

# Nucling, a Novel Apoptosis-associated Protein, Controls Mammary Gland Involution by Regulating NF- $\kappa$ B and STAT3\*

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**Background:** Nucling is identified as a novel regulator of apoptosis, but its roles in mammary gland remains unknown.

**Results:** Loss of Nucling led to an inhibited apoptosis and impaired mammary gland involution.

**Conclusion:** Nucling controlled NF- $\kappa$ B and STAT3 activities to mediate involution.

**Significance:** Elucidation of the physiological role of Nucling in this process may provide useful insight into breast cancer therapy.

Postpartum mammary gland involution is the physiological process by which the lactating gland returns to its pre-pregnant state. In rodent models, the microenvironment of mammary gland involution is sufficient to induce enhanced tumor cell growth, local invasion, and metastasis. Therefore, a deeper understanding of the physiological regulation of involution may provide in-depth information on breast cancer therapy. We herein identified Nucling as an important regulator of involution of the mammary gland. A knock-out mouse model was generated and revealed that postpartum involution were impaired in mice lacking Nucling. Involution is normally associated with an increase in the activation of NF- $\kappa$ B and STAT3, which is required for the organized regulation of involution, and was observed in WT glands, but not in the absence of Nucling. Furthermore, the loss of Nucling led to the suppression of Calpain-1, IL-6, and C/EBP $\delta$  factors, which are known to be essential for normal involution. The number of M2 macrophages, which are crucial for epithelial cell death and adipocyte repopulation after weaning, was also reduced in Nucling-KO glands. Taken together, the results of the present study demonstrated that Nucling played an important role in mammary gland involution by regulating NF- $\kappa$ B and STAT3 signaling pathways.

Mammary gland development continues undergoing dynamic changes after puberty with each cycle of pregnancy, lactation, and involution (1). Involution involves the regression of the lactation stage after weaning, and has been described as a two-step process. The first phase lasts 48 h, starts immediately upon weaning, and is associated with apoptosis and shedding cells in the lumen. The second phase begins 48 h after weaning and is characterized by marked changes in mammary gland architecture including the collapse of the lobular-alveolar

structure and repopulation of adipocytes (2, 3). During the first stage of involution, milk stasis has been shown to induce the expression and secretion of the pro-inflammatory cytokine leukemia inhibitory factor (LIF).<sup>4</sup> LIF binds to the heterodimer of the LIF receptor and gp130. The signal-transducing subunit gp130 is responsible for the intracellular activation of the JAK/STAT3 signaling pathway, which is indispensable for the initiation of involution (4–6). In the absence of STAT3, apoptosis was found to be markedly repressed and the first phase was abolished (7, 8). However, the activation of STAT3 is insufficient to induce involution in the absence of NF- $\kappa$ B signaling. During mammary gland development, the activation of NF- $\kappa$ B increases during pregnancy, decreases during lactation, and then increases again after weaning (9, 10). The loss of NF- $\kappa$ B signaling has been shown to result in a decrease in caspase-3 cleavage and delayed involution (11). In contrast, a previous study demonstrated that constitutively active IKK- $\beta$  increased NF- $\kappa$ B activation, leading to a reduction in milk protein levels and increases the level of apoptosis during involution (10).

Phagocytosis is a crucial contributor to the remodeling process and removal of cell debris and residual milk in the second phase of involution (12, 13). Furthermore, a recent study demonstrated that mammary gland involution was characterized by the recruitment of macrophages with M2 characteristics, which is crucial for epithelial cell death and adipocyte repopulation (14).

Nucling was originally isolated from murine embryonic carcinoma cells as a novel protein, and its up-regulated expression was observed during cardiac muscle differentiation (15). Nucling was also shown to recruit and transport the Apaf1-pro-caspase-9-apoptosome complex during stress-induced apoptosis (16, 17). In addition, we previously reported that Nucling mediated apoptosis by suppressing the expression of the anti-apoptotic molecule galectin-3 via NF- $\kappa$ B signaling (18). Apoptosis has been identified as a crucial part of involution in the mammary gland (13). However, it currently remains unknown whether Nucling controls apoptosis in mammary gland involution.

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<sup>4</sup> The abbreviations used are: LIF, leukemia inhibitory factor; Sim2s, Single-minded-2s; C/EBP $\delta$ , CAAT/enhancer binding protein  $\delta$ .

Previous studies reported that Nucling negatively regulated the translocation of NF- $\kappa$ B into the nucleus (19, 20, 21). However, Nucling is not only a suppressor of NF- $\kappa$ B in resting cells, but is also an up-regulator of NF- $\kappa$ B in stimulated cells (20). Furthermore, NF- $\kappa$ B signaling is required for normal mammary gland involution; therefore, it has not yet been determined whether Nucling is a suppressor or inducer of the activation of NF- $\kappa$ B in this context. We previously demonstrated that Nucling controlled the population of Kupffer cells in the liver, which act as phagocytes or antigen-presenting cells (22). However, the influence of Nucling on macrophage recruitment in mammary gland involution has not yet been elucidated.

Postpartum mammary gland involution has been identified as tumor promotional and is proposed to contribute to increased rates of metastasis and poor survival in postpartum breast cancer patients (23). Therefore, a deeper understanding of the regulation of involution is important for breast cancer therapy. The potential contribution of Nucling to mammary gland physiology has not yet been examined in detail. Therefore, the aim of the present study was to investigate the physiological role of Nucling in mammary gland involution and elucidate the possible signaling pathway. We demonstrated that Nucling was strongly expressed during involution, and the loss of Nucling resulted in delayed involution. Furthermore, mammary gland involution was controlled by Nucling through the regulation of crucial NF- $\kappa$ B and STAT3 signaling pathways.

## Experimental Procedures

**Induction of Involution and Tissue Processing**—Nucling-KO mice were described previously (16). Adult female mice (12–15 weeks of age) were mated. The induction of involution has been previously described (24). The thoracic glands were snap-frozen in nitrogen liquid to extract RNA and protein for quantitative real-time PCR and Western blotting. The inguinal glands were fixed in 4% paraformaldehyde and embedded in paraffin. The embedded tissues were sectioned at a thickness of 5  $\mu$ m for staining with hematoxylin/eosin, tunnel assays, and immunofluorescence.

**Whole Mount Carmine-stained Mammary Glands**—Inguinal mammary glands were fixed in Carnoy's solution, stained in carmine (Sigma catalog number C-1022) solution overnight, rinsed in alcohol, cleared in xylene, and mounted with permount under a coverslip. Whole mount images were taken using a Multizoom AZ100M microscope with an Imaging Software NIS-Elements (Nikon, Japan).

