The housekeeping gene *xanthine oxidoreductase* is necessary for milk fat droplet enveloping and secretion: gene sharing in the lactating mammary gland

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Xanthine oxidoreductase (XOR) is the rate-limiting enzyme in purine catabolism occurring in most cell types. However, this housekeeping gene is expressed at very high levels in a number of mammalian tissues including the lactating mammary epithelium, suggesting additional roles for *XOR* in these tissues. Mice with targeted disruption of *XOR* were generated to assess these potential additional roles. *XOR*–/– mice are runted and do not live beyond 6 wk of age. Strikingly, however, *XOR*+/– females, although of healthy appearance and normal fertility, are unable to maintain lactation and their pups die of starvation 2 wk postpartum. Histological and whole-mount analyses showed that in *XOR*+/– females the mammary epithelium collapses, resulting in premature involution of the mammary gland. Electron microscopy showed that XOR is specifically required for enveloping milk fat droplets with the apical plasma membrane prior to secretion from the lactating mammary gland. We present evidence that XOR may have primarily a structural role, as a membrane-associated protein, in milk fat droplet secretion and thus *XOR* provides another example of "gene sharing". About 5% of women experience primary lactation insufficiency. The above observations suggest that human females suffering from xanthinuria, a deficiency in XOR, are potential candidates for lactation problems.

[*Keywords*: Xanthine oxidoreductase; mammary gland; lactation; fat droplet; enveloping; gene sharing]

Received August 14, 2002; revised version accepted October 28, 2002.

Xanthine oxidoreductase (*XOR*) is a housekeeping gene that encodes a molybdenum iron-sulfur flavin hydroxylase found in all organisms. In vitro experiments show that XOR is a relatively nonspecific, multifunctional enzyme, capable of oxidizing a variety of substrates including purines, pyridine dinucleotides, pteridines, and aldehydes. Although it is one of the best studied enzymes in vitro, the complexities of its in vivo functions are less well understood. In vertebrates, a primary role of XOR is its participation in purine catabolism. The holo-enzyme exists as a homodimer and is composed of two identical and catalytically independent subunits, each containing two [2Fe–2S] groups, one FAD, and one molybdopterin (Rajagopalan and Handler 1967; Massey et al. 1969; Bray et al. 1975; Carpani et al. 1990; Cazzaniga et al. 1994; Hille and Nishino 1995).

XOR can be isolated as two distinct enzymatic forms from most biological sources: either as xanthine dehydrogenase (XD; EC 1.1.1.204) or as xanthine oxidase (XO; EC 1.1.3.22). The mammalian XD and XO forms are interconvertible in vitro (Waud and Rajagopalan 1976; Amaya et al. 1990). XD is the predominant enzymatic form found in normal tissues, while the XO form dominates in tissues subjected to injury. The different structural and catalytic activities as well as the preference for different oxidation substrates is derived from an additional NAD^+ binding site in XD. Hence, with XD, the reducing equivalents generated by the oxidation of substrates are transferred to NAD^+, whereas XO is unable to bind NAD^+ and exclusively uses O_2 as its electron acceptor. The latter reaction results in the production of reactive oxygen species (ROS) as a metabolic byproduct. ROS are produced excessively under certain pathophysiological conditions and, for this reason, the conversion of XD to XO is of major medical interest. XO has been implicated in many diseases associated with oxygen-radical-induced tissue damage, contributing to post-ischemic reperfusion injuries, as well as aging (Fridovich 1970; Parks and Granger 1983; McCord 1985; Granger et al. 1986; Engerson et al. 1987; Chung et al. 1999).

Besides its role as a housekeeping gene, *XOR* is highly expressed in liver and kidney, the main organs involved

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1032702.

in purine catabolism and nitrogen elimination. Furthermore, it is found at high levels in a number of other mammalian tissues and organs, including the mammary gland (Jarasch et al. 1981; Kurosaki et al. 1995, 1996). This suggests that XOR plays additional roles in these tissues beyond its metabolic function(s). In humans, deficiencies of the enzyme are known to result in xanthinuria, an autosomal-recessive disorder leading to kidney stone formation, urinary tract disorders, and muscle diseases (Dent and Philport 1954). However, no other histological or physiological symptoms have been associated with reduced activity of XOR.

It has long been known that cow milk contains high levels of XOR activity. Expression of *XOR* in the lactating mammary gland is restricted to the secretory epithelium. In vitro analyses show that the transcription, translation, and enzymatic activity of XOR are up-regulated during late pregnancy and throughout lactation. XOR has therefore been used as a marker for terminal differentiation of mammary epithelial cells. Interestingly, XOR expression levels positively correlate with the secretory activity of the mammary epithelial cells. Furthermore, XOR expression is highly induced by lactogenic hormones (Schardinger 1902; Massey et al. 1969; Hayden et al. 1991; Abadeh et al. 1992; Hunt and Massey 1992; Linder et al. 1999; McManaman et al. 1999, 2000). These observations support the idea that XOR has a specific role in the mammary gland during lactation. By generating mice containing a targeted disruption in *XOR*, it has been possible to study this role. We demonstrate that female mice heterozygous for a *XOR* loss-of-function mutation are unable to maintain lactation due to failure to secrete fat droplets into the milk.

Results

Generation of mice deficient for XOR

XOR is the product of a single gene containing 36 exons. Using homologous recombination in embryonic stem (ES) cells (Capecchi 1989) mice carrying a null mutation for *XOR* were generated by inserting, in frame, the *Green Fluorescent Protein* (*GFP*) gene and a *loxP/tACE-Cre/neo^r/loxP*-cassette into the third exon of *XOR* (Fig. 1A). The mutant *XOR* gene produces a truncated protein composed of exons 1, 2, and a portion of exon 3 fused to GFP. The *Cre/neo^r* cassette in the mutant *XOR* allele is automatically excised from the locus in sperm produced by the chimeric animals that were generated from these ES cells (Bunting et al. 1999).

Pups from XOR+/– *females die at approximately 12 days postpartum*

Mice homozygous (–/–) for the mutation in *XOR* are runted and do not live beyond 6 wk of age. *XOR* heterozygous (+/–) mice are viable and show normal fertility, litter size, and maternal behavior. However, all pups from *XOR*+/– females, regardless of genotype, die around day 12 postpartum (P12) of malnourishment. In contrast to controls, dissected pups born to these females were found to be devoid of adipose tissue, such as brown fat and mammary fat pads (Fig. 1B–E). In addition, skeletal muscles were reduced in volume, consistent with starvation (data not shown). The bladders of the pups from *XOR*+/– mothers were normal and filled with urine (data not shown), indicating that dehydration was not the cause of death. To further analyze this process, the body weight of litters nursed by either wild-type or *XOR*+/– mice was monitored between birth and P13 (Fig. 1F). Until P4, the average body weight of the pups nursed by *XOR*+/– mothers was not significantly different from control pups. Pups nursed by *XOR*+/– mothers began to appear visibly malnourished by P6. By P12, when pups from the mutant mothers begin to die, the pups were emaciated, and by P13, the remaining pups showed less

