ARTICLE

Expression of the Whey Acidic Protein (Wap) Is Necessary for Adequate Nourishment of the Offspring But Not Functional Differentiation of Mammary Epithelial Cells

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Summary: Whey acidic protein (WAP) is the principal whey protein found in rodent milk, which contains a cysteine-rich motif identified in some protease inhibitors and proteins involved in tissue modeling. The expression of the *Wap* gene, which is principally restricted to the mammary gland, increases more than 1,000-fold around mid-pregnancy. To determine whether the expression of this major milk protein gene is a prerequisite for functional differentiation of mammary epithelial cells, we generated conventional knockout mice lacking two alleles of the *Wap* gene. Wap-deficient females gave birth to normal litter sizes and, initially, produced enough milk to sustain the offspring. The histological analysis of postpartum mammary glands from knockout dams does not reveal striking phenotypic abnormalities. This suggests that the expression of the *Wap* gene is not required for alveolar specification and functional differentiation. In addition, we found that Wap is dispensable as a protease inhibitor to maintain the stability of secretory proteins in the milk. Nevertheless, a significant number of litters thrived poorly on Wap-deficient dams, in particular during the second half of lactation. This observation suggests that Wap may be essential for the adequate nourishment of the growing young, which triple in size within the first 10 days of lactation. Important implications of these findings for the use of *Wap* as a marker for advanced differentiation of mammary epithelial cells and the biology of pluripotent progenitors are discussed in the final section. genesis 43:1–11, 2005. © 2005 Wiley-Liss, Inc.

Key words: whey acidic protein (WAP); mammary gland; differentiation; gene targeting

INTRODUCTION

The proliferation and differentiation of mammary epithelial cells is controlled by the synergistic action of peptide and steroid hormones as well as local growth factors (Hennighausen *et al.*, 1998, 2001; Topper *et al.*, 1980). Loss-of-function studies in mouse models have demonstrated that prolactin (PRL) signaling though the Jak2/Stat5 pathway plays a central role in this process. PRL (Horseman *et al.*, 1997), the PRL receptor (PRL-R) (Ormandy *et al.*, 1997), the Janus kinase 2 (Jak2) (Shillingford *et al.*, 2002; Wagner *et al.*, 2004), and the signal transducers and activators of transcription 5 (both Stat5a and Stat5b) (Cui *et al.*, 2004; Liu *et al.*, 1997; Teglund *et al.*, 1998) exhibit an unexpected level of specificity during mammogenesis. Indispensable functions of these proteins in the mammary gland are restricted to alveolar proliferation and differentiation during pregnancy. The PRL signaling cascade has many transcriptional targets; among them, genes that regulate cell proliferation and cell adhesion as well as milk protein genes (Gass *et al.*, 2003). The expression of milk protein genes, however, varies slightly between caseins and whey proteins. In mice, casein transcription increases rather early during pregnancy, whereas high levels of expression of the whey acidic protein (Wap) and α-lactalbumin are restricted to the last phase of pregnancy (Pittius *et al.*, 1988; Robinson *et al.*, 1995). The PRL-induced activation and nuclear localization of Stat5a seems to be imperative

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for the transcriptional activation of the *Wap* gene, but not for the expression of β-*casein* (Liu *et al.*, 1997). Stat5 target sequences are part of composite response elements within the *Wap* gene promoter. These elements encompass Stat5, nuclear factor 1, and glucocorticoid receptor binding sites that confer a mammary gland-specific and hormonally regulated expression of *Wap* (Li *et al.*, 1995). Beside hormones and local growth factors, a proper expression of *Wap* also requires cell-to-cell contact and the correct three-dimensional structure of an alveolus (Chen *et al.*, 1989). Wap, therefore, is commonly applied as an advanced differentiation marker for mammary epithelial cells, and its upregulation heralds the appearance of alveolar cells secreting milk (Robinson *et al.*, 1995).

Wap is a major protein in the whey fraction of milk from rodents (Campbell *et al.*, 1984; Hennighausen *et al.*, 1982), rabbits (Devinoy *et al.*, 1988), pigs (Simpson *et al.*, 1998), camels (Beg *et al.*, 1986), and marsupials (Simpson *et al.*, 2000). It has been shown recently that the human genome also contains a *Wap* gene sequence with critical point mutations within the coding region, including the ATG start codon, that may account for the absence of Wap from human milk (Rival-Gervier *et al.*, 2003). The *Wap* transcript was first cloned by Hennighausen and Sippel (1982) from lactation-specific mRNAs of mouse mammary glands. Based on the sequence, the authors determined that *Wap* is a member of the family of four-disulfide-core (4-DSC) proteins that show a cysteine pattern, which is very similar to the hypothalamic carrier protein neurophysin (i.e., a component of pro-oxytocin and pro-vasopressin). The following identification of genes in human and mice, which encode proteins with a Wap-like 4-DSC domain (i.e., the "WAP motif"), led to their classification as the *Wap* gene family. Unlike Wap, which is exclusively expressed in the late-pregnant and lactating mammary gland, many of the WAP-related proteins are present in a variety of tissues, in which they are suggested to function as protease inhibitors. For further information about individual members of the *Wap* gene family please refer to a recent comprehensive review by Simpson and Nicholas (2002).

Despite numerous studies on the transcriptional regulation of the *Wap* gene during pregnancy and lactation, the biological function(s) of Wap in vivo remain, at least in part, elusive. The overexpression of *Wap* under its native regulatory elements, or the mouse mammary tumor virus long terminal repeat (MMTV-LTR), or the ubiquitously active chicken β-*actin* promoter led to premature differentiation and impaired alveolar development in transgenic mice and pigs (Burdon *et al.*, 1991; Hennighausen *et al.*, 1994; Nukumi *et al.*, 2004; Shamay *et al.*, 1992). These studies emphasize the notion that the correct temporal and spatial regulation of *Wap* is tightly linked to advanced differentiation and that Wap might play an important role in this process. Thus far, a conventional knockout model has not been available to test this assumption. Ludwig *et al.* (2001) recently generated the first mouse model with a genetically engineered *Wap* allele by targeting the Cre recombinase coding region into the 5-prime untranslated region (UTR) of the *Wap* locus. This genetic alteration of the *Wap* locus had apparently no phenotypic consequences. A reduction of the *Wap* mRNA or the Wap protein, however, had not been demonstrated in this animal model.

