Loss of EZH2 results in precocious mammary gland development and activation of STAT5-dependent genes

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ABSTRACT
Establishment and differentiation of mammary alveoli during pregnancy are controlled by prolactin through the transcription factors STAT5A and STAT5B (STAT5), which also regulate temporal activation of mammary signature genes. This study addressed the question whether the methyltransferase and transcriptional co-activator EZH2 controls the differentiation clock of mammary epithelium. Ablation of Ezh2 from mammary stem cells resulted in precocious differentiation of alveolar epithelium during pregnancy and the activation of mammary-specific STAT5 target genes. This coincided with enhanced occupancy of these loci by STAT5, EZH1 and RNA Pol II. Limited activation of differentiation-specific genes was observed in mammary epithelium lacking both EZH2 and STAT5, suggesting a modulating but not mandatory role for STAT5. Loss of EZH2 did not result in overt changes in genome-wide and gene-specific H3K27me3 profiles, suggesting compensation through enhanced EZH1 recruitment. Differentiated mammary epithelia did not form in the combined absence of EZH1 and EZH2. Transplantation experiments failed to demonstrate a role for EZH2 in the activity of mammary stem and progenitor cells. In summary, while EZH1 and EZH2 serve redundant functions in the establishment of H3K27me3 marks and the formation of mammary alveoli, the presence of EZH2 is required to control progressive differentiation of milk secreting epithelium during pregnancy.

INTRODUCTION
Cyclical development of mammary tissue can be divided into at least four phases (1,2), establishment of the ductal tree during puberty, formation of alveoli during pregnancy, lactation and tissue remodeling upon weaning. While proliferation of ductal epithelium is under the control of ovarian steroid hormones (3,4), proliferation and differentiation of alveolar epithelium are induced by prolactin (PRL) through the transcription factors STAT5 (5–7) and ELF5 (8). Remodeling of mammary tissue after cessation of lactation is associated with loss of STAT5 activity and gain of activated STAT3, which triggers cell death (9,10). Coordinated proliferation and differentiation of alveolar epithelium are characterized by the sequential activation of a distinct set of approximately 750 genes, whose expression is under strict dose dependent control by STAT5 (11).

Establishment and maintenance of differentiation programs are, at least in part, controlled by the introduction of epigenetic marks, such as specific histone modifications that ensure the precise execution of developmental processes in various tissues (12). Notably, polycomb repressive complexes (PRC) orchestrate lineage-specific proliferation and differentiation, possibly by suppressing untimely transcriptional programs (13–15). The polycomb repressive complex 2 (PRC2) contains four core subunits, including the histone methyltransferase EZH2, which is responsible for the trimethylation of lysine 27 of histone H3 (H3K27me3) (16,17). Research in embryonic stem cells (ESCs) demonstrated that PRC2 and H3K27me3 transcriptionally repress key genes controlling lineage specification (17–25). Inactivation of the Ezh2 gene in specific cell lineages in the mouse has provided additional insight into its ability to control specific functions in B cells (26), T helper cells (27), hematopoietic stem cells (28), cardiomyocytes (29–31), neural crest-derived cartilage and bone (32), neurons (33–35),

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epidermal progenitors and hair follicle stem cells (14), pancreatic islet cells (36), the lymphoid germinal center (37), muscle (13,38,39) and mammary epithelium (40,41). In general, loss of EZH2 affects stem cell activity and cell differentiation. Loss of EZH2 in a given lineage frequently results in the activation of genes characteristic of unrelated cell lineages, suggesting that the presence of EZH2 is necessary to ensure cell identity and maintenance. Although a large body of research has focused on the role of EZH2 as a histone methyltransferase, it also catalyzes methylation of non-histone proteins (42,43) and has the ability to recruit nuclear receptors to specific promoters (44). It has also become clear that EZH1 and EZH2 use different mechanisms to control repressive chromatin (16) and that EZH2 can control the genome outside its enzymatic role (45,46).

Although genetic studies suggest that EZH2 is the predominant methyltransferase required to establish H3K27me3 marks, H3K27me3 marks have been found in cells lacking EZH2, suggesting a compensatory role for EZH1 (17,47). This is supported by genetic studies in which both EZH1 and EZH2-containing complexes were inactivated in Merkel cells, skin and hematopoietic stem cells (28,47,48).

Inactivation of Ezh2 in mammary stem cells in the mouse has been reported to result in the overall loss of H3K27me3 marks and concomitant impairment of mammary development during pregnancy, which led to lactation failure (40,41). Due to potential compensatory functions of EZH1, the full contribution of EZH2 in the biology of the mammary gland remains to be understood. To address these questions, mammary development and alveolar differentiation were investigated in mice that lack either Ezh1 or Ezh2, or both specifically in mammary stem cells.

**MATERIALS AND METHODS**

**Mice**

Ezh2f/f (26) and MMTV-Cre transgenic mice (line A) (49) were used to generate mice lacking EZH2 in mammary stem cells (E2KO). Ezh1 knockout mice (E1KO) generated by the laboratory of Thomas Jenuwein were described earlier (47). Stat5f/f (5) and MMTV-CreA mice were used to generate mice lacking STAT5 in mammary stem cells (S5KO). To generate the mice lacking both EZH2 and STAT5 (E2S5 DKO), Ezh2f/fStat5f/fMMTV-CreA females were bred with Ezh2f/fStat5f/fMMTV-CreA males. Mammary tissue lacking both EZH1 and EZH2 (E1/2 DKO) was generated by transplanting mammary anlagen from Ezh1f/f−/Ezh2f/fMMTV-CreA dams into cleared fat pads of wild type recipients. All animals were handled and housed in accordance with the guidelines of the NIH and experiments were approved by the ACUC of NIDDK.