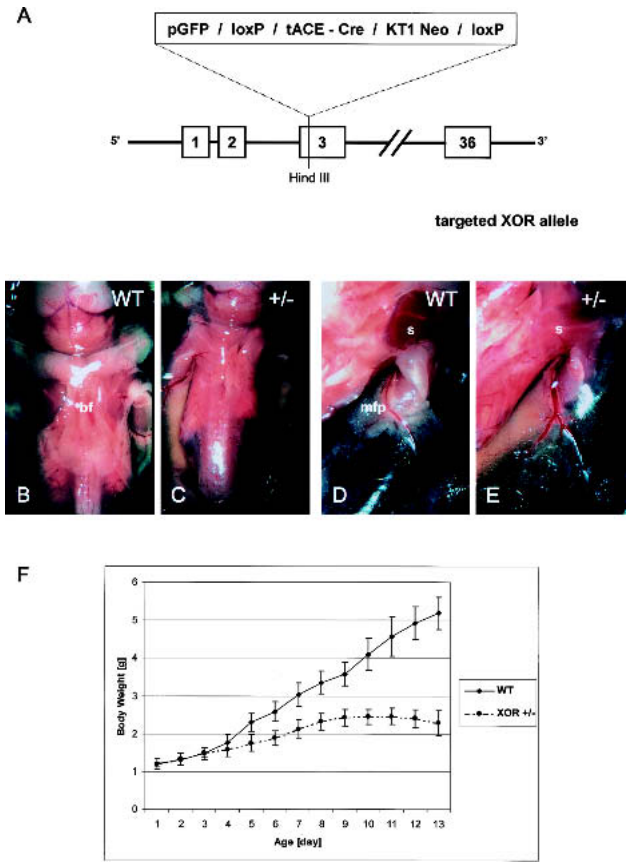

Figure 1. Targeting strategy for disruption of the *XOR* gene and pathology of pups raised by *XOR*+/– mothers. (*A*) A cassette containing *pGFP/loxP/tACE-Cre/neo^r/loxP* was inserted into the HindIII restriction site of the third exon of *XOR* genomic sequence, creating a loss-of-function mutation. Exons are shown as boxes and identified numerically. (*B–E*) Pups of wild-type (WT) and *XOR*+/– mothers at P12. Pups from *XOR*+/– mothers do not have the body fat stores of brown fat (*C*) and mammary fat pads (*E*) that age-matched pups from wild-type mothers have (*B*,*D*). bf, brown fat; mfp, mammary fat pad; s, scapula. (*F*) Average body weight increase of pups nursed by wild-type (WT) and *XOR*+/– mice. Pups of *XOR*+/– mothers start to die at lactation day 12. Each data point represents the average of 3 litters each with 6 pups (n = 18). Data are means ± S.D. Magnification: *B*,*C*, 2.5×; *D*,*E*, 4×.

than 50% of the body weight of the control litter. Fostering experiments showed that pups from *XOR*+/– mice can be raised normally by wild-type mothers (data not shown).

Mammary glands of XOR+/– *mice undergo tissue destruction during lactation*

The development of the mammary glands in wild-type and *XOR*+/– mice was examined in H&E stained paraffin sections and whole-mount preparations during late pregnancy and throughout lactation. No differences were found during pregnancy (data not shown) or at lactation day 1. The development of epithelial lobular-alveolar structure and the amount of adipocyte tissue appeared to be the same in wild-type and *XOR*+/– mammary glands on this day (Fig. 2A,B,I,J). By lactation day 5, the mammary epithelium of wild-type and *XOR*+/– glands proliferated and showed many expanded alveoli, indicating active milk secretion (Fig. 2C,D,K,L). The epithelium from *XOR*+/– mammary glands appeared similar; however, more adipocytes, which shrink in volume to provide fatty acids for milk fat synthesis, were still visible (Fig. 2D). In addition, within numerous mammary alveoli of the *XOR*+/– mice cell blebbing was detected; some of those cytoplasmic fragments were associated with milk fat droplets (Fig. 2D,L, arrows). At lactation day 11, the wild-type mammary alveoli expanded further, correlating with increased milk synthesis, secretion, and pup size (Fig. 2E,M). The *XOR*+/– mammary epithelium, however, showed severe cell shedding and tissue fragmentation associated with loss of epithelial tissue volume and premature tissue involution (Fig. 2F,N). By lactation day 13, severe tissue remodeling in the *XOR*+/– mammary epithelium resulted in the collapse of many mammary alveoli and less epithelial tissue remained throughout the mammary gland (Fig. 2H,P). The majority of the epithelial tissue appeared to be lactation deficient. However, some of the alveoli still seemed ex-

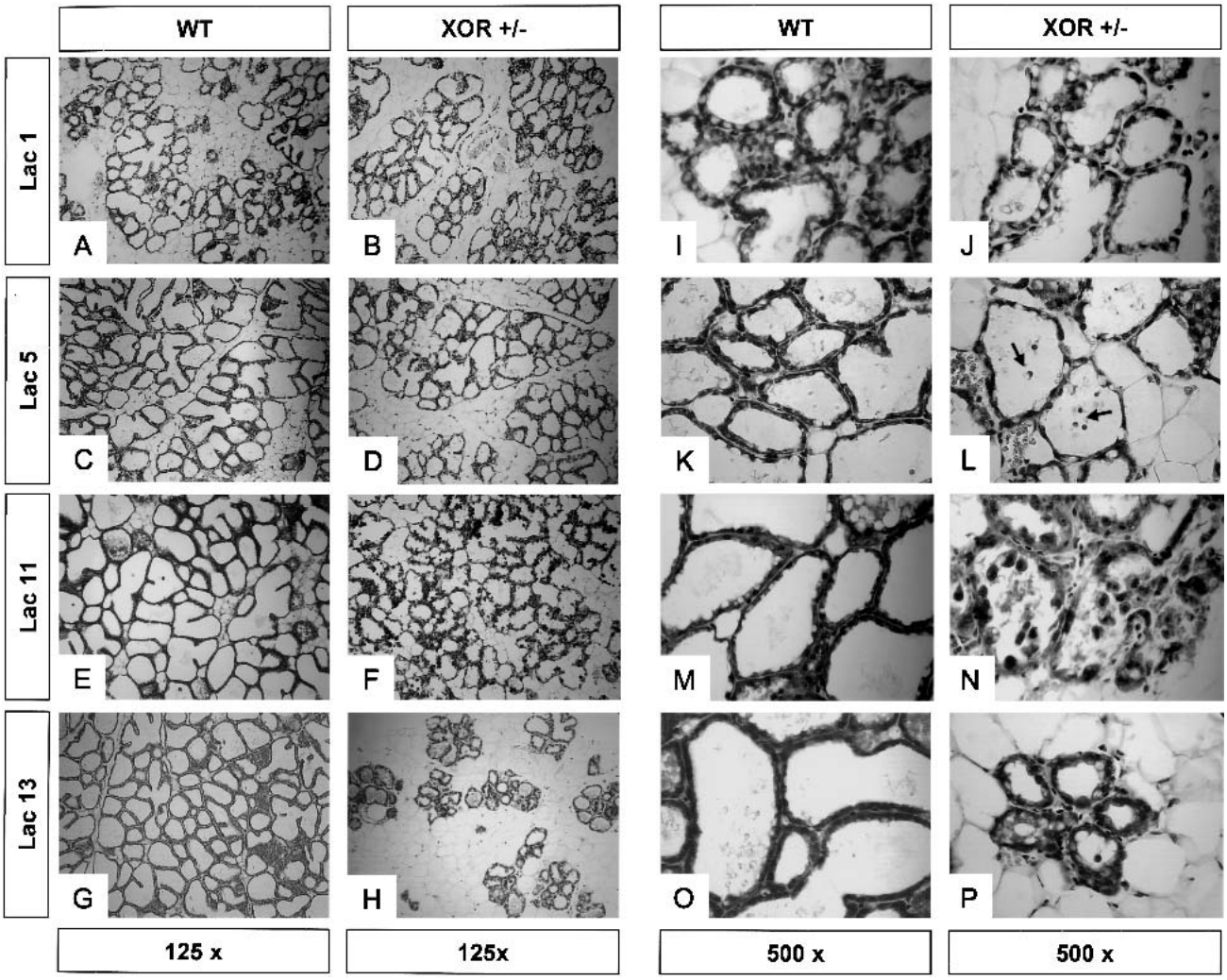

Figure 2. Histological analysis of normal and *XOR*+/– mammary glands. (*A–P*) Paraffin sections of mammary glands taken at various times during lactation and stained with hematoxylin and eosin. *XOR*+/– mammary epithelium undergoes destruction within the first half of lactation. Glands at lactation days 1 (*A,B,I,J*), 5 (*C,D,K,L*), 11 (*E,F,M,N*), and 13 (*G,H,O,P*). WT mammary glands are shown in *A,C,E,G,I,K,M,O*. *XOR*+/– mammary glands are shown in *B,D,F,H,J,L,N,P*. Magnification, 125× and 500×.