In this report, we describe the generation and analysis of conventional Wap knockout mice. Our main objective was to determine 1) whether the expression of this major milk protein is a prerequisite for functional differentiation of mammary epithelial cells; 2) whether Wap is an essential nutritional component of mouse milk to support the growth of the suckling offspring; and 3) whether Wap has a biologically relevant function as a protease inhibitor, as suggested from its structural analysis. Homozygous Wap knockouts developed normally until adulthood. Mutant females gave birth to normal litter sizes and, initially, produced enough milk to nurse the offspring. The histological analysis of postpartum mammary glands from knockout dams did not reveal striking phenotypic abnormalities associated with Wap deficiency. Our observations suggest that the expression of the whey acidic protein is not required for functional differentiation of mammary epithelial cells. Important implications for the use of the transcriptional activity of *Wap* as a marker for advanced differentiation of mammary epithelial cells and the biology of pluripotent progenitors are discussed in the final section. Despite the absence of a phenotype on the histological level, pups suckling on Wap-deficient dams, regardless of their genotype, were malnourished, and many of them could not be weaned after a normal lactation period of about 21–25 days postpartum. Hence, the secretion of the whey acidic protein into the milk, in particular during the second half of lactation, is important for adequate nourishment to meet the needs of the growing young. Finally, our experiments show that Wap is not an essential protease inhibitor to maintain the stability of other secretory proteins in the milk.

RESULTS

Targeted Deletion of the *Wap* Gene by Homologous Recombination

To study the in vivo function of the whey acidic protein, we replaced the first coding exon of the *Wap* locus with a neomycin resistance gene (Fig. 1A). In brief, a BAC clone encompassing the *Wap* locus was isolated from a mouse 129SvJ genomic library (Incyte Genomics, Wilmington, DE). Two contiguous DNA fragments harboring the entire *Wap* gene and more than 4 kb of flanking sequence on either end were subcloned into pZErO (Invitrogen, La Jolla, CA). A *Kpn*1/*Eco*R1 fragment harboring the entire coding region of *Wap* was sequenced (GenBank AY923114). A comparison of the 129SvJ sequence to a previous release of the *Wap* locus from the GR strain (U38816) revealed substantial inconsistencies within noncoding regions (introns and 3′ flanking

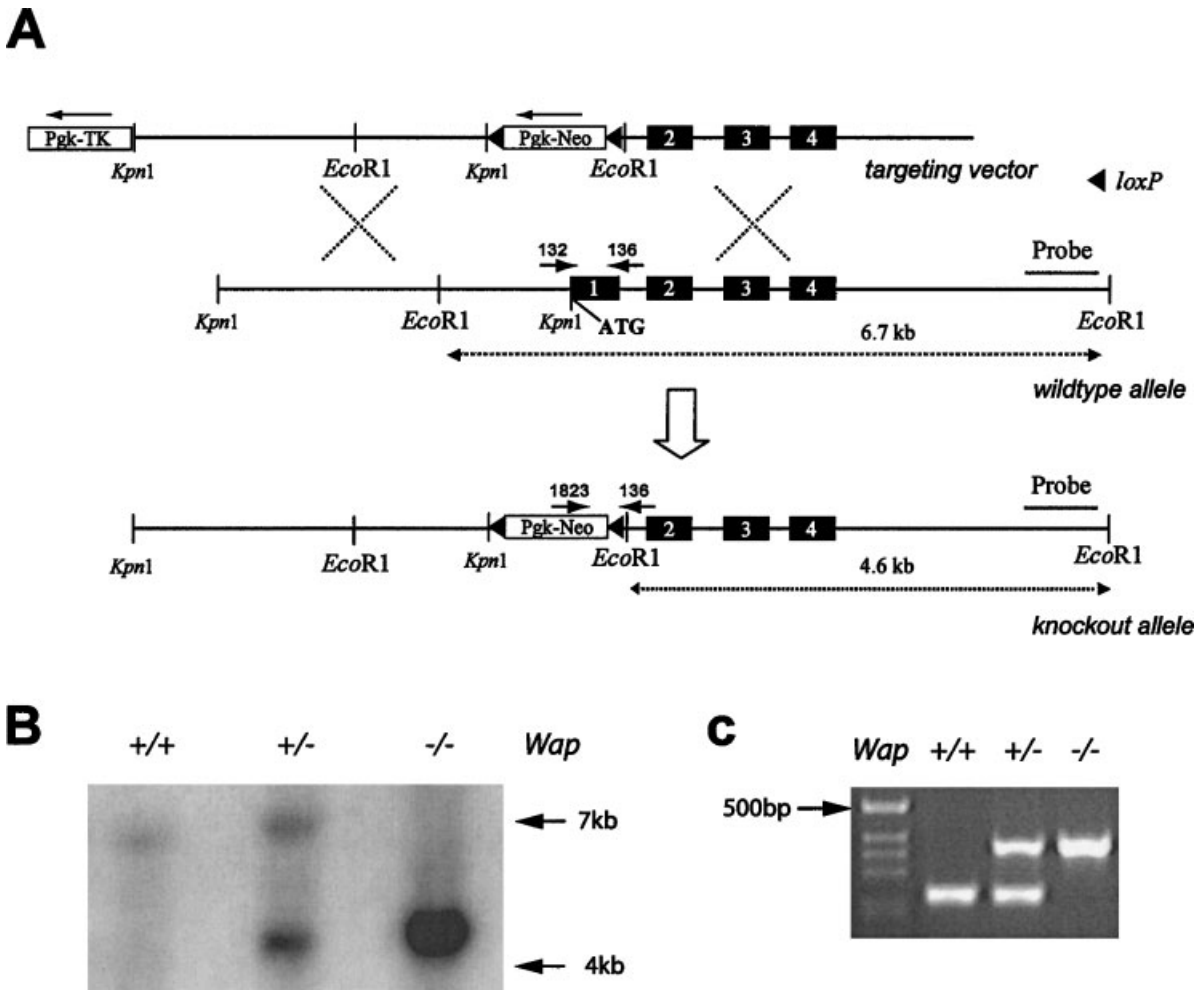


FIG. 1. Targeted deletion of the *whey acidic protein* (*Wap*) gene. **A:** Strategy to replace the first coding exon of *Wap* with a floxed PGK-neomycin resistance gene using homologous recombination in embryonic stem cells. **B:** Southern blot analysis using an *Eco*R1 restriction digest of genomic DNA in combination with a 3-prime external probe (see **A** for location) to verify the presence of the wildtype and/or knockout alleles in heterozygous (+/−) and homozygous mutants (−/−) as well as wildtype controls (+/+). **C:** PCR analysis on genomic DNA of tail biopsies for genotyping of Wap-deficient mice and their heterozygous and homozygous wildtype controls. Arrows indicate the location of the PCR primers (132/136/1823) in **A**.

region). More important, we found three single nucleotide polymorphisms within the coding region of exons 1, 2, and 3 that resulted in amino acid substitutions (R11L, Q35P, and T90M). Two of the three polymorphisms (11 and 35) are consistent with sequence variations that have been documented previously (see Swiss-Prot, P01173). A targeting vector was constructed by replacing the first coding exon and subsequent intron/exon junction with a PGK-neomycin (PGK-neo) selectable marker flanked by *lox*P sites. The PGK-tk cassette was used for negative selection against random integration events. The *Wap* promoter and 5-prime UTR were not genetically modified to allow a further manipulation of the targeted *Wap* locus in embryonic stem (ES) cells. For instance, targeted ES cells can be used for the site-specific insertion of a coding sequence to direct the expression of heterologous proteins to the mammary gland of lactating females (Cre-mediated knockin mutants). Nine correctly targeted ES cell clones were identified by Southern blot using *Eco*R1 restriction digest in combination with a 3-prime probe that was not part of the targeting vector. Although more than 8 kb of homology sequence was used to construct the targeting vector, only about 5% of the neo^{pos}/tk^{neg} clones were correctly targeted. This might be the result of using non-isogenic DNA or the fact that the *Wap* locus is proximal to the centromere of chromosome 11.

