CHAPTER 6:
BCL11A IS ESSENTIAL FOR MAINTENANCE OF LUMINAL SECRETORY CELL FATE

6.1 Introduction

6.1.1 Mammary gland development during pregnancy

The mammary gland undergoes extensive proliferation and attains terminal differentiation only after pregnancy. The functional differentiation state is suggested to be defined predominantly by the hormonal status of the animal (Hennighausen and Robinson, 1998). Embryonic development in the mice sets up a rudimentary network of ducts which undergoes extensive growth and arborisation during puberty, stimulated by hormones such as estrogen and progesterone. This generates the mature mammary ductal network that will ultimately serve as channels for milk transport during lactation. These ducts are lined by a single layer of luminal epithelial cells and are surrounded by a sleeve of myoepithelial cells. During pregnancy, additional ductal branching occurs and extensive lobulo-alveolar proliferation which eventually fills the fat pad completely at parturition. Cell division occurs in both the ductal and alveolar cell populations throughout the duration of gestation period and persists through the early phase of lactation. In addition, the luminal alveolar cells undergo functional terminal differentiation to attain a secretory phenotype that produces and secretes milk into the lumen of ducts and the contractile forces generated by the myoepithelium enable the transport of milk to the nipple to feed the newborn pups.

Systemic hormones such as estrogen, prolactin and progesterone play a key role in the regulation of the mammary gland during pregnancy and lactation. Estrogen is required for ductal elongation and arborisation during postnatal mammary development and lobulo-alveolar development during pregnancy (Mallepell et al., 2006). Similarly, progesterone is also required for side branching and alveolar differentiation in the mice (Lydon et al., 1995). During pregnancy, additional systemic local and intracellular signals are required for alveolar proliferation and differentiation. Prolactin signalling is essential for the proliferation and functional differentiation of lobulo-alveolar structures during

pregnancy (Horseman et al., 1997; Liu et al., 1997; Ormandy et al., 1997; Topper and Freeman, 1980). In addition, colony-stimulating factor-1 (Pollard and Hennighausen, 1994) and oxytocin (Nishimori et al., 1996; Young et al., 1996) are also required for alveolar development and milk secretion. Therefore, regulation of mammary gland development during pregnancy is of utmost importance to ensure that it is functionally ready for lactation.

6.1.2 Differentiation of luminal epithelial cells

With the recent publications on roles of transcription factors in determining luminal cell fate in the mammary gland (Asselin-Labat et al., 2007; Khaled et al., 2007; Kouros-Mehr et al., 2006), it is now clear that functional differentiation of the mammary gland is not defined solely by systemic hormones and local growth factors. It is apparent that transcription factors are also key mediators of the differentiation process. Gata-3 is the first transcription factor shown to be a critical determinant of luminal cell fate. Loss-of-function analyses showed that *Gata-3* is essential for differentiation and maintenance of luminal cell fate (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). In addition, luminal differentiation and alveolar morphogenesis were reduced in both *Stat6* and *IL4/IL13* doubly deficient mice during pregnancy, suggesting that Stat6 signalling is also important for luminal differentiation during pregnancy (Khaled et al., 2007). Recently, the prolactin-regulated Ets transcription factor Elf5 was shown to specify alveolar cell fate (Oakes et al., 2008). Even though both *Gata-3* and *Elf5* deficiencies led to accumulation of $CD29^{lo}CD24^{+}CD61^{+}$ luminal progenitor populations, some distinct differences exist. In addition to the accumulation of luminal progenitors, conditional deletion of *Gata-3* also resulted in disruptions to the ductal epithelium in virgin mice. In contrast, the effects of *Elf5* deficiency were restricted to alveolar cell fate specification during pregnancy. It was also observed that *Gata-3* and *Elf5* were expressed in different mammary luminal epithelial cells (Oakes et al., 2008) and the $CD29^{lo}CD24^{+}CD61^{+}$ luminal progenitor population may be a heterogenous population consisting of *Gata-3*- and *Elf5*-responsive progenitors. These studies imply that the combinatorial effects of different transcription factors are required to specify and maintain luminal cell fate and

highlight the complexity of the gene regulatory networks required to establish mammary cell fate.

As discussed in Chapter 5, *Bcl11* genes play important roles in the virgin gland development. Expression of *Bcl11a* was up-regulated during gestation and was detected in luminal ductal cells and differentiated alveolar cells (Figure 4.14). In addition, expression of *Bcl11a* was detected in the terminally differentiated secretory luminal cells during lactation (Figure 4.15). In contrast, expression of *Bcl11b* was detected only in the basal layer of ducts but not in the differentiated luminal alveolar cells (Figure 4.14). These differential expression patterns imply that *Bcl11a* may also have important functions in maintenance of terminally differentiation secretory luminal cell fate. In this Chapter, the main question that I will address is whether *Bcl11* genes are essential for the maintenance of terminally differentiated secretory luminal cell fate. I crossed both $Bcl11a^{flox/flox}$ and $Bcl11b^{flox/flox}$ conditional knockout mice to the BLG-Cre mice where Cre is specifically expressed in the lactation mammary gland (Selbert et al., 1998) and examined the effects of Cre-mediated excision of *Bcl11* genes in the lactation glands.

6.2 Results

6.2.1 *BLG-Cre;* $Bcl11a^{flox/flox}$ females have severe lactational defects

BLG-Cre expression was found to closely reflect the kinetics of BLG expression (Selbert et al., 1998). Low levels of recombination were detected in the virgin glands (7%), with increasing levels observed throughout gestation and parturition (20-30%). I first analyzed mammary glands from *BLG-Cre*; $Bcl11^{flox/flox}$ and control (*BLG-Cre*; $Bcl11^{flox/+}$) females at late gestation (16.5-17.5 dpc). No obvious histological defects were detected in the mammary glands from either *BLG-Cre*; $Bcl11a^{flox/flox}$ (Figure 6.1A2 and A4) or *BLG-Cre*; $Bcl11b^{flox/flox}$ (Figure 6.1B2 and B4) females compared to their respective *BLG-Cre*; $Bcl11^{flox/+}$ heterozygous control glands (Figure 6.1A1 and A3; B1 and B3). Lobulo-alveolar structures were abundant and sections showed that these structures contained lipid droplets and secretory vesicles, suggesting that lobulo-alveolar development was not significantly affected during gestation when low levels of Cre recombinase was expressed.

The highest levels of BLG-Cre expression were detected during lactation and quantification at mid-lactation determined the recombination efficiency to be approximately 70-80% (Selbert et al., 1998). Hence the effects of BLG-Cre mediated deletion of *Bcl11* genes were studied during lactation. I found that *BLG-Cre; Bcl11a*$^{flox/flox}$ females were unable to nurse their pups since no pups were successfully weaned. This result suggests a severe lactational insufficiency or impairment of the *BLG-Cre; Bcl11a*$^{flox/flox}$ females. To confirm this observation, I decided to monitor the weights of the pups from control *BLG-Cre; Bcl11a*$^{flox/+}$ and *BLG-Cre; Bcl11a*$^{flox/flox}$ females from postnatal day 0 (P0). Pups from *BLG-Cre; Bcl11a*$^{flox/flox}$ dams had little or no milk in their stomach from postnatal day 1 (P1) and displayed a significant reduction in weights over the observation period compared to pups from *BLG-Cre; Bcl11a*$^{flox/+}$ females (Figure 6.2). These pups were severely dehydrated with loose skin and were much smaller in size compared to age-matched pups nursed by control *BLG-Cre; Bcl11a*$^{flox/+}$ females. Most of these pups died by postnatal day 7 (P7) if not rescued (Figure 6.3B).

To rule out possible suckling defects of pups contributing to their inability to feed and hence resulting in a reduction in body weights, P1 and postnatal day 6 (P6) pups from *BLG-Cre; Bcl11a*$^{flox/flox}$ females were fostered to the control *BLG-Cre; Bcl11a*$^{flox/+}$ lactation females. The body weights of the fostered pups were monitored over a period of five days. All pups that were fostered to control *BLG-Cre; Bcl11a*$^{flox/+}$ females thrived with milk in their stomach and their weights increased constantly over the period observed (Figure 6.3A and B). This is in contrast to the original observation that the pups that remained with the *BLG-Cre; Bcl11a*$^{flox/flox}$ lactation females are usually dead by P7 (Figure 6.3A and B). These observations demonstrated that the pups from *BLG-Cre; Bcl11a*$^{flox/flox}$ females suckled normally. Hence the reduction in body weights of the pups were not due to suckling defects of the pups. Therefore loss of *Bcl11a* led to lactational deficiency in the *BLG-Cre; Bcl11a*$^{flox/flox}$ females, resulting in the lactating females being unable to feed their pups, suggesting that *Bcl11a* is critical to the maintenance of lactation state of the mammary glands.