**Quantitative Real-time PCR**—Total RNA for relative quantification was isolated using Isogen (Nippogene catalog number 211-02501) following the manufacturer's recommendations. First-strand cDNA was synthesized using a Superscript VILO<sup>TM</sup> cDNA Synthesis Kit (Invitrogen catalog number 11754-050). Quantitative RT-PCR was performed with SYBR Premix Ex Taq<sup>TM</sup> II (Takara) using a 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. GAPDH used as an internal control. The following primers were used: IL-6, 5'-TCCATCCAGTTGCCCTTCT-TGG-3' and 5'-TCTGCAAGTGCATCATCGTTG-3';  $\beta$ -casein, 5'-GCTCAGGCTCAAACCATCTC-3' and 5'-TGTGG-AAGGAAGGGTGTCTAC-3'; *Calpain-1*, 5'-ACCACATTTTA-

CGAGGGCAC-3' and 5'-GGATCTTGAAGTGGGGTTT-3'; *C/EBP $\delta$* , 5'-ACCCGCGGCCTTCTACGA-3' and 5'-GCGCCCTTTTCTCGGACTGT-3'; *LIF*, 5'-GGCTCTGGA-ACCTTGGGCAAACACTG-3' and 5'-GCCTGCACTGCTCC-AACCTCCTGTA-3'; *gp-130*, 5'-TCGGAGGAGCGGCCAGA-AGAC-3' and 5'-ATCAGCCCCCGTGCCAAGAGC-3'; Sim2s, 5'-AACCAGCTCCCGTGTTTTGAC-3' and 5'-CTCTGAGGA-ACGGCGAAAA-3'; GAPDH, 5'-TGTGTCCGTCGTGGATC-TGA-3' and 5'-CCTGCTTACCACCTTCTTGA-3'.

**Tunnel Staining**—Paraffin-embedded sections were dewaxed, rehydrated, and stained according to the manufacturer's protocol of the In Situ Cell Death Detection Kit with fluorescein (catalog number 11-684-795-910; Roche Applied Science).

**Immunofluorescence**—Slides were deparaffinized with xylene and dehydrated with graded alcohol, and boiled for 15 min in 10 mM sodium citrate, pH 6.0. The sections were blocked with 5% normal goat serum and incubated with rabbit anti-mouse arginase-1 antibody (ab91279), and rabbit anti-mouse Ym1 antibody (ab93034) at 4  $^{\circ}$ C for 24 h. The antibodies were diluted following the manufacturer's recommendations. The sections were then incubated with Alexa Fluor 594 goat anti-rabbit IgG antibody (Invitrogen), 1:400, diluted in blocking solution for 1 h at room temperature. Slides were coverslipped in mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA). Images were viewed by using BZ-9000 Fluorescence Microscope (KEYENCE Corp. of America).

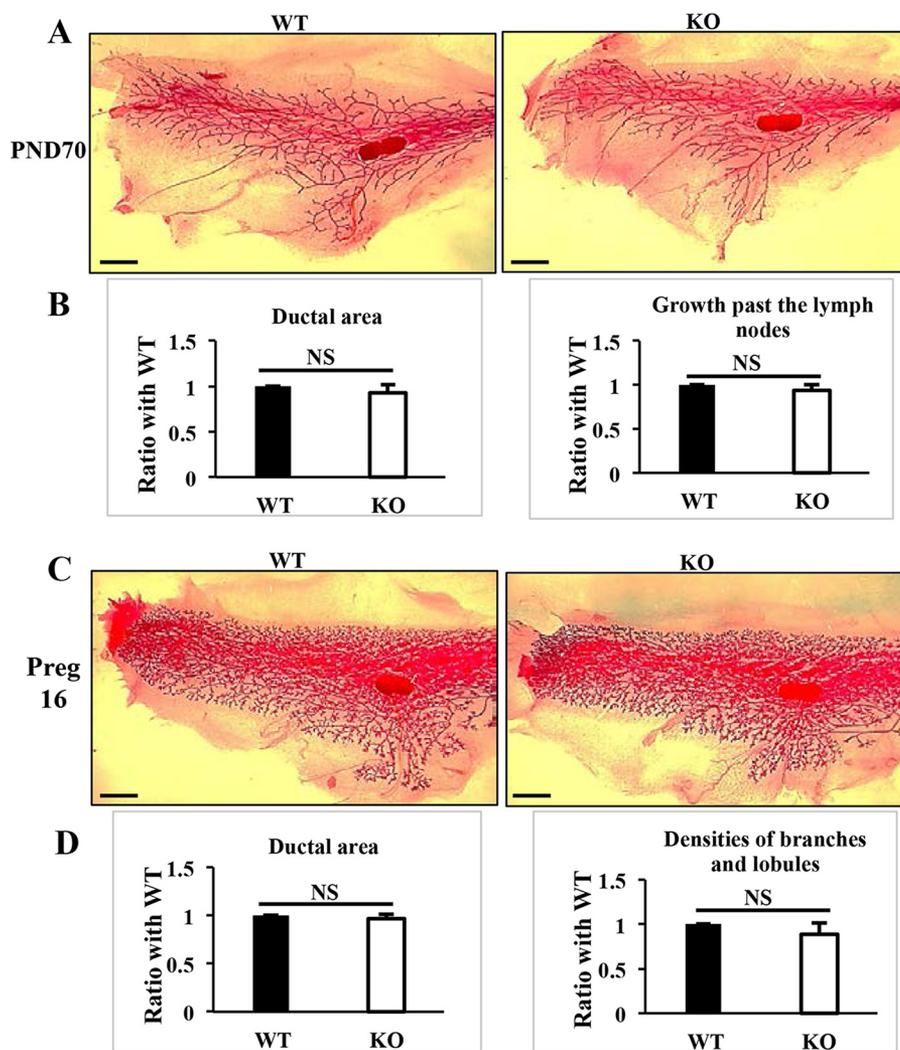
**Protein Isolation and Western Blotting**—Total protein was extracted from snap-frozen glands by homogenization in 2 ml of extraction buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate) and 1 mM phenylmethylsulfonyl fluoride with a protease inhibitor (catalog number 05892970001, Roche) and phosphatase inhibitor (catalog number 04960845001, Roche). Proteins were isolated as described previously (25). Protein concentrations were determined using the BCA<sup>TM</sup> Protein Assay Kit (Pierce). Western blotting was performed as described previously (26). The following antibodies were used: anti-mouse middle portion of Nucling (m.Nucl.mid) (16); a phospho-NF- $\kappa$ B-p65 (Ser-468) antibody (Cell Signaling); a phospho-STAT3 (Tyr-705) mouse antibody (Cell Signaling); caspase-3 antibody (Santa Cruz); a STAT3 antibody (sc482 Santa Cruz); anti-liver Arginase antibody (ab91279, Abcam); monoclonal anti- $\beta$ -actin (catalog number A5441, Sigma); HRP anti-mouse IgG (catalog number NA9310, GE Healthcare); and HRP anti-rabbit IgG (catalog number NA934, GE Healthcare).

**Data Analyses**—Images of whole mount carmine staining and immunoblots were quantified using ImageJ software. Relative mRNA levels were quantified by using the  $2^{-\Delta\Delta C_t}$  method. Statistical analyses were conducted using a two-tailed Student's *t* test.