Four ES cell clones (#59, #65, #69, and #107) were expanded and used for the production of 27 highly chimeric mice. Three ES cell lines (#59, #65, and #107) were transmitted though the germline of chimeric males. Subsequently, we bred the null allele of all three substrains into homozygosity (Fig. 1B,C). The mating of heterozygous mice resulted in homozygous mutants according to the expected Mendelian ratio of 25%, suggesting that Wap deficiency did not result in embryonic

lethality. Homozygous mutants developed normally until adulthood, and neither males nor females exhibited phenotypic abnormalities by 12–14 months of age.

Normal Mammary Gland Development in Wap-Deficient Females

The expression of the endogenous *whey acidic protein* locus is principally restricted to the mammary gland. During the course of mammary development from the virgin to the fully lactating female the steady-state levels of the *Wap* mRNA increases about 10,000-fold, with the most pronounced increase occurring around mid-pregnancy (Hennighausen *et al.*, 1991). Wap-deficient mice were fertile and exhibited a normal mating behavior. Females gave birth to normal litter sizes comparable to their wildtype controls ($Wap^{+/+}$ 6.9 ± 2.9; $Wap^{+/-}$ 7.2 ± 3.1; $Wap^{-/-}$ 6.8 ± 2.8, n = 162). Postpartum dams produced milk that was clearly visible in the abdominal region of the pups (data not shown). A histological examination of the mammary glands at day 1 of lactation revealed no phenotypic abnormalities between the knockouts and their age-matched lactating controls (Fig. 2A, upper panel). The whey acidic protein, which was abundant in secretory alveoli of lactating wildtype controls, was completely absent in Wap knockout dams as determined by immunohistochemistry (Fig. 2A, lower panel). As expected, the prolactin signaling cascade though Stat5a, which is imperative for the transcriptional activation of *Wap*, was not impaired in the mutants, as demonstrated by nuclear localization and phosphorylation of Stat5 (Fig. 2B, upper panels). Wap-deficient mammary epithelia exhibited a typical expression of ductal (Nkcc1) and alveolar (Npt2b) cell type-specific markers in virgin and lactating females (Fig. 2B, lower panels), suggesting that the lack of the whey acidic protein does not affect cell specification and functional differentiation.

We further examined the transcriptional activation of β-*casein* (*Csnb*), α-*lactalbumin* (*Lalba*), *Wdnm1,* and *Wap* mRNA in two wildtype females as well as three heterozygous and three homozygous knockouts at day 1 of lactation (Fig. 3A). Each heterozygous and homozygous mutant represents a pair from a substrain derived from the three targeted ES cell lines that were passed through the germline of chimeric males (lines #59, #65, and #107 in that order). The northern blot analysis demonstrated that the targeting strategy led to a complete transcriptional repression of the *Wap* locus. Furthermore, Wap deficiency had no effect on β-*casein* or α-*lactalbumin* mRNA expression. Like Wap, Wdnm1 is a member of the four-disulfide-core-domain (4-DSCD) protein family that is developmentally regulated in the mammary gland (Simpson *et al.*, 2002). The expression of *Wdnm1*, however, is not linked to terminal differentiation and precedes the expression of *Wap*. The *Wdnm1* mRNA is not upregulated in Wap-deficient mammary epithelia. In addition, we did not observe significant differences in *Wap* mRNA expression between heterozygous mutants and wildtype controls, which indicated that *Wap* haploinsufficiency resulted in the upregulation of the remaining wildtype allele. The absence of the whey acidic protein in mammary gland tissues from lactating females of the three Wap knockout substrains was verified by western blot analysis (Fig. 3B). In analogy to the northern blot results, heterozygous Wap knockout females exhibited levels of the whey acidic protein that were equivalent to wildtype controls. In addition, we examined the amount of caseins and whey proteins in the milk of nursing females around day 15 of lactation using SDS-polyacrylamide gel electrophoresis and Coomassie blue staining (Fig. 3C). Although the whey

