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Rosa Zaragoza, Ana Bosch, Concha García, Juan Sandoval, Eva Serna, et al.. Nitric oxide triggers mammary gland involution after weaning: Remodeling is delayed but not impaired in mice lacking inducible nitric oxide synthase.. *Biochemical Journal*, 2010, 428 (3), pp.451-462. 10.1042/BJ20091091 . hal-00486857

HAL Id: hal-00486857

<https://hal.science/hal-00486857>

Submitted on 27 May 2010

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Nitric oxide triggers mammary gland involution after weaning: Remodeling is delayed but not impaired in mice lacking inducible nitric oxide synthase.

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Short Title: NOS-2 KO mice show delayed mammary gland involution after weaning

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Accepted Manuscript

SYNOPSIS

During mammary gland involution different signals are required for apoptosis and tissue remodeling. To explore the role of NO in the involution of mammary tissue after lactation, NOS-2^{-/-} mice were used. No apparent differences were observed between NOS-2^{-/-} and WT animals during pregnancy and lactation. However, upon cessation of lactation a notable delay in involution was observed, compared to WT mice. KO mice showed increased phosphorylation of STAT5 during weaning, concomitant with increased β -casein mRNA levels when compared to weaned WT glands, both hallmarks of the lactating period. In contrast, activation of STAT3, although maximal at 24h weaning, was significantly reduced in NOS-2^{-/-} mice. STAT3 and NF κ B signaling pathways are known to be crucial in the regulation of cell death and tissue remodeling during involution. Indeed, activation of both STAT3 and NF κ B was observed in WT mice during weaning, concomitant with increased apoptotic rate. During the same period, less apoptosis, in terms of caspase 3 activity, was found in NOS-2^{-/-} mice and NF κ B activity was significantly reduced when compared to WT mice. Furthermore, the activation of NF κ B signaling pathway is delayed in KO mice when compared to WT mice. These results emphasize the role of NO in the fine regulation of the weaning process, since in the absence of NOS-2 the switch on of the cascades that trigger involution is hindered for a time, retarding apoptosis of the epithelial cells and extracellular matrix remodeling.

Keywords: Apoptosis, STATs, NF κ B, weaning, nitric oxide, ECM

Abbreviations footnote: WT, wild type; KO, knock out; STAT, signal transducer and activator of transcription; MMP, matrix metalloproteinase; ECM, extracellular matrix; NOS-2, inducible nitric oxide synthase.

INTRODUCTION

Mammary gland is a highly dynamic and specialized tissue which development takes place postnatally. During pregnancy there is epithelial cell proliferation and differentiation, regulated by progesterone and prolactin, resulting in the formation of secretory alveoli [1]. Prolactin signaling activates multiple regulatory networks within the mammary tissue, not only stimulating milk production by inducing milk protein genes, but also maintaining the viability of mammary epithelial cells [2]. At weaning, involution of the mammary gland takes place. This process is associated with programmed cell death of secretory epithelial cells and remodeling of the lobulo-alveolar structure, resulting in ductal structures that closely resemble the pre-pregnant gland. Removing the suckling pups at the peak of lactation induces a forced weaning, allowing to study the molecular mechanisms and morphological features associated with involution. These events that occur after weaning are reversible for up to 2 days but become irreversible thereafter [3]. Apoptosis of the epithelial cells is induced by milk stasis within the alveolar lumen and is dependent upon mammary-derived signals [4]. This first stage is p53-dependent and protease-independent and there is not a substantial remodeling of the mammary gland architecture. It is characterized by alterations in the expression of cell cycle regulatory genes, apoptosis-related genes and down-regulation of milk proteins gene expression. During the second, irreversible phase of involution, caused by a decrease in lactogenic hormones, the structure of the mammary gland is remodeled involving the destruction of the basement membrane by matrix metalloproteinases (MMPs), phagocytic clearance of the apoptotic bodies and re-differentiation of adipocytes [5]. Within two weeks the gland returns to a pre-pregnant state in preparation for a subsequent pregnancy.