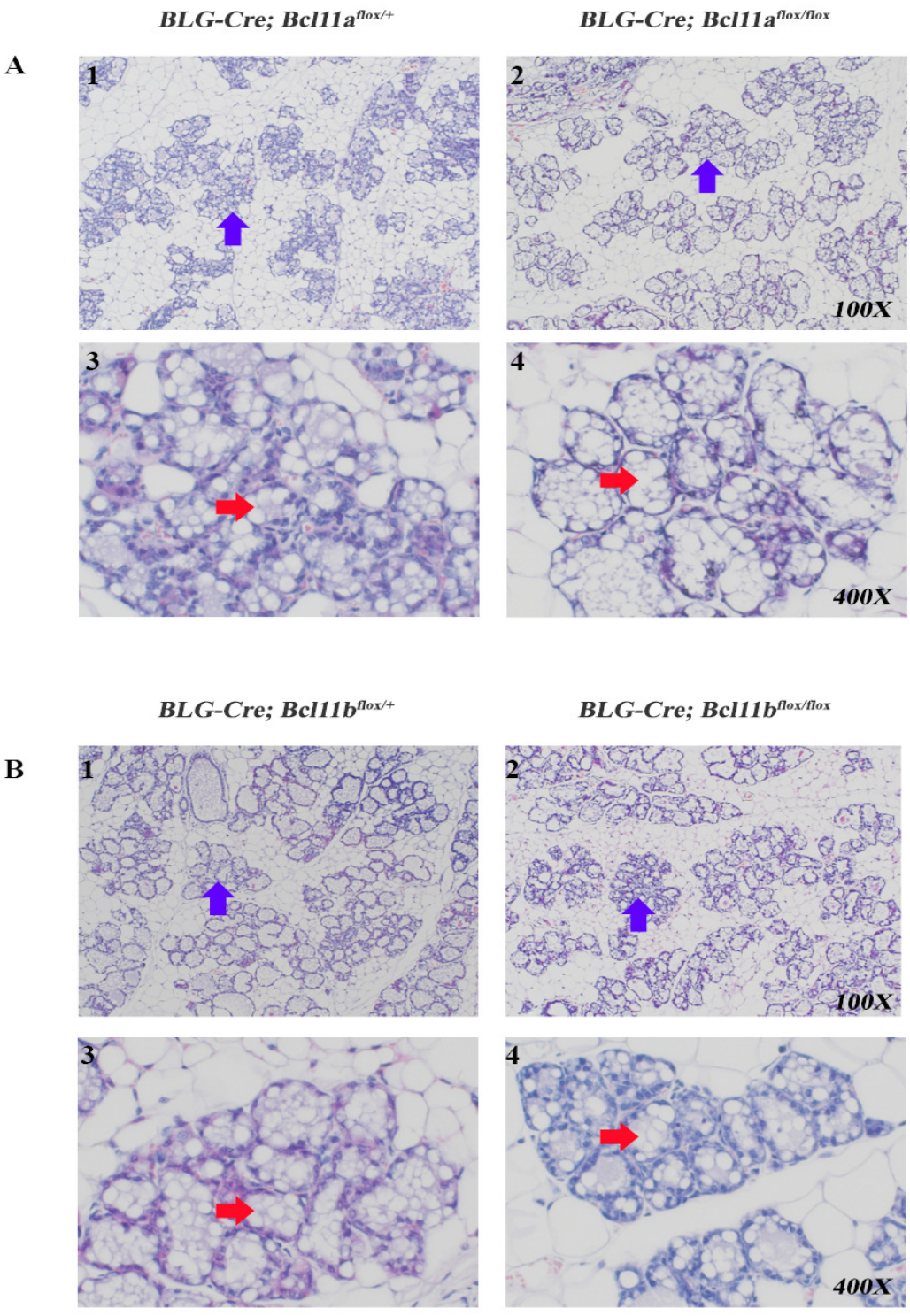


Figure 6.1. Histological analysis of mammary glands from *BLG-Cre; Bcl11*$^{flox/flox}$ and control females. H & E stained sections of day 16.5-17.5 gestation mammary glands from **(A1 and A3)** *BLG-Cre*; *Bcl11a*$^{flox/+}$; **(A2 and A4)** *BLG-Cre*; *Bcl11a*$^{flox/flox}$; **(B1 and B3)** *BLG-Cre*; *Bcl11b*$^{flox/+}$ and **(B2 and B4)** *BLG-Cre*; *Bcl11b*$^{flox/flox}$ females. Panels **A3**, **A4**, **B3** and **B4** are higher magnifications of panels **A1**, **A2**, **B1** and **B2** respectively. Blue arrows indicate lobulo-alveolar structures. Red arrows indicate fatty droplets.

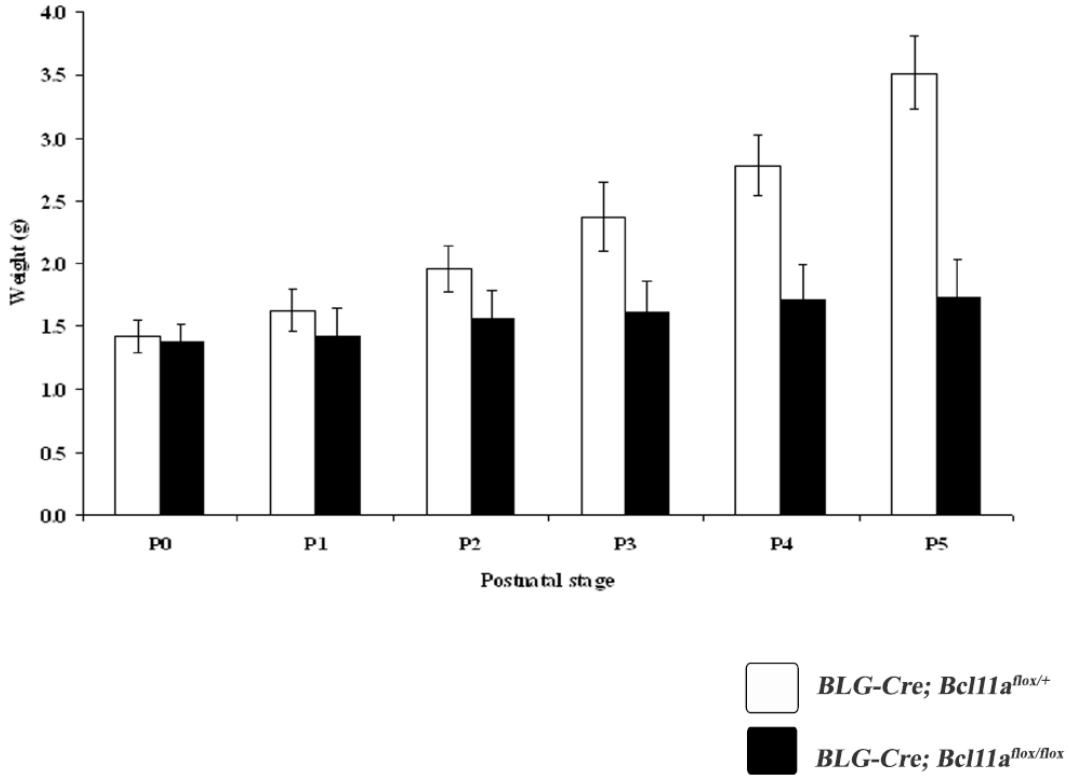
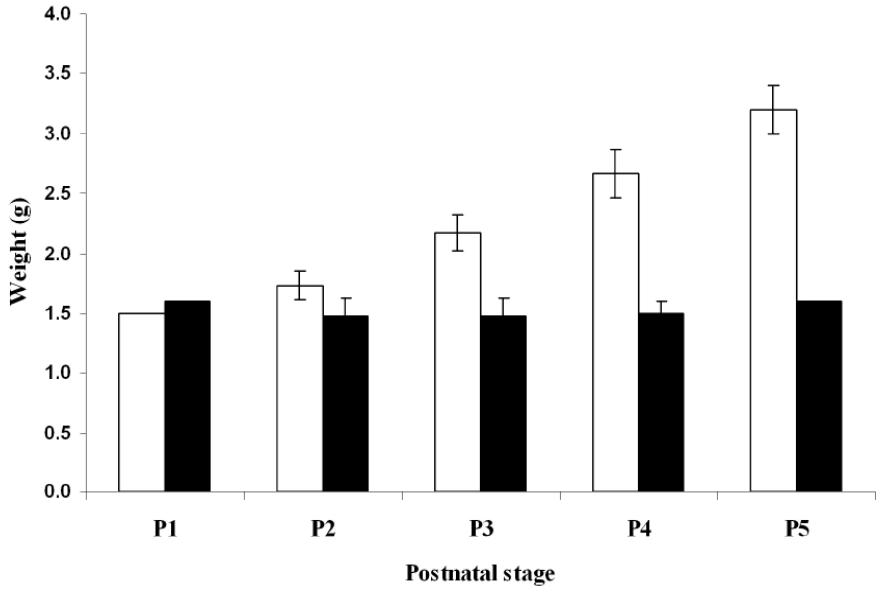
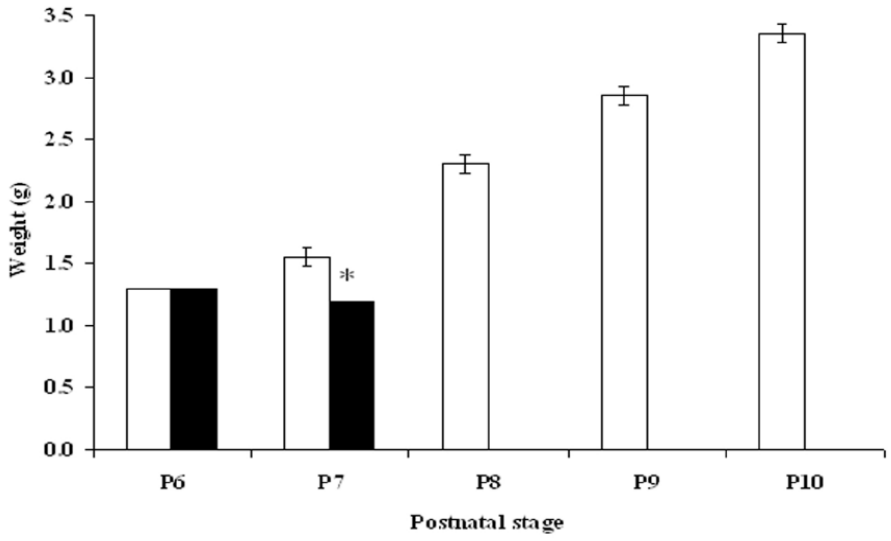


Figure 6.2. Graph showing average weights of pups from *BLG-Cre; Bcl11a*$^{flox/+}$ and *BLG-Cre; Bcl11a*$^{flox/flox}$ females. White and black bars represent average weights of pups from *BLG-Cre; Bcl11a*$^{flox/+}$ and *BLG-Cre; Bcl11a*$^{flox/flox}$ females respectively. Errors bar denote standard deviation obtained from analyzing 71 and 45 pups of *BLG-Cre; Bcl11a*$^{flox/+}$ and *BLG-Cre; Bcl11a*$^{flox/flox}$ females respectively.

A



B



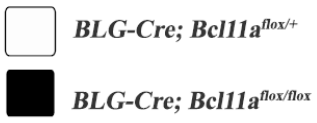
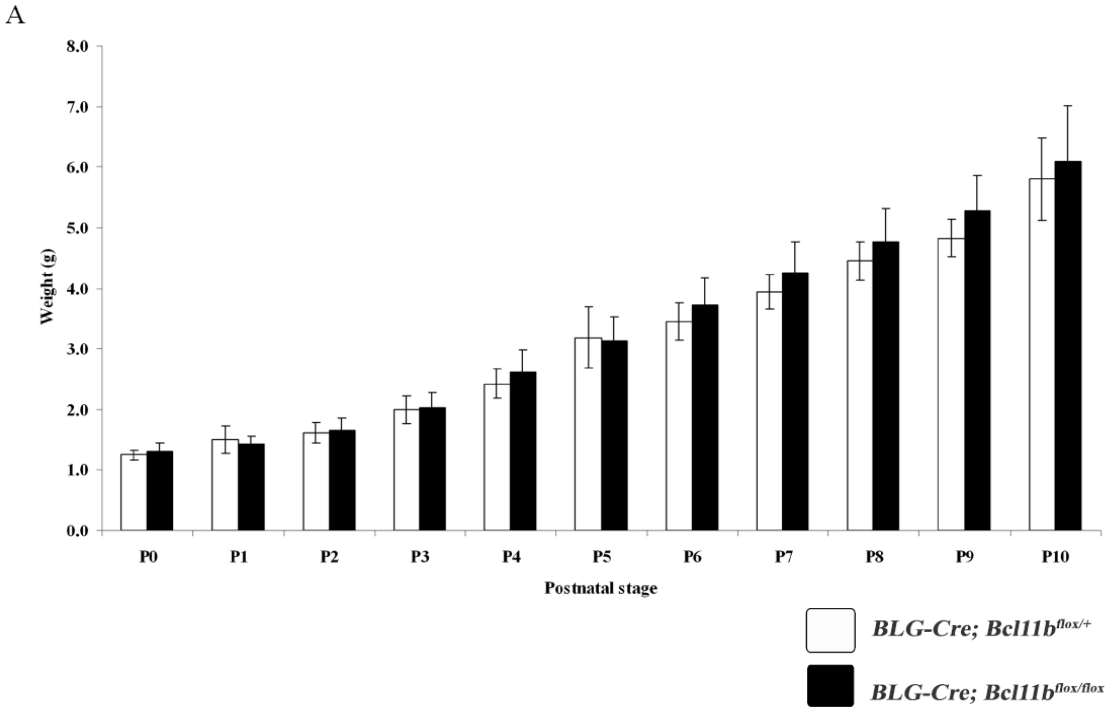


Figure 6.3. Graphs showing average weights of pups fostered from *BLG-Cre; Bcl11a$^{flox/flox}$* to *BLG-Cre; Bcl11a$^{flox/+}$* females. Pups from *BLG-Cre; Bcl11a$^{flox/flox}$* females are fostered to *BLG-Cre; Bcl11a$^{flox/+}$* females from **(A)** P1 and **(B)** P6. Their weights are monitored for 5 days and compared to littermates which remained with *BLG-Cre; Bcl11a$^{flox/flox}$* females. White and black bars represent average weights of pups fostered to *BLG-Cre; Bcl11a$^{flox/+}$* and *BLG-Cre; Bcl11a$^{flox/flox}$* females respectively. Error bars denote standard deviation obtained from analyzing 9 pups. * Pups found dead at P7.