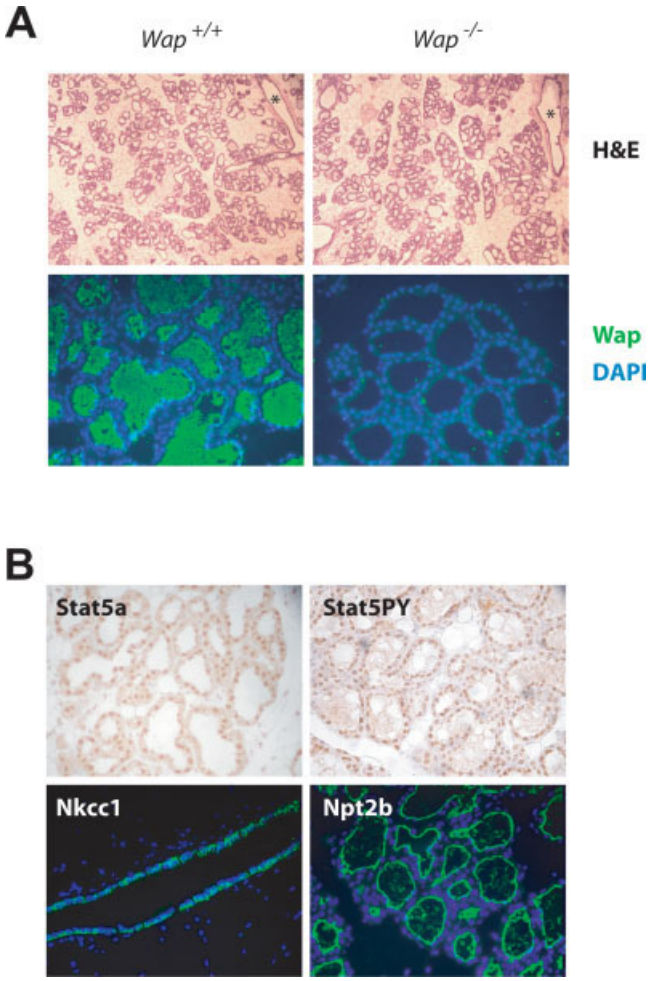


FIG. 2. Mammogenesis in Wap-deficient females (−/−) and their wildtype (+/+) controls. **A:** Upper panel: histological examination of mammary tissue at day 1 of lactation (hematoxylin and eosin staining, magnification 40×); lower panel: immunostaining of the whey acidic protein (Wap) in secretory alveoli of the mammary gland at day 1 of lactation (magnification 200×). **B:** Upper panel: Examination of Stat5a and Stat5-pY nuclear localization in Wap-deficient dams at day 1 of lactation (magnification 200×); lower panel: immunostaining of ductal (Nkcc1) and alveolar (Npt2b) differentiation markers in nonpregnant (left) and lactating (right) $Wap^{-/-}$ females (magnification 200×).

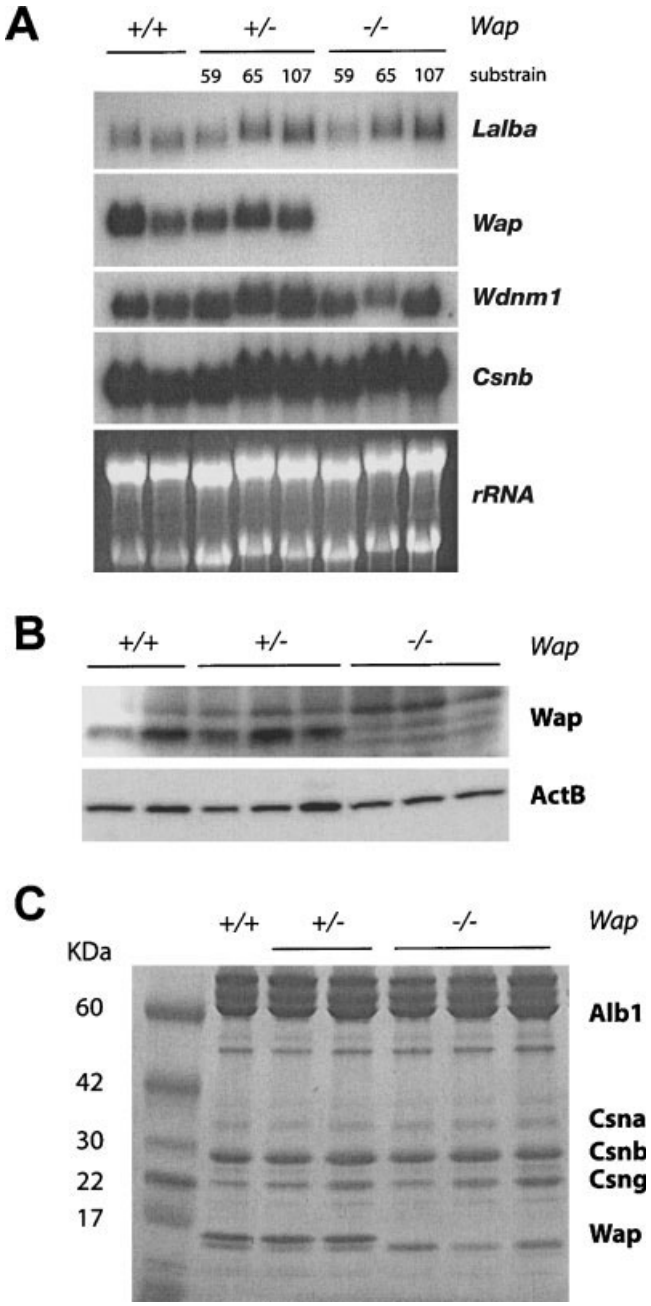


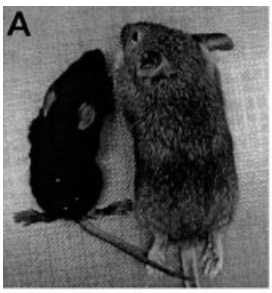
FIG. 3. Expression of major milk protein genes in the mammary gland (**A,B**) and milk (**C**) of Wap-deficient females (−/−) as well as heterozygous (+/−) and homozygous wildtype (+/+) controls at day 1 (**A,B**) and day 15 (**C**) of lactation. **A:** Northern blot analysis of β-*casein (Csnb), Wdnm1*, the *whey acidic protein (Wap*), and α-*lactalbumin (Lalba*) mRNA. The 18S and 28S ribosomal RNA serves as a loading control. **B:** Western blot analysis to verify the absence of the whey acidic protein in the same samples shown in **A**. Detection of the β-actin (ActB) protein serves as a loading control. **C:** SDS-polyacrylamide gel electrophoresis and Coomassie blue staining of caseins and whey proteins in the milk of nursing females at day 15 of lactation.

acidic protein was clearly absent in the milk from knockout females, we did not detect any other significant variations in the amount of major milk protein fractions, in particular the caseins. This suggests that whey acidic protein is not essential for casein synthesis or the stability of proteins in the milk.

Presence of Wap in Milk Is Important for Adequate Nourishment of Offspring During the Second Half of Lactation

As shown above, Wap-deficiency had no effect on the terminal differentiation of secretory mammary epithelial cells. Nevertheless, we noted that many litters thrived poorly on $Wap^{-/-}$ dams, resulting in either lethality of pups or reduced body weight (Fig. 4A), in particular during the second half of the lactation period. This resulted in an unusually long lactation period of many WAP-deficient females, and their offspring could not be weaned before 4 or even 5 weeks of age. In some cases, pups of the substrain #59 nursed for 6 weeks before they could be separated from their mothers. Litters from control mice were weaned after a lactation period of 21–25 days. To confirm these subjective observations, we closely monitored the growth rate of the offspring from more than 50 lactating females of all genotypes in all three substrains (>500 pups) during their first and second lactation cycle (Fig. 4B,C). Entire litters were weighed at birth and every second day within the first 20 days of lactation. In consideration of the effects of small litter sizes on the average weight gain of the young, we excluded from this study lactating dams with fewer than six pups. The average birthweights of the pups were statistically indistinguishable between the genotypes of the mothers regardless of the genetic makeup of the offspring (i.e., whether the dams were mated with wildtype, heterozygous, or homozygous knockout males). This suggested that any intrauterine effects of Wap-deficiency were negligible. Beginning at day 4 of lactation, the pups suckling on Wap-deficient dams exhibited a growth disadvantage, which was statistically significant during the second half of lactation. The inclusion of the second lactation cycle into the analysis made the statistical differences more evident due to a greater number of measurements (Fig. 4C). It was previously reported that, in a number of genetically engineered strains, the extent of an abnormal mammary phenotype is more prominent during the first as compared to subsequent lactation periods (Liu *et al.*, 1998; Ormandy *et al.*, 1997). Our results, therefore, suggested that the abnormal phenotype caused by Wap deficiency is not reversible during the second lactation cycle.

