

Zinc Finger Homeodomain Factor *Zfhx3* Is Essential for Mammary Lactogenic Differentiation by Maintaining Prolactin Signaling Activity*

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The zinc finger homeobox 3 (ZFHX3, also named ATBF1 for AT motif binding factor 1) is a transcription factor that suppresses prostatic carcinogenesis and induces neuronal differentiation. It also interacts with estrogen receptor α to inhibit cell proliferation and regulate pubertal mammary gland development in mice. In the present study, we examined whether and how *Zfhx3* regulates lactogenic differentiation in mouse mammary glands. At different stages of mammary gland development, *Zfhx3* protein was expressed at varying levels, with the highest level at lactation. In the HC11 mouse mammary epithelial cell line, an *in vitro* model of lactogenesis, knockdown of *Zfhx3* attenuated prolactin-induced β -casein expression and morphological changes, indicators of lactogenic differentiation. In mouse mammary tissue, knock-out of *Zfhx3* interrupted lactogenesis, resulting in underdeveloped glands with much smaller and fewer alveoli, reduced β -casein expression, accumulation of large cytoplasmic lipid droplets in luminal cells after parturition, and failure in lactation. Mechanistically, *Zfhx3* maintained the expression of *Prlr* (prolactin receptor) and *Prlr*-*Jak2*-*Stat5* signaling activity, whereas knockdown and knock-out of *Zfhx3* in HC11 cells and mammary tissues, respectively, decreased *Prlr* expression, *Stat5* phosphorylation, and the expression of *Prlr*-*Jak2*-*Stat5* target genes. These findings indicate that *Zfhx3* plays an essential role in proper lactogenic development in mammary glands, at least in part by maintaining *Prlr* expression and *Prlr*-*Jak2*-*Stat5* signaling activity.

Development of the mammary gland occurs in connection with sexual development and reproduction and is regulated by multiple hormones (e.g. estrogen, progesterone, and prolactin) and growth factors (EGF, FGF, insulin-like growth factor, etc.). It can be divided into six distinct stages: embryonic, prepubertal, pubertal (the linear phase), pregnancy, lactation, and involution (the cyclic phase) (1, 2). Unlike most other organs, 5 of the 6 stages of mammary gland development occur postnatally, providing an ideal model for studying genes in both normal development and neoplastic progression. Mammary glands

retain plasticity for undergoing the cyclic phase, which implies the existence of specific molecules that are able to integrate a variety of signals from hormones and growth factors. For example, the *STAT* genes have been demonstrated to play roles in both hormone response and growth factor signaling (3). At present, a few factors have been identified and characterized for their roles in mammary gland development in response to hormonal signaling, but many more remain to be discovered (4, 5).

The homeobox gene family contains the homeobox sequence that encodes for the homeodomain, a DNA-binding domain about 60 amino acids long. More than 200 homeodomain-containing proteins have been identified and characterized in a variety of species, most of which act as transcription factors in a wide range of critical activities during normal development and tumorigenesis (6). Several homeobox genes are expressed in mammary epithelial cells, and their functions as regulators of mammary gland development have been established using genetically modified mice (7, 8). For example, loss of *Msx2*, *Hoxc6*, *Hoxa9*, and *Pax2* in the mammary gland leads to a series of defects during mammary gland development, including failures in side branching and lobulo-alveolar development (8, 9).

Zinc finger homeobox 3 (ZFHX3), also named ATBF1 for AT motif binding factor 1, is a large transcription factor with 23 zinc finger and 4 homeodomains (10, 11). Very few homeobox genes have more than 1 homeobox (12), and the existence of 4 homeoboxes in *ZFHX3* suggests that it has a dynamic function in biological processes (13). For example, ZFHX3 is necessary for neuronal and myogenic differentiation in cell culture models (11, 14–16), and deletion of *Zfhx3* in mice causes developmental defects, interrupts epithelial homeostasis, and induces neoplastic morphology in mouse prostates (17–19). In mouse mammary glands, we previously demonstrated that *Zfhx3* mRNA expression varies at different stages during development, reaching the highest level at lactation (20), and that *Zfhx3* regulates pubertal mammary gland development (20). In addition, both estrogen and progesterone, two hormones essential for normal mammary gland development, induce or enhance the transcription of ZFHX3 in human and mouse mammary epithelial cells (21, 22), although estrogen also causes protein degradation of ZFHX3 when too much estrogen is present (21). Taken together with the observation that deletion of *Zfhx3* in

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TABLE 1
Sequences of shRNAs against mouse *Zfhx3*/human *ZFH3* in Sigma PLKO.1 vector

Name	Sequence	Sigma ID
shZfhx3-8	CCGGGCCAGGAAGAATTACGAGAATCTCGAGATTCCTCGTAATCTTCTGGCTTTTGG	TRCN0000075408
shZfhx3-9	CCGGCCCCGACAATAAGCCGTTAACTCGAGTTAAACGGCTTATTGTCCGGGTTTTTG	TRCN0000075409
shZfhx3-11	CCGGCCGGAAGAAGTTAGCGGATATCTCGAGATATCCGCTAACTTCTCCGGTTTTTG	TRCN0000075411
shZfhx3-12	CCGGGCCATGCTCTTAGACTGTGATCTCGAGATCACAGTCTAAGAGCATGGCTTTTTG	TRCN0000075412
shZFH3-2	CCGGCCGATGCTCTTAGACTGTGATCTCGAGATCACAGTCTAAGAGCATGGCTTTTT	TRCN0000013561
shZFH3-3	CCGGGCCAGGAAGAATTATGAGAATCTCGAGATTCCTCATAATCTTCTGGCTTTTT	TRCN0000013558
shZFH3-5	CCGGCCCTTTAGTTTCCACAGCTAACTCGAGTTAGCTGTGGAACATAAGGGTTTTT	TRCN0000013560

mouse prostates alters the transcription level of Prlr (17), a key regulator of lactogenic differentiation in the mammary gland, we hypothesize that ZFH3 is more relevant to lactogenic differentiation during mammary gland development.

In this study, we examined whether and how *Zfhx3* regulates lactogenic development in the mammary gland using both a cell culture model and mouse model. In the HC11 mouse mammary epithelial cell line, where prolactin induces morphological and molecular changes indicative of lactogenic differentiation, knockdown of *Zfhx3* expression attenuated the effect of prolactin. Consistently, deletion of *Zfhx3* in mouse mammary glands prevented proper alveologenesis and lactogenic differentiation. Mechanistically, the effect of *Zfhx3* was mediated at least in part by regulating the Prlr-Jak2-Stat5 signaling axis.

Experimental Procedures

Mice—Wild type C57BL/6 mice were purchased from the Academy of Military Medical Sciences (Beijing, China), and breeding was carried out following standard procedures for the collection of mammary tissues at different developmental stages. Breeding, genotyping, and preparation of mice with MMTV-Cre-mediated mammary-specific knock-out of *Zfhx3* were previously described (20). All mice were fed with pathogen-free food and water and were closely monitored and humanely euthanized. At least four mice were used for each genotype at a time point for different analyses, including whole mount analysis, immunohistochemical staining, and real time PCR. All mouse experiments were approved by the Institutional Animal Care and Use Committee at College of Life Sciences of Nankai University.

Cell Culture—The human embryonic kidney cell line 293T and human breast cancer cell line MCF-7 were cultured in DMEM (Invitrogen) supplemented with 10% FBS. Human breast cancer cell lines MDA-MB-231 and T-47D were cultured in RPMI 1640 medium supplemented with 10% FBS. For prolactin treatment, T-47D cells were first incubated in serum-free medium overnight and then treated with 500 ng/ml recombinant human prolactin (R&D Systems) for indicated times.