panded due to milk secretion and many of the mammary epithelial cells still contained fat droplets, indicating that mammary tissue destruction was independent of a defect in lactogenic hormones. Overall, tissue damage in the *XOR*+/– mammary gland correlates with the time course of pup starvation. An analysis of whole-mount preparations of wild-type and *XOR*+/– mammary glands at lactation day 13 revealed again the overall collapse of the *XOR*+/– epithelial tissue (Fig. 3A,B). However, only the secretory portion of the gland, the milk secreting alveoli, were affected, while the ducts, which mainly transport the secretions to the exterior of the gland, appeared normal (Fig. 3B, red arrows).

The enzymatic activity of XOR is reduced in terminally differentiated XOR+/– *mammary glands*

Previous studies have shown that the enzymatic activity and expression level of XOR increase during late pregnancy and remain high throughout lactation (Kurosaki et

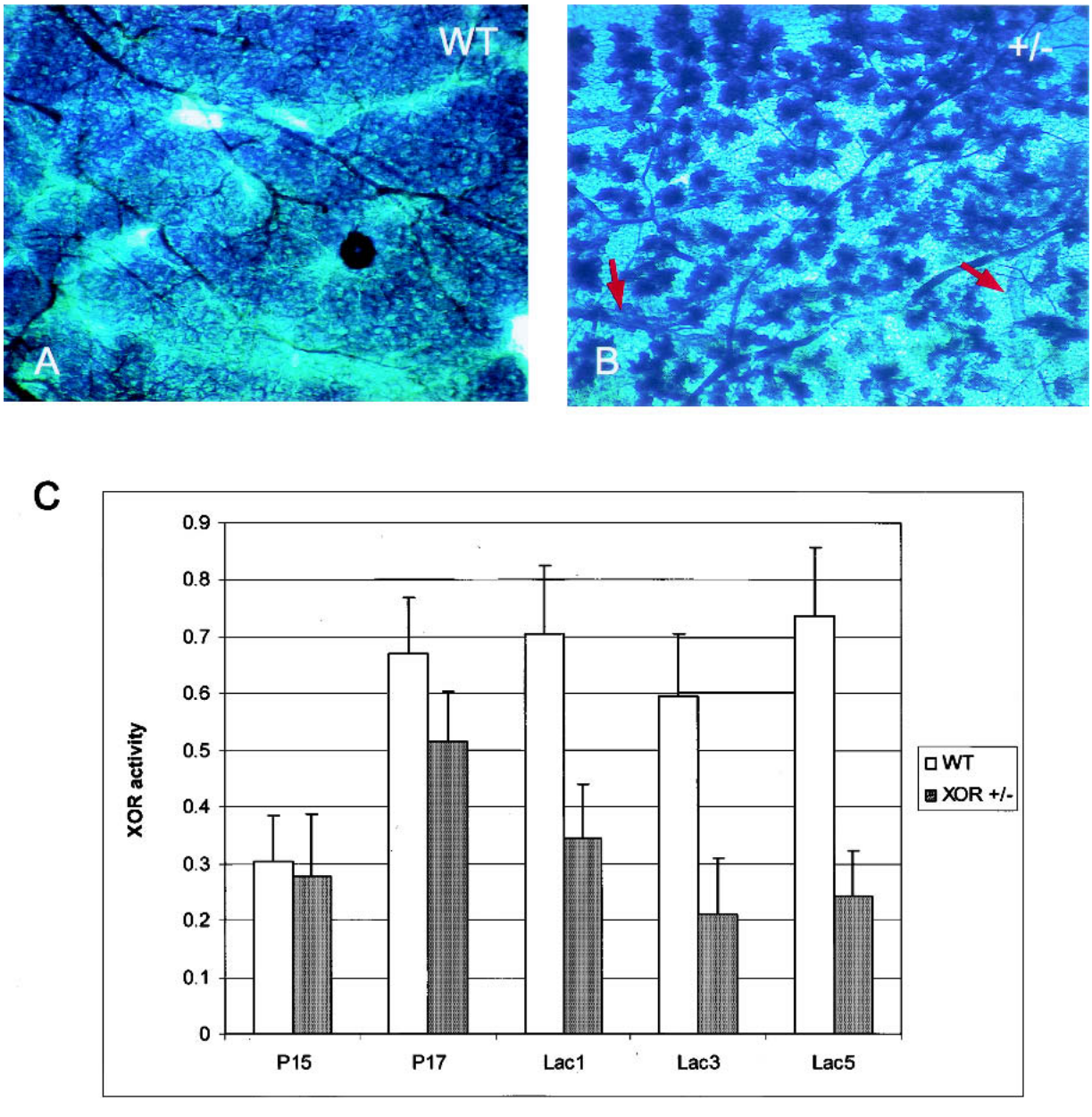

Figure 3. Whole-mount analysis and XOR enzymatic activity of wild-type and *XOR*+/– mammary glands. (*A*,*B*) Whole-mount analysis of wild-type (WT, *A*) and *XOR*+/– (*B*) mammary glands at lactation day 13. The milk-secreting alveoli are collapsed in *XOR*+/– mammary glands compared to wild-type, while the ducts are unaffected (red arrows). Tissue was stained with indigo carmine alum. (*C*) Reduced XOR enzymatic activity in terminally differentiated *XOR*+/– mammary glands. XOR activity in mammary glands of wild-type (WT) and *XOR*+/– mice was measured during late pregnancy (P) and early lactation (Lac). XOR activity is expressed as micromolar of isoxantho-pterin formed/minutes per milligrams of total mammary gland protein. Each value is the mean ± S.D. of eight separate tissue extracts. Magnification: *A*,*B*, 31.25×.

al. 1996; McManaman et al. 1999). To determine whether the absence of one allele of *XOR* results in reduced enzymatic activity of XOR in the mammary gland, the level of XOR activity was determined at multiple time points throughout late pregnancy and early lactation. At pregnancy day 15, wild-type and *XOR*+/– mammary glands showed similar levels of enzymatic activity (Fig. 3C). During later pregnancy, XOR activity was increased in accordance with earlier reported analyses. However, enzymatic activity increased less in *XOR*+/– mammary glands than in wild-type glands. In addition, while XOR activity remained high in the wild-type mammary glands throughout lactation, the enzymatic activity in *XOR*+/– glands steadily decreased postpartum, which correlates with the destruction of lactating mammary epithelium in the *XOR*+/– mice. Additional analyses of XOR activity in various organs of *XOR*+/– mice showed that the enzymatic activity of XOR is about 50% less than in wild-type tissue (A.J. Scriven and M.R. Capecchi, in prep.).