In recent years, the use of genetically modified mice and genetic and microarray approaches have allowed us to identify the essential components of the distinct phases of involution [6-8]. An up-growing number of genes are being implicated in apoptosis regulation during involution [9, 10] and several signaling pathways have been involved in first phase involution. One of these primary pathways is the signal transducer and activator of transcription (STAT) pathway, which is activated in response to cytokines and growth factors. This activation leads to the phosphorylation of specific STAT proteins that translocate to the nucleus and activate transcription of their target genes. Seven members of the STAT gene family have been identified. Two members of this family, STAT5A and STAT5B (collectively called STAT5) that share 96% similarity at the amino acid level, have been identified as prolactin activated transcription factors. STAT5 is required for lobuloalveolar development and lactation, being a critical regulator of milk protein gene expression. Indeed, a role for STAT5 in preventing apoptosis and remodeling in mammary gland has been inferred from its rapid decline at the onset of involution [11]. During weaning, STAT5 activity declines as a direct consequence of a fall in systemic lactogenic prolactin levels and STAT3 increases mainly in response to leukemia inhibitory factor (LIF). In vivo studies have shown that STAT3 is essential for the initiation of apoptosis and involution, involving the direct suppression of intrinsic cell survival signals mediated by ERK 1/2 and AKT [12-14].

However, other pathways such as the nuclear factor (NK)- κ B/ I κ B kinase (IKK)/ death receptor pathway are also critical to regulate apoptosis in mammary gland. Using transgenic mice that express a constitutively active form of IKK2, the upstream kinase in the classical NF κ B signaling cascade, it has been demonstrated that NF κ B activation during lactation and involution results in a reduction in milk levels and increased cleavage of caspase-3, exerting a pro-apoptotic effect [15]. In fact, activation of NF κ B is one of the

most rapid transcription factor responses in the involution of the mammary gland, with detectable DNA binding within 2h following litter removal [16]. Downstream targets of this pathway will be activated during weaning, ensuring the transition to the second phase of involution. Indeed, we have previously shown an increase of inducible nitric oxide synthase gene expression (iNOS or NOS-2), regulated by NF κ B, during weaning [8], resulting in an increase in nitric oxide (NO) levels. NO and NOS activities have been described in the human, rat and mouse mammary gland [8, 17-19], suggesting that NO plays important roles in the growth, development and involution of the mammary gland. In fact, during mammary gland involution, high levels of NO are produced, due not only to elevated NOS-2 but also NOS-1 and NOS-3 are highly expressed in involuting mammary gland [20]. This is consistent with increased tyrosine nitration in the weaned gland, which is limited to a few specific proteins. Among these nitrated proteins cathepsin D has been identified, being this modification responsible for the increase in the protease activity of cathepsin D observed during weaning [21]. According to the observations mentioned above, NO might have an apoptotic role in mammary involution, being involved in tissue remodeling. In order to elucidate the possible global impact of NO on the involutory process, C57BL/6J wild-type (WT) and NOS-2 knockout (KO) mice were used. A delay in the weaning process has been observed in mice lacking NOS-2 when compared to the WT.

EXPERIMENTAL

Animals and tissue extraction. NOS-2 KO mice [22] and WT control strain (C57BL/6) were obtained from Taconic (Ejby, Denmark). Genotyping to verify the absence or presence of the NOS-2 gene was accomplished by PCR of DNA from tail biopsies. The animals were given food and water *ad libitum* and housed in individual cages in a controlled environment (12h light/ 12h dark cycle). Experimental protocols were approved by the Research Committee of the School of Medicine (University of Valencia, Valencia, Spain). The mice were cared for and handled in conformance with National Institutes of Health guidelines and the *Guiding Principles for Research Involving Animals and Humans* approved by the Council of the American Physiological Society.

Following parturition, litters were maintained with at least 7 pups. Then, at the peak of lactation (days 9-11), the mice were divided into different groups: control lactating mice, and weaned mice which pups were removed 10 days after delivery to initiate involution. The weaning took place for 6, 24, 48 and 72 h before death (at least 5 animals for each condition were used). Mice were given intraperitoneally a single dose of pentobarbital sodium (60 mg/kg body wt) freshly dissolved in 0.9% NaCl (Braun Medical, Rubi, Spain). Then, the inguinal mammary glands were removed and quickly freeze-clamped in liquid N₂.