6.2.2 *BLG-Cre; Bcl11b$^{flox/flox}$* females are able to nurse their pups

In contrast to the *BLG-Cre; Bcl11a$^{flox/flox}$* females, *BLG-Cre; Bcl11b$^{flox/flox}$* females were able to nurse their pups. Pups from *BLG-Cre; Bcl11b$^{flox/flox}$* females were healthy and there were no significant differences in weights of these pups compared to those from the control *BLG-Cre; Bcl11b$^{flox/+}$* females (Figure 6.4A). All the pups from *BLG-Cre; Bcl11b$^{flox/flox}$* females were weaned successfully. As shown in Chapter 4.2.6.2, expression of *Bcl11b* in the mammary glands was undetectable during lactation. Thus deletion of *Bcl11b* in the lactation gland did not affect the lactational capabilities of the glands. Whole mount carmine alum and histological analysis of day 2 lactation mammary glands showed no obvious differences between *BLG-Cre; Bcl11b$^{flox/flox}$* and the control *BLG-Cre; Bcl11b$^{flox/+}$* females (Figure 6.4B-C). The lobulo-alveoli in the *BLG-Cre; Bcl11b$^{flox/flox}$* lactation glands were stretched with large lumens and contained fat droplets and secretory vesicles (Figure 6.4C). In addition, molecular analysis also failed to reveal any significant differences in these *Bcl11b*-deficient glands from the wild-type controls (See below, Figures 6.10-6.12). These results suggest that *Bcl11b* is not critical in the lactation gland.



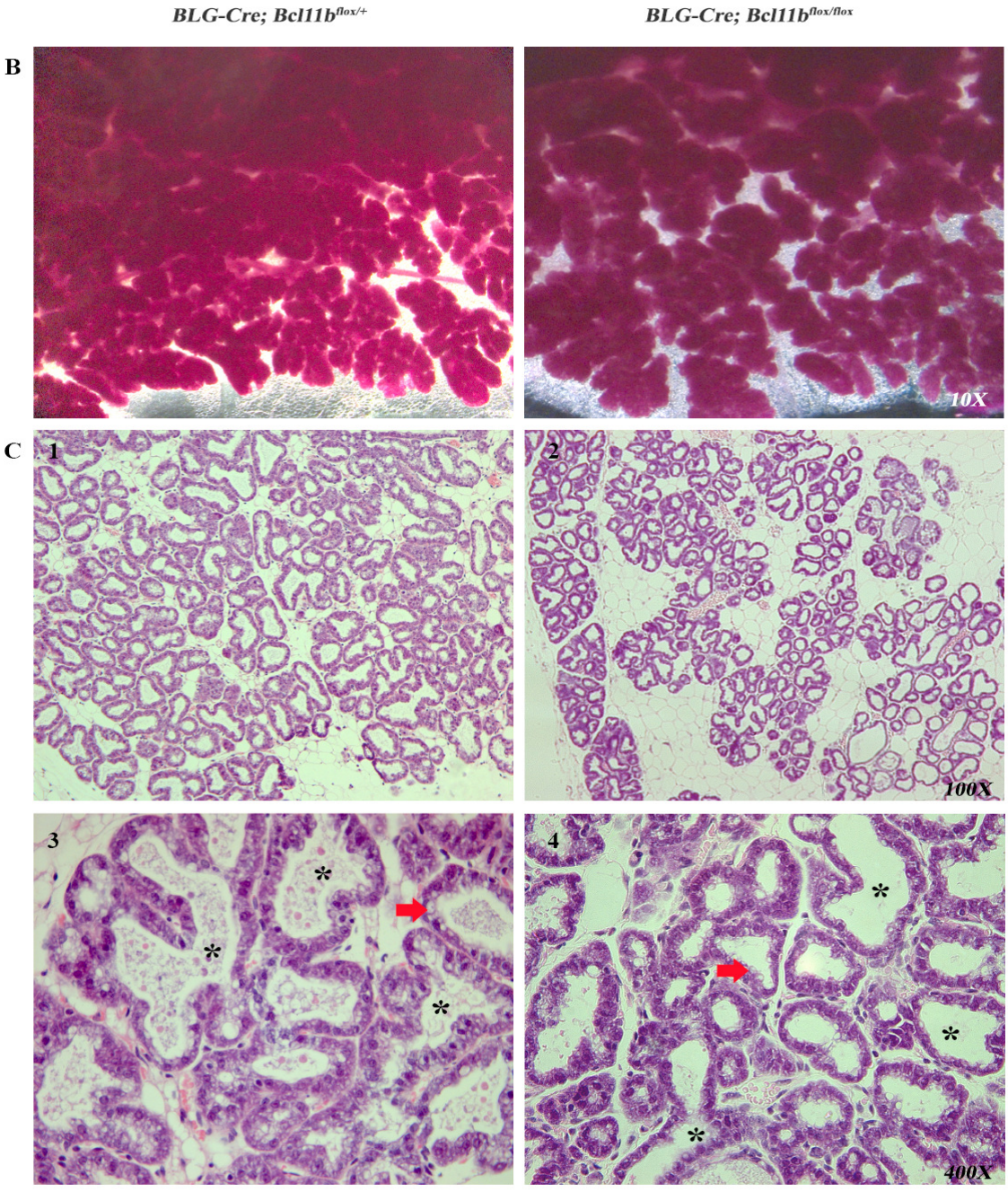


Figure 6.4. Analysis of *BLG-Cre; Bcl11b*$^{flox/flox}$ and control females. **(A)** Graph showing average weights of pups from *BLG-Cre; Bcl11b*$^{flox/+}$ and *BLG-Cre; Bcl11b*$^{flox/flox}$ females. White and black bars represent average weights of pups from *BLG-Cre; Bcl11b*$^{flox/+}$ and *BLG-Cre; Bcl11b*$^{flox/flox}$ females respectively. Errors bar denote standard deviation obtained from analyzing 67 and 60 pups of *BLG-Cre; Bcl11b*$^{flox/+}$ and *BLG-Cre; Bcl11b*$^{flox/flox}$ females respectively. **(B)** Whole mount carmine staining of day 2 lactation mammary glands isolated from *BLG-Cre*; *Bcl11b*$^{flox/+}$ and *BLG-Cre*; *Bcl11b*$^{flox/flox}$ females. **(C1-4)** H & E stained sections of day 2 lactation mammary glands isolated from *BLG-Cre*; *Bcl11b*$^{flox/+}$ and *BLG-Cre*; *Bcl11b*$^{flox/flox}$ females. **C3** and **C4** are higher magnification images of lobulo-alveolar structures of **C1** and **C2** respectively. Red arrows indicate fatty droplets within alveolar structures. * indicates examples of stretched alveolar structures.

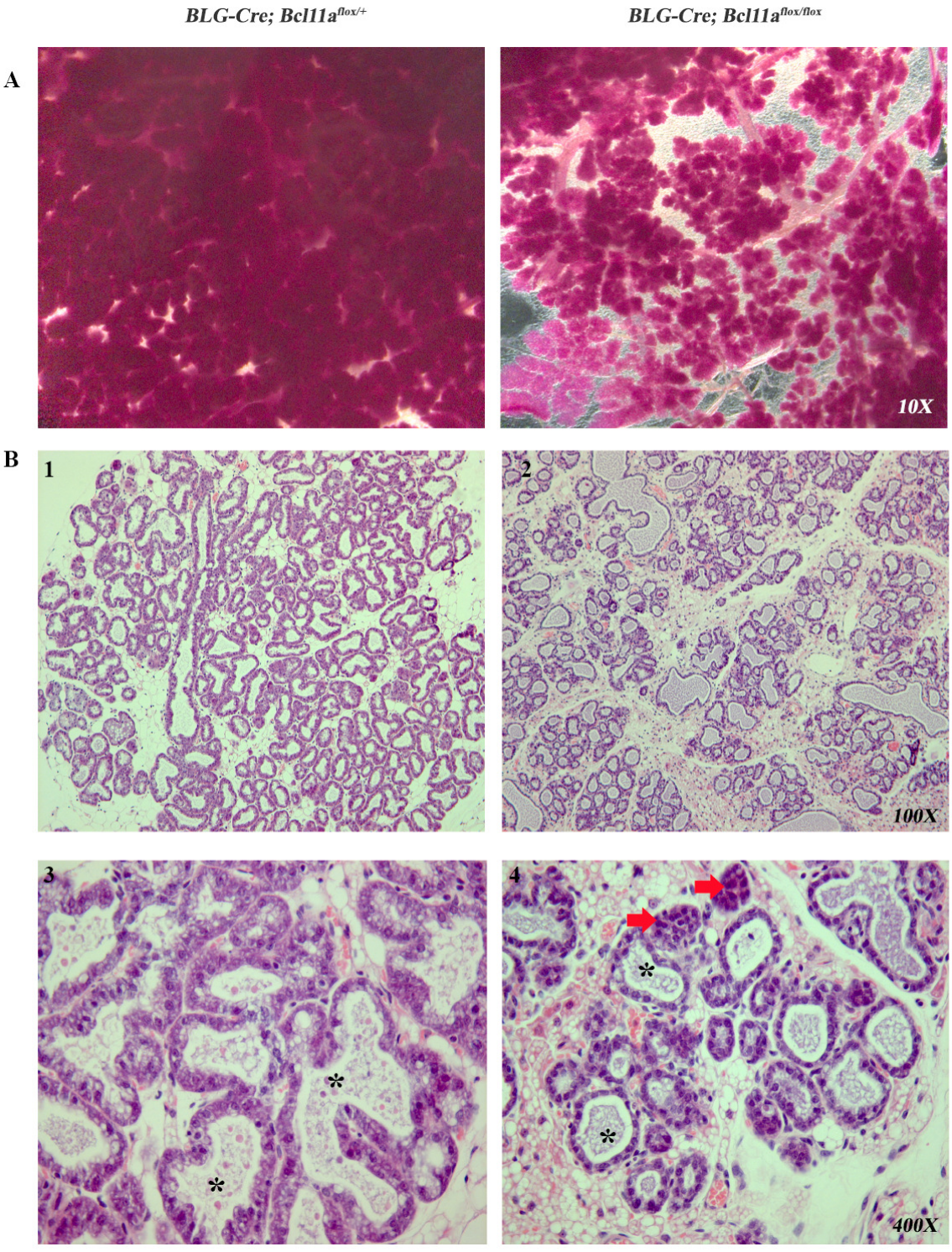
6.2.3 *Bcl11a* is essential in the lactation gland