Milk fat droplets accumulate in the mammary epithelium of XOR+/– *mice*

Toluidine blue stained semithin sections showed that already by lactation day 1, milk secretion was perturbed in *XOR*+/– mice. Most fat droplets in wild-type mammary glands were secreted and accumulated in the lumen of the alveoli (Fig. 4A). Only a few wild-type epithelial cells contained cytoplasmic fat droplets. However, in the *XOR*+/– mammary gland, secretion of milk fat droplets was perturbed and cytoplasmic fat droplets accumulated in most of the mammary epithelial cells (Fig. 4B). Few fat droplets were successfully secreted into the lumen of the *XOR*+/– alveoli, although some appeared to be forced out (Fig. 4B, red arrows). In addition, the accumulated fat droplets in the *XOR*+/– epithelial cells were often very large, presumably due to ongoing fat droplet synthesis and fusion with smaller droplets. Still, the lumen of *XOR*+/– mammary glands appeared to be rich in colloidal particles, known as the casein micelle (Fig. 4B, dark dots in the lumen of the mammary gland alveoli), suggesting that protein secretion is normal and only fat droplet secretion is defective. To determine if protein secretion is altered in *XOR*+/– mammary glands, milk protein content from *XOR*+/– and wild-type mice was compared. Electrophoretic separation of milk samples from lactation days 2–6 showed no difference in major milk protein content (data not shown).

The accumulation of milk fat droplets in the *XOR*+/– mammary tissue was also verified following food deprivation. Milk fat is the major calorie supply for newborns, and its synthesis is very sensitive to the mother's calorie intake and/or body fat. Rodents have very little metabolic reserve in comparison to their rate of milk synthesis, and prolonged food deprivation leads to a state of starvation with a significant effect on the synthesis of all milk components (Williamson 1990). After depriving wild-type and *XOR*+/– mice of food for about 30 h starting at lactation day 1, wild-type mammary epithelium

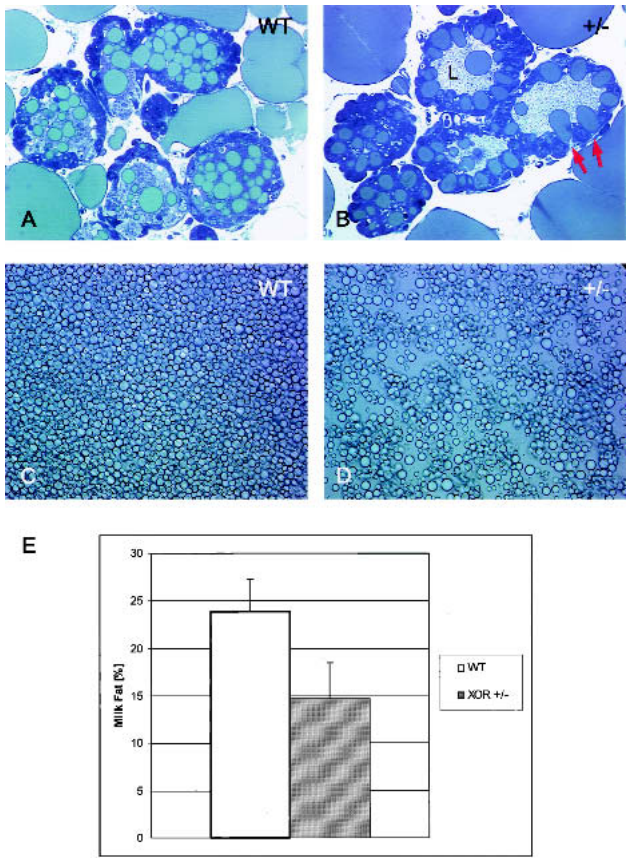

Figure 4. Milk fat droplets accumulate in *XOR*+/– mammary epithelium and less fat is formed in *XOR*+/– milk. (*A*,*B*) Histological analysis of resin-embedded mammary glands of wild-type (WT, *A*) and *XOR*+/– (*B*) mice at lactation day 1. The red arrows indicate *XOR*+/– epithelial cells that try to force out accumulated fat droplets. L, lumen of the mammary alveoli. (*C*,*D*) Lactating wild-type (WT) and *XOR*+/– mice were milked for milk fat analysis. Light microscopic analysis of milk samples show that *XOR*+/– (*B*) milk contains fewer fat droplets than milk from wild-type (WT, *A*) mice. (*E*) Creamatocrit analysis estimates 23.9% of average milk fat in WT mice, while only 14.7% in milk of *XOR*+/– mice. Values are the mean ± S.D. of 10 separate milk samples from lactation day 2. Magnification: *A*–*D*, 500×

was completely depleted of fat droplets, owing to the absence of additional milk fat synthesis. Previously synthesized milk fat droplets were secreted and some were still found in the lumen. However, the *XOR*+/– mammary epithelium still contained a significant number of fat droplets, again consistent with a defect in their secretion (data not shown). Further analyses of milk fat using microscopy and creamatocrit analysis show a reduction of milk fat content in the *XOR*+/– milk compared to wild-type milk (Fig. 4C–E).

Defective enveloping of milk fat droplets with the apical epithelial plasma membrane in XOR+/– *mammary glands*

Most milk components are secreted from the mammary epithelium by exocytosis, while milk fat droplet secre-

tion occurs by a unique mechanism. Cytoplasmic fat droplets are surrounded by a monolayer derived from the membranes of the ER in which fat droplet synthesis takes place. During the process of milk secretion, fat droplets become further enveloped by a bilayer of phospholipids derived from the apical plasma membrane from where they bud into the lumen of the mammary alveoli. To analyze the fat droplet secretion defects in the *XOR*+/– mammary gland on a subcellular level, transmission electron microscopy (TEM) was performed. TEM analyses of lactating mammary glands at lactation days 1–5 show that the mechanism of milk fat droplet enveloping is perturbed in *XOR*+/– mammary glands. In wild-type glands, each fat droplet was tightly enveloped with the apical membrane prior to secretion. Once a milk fat droplet was successfully secreted, the bilayer membrane was clearly visible around the floating fat droplet (Fig. 5A, arrows). In contrast, in *XOR*+/– mammary glands, some fat droplets became severely deformed during the process of secretion and were enveloped by a very abnormal membrane (Fig. 5B, black arrow). Figure 5C shows complete absence of milk fat droplet enveloping by the apical membrane. The entire fat droplet is still surrounded by cytoplasm as it was forced out of the cell (black arrow). In other cases, fat droplets burst while being secreted (Fig. 5D, black arrows). Some fat droplets seemed to be partially enveloped, yet other areas showed cytoplasmic crescents (Fig. 5E, black arrows). Incomplete enveloping by the apical membrane was also seen with deformation of the fat droplet where it remained nonenveloped (Fig. 5F).

TEM examination of lactating wild-type and *XOR*+/– mammary alveoli also showed numerous abnormal milk fat droplets as well as various cell fragments floating in the milk of lactating *XOR*+/– mice. Milk fat droplets from wild-type mammary glands were tightly enveloped with epithelial plasma membrane, which subsequently stabilizes the free-floating droplets (Fig. 5G). Numerous milk fat droplets in *XOR*+/– mammary glands contained cytoplasmic crescents of various sizes (Fig. 5H,I, black arrows). Fat droplets with cytoplasmic crescents also occur naturally in the milk of many mammalian species (Huston and Patton 1990) but wild-type mice showed very few. Furthermore, milk fat droplets with very abnormal surrounding membranes (Fig. 5I, white arrows), partially enveloped milk fat droplets (Fig. 5J, white arrows), burst milk fat droplets (Fig. 5J,K, black arrows), as well as many budded cell fragments, often containing secretory vesicles filled with casein micelles (Fig. 5L; black arrows point to cell fragments, white arrow points to secretory vesicle) were found in milk of *XOR*+/– mice.