In Vitro Lactogenic Differentiation of HC11 Cells—Mouse mammary epithelial cell line HC11 was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 1× GlutaMAX (Invitrogen), 5 μg/ml insulin (Roche), and 10 ng/ml epidermal growth factor (EGF, R&D). To induce cell differentiation, HC11 cells were grown to confluence, incubated in medium deprived of EGF for 24 h, and then treated for 5 days with a combination of lactogenic hormones including 1 μM dexamethasone (Sigma), 5 μg/ml insulin, and 5 μg/ml recom-

binant mouse prolactin (R&D), referred to as DIP.² The mammospheres were photographed by phase contrast microscopy using a Olympus CKX41SF inverted microscope (Olympus, Tokyo, Japan) with a Canon EOS 600D digital camera (Canon, Tokyo, Japan).

Lentivirus Production and Cell Transduction—MISSIONTM shRNAs targeting human *ZFH3* and mouse *Zfhx3* in pLKO.1-puro plasmid and empty vector control plasmid were purchased from Sigma. The insert sequences for these shRNAs are listed in Table 1. Lentiviral particles were produced in 293T cells by cotransfecting pLKO.1 with pMD2.G and psPAX2 plasmids using the FuGENE 6 transfection reagent (Promega, Beijing, China) according to the manufacturer's protocol. On the day after transfection, the medium was replaced, and the cells were incubated for an additional 2 days before viral supernatant was collected. Human T-47D or mouse HC11 cells were seeded in 6-well culture plates and grown to ~70% confluency, at which time the culture medium was replaced with fresh medium containing 8 μg/ml of Polybrene (Sigma), and 1 ml of lentiviral solution was added dropwise. 6–12 h after viral infection, the virus-containing medium was replaced with fresh medium again, and on the following day 2 μg/ml of puromycin (Sigma) was added to the medium for 3–5 days to select cells stably expressing shRNAs. RNA was extracted, and protein lysate was prepared for analysis. Cells stably expressing the desired shRNAs were maintained in medium containing 1 μg/ml of puromycin.

Reverse Transcription and Real Time PCR—Total RNA was used for cDNA synthesis with the Moloney murine leukemia virus reverse transcriptase system (Promega). RT-PCR was performed on the Mastercycler ep Realplex real time PCR system (Eppendorf, Hamburg, Germany) using the SYBR premix Ex Taq (TaKaRa Bio Inc., Tianjin, China). Human *GAPDH* or mouse *β-actin* gene was used as the internal control in real time PCR. Quantification of gene expression levels was determined using the comparative $\Delta\Delta CT$ method. Primer sequences used in real time PCR for each gene are listed in Table 2 and were from the PrimerBank (23) or the indicated references.

Immunoblotting—Cell lysate preparation and Western blotting were performed as previously described (20). Briefly, cells or tissues were lysed using radioimmune precipitation assay buffer supplemented with protease inhibitor (cOmplete, Roche) and phosphatase inhibitor (PhosStop, Roche). Protein concentrations were determined using a Pierce BCA protein

²The abbreviations used are: DIP, dexamethasone, insulin, and prolactin; H&E, hematoxylin and eosin; IHC, immunohistochemical; PRLR, prolactin receptor.

TABLE 2
Primer sequences for real time PCR

Gene	Sequence (5'-3'), forward/reverse	Reference
hZFHx3	TGTTCCAGATCGAGATGGGAAT / CTTTCCCAGATCCTCTGAGGTTT	Ref. 20
hGAPDH	GGTGGTCTCCTCTGACTTCAACA / GTTGTCTAGCCAAATTCGTGTG	Ref. 20
mZfhx3	AGAGCAAGAGGGCAGCGTCATC / CGGTCACGTCAGCGTGTCTATAC	Ref. 19
mCsn2	ACATCCAGCCTATGCTCAAC / GAGGGGCATCTGTTTGTGCT	Ref. 23
mCtgf	GGGCCTCTTCTGCGATTTC / ATCCAGGCAAGTGCATTGGTA	Ref. 23
mPrlr total	GTGGAATCCTGGGTCAGATG / GGGCCACTGGTTTGTAGTC	Ref. 80
mPrlr long	ATAAAAGGATTTGATACATCATCTGCTAGAG / TGTTCATCCACTTCCAAGAACTCC	Ref. 80
mId2	CGACCCGATGAGTCTGCTCTA / GACGATAGTGGGATGCGAGTC	Ref. 23
mElf5	TGGACTCCGTAACCCATAGCACCT / ATTGCTTAAGGGCTGATGGCATCC	Ref. 81
mLmo4	CCCAAGGCAACGTGTATCAT / GGGCTGTGGGCTATCATGT	Ref. 75
mCebpb	AACTCTCTGCTTCTCCCTCTG / AAGCCCGTAGGAACATCTTT	Ref. 82
mActin	GATCTGGCACCACACCTTCT / GGGGTGTTGAAGGTCTCAAA	Ref. 19

assay kit (Thermo Fisher) according to the manufacturer's protocol. Cell lysates containing equal amounts of proteins were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (PALL, Beijing, China), incubated with antibodies, and visualized using WesternBright ECL (Advansta, CA). Actin or tubulin was used as a loading control. For the detection of Zfhx3 protein, which has an estimated molecular mass of 406 kDa, 4% SDS-PAGE was run at 100 V for 3.5 h. The spectra multicolor high range protein marker (Thermo Fisher, catalog no. 26625) was used in those 4% gels, and the estimated molecular mass of Zfhx3 (406 kDa) is indicated in relevant figure panels because the highest band in the molecular mass marker (300 kDa) was still too distant from the Zfhx3 band. For the detection of other proteins, 8–12% gels were used with the PageRuler Plus Prestained protein marker (Thermo Fisher, catalog no. 26616), and molecular mass markers in kDa are indicated in applicable figure panels. The antibodies and dilution factors used in this study are listed in Table 3.

Whole Mount Preparation and Histological Analysis of Mammary Glands—Whole mount analysis of mouse mammary glands was performed as previously described (20) except that whole mount glands were stained with carmine alum instead of hematoxylin. Briefly, the fourth abdominal mammary gland on the left was dissected at lactation day 2, placed between two glass slides and spread by placing weights on top of the slide for 10 min, followed by fixing in 3.7% formalin overnight. Tissues were hydrated, stained with carmine alum, and cleared with xylene. For histological analysis, formalin-fixed tissues were processed for sectioning, and stained with hematoxylin and eosin (H&E).

Immunohistochemistry—Immunohistochemical staining was performed on sections of formalin-fixed, paraffin-embedded tissues. Briefly, tissue sections were first deparaffinized and rehydrated following standard procedure. Antigen retrieval was carried out by heating in sodium citrate buffer or Tris-EDTA buffer using a pressure cooker for 3 min at full pressure. Sections were incubated with primary antibodies at 4 °C overnight and then with the HRP solution and the DAB-chromogen. All sections were counterstained in hematoxylin, dehydrated in ethanol, cleared in xylene, and covered with a coverslip with Eukitt quick-hardening mounting medium (Sigma). Antibodies, dilution factors, and antigen retrieval buffers are listed in Table 3.

Statistical Analysis—Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA).

A two-tailed Student's *t* test was used to determine statistical differences between two groups, and one-way analysis of variance was used to compare three or more groups. *p* values less than 0.05 were considered statistically significant.

Results

Expression of Zfhx3 Protein during Mammary Gland Development—We previously measured Zfhx3 mRNA expression at different stages of mammary gland development and found that the Zfhx3 mRNA level varies at different stages, being the highest in late pregnancy and lactation (20). Here we determined Zfhx3 protein expression. Mammary glands were collected from mice at postnatal weeks 3, 6, and 9; pregnancy days 2 and 18; lactation days 1, 7, 14, and 20; and involution day 7. Western blotting demonstrated that the Zfhx3 protein level increased from weeks 3 to 9 after birth, further increased to an intermediate level during pregnancy, increased to and maintained a maximum level during lactation, and then dramatically reduced during involution (Fig. 1A). Even during lactation, the Zfhx3 protein level kept increasing, being higher at days 14 and 20 than at days 1 and 7 (Fig. 1A). This pattern of expression is almost identical to that for Zfhx3 mRNA expression (20).

