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Letter to the Editor

XIAP-deficiency leads to delayed lobuloalveolar development in the mammary gland

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Dear Editor,

Inhibitors of apoptosis proteins (IAPs) were originally identified in baculoviruses where they prevent apoptosis of the host cell, thereby allowing viral propagation.[1] Cellular homologues of the viral IAP have been identified in yeast and metazoans. IAPs share a conserved structure known as the baculovirus IAP repeat (BIR) domain, which is an ~80 amino acid zinc-finger motif. These proteins include DIAP1 and DIAP2 in *Drosophila*, XIAP, cIAP1, cIAP2 and ML-IAP as well as the more distantly related family members NAIPs, Survivin and BRUCE in vertebrates.[2] Some IAP proteins can act as potent inhibitors of apoptosis and appear to be the only endogenous inhibitors that directly inhibit both initiator and effector caspases.

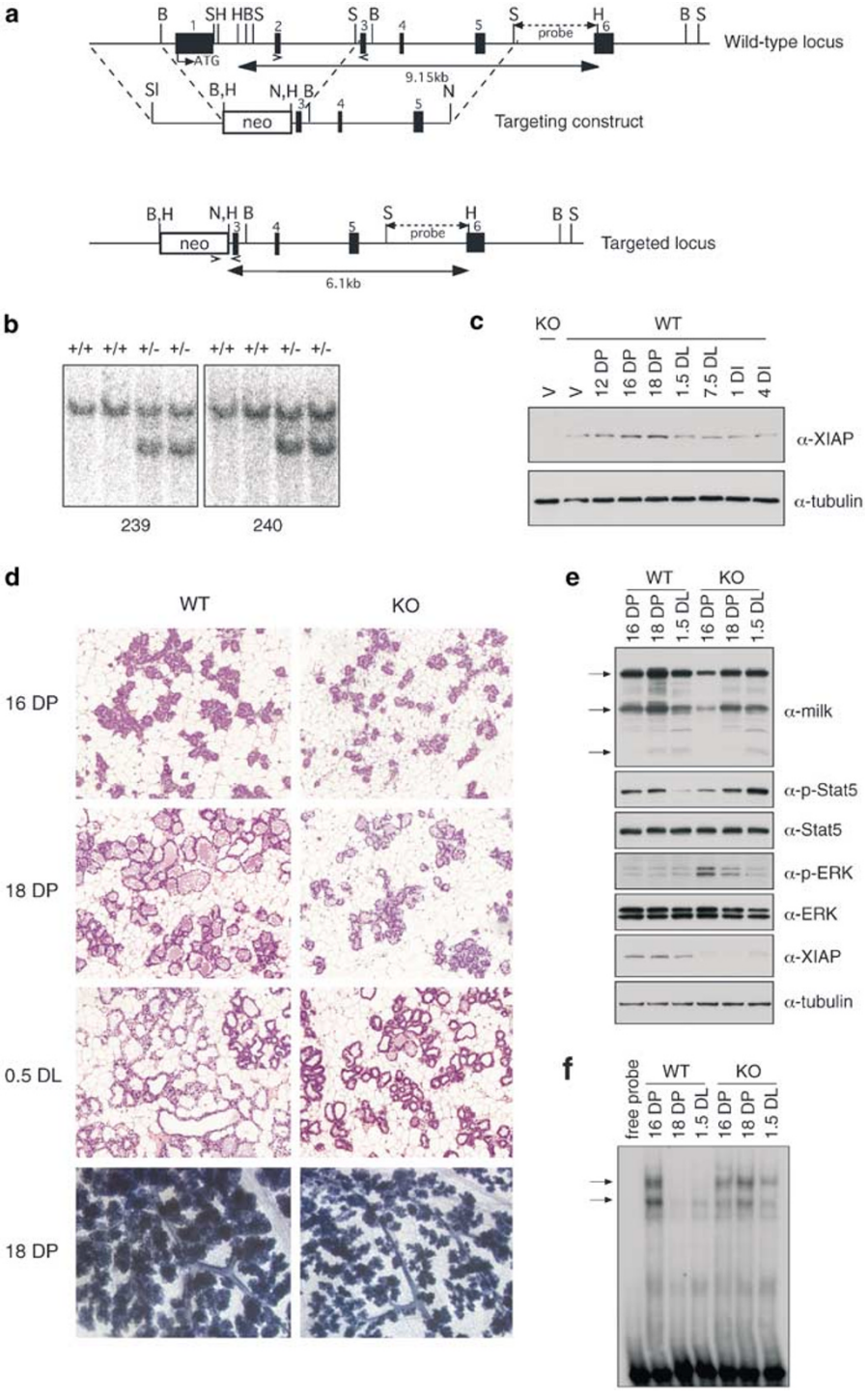
The most extensively studied IAP is X-linked inhibitor of apoptosis protein (XIAP).[3] This protein comprises three BIR domains at its N-terminus and a RING domain that has E3 ubiquitin ligase activity at its C-terminus. Cells transfected with XIAP are protected from apoptosis in response to a range of stimuli. XIAP directly binds to and inhibits caspases 3, 7 and 9. Although XIAP is ubiquitously expressed, *XIAP*-deficient mice were not reported to have any gross phenotypic abnormalities or any profound defects in programmed cell death following the induction of apoptosis by a variety of stimuli.[4] Recently, however, XIAP-deficient sympathetic neurons were reported to be more susceptible to cytochrome *c*-induced cell death.[5]