Secreted milk fat droplets in early lactating XOR+/– *mice show various membrane phenotypes*

TEM analyses of milk fat droplet membranes revealed various membrane phenotypes in *XOR*+/– milk. Some droplets appeared correctly enveloped with the apical plasma bilayer as found in wild-type (Fig. 6A, white arrow). Others contained the cytoplasmic fat droplet monolayer with unidentified associated structures (Fig. 6A, white arrowhead). As the enlargement in Figure 6B shows, these rounded structures appear to be under the fat droplet monolayer and are not associated with this membrane (white arrowhead). Therefore, they are not likely to be secreted milk proteins. Milk fat droplets that did not contain any membrane at all and burst through the cytoplasmic fat droplet monolayer, were also observed (Fig. 6A, black arrow). In some instances, different membrane phenotypes could be identified on a single *XOR*+/– milk fat droplet (Fig. 6C). Normal bilayer membrane (white arrow), a region with the unidentified associated structure (white arrowhead), and a small cytoplasmic crescent (black arrow) were all observed together. Figure 6D shows a milk fat droplet that was partly enveloped, but as there was still some cytoplasm between the apical membrane and the fat droplet, the envelopment was not stable and the membrane seems to be breaking off (arrow).

Defective enveloping of milk fat droplets in lactating XOR+/– *mammary glands causes tissue necrosis and premature involution of the mammary epithelium*

As light microscopy analyses demonstrated, the defect in milk fat droplet secretion in the *XOR*+/– mammary gland severely impacts the epithelial cells, causing the destruction of the epithelium in several ways. TEM analysis detected entirely destroyed mammary epithelial cells within secretory *XOR*+/– alveoli at a subcellular level. Figure 6E shows a section of a milk secreting *XOR*+/– alveolus with several epithelial cells that have deteriorated by lactation day 3 (arrows). The enlargement of burst epithelial cells in Figure 6F shows the large amount of mitochondria and ER membranes that are typical for lactating mammary epithelial cells (arrows). This again demonstrates on a subcellular level the tremendous damage the defect in milk fat droplet enveloping causes to the entire lactating mammary gland, resulting in tissue necrosis, premature involution of the epithelium, starvation, and death of the offspring.

Discussion

A genetic functional analysis of *XOR*'s role in mammalian lactation is provided. We demonstrate that XOR is required in the lactating mammary gland for milk fat droplet enveloping with the apical epithelial plasma membrane and subsequent secretion via budding from the terminal differentiated mammary epithelium. The defect in milk fat droplet secretion in the *XOR*+/– mice causes the rupture of the lactating mammary epithelium, resulting in premature involution of the mammary gland and starvation of the offspring during the second week of lactation.

The mechanism of milk fat droplet secretion is unique to the mammary epithelium. Unfortunately, the cellular mechanisms of fat droplet synthesis and secretion are still poorly understood (Keenan 2001). Fat droplet syn-

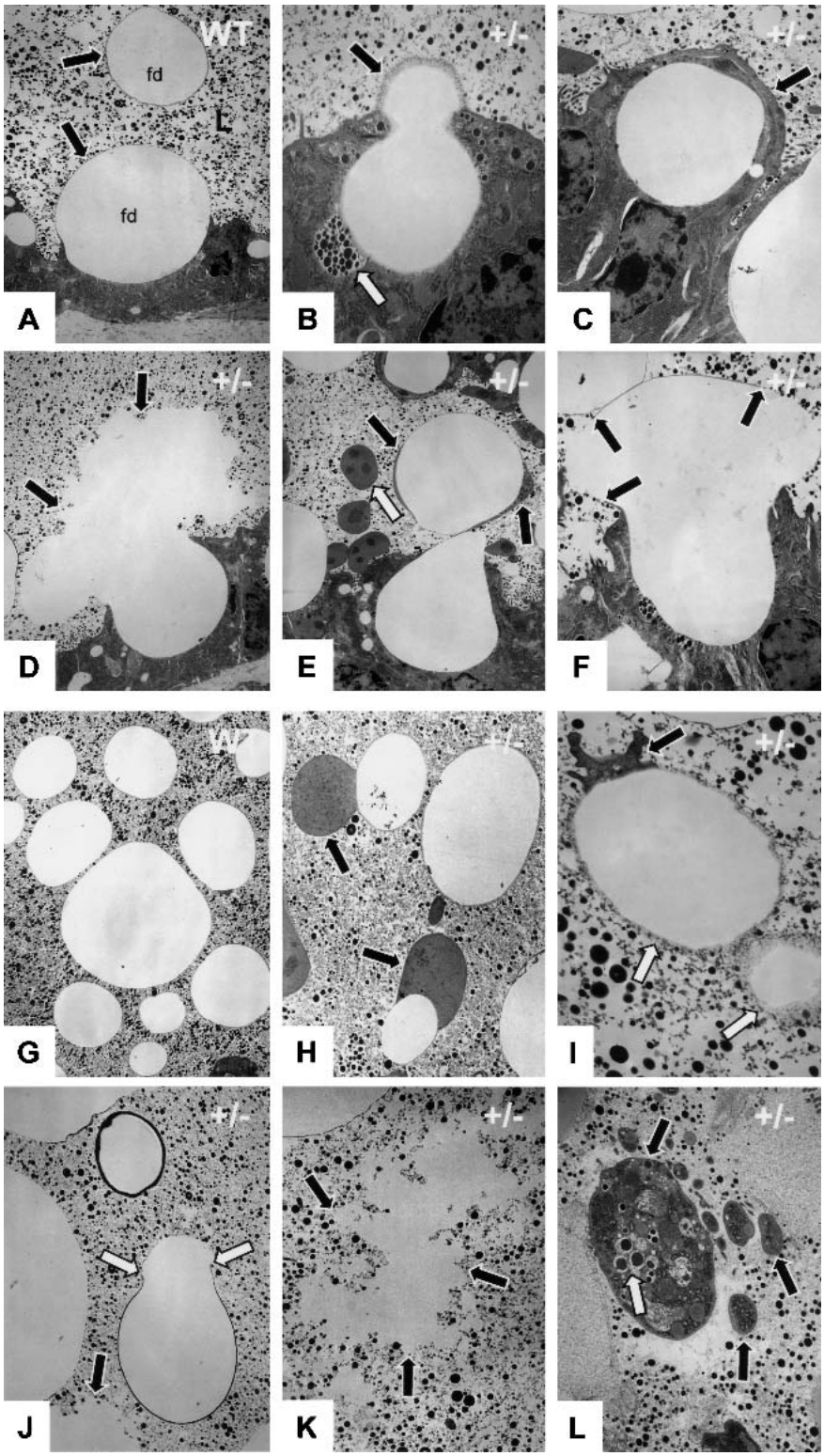

Figure 5. The mechanism of milk fat droplet enveloping with the apical plasma membrane is disturbed in *XOR*+/– mammary glands. (*A–F*) TEM analysis of wild-type (WT, *A*) and *XOR*+/– (*B–F*) mammary glands at lactation day 3. Defective enveloping results in several fat droplet secretion phenotypes in the *XOR*+/– mammary glands. L, lumen of the mammary gland filled with casein micelles. (*G–L*) TEM analysis of milk in mammary alveoli at lactation day 3 from wild-type (WT, *G*) and *XOR*+/– (*H–L*) mammary glands. Various deformed fat droplets and numerous cell fragments are found in milk from *XOR*+/– mice. Magnification: *A–F*, 6000×–10,000×; *G–L*, 5000×–15,000×.