We also performed IHC staining to detect Zfhx3 protein in the mammary gland at each developmental stage (Fig. 1B). We observed that Zfhx3 was expressed in both the epithelial cells and some types of stromal cells and was mainly localized in the nucleus. In a typical terminal end bud structure at postnatal week 3 (prepuberty) mammary gland, we could see two morphologically distinct cell types: the single-layered cap cells (outer layer, giving rise to basal cells) and multiple-layered body cells (centrally located, giving rise to luminal cells) (2). We observed that a significant portion of body cells in the terminal end bud were Zfhx3-negative (indicated by *arrows* in Fig. 1). During the pubertal and the early pregnancy stages when ductal morphogenesis occurs, we observed that some of the ductal luminal cells were also Zfhx3-negative (indicated by *arrows* in Fig. 1), but in contrast nearly all the mature alveolar luminal cells (indicated by *arrowheads* in Fig. 1) from the late pregnancy stage to the lactation stage were Zfhx3-positive, and at day 7 of involution Zfhx3 expression dramatically decreased. This pattern of expression strongly suggests that Zfhx3 may play a role in alveologenesis and lactogenic hormone-induced differentiation of mammary epithelial cells at lactation.

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TABLE 3

Antibodies, dilution factors, and antigen retrieval buffer used in Western blotting (WB) and/or IHC staining

Protein	ID (lot number)	Application and dilution	Antigen retrieval buffer
ZFHX3	Anti-ZFHX3 (1–160 amino acids) (27)	WB (1:800)	
ZFHX3	Anti-ZFHX3 (2107–2147 amino acids) (27)	IHC (1:1000)	Citrate, pH 6
STAT5	sc-835 (G0111, Santa Cruz)	WB (1:500)	
p-STAT5(Y694)	#9351 (Lot 7 of Ref 06/2012, Cell Signaling Technology)	WB (1:1000); IHC (1:1000)	Citrate, pH 6
PRLR	sc-20992 (J1910, Santa Cruz)	WB (1:1000); IHC (1:200)	Tris-EDTA, pH 9
CSN2	sc-17969 (C1605, Santa Cruz)	WB (1:200); IHC (1:200)	Citrate, pH 6
Tubulin	sc-23949 (B0408, Santa Cruz)	WB (1:3000)	
Actin	A2228 (Sigma)	WB (1:5000)	

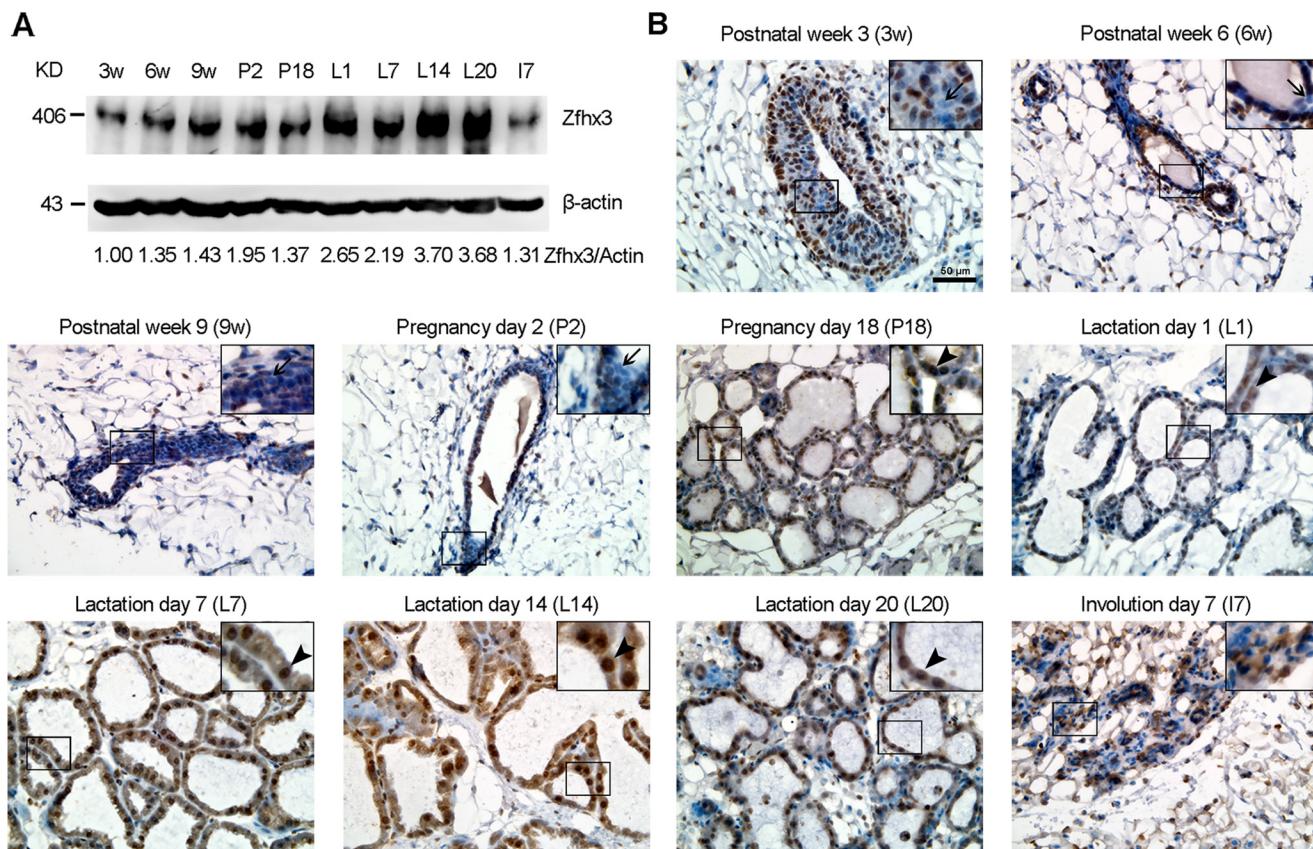


FIGURE 1. Dynamic expression of Zfhx3 protein at different stages of postnatal mammary gland development. *A*, detection of Zfhx3 protein by Western blotting in C57BL/6 female mouse mammary tissues at indicated developmental stages. β -Actin served as the loading control. 3w, postnatal week 3; 6w, postnatal week 6; 9w, postnatal week 9; P2, pregnancy day 2; P18, pregnancy day 18; L1, lactation day 1; L7, lactation day 7; L14, lactation day 14; L20, lactation day 20; I7, involution day 7. The data are representative of three mice per group. Band intensities were quantified using the ImageJ program, and ratios of Zfhx3/ β -actin, normalized to that of postnatal week 3, are shown at the bottom. *B*, IHC staining in mammary glands of C57BL/6 female mice at indicated developmental stages. Arrows show Zfhx3-negative body cells in the terminal end bud (postnatal week 3, prepuberty) or Zfhx3-negative ductal luminal cells at postnatal weeks 6 and 9 (puberty) and pregnancy day 2 (early pregnancy); arrowheads show mature alveolar luminal (Zfhx3-positive) cells in pregnancy day 18 (late pregnancy) and lactation days 1, 7, 14, and 20 (lactation). A representative image from three mice is shown for each stage. Scale bar, 50 μ m.