Next, we examined whether the genotype of the young had any influence on the postnatal growth retardation. For this purpose, we mated Wap heterozygous knockout females with heterozygous males ($Wap^{+/-} \times Wap^{+/-}$). One hundred forty-nine mice from the resulting litters were weighed and genotyped at the age of 4 weeks (Fig. 4D). Since gender influences weight gain, we compared males and females separately. We did not observe statistically significant differences in the average weight among the various genotypes that were all nursed by Wap heterozygous knockout dams. As men-



D

genotype of pups	average weight of pups	
	Male	Female
Wap +/+	15.8 ±1.9 (n=13)	14.5 ±1.2 (n=27)
Wap +/-	14.8 ±0.9 (n=39)	13.1 ±1.5 (n=37)
Wap -/-	17.0 ±1.3 (n=18)	13.0 ±2.2 (n=15)

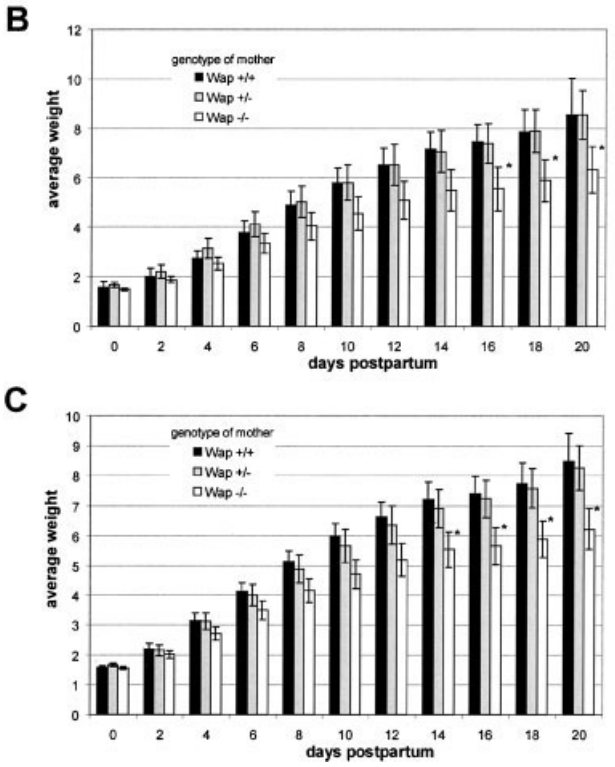


FIG. 4. Wap deficiency and growth rate of the nursing young. **A:** Comparison of the size of a malnourished pup suckling on a $Wap^{-/-}$ dam (left) and a normal weanling nursed on a $Wap^{+/-}$ heterozygous control dam (right) at day 21 of lactation. **B,C:** Average weight of pups during the first 20 days of lactation grouped by the genotype of the lactating mother and the first (**B**) or the first and second (**C**) lactation cycle of the dam. Error bars represent the confidence interval ($\alpha = 0.05$). **D:** Average weight of the offspring resulting from Wap heterozygous knockout breedings ($Wap^{+/-} \times Wap^{+/-}$) grouped by gender and genotype. Litters were weighed and genotyped at the age of 4 weeks.

tioned above, many pups nursing on $Wap^{-/-}$ dams were significantly smaller and could not be weaned by 4 or even 5 weeks of age. In summary, the combined studies suggested that the retarded development of the young is solely caused by the ablation of the whey acidic protein in the mammary gland and milk of $Wap^{-/-}$ lactating dams.

Wap Is Redundant as a Protease Inhibitor in the Milk

Many 4-DSC domain proteins (i.e., those carrying the "WAP motif") are suggested to function as protease inhibitors in various tissues. The analysis of the major protein fractions in the milk of Wap knockout mice and their controls (Fig. 3C) suggested that the founding member of this protein family is not essential for the inhibition of proteases. Wap deficiency did not alter the stability and equal quantity of caseins in the milk. To validate this observation, we performed a colorimetric assay to measure the endogenous protease activity in milk from Wap-deficient dams and their wildtype controls at day 15 of lactation (Fig. 5A). The protease assay was carried out at two time points: 1) immediately after retrieving the milk (day 1), and 2) after storing the milk overnight in a refrigerator (day 2). As expected, refrigerated milk exhibited a slightly higher endogenous protease activity in both the knockout and wildtype controls. At both time points, milk deficient in the whey acidic protein exhibited a marginally elevated protease activity. These differences, however, were statistically insignificant. Next, we examined whether Wap deficiency changes the capability of milk to buffer increasing amounts of exogenous proteases (Fig. 5B). This is an important characteristic of milk, which influences its digestibility in the stomach and gut of the suckling offspring. In comparison to the standard curve (reaction buffer control, no milk), milk samples from Wap-deficient dams and their controls were able to buffer low concentrations of exogenous trypsin in a very similar manner in our experimental setting, and the differences between both study groups were not statistically significant. Collectively, our results suggested that the whey acidic protein is not an essential protease inhibitor, and its absence from milk does not alter the stability of major milk proteins, the activity of endogenous proteases, or the ability of milk to buffer low concentrations of an exogenous protease in a significant manner.

Wap Deficiency in the Milk Does Not Affect the Glutathione (GSH) Antioxidant System in the Offspring

Whey protein concentrates serve as nutritional supplements during times of high physical activity, stress, and illness. Whey proteins are rich in cysteine, which is a crucial limiting amino acid for intracellular glutathione (GSH) synthesis. The GSH antioxidant system is the principal protective mechanism of the cell, and it is an essential factor in the development of an immune response. Therefore, whey proteins serve as effective and safe cysteine donors for GSH replenishment during GSH depletion in immune compromised states. Due to their positive effects on the immune system, whey proteins have also been suggested to possess anticancer activities (Bounous *et al.*, 1991). Since pups, regardless of their genetic backgrounds, thrive poorly on Wap knockout dams, we hypothesized that Wap deficiency in the milk might affect GSH levels in the offspring, resulting in poor general health. To address this issue, we measured GSH levels in the spleen and thymus of four severely malnourished pups from Wap-deficient dams and four pups of

