Stat5a is mandatory for adult mammary gland development and lactogenesis

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Prolactin (PRL) induces mammary gland development (defined as mammapoiesis) and lactogenesis. Binding of PRL to its receptor leads to the phosphorylation and activation of STAT (signal transducers and activators of transcription) proteins, which in turn promote the expression of specific genes. The activity pattern of two STAT proteins, Stat5a and Stat5b, in mammary tissue during pregnancy suggests an active role for these transcription factors in epithelial cell differentiation and milk protein gene expression. To investigate the function of Stat5a in mammapoiesis and lactogenesis we disrupted this gene in mice by gene targeting. Stat5a-deficient mice developed normally and were indistinguishable from hemizygous and wild-type littermates in size, weight, and fertility. However, mammary lobuloalveolar outgrowth during pregnancy was curtailed, and females failed to lactate after parturition because of a failure of terminal differentiation. Although Stat5b has a 96% similarity with Stat5a and a superimposable expression pattern during mammary gland development it failed to counterbalance for the absence of Stat5a. These results document that Stat5a is the principal and an obligate mediator of mammapoietic and lactogenic signaling.

[*Key Words:* Stat5 proteins; epithelial cell differentiation; lactogenesis; mammapoiesis]

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Lobuloalveolar proliferation and differentiation of the mammary gland during pregnancy requires the coordinated action of steroid and peptide hormones (Topper and Freeman 1980). In the cast of characters, prolactin (PRL) appears to be a central player in the promotion of mammapoiesis and lactogenesis (Vonderhaar 1987). PRL was first isolated and identified as a lactogen in 1933 (Riddle et al.), and it can induce lobuloalveolar growth in hypophysectomized virgin rats (Lyons 1958). Induction of terminal differentiation of mammary alveolar cells prior to and at parturition, as indicated by the transcriptional activation of milk protein genes, also requires the presence of prolactin (Topper and Freeman 1980; Pittius et al. 1989; Burdon et al. 1991a).

Binding of PRL to the PRL receptor (PRLR) induces receptor dimerization and activation of Janus kinase 2 (JAK2) (Darnell et al. 1994; Schindler and Darnell 1995). JAK2, in turn, phosphorylates transcription factors that belong to the family of signal transducers and activators of transcription (STAT) (Ihle and Kerr 1995; Schindler and Darnell 1995). Phosphorylation of specific tyrosine residues confers DNA-binding activity to STAT proteins and results in the transcriptional activation of genes containing γ -interferon activation sites (GAS) (TTCNNGAA). In vivo experi-

ments in transgenic animals have shown that the GAS sites in the β -lactoglobulin (BLG) (Burdon et al. 1994) and whey acidic protein (WAP) genes (Li and Rosen 1995) are required for maximal transcription during lactation. PRL has been shown in tissue culture cells to signal through Stat1, Stat3, and Stat5 (DaSilva et al. 1996) and to activate promoters containing the β -casein gene GAS site (Gouilleux et al. 1994b; Wakao et al. 1994; Liu et al. 1995). In the functional postpartum mammary gland high levels of activated Stat5 can be found, whereas only small amounts of phosphorylated Stat1 and Stat3 are detected (Liu et al. 1996). This led us to hypothesize that the activation of Stat5 is a critical step in the terminal differentiation of mammary secretory epithelial cells (Liu et al. 1996). The two isoforms of Stat5 (a and b) show 96% similarity (Liu et al. 1995) and form heterodimers upon phosphorylation (Liu et al. 1996). Phosphorylation of both isoforms is very low in mammary tissue of virgins and during early pregnancy but rises sharply after day 14 of pregnancy, supporting the hypothesis that Stat5 is necessary for alveolar proliferation and differentiation and is a key transcription factor of the WAP gene (Liu et al. 1996). Stat5a and Stat5b are expressed in most if not all tissues, and they can be activated in tissue culture cells by many cytokines and growth modulators, including epidermal growth factor (EGF), growth hormone (GH), erythropoietin (EPO), granulocyte macro-

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phage colony stimulating factor (GM-CSF), different interleukins, and PRL (Ihle and Kerr 1995). This suggests that Stat5 transcription factors are components of different signaling pathways leading to cell growth and differentiation.