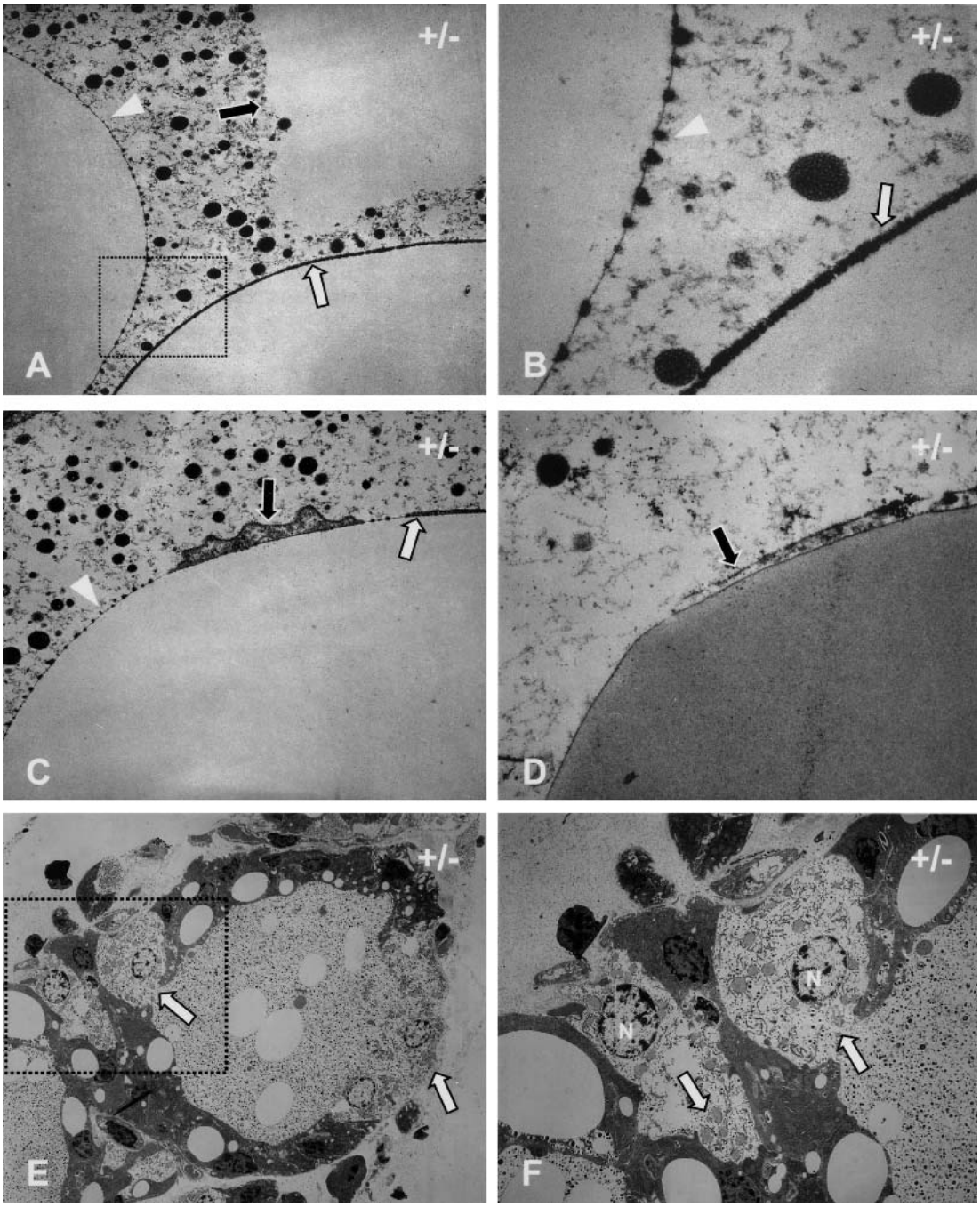

Figure 6. Various membrane defects in milk fat droplets from *XOR*+/– mice. (*A–D*) TEM analysis of milk fat droplets from *XOR*+/– mammary glands at lactation day 3. (*A*) The white arrow points to a bilayer membrane of correctly enveloped fat droplet areas as found in wild-type. The black arrow points to a completely unenveloped droplet. The white arrowhead points to an unidentified membrane structure discussed in the text. (*B*) Higher-power view of the boxed area in *A*. (*C*) Three membrane phenotypes can be found on a single *XOR*+/– milk fat droplet. The white arrow points to a portion of normal membrane bilayer. The black arrow indicates a piece of cytoplasmic crescent. Another portion of the fat droplet is occupied by the unidentified membrane structure as seen in *A* and *B* (white arrowhead). (*D*) *XOR*+/– milk fat droplet with a portion of cytoplasmic crescent that is only loosely associated with the droplet. (*E*) TEM analysis of an entire alveolus in a *XOR*+/– mammary gland at lactation day 3. The arrows point to completely destroyed mammary epithelial cells. (*F*) Higher-power view of boxed area in (*E*). Numerous free-floating mitochondria and ER are seen in burst cells of *XOR*+/– mammary glands (white arrows). N, nucleus. Magnification: *A*, 30,000×; *B*, 70,000×; *C*,*D*, 50,000×; *E*, 1000×; *F*, 3000×.

thesis occurs de novo in the ER from which fat droplets are released as microlipids surrounded by a monolayer of phospholipids and associated proteins (Ghosal et al. 1994). Fat droplets presumably form in the hydrophobic

center of the ER bilayer and growth of the fat droplets appears to drive the opposing ER membrane monolayers apart, resulting in a bulge that buds into the cytoplasm (Long and Patton 1978; Zaczek and Keenan 1990). A transport system is proposed to bring the microlipid droplets to the apical membrane of the mammary epithelial cells. Throughout this transport process microlipids fuse to larger cytoplasmic lipid droplets (CLDs). The CLDs become tightly enveloped with the apical plasma membrane and finally bud off into the lumen of mammary alveoli (Mather and Keenan 1998). The present study demonstrates that milk fat droplet synthesis, fusion, and apical transport appear to be normal in the lactating *XOR*+/– mice. Only the final step, enveloping the milk fat droplets with apical membrane prior to secretion, is abnormal.

The molecules that mediate enveloping of milk fat droplets are proteins associated with and integrated into the apical mammary epithelial membrane as well as the proteins found on CLDs. Most of these proteins are not expressed in undifferentiated mammary glands. Numerous milk fat globule membrane (MFGM) proteins have been isolated and characterized from milk fat of several species, and their subcellular orientations have been determined (Mather 2000). Interestingly, XOR is the second most common protein among the major MFGM proteins, comprising as much as 13% of total proteins. It has been estimated that XOR concentration increases >150-fold in the MFGM over its levels in the cytoplasm. While several of the MFGM proteins are integral transmembrane proteins, XOR is a globular cytoplasmic protein tightly associated with the membrane (Jarasch et al. 1977; Mangino and Brunner 1977; Freudenstein et al. 1979; Franke et al. 1981; Mather et al. 1982; Keenan and Patton 1995; McManaman et al. 1999). The presence of XOR among MFGM proteins and its strong epithelial expression during lactation, provides strong supporting evidence that the *XOR*+/– lactation phenotype is due to a defect within the mammary epithelium and not for example, due to a systemic defect of lactogenic hormones. Hormonal defects are also unlikely in the *XOR*+/– mice, since reproduction occurs normally.

But what is the function of XOR during milk fat droplet secretion? The role XOR plays in fat droplet enveloping is unlikely to involve purine catabolism for several reasons: (1) Although XOR expression and enzymatic activity are greatly increased during late pregnancy and throughout lactation, uric acid, the end product of purine catabolism, is found only at low levels in the mammary gland; (2) whereas the dehydrogenase form (XD) of XOR is predominantly responsible for purine catabolism in all cells, it is the oxidase form (XO) of XOR that associates with MFGM and also predominates in milk. Studies have shown that milk XO has a very low activity towards purines, again indicating that the mammary-specific role of XOR is distinct from purine catabolism (Kurosaki et al. 1996). Interestingly, the MFGM itself is competent to induce the transition from the XD to the XO form, and this may be the important signal for the initiation of fat droplet enveloping and secretion (McManaman et al. 1999). A study comparing bovine milk XD and XO crystal structures suggests that the reversible transformation of XD to XO is associated with a conformational change of the molecule due to formation of disulfide bonds (Enroth et al. 2000).