**Histological analysis**

Whole mounts of mammary tissues from nonparous mice and from day 13 of pregnancy (p13) were fixed in Carnoy’s mix, hydrated and stained with carmine alum. Paraffin sections were stained with H&E by standard methods. For immunostaining, primary antibodies were incubated overnight at 4°C (anti-H3K27me3, Millipore, 1:200; anti-EZH2, Cell Signaling, 1:200; anti-phosphorylated STAT5, Cell Signaling, 1:200; anti-WAP, Santa Cruz Biotechnology, 1:200; anti-E-cadherin, BD Biosciences, 1:100).

**Western blotting**

For EZH2, total protein was extracted in RIPA buffer (50mM Tris-HCl (PH7.5), 150mM NaCl, 10% Glycerol, 50mM NaF, 0.5mM EDTA and 1% NP-40) and 20 μg of protein was loaded on 7% Tris-Acetate gel (Invitrogen) and probed with anti-EZH2 (Cell Signaling, 1:1000) and β-actin (Millipore, 1:10 000). For H3K27me3 detection, mammary epithelial cells (MECs) were prepared as described previously (50). Histones were extracted from MECs using 0.2M HCl and 0.5 μg of histones were loaded on 10–20% Tris-Glycine gradient gels (Invitrogen) and reacted with anti-H3K27me3 (Millipore, 1:2000) and anti-Histone H3 (abcam, 1:2000).

**RNA-seq and qRT-PCR**

RNA-seq experiments were performed and analyzed as described (11). Quantitative PCR was performed using the TaqMan probe-based system (Socs2, Mm00850544_g1; Csn2, Mm00839664_m1; Wap, Mm00839913_m1 and mouse ACTB Endogenous Control on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems)).

**ChIP-seq**

Frozen-stored mouse mammary tissue harvested at day 13 of pregnancy (p13) was broken into powder with a mortar and pestle. Cross-linking was performed with 1% formaldehyde for 10 min at RT followed by the addition of 0.125M glycine to stop cross-linking. Nuclei were extracted in Farnham lysis buffer (5mM PIPES PH8.0, 85mM KCI, 0.5% NP-40 and protease inhibitor). Chromatin was fragmented to 200–300 bp by sonication using a MISONIX Sonicator 3000 (QSonica, Newtown, CT). One mg of fragmented chromatin from tissue lacking EZH2 and one mg control was used for immunoprecipitation with antibodies. Antibodies against STAT5A (Santa Cruz, sc-1081, 10μg), EZH2 (Diagenode, C15410039, 10μg), EZH1 (a generous gift from Dr. V. Sartorelli (51), 10μg) and Pol II (abcam, ab5408, 10μg) were used for ChIP. For STAT5A and Pol II, antibodies were incubated with beads for 1 hr and then incubated with 1 mg of chromatin O/N. For EZH1 and EZH2, 1 mg of chromatin was incubated O/N with antibodies and then beads were added and incubated O/N. Biological replicates were performed for EZH1, EZH2, Pol II and STAT5A. Active Motif performed ChIP-seq with H3K27me3 antibody (Millipore, 07–449) using pooled tissues from three mice. The ChIP DNA fragments were blunt-ended and ligated to Illumina Indexed DNA adaptors and sequenced using the Illumina HiSeq 2000.

**ChIP-seq and RNA-seq analyses**

To align the sequenced reads to the mouse reference genome mm9, Bowtie2 (52) and STAR aligners (53) were used for
ChIP-seq and RNA-seq, respectively. The relative abundances of transcripts were estimated using the Cufflinks program (54). Genome-wide binding sites of proteins and their intensities were assessed using the HOMER application with default settings (55).

Quantification of peak levels from ChIP-seq data at promoters

Promoter regions were defined as 10-kbp flanking regions surrounding transcription start sites (TSSs) of genes. H3K27me3 ChIP-seq data sets from this study were processed in parallel with those of a previous study (GEO: SRP017714) (41). For scatter plot, the number of mapped reads on the promoter regions was counted using BEDTools (56) and normalized by the RPM (reads per million) method. The normalized values in ChIPed samples against the H3K27me3 antibody were further subtracted by the normalized values in corresponding input controls. Promoters with the value below 0 (lower than background) in all samples were discarded. The input-corrected RPM values of promoters were converted to z-score for comparison.

RESULTS

Expression of EZH2 in mammary epithelium is activated during pregnancy

The presence of EZH2 in mammary epithelium was established using immunofluorescence (Figure 1A). Little or no EZH2 was detected in ductal epithelium from nulliparous mice (v) and at day 6 of pregnancy (p6). At p6, EZH2 was present in a subset of alveolar luminal cells. EZH2 levels increased throughout pregnancy with near homogeneous nuclear occupancy observed at the onset of lactation (L1). Western blots further demonstrated that EZH2 levels peaked at mid pregnancy (Figure 1B). These findings are in agreement with published data (41).

Loss of EZH2 results in precocious mammary epithelial development

To identify the biological significance of EZH2 in the development and function of mammary epithelium, the Ezh2 gene was inactivated in mice carrying floxed Ezh2 alleles (26) and an MMTV-Cre transgene (49), which is active in mammary stem cells (41). These mice are referred to as E2KO mice throughout the manuscript. The absence of EZH2 was verified using western blots, immunofluorescence (Figure 1B and C) and ChIP-seq analyses (Figures 4 and 5). While EZH2 was detected in total mammary tissue from control mice throughout pregnancy, with a peak at mid pregnancy, it was almost completely absent in E2KO tissue (Figure 1B). The remaining EZH2 signal at p6 in E2KO tissue can be attributed to stromal cells that significantly contribute to mammary tissue at this stage. Immunofluorescence validated the absence of EZH2 in epithelial cells (Figure 1C). ChIP-seq experiments also confirmed the absence in E2KO tissue (see later, Figures 4 and 5).