Although Stat5a and Stat5b are homologous, it is unclear whether their functions are purely redundant, or unique in some respects. The only profound difference between Stat5a and Stat5b is within their carboxyl termini (Liu et al. 1995), which encompass a transcriptional activator sequence (Moriggl et al. 1996). To address the role of Stat5a in development, we have generated mice in which the Stat5a gene has been inactivated by homologous recombination. Stat5a-deficient mice are normal in appearance, size, weight, and fertility, suggesting that most cytokine signaling pathways are grossly intact. However, mammary development is impaired in Stat5a-deficient mice and females fail to lactate. These results demonstrate an obligate role for Stat5a in mammary growth and differentiation and reveal an unexpected level of specificity when activated under physiological conditions.

Results

Generation of Stat5a-deficient mice

Successful targeting of the Stat5a locus was achieved in 4 of the 120 G418-resistant clones (data not shown). Two independently targeted embryonic stem (ES) cell clones were used to produce male chimeras, and germ-line transmission of the disrupted allele was obtained. Heterozygous crosses produced F₂ offspring that segregated

alleles in a Mendelian 1:2:1 ratio as detected by Southern blot analysis (Fig. 1B). Homozygous Stat5a-deficient mice did not show any overt developmental abnormalities and were indistinguishable from their hemizygous or wild-type littermates on the basis of size, activity, or fertility.

Stat5a-deficient mice fail to lactate

To evaluate the contribution of Stat5a in mammary development and function, homozygous females were bred. Fertility, length of gravidity, and litter size in Stat5a-deficient mice were comparable to that seen in hemizygous and wild-type littermates. However, the 12 homozygous mothers analyzed up to this point were unable to nurse their young. Despite continued mothering and nurturing by the mothers and constant and vigorous suckling by the pups, no milk was detected in the stomachs of the pups. Upon fostering with hemizygous females, these pups thrived, which suggested that Stat5a-deficient females could not produce or eject milk. To test whether the females could produce and secrete milk after an extended period of suckling, we replaced the litter every 12 hr with a set of healthy and strong pups that were accepted by the female and continued to suckle. No signs of successful lactation were obtained, demonstrating that the glands were unable to produce and deliver milk.

Whole-mount analysis of mammary tissue from postpartum Stat5a-deficient mice demonstrated incomplete mammary development (Fig. 2A) compared to hemizygous littermates (Fig. 2B). Ductal development appeared normal,

Figure 2. Stat5a-deficient mice display aberrant mammapoiesis. Mammary tissue was biopsied from hemizygous (B,D,F,H) and Stat5a-deficient (A,C,E,G) mothers within 12 hr after parturition (A–C,G,H) or after 3 days of suckling (E,F). (A,B) Whole-mount image; (C,D) sections of the whole mounts shown in A and B; (E,F) sections of tissue after 3 days of suckling. To maintain suckling in the Stat5a-deficient mice, the mother was provided with a new litter every 12 hr. Whole mounts were stained with carmine red; sections were stained with hematoxylin and eosin (C–F) or with anti-WAP antibodies (G,H).

but lobuloalveolar outgrowth was severely reduced. Thin-section analyses confirmed that secretory tissue from Stat5a-deficient mice was sparse (Fig. 2C). In contrast to alveoli from wild-type or hemizygous mice (Fig. 2D), alveoli from Stat5a-deficient mice were petite and contained small lumina (Fig. 2C). Fat droplets were present within the cytoplasm indicating metabolic activity of the tissue. Continued suckling for 3 days by providing fresh sets of pups twice daily led to some additional lobuloalveolar development (Fig. 2E). However, alveoli and lumina remained small. In contrast, alveoli from hemizygous mice expanded even further (Fig. 2F). In summary, lobuloalveolar units of the Stat5a-deficient mice were underdeveloped and did not exhibit a secretory phenotype, even after maximal stimulation of PRL secretion induced by suckling.

Reduction of Stat5b activity in Stat5a-deficient mice

The absence of Stat5a and the presence of Stat5b in ho-

mozygous mice was verified using immunoprecipitations and Western blot analyses. Mammary extracts from postpartum hemizygous and homozygous null mice were subjected to immunoprecipitation with anti-Stat5a antibodies. The immunoprecipitate was separated in an SDS-polyacrylamide gel, transferred to a PVDF membrane and probed consecutively with anti-Stat5b antibodies to evaluate heterodimer formation, with anti-phosphotyrosine antibodies to estimate the amount of active Stat5a, and with anti-Stat5a to visualize the total amount of Stat5a (Fig. 3A). Stat5a was clearly present in mammary tissue from hemizygous mice but completely absent in the null mice. Stat5a-Stat5b heterodimers were observed in hemizygous, but not in homozygous null mice. As expected, Stat5a in hemizygous mice was tyrosine phosphorylated.

