

# Loss of protein kinase C delta alters mammary gland development and apoptosis

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As apoptotic pathways are commonly deregulated in breast cancer, exploring how mammary gland cell death is regulated is critical for understanding human disease. We show that primary mammary epithelial cells from protein kinase C delta (PKC $\delta$ )  $-/-$  mice have a suppressed response to apoptotic agents *in vitro*. In the mammary gland *in vivo*, apoptosis is critical for ductal morphogenesis during puberty and involution following lactation. We have explored mammary gland development in the PKC $\delta$   $-/-$  mouse during these two critical windows. Branching morphogenesis was altered in 4- to 6-week-old PKC $\delta$   $-/-$  mice as indicated by reduced ductal branching; however, apoptosis and proliferation in the terminal end buds was unaltered. Conversely, activation of caspase-3 during involution was delayed in PKC $\delta$   $-/-$  mice, but involution proceeded normally. The thymus also undergoes apoptosis in response to physiological signals. A dramatic suppression of caspase-3 activation was observed in the thymus of PKC $\delta$   $-/-$  mice treated with irradiation, but not mice treated with dexamethasone, suggesting that there are both target- and tissue-dependent differences in the execution of apoptotic pathways *in vivo*. These findings highlight a role for PKC $\delta$  in both apoptotic and nonapoptotic processes in the mammary gland and underscore the redundancy of apoptotic pathways *in vivo*.

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Apoptosis is an active process of cell death that has a key role in the development and maintenance of tissue homeostasis.<sup>1</sup> Mammary gland development occurs primarily in the post-natal period through the process of branching morphogenesis during puberty, and massive proliferation and secretory differentiation during pregnancy.<sup>2</sup> During puberty, terminal end buds (TEBs) form at the leading edge of the growing ducts and bifurcate to produce the branched ducts characteristic of the mature virgin. TEBs are composed of a layer of highly proliferative cap cells on their distal surface, surrounding a mass of luminal body cells that undergo high levels of apoptosis resulting in luminal hollowing.<sup>3,4</sup> Multiple B-cell leukemia/lymphoma 2 (Bcl-2) family members are expressed during branching morphogenesis, including antiapoptotic B-cell leukemia/lymphoma X (Bcl-x), Bcl-2 and B-cell leukemia/lymphoma w (Bcl-w), as well as the proapoptotic Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) proteins.<sup>5</sup> Mice in which the Bcl-2 gene is overexpressed have abnormal ductal development, whereas mice in which the proapoptotic protein, Bcl-2-interacting

mediator of death (Bim), is deleted show delayed apoptosis in the TEBs, supporting a role for apoptosis during ductal morphogenesis.<sup>3,6</sup>

In addition to early luminal hollowing, apoptosis is also important for the clearance of epithelial cells during mammary gland involution. Following the cessation of lactation, large numbers of secretory mammary epithelial cells (MECs) are deleted by apoptosis, returning the gland to its prepregnancy state.<sup>7,8</sup> Quantitative morphometric analysis of epithelial cell apoptosis and apoptotic cell clearance during mammary gland involution suggests that these processes occur rapidly after forced weaning and are largely complete by 72–96 h.<sup>8</sup> Gene array studies show an early transient increase in the expression of death receptor ligands and their receptors starting 12 h after weaning, while increased expression of regulators of the intrinsic apoptotic pathway, including Apaf1, Bcl-x, Bak and Bax, and suppression of the death inhibitory proteins, Bcl-2 and Bcl-w, was observed at 24–96 h of involution.<sup>5,9,10</sup> In mice, loss of Bax or overexpression of Bcl-2 results in suppression of alveolar cell apoptosis,<sup>11</sup>

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**Abbreviations:** PKC $\delta$ , protein kinase C delta; Bcl-2, multiple B-cell leukemia/lymphoma 2; TEBs, terminal end buds; Bcl-x, B-cell leukemia/lymphoma X; Bcl-w, Bcl-2 and B-cell leukemia/lymphoma w; Bax, proapoptotic Bcl-2-associated X protein;  $\delta$ KO, PKC $\delta$  gene has been disrupted

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whereas deletion of Bcl-x accelerates apoptosis during involution.<sup>12</sup>

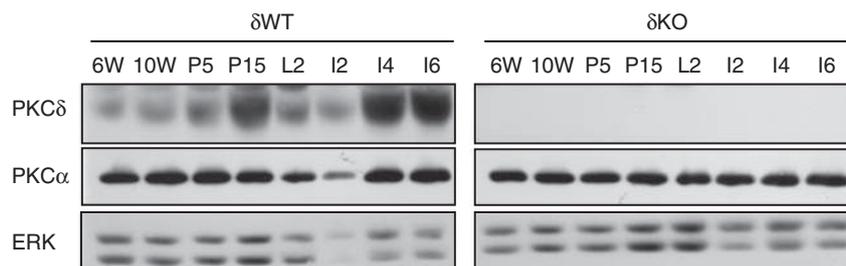
Protein kinase C $\delta$  (PKC $\delta$ ) is an ubiquitously expressed isoform of the PKC family of serine/threonine kinases.<sup>13</sup> Studies have identified diverse roles for this signaling molecule in control of immunity,<sup>14,15</sup> apoptosis,<sup>16</sup> and cell migration.<sup>17</sup> Furthermore, its reduced expression in some human tumors suggests that it may function as a tumor suppressor.<sup>18,19</sup> Our laboratory and others have shown an essential role for PKC $\delta$  in epithelial cell apoptosis induced by genotoxins, other cell toxins, and death receptors.<sup>16,20–22</sup> The central role PKC $\delta$  has in epithelial cell apoptosis suggests that PKC $\delta$  may contribute to the regulation of apoptosis in the mammary gland *in vivo*. In this study, we have explored this hypothesis using mice in which the PKC $\delta$  gene has been disrupted ( $\delta$ KO). We show that PKC $\delta$  regulates branching morphogenesis through nonapoptotic mechanisms in early mammary gland development. During mammary gland involution, however, the absence of PKC $\delta$  results in delayed apoptosis. Our studies also show that apoptosis in the thymus displays a similar differential sensitivity to apoptotic signals, suggesting a redundancy of apoptotic pathways *in vivo*.