## Results

**Nucling-KO Mice Exhibited Normal Mammary Gland Development, but Defective Involution**—Before addressing the importance of Nucling during involution, we investigated whether this gene was required for mammary gland puberty, pregnancy, and lactation. We analyzed the whole mount mammary gland outgrowths of Nucling-KO and WT animals at 10

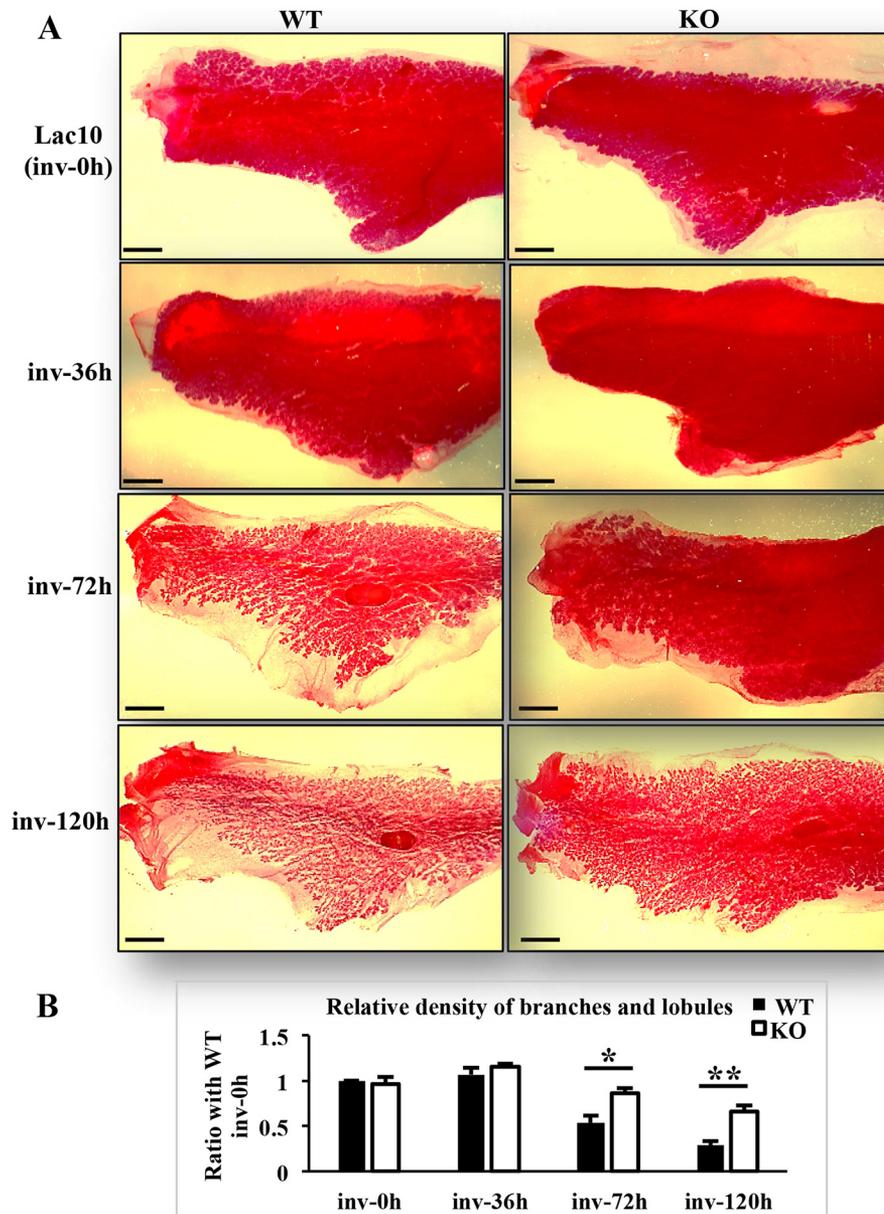
## Nucling Regulates Mammary Gland Involution



**FIGURE 1. Nucling-KO mammary glands showed normal morphologies in pubertal and pregnant mice.** Representative whole mount carmine-stained mammary glands from WT and Nucling-KO mice at (A) PND70 and (C) pregnancy day 16. Scale bars represent 2,000  $\mu\text{m}$ . B, quantification of the ductal area and growth past the lymph node at PND70 (postnatal day 70). D, quantification of the ductal area and the density of branches/lobules at pregnancy day 16. The ductal area was measured as the area occupied by the entire mammary gland at PND70 and pregnancy day 16. The length of growth past the lymph node was measured by determining the distance from the lymph node to the tip of the longest duct. The density of branches/lobules was measured as the density of the area occupied by the entire mammary gland at pregnancy day 16. Data were normalized with those of WT mice and represent mean  $\pm$  S.D. ( $n = 5$  per genotype, per age group). NS, not significant.

weeks and confirmed that the loss of Nucling did not affect mammary gland development in pubertal mice (Fig. 1, A and B). Furthermore, whole mount analyses of Nucling-KO mice showed a normal pregnancy, as indicated by similar ductal areas and densities of branches/lobules at pregnancy to those of WT mice (Fig. 1, C and D). To exclude the possibility that Nucling was essential for lactation, whole mount staining of glands at lactation day 10 demonstrated the identical gross morphologies of Nucling-KO mice and the controls (Fig. 2A). Furthermore, pups derived from Nucling-KO females were stable and reached adulthood without any signs of a decrease in weight or health. These results confirmed the normal lactation of Nucling-KO mice. However, a decrease in the densities of branches/lobules were slower in Nucling-KO glands than in WT glands 72 and 120 h after weaning (Fig. 2, A and B). This result suggested that Nucling may play an important role in the regulation of mammary gland involution.

*Nucling-KO Mammary Glands Displayed Delayed Involution*—As Nucling affected the regression of mammary glands after weaning, we analyzed histological changes in Nucling-KO glands using H&E staining. No significant differences were observed in the histoarchitectural morphology of lactating glands in Nucling-KO mice and WT mice. However, the loss of Nucling had marked effects on involution. Fewer epithelium cells were shed into the lumen in Nucling-KO mice at 36 h of involution (Fig. 3A). As expected, the delayed involution observed in Nucling-KO mice correlated with a significantly lower number of apoptotic epithelial cells at 36 h of involution, as assessed by a TUNEL assay (Fig. 3, B and C). After 72 h of weaning, WT mammary glands had undergone extensive tissue remodeling, characterized by the collapse of the lobular-alveolar structure. In contrast, open alveolar structures persisted and the reappearance of adipocytes was slower in Nucling-KO mice than in WT mice (Fig. 3A). This was accompanied by a decrease