Mammary extracts from the same mice were analyzed for the presence of Stat5b (Fig. 3B). Stat5b, its heterodimerization with Stat5a, and the phosphorylation were clearly observed in hemizygous mice. In contrast, the level of Stat5b and its phosphorylation were severely reduced in Stat5a-deficient mice (Fig. 3B). Stat5b levels increased after 3 days of continued suckling, and phosphorylation was apparent (Fig. 3B). To control for loading, mammary extracts were separated in an SDS-polyacrylamide gel, blotted onto a PVDF membrane, and probed directly with anti-Stat5b antibodies. Again, Stat5b was present in much lower levels in the Stat5a-deficient mice than in hemizygous littermates (Fig. 3B). Because Stat3 can also be activated by PRL, we tested whether increased Stat3 activity could compensate for the absence of Stat5a. However, phosphorylation of Stat3

Figure 3. Activation of Stat5a and Stat5b. Mammary tissue from postpartum mice was analyzed for the presence of Stat5a-Stat5b heterodimers, tyrosine-phosphorylated Stat5a, tyrosine-phosphorylated Stat5b, the presence of Stat5a and Stat5b, the presence of tyrosine-phosphorylated Stat3, and Stat5b protein in a Western blot. (Lanes a,b) Mammary tissue from littermates within 12 hr after parturition; (lanes e,f) same littermates as shown in lanes a and b but after 3 days of suckling (the histology shown in Fig. 2 is from these mice). To guarantee suckling, the litter of the Stat5a $-/-$ female was switched every 12 hr with that of the Stat5a $+/-$ female. (Lanes c,d) Mammary tissue from a second set of littermates within 12 hr after parturition.

was not altered significantly in the Stat5a-deficient mice (Fig. 3A; note that the blot with Stat3 was exposed ~500 times longer than that for Stat5a and Stat5b).

Milk protein genes are expressed in Stat5a-deficient mice

It has been established in tissue culture cells that mammary gland-specific factors (MGF) (now Stat5a and Stat5b) activate the β -casein gene promoter that contains a GAS site (Schmitt-Ney et al. 1991; Gouilleux et al. 1994a; Standke et al. 1994; Liu et al. 1995; Moriggl et al. 1996). Support for an in vivo role of STATs in milk protein gene expression comes from experiments with WAP (Li and Rosen 1995) and BLG (Burdon et al. 1994) transgenes in which the GAS sites had been inactivated. The consequence of a complete absence of Stat5a and reduction of Stat5b on milk protein gene expression was evaluated in Stat5a-deficient mice. RNA was isolated from hemizygous and homozygous null mice, both within 12 hr after parturition, and also after 3 days of suckling, followed by an analysis for the presence of milk protein RNAs (Fig. 4). Within 12 hr after parturition, steady-state levels of α -lactalbumin and WDNM1 (another major milk protein) RNA were similar in wild-type, hemizygous, and null mice. However, β -casein RNA was slightly reduced in null mice, and WAP RNA was reduced to ~10% (Fig. 4). Upon continued suckling, WAP and β -casein RNA levels remained constant. WDNM1 and α -lactalbumin levels remained constant in null mice but declined in hemizygous mice (Fig. 4). In summary, expression of milk protein genes, with the exception of the WAP gene, is not affected by the complete absence of Stat5a and reduction of Stat5b.

Mammary protein composition in Stat5a-deficient mice

Total mammary protein from postpartum mice was separated in an 18% SDS-polyacrylamide gel and either stained with Coomassie blue (Fig. 5A) or blotted onto a PVDF membrane followed by analysis with anti-WAP antibodies (Fig. 5B). The protein pattern in Stat5a-deficient mice was indistinguishable from that in wild-type and hemizygous mice (Fig. 5A). The only clear difference was seen in the amount of WAP (Fig. 5B). In agreement with the Northern blots, WAP levels were greatly reduced in the Stat5a-deficient mice (Fig. 5B).

Secretion of milk proteins

Translation of milk protein RNAs results in protein precursors containing signal peptides (Hennighausen and Sippel 1982b). As part of the secretion process these signal peptides are cleaved as the nascent protein chain is translocated into the endoplasmic reticulum. Western blot analysis confirmed that WAP was synthesized and processed in mammary tissue of Stat5a-deficient mice, as evidenced by the cleaving of the signal peptide (Fig. 5). To evaluate the ability of alveolar cells to secrete milk

proteins, immunocytochemical analyses were performed (Fig. 2G,H). Using anti-WAP antibodies, we observed WAP in the lumina of Stat5a-deficient mice (Fig. 2G), demonstrating that milk proteins were secreted. Because of continued emptying of the glands in hemizygous mice, only little WAP was detected (Fig. 2H).