Zfhx3 Is Necessary for Prolactin-induced Lactogenic Differentiation in HC11 Mammary Epithelial Cells in Culture—We first used the HC11 cell line model to test Zfhx3 function in lactogenic differentiation induced by lactogenic hormones. HC11 is a clone of the COMMA-1D mouse mammary epithelial cell line (24), derived from mammary tissue of BALB/c mice in the middle of pregnancy (25). Treatment with prolactin in the presence of insulin and glucocorticoid hormone dexamethasone (the DIP treatment) makes cultured HC11 cells produce milk protein β -casein, a marker of lactogenic differentiation (26), and form mammospheres in high density, making this cell line a widely used *in vitro* model of mammary gland differentiation in the context of prolactin and glucocorticoid stimulation (24).

First, we measured the expression of Zfhx3 in HC11 cells. Compared with three human breast cancer cell lines with low (MDA-MB-231), intermediate (T-47D), and high (MCF-7) levels of ZFHX3 (27), HC11 cells showed an expression level higher than T-47D cells but lower than MCF-7 cells, as detected by Western blotting (Fig. 2A). We then grew HC11 cells to confluence, incubated cells in EGF-depleted medium for 24 h, and treated them with a combination of lactogenic hormones including DIP to induce β -casein expression. Consistent with previous studies (28), an induction of β -casein was detected at both the mRNA and protein levels as expected (Fig. 2B, left and right panels). Meanwhile, hormone treatment did not alter Zfhx3 expression at either the mRNA or protein level (Fig. 2B, middle and right panels). Immunofluorescence staining

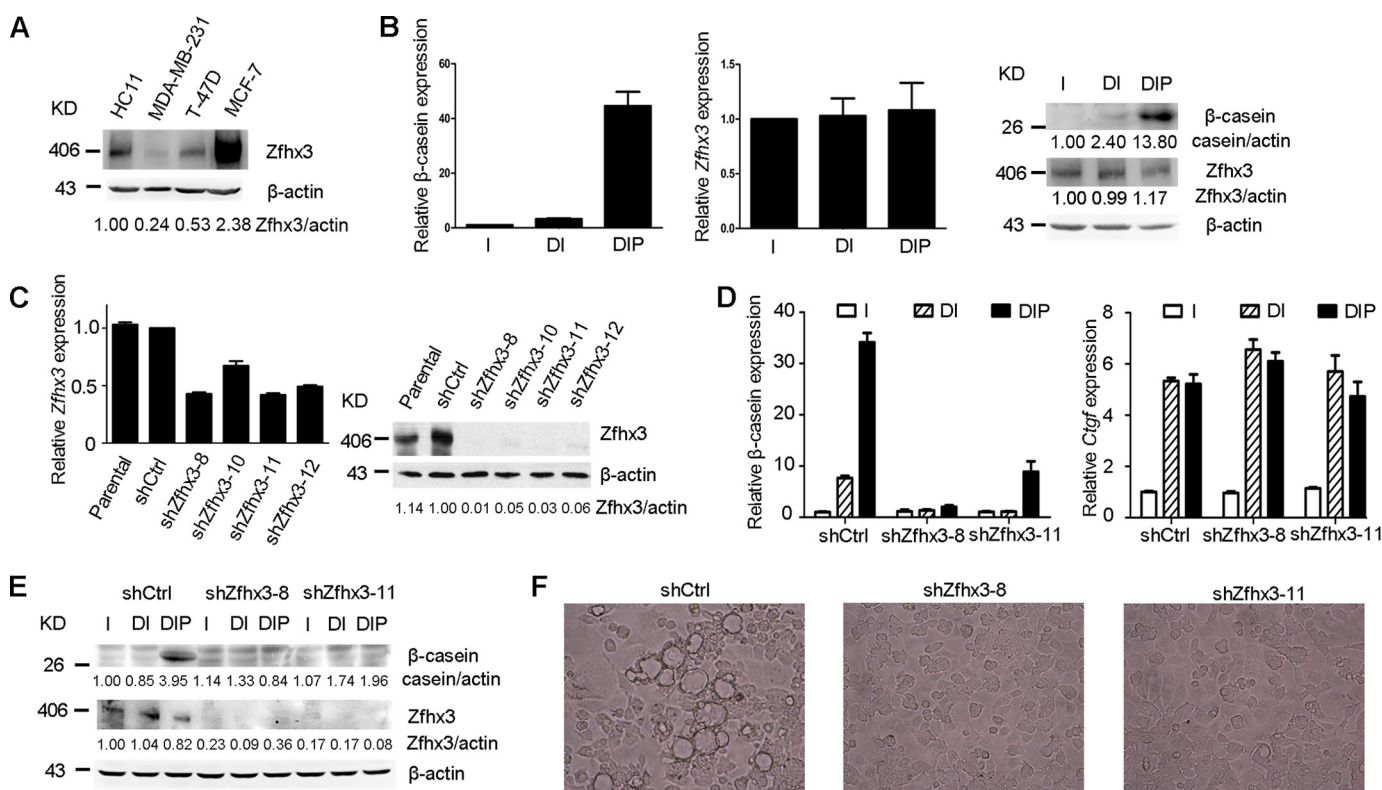


FIGURE 2. Knockdown of *Zfhx3* impairs prolactin-induced differentiation of the mouse HC11 mammary epithelial cells. *A*, detection of *Zfhx3* protein expression by Western blotting in HC11 and three human breast cancer cell lines that show low, moderate, and high expression of *ZFHX3*, with β -actin as the loading control. *B*, detection of the expression of β -casein and *Zfhx3* in lactogenic hormone-treated HC11 cells through RT-PCR (left and middle panels) and Western blotting (right panel). *C*, lentiviral expression of shRNAs against *Zfhx3* (shZfhx3-8, -10, -11, and -12) knocked down *Zfhx3* expression in HC11 cells, as detected by RT-PCR (left panel) and Western blotting (right panel). Parental HC11 cells were used as a positive control. *D* and *E*, knockdown of *Zfhx3* attenuated prolactin-induced β -casein expression while not affecting the expression of *Ctgf*, a target gene of dexamethasone but not prolactin, as detected by RT-PCR (*D*) and Western blotting (*E*). *F*, prolactin-induced bubble-like structures, an established indicator of lactogenic hormone-induced differentiation, disappeared upon the knockdown of *Zfhx3* in HC11 cells. Cells in *B* and *D–F* were treated with insulin (I), insulin plus dexamethasone (DI), or DIP for 5 days; in *F* only the DIP groups are shown. All Western blots were quantified using the ImageJ program. Ctrl, control.

showed that *Zfhx3* was localized in the nucleus regardless of hormone treatment (data not shown). These results validated the HC11 model for *Zfhx3* study.

We then knocked down *Zfhx3* expression by lentivirus-mediated stable expression of shRNAs against *Zfhx3* in HC11 cells. All four shRNAs induced significant knockdown of *Zfhx3*, as confirmed by real time PCR for *Zfhx3* mRNA and Western blotting for its protein (Fig. 2C). Two of the shRNAs, shZfhx3-8 and shZfhx3-11, were more effective in *Zfhx3* knockdown and thus were used for further experimentation. At day 5 of DIP treatment, β -casein mRNA (Fig. 2D, left panel) and protein (Fig. 2E) were significantly induced in the control shRNA group, whereas knockdown of *Zfhx3* almost abolished the induction of β -casein (Fig. 2, D and E). Meanwhile, the connective tissue growth factor gene (*Ctgf*), which enhances lactogenic differentiation and only responds to dexamethasone but not to prolactin in its induction (29, 30), did not show an expression change upon the knockdown of *Zfhx3* (Fig. 2D, right panel). Parallel to β -casein induction, DIP-treated HC11 cells formed mammospheres during differentiation at high density in culture (Fig. 2F, left panel) as previously reported (31), but the sphere formation was interrupted by *Zfhx3* knockdown (Fig. 2F, middle and right panel). These results suggest that *Zfhx3* is necessary for DIP to induce lactogenic differentiation in HC11

cells, and the effect only involves the prolactin signaling, not the glucocorticoid signaling.