**FIGURE 2. Nucling-KO mammary glands displayed a defective morphology for postlactational involution.** *A*, representative whole mount carmine-stained mammary glands from WT and Nucling-KO mice at lactation day 10 (involution 0 h) and at 36, 72, and 120 h of involution. Scale bars indicate 2,000  $\mu\text{m}$ . *B*, quantification of the density of branches/lobules in the mammary gland. The density of branches/lobules was measured as the density of area occupied by the entire mammary gland at 0, 36, 72, and 120 h of involution. Data were normalized with WT mice at 0 h of involution and represent mean  $\pm$  S.D. ( $n = 5$  per genotype, per age group); \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

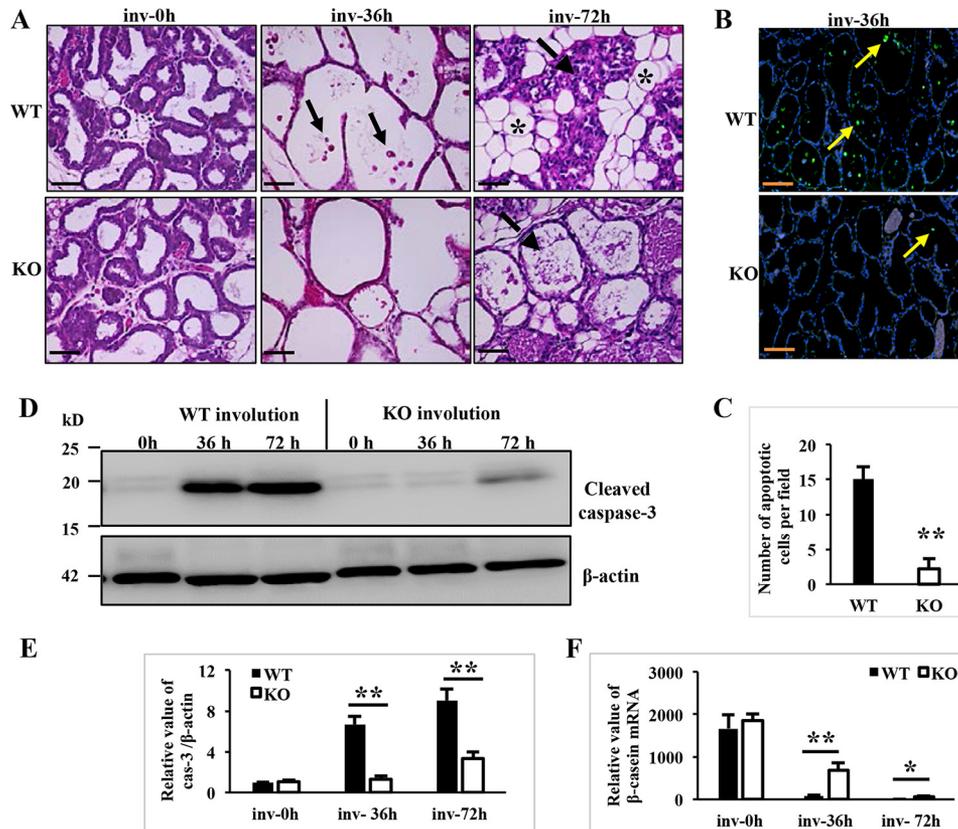
in the presence of cleaved caspase-3 in Nucling-KO mice (Fig. 3, *D* and *E*). The other indication of mammary gland involution is a reduction in milk production. Therefore, RT-PCR was performed to determine whether Nucling affected the expression of  $\beta$ -casein mRNA after weaning. Reductions in  $\beta$ -casein mRNA levels in mammary glands occurred later in Nucling-KO mice than in WT mice (Fig. 3*F*). Taken together, these results showed that the loss of Nucling during involution delayed involution of the mammary gland.

**Overexpression of Nucling during Mammary Gland Involution**—Because Nucling is important for the regulation of involution, we attempted to elucidate how Nucling is expressed in the mammary gland. Therefore, the expression of Nucling was examined in the mammary gland at various developmental

stages by Western blotting. As shown in Fig. 4, *A* and *B*, Nucling was weakly expressed in pubertal glands. Its expression was up-regulated in pregnancy, down-regulated in the lactation stage, and increased after the glands underwent involution.

**The NF- $\kappa$ B Signaling Pathway Was Impaired during Mammary Gland Involution in Nucling-KO Mice**—We found that Nucling was strongly expressed during involution (Fig. 4, *A* and *B*). Therefore, in an attempt to reveal the molecular mechanisms underlying delayed involution in Nucling-KO mice, we investigated the influence of Nucling on NF- $\kappa$ B activity by analyzing the phosphorylation levels of NF- $\kappa$ B-p65 in WT and Nucling-KO glands using Western blotting. The results obtained showed that the level of phosphorylated NF- $\kappa$ B-p65 was lower in Nucling-KO mice than in WT during involution

## Nucling Regulates Mammary Gland Involution



**FIGURE 3. Loss of Nucling resulted in delayed involution.** *A*, H&E-stained mammary sections from WT and Nucling-KO mice at 0, 36, and 72 h of involution. *Black arrows* indicate the shedding of epithelial cells into the alveolar lumen at 36 h of involution. *Dashed arrows* show the lobular-alveolar structure. *Asterisks* represented adipocytes. *Scale bars* indicate 100  $\mu$ m. *B*, TUNEL-stained mammary gland sections from WT and Nucling-KO mice at 36 h of involution. *Yellow arrows* represented apoptotic epithelial cells. *Scale bars* indicate 300  $\mu$ m. *C*, quantification of apoptotic cells in TUNEL-stained mammary gland sections. The number of TUNEL-positive cells in 5 random fields at  $\times 200$  magnification was counted. *D*, Western blot analysis of cleaved caspase-3 expression in WT and Nucling-KO mammary glands at 0, 36, and 72 h of involution. *E*, quantification of immunoblots for cleaved caspase-3/ $\beta$ -actin. The *bar graph* shows the relative density of the bands normalized to WT at 0 h of involution. *F*, quantification of the mRNA expression of  $\beta$ -casein in the mammary glands of WT and Nucling-KO mice at 0, 36, and 72 h of involution by quantitative RT-PCR. Data were normalized with those of WT mice at involution of 72 h and represent mean  $\pm$  S.D. Data were represented as mean  $\pm$  S.D. ( $n = 3$  per genotype, per age group). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

(Fig. 4, *C* and *D*). We next attempted to determine whether this suppression correlated with a decrease in the expression of NF- $\kappa$ B target genes, which are known to play a role during involution. When we assessed IL-6 and Calpain-1 mRNA levels by RT-PCR, we found a decrease in gene expression in Nucling-KO glands (Fig. 4, *E* and *F*). These results suggested that delayed involution in Nucling-KO mice was mediated, at least in part, by the Nucling-dependent regulation of the NF- $\kappa$ B signaling pathway.