Milk production. Milk production was measured as previously described by Sampson and Jansen [23]. Briefly, the following equation relating pup milk yield to pup weight and weight gain was used to estimate milk production: yield = 0.0322 + 0.0667 (weight) + 0.877 (gain), where yield is daily yield per pup (g·day⁻¹·pup⁻¹), weight is pup weight (g), and gain is daily pup weight gain (g/day).

Tissue preparation and histopathology. Inguinal mammary glands from lactating and weaned WT and KO mice were embedded in paraffin after removal and fixated in 4% p-formaldehyde. Tissue sections of 5-7 μ m were cut and stained with haematoxylin and eosin for histological studies.

Fluorometric determination of nitrite. Samples from control lactating and 24h weaned mice were prepared by homogenizing 0,1g of tissue in 500 μ l Tris-HCl 50mM, pH 7.5 followed by a centrifugation at 12000g for 20min. The supernatant was further centrifuged

at 100000g for 30min. Then it was ultrafiltered using a 30kDa molecular weight cut off filter (Microcon® YM-30; Millipore, Bedford, MA). 100µl of the filtrate were assayed for nitrite as described previously [8].

Quantification of serum prolactin levels. Blood from WT and KO mice was collected on day 10 of lactation (n=6) and plasma prepared. Measurement of prolactin levels in plasma was conducted using an Enzyme immune assay kit from SPIbio (Montigny, France) following the manufacturer's instructions.

Protein extraction and immunoblotting analysis. Total protein was extracted from snap-frozen mammary glands (0.1 g) by homogenization in a buffer containing detergents and protease inhibitors as previously described [24]. Equal amounts of protein (15 µg), quantified by a Bradford-based assay (Bio-Rad protein assay, Bio-Rad Laboratories, Munich, D), were denatured with Laemmli sample buffer, separated by SDS-PAGE electrophoresis and then electroblotted onto a nitrocellulose membrane (Protran®, Whatman, Kent, UK). After addition of the corresponding primary antibody, immunocomplexes were revealed by using a peroxidase-conjugated secondary antibody (Santa Cruz Biotech., Santa Cruz, CA, USA), as appropriate, and subsequent peroxidase-induced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech., Buckinghamshire, UK). The intensity of the bands was measured by densitometry using Image J, a public domain Java image processing program.

The primary antibodies and their source used for blotting are specified as follows: STAT-3 (Upstate, Lake Placid, NY, USA); p-STAT-3 and p-STAT-5 (Cell Signaling Technology Inc., MA, USA); STAT-5, IκBα and IκBβ (Santa Cruz Biotech.). Equal loading was confirmed reprobing the blot against α-tubulin (Santa Cruz Biotech.) and by Red Ponceau staining.

Measurement of caspase-3 activity. Caspase-3 activity was measured in mammary tissue lysates using the Caspase-3/ CPP32 Colorimetric Assay Kit (BioVision, Mountain View, CA, USA). Mammary gland was homogenized in lysis buffer, incubated on ice for 10min and centrifuged at 10000g for 15min. Supernatants from tissue homogenates (200µg in 50µl) were used to measure caspase-3 activity following the manufacturer's instructions.

Gelatin zymography. Gelatin zymography was carried out as previously described [25]. In brief, protein lysates (150µg/ sample) were diluted in non-reducing SDS sample buffer (62,5mM Tris-HCl pH 6.8; 25% Glycerol; 4% SDS and 0,001% bromophenol blue) and separated on 10% SDS-PAGE mini-gels containing 1 mg/ml gelatin (Bio-Rad). After electrophoresis, the gels were washed for 30min at room temperature in renaturing buffer (10mM Tris-HCl pH 7.5; 2,5% Triton X-100) followed by incubation in enzyme reaction buffer (50mM Tris-HCl pH 7.6; 200mM NaCl; 5mM CaCl₂; 0.02% Brij-35) for 36h at 37°C. To visualize gelatinolytic bands, gels were stained with Coomassie Brilliant Blue R-250. Gelatinolytic activities were detected after destaining by clear bands indicating the lysis of the substrate.