As discussed in Chapter 6.2.1, *BLG-Cre; Bcl11a*$^{flox/flox}$ dams were unable to nurse their pups. Therefore, in order to confirm that this lactational defect was a functional consequence of the loss of *Bcl11a* in the lactation gland, whole mount and histological analyses of the mammary glands of *BLG-Cre; Bcl11a*$^{flox/flox}$ dams were carried out. As shown in Figure 6.5A, the mutant *BLG-Cre; Bcl11a*$^{flox/flox}$ glands had a substantial reduction in the number and size of milk-producing lobulo-alveoli compared to control *BLG-Cre; Bcl11a*$^{flox/+}$ mammary glands during early lactation (day 2) (Figure 6.5A). Histological analysis also showed that the control mammary glands were populated with numerous alveolar structures (Figure 6.5B1). In contrast, the mutant mammary glands had much fewer alveolar structures (Figure 6.5B2). Closer examination revealed that the alveoli in the control glands were stretched with large lumens (Figure 6.5B3). However the alveoli in the mutant glands contained lesser fat droplets and secretory vesicles (Figure 6.5B4). Numerous collapsed alveolar structures in the *Bcl11a*-deficient glands were also visible (Figure 6.5B4). By day 5 lactation, the alveolar structures in the control females had expanded and populated the entire mammary gland (Figure 6.5C1). In contrast, the mutant glands displayed sparse number of lobulo-alveoli with a large area of fat cells still visible in the mammary gland (Figure 6.5C2). In addition, the alveolar cells of the control glands had a thick luminal layers filled with secretory vesicles and fat droplets (Figure 6.5C3). However, most of alveoli in the mutant glands had thin luminal layers and reduced fat droplets and secretory vesicles (Figure 6.5C4). As development of lobulo-alveolar structures was unaffected in the *BLG-Cre; Bcl11a*$^{flox/flox}$ glands during pregnancy (Figure 6.1A), these observations demonstrated that loss of *Bcl11a* in the lactation glands affected the terminal differentiation state of the lobulo-alveoli, resulting in the observed lactational defects and hence a failure of *BLG-Cre; Bcl11a*$^{flox/flox}$ females to nurse their pups.

BLG-Cre-mediated excision of *Bcl11* genes was detected by PCR amplification of genomic DNA of the mammary glands (Figure 6.6A) and quantification using quantitative real time PCR (qRT-PCR) showed the deletion efficiency to be ~75-78%. Analysis of these glands using qRT-PCR showed ~95% reduction in the levels of *Bcl11a* mRNA in *BLG-Cre*; *Bcl11a*$^{flox/flox}$ mammary tissues relative to that in control glands

(Figure 6.6B). Thus the lactational defects observed in *BLG-Cre*; *Bcl11a*$^{flox/flox}$ females were a functional consequence of loss of *Bcl11a* in the differentiated luminal cells. To confirm the lobulo-alveolar defects at the molecular level, I examined the RNA levels of milk protein genes using RT-PCR. As shown in Figure 6.7A, there were slight reductions in the mRNA levels of α-*casein*, *β-casein* and *α-lactalbumin* but a substantial reduction in the mRNA levels of *whey acidic protein* (WAP) in the *Bcl11a*-deficient mammary tissues (Figure 6.7A). This observation was confirmed in the Western analysis as WAP protein was undetectable in the *Bcl11a*-deficient mice (Figure 6.7B). In addition, immunostaining with an antibody to β-casein showed a significant reduction in the levels of β-casein in the *Bcl11a*-deficient glands (Figure 6.7C). No changes in the milk transcripts or protein levels were observed in the *Bcl11b*-deficient lactation glands compared to control glands (Figure 6.7A-B). Therefore, loss of *Bcl11a* but not *Bcl11b* in the lactation glands resulted in severe reduction in milk proteins, leading to the lactational defects and affected the ability of *BLG-Cre*; *Bcl11a*$^{flox/flox}$ females to nurse their pups.

During early lactation, mammary epithelial cells undergo rapid proliferation. In the *Bcl11a*-deficient day 2 lactation mammary glands, staining with antibody to Ki67 showed a complete absence of proliferating cells (Figure 6.8A). Thus, deletion of *Bcl11a* in the terminally differentiated luminal cells also resulted in proliferation defects. Immunostaining with antibody to smooth muscle actin (SMA) on the other hand did not detect any obvious changes in the basal layer of the *Bcl11a*-deficient lactation glands (Figure 6.8B). This was expected as BLG-Cre-mediated excision should occur only in the luminal cells. In summary, whole mount, histological and molecular analyses demonstrated that loss of *Bcl11a* in the lactation glands resulted in severe lactational defects that affected milk production and proliferation of the luminal alveolar cells.


BLG-Cre; Bcl11a flox/+
BLG-Cre; Bcl11a flox/flox
A
10X
B
1
2
100X
3
4
400X

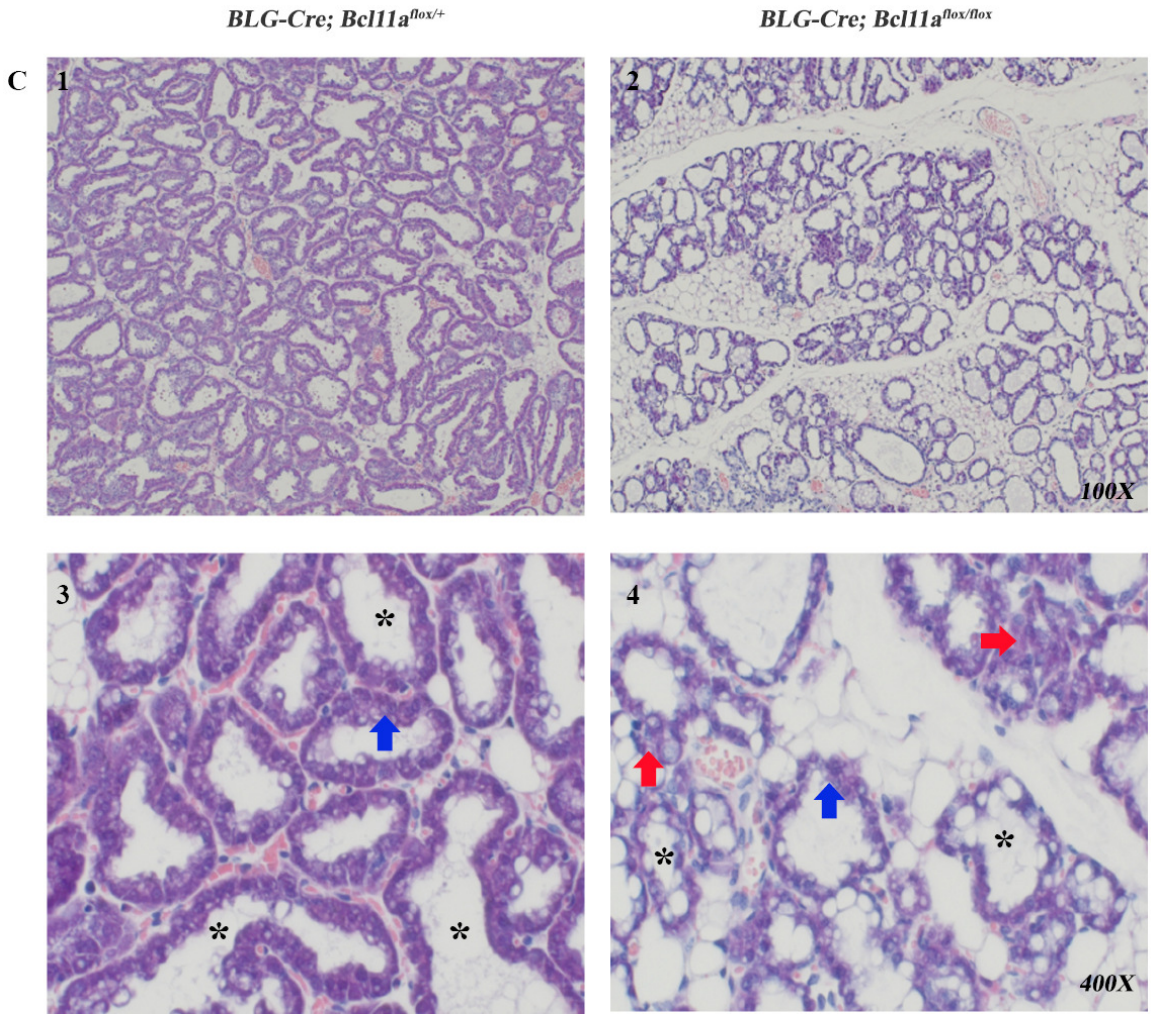


Figure 6.5. Histological analysis of *BLG-Cre; Bcl11a*$^{flox/flox}$ and control females. **(A)** Whole mount carmine staining of day 2 lactation mammary glands isolated from *BLG-Cre*; *Bcl11a*$^{flox/+}$ and *BLG-Cre*; *Bcl11a*$^{flox/flox}$ females. **(B-C)** H & E stained sections of day 2 **(B1-4)** and day 5 **(C1-4)** lactation mammary glands isolated from *BLG-Cre*; *Bcl11a*$^{flox/+}$ and *BLG-Cre*; *Bcl11a*$^{flox/flox}$ females. **B3** and **B4** are higher magnification images of lobulo-alveolar structures of **B1** and **B2** respectively; **C3** and **C4** are higher magnification images of lobulo-alveolar structures of **C1** and **C2** respectively. Red arrows indicate collapsed alveolar structures. Blue arrows show the thick luminal layer of alveolar structures in *BLG-Cre*; *Bcl11a*$^{flox/+}$ compared to that of *BLG-Cre*; *Bcl11a*$^{flox/flox}$ lactation glands. * indicates the lumen of alveolar structures. The lumens of alveoli in *BLG-Cre*; *Bcl11a*$^{flox/+}$ lactation glands are large and distended with secretory vesicles while lumens of alveoli in *BLG-Cre*; *Bcl11a*$^{flox/flox}$ lactation glands are small.