**Reduction in M2 Macrophage Recruitment in Mammary Gland Involution in Nucling-KO Mice**—Macrophages with an alternatively activated M2 phenotype have been shown to play a role in involution (14). We also previously reported that Nucling affected the number of Kupffer cells in the liver (22). Therefore, we attempted to elucidate the contribution of Nucling to M2 macrophage recruitment during mammary gland involution. To achieve this, we examined the expression of Arginase-1 and Ym1, markers of M2 macrophages (14, 27). At 36 h of involution, protein levels of Arginase-1 were similar in WT and Nucling-KO mice. However, after 72 h of weaning, Nucling-KO glands expressed a significantly lower level of Arginase-1 than WT glands (Fig. 5, *A* and *B*). Then, to determine the fluctuations in M2 macrophage numbers within the mammary gland, we performed immunofluorescence analyses

on mammary gland tissue with Arginase-1 and Ym1. We found that there was a significantly fewer number of Arginase-1 and Ym1 positive cells per unit area in Nucling-KO mice than in WT mice at 72 h of involution (Fig. 5, *C–F*). These results suggested that Nucling directly or indirectly influenced the number of M2 macrophages in the mammary gland during the second phase of involution.

**The Loss of Nucling Blocked STAT3 Activation by Repressing gp130 Expression**—A recent study reported that STAT3 activity induced a M2 macrophage population during involution (27). Furthermore, the activation of STAT3 after weaning was required for normal mammary gland involution (7, 8). Therefore, the phosphorylation levels of STAT3 was evaluated by Western blotting to determine whether a decline in the number of M2 macrophages in Nucling-KO mice was related to a decrease in the activation of STAT3. Although the expression of STAT3 in WT mice was similar to that in Nucling-KO mice during involution (Fig. 6, *A*, *top panel*, and *B*), the loss of Nucling resulted in the suppression of STAT3 phosphorylation during involution (Fig. 6*A*, *lower panel*). The quantification of *p*-STAT3 revealed a significant decrease in the Nucling-KO gland at 36 and 72 h of involution, illustrating the activation of STAT3 by Nucling (Fig. 6*C*). This result was also confirmed by examining the mRNA expression level of C/EBP $\delta$ , which is a

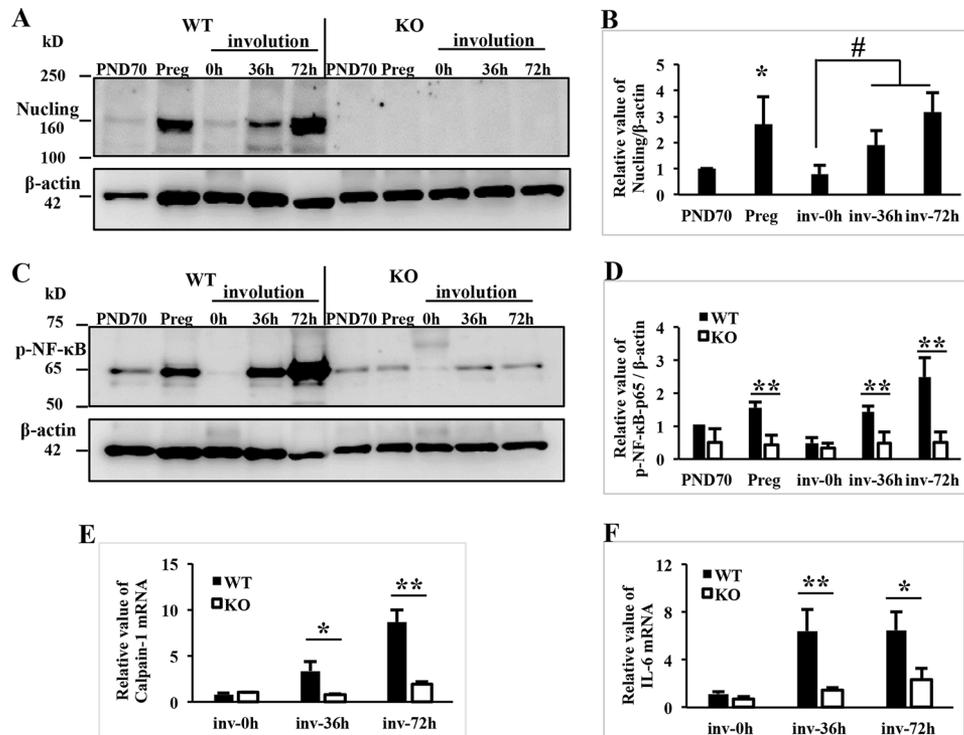


FIGURE 4. **NF- $\kappa$ B signaling was impaired during involution in Nucling-KO mice.** *A*, Western blot analysis of protein extracted from the mammary glands of WT and Nucling-KO mice at postnatal day (PND) 70, pregnancy day 16, and at 0, 36, and 72 h of involution was performed using antibodies against Nucling. *B*, quantification of immunoblots for Nucling/ $\beta$ -actin. The bar graph showed the relative density of the bands normalized to PND70 of WT. *C*, Western blot analyses of *p*-NF- $\kappa$ B-p65 expression during mammary gland development and involution. Protein of the total cell (include cytosolic and nuclear fractions) was extracted from the mammary gland of WT and Nucling-KO mice at PND70, pregnancy day 16, and at 0, 36, and 72 h of involution. *D*, quantification of immunoblots for *p*-NF- $\kappa$ B-p65/ $\beta$ -actin. The bar graph showed the relative density of the bands normalized to WT PND70. *E*, A quantitative RT-PCR analysis of (*E*) Calpain-1 and (*F*) IL-6 mRNA expression at 0, 36, and 72 h of involution is shown. Data were normalized with those of WT mice at 0 h of involution and represent mean  $\pm$  S.D. Data were represented as mean  $\pm$  S.D. ( $n = 3$  per genotype, per age group). \*,  $p < 0.05$ ; #,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

common target gene of STAT3. *C/EBP $\delta$*  mRNA levels were lower in Nucling-KO than in WT mice (Fig. 6*D*). Taken together, these results suggested that delayed involution in Nucling-KO mice was mediated not only through the regulation of NF- $\kappa$ B, but also STAT3 activity.

To elucidate the mechanism through which the loss of Nucling inhibited the activation of STAT3, the expression of LIF was examined in mammary glands. As indicated in Fig. 7*A*, LIF mRNA expression was induced after weaning in WT and Nucling-KO mice. No significant differences were observed in LIF mRNA levels at 36 h of involution between WT and Nucling-KO. LIF mRNA expression levels were significantly higher in Nucling-KO mice than in WT mice after 72 h of weaning. This result suggested that the suppression of STAT3 activity in Nucling-KO mice was independent of LIF expression. Therefore, we continued to investigate the expression of gp130 mRNA because of the interaction between gp130 and the LIF-specific receptor, which is required for the activation of STAT3 (6). We found that gp130 mRNA levels were up-regulated after weaning in WT glands, but not in the absence of Nucling (Fig. 7*B*). Therefore, the inhibition of gp130 expression may be the mechanism through which the activation of STAT3 is blocked in Nucling-KO mice during mammary gland involution.