Nuclear extract isolation. Isolation of nuclei from mouse mammary gland WT and KO and different weaning conditions was carried out with the Nuclear Extract Kit (Active Motif, CA, USA) following instructions by manufacturer. Briefly, 0,3 g of mammary gland tissue was treated with 750µL of hypotonic buffer supplemented with 1µL/mL DTT 1M, detergent 1µL/mL and 50µL/mL of phosphatase inhibitor. After incubation on ice for 15 minutes the samples were centrifuged for 10 minutes at 850g at 4°C. The pellet was resuspended in complete lysis buffer (which included 1mM DTT, protease and phosphatase inhibitors) and incubated on ice for 30 min under gentle shaking. The samples were then

centrifuged at 4°C for 10 min at 14000g. The supernatant fraction (the nuclear protein extract) was stored at -80°C or used immediately.

Determination of STAT3 and NFκB activity in nuclear extract. Mammary gland nuclear extracts (5 μg) were assayed for STAT3 or NFκB DNA binding activity using the TransAM STAT family kit or NFκB p65 chemi transcription factor assay kit, respectively (Active Motif), according to the manufacturer's instructions. Assays were performed in the absence or presence of 20 pmol of competitor oligonucleotide that contains either a wild type or mutated STAT or NFκB consensus binding site. The plate was read using a microplate luminometer (SPECTRAMax GEMINI XS, Molecular Devices, CA, USA).

Chromatin Immunoprecipitation (ChIP) assays. Chromatin from mammary tissue was fixed and immunoprecipitated as described previously [25]. The antibodies used in this case were p65 and p50 (both from Santa Cruz Biotech.).

RNA extraction. Total RNA from mammary tissue was extracted by TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) followed by additional column purification (RNeasy, Qiagen, Crawley, UK). RNA quantity and purity were determined using the NanoDrop ND-2000 (Nanodrop Technologies, Wilmington, DE, USA), and RNA integrity was assessed by determining the RNA 28S/18S ratio using RNA 6000 Nano Labchips in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Real time PCR analysis. RNA (500 ng) was reverse transcribed into cDNA at 25°C for 10 min and 37°C for 2 h using the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA products were amplified by qPCR using the GeneAmp Fast PCR Master Mix (Applied Biosystems). All reactions were carried out in triplicate. Quantitative real-time PCR was run in the 7900HT Fast Real-Time PCR System. Pre-developed Taqman primers specific for β-casein, LIF, Bcl-3, MMP-2, MMP-9 and 18S were purchased from Applied Biosystems. Results were normalized according to 18S quantification in the same sample reaction. The threshold cycle (C_T) was determined, and then the relative gene expression was expressed as follows:

$$\text{Relative amount} = 2^{-\Delta(\Delta C_T)}$$

where $\Delta C_T = C_T \text{ target} - C_T \text{ 18S}$, and $\Delta(\Delta C_T) = \Delta C_T \text{ weaned} - \Delta C_T \text{ control}$.

Microarray hybridization and analysis. Mammary gland gene expression analysis was performed according to the Affymetrix-recommended protocol using Affymetrix Mouse ST v1.0. GeneChips containing probes for over 28,000 well-annotated genes. Total RNA (300ng per sample) from 48h weaned WT and KO mammary gland, was labeled using the Affymetrix GeneChip cDNA Synthesis and Amplification kit protocol and hybridized to the arrays as described by the manufacturer (Affymetrix, SantaClara, CA). Arrays were scanned on an Affymetrix GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). GeneChip Operating Software supplied by Affymetrix was used to perform gene expression analysis. Three mice per group were examined. Data (.CEL files) were analyzed and statistically filtered using software Partek Genomic Suite 6.4 (Partek Inc., St. Louis, MO). Input files were normalized with the RMA algorithm for gene array on core meta probesets. A one-way ANOVA was performed with the Partek Genomics Suite program across all samples. Statistically significant genes were identified using mixed model analysis of variance with a false discovery rate (Benjamini-Hochberg test) of $p < 0.05$. Fold-change values $< |\pm 1.5|$ were removed.