Our study suggests that XOR has a function in the mammary gland distinct from its catabolic function(s), and moreover that this function may be entirely structural and nonenzymatic. This hypothesis is further supported by the observed severity of the phenotype associated with the haploinsufficiency of XOR. The time course for the loss of lactation and of fat droplet secretion is consistent with progressive exhaustion of a membrane-associated structural component, rather than a reduction by 50% of an enzymatic activity.

The catalytic activity of XOR depends on the molybdenum cofactor and the experimental incorporation of tungsten as a competitive antagonist of molybdenum yields enzymatically inactive XOR in various animal systems. Feeding rats on a tungsten-supplemented molybdenum-free diet results in synthesis of inactive XOR and reduces liver XOR enzymatic activity to less than 10% of initial values. However, although XOR enzymatic activity is presumably also reduced in their mammary glands, neither the delivery of normal litters nor the growth of pups is affected in tungsten-treated mothers. Liver XOR activity is undetectable in those pups while nursed by tungsten-treated mothers, but rises dramatically after weaning, since the administration of molybdenum rapidly reconstitutes the enzymatic activity of XOR. These studies suggest that the pups receive tungsten and inactive de-molybdo XOR through milk, yet it had no impact on the maternal mammary glands (Cohen et al. 1974; Johnson et al. 1974). Feeding tungsten to lactating goats and cows also shows a strong decrease of milk XOR enzymatic activity in both cases without any influence on milk yield (Owen and Proudfoot 1968). Interestingly, milk contains by nature a mixture of active and inactive (de-molybdo) XOR (McGartoll et al. 1970; Ventom et al. 1988). While the enzymatic activity of milk XOR changes over time, the level of XOR protein remains relatively constant (Brown et al. 1994, 1995). Oral administration of sodium molybdate to cows and goats increases only the molybdenum content in milk, but does not alter the activity of milk XOR and also does not cause changes in milk fat content or milk yield (Kiermeir and Capellari 1958; Hart et al. 1967). Furthermore, in vitro experiments have shown that application of the XOR enzymatic inhibitor allopurinol does not affect terminal differentiation of mammary cells (Hayden et al. 1991). All the above results are consistent with a nonenzymatic role for XOR protein in milk fat droplet secretion.

TEM analysis of isolated MFGM reveals the existence of an intervening coat material between the inner face of the MFGM bilayer and the outer face of the fat droplet monolayer. The coat material is a protein layer composed of plasma membrane proteins associated with selected cytosolic proteins, as well as proteins from the surface of the cytoplasmic lipid droplets. XOR and bu-

tyrophilin (BTN) have been identified as the major proteins forming this coat material (Franke et al. 1981). BTN, a member of the Ig superfamily, is a type I membrane glycoprotein unique to the mammary gland, and the most abundant protein of bovine and guinea pig MFGM. Interestingly, coimmunoprecipitation studies with the cytoplasmatic domain of BTN suggest specific binding to XOR, and correlate with previous findings that XOR is tightly associated with the cytoplasmic surface of the apical epithelial membrane (Ishii et al. 1995; Keenan and Patton 1995). BTN can also be covalently bound to XOR by bifunctional cross-linking agents (Valivullah and Keenan 1989). The aggregation of a BTN/XOR complex may subsequently trigger the binding of additional molecules to form the protein core. The major fat droplet surface protein, likely to be involved in protein core formation during envelopment, is the adipocyte differentiation-related protein (ADRP). ADRP is frequently associated with the surface of cytoplasmic lipid droplets, and together with XOR and BTN is a major component of the protein core (Heid et al. 1996). Immunohistochemical analysis of early lactating mammary tissue, as well as of milk from wild-type and *XOR*+/– mice, revealed no differences in the expression of ADRP and MUC-1, a MFGM protein of the mammary epithelium (data not shown). It has been reported that BTN and XOR are expressed in constant molar proportions in bovine MFGM and these proportions vary with breed and stage of lactation. Particularly, XOR as a homodimer may simultaneously bind two BTN molecules and therefore be responsible for the observed accumulation of BTN molecules on budding membrane areas (Mather et al. 2001). A likely explanation for the up-regulation of XOR during lactation is the high cellular demand for this structural molecule, since it is continuously lost from the cell with each budding fat droplet. The TEM analyses in Figures 5 and 6 show partially enveloped fat droplets in *XOR*+/– mammary glands. An attractive hypothesis is that only the membrane with enough localized XOR protein can successfully envelop a milk fat droplet. Other areas with less or no XOR may be either missing the apical membrane or are enveloped by cytoplasmic crescents of various lengths.

Our current model for the role of XOR in milk fat droplet enveloping is as follows: after parturition, the MFGM converts a significant amount of XOR from the XD to the XO form. This conformational change may allow XO to then interact with the cytoplasmic portion of BTN from the apical plasma membrane, leading to an accumulation of BTN at future membrane budding areas. BTN and XO may interact directly or indirectly with surface proteins from the CLDs, presumably ADRP. Additional cytosolic proteins, proteins from the surface of CLDs, and proteins from the apical membrane may interact with BTN, XO, and ADRP to form an entire protein core between the apical membrane and the surface of the CLDs. The formation of the protein core presumably triggers the binding of the apical membrane to the CLD, resulting in tight enveloping and subsequent budding of the milk fat droplets from the mammary epithelial cells (Heid et al. 1996, 1998; Mather and Keenan 1998; McManaman et al. 1999). Since XOR molecules are constantly lost from the lactating mammary epithelium and are therefore in high demand, the reduction in the amount of XOR protein in *XOR*+/– mice leads to numerous defects in milk fat droplet enveloping, the destruction of the mammary epithelium, and premature involution of the mammary gland.

In the context of evolution, we suggest that the housekeeping enzyme XOR became one of the key (structural) proteins that allow fat droplets to be secreted and thereby creating a new cell function in a new cell type of the class mammalia. *XOR* is not the only housekeeping gene that performs an additional role in evolution. Studies on vertebrate and invertebrate lens proteins show that several metabolic housekeeping genes have been recruited as taxon-specific structural lens proteins (crystallins), the major proteins in refractive eye lenses. This phenomenon, the use of a gene and its encoded protein for more than one function, has been termed "gene sharing" (Wistow and Piatigorsky 1987; Piatigorsky and Wistow 1989). While some of the crystallin proteins retain full enzymatic activity, others have reduced or unknown enzymatic activity (Zigler and Rao 1991). Just as for XOR in milk fat droplet secretion, the enzymatic activity of crystallin proteins may not be required for the additional structural role in the lens. Gene sharing of *XOR* in the lactating mammary gland, an evolutionarily young organ, provides a striking example of a gene acquiring multiple functions during its evolution. How the additional role of XOR in milk fat droplet secretion and its presence in milk may have impinged on the evolutionary development of the mammary gland will be addressed in a separate communication (C. Vorbach and M.R. Capecchi, in prep.).

As mentioned above, humans with mutations in *XOR* suffer from xanthinuria; however, lactation problems have not been reported in these patients. This is particularly striking, since already a heterozygous mutation of *XOR* in mouse causes the destruction of the lactating mammary epithelium, mortally affecting the ability to nurse. A potential explanation for this difference between mice and humans is that mice have a milk fat content of 20%–30%, whereas human milk contains only about 4% fat. This suggests that a mutation in the *XOR* gene and a defect in milk fat droplet secretion may have a much stronger impact on the lactating murine mammary gland than on human lactation. Furthermore, the nature of the mutation (point mutations, deletions) should differentially affect the functionality of XOR in the mammary gland. Still, about 5% of women experience primary lactation insufficiency (Neifert 2001), and *XOR* is a potential candidate gene for contributing to this deficiency.