Furthermore, a previous study reported that Single-minded-2s (*Sim2s*) regulates mouse mammary gland involution by suppression of STAT3 and NF- $\kappa$ B (28). Therefore, RT-PCR were performed to determine whether Nucling affects the

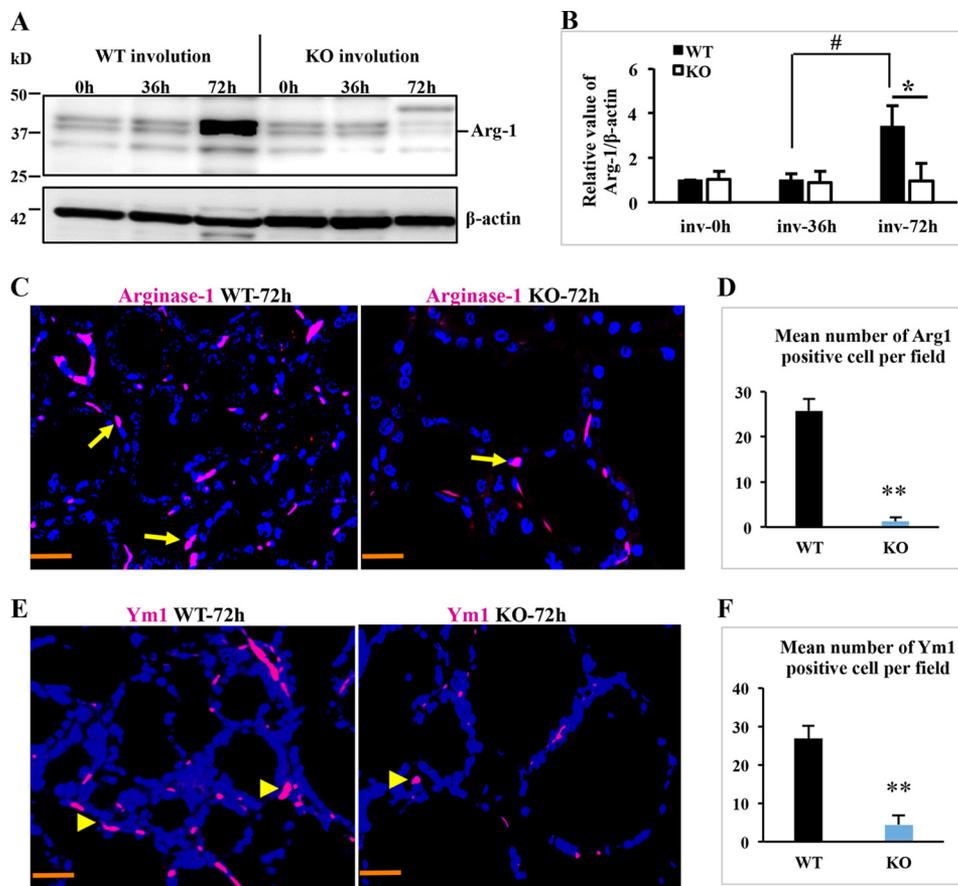
expression of *Sim2s* mRNA after weaning. We found that loss of Nucling did not affect the reductions in *Sim2s* mRNA levels in mammary glands after weaning (Fig. 8). This result suggests that Nucling may be independent from the *Sim2s* signaling pathway in mammary gland involution.

### Discussion

This study is the first to demonstrate that Nucling plays a physiological role in mammary gland involution. Furthermore, we showed that Nucling controlled postlactational involution through not only the regulation of NF- $\kappa$ B, but also STAT3 signaling (Fig. 9).

Mammary gland proliferation is mostly observed in pregnancy (29). NF- $\kappa$ B activity is essential for the proliferation of mammary glands during pregnancy. For example, the activities of NF- $\kappa$ B-p65 and NF- $\kappa$ B-p52 are suppressed in *IKK $\alpha$* -deficient mice, thereby leading to incomplete pregnancies and the lack of lactation (30). Mice lacking RANK also exhibited impaired lobular-alveolar development in pregnancy and defective lactation (31). RANK has been shown to mediate the activation of both classical and alternative NF- $\kappa$ B signaling pathways (32). However, another study reported that the inhibition of *I $\kappa$ B $\alpha$*  phosphorylation in transgenic mice with targeted superrepressor *I $\kappa$ B $\alpha$*  expression decreased NF- $\kappa$ B-p65 activity but did not affect NF- $\kappa$ B-p52 activity, and this mouse model showed the completion of pregnancy development (33). These findings suggest that defective lactation due to preg-

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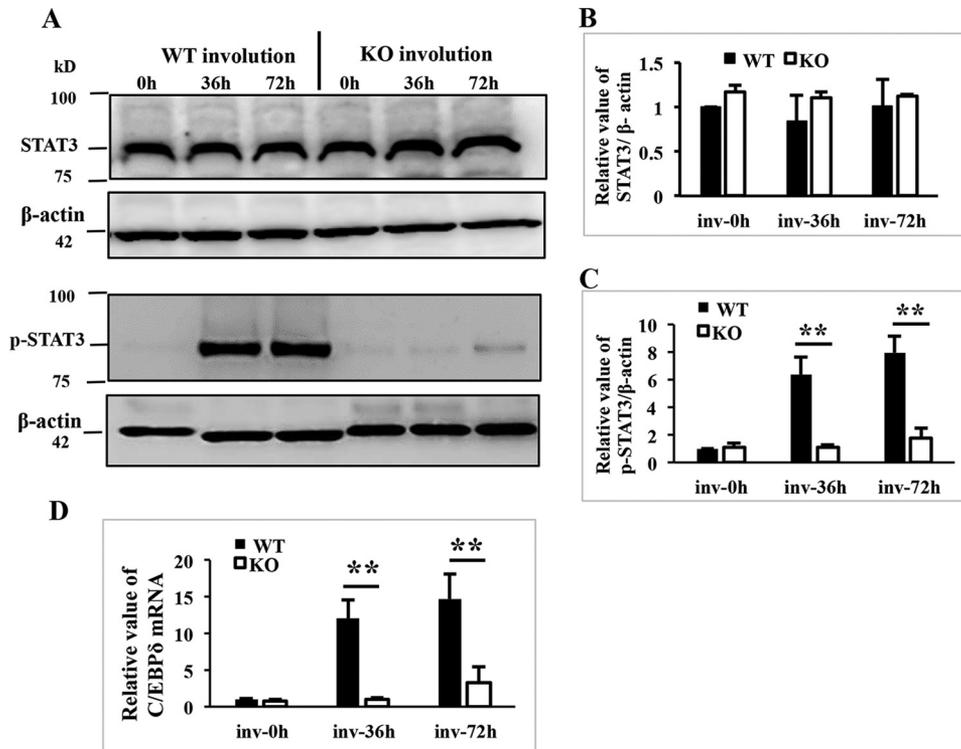
**FIGURE 5. In the absence of Nucling, the number of M2 macrophages was decreased during involution.** *A*, Western blot analysis of Arginase-1 expression during involution. Protein samples were prepared from the mammary glands of WT and Nucling-KO mice at 0, 36, and 72 h of involution. *B*, quantification of immunoblots for Arginase-1/β-actin. The bar graph shows the relative density of the bands normalized to WT at 0 h of involution. Data were represented as mean ± S.D. ( $n = 3$  per genotype, per age group). #,  $p < 0.05$ ; \*,  $p < 0.05$ . Immunofluorescence of Arginase-1 (*C*) and Ym1 (*E*) as markers of M2 macrophages in mammary glands at 72 h of involution. Yellow arrows indicate Arginase-1-positive cells and yellow arrowheads indicate Ym1-positive cells. The scale bar represents 100 μm. Quantification of the number of Arginase-1-positive cells (*D*) and Ym1-positive cells (*F*) in the mammary glands of WT and Nucling-KO mice at 72 h of involution. The number of Arginase-1 and Ym1-positive cells were counted in 6 randomly selected fields of each mice at ×400 magnification; values are mean ± S.D. ( $n = 3$  per genotype, per age group); \*\*,  $p < 0.01$ .