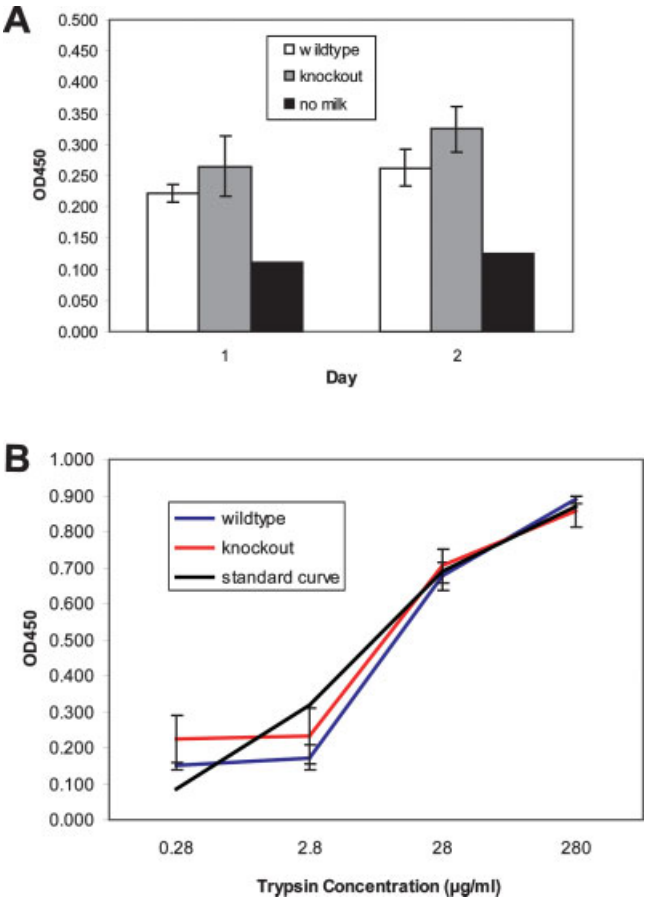


FIG. 5. Colorimetric protease assay. **A:** Determination of the endogenous protease activity in milk from Wap-deficient dams and their wildtype controls at day 15 of lactation. The assay was carried out as described in Materials and Methods immediately after retrieving the milk (day 1) and after storing the milk overnight in a refrigerator (day 2). The background absorbance of the colorimetric assay was established by a "no milk" (reaction buffer only) control. **B:** Mean protease activity of trypsin in the milk from Wap knockout females and their wildtype controls. Note that although milk lacking Wap has a slightly higher endogenous protease activity compared to milk from wildtype controls ($P > 0.05$), both milk types are equally able to buffer trypsin at a low concentration in comparison to the "no milk" (reaction buffer only) standard curve. Error bars in **A** and **B** represent confidence intervals ($\alpha = 0.05$).

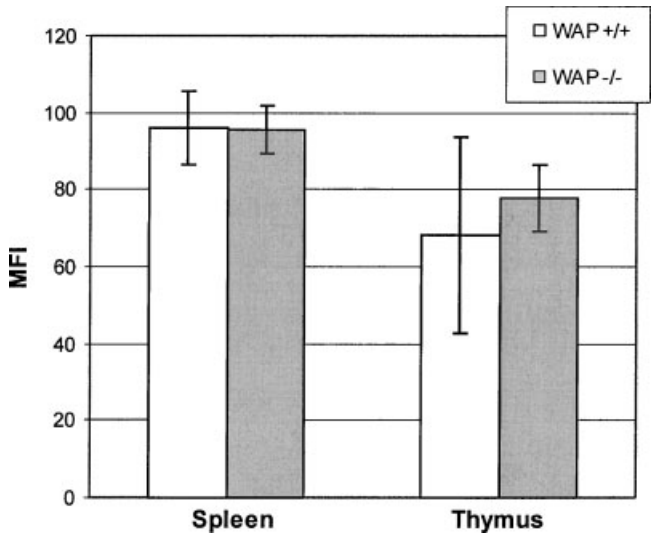


FIG. 6. Glutathione levels in splenocytes and thymocytes derived from four 15-day-old severely malnourished pups suckling on Wap-deficient dams ($Wap^{-/-}$) and from four average-size pups nursing on wildtype control females ($Wap^{+/+}$). Glutathione levels within individual cells were measured as fluorescence by flow cytometry. The mean fluorescent intensity (MFI) represents at least 10^4 individual measurement points per animal and tissue type. Error bars represent the confidence intervals ($\alpha = 0.05$) between animals grouped by tissue type and genotype of the lactating mother.

average size suckling on wildtype control females at day 15 of lactation. Using a flow cytometry-based glutathione assay (Roederer *et al.*, 1991), we were unable to detect statistically significant differences in the amount of intracellular GSH levels in either splenocytes or thymocytes (Fig. 6). Hence, while whey acidic protein in the milk is required for adequate nourishment of the offspring, it is not a major nutritional source for cysteine to establish and maintain the GSH antioxidant system in the offspring.

DISCUSSION

The mammary gland-specific expression of the *Wap* gene increases more than 1,000-fold from the virgin state to late pregnancy and lactation (Pittius *et al.*, 1988). It had been hypothesized that Wap is a protease inhibitor, and it may have a role in remodeling the mammary gland at specific stages of the lactation cycle (Simpson *et al.*, 2002). The generation and analysis of mice lacking *Wap*, however, reveal that this milk protein gene is not required for functional differentiation. This is a surprising observation, since the expression of this gene is frequently applied as an advanced differentiation marker in a variety of in vivo and 3D cell culture model systems. Clearly, Wap is not required to suppress alveolar proliferation during functional differentiation, as suggested from various transgenic studies in mice and pigs (Burdon *et al.*, 1991; Hennighausen *et al.*, 1994; Nukumi *et al.*, 2004; Shamay *et al.*, 1992). In addition, we found that Wap is dispensable as a protease inhibitor to maintain the stability of abundant secretory proteins in the milk as anticipated from its 3D structure and suggested functions of other Wap-like 4-DSC domain proteins in different tissues. Our findings are in compliance with unpublished studies by Simpson and Nicholas (University of Melbourne, Australia) that show that HPLC-purified whey acidic protein from the tammar wallaby did not exhibit evidence of inhibition of either trypsin or chymotrypsin in a colorimetric assay that uses a 10-fold excess of Wap over the two proteases (Kaylene Simpson, pers. commun.). Moreover, Simpson and Nicholas discussed that all four disulfide bridges are required for a protease activity, and they questioned whether domain I of the mouse whey acidic protein is functionally active due to the absence of cysteine residues 1 and 8 to complete the

folding unit (Simpson *et al.*, 2002). While Wap might not act as a general protease inhibitor, the authors hypothesized that this protein might function as a specific inhibitor of unidentified milk proteases. Our observations in Wap knockout mice do not exclude this possibility, but it is evident that other, less abundant protease inhibitors are able to compensate for the loss of Wap in the milk.