## Results

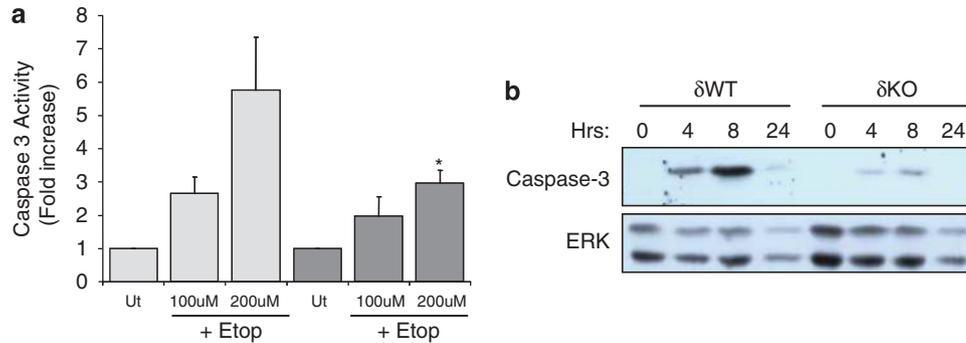
**PKC $\delta$  expression in the mouse mammary gland.** Development and remodeling of the mammary gland during puberty and involution require apoptosis, and thus this tissue is a useful model for exploring regulation of cell death *in vivo*. As shown in Figure 1, PKC $\delta$  protein is expressed at all stages of the mammary gland developmental cycle, with the highest levels seen during mid-pregnancy and during involution. As expected, no PKC $\delta$  expression was detected in tissues from  $\delta$ KO mice. In contrast to PKC $\delta$ , the expression of PKC $\alpha$  does not vary during the mammary developmental cycle (Figure 1). The mammary gland is composed of epithelial, adipose, and connective tissue. Expression of PKC $\delta$  in MECs was verified by immunoblot of primary MECs isolated from postpubertal  $\delta$ WT and  $\delta$ KO mice (data not shown). Differential regulation of PKC $\delta$  expression in the mammary gland suggests that it may contribute to the dynamic changes seen in this gland during pregnancy and involution.

**Suppression of apoptosis in primary MECs from  $\delta$ KO mice.** We have previously shown that the loss of PKC $\delta$  protects salivary gland cells *in vivo* from irradiation-induced cell death; however, the contribution of PKC $\delta$  to developmental or physiological programs of cell death is not known.<sup>22</sup> To assess whether PKC $\delta$  is required for apoptosis in MECs, we investigated the effects of etoposide and integrin detachment on MECs isolated from  $\delta$ KO or  $\delta$ WT mice. Primary MECs were cultured *in vitro* and treated with etoposide. Activation of caspase-3 in MECs from  $\delta$ KO mice was suppressed by about 50% compared with MECs isolated from  $\delta$ WT mice, similar to what we have previously observed in primary salivary epithelial cells<sup>22</sup> (Figure 2a). Loss of integrin engagement (anoikis) induces apoptosis in epithelial cells and may be an important apoptotic mechanism during mammary gland involution. To determine if anoikis is a PKC $\delta$ -dependent process, MECs were detached from the culture dish and plated on poly-hema-coated plates to prevent reattachment. Caspase-3 activation occurs within 4 h after plating; however, activation of caspase-3 was greatly diminished in  $\delta$ KO MECs as compared with  $\delta$ WT MECs (Figure 2b). Together, this suggests that PKC $\delta$  regulates multiple apoptotic pathways relevant to mammary gland development and maintenance.

**Development of the mammary gland during puberty in  $\delta$ KO mice.** Before the onset of puberty the mouse mammary gland consists of quiescent rudimentary ducts. Under the influence of pubertal hormones TEBs form at the leading edge of the growing ducts and undergo several bifurcation events to produce the ducts characteristic of the mature virgin. Body cells within the TEB undergo apoptosis resulting in a hollow duct for milk flow. To determine if PKC $\delta$  contributes to branching morphogenesis, we examined glands from  $\delta$ KO and  $\delta$ WT mice at 4, 5, 6 and 10 weeks of age (Figure 3). As seen in Figure 3A, the morphology of mammary glands from  $\delta$ KO and  $\delta$ WT mice at these developmental time points appears to be similar. Immunohistochemical analysis of proliferation and apoptosis in TEBs from 5-week-old  $\delta$ WT and  $\delta$ KO mice also showed comparable Ki-67 (proliferation) and active caspase-3 (apoptosis) staining (Figure 3B). However, examination of the ductal structure in mammary gland whole mounts revealed a ductal architecture that is more



**Figure 1** Expression of PKC $\delta$  in the mammary gland. Mammary glands were harvested from  $\delta$ WT or  $\delta$ KO mice at the time points indicated and PKC $\delta$  or PKC $\alpha$  expression was determined by immunoblot analysis as described in Materials and Methods section. Blots were stripped and reprobed for total ERK expression (p44 and P42) as a loading control. Samples are as follows: 6 and 10 week virgin mice (6W, 10W); pregnancy day 5 and 15 (P5, P15); lactation day 2 (L2), and involution days 2, 4 and 6 (I2, I4 and I6). Representative data from three or more mice per time point are shown



**Figure 2** PKC $\delta$  regulates apoptosis in MECs *in vitro*. MECs were prepared from  $\delta$ WT and  $\delta$ KO mice as described in Materials and Methods section. (a) Primary MECs were treated with etoposide for 16 h, harvested and caspase-3 activity assayed as described in Materials and Methods section;  $\delta$ WT, light gray bars;  $\delta$ KO, dark gray bars; Ut=untreated. Results are the average of triplicate measurements  $\pm$  S.E.M. A representative experiment is shown ( $n = 3$ ); \* $P < 0.02$  by Student's two-tailed *t*-test. (b) Primary  $\delta$ WT or  $\delta$ KO MECs were plated on polyhema-coated dishes to prevent attachment and harvested at the indicated times. Top, immunoblot for active caspase-3; bottom, blots were stripped and immunoblotted for ERK as control for protein loading and transfer. Representative immunoblots are shown ( $n = 3$ )