nancy incompleteness occurs if both classical and alternative NF-κB signaling pathways are impaired. In the present study, we found that Nucling-KO mammary glands showed the normal morphologies of pregnancy and lactation; however, the activation of NF-κB-p65 activity was suppressed during pregnancy. Therefore, this result may be attributed to the induction of NF-κB-p52 activity, which may not be affected by the loss of Nucling. Alternately, another mechanism may compensate for the inhibition of NF-κB-p65 activity during pregnancy in Nucling-KO mice, but further investigations are needed to determine this. In the present study, we only focused on the effects of Nucling on postlactational involution of the mammary glands.

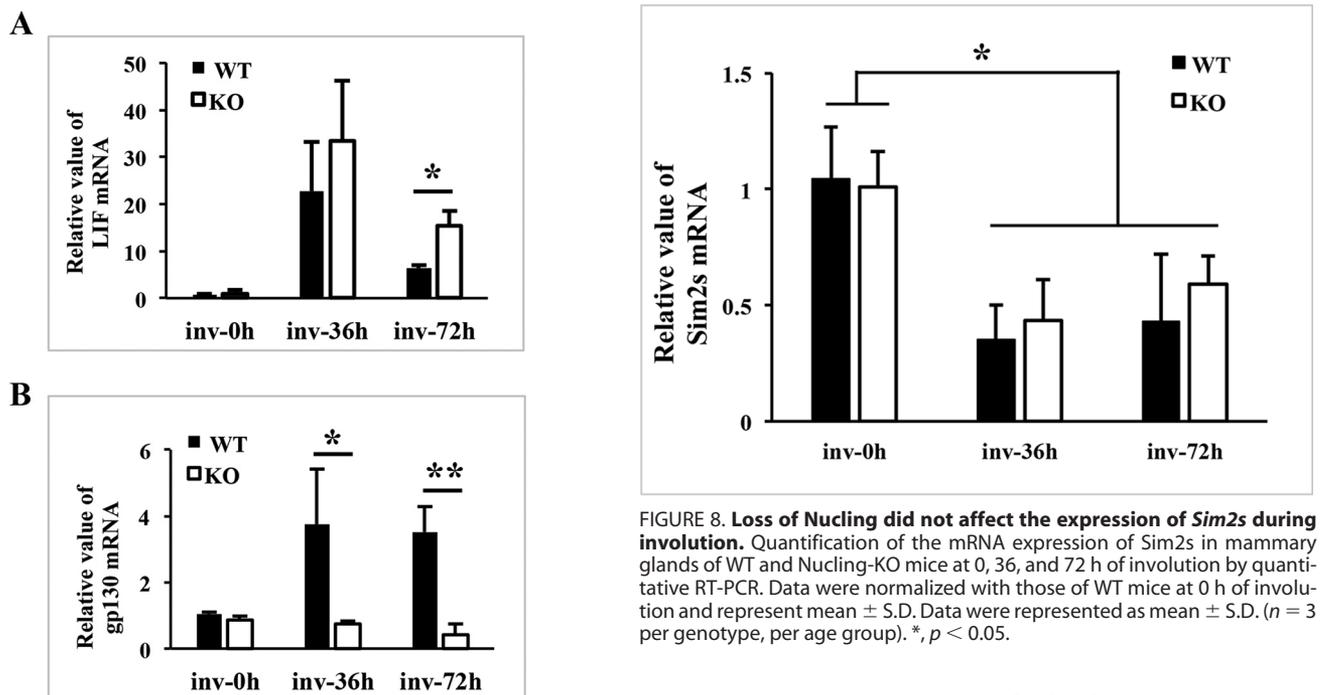
We previously reported that Nucling not only inhibited the activation of NF-κB in resting cells, but, in contrast, also induced the activation of NF-κB in stimulated cells (19, 20). These findings indicated that the effects of Nucling on NF-κB signaling were dependent on the status of the cell. In the present study, we demonstrated that the loss of Nucling inhibited the activation of NF-κB, leading to suppressed expression of NF-κB target genes such as Calpain-1 and IL-6. A previous study reported that NF-κB bound to the promoter region of the Calpain-1 gene and that Calpain-1 mediated epithelial cell death

during mammary gland involution though mitochondrial and lysosomal destabilization (34). IL-6 null mice also exhibited delayed involution and decreased apoptosis (35). We herein found that the pattern of Nucling expression correlated with the activation of NF-κB in the mammary gland from puberty to the involution stage. These results suggest that there is an intimate interaction between Nucling and NF-κB signaling in the mammary gland. We propose that Nucling is one of the target genes of the NF-κB signaling pathway; Nucling is essential for the normal activation of NF-κB, and, together these play an important role in the regulation of postlactational involution.

Our study also revealed that the activation of STAT3 was inhibited during mammary gland involution in the absence of Nucling, resulting in suppressed expression of C/EBPδ mRNA, which is induced by STAT3 signaling (36). C/EBPδ is known to be important in the apoptotic response in mammary glands as well as in diminished involution in C/EBPδ-deficient mice (37). STAT3 was previously shown to be activated after weaning and acts as a critical regulator of apoptosis in involution (7, 8). In addition, LIF appears to be the primary activator of STAT3-mediated apoptosis in the mammary gland, because involution was delayed in LIF-deficient mice and STAT3 was not activated (4, 5). STAT3 activity was suppressed despite the induction



**FIGURE 6. Blockage of STAT3 activity during involution in Nucling-KO mice.** A, Western blot analysis of STAT3 and phosphorylated STAT3 expression during involution. Protein samples were prepared from the mammary glands of WT and Nucling-KO mice at 0, 36, and 72 h of involution. B, the quantification of immunoblots for STAT3/ $\beta$ -actin. The bar graph shows the relative density of the bands normalized to WT at 0 h of involution. C, quantification of immunoblots for p-STAT3/ $\beta$ -actin. The bar graph shows the relative density of the bands normalized to WT at 0 h of involution. D, quantification of the mRNA expression of C/EBP $\delta$  in mammary glands of WT and Nucling-KO mice at 0, 36, and 72 h of involution by quantitative RT-PCR. Data were normalized with those of WT mice at 0 h of involution and represent mean  $\pm$  S.D. Data were represented as mean  $\pm$  S.D. ( $n = 3$  per genotype, per age group). \*\*,  $p < 0.01$ .