Despite normal mammogenesis, a significant number of litters thrived poorly on Wap-deficient dams, in particular during the second half of lactation. This phenotype was intrinsic to the genotype of the lactating dam but not to the nursing offspring. We also excluded the possibility that Wap deficiency affects the immune system of the young through reduced amounts of cysteine in the milk and subsequent glutathione deprivation. The growth retardation observed during the second half of pregnancy might be caused by a reduced milk protein supply that does not fully meet the needs of the growing litter, which triples in size within the first 10 days of lactation. Thus far, we have not experimentally addressed whether Wap is required for the maturation of the digestive system of the offspring. While extracting milk from the mammary glands at day 15 of lactation for the protease assays, we also noticed that the mammary glands of a subset of Wap knockout dams contained less milk compared to their wildtype controls. The skin of 1-week-old malnourished pups was dry, which is another indication of a lack of fluids. A histological analysis of these glands revealed that, although well-differentiated secretory acini were present in all specimens, individual alveoli appeared to be smaller (data not shown). The histological appearance, however, varied quite considerably between knockout animals, and a number of Wap-deficient mammary glands were indistinguishable from their wildtype controls. We therefore hypothesize that another modifier locus might be responsible for this phenotypic variation among knockouts at the histological level since all studies were performed in a C57Bl6/129Svev mixed genetic background. To address this issue, we are currently backcrossing the Wap knockout allele into various inbred strains, which is the basis for mapping loci that modify this phenotypic variation.

The surprising finding that Wap is not required for terminal differentiation has implications for the biology of pluripotent mammary progenitor cells. Using a *Wap-Cre/Rosa-LacZ* double transgenic reporter system, we recently discovered parity-induced mammary epithelial cells (PI-MECs), which are abundant in nonpregnant, parous females and are virtually absent in nulliparous (virgin) animals (Wagner *et al.*, 2002). In synchrony with the endogenous locus, the *Wap* gene promoter-driven Cre recombinase transgene specifically targets hormone-responsive alveolar cells undergoing an advanced differentiation program during the second half of pregnancy and during lactation. Through the Cre-mediated excision of a transcriptional *Stop* sequence between the promoter and the *LacZ* gene, the transient upregulation of Cre recombinase permanently activates a ubiquitously expressed *Rosa-LacZ* reporter transgene (Soriano, 1999), whose expression (unlike the *Wap-Cre* construct) is not dependent on the differentiation status of a given cell. Hence, the constitutive activation of the reporter transgene labels differentiating cells during pregnancy and lactation and their descendents (i.e., cells that are apoptosis-resistant and that silence the *Wap* locus during mammary gland remodeling following a normal pregnancy-lactation cycle). Thus, the LacZ-expressing cells in the remodeled gland represent an epithelial subtype (PI-MECs), which is not present in the virgin gland. Unexpectedly, PI-MECs exhibited various features of multipotent mammary epithelial stem cells. Upon transplantation into an epithelial-deprived mammary gland fat pad, these LacZ-expressing epithelial progenitors are able to self-renew and contribute to ductal and alveolar morphogenesis in the reconstituted mammary gland (Wagner *et al.*, 2002). Our observation suggested that a subset of hormone-responsive cells expressing advanced differentiation markers (i.e., *Wap*) maintains or regains characteristics of stem cells. This potentially paradigm-shifting observation has been scrutinized in the peer-review process at a study section at the NIH Center for Scientific Review. The critique mainly focuses on the hypothetical limitation of the labeling methodology that a Cre-lox-based cell fate-mapping technique cannot discriminate cells with high or low levels of transient *Wap-Cre* expression. This critique is, therefore, centered on the commonly accepted paradigm that the presence of the whey acidic protein and, more importantly, the level of *Wap* expression determine the advanced or "terminal" differentiation status of a given cell. To test whether this critique is legitimate was another strong motivator to generate the Wap knockout model. The results of this study clearly demonstrate that *Wap* is not required for terminal differentiation. In conclusion, the upregulation of *Wap* might be a valid indicator for an advanced differentiation profile of the *entire* mammary gland. A difference in *Wap* expression between individual cells, however, may not serve as an indicator for the terminal differentiation status and the fate of a cell during involution (i.e., whether a cell will live or die). Therefore, the Cre-lox-based cell fate-mapping technique is, indeed, a valid method to label differentiating, pregnancy hormone-responsive cells that activate *Wap* regulatory elements.

MATERIALS AND METHODS

Construction of the *Wap* Targeting Vector

BAC clone encompassing the *Wap* locus was isolated from a mouse 129SvJ genomic library (Incyte Genomics). Contiguous *Kpn*1 and *Kpn*1/*Eco*R1 fragments harboring 4.5 kb of the promoter, the entire *Wap* gene, and more than 1.8 kb of the 3′ sequence were subcloned into pZErO (Invitrogen). Sequencing all exons and introns as well as part of the 3′ flanking DNA was pivotal for determining the targeting strategy and the design of the 3′ outside probe. A 3.7-kb fragment containing exons 2 to 4 and part of the 3′ sequence was

amplified using Pfx polymerase (5′-CCG CTC GAG CGA ATT CTC ACC TTA CTA CCG GGT GTG-3′; 5′-CCG CTC GAG GCG GCC GCG AGT GGA TGG AAC CTT AAT TGA AG-3′), introducing an *EcoR*1 site 5′, a *Not*1 site 3′, and two *Xho*I sites on either end of the amplification product. The PCR product was cut with *Xho*I, cloned sticky into the *Xho*I site of pZErO, and sequenced. The 3′ targeting arm was released by *Xho*I/*Not*1 digest and directionally cloned into the *Xho*I and *Not*1 sites downstream of the floxed neomycin cassette of pLoxpNeo (Xu *et al.*, 1999). The targeting vector was completed by subcloning a 4.5 kb *Kpn*1 fragment, containing the promoter and the 5′ UTR of *Wap,* into the *Kpn*1 site upstream of the floxed neomycin cassette. The correct orientation of the 5′ arm was verified by PCR (5′-TAG AGC TGT GCC AGC CTC TTC-3′; 5′-GAT CTA TAG ATC TCT CGT GGG ATC-3′), and all cloning junctions of the final targeting vector were sequenced. The targeting vector was linearized using *Not*I, phenol-chloroform extracted, and electroporated into R1 cells (20 μg DNA per 10^7 ES cells). The selection and expansion of ES cell clones were performed at the Germline Mutation Core Facility (GMCF) at NCI.