In summary, we have shown that mice heterozygous for a loss-of-function mutation in *XOR* are unable to maintain lactation. The disruption of lactation is caused by a *XOR*-dependent defect in the enveloping of milk fat droplets with the apical mammary epithelial membrane, thereby markedly interfering with secretion of those

droplets into the milk, causing tissue necrosis and induction of premature involution of the mammary gland.

Materials and methods

Cloning of a targeting vector for xanthine oxidoreductase

XOR was cloned from a 129SvJ Lambda Fix II genomic library (Stratagene). Based on the published *XOR* cDNA sequence (Terao et al. 1992), synthetic oligonucleotide probes homologous to regions of exons 3 and 5, were used in the library screen ensuring that the recovered clones contained the most proximal redox active center of XOR. The oligonucleotide probe for exon 3 had the sequence: 5′-TGTGCGGGACCAAGCTTGGCTGTG GAGAAGGTGGCT-3′. The oligonucleotide probe for exon 5 had the sequence 5′-CATTCTCGATCTCCTCGACAGTAGG CTCAGGCTTGTTTCGGAG-3′. An 8.8-kb EcoRI fragment, surrounding exon 3, was isolated from the genomic library and used as the homologous region in the targeting vector. A replacement vector was generated by inserting a *GFP/loxP/tACE-Cre/neo^r/loxP* cassette at a unique HindIII site in exon 3 (Bunting et al. 1999). The linearized targeting vector was electroporated into R1 ES cells (Nagy et al. 1993) and ES cells enriched for the targeting event were selected with the drugs G418 and FIAU. These ES cells were screened for accurate gene targeting via Southern blot analysis. Correctly targeted ES clones were injected into C57BL/6J-derived blastocysts and then transferred to uteri of pseudopregnant mice. Chimeric males estimated to contain >80% ES cell contribution were mated with BL6 females to obtain germline transmission at the mutant *XOR* allele (A.J. Scriven and M.R. Capecchi, in prep.).

Body weight analysis and dissection of pups

Each of six litters (three from wild-type and three from *XOR*+/– mothers) was reduced to the same size of six pups and the body weight increase was documented for each pup from lactation day 1 to 13. The average body weight of the wild-type and *XOR*+/– litter (n = 18) was calculated as means ± S.D. for each lactation day. Pups were euthanized with CO_2, dissected, and analyzed macro- and microscopically.

Histology

Mammary glands #4 were removed from wild-type and *XOR*+/– mice, immediately cut into four pieces, and fixed in 4% Paraformaldehyde/PBS overnight. The fixed glands were washed for 3 × 10 min in PBS and paraffin embedded (Paraplast X-tra, CMS) with the help of a Modular Vacuum Processor (Instrumentation Laboratory) and a Tissue Embedding Center (Reichert-Jung) according to standard methods. Six-micrometer tissue sections were mounted on pretreated glass slides (Superfrost Plus Micro Slides, VWR), mounted with D.P.X. (Aldrich) and observed using a Leitz Ortholux II with a 3CCD digital color camera (DAGE-MTI).

Whole-mount analysis

Mammary glands #3 were removed from wild-type and *XOR*+/– mice at different lactation days and immediately spread onto glass slides. After drying the glands to the slides for about 1 min, the glands were fixed in Carnoy's fixative (3 vol 100% EtOH, 1 vol glacial acetic acid) overnight. The glands were washed in 70% EtOH for 20 min, rinsed in water, and stained with carmine alum stain (2.5 g alum potassium sulfate, 1 g indigo carmine, 500 mL water) overnight, washed in water, dehydrated, and mounted.

Semithin sections and electron microscopy

Mammary glands #4 were removed from wild-type and *XOR*+/– mice, cut in very small pieces, and fixed overnight (phosphate buffer at pH 7.4 containing 2% glutaraldehyde and 1% paraformaldehyde). The tissue was postfixed in 2% osmium tetroxide buffered with phosphate buffer at pH 7.4 for 2–4 h, dehydrated and embedded with the EMbed 812 Kit (Electron Microscopy Sciences). Blocks were randomly sectioned (1µm-thick) and stained with 1% toluidine blue/1% sodium borate in water for light microscopy analysis (Leitz Ortholux II with a 3CCD digital color camera, DAGE-MTI). For electron microscopy, random areas of mammary glands were thin sectioned with the aid of a diamond knife mounted on an ultramicrotome (LKB Nova; Leica). The thin sections (60–80 nm) were counterstained with uranyl acetate and lead citrate before the ultrastructural samples were observed, using a transmission electron microscope (Hitachi H-7100, University of Utah, Research Microscopy Facility).

Enzymatic activity

Mammary glands #4 from wild-type and *XOR*+/– mice were collected and shock frozen with liquid nitrogen. Enzymatic analysis was performed according to (Beckman et al. 1989) with minor modifications. The tissue was homogenized in 2 mL of 50 mM potassium phosphate at pH 7.4/0.1 mM EDTA and protease inhibitor (Complete, Roche) followed by sonication. The cell lysates were centrifuged at 18,000 g for 30 min at 4°C. Of the supernatant, 100 µL was diluted to 2 mL with 50 mM potassium phosphate/0.1 mM EDTA. Fluorescence was monitored at 390 nm with the excitation wavelength set at 345 nm. After achieving a stable baseline, 20 µL of 1mM pterin and 20 µL of 1mM methylene blue were added as an electron acceptor to assay total XOR activity for 2 min. To inhibit and to confirm the specificity of the reaction, 20 µL (2 mM) allopurinol was added and the XOR activity reached a plateau. 20 µL (1 µM) isoxanthopterin was added as an internal standard after its exact concentration was determined spectrally (E_{336} = 13.0 mM^{-1}/cm^{-1}). XOR activity was expressed as micromolar of isoxanthopterin formed/minutes per milligram protein. The total protein concentrations in the above-described supernatants were determined by dye-binding assay (Bio-Rad Laboratories) according to manufacturer's instructions.

Milk analysis

Pups were removed for 2–5 h before milking the mothers. Mice were anesthetized with avertin (450 µL/25 g body weight) and injected intraperitoneally with Oxytoxin (0.3 IU; Sigma) in 200 µL PBS. After a 5–10-min incubation period milk was withdrawn with a Pasteur pipette attached to tubing and a mouthpiece. For creamatocrit analysis, an estimation of total milk lipid content, 10 wild-type and 10 *XOR*+/– mice were milked on lactation day 2. The milk was spun in a haematocrit centrifuge and the fat content was estimated using *Spiracrit*, a micro-hematocrit capillary tube reader (Lancer; Lucas et al. 1978). For milk protein analysis, three wild-type and three *XOR*+/– mice were milked on 5 consecutive days and 0.5 µL milk was analyzed with SDS-PAGE using a 15% resolving and a 5% stacking gel.

Acknowledgments

We thank Marjorie Allen, Carol Lenz, Gail Peterson, Jim Hayes, Sheila Barnett, and Julie Tomlin for ES culture work and blastocyst injection, the vivarium staff for help with animal care, Nancy Chandler for help with electron microscopy, Tom Hueckstead for help with the enzymatic assay, Gurmail Gill for help with the creamatocrit analysis, Thomas Keenan for the ADRP antibody, Sandra Gendler for the MUC-1 antibody, and members of the Capecchi lab for critical reading of the manuscript.

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