We have found that XIAP expression is developmentally regulated in the mammary gland (see below) and therefore investigated a potential role for this gene during mammopoiesis using targeted mice. Homologous recombination in embryonic stem (ES) cells was used to disrupt the murine *XIAP* locus, which comprises six coding exons (Figure 1a). The targeting vector was designed to replace exons 1 and 2 with a PGK-neomycin resistance cassette, thus deleting the initiation codon, the first two BIR domains and the majority of the third BIR domain (Figure 1a). Several clonal $G418^R$-targeted ES cell lines were generated and correct integration was verified by Southern blot analysis using the indicated probe (Figure 1b). Chimeric mice generated from two independent ES cell lines (239 and 240) gave germ-line transmission and were used to generate heterozygous *XIAP* female mice. These were intercrossed to yield male and female mice lacking *XIAP*. To confirm that deletion of exons 1 and 2 within the *XIAP* locus gave rise to animals deficient in XIAP, we performed Western blot analysis using a monoclonal XIAP antibody. XIAP was not detectable in *XIAP* mutant tissues including the liver, kidney, spleen and mammary gland, whereas it was readily visualized in tissues from wild-type mice (Figure 1e and data not shown). Therefore, targeted disruption of the *XIAP* gene generated a null mutation. Consistent with previous findings,[4] *XIAP*-deficient mice appeared healthy with no gross defects.

XIAP expression was examined in the mouse mammary gland by Western blotting. The expression was found to vary during mammary gland development, with highest levels evident in late pregnancy (Figure 1c). We therefore examined whether mammary gland development was normal in *XIAP*-null mice. No defects were evident in the mammary glands of virgin ($n=3$), lactating ($n=8$) or involuting ($n=8$) female mice. However, histological and wholemount analyses of mammary glands from two independent strains of *XIAP*-deficient mice at different stages of pregnancy revealed a defect during late pregnancy (Figure 1d). Reduced lobuloalveolar development was evident in the mammary glands of all $XIAP^{-/-}$ mice at day 16 ($n=3$), day 17 ($n=3$) and day 18 ($n=5$) of pregnancy, compared with that seen in wild-type control animals. The density of lobuloalveoli was significantly decreased and the lumens were less dilated, suggesting reduced milk protein synthesis and/or secretion (Figure 1d). The delay in lobuloalveolar development was also evident at 0.5 day lactation but was no longer apparent by day 7 of lactation. Consistent with these observations, females were found to lactate normally. RT-PCR analysis of cIAP-1 and cIAP-2 expression revealed that mRNA levels were similar in mutant and wild-type mammary glands during late pregnancy and early lactation (data not shown). Therefore, compensatory upregulation of these IAP genes does not appear to occur in the mammary glands of *XIAP*-null mice, suggesting an alternative mechanism for the transient nature of the phenotype observed. Incorporation of BrdU was used to assess the number of proliferating cells at 16 and 18 days of pregnancy in $XIAP^{-/-}$ and wild-type mice. However, no marked alteration in the number of proliferating cells was evident between wild-type and mutant glands (data not shown). Nor was there any significant change in the number of apoptotic cells as detected by TUNEL staining (data not shown). These data indicate that proliferation and apoptosis are not substantially altered in cells in $XIAP^{-/-}$ mammary glands, suggesting that mammary differentiation may be aberrant in these mice.

To determine whether the condensed acini observed in mammary glands of *XIAP*-deficient mice reflected a decrease

in milk protein synthesis, Western analysis was performed on whole-cell mammary extracts derived from at least three *XIAP*-deficient and control mice at different developmental stages. A representative Western blot is shown in Figure 1e. Immunoblotting with anti-mouse milk antisera revealed a decrease in milk production at days 16 and 18 of pregnancy relative to that observed in wild-type mammary glands. Diminished production of α and β casein and whey acidic protein (WAP) was evident in these mammary glands, while little difference in milk protein levels was evident by day 1.5 of lactation. The expression of milk proteins is regulated by Stat5 transcription factors, which are activated upon phosphorylation on a specific tyrosine. Consistent with diminished milk production, a decrease in the level of phospho-Stat5 was observed in mutant mammary glands at day 16 of pregnancy whereas the level of total Stat5 protein remained unchanged.