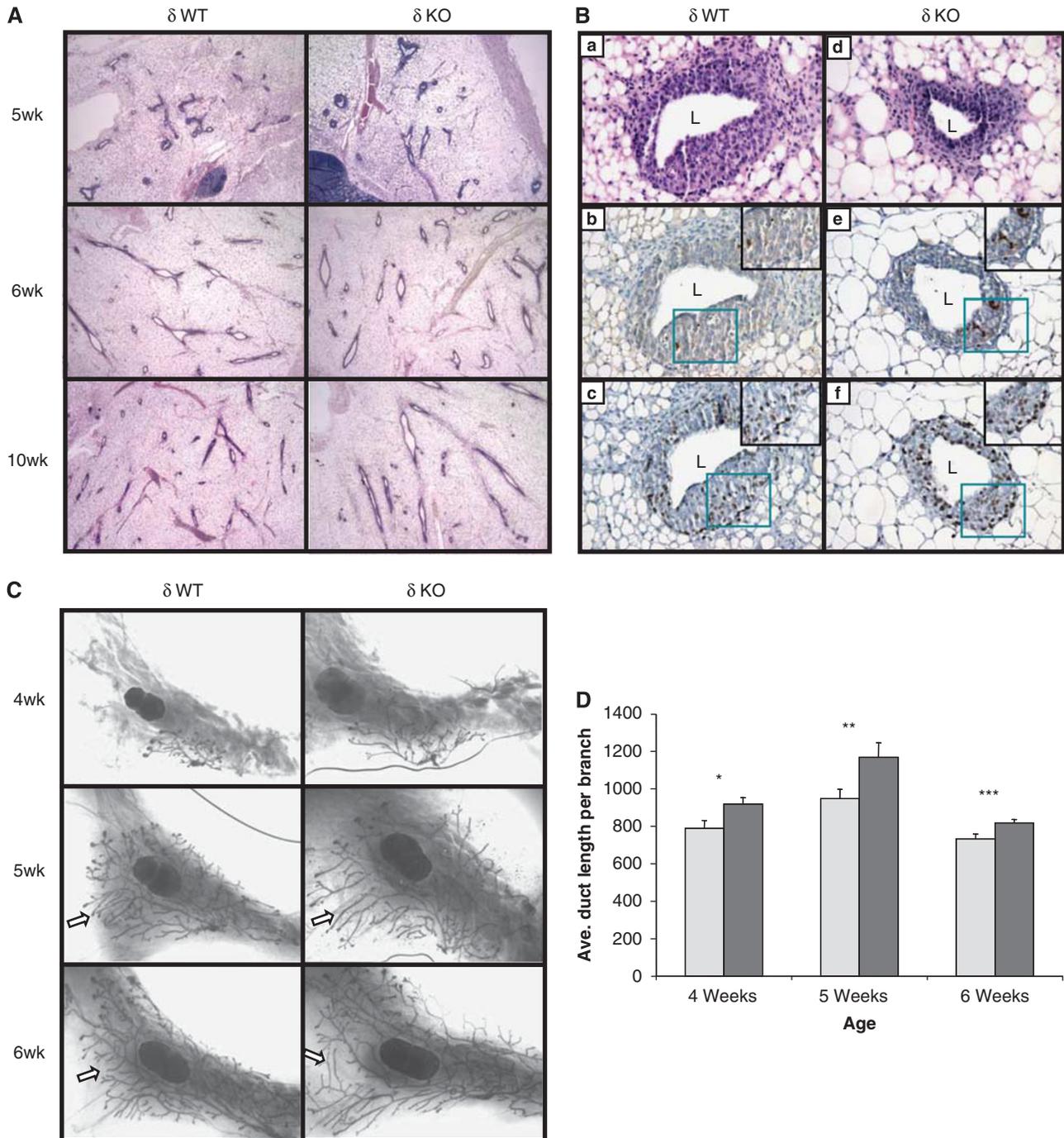
simplistic and open in  $\delta$ KO mammary glands relative to  $\delta$ WT mammary glands (Figure 3C). Notably, ductal branching appeared to occur less frequently in  $\delta$ KO mammary glands compared with  $\delta$ WT glands, as relatively long ducts without side branches were commonly observed in the  $\delta$ KO mammary glands (Figure 3C; arrows). When these effects were quantified we found a small, but significant increase in the distance between primary branch points at 4, 5 and 6 weeks (Figure 3D). As overall duct length did not differ between the  $\delta$ KO and WT mice (data not shown), we conclude that branching occurs less frequently in the mammary glands of  $\delta$ KO mice, indicating that PKC $\delta$  contributes to branching morphogenesis *in vivo*. However, other pathways eventually compensate for the loss of PKC $\delta$ , as the defect in branching in the  $\delta$ KO mice was transient, with no observable differences at 10 weeks (data not shown). Importantly, as apoptosis is not reduced, the decreased branching observed in  $\delta$ KO mice during pubertal development indicates an apoptosis-independent function for PKC $\delta$  in mammary gland development.

**Mammary gland involution in  $\delta$ KO mice.** To determine if PKC $\delta$  contributes to apoptosis during mammary gland involution, we used a forced-weaning model in which pups were removed from dams 9 days after parturition. In this model, lactation is fully established before pup removal and involution occurs over approximately 2–3 weeks, after which the gland resembles its prepregnant state. Early involution (days 1–4) is associated with suppression of milk protein genes and loss of up to 80% of the secretory epithelial cells.<sup>8</sup> Latter stages are characterized by extensive tissue remodeling. Mammary gland tissue was harvested from  $\delta$ WT and  $\delta$ KO mice on days 1 through 8 after weaning (I1 to I8) and apoptotic cells were identified by staining with an antibody that detects active caspase-3. At day 1 of involution (Figure 4a; I1), there was some shedding of apoptotic epithelial cells into the lumen, although this is more evident by involution day 2 (Figure 4a; I2). In mammary glands from  $\delta$ WT mice, the proportion of cells showing caspase-3 activation peaks at involution day 4 and declines thereafter. Meanwhile, mammary glands from  $\delta$ KO mice have peak levels of apoptosis at involution day 6, a 2-day delay

compared with  $\delta$ WT. Quantification of active caspase-3-positive cells (Figure 4b) shows that apoptosis is significantly reduced in mammary glands from  $\delta$ KO mice compared with  $\delta$ WT mammary glands at day 2 (36% decrease), day 4 (21% decrease) and day 8 (38% decrease) of involution. In contrast, at involution day 6, activation of caspase-3 in  $\delta$ KO mice is significantly increased relative to  $\delta$ WT mammary glands. These studies suggest that the kinetics of activation of caspase-3 during involution is delayed in the  $\delta$ KO mice relative to  $\delta$ WT mice.