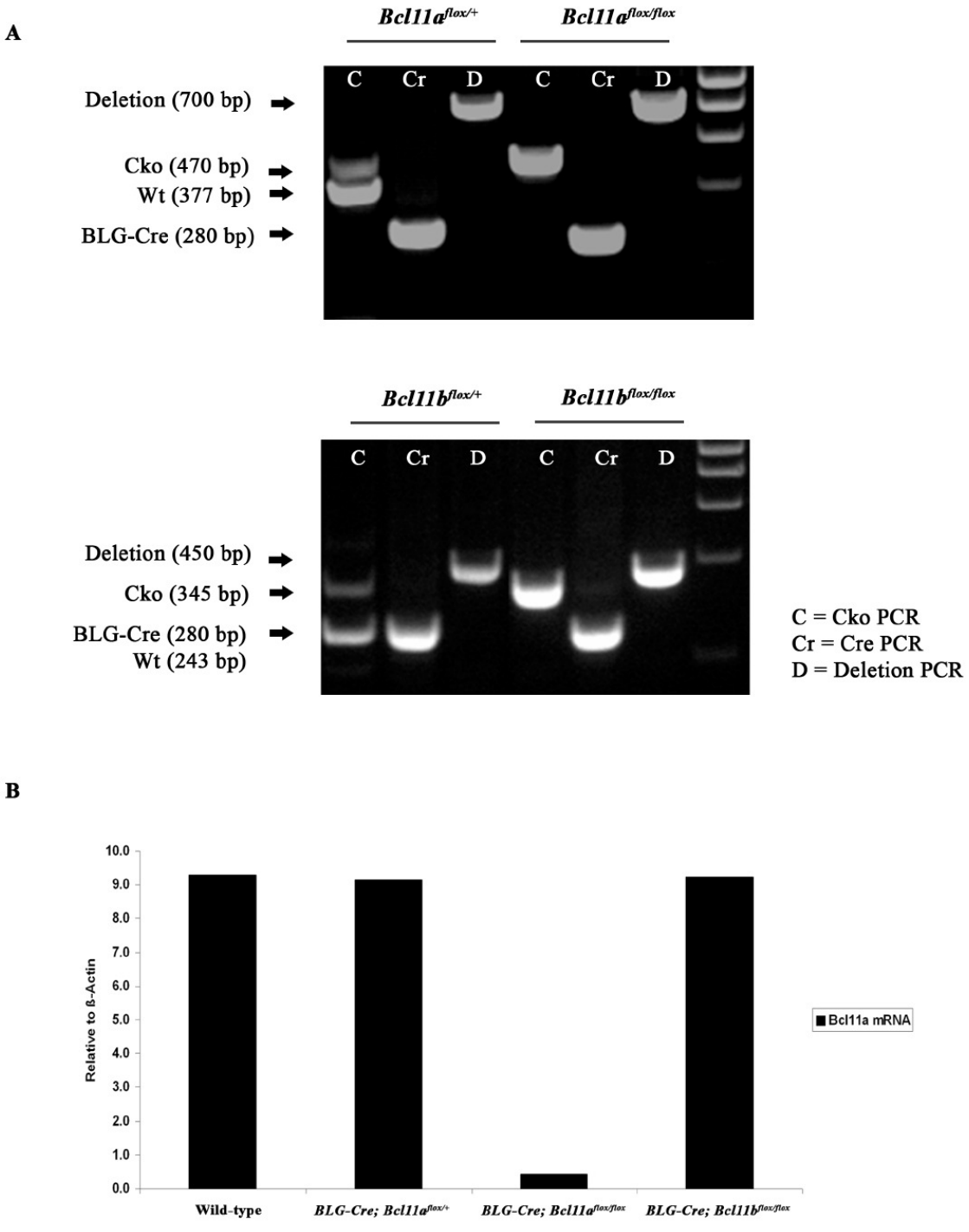


Figure 6.6. Detection of deletion of *Bcl11a* and *Bcl11b* after *BLG-Cre* expression. **(A)** Gel image showing PCR products obtained with primers using genomic DNA extracted from day 2 lactation mammary glands. Primers used are as described in Figure 5.4A and 5.12A. Cko: conditional band; Wt: Wild-type band; BLG-Cre: Cre band; Deletion: Deletion band. C: Cko PCR; Cr: Cre PCR; D: Deletion PCR. **(B)** Quantification of levels of *Bcl11a* transcripts in day 2 lactation mammary glands using quantitative real time PCR. Levels are normalized to β-actin.

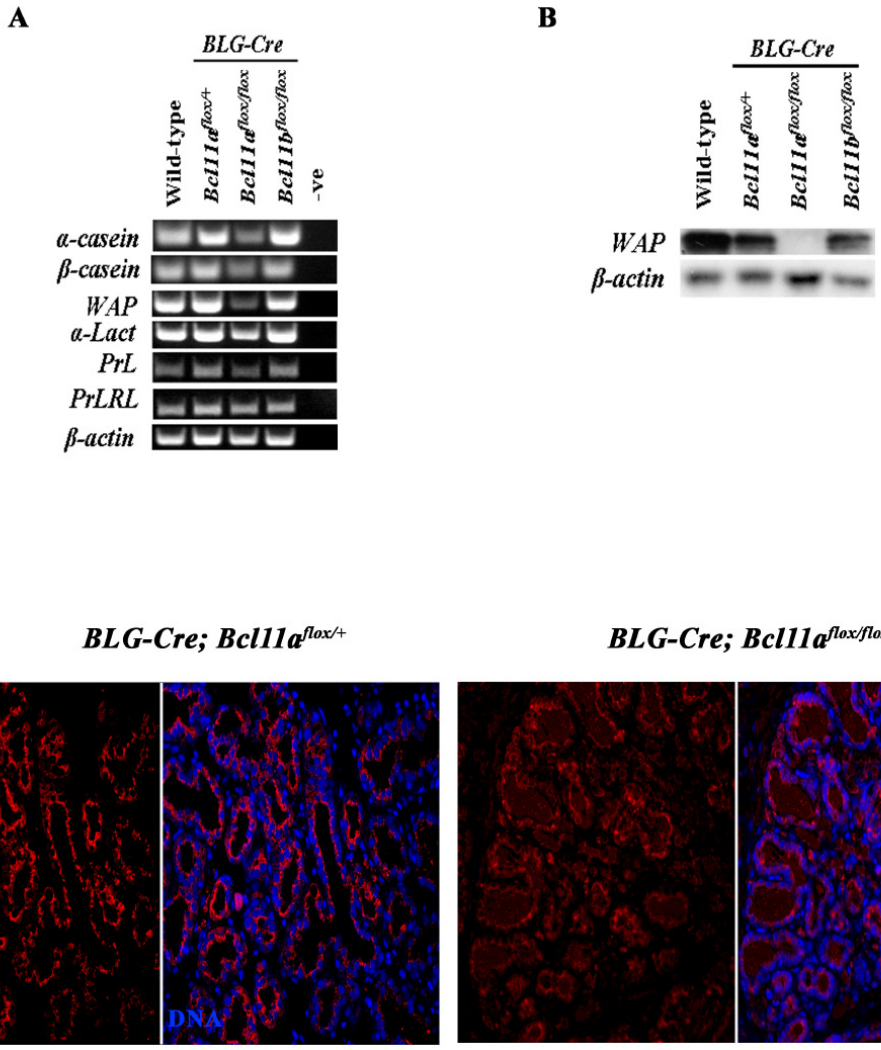


Figure 6.7. Analysis of milk transcripts and protein levels in *BLG-Cre; Bcl11*$^{flox/flox}$ and control females. (A) RT-PCR analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11*$^{flox/+}$) and mutant (*BLG-Cre*; *Bcl11*$^{flox/flox}$) females using milk protein primers. β-actin is used as a control. –ve indicates no template control. **(B)** Immunoblot analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11*$^{flox/+}$) and mutant (*BLG-Cre*; *Bcl11*$^{flox/flox}$) females with antibody to WAP. β-actin is used as a loading control. **(C)** Immunostaining of day 2 lactation mammary glands of *BLG-Cre*; *Bcl11a*$^{flox/+}$ and *BLG-Cre*; *Bcl11a*$^{flox/flox}$ females with antibody to β-casein.

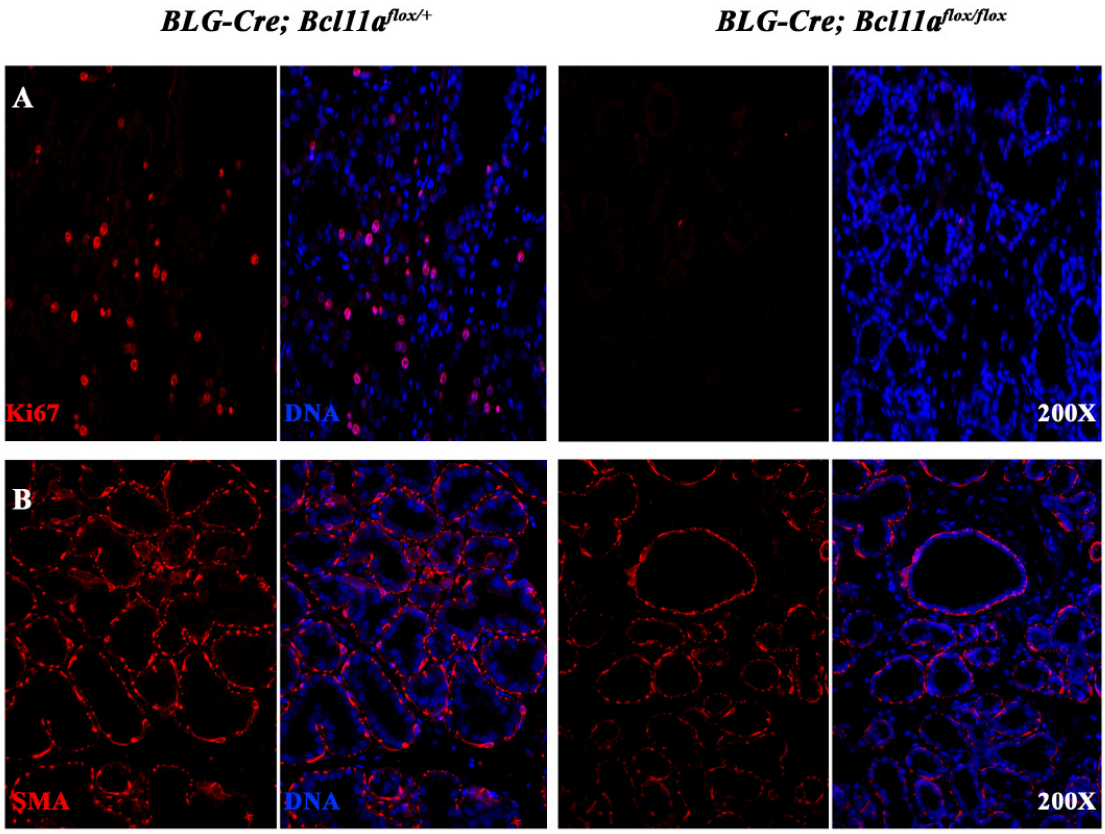


Figure 6.8. Immunostaining of day 2 lactation mammary glands of *BLG-Cre; Bcl11a*$^{flox/+}$ and *BLG-Cre; Bcl11a*$^{flox/flox}$ females. Immunostaining using antibodies to **(A)** Ki67 and **(B)** smooth muscle actin (SMA).