Deletion of *Zfhx3* Attenuates Alveologenesis and Lactogenic Differentiation in Mouse Mammary Glands—To more directly test the necessity of *Zfhx3* for lactogenic differentiation, we bred mice with floxed *Zfhx3* alleles to MMTV-Cre mice in which the Cre recombinase is specifically expressed in mammary epithelial cells under the MMTV promoter, as described in our previous study (20). 9-week-old MMTV-Cre/*Zfhx3*^{+/+}, MMTV-Cre/*Zfhx3*^{fl/+}, and MMTV-Cre/*Zfhx3*^{fl/fl} female mice were mated and sacrificed at lactation day 2. Whole mount analysis showed that *Zfhx3* deletion led to defects in alveologenesis, as indicated by reduced alveolar density and size (Fig. 3A). H&E staining of tissue sections demonstrated that *Zfhx3* deletion caused the accumulation of large cytoplasmic lipid droplets in the luminal layer (Fig. 3B), which has been established as an indicator of failure in secretory activation in multiple lines of genetically engineered mice (32). We estimated the proportion of areas occupied by alveoli and the mean area of a single lumen in H&E-stained tissue sections using the ImageJ program. A significant decrease was detected in both parameters when *Zfhx3* was deleted (Fig. 3, C and D).

We also measured the milk protein gene β -casein expression in these mice. The mRNA level of β -casein was significantly

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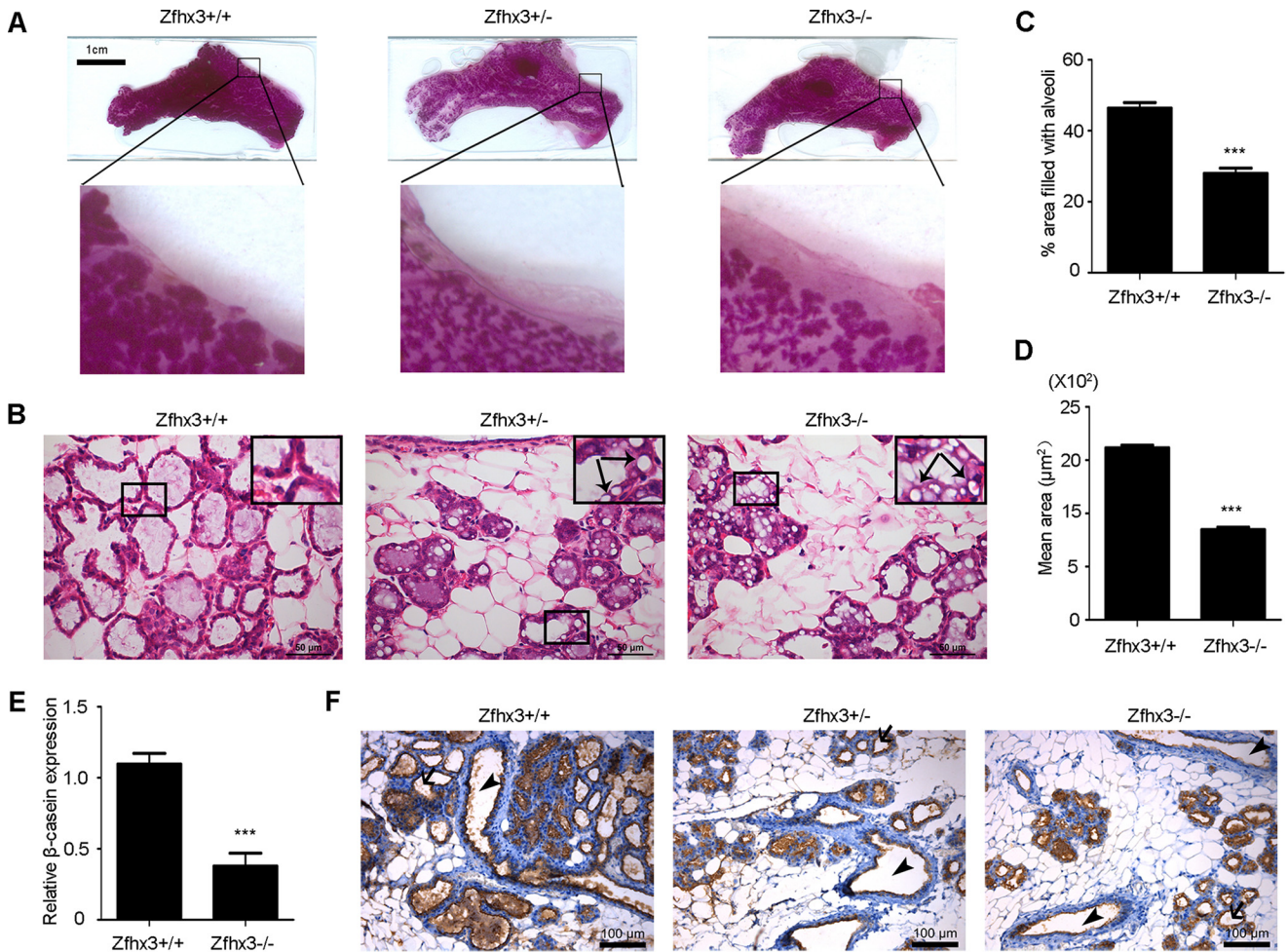


FIGURE 3. Deletion of *Zfhx3* interferes with the lactogenic development of mouse mammary gland. *A* and *B*, underdeveloped mammary glands in *Zfhx3* depleted females at lactation day 2, as seen in images of mammary glands from whole mount preparation (*A*) and H&E staining of tissue sections (*B*). *Arrows* in *B* indicate large cytoplasmic lipid droplets in the luminal epithelial cells of *Zfhx3*-deleted mammary glands. *C*, quantification of the total area filled by lactating alveoli in the H&E-stained sections using ImageJ. At least four mice were analyzed, and for each section, at least 20 images ($\times 20$) were used to analyze the area occupied by alveoli. *D*, quantification of the mean area of a single alveoli in the mammary glands using ImageJ. A total of at least 1000 alveoli were analyzed per genotype. For *C* and *D*, the data are means \pm S.E. *E*, expression of β -casein by real time PCR; the data are means \pm S.E. *F*, IHC staining of β -casein at lactation in the mammary glands; *arrows* show empty alveolar lumen, and *arrowheads* show empty ducts. Statistical significance was calculated by two-tailed Student's *t* test. ***, $p < 0.001$.

reduced by *Zfhx3* deletion, as detected by real time PCR (Fig. 3*E*), consistent with the findings from HC11 cells. The protein expression of β -casein, as detected by IHC staining, was hardly detectable in some alveolar lumen or the mammary ducts, and the staining intensity was indistinguishable between wild type mice and those with *Zfhx3* deletion (Fig. 3*F*), which could be due to quick emptying of the glands, as reported for IHC staining of the WAP milk protein in the *Stat5a* knock-out mammary glands at lactation day 1 (33). Lobulo-alveolar hypoplasia was evident in all four mice with homozygous deletion of *Zfhx3*.

Deletion of one *Zfhx3* allele caused an intermediate abnormality in the size and density of alveoli, accumulation of cytoplasmic lipid droplets (Fig. 3*B*, middle panel). In addition, on day 2 after birth, although all litters from wild type mothers survived, most litters from mice with one *Zfhx3* allele and all from *Zfhx3*-null mice died, likely because of dehydration. These findings indicate that *Zfhx3* is required for proper development, maintenance, and function of structurally organized secretory lobulo-alveolar units in mammary glands.