To determine if delayed activation of caspase-3 correlates with a delay or suppression of mammary gland involution, we examined mammary gland histology in  $\delta$ WT and  $\delta$ KO mice up to 21 days after weaning. Lactating glands from both genotypes show normal alveolar development and secretory activation (Figure 5, L9). Following pup withdrawal, milk accumulates within the glands (Figure 5, I1 and I2). Although the histology of mammary glands from  $\delta$ WT and  $\delta$ KO mice appears similar at these stages, lipid droplet accumulation in the  $\delta$ KO glands at I1 was consistently greater compared with glands from  $\delta$ WT mice (see inset). By involution day 3, most of the alveolar structures had collapsed (Figure 5, I3). Mid-to-late involution is characterized by remodeling of the gland, which is complete by about 21 days. A comparison of mammary gland histology at involution days 4, 6, 8, 14 and 21 in  $\delta$ WT and  $\delta$ KO mice shows a loss of secretory alveoli, a reduction in ductal structures, and an increased ratio of fat to epithelial cells. Histologically, mammary glands from  $\delta$ WT and  $\delta$ KO mice appear to be very similar, and glands from both genotypes are fully regressed by day 21 (Figure 5, I21). However, it was noted that a subset of mammary glands from  $\delta$ KO mice at involution days 4, 6 and 8 tended to have slightly more epithelial cells and to be more pleiomorphic with regard to adipocyte size and shape compared with glands from  $\delta$ WT mice, suggesting that PKC $\delta$  may have a role in the composition or organization of the mammary gland stroma.

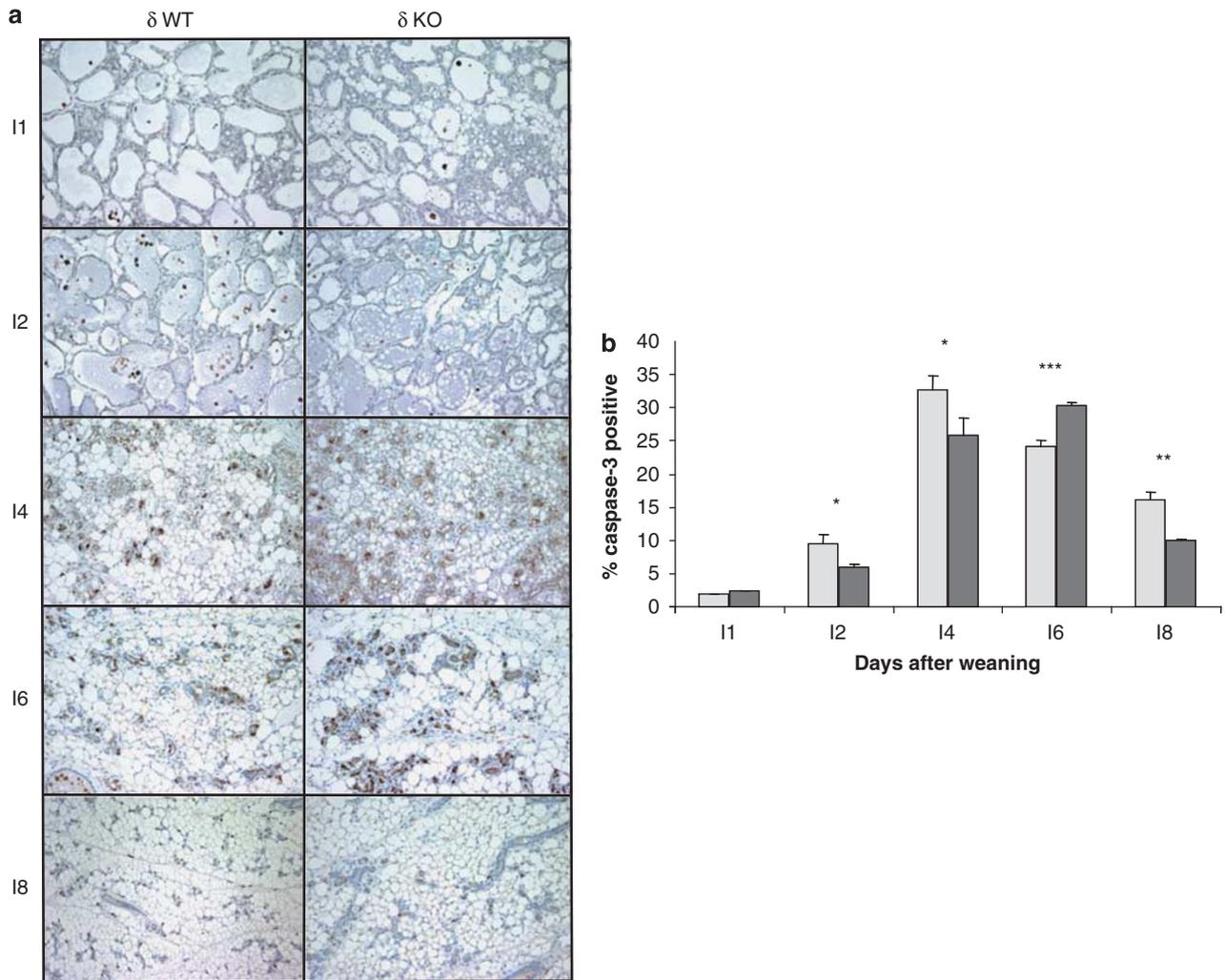
Signal transducer and activator of transcription 3 (STAT3) is a critical regulator of mammary gland involution, and mice with a conditional deletion of STAT3 in the mammary gland show impairment of epithelial cell apoptosis and delayed involution.<sup>23–25</sup> STAT3 activation, as determined by