E2KO females were fertile, gave birth to normal-sized litters (n > 10) and were able to support their pups. This demonstrates that functional mammary tissue develops during pregnancy in the absence of EZH2. However, it is unknown whether EZH2 contributes to the temporal differentiation program of mammary epithelium, i.e. the sequential activation of mammary-specific genes during pregnancy. To evaluate the contribution of EZH2 to this program, mammary tissue was analyzed at day 13 of pregnancy (p13). This specific time point was chosen as it marks a stage of differentiation that is characterized by the presence of immature, non-secreting alveoli. Steady-state levels of differentiation-specific mRNAs, such as those encoding milk proteins, are low at this stage and increase up to 1000-fold throughout the remainder of pregnancy. Whole mount analysis revealed enhanced mammary alveolar content in E2KO mice compared to controls (Figure 1D, a–d) and histological sections revealed signs of precocious differentiation of alveolar epithelium lacking EZH2 (Figure 1D, e and f). In contrast to controls, only E2KO alveoli displayed overt lumina and small lipid droplets. Notably, whey acidic protein (WAP), a milk protein normally detected in late pregnancy, was already present in E2KO mammary epithelium at p13 (Figure 1E).

EZH2 is not required for ductal development and the activity of mammary stem and progenitor cells

While temporal alveolar development during pregnancy was accelerated in the absence of EZH2, outgrowth of mammary ducts during puberty was unaffected (Supplementary Figure S1A), which is in agreement with the absence of tangible EZH2 expression in ductal epithelium from virgin mice. At six weeks of age outgrowth of mammary ducts had passed the lymph node in the inguinal glands in both wild type and E2KO mice (n = 5) and at 10 weeks mammary fat pads were completely filled regardless of the genotype. A recent study reported delayed outgrowth of the ductal tree, which was partially rescued by 12 weeks of age (41). A comprehensive comparison of data from our study and published data are presented in Supplementary Table S1.

The possibility that EZH2 controls mammary stem cell activity was addressed with limiting dilution transplantation experiments of MECs prepared from mature virgin mice. E2KO and control cells, injected into contralateral fat pads, generated ductal outgrowths to the same extent (Supplementary Figure S1B). Injection of at least 50 000 cells into 32 recipients yielded 22 and 23 outgrowths from wild type and E2KO MECs, respectively. Out of 16 fat pads injected with 500–10 000 wild type and E2KO cells, 7 outgrowths were obtained with either genotype. Whole mount analysis of fat pads transplanted with 1000 and 5000 cells from each genotype showed similar ductal outgrowth (Supplementary Figure S1B). No outgrowths were observed upon transplanting 500 cells from either genotype. These results demonstrate a normal repopulating ability of mammary stem and progenitor cells in the absence of EZH2. In contrast to our findings, a 14-fold decrease in the repopulating frequency of enriched E2KO stem / progenitor cells has been reported (41).
LossofEZH2, butnotEZH1, results in temporally advanced activation of STAT5-controlled genes during pregnancy

The differentiation status of alveoli in the presence and absence of EZH2 was further assessed by RNA-seq analysis from mammary tissue isolated at p13. Steady-state mRNA levels of approximately 398 genes were elevated more than 2-fold in the absence of EZH2 compared to wild type control tissue (Figure 2A and Supplementary Table S2). Moreover, mRNA levels corresponding to approximately 783 genes were decreased by more than 50%. To determine whether these up- and down-regulated genes are also controlled during pregnancy in wild type tissue, we analyzed their expression at mid-pregnancy (p13) and late-pregnancy (p18). Approximately 70% of genes up-regulated in p13 Ezh2-null tissue were also induced in wild type tissue between p13 and p18 (Figure 2B), further supporting the notion that loss of EZH2 enhances the predetermined differentiation program. Approximately 84% of genes, with reduced expression in Ezh2-null tissue are also down-regulated in wild type tissue at late pregnancy (Figure 2B). Since the strong accumulation of milk protein mRNAs during late pregnancy can result in a dilution effect of less abundant mRNAs, it is likely that many of the genes with reduced expression fall into this category.

Gene Ontology (GO) analyses of genes up-regulated in the absence of EZH2 identified biological processes associated with mammary gland development and function, in-
**Figure 2.** Expression of STAT5 target genes is enhanced in the absence of EZH2. (A) Heat map of 1181 genes expressed differentially in mammary tissue at day 13 of pregnancy (p13) in the presence and absence of EZH2 and in p18 wild type tissue. Genes expressed below 5 FPKM in E2KO tissue were removed. The heat map shows 398 genes activated more than 2-fold in E2KO tissue compared to wild type. (B) Pie charts showing overlap of up- or down-regulated genes in E2KO tissue with genes differentially expressed in wild type mice between p13 and p18. GO analysis of up- and down-regulated genes at p13 in E2KO tissue compared to wild type tissue. (C) Differentially expressed genes (DEGs) between p13 and p18 wild type tissue at p13 in the absence and presence of EZH2 (right panel). Three mice from each genotype were analyzed. FDR-adjusted P-value cutoff < 0.05. (D) Coverage plot (top) of normalized tags from RNA-seq shows differential expression throughout gene structure (bottom). Numbers indicate expression fold changes in the absence of EZH2 compared to control. (E) qRT-PCR results of Socs2, Wap and Csn2 mRNA levels at p13 are shown, confirming the RNA-seq data (n = 6). Relative gene expression was obtained from β-actin.
cluding milk secretion, ion transport and response to cytokines (Figure 2B). Genes highly activated during pregnancy, such as Wap, Csn2, Csn1s2a and Cidea, are specifically expressed in mammary epithelium and associated with its differentiation status. Functional categories of down-regulated genes covered cell adhesion, epithelial proliferation, apoptotic process and developmental growth. Fgf10, Esr1, Igf1, Ghr and Wnt2, which are normally expressed mainly during puberty and less during pregnancy and lactation, are involved in these categories. Precocious activation or reduction of these genes at p13 further corroborated the advanced morphological differentiation of alveolar luminal cells in the absence of EZH2.