Interestingly, the level of phosphorylated Stat5 was consistently higher at day 1.5 of lactation on comparison of knockout and wild-type animals. We next analysed the activation status of extracellular signal-regulated kinase (ERK) in the mammary glands of mutant mice. Using an antibody specific for the dually phosphorylated active protein, Erk activation was found to be elevated in *XIAP* mutant mammary glands at day 16 of pregnancy, whereas the level of total ERK protein remained unaltered in mutant and wild-type mice. These findings are compatible with the delay in terminal differentiation that is apparent in the mammary glands of mutant mice.

NF-κB has been shown to exhibit stage-specific changes in activity during mammopoiesis, with prominent DNA-binding activity during pregnancy, virtually undetectable activity during lactation but restored DNA-binding accompanying involution.[6] We therefore examined whether NF-κB DNA-binding activity was altered in *XIAP* mutants using electromobility shift assays (EMSA). In agreement with previous reports, NF-κB activity in mammary glands from wild-type animals was downregulated by day 18 of pregnancy. In contrast to wild-type mice, the peak of NF-κB activity in mammary glands from *XIAP*-deficient mice was shifted to day 18 of pregnancy (Figure 1f). The apparent altered kinetics of NF-κB DNA-binding activity are likely to reflect the delay in differentiation that occurs in *XIAP* mutants.

Our data imply a role for XIAP in the developing mammary gland that is distinct from its antiapoptotic role. XIAP appears to be required for the normal rate of alveolar differentiation in late pregnancy, but is not required for involution during which the mammary gland regresses by extensive apoptosis. The levels of milk protein and phospho-Stat5a, both markers of differentiation in the mammary gland, were diminished in *XIAP* mutants during pregnancy. Concomitantly, higher levels of activated Erk were evident in mutant mammary glands. We did not find any evidence of XIAP directly regulating cell proliferation in the mammary gland, despite the observation that XIAP can inhibit cell proliferation of human endothelial cells *in vitro*.[7]

IAPs appear to be multifunctional proteins that, in addition to regulating apoptosis, are also involved in processes including receptor-mediated signalling, the cell cycle and ubiquitination. XIAP-deficient fibroblasts and liver tissue were demonstrated to contain reduced copper levels, suggesting a role for XIAP in the regulation of copper homeostasis through downregulation of the MURR1 protein.[8] XIAP has also been shown to have a role in BMP signalling in Xenopus, in which it links the BMP receptor (type 1) to the downstream signalling molecule TGF-β-activated kinase 1-binding protein 1 (TAB1).[9] It has also been reported to function as a cofactor for TGF-β signalling via the TGF-β type 1 receptor and Smad4.[10] Other members of the IAP family such as c-IAP1 and c-IAP2 associate with TRAFs in the TNF signalling pathway, and may play a role in activation of the stress-induced kinase JNK. XIAP can also activate JNK but appears to do so in a manner independent of interacting with components of the TNFR signalling pathway.[11] Finally, XIAP has been shown to activate the transcription factor NF-κB in endothelial cells.[12] Here we demonstrate that NF-κB activation still occurs in *XIAP* mutant mammary glands but peaks by day 18 of pregnancy when activation is normally downregulated. This finding is likely to reflect the delay in functional differentiation that occurs in mutant mice and indicates that XIAP may regulate NF-κB differently in different types of cells. Huh *et al*.[13] have recently described a role for DIAP1-inhibitable caspases in differentiation of reproductive organs in Drosophila. It is possible that XIAP plays a similar role in the mammary gland.

Targeted disruption of several genes implicated in mammopoiesis has revealed that many of these affect lobulo-alveolar development during pregnancy.[14] These include the prolactin receptor, Stat5a, C/EBP β, Id-2, the EGF family member HRG1 and the progesterone receptor. While many