Zfhx3 Is Required for Prolactin-induced Tyrosine Phosphorylation of *Stat5*—During lactogenic differentiation, prolactin binds to its receptor (PRLR), which then recruits the JAK2 kinase to phosphorylate *STAT5* at Tyr^{694/699} to transduce the signal into the nucleus, where lactogenic genes including milk protein β -casein are induced (34–37). We therefore analyzed the phosphorylation status of *Stat5* to determine whether *Zfhx3* regulates alveologenesis and lactogenic differentiation by the canonical PRLR-JAK2-*STAT5* signaling pathway.

In the HC11 cell system, treatment with prolactin for 7.5 min clearly induced *Stat5* phosphorylation as expected, and the phosphorylation was still detectable after 2.5 h of treatment and hardly seen after 24 h (Fig. 4*A*). When *Zfhx3* was knocked down in HC11 cells, prolactin-induced *Stat5* phosphorylation was dramatically decreased, whereas total *Stat5* showed similar expression levels (Fig. 4*B*). In the T-47D human breast cancer cell line, which expresses a higher level of PRLR (38), prolactin treatment also significantly induced *STAT5* phosphorylation as in HC11 cells (Fig. 4*C*). Stable knockdown of *ZFHX3* expres-

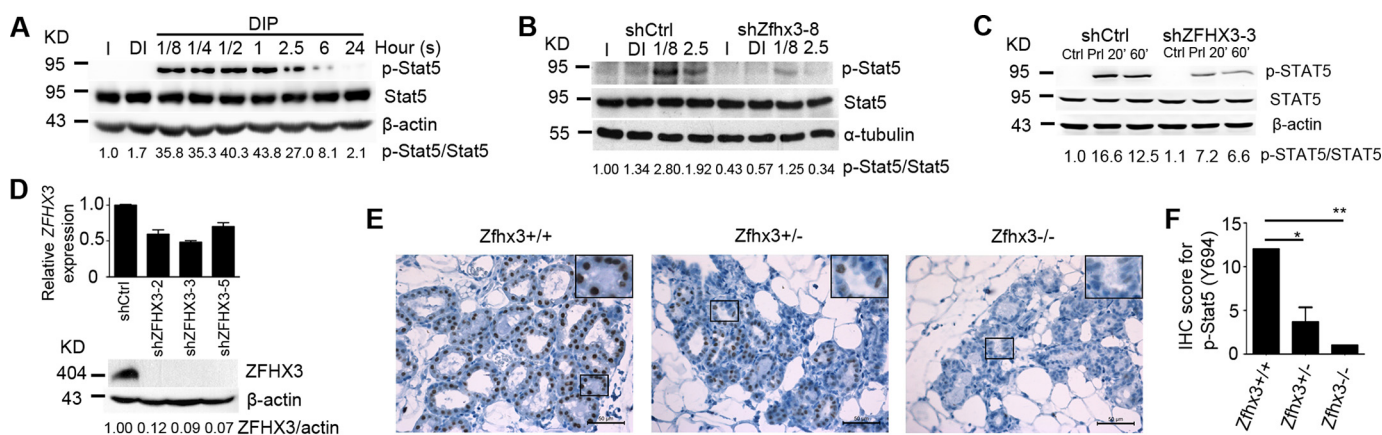


FIGURE 4. Zfhx3 is necessary for prolactin-induced phosphorylation of STAT5. *A*, parental HC11 cells were treated with 5 μ g/ml prolactin in a time-dependent manner and Stat5 phosphorylation at tyrosine 694 (p-Y694-Stat5) was detected by Western blotting. *B*, mouse HC11 mammary epithelial cells with stable knockdown of *Zfhx3* (shZfhx3-8) or control (shCtrl) were treated with insulin (I), insulin plus dexamethasone (DI), or DIP for 7.5 min or 2.5 h and then subjected to Western blotting to detect p-Y694-Stat5 and total Stat5. *C* and *D*, stable knockdown of ZFHX3 in human breast cancer cell line T-47D also reduced prolactin (Prl)-induced STAT5 phosphorylation, as detected by Western blotting. Knockdown efficiency was confirmed by RT-PCR and Western blotting (*D*). *E* and *F*, IHC staining of p-Y694-Stat5 in lactation day 2 mammary glands (*E*). IHC score is shown as means \pm S.E. (*F*) and was produced by multiplying the percentage of positive cells (scored as 0 for negative, 1 for < 10%, 2 for 11–50%, 3 for 50–80%, and 4 for > 80%) with the intensity of staining (0 for negative, 1 for weak, 2 for moderate, and 3 for strong). Statistical significance was calculated by one-way analysis of variance followed by Bonferroni's test. *, $p < 0.05$; **, $p < 0.01$. All Western blots were quantified using the ImageJ program.

sion using shRNA in T-47D cells, which was confirmed by real time PCR and Western blotting (Fig. 4D), compromised the phosphorylation of STAT5 (Fig. 4C). Similar results were obtained using a different shRNA(s) in both HC11 and T-47D cells (data not shown). These results showed that *Zfhx3* is necessary for STAT5 phosphorylation not only in the normal mammary epithelial cells but also in breast cancer cell lines, indicating that ZFHX3 protein may play a role in both normal development and tumorigenesis of the breast.

In mouse mammary tissues at lactation day 2 with different *Zfhx3* deletion status, IHC staining demonstrated that although most epithelial cells had intense staining for phospho-Stat5 (Fig. 4E, left panel), homozygous deletion of *Zfhx3* almost eliminated the staining (Fig. 4E, right panel). Deletion of one *Zfhx3* allele showed an intermediate effect in both the p-Stat5 staining intensity and the number of p-Stat5-positive cells (Fig. 4E, middle panel). The IHC staining index (IHC score) for p-Stat5, which was produced by multiplying the percentage of positive cells (scored as 0 for negative, 1 for < 10%, 2 for 11–50%, 3 for 50–80%, and 4 for > 80%) with the intensity of staining (0 for negative, 1 for weak, 2 for moderate, and 3 for strong), was significantly lower in *Zfhx3*-null tissue than in normal tissue (Fig. 4F). These observations suggest that *Zfhx3* plays a role in the activation of Stat5 by prolactin-Prlr signaling.

Zfhx3 Plays a Role in the Expression of Prlr—In a previous work, Yan et al. (39) carried out ChIP-seq experiments using ChIP grade antibodies of more than 100 transcription factors, including ZFHX3. Reanalyzing their data using the ChIP-Enrich tool (40) with annotation of all peaks to the nearest transcription start sites, we found that a number of potential target genes of ZFHX3, including PRLR, played crucial roles in mammary gland development (data not shown). We therefore tested whether ZFHX3 regulates PRLR expression to modulate prolactin-PRLR signaling. We found that in mouse mammary glands at lactation day 2, deletion of *Zfhx3* decreased the *Prlr* mRNA level, as detected by real time PCR (Fig. 5A). The

decrease in *Prlr* mRNA level by *Zfhx3* deletion was already significant at postnatal week 9 (data not shown), suggesting that *Prlr* down-regulation is independent of lactogenesis. We also detected *Prlr* protein in mouse mammary tissues by IHC staining and found that deletion of *Zfhx3* also down-regulated *Prlr* protein at lactation day 2 (Fig. 5, B and C). Down-regulation of *Prlr* was also induced by the knockdown of *Zfhx3* in HC11 cells at both the mRNA level (Fig. 5D) and the protein level (Fig. 5E). Consistent with the down-regulation of *Prlr*, the expression of two established players downstream of prolactin-Prlr signaling, *Elf5* and *Id2*, was also down-regulated by *Zfhx3* knock-out in mouse mammary glands at lactation day 2 (Fig. 5F), although the expression of two other prolactin signaling molecules, *Lmo4* and *Cebpb*, was not altered by *Zfhx3* deletion (Fig. 5F). Using the cBioPortal web tool (41, 42), we analyzed the expression of ZFHX3 and PRLR mRNAs in 1100 breast cancer samples (TCGA, Provisional), and found a positive correlation between the expression of the two genes (Fig. 5G). These results indicate that ZFHX3 plays a crucial role in the expression of PRLR in mammary epithelial cells, which could mediate the effect of *Zfhx3* on PRLR-JAK2-STAT5 signaling and mammary gland development.

