. Treatment of ovariectomized mice with E led to up regulation of *Cuzd1* transcripts in the uterus and mammary gland (Fig. A.1A). Protein expression of CUZD1 is also up regulated in the mammary epithelium following treatment with E (Fig. A.1B). In human breast cancer cells, we observed a correlation between *CUZD1* expression and ER-positive status, which led us to further investigate E regulation of *CUZD1* in MCF7 cells. In MCF7 cells, *CUZD1* is up regulated following treatment with E and *CUZD1* expression peaks at 10 h (Fig. A.2A). To determine if this up regulation is mediated by ER, we treated cells with ICI, a selective ER inhibitor. Indeed, treatment with ICI prevented E-induced up regulation of *CUZD1* in MCF7 cells (Fig. A.2B). Finally, analysis of the *CUZD1* gene 10kb upstream of the transcription start site revealed multiple potential ER binding sites. ChIP analysis using an antibody directed against ERα revealed enrichment of a ERα binding site at a region 7kb upstream of the transcriptional start site of the *CUZD1* gene (Fig. A.3). These data strongly suggest that *Cuzd1* expression is induced by ERα, although further functional analysis is necessary to determine the mechanism of this regulation.

Molecular Interactions Within the CUZD1-STAT5 Complex

Our studies have identified CUZD1 as a component of a cellular multi-protein complex containing JAK1/2 and STAT5. The exact molecular interactions within this complex, however, remain poorly understood. We have hypothesized that CUZD1 may act as a scaffolding protein that interacts with JAKs and STAT5 to stabilize the complex to facilitate STAT5 phosphorylation. Interestingly, CUZD1 can translocate to the nucleus in response to growth factor stimulation and co-localizes with STAT5. However, we have not yet determined whether CUZD1 interacts directly with STAT5. We have performed preliminary mutagenesis studies to determine the functional domains of CUZD1 important for its nuclear localization, although specific amino acid sequences essential for this function are yet to be identified. Analysis of the effect of point mutations within the CUB and ZP protein motifs of CUZD1 on its interaction with STAT5 would offer further insight into its physical relationship between STAT5 as well as the mechanism of its nuclear translocation. The results of these experiments would shed much needed light on the specific details of the STAT5-CUZD1 interaction.

Additional Interacting Protein Partners of CUZD1

Mass spectrometry of proteins co-immunoprecipitated with CUZD1 from mammary epithelial cell lysates indicated that there are dozens of interacting partners of CUZD1, providing multiple potential avenues to investigate its mechanism of action. One interesting candidate from this list is PRMT5, an arginine methyltransferase that is known to modify histones post-translationally and remodel chromatin. The co-immunoprecipitation of FLAG-CUZD1 and PRMT5 was confirmed in MCF7 cells overexpressing FLAG-CUZD1, but the biological significance of this interaction has not been investigated. (Fig. A.4). Microarray analysis of *Cuzd1*$^{-/-}$ versus Cuzd1$^{+/-}$ mammary epithelium identified many genes that are differentially regulated in the absence of CUZD1. Association of PRMT5 with a nuclear CUZD1- STAT5 complex could be vital for this differential transcriptional regulation. Interestingly, PRMT5 has been shown to interact with JAK1, JAK2, and EGFR, in addition to regulating genes involved in cancer cell proliferation (2–4). The ErbB signaling pathways that are potentially altered by the interactions between CUZD1 and PRMT5 could, therefore, be an interesting topic of future exploration.

How are EGF Family Ligands Regulated by CUZD1?

An interesting aspect of CUZD1's control of the ErbB signaling pathway is its regulation of a specific subset of the EGF family ligands: Ereg, Epgn, and Nrg1. Although CUZD1 is essential for the proliferation

of the mammary epithelium during pregnancy and lactation, it does not control the expression of the EGF family member amphiregulin, which directs ductal elongation and branching that occur during the pubertal phase of mammary gland development (5). The specificity of regulation of a subset of the EGF family ligands by CUZD1 could be rooted in the unique expression pattern of CUZD1 during development, with the highest expression and nuclear localization of CUZD1 occurring during pregnancy and lactation. Additionally, this specificity may be due to the interaction of CUZD1 with other protein partner(s). It is conceivable that CUZD1 can interact with multiple transcription factors or chromatin modulators that differentially target and regulate the genes encoding the EGF family growth factors at precise time points during mammary gland development. Further characterization of the protein complexes containing CUZD1 will provide a more detailed understanding of its regulation of the EGF family ligands.