**Figure 3** The loss of PKC $\delta$  leads to defective mammary gland branching morphogenesis. Immunohistochemistry, whole mounts and analysis of branching frequency were performed as described in Materials and Methods section. **(A)** Representative pictures ( $\times 40$ ) of mammary glands from 5, 6 and 10 weeks virgin  $\delta$ WT and  $\delta$ KO mice stained with H&E. **(B)** Sections ( $\times 400$ ) through one  $\delta$ WT (a–c) and one  $\delta$ KO (d–f) TEB stained with H&E (a, d), or with antibodies to cleaved caspase-3 (b, e) or Ki67 (c, f). Inset, digital magnification of area boxed in blue to show cleaved caspase-3-positive cells (b, e) or Ki67-positive cells (c, f). **(C)** Representative whole mounts of 4th mammary glands from 4-, 5- and 6-week-old  $\delta$ WT and  $\delta$ KO mice stained with carmine alum. Arrows identify areas of decreased branching in  $\delta$ KO mammary glands as compared with  $\delta$ WT. **(D)** The distance between branch points was quantified from 4-week ( $n = 7$ ), 5-week ( $n = 6$ ) and 6-week ( $n = 5$ ) old  $\delta$ WT (light gray bars) and  $\delta$ KO (dark gray bars) mice. The difference between branching frequency in  $\delta$ WT and  $\delta$ KO is significant over 4 weeks ( $*P < 0.02$ ), 5 weeks ( $**P < 0.03$ ), and 6 weeks ( $***P < 0.03$ ) by Student's two-tail *t*-test

phosphorylation on Y705, was analyzed to assess if the delay in caspase-3 activation during involution in  $\delta$ KO glands is due to aberrant STAT3 signaling. Similar levels of activated STAT3 are seen in mammary glands from  $\delta$ WT and  $\delta$ KO

mice at L2 and I2, and slightly reduced in mammary gland tissue from  $\delta$ KO mice at I4 (Figure 6a). To further address whether STAT3 activation is altered in the mammary glands of  $\delta$ KO mice, we analyzed STAT3 Y705 phosphorylation in

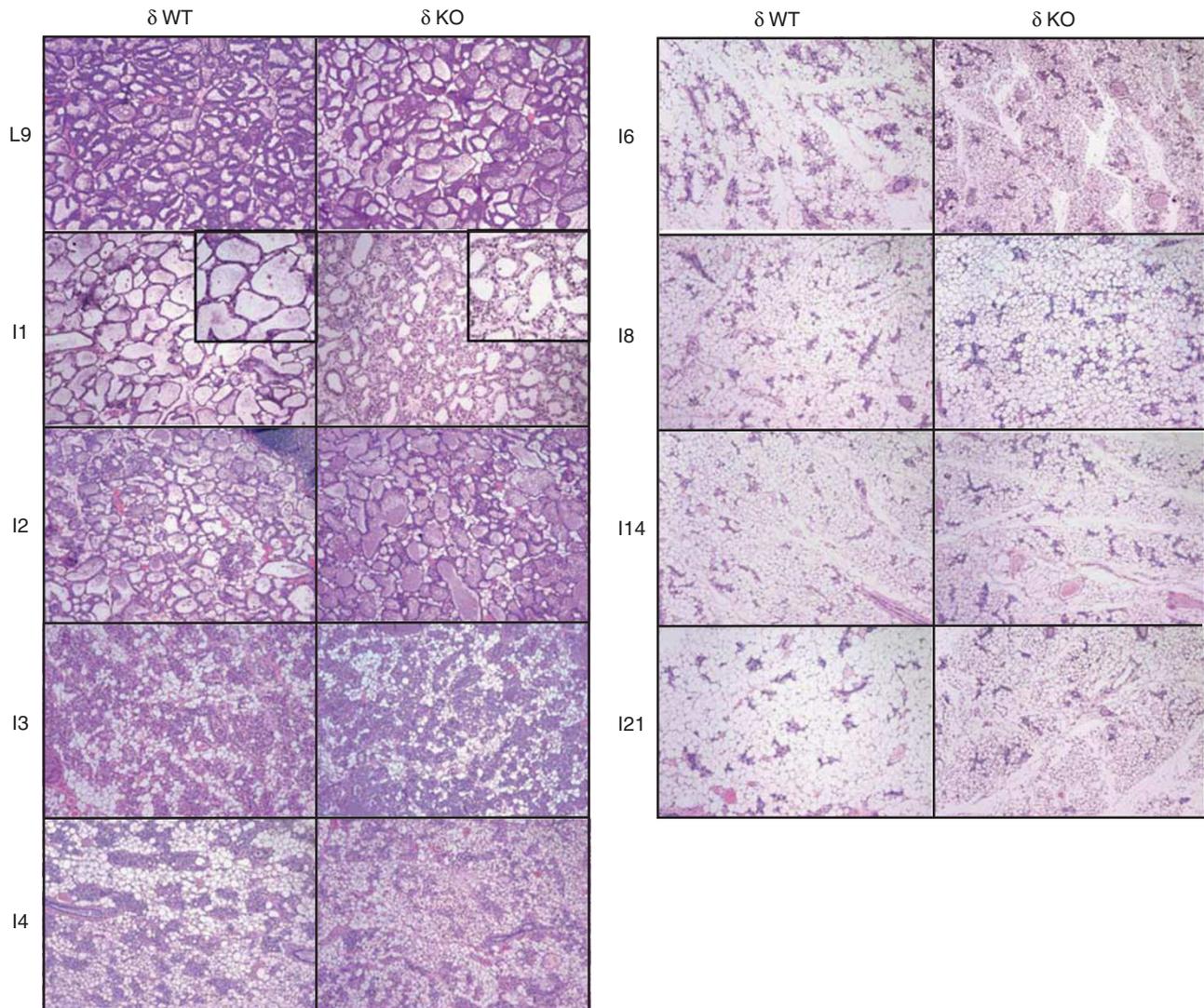


**Figure 4** Loss of PKC $\delta$  delays activation of caspase-3 during early involution. (a) Mammary gland tissue was harvested from  $\delta$ WT and  $\delta$ KO mice at involution days 1–8 (I1 to I8), sectioned, and stained with an antibody to active-caspase-3 to identify apoptotic cells. Representative pictures ( $\times 200$  magnification) of each time point are shown ( $n = 6$  or more mice per time point). (b) Caspase-3-positive cells were quantified as a percent of total epithelial cells per section. A minimum of 1000 cells from five fields were counted per mouse; six or more mice were analyzed per time point.  $\delta$ WT, light gray bars;  $\delta$ KO, dark gray bars; \*differs from  $\delta$ WT  $P < 0.05$ , \*\*\* $P < 0.001$ , \*\* $P < 0.03$  by Student's two-tailed  $t$ -test

primary MECs treated with leukocyte inhibitory factor (LIF) and interleukin-6 (IL-6), cytokines critical for activation of STAT3 in the mammary gland during involution.<sup>26</sup> STAT3 activation was nearly identical in  $\delta$ WT and  $\delta$ KO MECs treated with IL-6 or LIF (Figure 6b). Taken together, our studies indicate that loss of PKC $\delta$  results in delayed activation of caspase-3 during involution, and that this is not due to a defect in STAT3 activation.