Figure 1 (**a**) Generation of *XIAP*-null mice. The genomic DNA locus surrounding the first six coding exons (black boxes) of the *XIAP* gene and the targeting construct containing the mutated *XIAP* gene locus are shown. The resultant targeted *XIAP* locus is shown below. The 3′ hybridization probe used to identify mutant ES clones is depicted. Restriction sites for *BamH*I (B), *Hind*III (H), *Not*I (N), *Sac*I (S) and *Sal*I (SI) are indicated. Arrows (> <) indicate PCR primers used for typing mice. A 16 kb genomic clone was isolated from a 129^{sv} mouse genomic library using *XIAP* cDNA as a probe and then used to construct the targeting vector as follows: a 1.8 kb *Sal*I/*BamH*I fragment was cloned into the *Sal*I/*Hind*III sites of pPGKneoTK. A 4.0 kb *Sac*I fragment was subsequently cloned into the *Not*I site of this construct, thus replacing the 4.9 kb region with a neomycin resistance expression cassette. The targeting vector was linearized with *Sal*I and electroporated into W9.5 ES cells. (**b**) G418-resistant colonies were screened by Southern blotting of *Hind*III digested genomic DNA probed with a 2.1 kb *Sac*I/*Hind*III fragment that lies immediately 3′ of the targeting vector. Correctly targeted ES cell clones were injected into C57BL/6 blastocysts. Genotyping of mice backcrossed onto C57BL/6 was performed by PCR, using the forward primer (5′ CTT GGG AAC AGC ATG CGA AG 3′) and Neo (5′ TCC TCG TGC TTT ACG GTA TC 3′), and reverse primer (5′ GAT TCC TCA AGT GAA TGG GT 3′). Mice were backcrossed onto a C57BL/6 background for 10 generations. We analysed both strain 239 and 240. (**c**) Protein lysates from mammary glands of wild-type (WT) and *XIAP* knock-out (KO) mice were subjected to SDS-PAGE and immunoblotted using anti-XIAP monoclonal antibody (MBL, Nagoya, Japan).[15] Proteins were visualized with peroxidase-coupled secondary antibody using the ECL detection system. Equal loading was confirmed by reprobing with an α-tubulin-specific antibody (Sigma). V, virgin; DP, day pregnancy; DL, day lactation; DI, day involution. (**d**) Representative histological sections of WT and *XIAP* KO mammary glands at 16 DP, 18 DP and 0.5 DL are shown. *XIAP* heterozygous mice were back-crossed with C57BL/6 mice for 10 generations in order to generate $XIAP^{-/-}$ mice on a C57BL/6 background. The controls were C57BL/6 mice age-matched with the $XIAP^{-/-}$ mice. Representative wholemounts of the inguinal mammary glands of wild-type and *XIAP*-null mice at 18 days of pregnancy are shown. For histological examination, tissues were fixed in 10% (v/v) formalin in phosphate-buffered saline (PBS), embedded in paraffin and sections (2 μm) prepared and stained with haematoxylin and eosin. For wholemount examination, tissues were fixed in Carnoy's solution and stained with haematoxylin. (**e**) Protein extracts prepared from mammary glands of WT and *XIAP* KO mice at the indicated developmental stages were analysed by Western blotting using anti-XIAP, rabbit anti-milk polyclonal antiserum (Accurate Chemical & Scientific Corporation), anti-phospho-Stat5a/b (UBI) and anti-phospho-Erk1/2 (T202/Y204) antibodies (NEB). Equal loading was confirmed by reprobing with anti-α-tubulin, anti-Stat5a/b rabbit polyclonal (Santa Cruz) and anti-Erk1/2 antibodies (NEB). At least three independent animals of each genotype were analysed at each stage and a representative blot is shown. The milk proteins α and β casein (46 and 30 kDa) and whey acidic protein (14 kDa) are indicated by arrows. (**f**) EMSA showing NF-κB-specific complexes in mammary gland extracts. The NF-κB binding site within the H-$2K^b$ promoter (5′-CAG GGC TGG GGA TTC CCA TCT CCC ACA GTT TCA CTT C-3′) was 5′ end-labelled with ^{32}P-ATP to provide probe. Whole-cell extract (15 μg) was incubated in binding buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 0.1%NP40) containing 2 μg dI.dC (Amersham-Pharmacia) and 10 μg BSA for 10 min on ice, prior to the addition of probe, and incubated for a further 20 min at RT. Samples were fractionated on a 4% nondenaturing polyacrylamide gel. Competition studies using unlabelled wild-type and mutant probes verified that the bands were specific NF-κB complexes (data not shown)

of these severely impaired lactogenesis, in some cases there was sufficient development to allow lactation to proceed. For example, HRG1-null and the PrlR heterozygous females are still capable of lactation, although the latter is dependent on parity and strain background. We show here that alveolar differentiation is defective in *XIAP* mutants but that the mice are still capable of lactation. By day 7 of lactation, the mammary glands appear essentially normal, indicating that XIAP is required for a specific window during late pregnancy or that other factors compensate for its loss during lactation.

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