Discussion

Postnatal mammary gland development is a multistage process involving a sequence of morphological and molecular changes (4, 32). The transition from pregnancy to lactation activates the secretion of all the nutrients required for the newborns, including milk proteins, lactose, and milk fat. Like other stages of mammary gland development, alveologenesis and lactogenic differentiation are controlled by hormones such as progesterone and prolactin and key transcription factors (4, 32). In this study, we performed histological and molecular analyses using *in vitro* and *in vivo* models to establish the *Zfhx3* transcription factor as a crucial regulator of normal alveologenesis and lactogenic differentiation. We further demonstrated that

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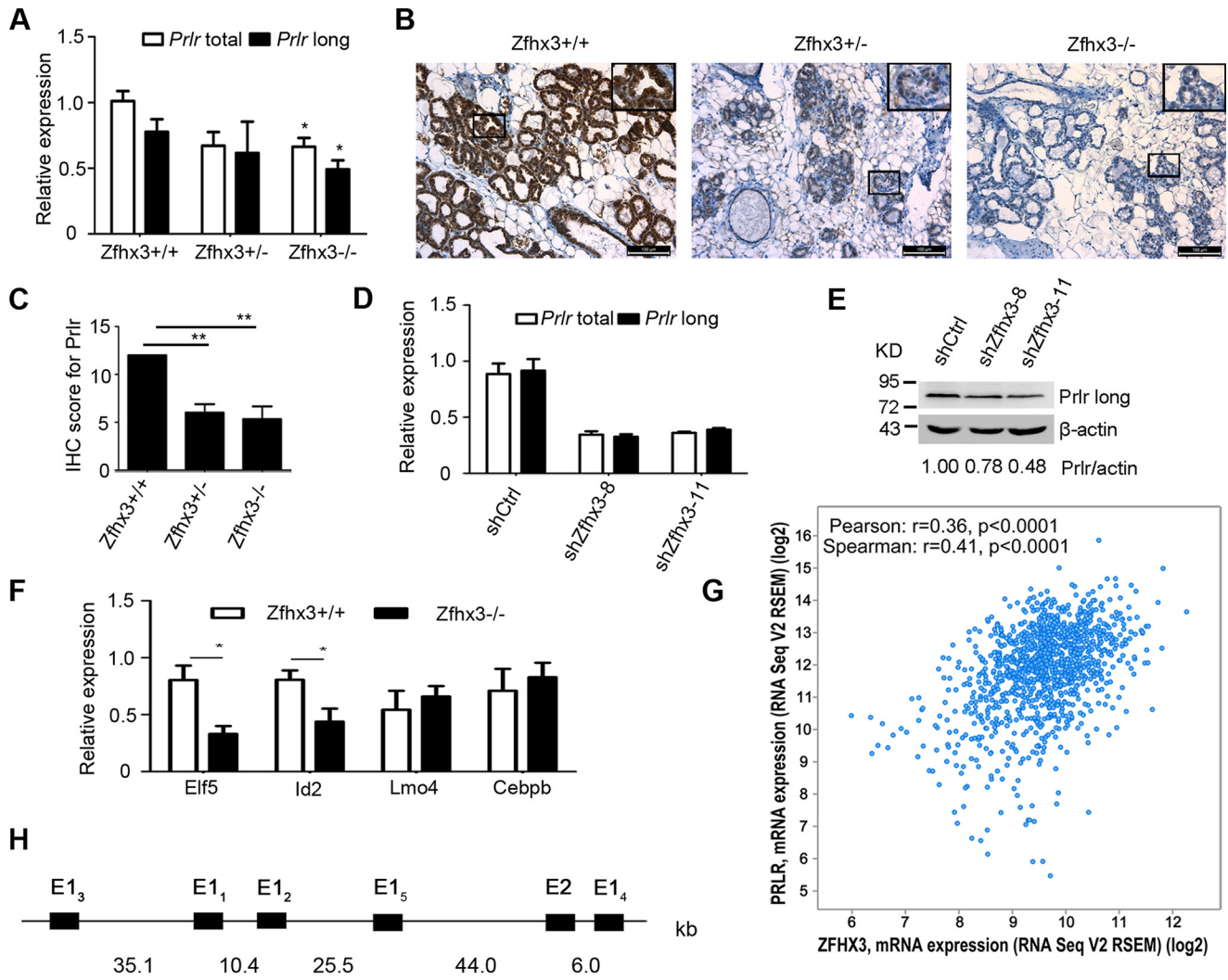


FIGURE 5. Zfhx3 is needed for the expression of Prlr in mouse mammary epithelial cells. A–C, deletion of *Zfhx3* down-regulates Prlr expression, as detected by RT-PCR analysis for *Prlr* mRNA (total and long form, A) or by IHC staining for Prlr protein (B) at day 2 of lactation. C, IHC score is shown as means \pm S.E., statistical significance was calculated by one-way analysis of variance followed by Bonferroni's test; **, $p < 0.01$. D and E, detection of Prlr expression in HC11 cells with stable knockdown of *Zfhx3* by RT-PCR (total and long form, D) or Western blotting (long form, E), band intensities in Western blot were quantified using the ImageJ program. F, *Zfhx3*-mediated gene regulation in mammary alveolar development. Gene expression analysis of mammary glands from MMTV-Cre/*Zfhx3*^{+/+} and MMTV-Cre/*Zfhx3*^{fl/fl} mice at day 2 of lactation. At least four mice per genotype were used; the data are means \pm S.E. Statistical significance was calculated by two-tailed Student's *t* test using GraphPad Prism 5; *, $p < 0.05$. G, the mRNA expression data of ZFHX3 and PRLR in breast invasive carcinoma (TCGA, Provisional) were obtained using cBioPortal. The case set contained 1100 tumor samples. Pearson or Spearman correlation coefficients (r) and two-tailed p value calculations to determine the significance of the correlations were calculated using GraphPad Prism 5. H, the 5' region of mouse *Prlr* and the location of five alternative first exons (represented by black boxes). E2, exon 2.

Zfhx3 functions by maintaining Prlr-Jak2-Stat5 signaling activity.

We first demonstrated that the *Zfhx3* protein level varied at different stages of mammary gland development and reached a peak in late pregnancy and lactation (Fig. 1), which is consistent with the mRNA level of *Zfhx3* reported previously (20). Based on transcriptomic analysis of non-human primate breast tissues at prepubertal, adolescent, adult luteal, pregnant, lactating, and postmenopausal stages, four distinct patterns of gene expression were noticed during reproductive life, including the juvenile pattern, adult pattern 1, adult pattern 2, and the lactational pattern (43). The pattern of *Zfhx3* expression fits between the adult pattern 1 and the lactational pattern, and genes with either pattern of expression likely play a role in prolactin signaling (43). This appears to be the case for *Zfhx3*.

Our functional studies indicated the essential role of *Zfhx3* in prolactin signaling and lactogenic differentiation. Prevention of *Zfhx3* expression compromised alveologenesis and lactogenic differentiation. In the mouse HC11 cell line, a well established and widely used *in vitro* model of lactogenic differentiation, knockdown of *Zfhx3* compromised prolactin-induced cell differentiation including β -casein expression (Fig. 2). More directly, when *Zfhx3* was deleted in mouse mammary glands, the number and size of alveoli decreased, milk protein secretion was attenuated, and litters died after birth, likely because of dehydration (Fig. 3). *Zfhx3* is essential for proper alveologenesis and lactogenic differentiation.