6.2.4 Loss of *Bcl11a* in lactation glands results in premature onset of involution

The JAK-Stat pathway plays an important role in development of the mammary gland during gestation and lactation. Activation of Stat5 transcriptional activities by phosphorylation is essential for normal lobulo-alveolar cell proliferation and differentiation (Liu et al., 1997). *Stat5*-deficient female mice showed curtailed mammary lobulo-alveolar outgrowth during pregnancy and a failure to lactate due to failed terminal differentiation of secretory luminal cells. To determine whether deletion of *Bcl11a* affected phosphorylation of Stat5 (p-Stat5), immunostaining with antibody to p-Stat5 was performed. Abundant p-Stat5 positive epithelial cells were detected in the control *BLG-Cre*; *Bcl11a*$^{flox/+}$ day 2 lactation glands, characteristics of alveolar differentiation (Figure 6.9A). Strikingly, no p-Stat5 positive epithelial cells were observed in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ day 2 lactation glands (Figure 6.9A), indicating a likely loss of lobulo-alveolar cells. Stat3 is an important mediator of the switch between survival and death signalling in mammary epithelial cells and activation of Stat3 is pivotal to the normal induction of involution (Abell et al., 2005; Chapman et al., 1999). In contrast to the loss of p-Stat5, phospho-Stat3 (p-Stat3, activated form of Stat3) was detected in many alveolar cells of the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ day 2 lactation glands (Figure 6.9B). Moreover, an increase in the number of cleaved Caspase-3 positive cells was observed in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ day 2 lactation glands (Figure 6.9C), indicating that loss of *Bcl11a* in the lactation glands resulted in activation of Stat3-mediated apoptosis. These results suggest that loss of *Bcl11a* resulted in premature onset of involution in the lactation glands. Despite the severe lactation defects, there was no change in the expression of prolactin and prolactin receptor, suggesting that *Bcl11a* may regulate Stat5 and Stat3 activities via different pathways in the lactation gland (Figure 6.6B).

To further study the premature onset of involution at the molecular level in the *Bcl11a*-deficient lactation glands, I first used RT-PCR to examine expression changes in key lactation and involution genes. I found that expression of *Stat5*, *Stat6*, *p85* and *Akt1* was down-regulated, and a dramatic up-regulation of *Lif*, *Stat3* and the *p55α/p50α* regulatory subunits of phosphoinositide-3-OH kinase [PI(3)K] in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ day 2 lactation glands (Figure 6.10A). Loss of *Bcl11a* in the lactation gland

therefore resulted in expression of genes that were normally only expressed during involution. To further probe the molecular changes resulting from the premature onset of involution in the *Bcl11a*-deficient glands, immunoblotting was performed to examine changes in components of the JAK-Stat pathway. As shown in Figure 6.10B, there was a decrease in Stat5 and a complete absence of p-Stat5 in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ lactation glands. No changes in the levels of Stat6 and p-Stat6 (phospho-Stat6) were found. As expected, a significant increase in the levels of Stat3 and p-Stat3 was observed in the *Bcl11a*-deficient lactation glands (Figure 6.10B). During involution, expression of *p55α* and *p50α* is induced by Stat3 to down-regulate PI(3)K-Akt-mediated survival signalling (Abell et al., 2005). The premature onset of involution in the *Bcl11a*-deficient lactation glands was confirmed by increase in levels of cleaved Caspase 3, p55α and p50α together with a decrease in total Akt (Figure 6.10B). Consistent with no obvious phenotypes in the *Bcl11b*–deficient lactation glands, I did not find any noticeable changes in the transcript or protein levels of the components of the JAK-Stat pathway (Figure 6.10). Taken together, these results strongly suggest that *Bcl11a* plays an essential role in maintaining the identity of the terminally differentiated secretory cells as loss of *Bcl11a* in secretory luminal cells results in premature activation of Stat3-mediated apoptosis in the lactation glands.

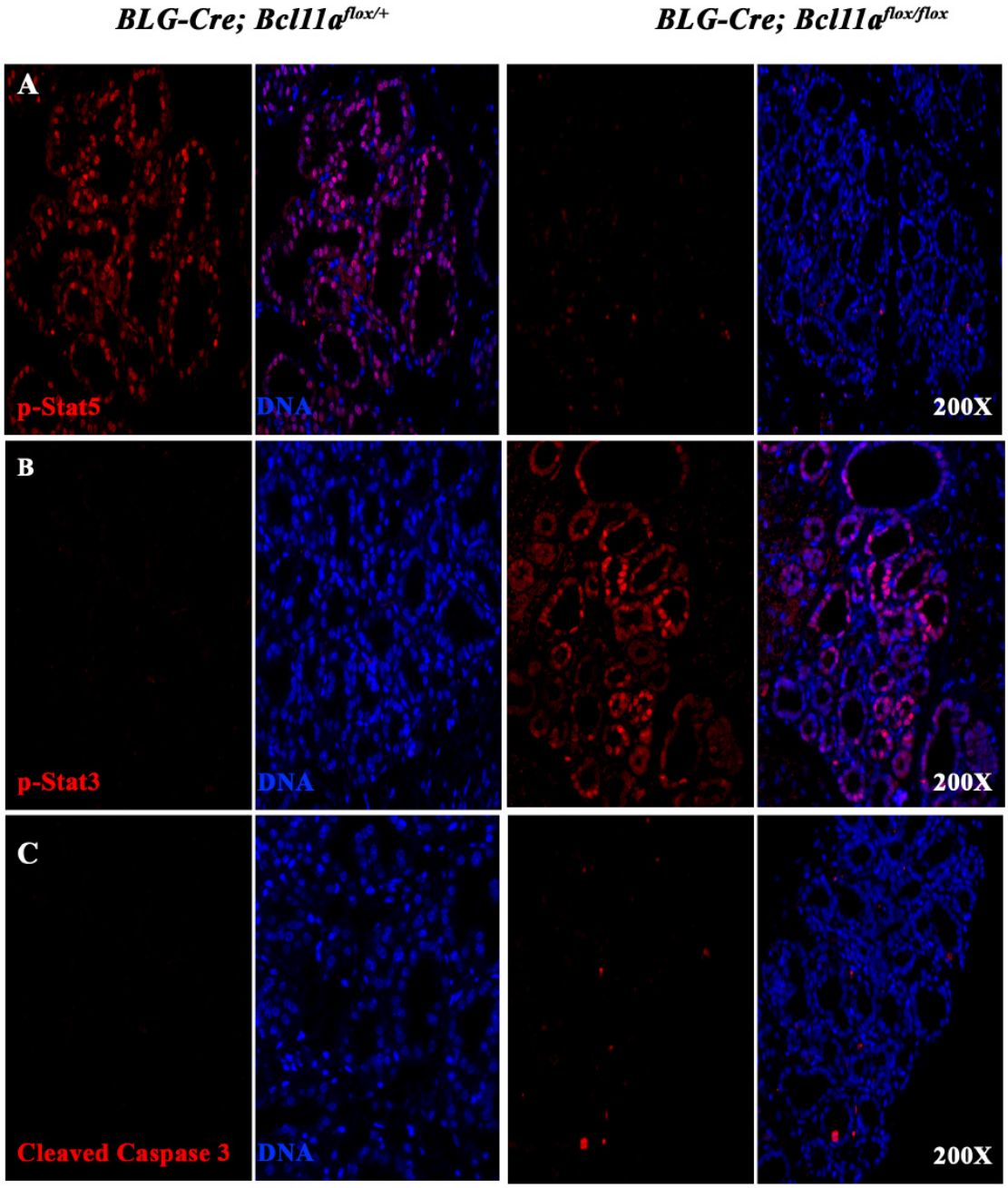


Figure 6.9. Loss of *Bcl11a* results in activation of Stat3-mediated apoptosis. Immunostaining using antibodies to **(A)** phospo-Stat5, **(B)** phospo-Stat3 and **(C)** cleaved Caspase-3.

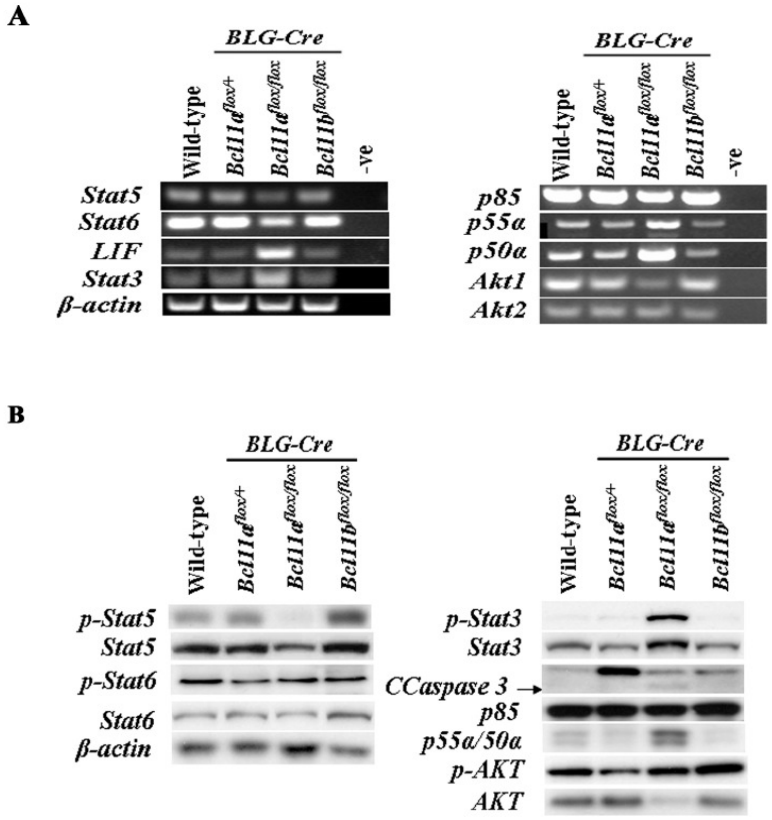


Figure 6.10. Analysis of Stat transcripts and protein levels in *BLG-Cre; Bcl11*$^{flox/flox}$ and control females. (A) RT-PCR analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11*$^{flox/+}$) and mutant (*BLG-Cre*; *Bcl11*$^{flox/flox}$) females. β-actin is used as a control. –ve indicates no template control. **(B)** Immunoblot analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11*$^{flox/+}$) and mutant (*BLG-Cre*; *Bcl11*$^{flox/flox}$) females with antibodies to components of JAK-Stat pathway. β-actin is used as a loading control. Note: The loading control panel for the immunoblot was the same as that in Figure 6.7B as experiments were carried out using the same protein samples.

6.2.5 Lactational defects in *Bcl11a*-deficient glands are not compensated by physiological levels of Gata-3

Gata-3 has been demonstrated to be essential for generation, differentiation and maintenance of luminal cells (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). Over-expression of *Gata-3* is sufficient to drive primary luminal progenitors to produce milk *in vitro* (Asselin-Labat et al., 2007). However, in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ lactation glands, Gata-3 mRNA and protein levels appeared unchanged (Figure 6.11A and 6.11B), indicating that loss of *Bcl11a* in the terminally differentiated luminal cells was not compensated by the physiological amounts of Gata-3. Like *Bcl11a*, *Gata-3* is highly expressed in the lactation gland (Asselin-Labat et al., 2007), the fact that 95% reduction in *Bcl11a* expression in the lactation gland (Figure 6.6B) did not significantly alter *Gata-3* expression shows that Bcl11a and Gata-3 function in distinct luminal cell populations in the lactation gland. The Ets transcription factor, *Elf5* has recently been shown to specify mammary alveolar cell fate (Oakes et al., 2008). *Elf5* expression was reduced in the *Bcl11a*-deficient lactation glands (Figure 6.11A). Interestingly, *Gata-3* and *Elf5* have been shown to be mostly expressed in different luminal cell populations, with *Elf5*-expressing cells predominantly being ERα-negative and *Gata-3*-expressing cells mainly ERα-positive. Interestingly, I noticed that loss of a single copy of *Bcl11a* resulted in up-regulation of *Bcl11b* expression but this increase was apparently insufficient to cause an obvious mammary phenotype in the heterozygous females (Figure 6.11A). Taken together, these data demonstrate that *Bcl11a* plays an essential role in maintaining the identity of the terminally differentiated secretory cells.