PCR array for NFκB signaling pathway. The effect of weaning on the expression of 84 genes associated with NFκB mediated signal transduction were examined using mouse NFκB signaling pathway RT² Profiler PCR array (SuperArray Bioscience, Frederick, USA)

according to the manufacturer's instructions. In brief, 1 μ g total RNA of control and 48h weaned mammary glands from WT and KO mice was converted into cDNA using the RT² First Strand Kit (SABiosciences). This cDNA was then added to the RT² SYBR Green/ROX qPCR Master Mix (SABiosciences). Next, each sample was aliquoted on Mouse NF κ B Signaling Pathway PCR Arrays (catalog number PAMM-025). All steps were done according to the manufacturer's protocol for the 7900HT Fast Real-Time PCR System. PCR amplification was conducted with an initial 10-min step at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). The fluorescent signal from SYBR Green was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold (C_T). Data normalisation was based on correcting all C_T values for the average C_T values of several constantly expressed housekeeping genes (HKGs) present on the array.

Statistical analysis. Data within the same genotype, were analyzed by a one-way ANOVA and significant differences were determined by a Tukey-Kramer test. Differences were considered significant at $p < 0.05$ and different superscript letters indicate significant differences. The letter "a" always represents the lowest value within the group. To compare WT and KO at the same time point, two-tailed Student's t-test was performed between both groups ($*p < 0.05$). Independent experiments were conducted with a minimum of three replicates per condition to allow for statistical comparison.

RESULTS AND DISCUSSION

NO generation in the weaned mammary gland

Nitric oxide (NO) might be a signaling modulator not only during involution but also during pregnancy and lactation where proliferation and differentiation of the mammary gland epithelial cells is maximal. Indeed, the three isoforms, NOS-1, NOS-2 and NOS-3, are expressed in normal mammary epithelium [17]; being upregulated during mammary gland involution [8, 20]. In order to gain a better understanding of the role played by NO in mammary gland involution, mice in which the expression of NOS-2 has been abolished were used for this study.

Nitrite levels as an end product of NO were measured in the mammary gland of control lactating and 24 hours weaned mice both in WT and NOS-2 KO strains. As shown in Table I, NO production is significantly increased in the involuting mammary gland after 24h of pup removal compared with control lactating glands both in WT and NOS-2^{-/-} mice. However, nitrite levels in NOS-2 KO mice are statistically lower than in WT littermates either in control lactating glands or in weaned glands. The increase observed in the involuting mammary gland of NOS-2^{-/-} mice may be due to the activation of NOS constitutive isoforms, which has already been described during the weaning state [20].

Histological Changes in the mammary gland.

In order to study the role of NO in the modulation of lactation and the onset of involution, we studied the histological integrity of mammary tissue. The inguinal mammary glands of WT and NOS-2 KO mice were blind-analyzed at day 10 of lactation and 6, 24, 48 and 72h of involution by an experienced pathologist. Histological analysis of tissue sections from WT mice showed a loss of epithelial cells and alveolar structure, as well as reappearance of adipose tissue from 24h of involution onward (Fig. 1A panels i, iii, v, vii and ix). On day 10 of lactation (Control), a comparative examination of mammary tissue sections showed that mammary gland development in the NOS-2^{-/-} mice was normal, with prominent secretory alveoli surrounded by adipocytes (Figure 1A, panels i and ii). This observation is in agreement with previous reports describing that in the absence of NOS-2, ductal development and branching in the mammary glands appeared unaffected [26].

Moreover, lack of NOS-2 did not alter the birth weight of newborn mice or significantly affect the duration of gestation when compared to WT mice [27].