Southern Blot Analysis

Genomic DNA from 178 ES cell clones was prepared using standard phenol/chloroform extraction. Fifteen μg of DNA was digested with *EcoR*I at 37°C overnight and separated on a 0.8% agarose gel. The DNA was denatured and blotted onto a nylon membrane (GeneScreen plus, NEN, Boston, MA), and hybridized overnight with a ^{32}P-labeled probe at 65°C using QuickHyb (Stratagene, La Jolla, CA). The 3′ external probe, ~550 bp in size, was generated by PCR (5′-CTT GAA GCC TTA GCT AAC GTG G-3′ and 5′-TGG GTT CTC CCA CAC CAA TGA C-3′). Membranes were washed in 0.1× SSC buffer containing SDS and exposed for 16–24 h to a Kodak XOMAT-AR film. The *Eco*R1 Southern analysis yielded two distinct bands of 6.7 kb (wildtype allele) and 4.6 kb in size (knockout allele).

Generation of Wap Knockout Mice and Genotyping Protocols

Four correctly targeted ES cell clones (#59, #65, #69, and #107) were used for the production of chimeric mice. The injection of ES cells into C57/Bl6 blastocytes was carried out at the Germline Mutation Core Facility (GMCF). The germline transmission of the *Wap* knockout allele [*Wap*$^-$ or Wap^{tm1Kuw}] was verified using the Southern blot assays described above. A single PCR assay using three primers (132 5′-TAG AGC TGT GCC AGC CTC TTC-3′; 136 5′-GTT CTC CAA GCC ACA CCC GG-3′; and 1823 5′-GTG CTG TCC ATC TGC ACG AGA C-3′) was developed to genotype mice that carry one or two *Wap* knockout alleles. Amplicons for the wildtype (230 bp) and null allele (330 bp) were clearly distinguishable on a 2% agarose gel. All animals used in the studies were treated humanely and in accordance with federal guidelines and institutional policies.

Histology and Immunohistochemistry

The fourth inguinal mammary glands of Wap knockout mice and their controls were resected, fixed overnight at 4°C in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), dehydrated, paraffin-embedded, and sectioned. Sections were rehydrated and stained with hematoxylin and eosin (H&E, Vector Laboratories, Burlingame, CA) for general histology. The immunohistological detection of the whey acidic protein, Nkcc1, Npt2b, Stat5a, and Stat5a/b (Tyr694/Tyr699) was performed as described previously (Nevalainen *et al.*, 2002; Shillingford *et al.*, 2002; Wagner *et al.*, 1997). An AlexaFluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR; Invitrogen) was used to visualize Wap, Nkcc1, and Npt2b. Slides were counterstained with DAPI, which was a component of the Vectashield mounting medium (Vector). Due to low immunofluorescence of the Stat5 proteins, we used biotinylated secondary antibodies to detect Stat5a and the phosphorylated form of Stat5a/b instead. Vectastain Elite ABC and DAB peroxidase substrate kits (Vector) were used to complete the color reaction. Slides were counterstained briefly with hematoxylin to visualize Stat5 negative nuclei. Brightfield and fluorescence images of histological slides were taken on a Nikon Labophot microscope equipped with a Nikon Coolpix 990 camera as well as FITC and DAPI filter sets.

Northern Blot Analysis

The isolation of total RNA from mammary tissue has been described previously (Wagner *et al.*, 1997). Twenty μg of total RNA was separated on a 1.5% formaldehyde gel and transferred to a GeneScreen Plus membrane. Transcripts of milk protein genes were detected by probing the membranes with ^{32}P end-labeled antisense oligonucleotides (*Wap* 5′-CAA CGC ATG GTA CCG GTG TCA-3′; *Csnb* 5′-GTC TCT CTT GCA AGA GCA AGG GCC-3′) or ^{32}P randomly labeled cDNA probes (*Wdnm1* and *Lalba*). The α-*lactalbumin* cDNA was a kind gift from Gertraud Wasner Robinson and Lothar Hennighausen (NIH). The cDNA probe for *Wdnm1* was cloned by RT-PCR from total RNA of lactating mammary tissue using an oligo $dT_{12\text{-}18}$ primer for reverse transcription and the following *Wdnm1*-specific primer pair for the PCR amplification: 5′-GTC AGA GCC AAC ATG AAG AC-3′; 5′-GGA TGC TAA GGA TAG TTT ATT TTA G-3′. The hybridization was performed overnight at 55°C (oligo probes) or 65°C (cDNA probes) using QuickHyb (Stratagene). Membranes were washed in 1× SSC buffer containing 0.1% SDS and exposed for 6–12 h to Kodak XOMAT-AR film.

Milk Protease Assay

Pups were removed from their lactating mothers several hours before both #4 inguinal mammary glands were retrieved. Milk was separated by slow-speed centri-

fugation through a cell strainer into a 50 ml Falcon tube. We normally obtained 100–500 μl milk from both controls and knockout dams; only a subset of lactating Wap-deficient dams had less than 100 μl. The milk was transferred into a 1.5-ml centrifuge tube and refrigerated. A small portion of the milk that contained red blood cells on the bottom of the Falcon tube was discarded. To measure the endogenous protease activity of milk, we used the colorimetric Protease Determine Quick Test (PDQ) assay from Athena Enzyme Systems Group (Baltimore, MD). This assay measures the activity of a wide range of proteases described in the manufacturer's protocol. In brief, the storage solution was removed from the PDQ vials after maintaining those for an hour at room temperature. Next, we added either 0.5 ml of the pH 8.0 Tris buffer (negative control) or our test samples containing 50 μl milk in 0.45 ml Tris buffer. Vials were incubated for 1, 2, or 3 h at 37°C. To stop the reaction and to amplify color, 1.5 ml of a 0.1 N NaOH solution was added to each vial. The liquid content was transferred to standard cuvettes to record the absorbance spectrophotometrically at 450 nm. Additionally, we prepared a 10-fold dilution series of trypsin (e.g., 280–0.28 μg/ml) in the supplied Tris buffer to construct a standard curve. The optical density at 450 nm is directly proportional to the enzyme activity. To determine the capacity of milk to buffer the activity of trypsin, we added 50 μl of milk into 0.45 ml of Tris buffer, which contained various dilutions of the protease in accordance with the standard curve.

Glutathione Assay

Splenocytes and thymocytes were derived from four 15-day-old, severely malnourished pups suckling on Wap-deficient dams and from four, average-size pups nursing on wildtype control females. Glutathione (GSH) levels within individual cells were measured using a flow cytometry-based assay (Roederer *et al.*, 1991). The mean fluorescent intensity (MFI) represents at least 10^4 measurement points per animal and tissue type.

Statistical Analyses

Mean values shown in Figures 4–6 are accompanied by a confidence interval (CI). Nonoverlapping CIs represent significant differences at a significance level of 0.05. The statistical significance was verified or tested for those groups with overlapping CIs using the *t*-test or nonparametric Mann-Whitney (U) test.

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