Discussion

Stat5a is expressed ubiquitously and can be activated by many cytokines, including interleukins, GH, PRL, EGF, and EPO (Ihle and Kerr 1995; Schindler and Darnell 1995; Liu et al. 1996). This has led to the speculation that its presence is critical for many signaling cascades in vivo. Here we demonstrate that the presence of Stat5a is mandatory for mammary development and lactogenesis.

Lobuloalveolar mammary development

Development of the mammary gland during the second half of pregnancy is characterized by the growth of secretory units or alveoli. Here we have demonstrated that alveolar development in postpartum Stat5a-deficient mice is curtailed and has the appearance of a gland at mid-pregnancy. The developmental abnormalities in

Figure 4. Expression of milk protein genes. Mammary tissue from wild-type, hemizygous, and homozygous mice was biopsied within 12 hr after parturition (lanes a–f) or after 3 days of suckling (lanes g,h) and probed for the presence of RNA encoding Stat5b, β -casein, WAP, α -lactalbumin, and WDNM1.

Figure 5. Protein composition in mammary tissue of Stat5a-deficient mice. Protein extracts from mammary tissue biopsies of wild-type, hemizygous, and homozygous mice isolated within 12 hr after parturition (lanes *a–e*) or after 3 days of suckling (lanes *f,g*) were analyzed by Coomassie blue staining (*A*) and probed for the presence of WAP using anti-WAP antibodies (*B*). The molecular masses (in kD) of a standard run in the same gel are indicated at *right*. The data shown in Figs. 2–5 were from the same mice.

Stat5a-deficient mice are consistent with a role of Stat5a in mammopoiesis predicted by previous studies that showed a sharp increase of its activity at mid-pregnancy (Liu et al. 1996). Although GH, PRL, and EGF can signal through Stat5a and Stat5b and induce alveolar growth in specific experimental settings, a large body of *in vivo* and *in vitro* studies implicates PRL [and perhaps placental lactogen (PL)] as a prime candidate for the control of mammary alveolar development and differentiation in late pregnancy (Vonderhaar 1987). Recent studies of mice, in which one allele of the PRLR has been inactivated, support the hypothesis that the PRL pathway is critical for alveolar development (Ormandy et al., this issue). However, other growth factors may perform auxiliary functions. Ectopic expression of GH in transgenic mice induces precocious mammary development and epithelial cell differentiation (Bchini et al. 1991), but a clear *in vivo* role of GH in mammary morphogenesis has not been established yet. The GH signaling pathway in Stat5a-deficient mice appears intact, as indicated by the normal developmental weight gain of male and females (data not shown). This is in stark contrast to Stat5b-deficient mice where reduced growth rates in males has been observed (H. Davey, pers. comm.). If GH contributes to mammary development, its effects may be conveyed through Stat5b. EGF can also enhance mammary development in organ cultures (Vonderhaar 1987). Because EGFR-deficient mice die within the first 3 weeks of life (Miettinen et al. 1995), a thorough analysis of their breast development has been difficult to perform.

Activation of milk protein genes

WAP and β -casein RNAs were detected in postpartum

mammary tissue from Stat5a-deficient mice, demonstrating that other signals must counterbalance the lack of Stat5a. Although Stat1 and Stat3 can be activated by PRL in tissue culture cells (DaSilva et al. 1996) their activity in lactating mammary tissue is at least 500-fold lower than that of Stat5a and Stat5b (Liu et al. 1996). Stat5b activity in Stat5a-deficient mice is greatly reduced. If Stat1, Stat3, or Stat5b compensate for Stat5a activity, it is clearly accomplished by a low level of active protein. Based on the expression of milk protein genes in Stat5a-deficient mice it is necessary to reevaluate the role of Stat5a and Stat5b, in particular for the β -casein gene. We propose that maximum β -casein gene expression can be accomplished with very little Stat5b in the absence of Stat5a. In contrast, high expression of the WAP gene requires high levels of Stat5a. The activity pattern of Stat5 during pregnancy does not correlate with β -casein gene expression but coincides with WAP gene expression (Liu et al. 1996). Differential regulation of the WAP and β -casein genes by prolactin has been reported earlier (Hobbs et al. 1982; Pittius et al. 1988; Burdon et al. 1991a), and one molecular basis has now been established as a differential requirement for Stat5a. The reduction of WAP gene expression in the absence of Stat5a is comparable to that seen in WAP transgenes with a mutated Stat5 binding site (Li and Rosen 1995). The residual WAP gene activity may be induced by other transcription factors such as NF1 and GR (Li and Rosen 1995) and Ets proteins (McKnight et al. 1995). Stat5b may be dispensable for WAP gene expression *in vivo* as Stat5b-deficient mice are able to lactate (H. Davey, pers. comm.).