Notably, genes that are part of the mammary signature (11), such as Lao1, Glycam1 and other milk protein genes were up-regulated at p13 in the absence of E2KO and induced in wild type tissue between p13 and p18 (Figure 2C). Mammary signature genes are under prolactin and STAT5 control and activated up to 10 000-fold during pregnancy (11). In contrast, expression of generic STAT5 target genes, such as Socs2 (57), was not altered (Figure 2D). Expression of Socs2, Wap and Csn2 was validated by qRT-PCR (Figure 2E). These findings suggest that the absence of EZH2 preferentially affects the expression of mammary-specific STAT5 target genes. The relationship between the transcription factor STAT5 and EZH2 will be addressed later.

Since EZH1 is also a methyltransferase with redundant functions to EZH2, we analyzed its physiological role during pregnancy. Loss of EZH1 did not result in an accelerated development of mammary epithelium during pregnancy (Supplementary Figure S2A) and mammary function was overtly normal. Based on RNA-seq data, 94 genes were up-regulated and 242 genes were down-regulated in E1KO tissue compared to wild type control. Only 26% of the up-regulated genes coincided with genes activated in the absence of EZH2 (Supplementary Figure S2B and Table S3). GO analysis (Supplementary Figure S2B) indicated that genes up-regulated in the absence of E1KO or E2KO are enriched for distinct biological processes, suggesting that they participate in different cellular functions. Meanwhile, 96% of the genes down-regulated in E1KO tissue are also reduced in the absence of EZH2. GO analysis pointed to genes associated with developmental process, cellular component morphogenesis and regulation of ion transport. However, hierarchical clustering of gene expression data from p13 wild type, E1KO and E2KO tissue indicated that the E1KO expression pattern was similar to that of wild type tissue (Supplementary Figure S2C). In addition, expression of genes differentially activated in the absence of EZH2 was generally not altered in the absence of EZH1 (Supplementary Figure S2D). Taken together, loss of EZH2, not EZH1, induced precocious differentiation during pregnancy and the activation of mammary signature genes.

Enhanced STAT5 binding in the absence of EZH2

STAT5 is a key transcription factor controlling mammary gland development and the activation of mammary-specific genes. Since precocious differentiation was observed in the absence of EZH2, STAT5A and EZH2 occupancy and H3K27me3 levels were assessed by ChIP-seq in E2KO and wild type p13 mammary tissue. Out of 398 up-regulated genes in E2KO tissue, promoters of 226 genes were occupied by EZH2. Approximately 68% of these EZH2 target genes contained at least one site within ± 10 kb of transcription start sites (TSSs) that was occupied by STAT5. Out of the 783 down-regulated genes, 351 genes coincided with EZH2 binding and 60% displayed STAT5 binding (Figure 3A). Enhanced STAT5 binding was observed near the 226 differentially expressed genes (DEG) in E2KO tissue, but almost no changes in STAT5 occupancy were detected in gene loci not induced despite the presence of at least one STAT5 binding site (Figure 3B). Visualization of several loci validated enhanced STAT5 binding to genes induced during mammary gland development, such as Glycam1 and Wap, but not at genes, such as Socs2, that are not affected by the absence of EZH2 (Figure 3C). In general, the pattern of enhanced STAT5 binding in the up-regulated genes was similar to that observed in wild type tissue at the onset of lactation (Figure 3C). In sum, enhanced STAT5 binding in E2KO mammary tissue was only observed on genes up-regulated in E2KO but not on generic STAT5 target genes, whose expression was not altered. Of note, enhanced STAT5 binding could be merely the consequence of precocious epithelial differentiation and not a direct result of absence of EZH2.

Surprisingly, there were few or no changes in genomewide H3K27me3 levels in the absence of the histone methyltransferase EZH2. For example, Non-DEGs, such as Socs2, are extensively covered by H3K27me3, both in the presence and absence of EZH2 (Figure 3C). Most notably, little or no H3K27me3 coverage was observed on DEGs that are part of the mammary signature, suggesting that they are not controlled by H3K27me3.

EZH2-regulated genes are partially controlled by STAT5

Precocious activation of differentiation-specific STAT5 target genes in the absence of EZH2 is associated with enhanced STAT5 occupancy to respective regulatory regions. However, it is not clear whether enhanced STAT5 binding to these genes is the direct result of EZH2 loss or a consequence of the induced differentiation program. To distinguish between these possibilities, mice were generated that lacked EZH2 and STAT5, individually or combined, and mammary development was evaluated at p13 (Figure 3D). While wild type tissue was characterized by the presence of ducts and immature alveoli, there was a complete lack of alveoli in the absence of STAT5 (Figure 3D, top panel) as published earlier (5). The histological appearance of mammary tissue in the combined absence of EZH2 and STAT5 was indistinguishable from Stat5-null tissue. Using immunofluorescence, phosphorylated STAT5 was detected in mammary epithelium from wild type and E2KO tissue but not in S5KO and E2SS DKO cells (Figure 3D, bottom panel). Histological analysis from biological replicates of mutant mice was included in Supplementary Figure S5.