In summary, our work to date has uncovered the molecular pathways of CUZD1 action in mammary epithelial cells during normal development and tumorigenesis. The identification of these mechanisms opens up further studies that would provide a more comprehensive understanding of CUZD1's regulatory role in cell proliferation and differentiation.

REFERENCES

1. Chen D, Xu X, Zhu L-J, Angervo M, Li Q, Bagchi MK, et al. Cloning and Uterus/Oviduct-specific Expression of a Novel Estrogen-regulated Gene (ERG1). Vol. 2014. 1999.
2. Branscombe TL, Frankel A, Lee JH, Cook JR, Yang Z, Pestka S, et al. PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. J Biol Chem. 2001 Aug;276(35):32971–6.
3. Pal S, Vishwanath SN, Erdjument-Bromage H, Tempst P, Sif S. Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. Mol Cell Biol. 2004 Nov;24(21):9630–45.
4. Stopa N, Krebs JE, Shechter D. The PRMT5 arginine methyltransferase: many roles in development, cancer and beyond. Cell Mol Life Sci. 2015 Jun;72(11):2041–59.
5. Kenney NJ, Smith GH, Rosenberg K, Cutler ML, Dickson RB. Induction of ductal morphogenesis and lobular hyperplasia by amphiregulin in the mouse mammary gland . Cell Growth Differ. 1996 Dec;7(12):1769–81.

FIGURES

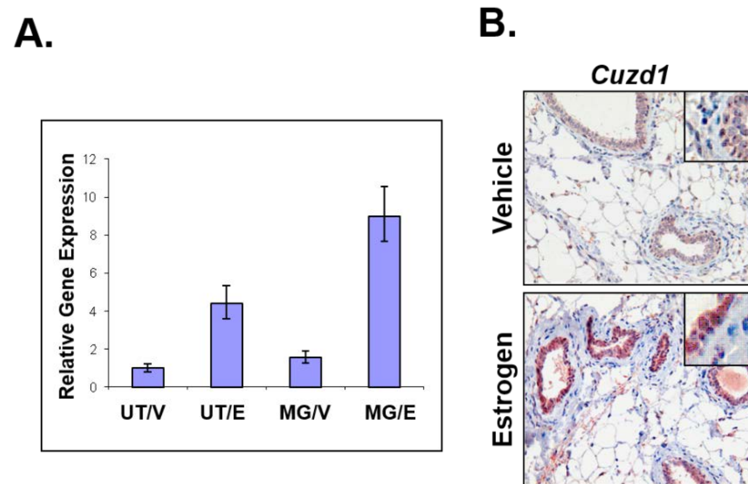

Figure A.1. E regulation of Cuzd1 expression in the mammary gland. A. mRNA expression of *Cuzd1* following E treatment. Ovariectomized mice were treated with Vehicle (V) or estradiol (E) at 40 ug/kg body weight (E). After 24 h, total RNAs were isolated from uteri (UT) and purified mammary epithelial cells (MG) and subjected to real-time PCR using gene-specific primers for Cuzd1 or 36B4 (control). Data are expressed as Relative Gene Expression ± SEM from three independent experiments. (Courtesy of Quanxi Li) **B. Localization of Cuzd1 and ERα in mammary glands in response to E administration.** Paraffin-embedded sections from mammary glands of adult intact mice following 5 days of treatment of vehicle collected at estrous stage of ovarian cycle (a and c) or estrogen (b and d) were subjected to immunohistochemical analysis using an antibody specific for Cuzd1 (a-b) or ERα (c-d). Magnification, 20X. (Courtesy of Quanxi Li and Athi Kannan)