Lactogenesis

Full mammary growth is not attained until the alveoli enlarge to their functional secretory state. Lactogenesis may thus be considered the final stage of mammopoiesis. An early sign of a shift to a secretory parenchymal phase of lactogenesis during mid-pregnancy is the formation of fat droplets in the alveolar epithelium, followed by further alveolar cell proliferation and considerable enlargement of the alveolar lumina. Although we have a clear phenotypic understanding of the lobuloalveolar defects in the absence of Stat5a, the cause of lactation failure is still elusive. The mechanisms by which alveolar lumina expand are not fully understood. One possibility for the failure of lumina to expand in Stat5a-deficient mice could be the lack of water flow into the lumen, which in turn is believed to be dependent on lactose. However, the phenotype of the Stat5a-deficient mice differs from that seen in the absence of α -lactalbumin (Stinnakre et al. 1994). These females do not produce lactose and therefore have thickened milk, but alveolar size and luminal expansion is apparently normal. Although the accumulation of milk proteins and fat droplets in alveolar cells of Stat5a-deficient mice are clear indicators of a successful completion of early steps in lactogenesis, little or no expansion of the lumina was detected, even after extended periods of suckling. Even though continued suck-

ling did not result in milk production and release, the expression of milk protein genes and the synthesis of milk proteins was maintained and the gland did not involute. This is in sharp contrast to other instances in which failure of complete mammapoiesis and lactogenesis has been observed. Similarly to the Stat5a-deficient mice, small lumina coupled with lactation failure has been observed in mammary tissue in which pRb and p53 was inactivated through the expression of the SV40 T-antigen (Li et al. 1996). However, deregulation of the cell cycle resulted in the induction of apoptosis in the epithelial cells and the expression of milk protein genes was sharply reduced within 24 hr after parturition (Li et al. 1996).

The lack of Stat5a clearly leads to the deregulation of one or several genes critical for normal mammapoiesis. Although the genes in question are still elusive, the WAP gene may be a promising candidate. WAP is a mammary protein belonging to a family of protease inhibitors containing a four-disulfide core, but its role in normal mammary function is unknown (Hennighausen and Sippel 1982a). Its expression during pregnancy is tightly regulated, and precocious activation in transgenic mice also results in a developmental arrest at mid-pregnancy, small lumina, and a *milchlos* phenotype (Burdon et al. 1991b). Interestingly, the overall protein pattern from Stat5a-deficient mammary tissue indicates that WAP is the only milk protein with reduced expression.

Genes and mammary development

Disruption of mammapoiesis and lactogenesis has been observed upon the deletion of several genes from the mouse genome. Lobuloalveolar outgrowth is severely inhibited in the absence of cyclin D1 (Sicinski et al. 1995), the progesterone (Lydon et al. 1995), and PRL (Ormandy et al., this issue) receptors, the activin/inhibin β B gene (Vassalli et al. 1994), and, as shown in this report, Stat5a. Although each of these gene products is part of signaling pathways leading to alveolar cell proliferation and differentiation, we do not know at this point whether they function in the same or distinct pathways.

It is intriguing to speculate why widespread and general signaling cascades involving transcription factors, cyclins, hormones, and growth factors are so critical for mammapoiesis and lactogenesis, whereas their requirements for the development of other tissues appears to be far less stringent. Because the mammary gland arrived late, if not last, in the phylogenetic scale of organ evolution, it may well be possible that redundant signaling pathways have not been developed in the mammary gland. There is experimental evidence to support such a view. Many cytokines, including EGF, GH, EPO, GM-CSF, and PRL, can activate Stat5a and Stat5b as well as Stat1 and Stat3 (for recent references, see Liu et al. 1996). Although PRL activates Stat5 and Stat3 to a similar extent in some lymphoid and breast cancer cells (DaSilva et al. 1996), Stat1 and Stat3 activities are barely detectable in the lactating mammary gland and do not correlate with prolactin levels (Liu et al. 1996). Thus, PRL-

mediated pathways activating Stat1 or Stat3 were perhaps never established in the mammary gland and therefore cannot compensate for the loss of Stat5a.