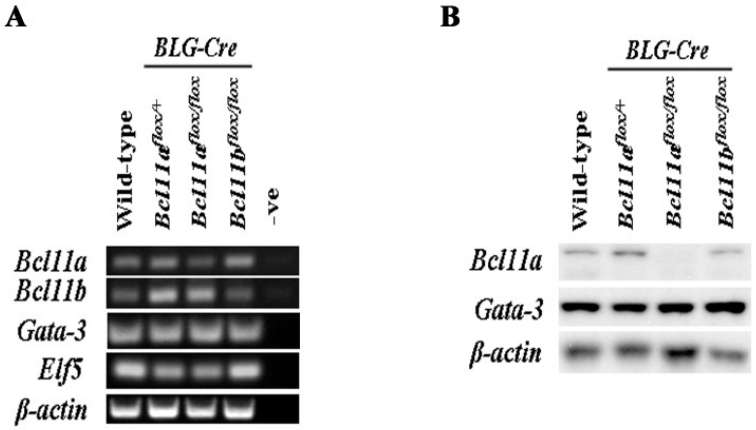


Figure 6.11. Analysis of transcription factors transcripts and protein levels in *BLG-Cre; Bcl11*$^{flox/flox}$ and control females. (A) RT-PCR analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11*$^{flox/+}$) and mutant (*BLG-Cre*; *Bcl11*$^{flox/flox}$) females. β-actin is used as a control. –ve indicates no template control. **(B)** Immunoblot analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11*$^{flox/+}$) and mutant (*BLG-Cre*; *Bcl11*$^{flox/flox}$) females with antibodies to Bcl11a and Gata-3. β-actin is used as a loading control. Note: The loading control panel for the immunoblot was the same as that in Figure 6.7B as experiments were carried out using the same protein samples.

6.2.6 Loss of *Bcl11a* in lactation glands also results in dysregulation of Notch signalling pathways

Besides the JAK-Stat pathway, several molecular pathways such as Notch signalling pathway are known to be involved in mammary lobulo-alveolar development. Constitutive activation of Notch signalling by expression of the intracellular domains of Notch1, Notch3, and Notch4 inhibits alveolar terminal differentiation and causes lactational defects (Gallahan et al., 1996; Hu et al., 2006; Jhappan et al., 1992; Smith et al., 1995). However, the roles of different Notch ligands and receptors in mammary development and cell fate determination remained unclear. Loss of *Bcl11a* in the virgin glands resulted in increased Notch1 and Jagged1 expression (Chapter 5). This is similar to the loss of *Bcl11a* in lymphocytes which showed up-regulation in *Notch1* transcripts (Liu et al., 2003b). To interrogate whether other components of the Notch signalling pathway were altered in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ lactation glands, RT-PCR was performed to detect changes in gene expression. All the known Notch ligands, except Delta-like ligand 3 (*Dll3*), were expressed in the lactation gland (Figure 6.12A). Remarkably, loss of *Bcl11a* led to dramatic up-regulation of *Dll3* expression in the lactation glands (Figure 6.12A). In contrast, the mRNA levels of *Jagged1* and *Dll1* (Delta-like ligand 1) were reduced while expression of *Dll4* (Delta-like ligand 4) was completely ablated (Figure 6.12A). No changes in the transcript levels of *Jagged2* were observed. Using immunoblotting (Figure 6.12C) and immunostaining (Figure 6.12D) with antibody to Jagged1, a dramatic reduction in Jagged1 protein levels in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ lactation glands was observed. This observation differs from that of the virgin gland where loss of *Bcl11a* resulted in an increase in Jagged1, suggesting that Jagged1 probably plays different roles in the virgin and lactation glands.

All four Notch receptors were expressed in the wild-type day 2 lactation gland (Figure 6.12A). Deletion of *Bcl11a* resulted in an increase in *Notch1* expression (Figure 6.12A). Interestingly, no obvious increase in the protein level of Notch1 was detected using immunoblotting but activation of Notch1 signalling pathway was confirmed by a slight increase in activated Notch1 (Notch1 intra-cellular domain, ICN) (Figure 6.12C) and up-regulation of downstream targets of Notch1 as discussed below (Figure 6.12B). Additionally, an obvious increase in Notch1 was detected using immunostaining with

antibody to Notch1 (Figure 6.12E). Surprisingly, loss of *Bcl11a* led to an absence of Notch3 expression at both transcript and protein levels (Figure 6.12A and 6.12C). Interestingly, Notch3 has been shown to promote lobulo-alveolar differentiation during late pregnancy (Hu et al., 2006) and could act as a repressor by blocking the ability of the Notch1 intracellular domain to activate expression through the Hairy enhancer of split (Hes) family of transcriptional repressors, *Hes1* and *Hes5* promoters (Beatus et al., 1999). In addition, a recent report proposed that in the human mammary epithelium, Notch3 is critical for luminal differentiation program *in vitro* (Raouf et al., 2008). No apparent changes in the expression of *Notch2* and *Notch4* were observed (Figure 6.12A).

To determine whether deletion of *Bcl11a* affected the downstream target genes of the canonical Notch pathway in the lactation mammary gland, I analyzed expression of *Hes* genes using RT-PCR. Most of the *Hes* genes, including *Hes1*, *Hes2*, *Hes3*, *Hes5* and *Hes6* were expressed at low levels in the control lactation glands (Figure 6.12B). However, significant increases in the expression of these *Hes* genes were detected in the *BLG-Cre*; *Bcl11a*$^{flox/flox}$ lactation glands, suggesting activation of the canonical Notch signalling pathway (Figure 6.12B). In contrast, *Hes7*, which was expressed at high levels in the control lactation glands, was completely absent in the *Bcl11a* mutant glands (Figure 6.12B). It is not clear at this moment whether the loss of expression of *Notch3*, *Dll4, Jagged1* and *Hes7*, and the gain of *Dll3* and *Notch1* expression in the *Bcl11a*-deficient glands are directly implicated in the phenotypes of the *Bcl11a*-deficient lactation glands. Nevertheless, these results demonstrate that loss of *Bcl11a* in the lactation mammary glands results in dysregulation of Notch signalling pathway, and indicate that different Notch receptors and ligands play different roles in mammary development and epithelial differentiation. In contrast, no changes in the transcript or protein levels of the components of the Notch pathway were observed in the *Bcl11b*–deficient lactation glands compared to control glands (Figure 6.12).

Taken together, deletion of *Bcl11a* but not *Bcl11b* in the lactation gland ablated the function of terminally differentiated secretory luminal cells and resulted in premature onset of involution. These data clearly demonstrate that *Bcl11a* is critical during lactation and maintains the differentiated functional state of the lactation gland at least partly through modulating Notch signalling.

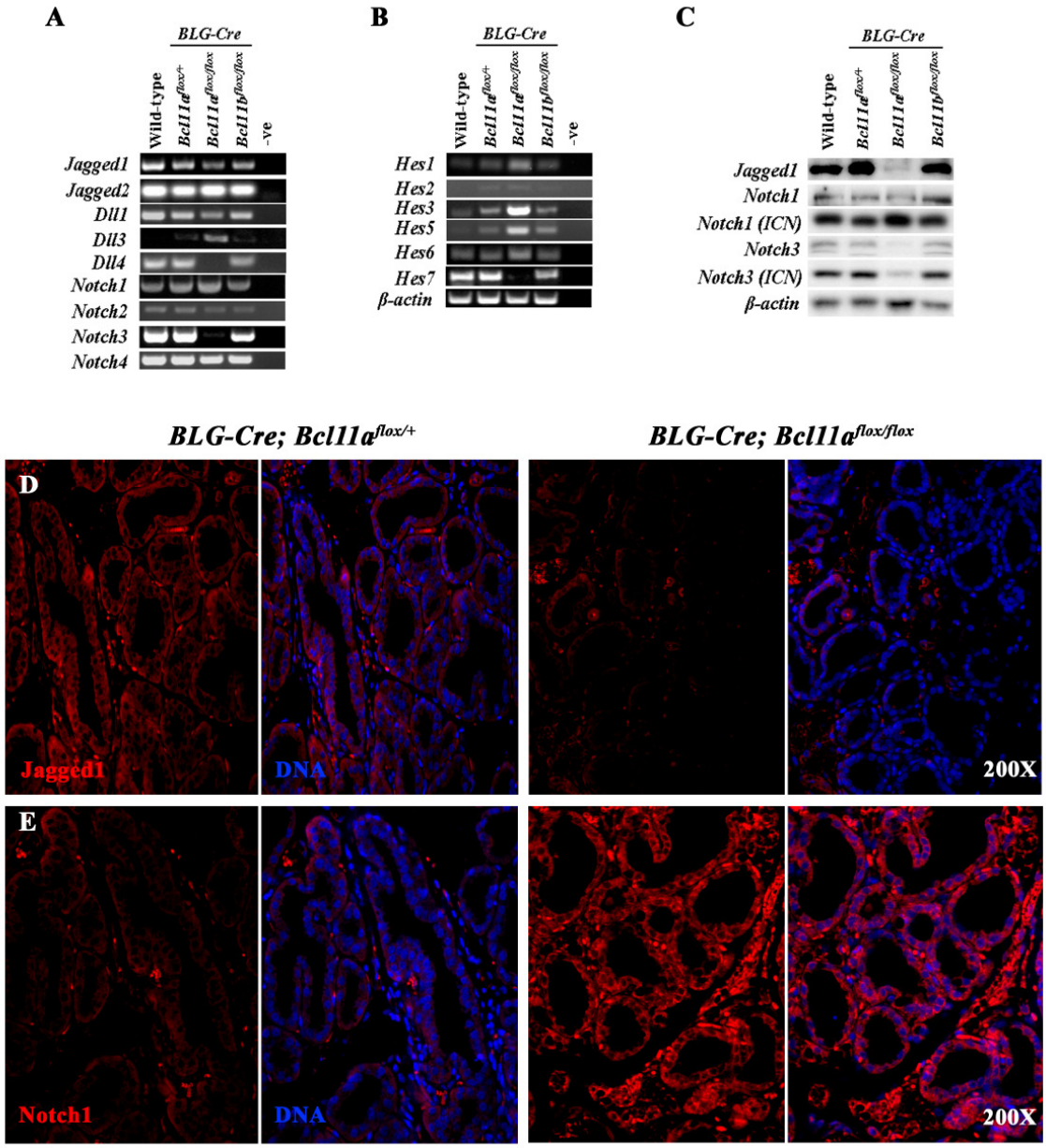


Figure 6.12. Analysis of Notch signalling pathway transcripts and protein levels in *BLG-Cre; Bcl11$^{flox/flox}$* and control females. (A-B) RT-PCR analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11$^{flox/+}$*) and mutant (*BLG-Cre*; *Bcl11$^{flox/flox}$*) females using primers to **(A)** *Notch* receptors/ligands and **(B)** *Hes*. β-actin is used as a control. –ve indicates no template control. **(C)** Immunoblot analysis of day 2 lactation mammary glands from control (*BLG-Cre*; *Bcl11$^{flox/+}$*) and mutant (*BLG-Cre*; *Bcl11$^{flox/flox}$*) females using antibodies to Notch1, Notch3 and Jagged1. β-actin is used as a loading control. **(D-E)** Immunostaining of day 2 lactation mammary glands from *BLG-Cre; Bcl11a$^{flox/+}$* and *BLG-Cre; Bcl11a$^{flox/flox}$* females using antibodies to **(D)** Jagged1 and **(E)** Notch1. Note: The loading control panel for the immunoblot was the same as that in Figure 6.7B as experiments were carried out using the same protein samples.