**Thymic apoptosis in  $\delta$ KO mice.** Although loss of PKC $\delta$  results in a delay in caspase-3 activation in the mammary gland, involution proceeds normally in these mice. In contrast, irradiation-induced caspase-3 activation is reduced by  $>60\%$  in the parotid glands of  $\delta$ KO mice.<sup>22</sup> This suggests that *in vivo* apoptotic pathways induced by agents that damage DNA are highly selective for PKC $\delta$ , whereas other inducers of apoptosis such as those that regulate development may be less selective. To further

explore the selectivity of apoptotic pathways for PKC $\delta$ , we compared caspase-3 activation in the thymus in response to irradiation and dexamethasone treatment. Glucocorticoids have been shown to induce apoptosis and cause acute thymic involution in rodents, and this pathway of involution may be relevant physiologically in response to stress and inflammation.<sup>27,28</sup>  $\delta$ KO or  $\delta$ WT mice were injected with dexamethasone, the thymus was harvested after 2 h, and active caspase-3 was assayed by immunohistochemistry. As seen in Figure 7a, dexamethasone is a potent inducer of apoptosis in the thymus of both  $\delta$ KO or  $\delta$ WT mice; however, no significant difference in caspase-3 activation was found. In contrast, in response to irradiation of the thymus, caspase-3 activation is potently induced in  $\delta$ WT, but not  $\delta$ KO mice (Figure 7b). This finding suggests that irradiation-induced apoptosis is highly dependent on PKC $\delta$ , whereas collateral apoptotic pathways may be recruited to regulate cell death in response to other signals, including developmental signals.



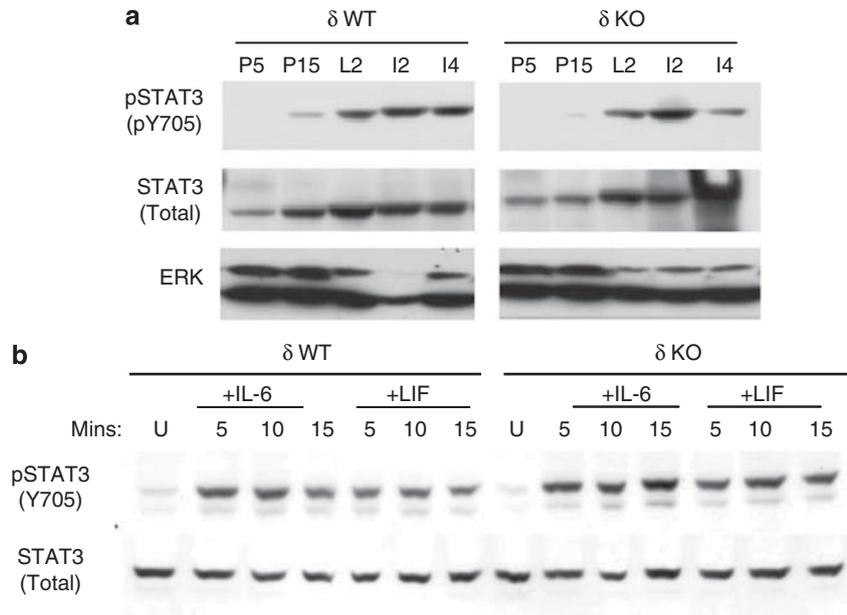
**Figure 5** Involution in mammary glands from  $\delta$ KO mice. Mammary glands from  $\delta$ WT and  $\delta$ KO mice at involution days 1–21 (I1 to I21) were stained with H&E as described in Materials and Methods section. Representative pictures ( $\times 100$  magnification) are shown from six or more mice analyzed per time point. Inset,  $\times 200$  magnification to show lipid accumulation

## Discussion

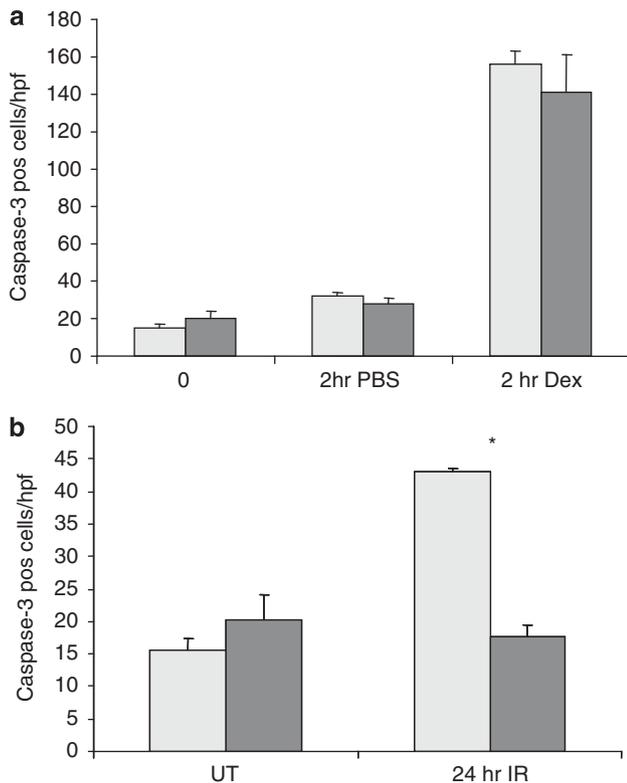
PKC $\delta$  is required for apoptosis in response to cell injury;<sup>21</sup> however, its contribution to apoptosis during development and tissue remodeling has not been addressed. In this study, we have explored a role for PKC $\delta$  in the apoptosis of primary MECs during branching morphogenesis and involution. *In vitro*, apoptosis in primary MECs derived from  $\delta$ KO mice is reduced, and *in vivo*, activation of caspase-3 during mammary gland involution is delayed in  $\delta$ KO mice. Loss of PKC $\delta$  also results in decreased branching during pubertal development of the mammary gland; however, this is likely due to a nonapoptotic function of PKC $\delta$ . These phenotypic changes are transient presumably reflecting the redundancy of apoptotic pathways *in vivo* and the value of proper mammary gland development to species survival.