The superimposable expression pattern of Stat5a and Stat5b during pregnancy and lactation in conjunction with the 96% sequence similarity between the two isoforms was a harbinger that Stat5b might be able to compensate for the loss of Stat5a. Unexpectedly, Stat5b protein levels, and even more pronounced the extent of phosphorylation but not the RNA levels, were greatly reduced in Stat5a-deficient mammary tissue. This demonstrates that efficient phosphorylation of Stat5b requires the presence of Stat5a and suggests that a docking of both molecules to the receptor complex is critical for maximum activation. The importance of Stat5a-Stat5b heterodimerization for mammary cell differentiation had been postulated previously (Liu et al. 1996). Reduced amounts of phosphorylated Stat5b were detected in Stat5a-deficient mammary tissue, and we did not observe a functional compensation of Stat5a deficiency, suggesting that the two molecules exhibit unique biochemical activities. This is supported by the phenotype of Stat5b-deficient mice, which differs dramatically from the Stat5a mice and includes reduced growth and fertility (H. Davey, pers. comm.). Stat5a and Stat5b exhibit similar if not identical transcription properties in tissue culture cells, and their only difference resides in the 15 carboxy-terminal amino acids, a region mediating transcriptional activation (Moriggl et al. 1996). However, the physiological properties of these homologous proteins are different when investigated in the living organism.

Materials and methods

Cloning of mouse Stat5a gene

A λ FIX II library (Stratagene, La Jolla, CA, cat. no. 946309) containing *Sau3AI* partially digested liver DNA isolated from 4- to 8-week-old female mice of the strain 129SVJ was probed with a PCR fragment amplified from Stat5a cDNA encoding amino acids 66–166 (Liu et al. 1995). Two overlapping clones encoding Stat5a were isolated and restriction mapped, and the exon regions were identified by hybridizing to various oligonucleotides derived from different regions of Stat5a cDNA. The 5' part of the gene used for making the knockout construct was sequenced.

Construction of targeting plasmid, transfection of ES cells, and generation of Stat5a-disrupted mice

A targeting construct (Fig. 1A) containing a 2.2-kb *EcoRI*-*XbaI* fragment from the 5'-upstream region and a 5.0-kb *XbaI*-*XbaI* fragment derived from the internal region of the Stat5a gene was interrupted by a phosphoglycerate kinase-*neo* (PGK-*neo*) gene in an opposite orientation relative to the Stat5a transcription, using pPNT (Tybulewicz et al. 1991). The plasmid was linearized and transfected into TC1 ES cells as described previously (Deng et al. 1996). After positive/negative selection with G418 and FIAU, we picked 120 colonies and screened them for correct targeting by Southern blot analysis using an external probe (Fig. 1A), and four clones were positively identified. Two of the four clones were injected into C57BL/6J blastocysts. One clone produced high-level chimeras that gave germ-line transmission

when bred to NIH Black Swiss or the 129SvEv inbred strain. Heterozygous mice were intercrossed to generate wild-type, heterozygous, and homozygous mutant mice. All animal experiments were carried out according to the NIH Guidelines.

Histology and immunocytochemistry

Mammary tissues were biopsied on the first day postpartum. Pups from the heterozygous and null mothers were circulated between mothers twice daily over a period of 3 days to maintain the suckling stimulus. After 3 days the females were sacrificed, and the remaining mammary tissue was taken. For whole-mount examination the tissues were fixed in Carnoy's solution for 2 hr and stained with carmine alum overnight as described previously (Kordon et al. 1995). The whole mounts were embedded and sectioned using standard methods. Mammary sections were stained with hematoxylin and eosin and used for histological analysis. Antibody staining of mammary sections with WAP antibodies has been described previously (Robinson et al. 1995).

Protein and RNA analyses

Protein extraction, Western blotting, and immunoprecipitation were performed as described previously (Liu et al. 1996). Antibodies for Stat5a, Stat5b, and phosphotyrosine have been described (Liu et al. 1996). Total cellular RNA was isolated, and Northern blot analyses were done as described (Robinson et al. 1995). The probes for Stat5b (Liu et al. 1995), WAP, β -casein, WDNM1, and α -lactalbumin have also been described previously (Robinson et al. 1995). Quantitation of WAP and β -casein mRNA levels were performed using a Fuji PhosphorImager.

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