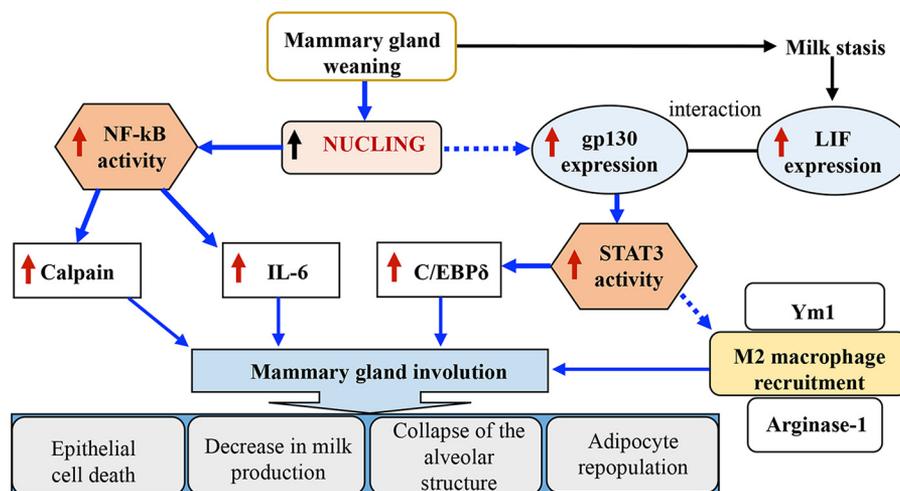


**FIGURE 7. Loss of Nucling resulted in suppression of gp130 expression.** Quantitative RT-PCR analysis of (A) LIF and (B) gp130 mRNA expression is shown. Total RNA samples were prepared from the mammary glands of WT and Nucling-KO mice during involution 0, 36, and 72 h. Data were normalized with those of WT mice at 0 h of involution and represented as mean  $\pm$  S.D. ( $n = 3$  per genotype, per age group). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

**FIGURE 8. Loss of Nucling did not affect the expression of Sim2s during involution.** Quantification of the mRNA expression of Sim2s in mammary glands of WT and Nucling-KO mice at 0, 36, and 72 h of involution by quantitative RT-PCR. Data were normalized with those of WT mice at 0 h of involution and represent mean  $\pm$  S.D. Data were represented as mean  $\pm$  S.D. ( $n = 3$  per genotype, per age group). \*,  $p < 0.05$ .

of LIF expression in Nucling-KO glands. Previous studies reported that, LIF activated STAT3 via gp130, which is normally up-regulated after weaning to activate STAT3 (5, 6, 38). Our results indicated that the loss of Nucling inhibited the gene expression of gp130. Therefore the down-regulation of STAT3 activation observed in Nucling-KO mice may have been due to

## Nucling Regulates Mammary Gland Involution



**FIGURE 9. Hypothetical model of the results of the present study.** Mammary gland involution is characterized by the apoptosis of epithelial cells, decreases in milk production, collapse of lobular-alveolar structures, and adipocyte repopulation. After weaning, Nucling expression is up-regulated through the NF- $\kappa$ B signaling pathway to mediate mammary gland involution by the expression of Calpain-1 and IL-6. Nucling also induces STAT3 activity via the gp130 expression to regulate involution by the modulation of M2 macrophage recruitment and expression of C/EBP $\delta$ . Therefore, Nucling controls mammary gland involution by regulating NF- $\kappa$ B and STAT3.

the inhibition of gp130 up-regulation during involution. Taken together, our results identified Nucling as an essential effector of STAT3 signaling via gp130 after weaning. The Nucling-dependent up-regulation of gp130 is regulated by a novel alternative pathway to the LIF-related pathway. However, the role of Nucling in the regulation of gp130 awaits further studies.

The absence of Nucling reduced the number of M2 macrophages present at 72 h of involution, indicating that Nucling is important for the recruitment of M2 macrophages. We attributed the presence of small numbers of M2 macrophages in Nucling-KO glands to two possible causes: it may be a consequence of the down-regulated expression of cytokines as well as lack of resident tissue macrophages within the mammary gland due to delayed involution when Nucling is deleted. It may also be due to the inhibited activation of STAT3 in Nucling-KO mice. A previous study demonstrated that STAT3 activity directly or indirectly modulated M2 macrophage populations in postlactational mammary gland involution (27).

A previous study found that Sim2s is an inhibitor of NF- $\kappa$ B and STAT3 activity in postlactational involution. Overexpression of Sim2s results in delayed involution (28). In contrast, herein, we revealed that Nucling activates NF- $\kappa$ B and STAT3 to mediate involution. After weaning, the decrease in the expression of Sim2s was insufficient to induce involution in the absence of Nucling. Therefore, the present study demonstrated that Nucling is a novel factor that plays an important physiological role in regulation of mammary gland involution.

At present, much of the evidence pointed to a correlation between delayed postlactational involution and increase in breast cancer formation (1). Nucling-KO mice have shown delayed involution but breast tumors were not observed in these mice. This may be due to the suppression of important NF- $\kappa$ B and STAT3 signalings, which are essential for progression of breast tumor. Moreover, we have observed that the absence of Nucling in postpartum female nude mice resulted in formation of breast tumor in four of 20 mice. Although there was no tumor bearing mice in more than 20 postpartum

females of nude mice. This suggests that cellular immunity suppressed the breast tumor development in Nucling-KO mice.

Postlactational involution is a complex multistep process that displays similar characteristics to the microenvironments present during wound healing and tumor progression (39). The activation of NF- $\kappa$ B and STAT3 are essential for the progression of breast cancer, and their inhibition has been shown to cause cell death and the suppression of breast tumors (40, 41). Because a regulatory connection was identified between NF- $\kappa$ B/STAT3 activity and Nucling in mammary gland involution in the present study, we postulated that this connection may extend to breast cancer. More specifically, if Nucling is strongly expressed in breast cancer, its inhibition may suppress the activation of NF- $\kappa$ B as well as STAT3, leading to the suppression of the pro-oncogenic activities of these transcription factors. Therefore, Nucling signaling needs to be examined in more detail in breast cancer, so that effective inhibitors of breast tumor progression can be developed in the future.

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