Branching morphogenesis requires a delicate balance between apoptotic and proliferative signals to effectively

establish ductal outgrowth and luminal hollowing. During puberty, mammary glands from  $\delta$ KO mice are similar in organization to  $\delta$ WT glands, with hollow lumens and correct TEB and ductal architecture. Proliferation and caspase-3 activation also appear to be similar in the TEBs of  $\delta$ KO and  $\delta$ WT mammary glands. This suggests that PKC $\delta$  either does not have a significant role in the maintenance of cell growth/death within the TEB or that loss of PKC $\delta$  is compensated for by other cell death mechanisms. Brugge and co-workers<sup>6</sup> have shown that loss of the proapoptotic factor, Bim, suppresses apoptosis within the TEB resulting in transient luminal filling; however, the ducts eventually hollow out by a caspase-independent mechanism and resemble wild-type mammary glands. Similarly, overexpression of the antiapoptotic proteins Bcl<sub>2</sub> or Bcl-X<sub>L</sub> suppresses apoptosis in three-dimensional cultures of MCF10A cells, but lumen formation still occurs.<sup>29</sup> As mammary gland development and function is critical to species survival, there is likely to be evolutionary pressure to provide



**Figure 6** STAT3 activation in mammary glands from  $\delta$ KO mice. (a) Protein lysates were prepared from  $\delta$ WT and  $\delta$ KO mammary glands and expression of phosphorylated STAT3 (pY705), total STAT3 and ERK were analyzed by immunoblot as described in Materials and Methods section. (b) Primary MECs from  $\delta$ WT and  $\delta$ KO mice were starved for 24 h in serum-free DMEM/F12 followed by stimulation with either IL-6 or LIF (50 ng/ml) for the indicated time; U = untreated. Cells were harvested and expression of pY705 STAT3 and total STAT3 was analyzed by immunoblot. Representative blots are shown; each experiment was repeated three or more times



**Figure 7** PKC $\delta$  and apoptosis in the thymus. Thymic involution was induced by dexamethasone (a) or irradiation (b) as described in Materials and Methods section; UT = untreated. Tissues were removed and apoptosis detected by immunohistochemistry for active caspase-3. Shown is the average of active caspase-3-positive cells per  $\times 200$  field. Six fields were counted per mouse;  $n = 5$  mice per each genotype for dexamethasone and four mice for irradiation. Significantly different from  $\delta$ WT, \* $P = 0.0001$ ;  $\delta$ WT, light gray bars;  $\delta$ KO, dark gray bars

alternative pathways to assure proper mammary gland function. In the context of ductal morphogenesis, these may include alternative regulators of apoptosis, as well as non-apoptotic cell death pathways such as autophagy.<sup>6</sup>

Whole mount stains of mammary glands from  $\delta$ KO mice during puberty show reduced branching during early ductal morphogenesis; however, by later stages these glands were indistinguishable from their WT counterparts. Altered branching morphogenesis has been observed in several other mouse models within the epithelial and stromal compartments. Although estrogen receptor,<sup>30</sup> progesterone receptor,<sup>31</sup> matrix metalloproteinase-2<sup>32</sup> and Src-1<sup>33</sup> have been shown to be essential for both branching and ductal outgrowth, other factors, such as eotaxin,<sup>34</sup> primarily regulate branching. Notably, within the diverse variety of mouse models that exhibit a branching phenotype, there are very few cases in which branching morphogenesis is halted altogether, highlighting the complexity of this process. While changes in proliferation and apoptosis can affect TEB shape and its ability to drive ducts through the fat pad, ductal elongation was not affected in  $\delta$ KO mice. This confirms that alterations in branching were not due to deficiencies in proliferation or cell death within the TEB. More importantly, these findings implicate PKC $\delta$  in a nonapoptotic role during ductal morphogenesis.

Our previous studies show that in salivary epithelial cells PKC $\delta$  regulates apoptosis upstream of cytochrome *c* release and caspase activation in response to genotoxins and other cell damaging agents.<sup>21,22</sup> In this study, we show that apoptosis induced by etoposide is similarly suppressed in primary MECs from  $\delta$ KO mice. Interestingly, activation of caspase-3 in anoikis, a process possibly relevant to physiological modes of apoptosis, is also suppressed in  $\delta$ KO MECs.

Our current studies show that PKC $\delta$  contributes to the activation of caspase-3 during mammary gland involution *in vivo*; however, suppression of caspase-3 activation is much greater in cultured primary MECs from  $\delta$ KO mice and in irradiated  $\delta$ KO salivary glands *in vivo*.<sup>22</sup> This may be explained in part by the fact that tissue cell death peaks by 24 h postirradiation, while involution occurs over 1–4 days, possibly allowing activation of redundant cell death programs to insure successful completion. Alternatively, several studies indicate that cell death during involution occurs in a series of sequential steps, perhaps mediated by distinct cell death pathways.<sup>7,10</sup> PKC $\delta$  predominantly facilitates intrinsic- or mitochondrial-mediated cell death, whereas microarray data suggest that death receptor or extrinsic cell death pathways regulate the initial wave of apoptosis during involution. If intrinsic pathways function as a back-up mechanism of cell death during involution, this may explain the relatively minor changes in involution seen in the  $\delta$ KO mouse.

Nuclear accumulation of PKC $\delta$  is an early event in genotoxin-induced apoptosis that results in the activation of caspase-3 and amplification of the apoptotic signal.<sup>35,36</sup> Our current studies suggest that apoptosis *in vivo* is differentially dependent on PKC $\delta$ . This suggests that upstream regulators of apoptosis, such as PKC $\delta$ , may be differentially activated in a signal-specific manner. In this regard, we show that dexamethasone induces caspase-3 activation in the thymus to a similar extent in both  $\delta$ KO and  $\delta$ WT mice, while in response to irradiation, caspase-3 activation is induced in  $\delta$ WT and not  $\delta$ KO mice. This supports the role of PKC $\delta$  in DNA damaging pathways, whereas alternative apoptotic pathways may be recruited to facilitate cell death in response to other stimuli.