However, histological analysis of NOS-2 KO mice during involution revealed a marked delay in mammary gland regression. Programmed cell death is induced in mammary gland within 12h of involution with a sharp increase thereafter [3]. As seen in figure 1A, at 24h post-weaning, NOS-2^{-/-} mice showed condensed lobulo-alveolar structures (panel vi), as WT mice did at 6h weaning (panel iii). Moreover, 48 hours after litter removal, mammary gland from WT mice showed an evident regression of alveoli that was not so obvious in KO animals (panels vii and viii). Indeed, the pattern of involution shown in KO mice after 48h weaning resembled that observed in WT mice after 24h weaning (Fig. 1A panels v and viii). Regardless of this “delay” observed in tissue regression of NOS-2^{-/-} mice, it is important to highlight that involution is not impaired, since the histological sections of WT and KO mice after 72h weaning were similar. At this time, adipocytes had already redifferentiated and replaced the mammary gland alveolar structures (Fig. 1A, panels ix and x). This observation suggests that the regulation of apoptosis is not entirely dependent on the levels of NO reached in the mammary gland after cessation of suckling; however, NO seems to be triggering the early signals of the involutory process.

It is well known that regression of the mammary gland does not occur immediately after litter removal, indeed involution is reversible for the first 24h, and for this reason no loss of gland weight is observed during this first phase of apoptosis but thereafter, in the second phase of involution when there is loss of epithelial cells [28]. Therefore, in order to investigate whether NO is involved in the modulation of those signals that trigger the first phase of involution, we determined the ratio between inguinal mammary gland to total mice weight. In Figure 1B is shown that after 24h weaning the mammary gland regressed and there was a marked decrease in the ratio mammary gland/ total weight in both WT and NOS-2^{-/-} mice. Nevertheless, in KO mice this ratio is significantly higher at 6 and 24h weaning than in WT mice. This effect could be the result of an increased milk secretion in KO mice, supporting a delay in mammary regression in these animals.

Alterations in the level of programmed cell death have been reported in other knockout mouse models that elicit a delay or disruption of involution [9, 10]. The delay in involution observed in the absence of NOS-2 correlates with a decrease in the cleavage, and thereby activation, of caspase 3 (Fig. 1C). The activity of caspase 3 was measured in WT and KO mammary glands at the peak of lactation and at different times after weaning. We could not observe any difference in caspase 3 activity after 24h of weaning; most probably, the extent of activation at this time is not sufficient to be detected. Nevertheless, this executioner caspase increased its activity at 48h after weaning in WT mice whereas the enhanced caspase 3 activation was not observed until 72h in the NOS-2^{-/-} mammary tissue. Apoptosis levels, in terms of caspase 3 activation, are also “delayed” in NOS-2 KO mice. From these results we can hypothesize that NO is not only responsible of the changes observed at the early stages of involution but that the effect of this molecule is extended far beyond the first phase of apoptotic cell death regulating somehow the second phase of tissue remodeling.

Prolactin levels in NOS-2^{-/-} mice

Since nitrite levels were reduced in NOS-2^{-/-} lactating mice (see Table I) and increased mammary gland weight was observed in those mice after weaning (Fig. 1B), we wondered if lactation could be affected. Prolactin is required for normal proliferation and differentiation of alveoli during pregnancy and for milk protein expression during lactation [1, 2]. It has been described that prolactin release by pituitary glands can be modulated by

NOS-2 [29]; in order to confirm this possibility, the amount of serum prolactin in WT and NOS-2 KO was measured to determine whether increased prolactin secretion could account for the delayed mammary gland involution observed in the absence of NOS-2. Serum prolactin was determined in six WT (165 ± 27 ng/ml; mean \pm SEM) and NOS-2^{-/-} mice (213 ± 16 ng/ml) at day 10 after delivery and a statistical difference was observed between both groups ($P=0.05$). In accordance with this, we observed that pups nourished by NOS-2^{-/-} mice show increased weight along the lactating period when compared with the litters suckled by WT mice (data not shown); although no differences were observed in birth weight as previously described [21]. When estimating the milk yield, taking into account pup weight and weight gain [23], milk production was slightly higher in KO, being statistically different around the peak of lactation (between days 8 and 11) (Fig. 2). Since Andric et al. [29] have described that NO inhibits prolactin secretion in pituitary cells, our results point to the possibility that this slight increase in serum prolactin levels in NOS-2^{-/-} could account for the delay in mammary gland involution, consistent with the increased mammary gland weight (Fig. 1B) and milk production (Fig.2), observed in this phenotype.