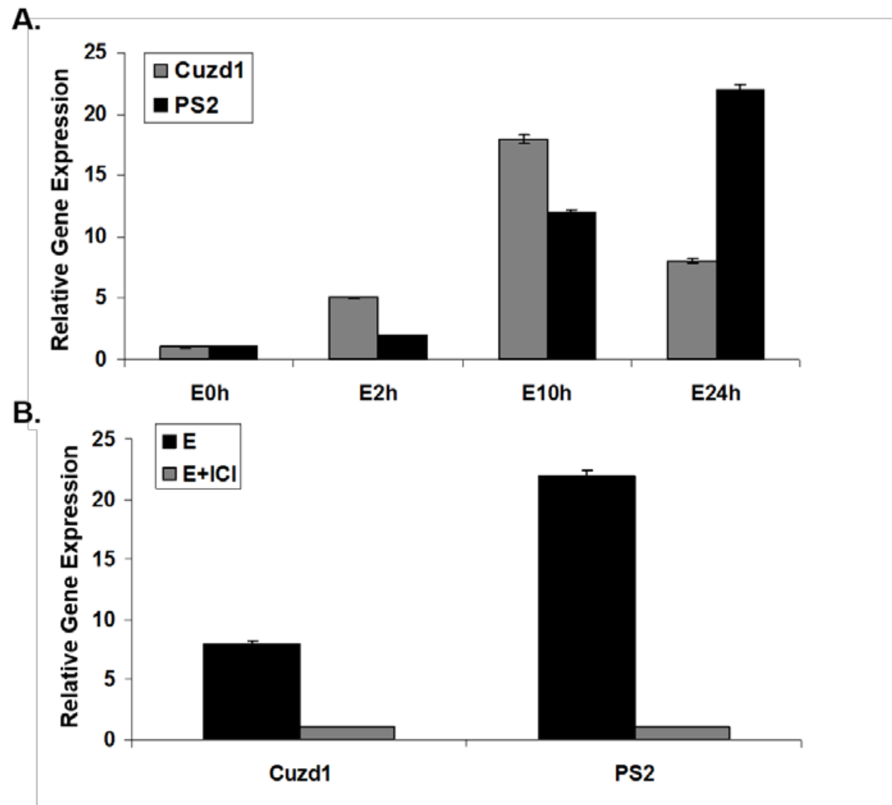

Figure A.2. Estrogen regulation of *CUZD1* in MCF7 breast cancer cells. A. *CUZD1* is upregulated following treatment with E. MCF7 cells grown in E-free culture medium were treated with E (10-8M) for indicated times. Cells were harvested at various time points, total RNA was isolated from these cells, and subjected to real-time PCR analysis using gene-specific primers to assess the expression the expression of *pS2* (positive control) and *CUZD1*. (Courtesy of Lavanya Anandan) **B. *CUZD1* upregulation via E is blocked by ICI.** MCF-7 cells were treated with E in the absence or presence of ICI 182,780 (10-6 M) for 24 h. Total RNA was isolated and subjected to real-time PCR analysis using gene-specific primers to assess the expression the expression of *pS2* (positive control) and *CUZD1*. (Courtesy of Lavanya Anandan)

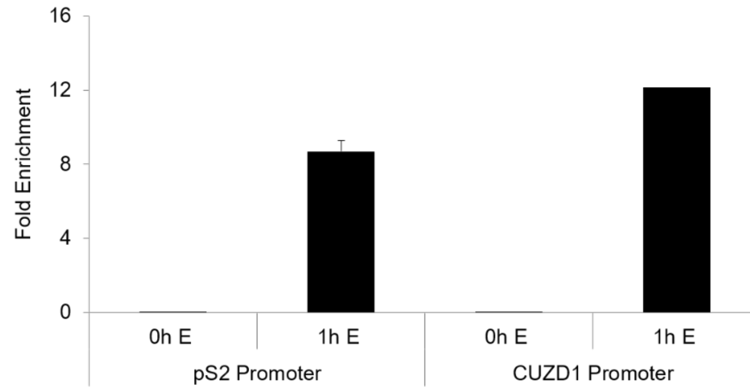

Figure A.3. Transcriptional regulation of *CUZD1* by ERα. Using Chromatin Immunoprecipitation, we identified enrichment in a partial ERα binding site 7kb upstream of the *CUZD1* transcriptional start site. MCF7 cells were serum starved for 48h and then treated with E for 1h. Cells were fixed, lysed, and protein/DNA complexes were isolated with an antibody specific to ERα. pS2 was used as a positive control.

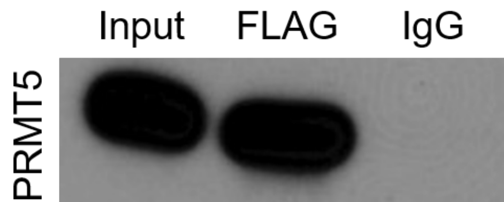

Figure A.4. PRMT5 co-immunoprecipitates with FLAG-CUZD1. MCF7 cells overexpressing FLAG-CUZD1 were treated with a cocktail of FBS/EGF/PRL for 6h, fixed, and lysed. Immunoprecipitation was carried out with an antibody specific for FLAG. A Western blot was run using these lysates and the membrane was probed with an antibody specific for PRMT5,