The loss of PKC $\delta$  results in small changes across both mammary gland pubertal growth and involution. This suggests that the role of PKC $\delta$  in the mammary gland is not restricted to apoptosis, as a reduction in apoptosis is not likely to explain the decreased ductal branching or decreased lactation we observe. Furthermore, subtle differences in lipid composition are evident in the mammary glands of  $\delta$ KO mice, suggesting that PKC $\delta$  may have additional roles in mammary gland homeostasis. This suggests that mammary gland development and involution are complex biological processes that are essential for the maintenance and propagation of mammalian species, thus apoptosis is likely to be regulated at multiple levels to assure its proper execution.

#### Materials and Methods

**Animals.** The PKC $\delta$   $-/-$  mouse ( $\delta$ KO) on the C57/Bl6 background has been previously described.<sup>15</sup> Animals were maintained at the University of Colorado Denver at Anschutz Medical Campus in accordance with Laboratory Animal Care guidelines and protocols. These studies were conducted with approval of the University of Colorado Denver Institutional Animal Use and Care Committee. Wild-type littermates ( $\delta$ WT) were used for all studies. For analysis of mammary gland involution, pups were removed from mothers at day 9 of lactation.

**Mammary gland preparation.** Mammary glands were harvested from  $\delta$ WT or  $\delta$ KO mice and processed for whole mount analysis or immunohistochemistry. For analysis of mammary gland whole-mounts, mammary glands (#4) were spread on microscope slides and fixed overnight in 10% formalin. Tissues were hydrated, stained with carmine alum overnight, dehydrated and cleared in xylene for 2 h. Slides were analyzed on a dissecting scope and digital pictures were acquired using

SPOT imaging software. For quantification of branching frequency, the total duct length of every primary branch, from nipple to distal TEB, was measured in microns and divided by the total number of branch points within the gland.

For histological and immunohistochemical analysis, contralateral #4 glands were fixed in 10% formalin, dehydrated, and embedded in paraffin. Five  $\mu$ m sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry for active caspase-3 and Ki67 were performed as previously described using primary antibodies purchased from Cell Signaling Technology, Beverly, MA, USA.<sup>22</sup> Digital images were acquired using SPOT imaging software.

**Immunoblot analysis.** Immunoblots were performed as previously described.<sup>20</sup> Super Signal West Pico Luminol/Enhancer Solution (Pierce, Rockford, IL, USA) was used for detection of the signal. Sources of antibodies were as follows: PKC $\delta$  and PKC $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA, USA), STAT3, pY705STAT3, active caspase-3, Ki-67 and extracellular signal-regulated kinase (Cell Signaling Technology).

**Primary cell culture.** Mammary glands removed from  $\delta$ WT or  $\delta$ KO mice (8–14 weeks) were minced and digested in Dulbecco's modified Eagle medium/F12 (DMEM/F12) containing 2 mg/ml collagenase-B, 100 U/ml Hyaluronidase, 100 U/ml penicillin-streptomycin (pen/strep), and 100 U/ml Gentamicin at 37° for 3 h. Primary MECs were isolated by a series of spins at 1500 and 800 r.p.m. and then plated on collagen 1 (Sigma-Aldrich, St. Louis, MO, USA)-coated plates ( $2.5 \times 10^5/\text{cm}^2$ ) in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1 mg/ml fetuin (Sigma-Aldrich), 2.5  $\mu$ g/ml insulin, 0.5  $\mu$ g/ml hydrocortisone, 2.5  $\mu$ g/ml epidermal growth factor (EGF), 25  $\mu$ g/ml gentamicin and 50 U/ml Pen/Strep. After 48 h, cells were transferred to growth media (DMEM/F12 supplemented with 2.55  $\mu$ g/ml insulin, 0.5  $\mu$ g/ml hydrocortisone, 2.5  $\mu$ g/ml EGF, 25  $\mu$ g/ml gentamicin, 50 U/ml pen/strep and 10% FBS). For induction of anoikis, cells were trypsinized and plated on dishes coated with Polyhema (Sigma-Aldrich). Before treatment with LIF or IL-6, MECs were plated on Matrigel (BD Biosciences, Bedford, MA, USA) for 24 h in DMEM/F12 supplemented with 10% fetal calf serum, 1 mg/ml fetuin (Sigma-Aldrich), 2.5  $\mu$ g/ml insulin, 0.5  $\mu$ g/ml hydrocortisone, 2.5  $\mu$ g/ml EGF, 25  $\mu$ g/ml gentamicin and 50 U/ml pen/strep, followed by incubation in serum-free growth media for an additional 24 h. LIF was purchased from Calbiochem (San Diego, CA, USA) and IL-6 was purchased from Chemicon (Billerica, MA, USA). DMEM/F12 and FBS were purchased from HyClone (Logan, UT, USA); all other reagents for tissue culture were from Invitrogen (Carlsbad, CA, USA) unless noted otherwise.

**Caspase-3 activity.** Caspase-3 activity was quantified using a Biomol Quantizyme Colormetric Assay Kit (BioMol, Plymouth Meeting, PA, USA) as previously described.<sup>22</sup> Briefly, caspase-3 activity in 30  $\mu$ g of cell lysate was measured by cleavage of Ac-DEVD-pNA colormetric substrate, and absorbance at  $A_{405}$  was quantified in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA) at 10 min intervals for 7 h.

**Apoptosis in the thymus.** For dexamethasone-induced involution, 6-week-old  $\delta$ WT or  $\delta$ KO male mice ( $n=5$ ) were intraperitoneally injected with 0.2 mg dexamethasone (Sigma-Aldrich) dissolved in 100  $\mu$ l endotoxin-free phosphate-buffered saline (PBS) or 100  $\mu$ l endotoxin-free PBS alone. Two hours after injection, mice were killed and the thymus glands harvested. For irradiation-induced involution, 6-week-old  $\delta$ WT or  $\delta$ KO male mice ( $n=4$ ) were subjected to 2Gy irradiation using a cobalt source. Twenty-hours after irradiation, mice were killed and the thymus glands harvested and processed for histology and immunohistochemistry as described.

**Miscellaneous.** Etoposide was purchased from Sigma-Aldrich and dissolved in dimethylsulfoxide.

#### Conflict of interest

Drs. Anderson, Neville and Reyland's work has been funded by the NIH. Dr. Nakayama, Ms. Allen-Petersen and Ms. Miller declare no potential conflict of interest.

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