Although the absence of EZH2 and STAT5 resulted in a complete lack of visible alveolar structures the possibility remained that the differentiation potential of ductal epithelium was affected. RNA-seq was used to gauge the differ-
**Figure 3.** EZH2 does not directly regulate STAT5 target genes. (A) Venn diagram showing the percentage of genes occupied with STAT5 in EZH2 targets. 226 EZH2 targets with increased expression levels in E2KO tissue and 351 EZH2 targets with decreased expression levels in E2KO were identified from combined analysis with RNA-seq and ChIP-seq. (B) Coverage plots of read density within ±1 kb of the center of STAT5 bindings in differentially expressed genes (DEGs) and non-DEGs in E2KO compared to wild type tissue. Asterisk indicates significantly enhanced STAT5 bindings in E2KO (Student’s t-test, \( P \)-value: 0.001665 in DEGs, 0.2852 in non-DEGs). (C) Genome browser snapshots illustrate enrichment of STAT5 (L1 and p13) and H3K27me3 (p13) on mammary-specific and pregnancy-induced genes. Asterisks indicate STAT5 binding sites, which show enhanced STAT5 access in the absence of EZH2 compared to control at p13 while H3K27me3 marks are nearly identical. STAT5 ChIP-seq data set at L1 was used as positive control for STAT5 binding sites. Numbers in boxes indicate the maximum height of normalized reads. (D) Histological analysis of mutant (E2KO, S5KO and E2S5 DKO) and control tissue at p13 (Top). Arrow heads indicate alveoli unit and arrows show ducts at p13 mammary tissue. Immunofluorescence with phosphorylated STAT5 (bottom). Arrows indicate positive signals for pSTAT5. (E) Hierarchical clustering of gene expression levels in control and mutant mice. (F) Pie chart showing recovered gene expression in E2S5 DKO compared to S5KO. (G) GSEA analysis based on 37 STAT5 target genes whose expression increased in E2KO tissue (NES, Normalized enrichment score).
Figure 4. H3K27me3 profiles are maintained in the absence of EZH2. (A) Representative pictures of H3K27me3 staining in control and E2KO tissue. (Arrow: mammary epithelial cells, arrow head: non-mammary epithelial cells such as immune cells as positive control). (B) Western blotting of H3K27me3 in mammary epithelial cells (MECs) isolated from p13 control and E2KO tissues (lanes 1–6). Histone extracts (lane 8) from control (C) and E2KO (lane 7) mouse embryonic fibroblasts (MEFs) are loaded to confirm the position of H3K27me3 band. (C) Scatter plot shows changes in H3K27me3 levels on promoters in E2KO compared to control at p13. (D) H3K27me3 occupancy of a 2Mbp locus of chromosome 9 including the generic STAT5 target gene Cish and chromosome 5 including the casein locus, which contains mammary specific STAT5 target genes. H3K27me3 ChIP-seq data sets from a previous study were integrated for comparison (41). LCs, luminal cells. (E) Genome browser snapshots of EZH1, EZH2 and H3K27me3 ChIP-seq on Cdkn2a/b, Hoxd and Six1. Enrichment of EZH1 the likely cause for the establishment H3K27me3 marks in E2KO cells.
Figure 5. Dual activity of EZH2. (A) Venn diagram showing the number of genes enriched with EZH1 (5836) or EZH2 (5380) in p13 mammary tissue. 4146 out of 5380 genes enriched with EZH2 were co-occupied by EZH1. (B) Pie chart showing the overlap between genes enriched with EZH1, EZH2 and H3K27me3. 84% of genes enriched with H3K27me3 overlapped with EZH1 or EZH2. (C) Pie chart of the percentage of genes enriched with H3K27me3 in EZH2 targets. 51% of EZH2 targets also coincide with H3K27me3 (canonical genes) and 49% of them have minimal H3K27me3 levels (non-canonical genes). Approximately 55–58% of both groups also bind EZH1. (D) GO analysis of canonical or non-canonical genes. (E) ChIP-seq density heat maps are shown for H3K27me3, EZH1, EZH2 and Pol II within canonical and non-canonical genes. Non-canonical genes have low H3K27me3 and high EZH2 occupancy in control tissue and increased EZH1 binding in E2KO tissue. Their expression is more than 2-fold increased in E2KO tissue compared to controls. Canonical genes are characterized by high H3K27me3 and high EZH2 occupancy in wild type tissue and increased EZH1 binding in E2KO tissue. These genes are generally expressed at low levels (below 5 FPKM in control and E2KO tissue). (F) Coverage plots of read density for H3K27me3, EZH1, EZH2 and Pol II within −10/+10 kb of transcription start sites (TSSs) of canonical and non-canonical genes. (G) Genome browser snapshots of H3K27me3, EZH1, EZH2 and Pol II ChIP-seq on the Hoxa cluster representing canonical genes and the Casein locus representing non-canonical genes.
entiation status of mammary epithelium with all four genotypes (wild type control, E2KO, S5KO and E2S5 DKO). Hierarchical clustering revealed an overall similar gene expression pattern in E2S5 DKO and S5KO tissue (Figure 3E). However, expression of a subset of genes differed between S5KO and E2S5 DKO tissue (Supplementary Table S4). Expression of mammary signature genes was elevated in E2S5 DKO tissue, suggesting EZH2 could rescue expression of these strictly STAT5-dependent genes. We specifically analyzed to what extent expression of genes with decreased activity in S5KO tissue would recover upon additional loss of EZH2. Expression of 93 genes displaying STAT5 binding to promoter and enhancer sites was decreased by more than 50% in S5KO tissue (Figure 3F). Notably, 40% of STAT5 target genes had increased expression in E2KO tissue. Gene set enrichment analysis (GSEA) of this gene set indicated that 59% of them recovered, albeit not fully, in the combined absence of STAT5 and EZH2 (Figure 3G). Among these genes were bona fide mammary-specific STAT5 targets, including those encoding milk proteins and membrane transporters. These results demonstrate that EZH2 directly regulates a subset of mammary specific STAT5 targets.