6.3 Discussion

In this Chapter, I showed that *Bcl11a* is critical for the maintenance of terminally differentiated luminal secretory cells. Expression of *Bcl11a* was detected in the luminal layer of lobulo-alveoli while expression of *Bcl11b* was virtually undetected during lactation. To delete *Bcl11a* and *Bcl11b* specifically in the luminal layers during lactation, I used the BLG-Cre mice (Selbert et al., 1998). Expression of BLG-Cre is low in the virgin mice but increases throughout gestation and is highest during lactation. Histological analyses of mammary glands from *BLG-Cre*; *Bcl11*$^{flox/flox}$ females at late gestation showed the presence of lobulo-alveolar structures with lipid droplets and secretory vesicles, suggesting that development and differentiation of alveoli during gestation was not significantly affected.

6.3.1 *Bcl11a* maintains terminally differentiated luminal secretory cells

Deletion of *Bcl11a* during lactation resulted in severe lactational defects in the mammary glands. *BLG-Cre*; *Bcl11a*$^{flox/flox}$ females were unable to nurse their pups. These pups had little or no milk in their stomach from P1, were severely dehydrated and usually died by P7 if not rescued. Consistent with the undetectable *Bcl11b* expression during lactation, the *BLG-Cre*; *Bcl11b*$^{flox/flox}$ females were able to nurse their pups and histological analyses showed no obvious lactational defects in the mammary glands. Histological analyses of lactation mammary glands from *BLG-Cre*; *Bcl11a*$^{flox/flox}$ females showed a drastic reduction in the number and size of lobulo-alveoli compared to the control females. The mutant lobulo-alveoli had small lumens and contained fewer fat droplets and secretory vesicles. Moreover, numerous collapsed alveolar structures were present within the *Bcl11a*-deficient glands. Severe lactational defects were exemplified by the decrease in transcripts of milk protein genes and the complete absence of p-Stat5 and WAP in the lactation glands. I found that a premature onset of involution had occurred in the *Bcl11a*–deficient lactation gland, which was confirmed by expression of involution genes and activation of Stat3-mediated apoptosis. Surprisingly, the levels of the key luminal differentiation transcription factor, *Gata-3* were not affected in the *Bcl11a*-deficient lactation glands, suggesting that physiological levels of *Gata-3* was

unable to compensate for loss of *Bcl11a*. This also suggests that Bcl11a and Gata-3 may function in distinct luminal progenitor populations. On the other hand, deletion of *Bcl11a* did result in a reduction in *Elf5* expression. This, together with the earlier observations that *Bcl11a* was expressed and necessary in the $Sca1^-$ progenitors (Chapter 4.19B and Appendix A.8), indicate that *Bcl11a* is likely to be expressed in the alveolar progenitors together with *Elf5* but not *Gata-3*. Loss of *Bcl11a* would thus lead to depletion of these alveolar progenitors and their differentiated derivatives. In addition, Bcl11a appeared to suppress *Bcl11b* in the lactation gland as loss of a single copy of *Bcl11a* resulted in up-regulation of *Bcl11b* expression. These results thus demonstrate that Gata-3 is not a key player in specifying and maintaining the alveolar secretory cell fate and that *Bcl11a* is essential for the maintenance of terminally differentiated luminal secretory cells.

6.3.2 Implications of different Notch signalling pathways

The Notch signalling pathway plays a pivotal role in several cell functions; such as cell fate decision, proliferation, differentiation, and cell death during development (Artavanis-Tsakonas et al., 1999; Radtke et al., 2004). Notch signalling is thought to be required for luminal cell development and lineage maintenance (Buono et al., 2006). However over-expression of activated *Notch1*, *Notch3* or *Notch4/Int3* in the mammary epithelium blocked or delayed alveologenesis and resulted in mammary tumour formation (Gallahan et al., 1996; Hu et al., 2006). This apparent discrepancy regarding the role of Notch in mammary development can be explained by the fact that Notch signalling is dosage-sensitive. For example, Notch is essential for T-cell development. However, abnormal high levels of Notch signalling block normal thymic T-cell development (Rothenberg, 2007a). Thus, these results suggest that regulating the levels of Notch signalling is critical to the balance between normal mammary development and tumorigenesis.

Our lab has shown that loss of *Bcl11a* in lymphocytes resulted in up-regulation of *Notch1* transcripts (Liu et al., 2003b). This study herein revealed that loss of *Bcl11a* in the lactation gland resulted in an increase in the levels of *Notch1* transcripts and activated Notch1 intracellular domain (ICN) as well as up-regulation of downstream targets of *Notch1* such as the *Hes* genes. An unexpected finding is that Bcl11a differentially

regulates Notch signalling in the mammary gland. In contrast to the up-regulation of Notch1, deletion of *Bcl11a* caused the loss of Notch3 in the lactation gland. Notch3 has been shown to promote lobulo-alveolar development during late pregnancy (Hu et al., 2006) and also plays a distinct role from NOTCH1 in ERBB2 negative breast cancer (Yamaguchi et al., 2008). In addition, Notch3 has been shown to be critical for the commitment of bi-potent progenitors to the luminal lineage *in vitro* (Raouf et al., 2008). In addition, expression of *Dll4* and *Hes7*, were completely absent in the *Bcl11a* mutant lactation mammary epithelium. Further work is required to address the function of each of these Notch components in normal mammary gland development. Nevertheless, my current data clearly implies that Notch1 and Notch3 may play distinct roles in luminal differentiation and maintenance of terminal differentiated luminal cell state and also highlight the diverse roles played by different components of Notch signalling pathway in mammary development.

6.3.3 Proposed working model of *Bcl11* genes during lactation

A precise regulation of the spatial and temporal control of *Bcl11a* and *Bcl11b* is required for normal mammary development. Up-regulation of *Bcl11a* and down-regulation of *Bcl11b* is required for terminal differentiation of secretory luminal cells during gestation and lactation. The JAK-Stat pathway plays critical roles during mammary gland development. During pregnancy and lactation, Stat5 is a key mediator of prolactin signalling, which is required for lobulo-alveolar development and lactogenesis (Cui et al., 2004; Liu et al., 1997). In contrast, Stat3-mediated apoptosis is essential for the involution process that removes redundant alveolar structures (Chapman et al., 1999). Thus Stat5 and Stat3 play reciprocal functions in the mammary gland and the balance of phosphorylated Stat5 and Stat3 levels is critical to the normal function of the gland. This study has demonstrated that *Bcl11a* prevents premature onset of involution in the lactation gland. Loss of *Bcl11a* thus led to the loss of p-Stat5 and gain of p-Stat3 positive luminal cells. My data also revealed that *Bcl11a* maintains secretory luminal cell fate by differentially regulating the Notch1 and Notch3 signalling pathways which may in turn regulate the levels of Stat3 and Stat5 during lactation (Figure 6.13). Loss of *Bcl11a* resulted in the absence of *Jagged1/Dll4/Notch3/Hes7* and ectopic expression of

Dll3/Notch1/Hes1/3/5/6 that cumulatively caused the activation of Stat3-mediated apoptosis in the lactation gland. Formal proof of the direct regulation and the functional consequences require further molecular studies and analyses of mutant mice that have gain or loss of different components of the Notch pathway. Unexpectedly, Gata-3, a key luminal transcription factor is not affected by loss of *Bcl11a*. This suggests that *Bcl11a* and *Gata-3* are likely to be expressed in distinct alveolar cell population. In conclusion, the results in Chapter 6 demonstrate that *Bcl11a* is essential for the maintenance of secretory luminal cell fate in the lactation mammary gland.

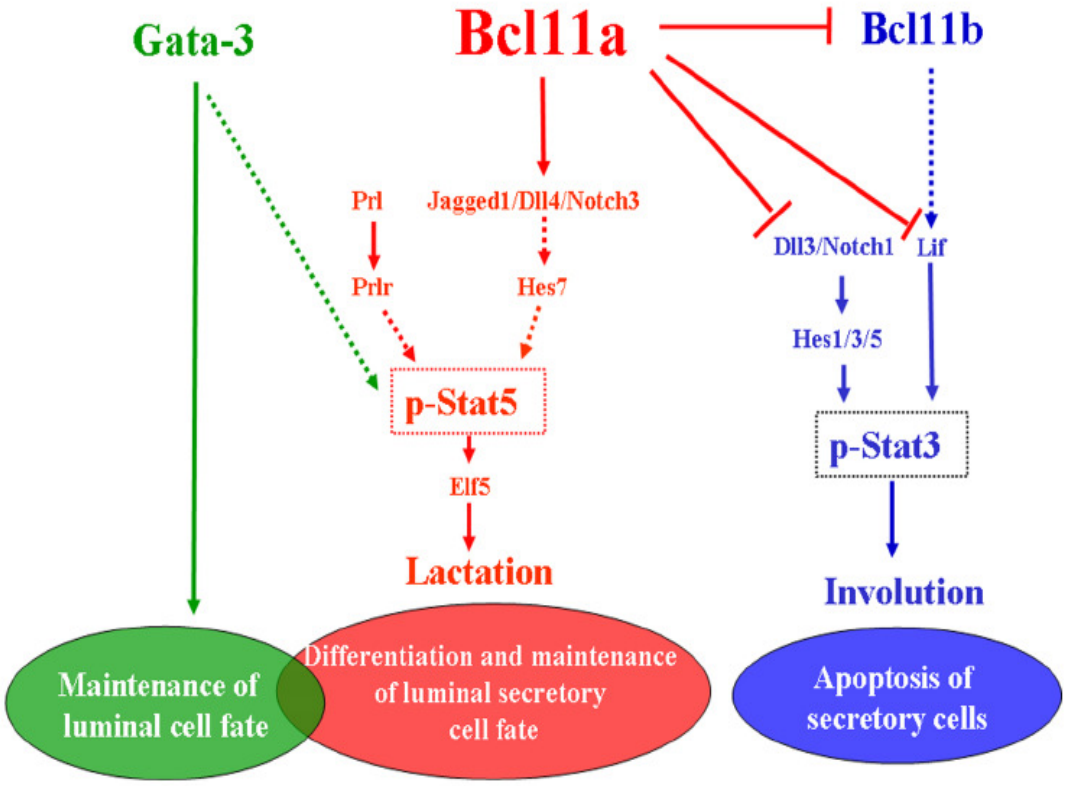


Figure 6.13. Proposed working model of the roles of *Bcl11* genes in mammary lineages. Putative model for role of *Bcl11a* in maintenance of secretory luminal cell fate in relationship to Notch and JAK-Stat pathways.