It has been established that lactation involves the expression of numerous genes and the secretion of their gene products in the milk (43, 44). Our previous study demonstrated that dele-

tion of *Zfhx3* in mouse prostates dysregulates a large number of genes, and those encoding for secretory and membrane proteins are the most affected gene groups (17). Using β -casein as a representative of milk proteins, we found that knockdown in HC11 cells and knock-out in mammary tissues indeed interrupted β -casein expression (Figs. 2 and 3), further indicating that *Zfhx3* is necessary for lactogenic differentiation and the production of milk.

Mechanistically, our findings suggest that *Zfhx3* regulates alveolar and lactogenic differentiation by maintaining prolactin-Prlr signaling activity, which is the single most potent regulator of alveologenesis and lactogenic differentiation (45, 46). In this signaling pathway, binding of prolactin to PRLR activates JAK2 kinase, which then phosphorylates the STAT5 transcription factor, leading to the up-regulation of the *Elf5* transcription factor and the expression of genes underlying alveolar and lactogenic differentiation (33, 47–51). We found that *Zfhx3* was not only needed for the expression of the milk protein β -casein (Figs. 2 and 3), an indicator of the PRLR-STAT5 signaling axis, it was also necessary for the phosphorylation of Stat5 in both mouse mammary tissues and cultured HC11 mouse mammary epithelial cells (Fig. 4). Furthermore, we found that *Zfhx3* was required for the expression of *Prlr*. For example, deletion of *Zfhx3* in mouse mammary tissues and knockdown of *Zfhx3* in cultured HC11 mouse mammary epithelial cells significantly down-regulated *Prlr* expression at both the mRNA and protein levels (Fig. 5). Consistently, a significant correlation was detected between mRNA levels of *ZFH3* and *PRLR* in hundreds of human breast cancer samples (Fig. 5G). Therefore, maintaining PRLR expression and PRLR-JAK2-STAT5 signaling activity could be the major mechanism by which *ZFH3* regulates alveolar and lactogenic development of the mammary gland.

The promoter of *PRLR* is complicated, and so is its transcriptional regulation. For example, in addition to the abundantly expressed and widely conserved exon 1 among different species, the *PRLR* gene has multiple additional first exons because of alternative splicing in different tissues (52). Different tissues can have different 5'-regions of the *Prlr* gene (53). Human *PRLR* also has multiple first exons that employ distinct mechanisms for controlling *PRLR* transcription (54). In mice, five alternative first exons have been identified for *Prlr*, including *E1₁*, *E1₂*, *E1₃*, *E1₄*, and *E1₅* (Fig. 5H) (55). Little is known about how the putative promoters upstream of these first exons drive *Prlr* transcription and what factors bind to the promoter sequences. We cloned the putative promoters of the five first exons and measured their promoter activities using dual luciferase reporter assay. Except for the *E1₅* promoter, which showed a slight change in promoter activity, none of the other four putative promoters showed detectable changes when *Zfhx3* was knocked down in HC11 cells (data not shown). It remains to be determined whether *Zfhx3* directly binds to *Prlr* promoter, and if so where it binds. Porcine *Prlr* promoter, which is the best studied so far among different species for the potential use of *Prlr* as a marker for reproductive performance (56–59), has 12 alternative first exons (52). Similar to porcine *Prlr*, the 5' region of mouse *Prlr* is also quite large, spanning more than 120 kb (Fig. 5H). It is thus possible that mouse *Prlr*

has more alternative first exons. Furthermore, whereas STAT5 can bind to an interferon- γ -activated sequence (GAS) in the *PRLR* gene to regulate its transcription (60), it remains to be determined whether *ZFH3* interacts with STAT5 on the same promoter region. Currently we are in the process of examining how *ZFH3* regulates the transcription of *PRLR* in mammary epithelial cells.

Regulation of the PRLR-JAK2-STAT5 signaling by *ZFH3* could play an important role in human breast cancer, because the level of phosphorylated STAT5 gradually decreases during breast carcinogenesis, whereas the total expression level of STAT5 does not appear to change. The decrease of phosphorylated STAT5 is correlated with poorer patient survival and predicts both poor clinical outcome and increased risk of anti-estrogen therapy failure (61–65). PRLR has been shown to suppress epithelial-mesenchymal transition and invasiveness of breast cancer cells (66), and PRLR expression is significantly down-regulated in invasive breast cancer, the down-regulation being associated with lymph node metastasis, lower tumor grade, and metastasis-free survival in breast cancer patients (67). Consistent with the findings of p-STAT5 and PRLR expression, the *ZFH3* mRNA level has also been shown to be frequently down-regulated in human breast cancer (68), which is likely mediated by hemizygous deletion (69), and again down-regulation was correlated with a worse patient survival (68). Indeed, a tumor suppressor function has been established for *ZFH3* in prostate cancer, because mutation of *ZFH3* is frequent in human prostate cancer (70, 71), and deletion of *Zfhx3* in mouse prostates induced neoplastic lesions (17). These findings suggest that *ZFH3*-mediated PRLR-JAK2-STAT5 activity plays a suppressive role in breast cancer development. Currently we are testing this hypothesis by investigating whether *Zfhx3* deletion induces neoplastic lesions in mouse mammary glands.

In addition to *Prlr*, other molecules could also mediate the effect of *Zfhx3* in alveolar and lactogenic development of the mammary gland. For example, deletion of *Zfhx3* in mouse mammary glands led to the down-regulation of *Elf5* and *Id2* (Fig. 5F), both of which have established roles in mammary gland development. *Id2* mediates the function of RANKL in the survival and proliferation of mammary epithelial cells (72, 73), and deletion of *Id2* leads to lactation defects in mice (74). The *Elf5* transcription factor has a similar effect on mammary gland development as *Zfhx3*, and deletion of *Elf5* leads to failure of Stat5 activation and defects in alveologenesis (75). *Elf5* has also been demonstrated to regulate mammary gland stem/progenitor cell fate (76), although such a role has not been examined for *Zfhx3*. Both *Elf5* and *Zfhx3* are transcription factors, but whether they directly interact on gene promoters remains to be clarified. Recently *Zfhx3* was also shown to modify circadian function by directly activating circadian genes *Cry2*, *Per1*, and *Per2* (77). Interestingly, both *Per1* and *Per2* have been implicated in the development and differentiation of mouse mammary gland (78, 79). Therefore, it is more likely that *Zfhx3* regulates alveologenesis and lactogenic differentiation by interacting with or regulating the expression of other factors.

In summary, we found in this study that the *Zfhx3* protein level varied during mammary gland development, reaching the

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highest level in late pregnancy and lactation. Knockdown of *Zfhx3* in HC11 mammary epithelial cells and deletion of *Zfhx3* in mouse mammary tissues attenuated prolactin-induced alveologenesis and lactogenic differentiation, as indicated by morphological and molecular alterations. Mechanistically, knockdown and knock-out of *Zfhx3* decreased the expression level of *Prlr*, *Elf5*, *Id2*, and phosphorylated *Stat5*. These findings suggest that *Zfhx3* plays an essential role in proper development of the mammary gland in pregnancy and lactation by maintaining the activity of the PRLR-JAK2-STAT5 signaling pathway.

Author Contributions—D. Z., M. L., L. F., and J.-T. D. conceived the project. D. Z., G. M., T. N., and J.-T. D. designed all experiments and analyzed all data. D. Z., G. M., X. Z., Y. H., X. H., L. F., X.-Y. D., and Q. Z. prepared and performed experiments. D. Z. and J.-T. D. wrote the manuscript.

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