Presence of H3K27me3 marks in mammary tissue is independent of EZH2

Since EZH2 has been proposed to be the defining H3K27 methyltransferase in mammary epithelium (41) it is possible that precocious activation of mammary-specific genes in the absence of EZH2 could be the result of loss of H3K27me3 marks. The H3K27me3 status of wild type and E2KO tissue at p13 was initially assessed by immunofluorescence. Nuclear staining of H3K27me3 in mammary epithelial cells was equivalent in the presence and absence of EZH2 (Figure 4A). Moreover, western blots demonstrated the presence of H3K27me3 in mammary epithelial cells (MECs) isolated from E2KO tissue (Figure 4B).

To ascertain H3K27me3 occupancy at specific gene loci, genome-wide H3K27me3 marks were assessed by ChIP-seq. Scatter plot analyses demonstrated no significant differences in the H3K27me3 tag density between control and E2KO tissue (Figure 4C). Parameters used to analyze ChIP-seq data were identical to those applied in a study that had established H3K27me3 maps from enriched luminal cells (41). Consistent with western blot results, H3K27me3 marks throughout the genome were comparable between control and E2KO mammary tissue harvested at p13. H3K27me3 profiles at specific loci, as exemplified by a 2Mbp region on chromosome 9 that includes the STAT5 target Cish, were indistinguishable between mammary tissue from control and E2KO mice (Figure 4D, upper panel). A direct comparison of data from this study and those from enriched luminal cells published earlier (41) demonstrated equivalent H3K27me3 patterns (Figure 4D). However, the study by Pal and colleagues did not feature H3K27me3 ChIP-seq data from cells lacking EZH2 (41).

While the 2Mb locus including the Cish gene was characterized by extensive H3K27me3 coverage, minimal occupancy was observed at mammary-specific loci, such as the one encoding the casein genes (Figure 4D, lower panel) and other mammary-specific genes (Figure 3C). H3K27me3 occupancy was equivalent in the presence and absence of EZH2. Again, the pattern obtained from tissue at p13 was similar to that obtained from enriched luminal cells from wild type mice (41). The paucity of H3K27me3 marks was specific to mammary signature genes, such as those activated during pregnancy, and did not spread into neighboring genes as can be seen in the casein locus (Figure 4D, lower panel). H3K27me3 enrichment was observed on widely expressed STAT5 target genes, such as Socs2, but not on mammary-specific STAT5 target genes, such as Spp1 and Wap (Figure 3C). Strong, yet EZH2-independent, H3K27me3 was observed at loci encoding patterning genes, such as the Hoxd gene cluster (Figure 4E) (58). Furthermore, bona fide PRC2 target genes, such as Cdkn2a, Cdkn2b (41) and Six1 (29) displayed equivalent levels of H3K27me3 levels in E2KO and control tissue (Figure 4E). EZH2 ChIP-seq validated the absence of EZH2 in E2KO tissue (Figure 4E). These results demonstrate that EZH2 is not required for the presence of H3K27me3 marks in mammary epithelium.

Recruitment of EZH1 and Pol II is enhanced in the absence of EZH2

The presence of genome-wide and gene-specific H3K27me3 marks in E2KO mammary tissue suggested a substituting role for EZH1. To address this hypothesis, genome-wide binding maps were established for both EZH1 and EZH2. Biological replicates for ChIP-seq were performed (Supplementary Figure S4A) and the specificity of EZH1 antibodies was validated by EZH1 ChIP-seq in E1KO tissue (Supplementary Figure S4B). ChIP-seq of p13 wild type mammary tissue resulted in the identification of 5380 gene loci enriched for EZH2 and 5836 gene loci in E2KO tissue were enriched for EZH1 (Figure 5A). Approximately 77% of gene loci enriched for EZH2 were also occupied by EZH1 (Figure 5A) and 84% of the 3748 gene loci enriched for EZH1 were also occupied by EZH1 or EZH2 (Figure 5B). This is further indication that these two methyltransferases likely serve redundant functions in the establishment of H3K27me3 marks.

The possibility that EZH1 could compensate for the loss of EZH2 was investigated by establishing the EZH1 occupancy in the absence of EZH2 (Figure 5E). Increased EZH1 occupancy was observed in E2KO tissue at genes that include bona fide PRC2 targets, such as Cdkn2a, Cdkn2b, Hoxd cluster and Six1 (Figure 4E) and mammary-specific genes, such as the casein locus (Figure 5G). Notably, mammary-specific loci failed to accrue H3K27me3 marks despite increased EZH1 occupancy, suggesting that neither EZH1 nor EZH2 exert K27 methyltransferase activity at those loci.

A recent study reported that EZH2 is involved in gene regulation through the non-PRC2 function as a transcriptional coactivator (44). To assess the dual roles of EZH2 as activating and repressive function at target genes, RNA-seq and ChIP-seq performed in the presence and absence of EZH2 were analyzed by using the BETA application, which can predict functions of a DNA-binding protein through the integrative analysis of RNA-seq and ChIP-seq (59). Briefly, if the function of a given protein is either activator
or repressor, cumulative fraction of its target genes should show substantial difference in a plot. However, analysis revealed that similar fractions of EZH2 target genes were observed in case of EZH2 (Supplementary Figure S4D). Therefore, the result clearly showed that EZH2 acts not only as a repressor, but also as an activator of transcription and might be associated with a redundant protein, EZH1.

To examine K27 methyltransferase-dependent and independent roles of EZH2 systematically, the genome was divided into two groups according to observed enrichment of H3K27me3 and EZH2 (Figure 5C). One group, predominantly occupied by EZH2 and H3K27me3 marks, was named ‘Canonical genes’. The other group named ‘Non-canonical genes’ was highly enriched for EZH2 but few or no H3K27me3 marks were detected. One half of the genes enriched for EZH2 belong to the canonical group and the other half were non-canonical genes. As expected, the majority of canonical (85%) and non-canonical (69%) genes were also occupied by EZH1, suggesting that EZH1 is not only a methyltransferase but also a co-activator for a subset of genes in mammary tissues. GO analysis indicated that the canonical- and non-canonical genes participated in distinct biological processes (Figure 5D). The canonical genes were associated with various developmental processes and the non-canonical group, including Med20, Med23, Glycam1, Lao1 and Caseins, is associated with transcriptional regulation and mammary biology. These results suggest that EZH2 not only mediates methylation on histone H3 lysine 27 but also regulates expression of their target genes as a co-activator. Furthermore, detailed analyses of genome-wide enrichments of EZH1, EZH2 and H3K27me3 in E2KO and wild type mammary tissues supported a compensatory role of EZH1.

EZH1 can control transcription by recruiting RNA polymerase II (Pol II) in muscle cells (51). Increased EZH1 occupancy to mammary-specific genes observed in E2KO mammary tissue suggests that EZH1 might recruit Pol II thereby activating specific target genes. To address this hypothesis Pol II occupancy was assessed using ChIP-seq (Figure 5E). A total of 56 non-canonical genes and 614 canonical genes were identified. As expected, genes highly up-regulated in E2KO tissue belong to the non-canonical group whereas relatively repressed genes (below 5 FPKM) were in the canonical group (Supplementary Table S5). For example, the Hoxa cluster containing canonical genes showed little or no increase of Pol II occupancy in the absence of EZH2, while the casein locus displayed enhanced Pol II loading. Accordingly, coverage plots of read density for H3K27me3, EZH2, EZH1 and Pol II showed that enhanced occupancy by Pol II was only observed in non-canonical but not in canonical genes (Figure 5F). Interestingly, increased peak density of EZH2, EZH1 and Pol II in the non-canonical group was higher in gene bodies than in promoter/upstream sequences, while peak density of canonical genes was almost identical in these regions. Although Pol II binding was detected in these genes, their expression levels remained below 5 FPKM and coincided with strong H3K27me3 marks, suggesting that Pol II binding is not sufficient to activate their target genes (60). Overall, these results suggest that EZH1 compensates for the loss of EZH2 and establishes H3K27me3 marks at PRC2-targeted loci and maintains the expression of mammary specific genes through the recruitment of RNA Pol II.

Mammary alveolar development is attenuated in the combined absence of EZH1 and EZH2

Since this study provided evidence that mammary-specific genes are co-occupied by EZH1 and EZH2, and of increased EZH1 occupancy in the absence of EZH2, it was fair to suggest redundant functions of these proteins in the development and differentiation of mammary epithelium during pregnancy. To test this hypothesis, mammary tissue devoid of both EZH1 and EZH2 was investigated. Breeding efforts to generate mice carrying two germine Ezh1-null alleles, two floxed Ezh2 alleles and the MMTV-Cre transgene failed. Since the MMTV-Cre transgene is also active in a restricted set of non-mammary cells it is likely that the combined loss of EZH1 and EZH2 in these cells is incompatible with survival of mice.

To bypass this stumbling block, mammary anlagen from Ezh1−/− Ezh2−/−;MMTV-CreL (E1/2 DKO) embryos were transplanted into cleared fat pads of wild type recipients. After two months, transplanted tissue was harvested from nulliparous mice and at p13 and subjected to histological analysis (Figure 6). Reduced puberty-mediated ductal outgrowth was obtained and no visible alveologenesis was observed with E1/2 DKO cells. The lack of differentiated secretory alveoli at p13 and the presence of disorganized cellular structures indicated a perturbed morphogenetic program. Immunofluorescence verified the absence of H3K27me3 in E1/2 DKO epithelial cells while staining was present in surrounding immune cells serving as positive control (Figure 6). On the other hand, H3K27me3 was present in mammary epithelial cells of non-parous (Supplementary Figure S3A and B) and in p13 E1KO and E2KO single KO mice (Supplementary Figure S3C). Cells positive for pSTAT5 were observed in wild type tissue but not in E1/2 DKO cells (Figure 6). These results demonstrate that no H3K27me3 marks are formed in mammary epithelium in the combined absence of EZH1 and EZH2 and no normal alveolar development is attained.

Comparison with previously published data

Supplementary Table S1 details experiments and findings from this study and a previous publication (41) using mice with a mammary-specific deletion of Ezh2. Both studies were based on mice carrying floxed Ezh2 alleles (26) and MMTV-Cre transgenic mice (49) that have been used extensively to delete sequences flanked by loxP sites in mammary stem cells. While mice used by Pal and colleagues were in a C57BL/6 background, ours were in a mixed background containing C57BL/6, 129 and FVB/N. While some data are in agreement in both studies, others differed considerably. Both studies established that EZH2 expression is induced in mammary epithelium during pregnancy. However, functional, histological and molecular studies on mammary-specific E2KO mice yielded substantially different results. While mutant mice generated in the Pal study were unable to rear their litters, dams in our study successfully raised their litters.
In the present study the absence of EZH2 was established by immunofluorescence, western blots and ChIP-seq. The existence of H3K27me3 marks in wild type and E2KO tissue was validated by immunofluorescence, western blot and ChIP-seq. In contrast the study by Pal et al. used western blots to determine H3K27me3 levels. They validated decreased H3K27me3 occupancy for specific loci such as Cdkn2a and Cdkn2b by ChIP-qPCR. However, they did not include H3K27me3 ChIP-seq data from E2KO tissue thus making it difficult to assess this histone modification in the absence of EZH2. Analysis of H3K27me3 ChIP-seq in wild type tissue at mid pregnancy demonstrated that the overall pattern of H3K27me3 marks from our study was similar to that of enriched luminal cells described by Pal et al. Loci, which contain PRC2 target genes, such as Cdkn2a and Cdkn2b, have strong H3K27me3 marks while H3K27me3 marks are mostly absent in mammary specific loci such as the Casein locus.

Our study also contains EZH1 ChIP-seq data, which suggest that EZH1 can compensate for the loss of EZH2 to establish and maintain genome-wide H3K27me3 marks. In addition, we performed RNA Pol II ChIP-seq experiments, which demonstrate that the absence of EZH2 triggers not only enhanced EZH1 binding but also recruitment of RNA Pol II to mammary specific loci, which agrees with elevated expression of mammary-specific genes. Since the study by Pal and colleagues did not include ChIP-seq data for EZH1, EZH2 and RNA Pol II, it is not possible to address a compensatory role by EZH1 in their mice.

**DISCUSSION**

This study demonstrated that EZH2 is not required for functional mammary gland development and mammary stem cell activity but its presence ensures correct timing of mammary alveolar differentiation during pregnancy. Precocious differentiation of mammary alveoli occurs in the absence of EZH2, which coincides with the recruitment of...
However, this morphogenetic role might be confined to specific cell types as epidermal cells hyperproliferate in the absence of EZH1 and EZH2 (47). While there is no compelling evidence for a role of EZH2 in normal mammary stem cells, its overexpression might contribute to the biology if tumor initiating cells (64).

Precocious alveolar development and differentiation of mammary epithelium in the absence of the histone methyltransferase EZH2 had suggested a contribution of H3K27me3 in the temporal regulation of genetic programs. However, genome-wide H3K27me3 profiles were unaffected by the absence of EZH2 and the intensity was even elevated at specific genes. Enhanced recruitment of EZH1 in E2KO cells could certainly explain the presence of H3K27me3 marks, an issue not addressed in the previous study (41). Notably, elevated Pol II occupancy of mammary specific genes coincided with EZH1 recruitment in the absence of EZH2 and paralleled with elevated expression of the respective genes. Sartorelli and colleagues (51) had suggested that EZH1 recruits RNA Pol II to muscle specific genes and thereby controls their expression independent of the H3K27me3 status. Although this mechanism shows similarity to mammary epithelium, there are distinct differences to muscle. Most notably, loss of only EZH1 does not adversely affect mammary development and differentiation, suggesting that it plays a less prominent role than in muscle cells. In addition, EZH1 recruitment in mammary tissue preferentially affects genes that are under STAT5 control. Notably, while EZH1 is known to directly repress gene expression in some cell lines through compacting chromatin (16), in organs, such as mammary tissue and muscle, it potentially acts as a transcriptional activator.

STAT5 occupancy of mammary-specific genes was enhanced in the absence of EZH2, coinciding with their increased expression. Elevated STAT5 signaling could be the result of increased pSTAT5 levels, enhanced STAT5 binding due to the acquisition of additional transcription factors, such as the steroid hormone receptors (44), or enhanced STAT5 binding to specific targets upon loss of EZH2. Involvement of EZH2 in STAT5 signaling can also be inferred from studies, where loss of EZH2 in T cells results in increased IFNγ production and skewing of T cell populations (65). Loss of EZH2 has also been linked to the regulation of lineage specifying genes in T cells (27) and a role in controlling gene expression in specific neuronal subtypes (66) has been shown. A direct interaction between STAT5 and EZH2 has been reported in B cells where STAT5 tetramers recruit EZH2 to a specific site in the Igk locus and thereby repress germline transcription (67). However, there is no evidence for a direct STAT5-EZH2 interaction in mammary epithelium and a steric hindrance might be more plausible. Enhanced STAT5 binding to genes induced in the absence of EZH2 is possibly not an initiating event of precocious differentiation as limited gene activation can also be seen in the combined absence of STAT5 and EZH2. We therefore propose that enhanced STAT5 occupancy to mammary signature genes is the result of precocious differentiation in the absence of EZH2. It has been reported that EZH2 methylates a specific lysine residue in STAT3 and thereby increases its phosphorylation status and activity in glioma cells (43). However, we have not detected methylated STAT5 in mammary epithelium (unpublished data).
Figure 7. Model defining roles of EZH2 in mammary epithelial cells. Absence of EZH2 induces precocious differentiation. Milk protein genes, which are normally expressed in late pregnancy, are already expressed during mid-pregnancy in E2KO mammary epithelium. EZH2 acts as methyltransferase on Histone H3 Lysine 27 at silent loci in mammary epithelium. EZH1 compensates for loss of EZH2 to maintain H3K27me3 marks in PRC2 target genes such as Cdkn2a, Cdkn2b, Hoxa cluster and Six1. EZH2 is a transcription regulator for mammary specific genes, which lack significant H3K27me3 marks. Loss of EZH2 results in enhanced recruitment of STAT5 and EZH1 to these loci. EZH1 recruits RNA Pol II to increase expression of mammary specific genes. This model represents steady state situations and it is possible that enhanced STAT5 binding is the consequence of precocious differentiation and not necessarily a direct effect of EZH2 loss.

In summary, the presence of EZH2 in mammary alveolar epithelium ensures the precise execution of a defined differentiation program leading to lactation. Likely, this function is independent of the genomic H3K27me3 status, which is not adversely affected by the absence of EZH2. Rather our study proposes that the presence of EZH2 enables the controlled recruitment of EZH1, STAT5 and RNA Pol II to mammary specific genes.

ACCESSION NUMBERS
The data presented in this article have been deposited to GEO under accession number (GSE70440).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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