Effect of NOS-2 absence on the Prolactin-dependent STAT5 pathway.

STAT5 is strongly activated by prolactin at the end of pregnancy and during lactation, being essential for lobulo-alveolar development and the expression of milk protein genes, and is rapidly inactivated at the early stages of weaning. [14]. It has been reported that STAT5 deficient mice are unable to lactate [30] supporting also the idea of its prominent role in the maintenance of lactation in mammary gland. Moreover, constitutively activated-STAT5 (ca-STAT5) transgenic mice show a delay in postlactational apoptosis [31]. The appearance of NOS-2^{-/-} mammary gland at 24h of involution is reminiscent of that found in ca-STAT5 mice. Western blotting was used to examine the phosphorylation of STAT5 following lactation and involution (Figure 3A). The expression levels of STAT5 did not differ between NOS-2^{-/-} and WT mice and, as previously reported there is a progressive down-regulation of this protein after forced weaning. Interestingly, while phosphorylation of Tyr⁶⁹⁴-STAT5 was elevated in both WT and KO mice at the peak of lactation, it persisted during weaning only in NOS-2^{-/-} mice. The increased prolactin levels observed in NOS-2^{-/-} could be modulating the JAK/STAT signaling pathway that avoids phospho-STAT5 down-regulation during the first phase of involution. However, it is important to highlight that NO might also have a direct effect on STAT5 activity, that could also account for the diminished phosphorylated STAT-5 observed in WT mice after weaning. Indeed, NO has been described to inhibit STAT5 signaling pathway in macrophages either by decreasing STAT5a and STAT5b protein levels [32] or by reducing tyrosine phosphorylation [33].

Following tyrosine phosphorylation, STAT5 dimerizes and, upon translocation to the nucleus, it binds to promoters of responsive genes. The levels of STAT5 expression and activation have been demonstrated to affect the extent of milk protein expression and secretion during lactation [13, 14, 34]. Since it has been demonstrated that casein promoter is more sensitive to STAT5 transactivation than other milk proteins [31], we studied the pattern of β -casein expression at the peak of lactation and during the time-course of involution. Increased mRNA levels of this gene were observed at the peak of lactation and after 6h weaning, which is not surprising since β -casein mRNA half life is around 9h at the peak of lactation [35] and it has been described that the levels of mRNA for α -, β -, and γ -caseins remain high for at least 12h after weaning, decreasing thereafter [36]. However, accompanying the inactivation of STAT5, the β -casein gene expression was decreased at 24h weaning in WT mice (Fig. 3B). In accordance with the sustained phosphorylation of STAT5 noted in NOS-2^{-/-} mice during involution (Fig.3A), β -casein gene expression,

although reduced during involution, was significantly higher in NOS-2 KO mice at 24 and 48h than in littermate WT mammary glands (Fig. 3B). These results further support the hypothesis that the NOS2^{-/-} mammary tissue is held in a functional stasis for the early involution despite the loss of the suckling stimulus.

STAT3 in wild-type and NOS-2 knockout mice.

It has been reported that STAT5 and STAT3 are inversely activated during the course of a lactation/involution cycle [13,14]. Furthermore, it has been demonstrated that blocking STAT3 activation induces a strong delay in mammary gland involution [12]. Thus, we analyzed STAT3 phosphorylation at the peak of lactation and during involution by western blot (Figure 4A, upper panel). As previously published, phospho(Tyr)-STAT3 levels were induced after 24h weaning and declined thereafter in WT mice. Interestingly, STAT3 phosphorylation was not delayed in NOS-2 KO mice, showing a peak after 24h weaning. On the other hand, as reported by others in WT mice [37], STAT3 protein levels are induced during lactation and remained up-regulated during involution in both, WT and KO mice (Figure 4A middle panel). Finally, as shown in figure 4B, the STAT3 DNA-binding activity, further confirmed that STAT3 was activated to a considerably lesser extent in KO mice (5±0.5 fold increase in WT vs. 3±0.4 fold increase in KO mice at 24h weaning). Although we cannot rule out the possibility that NO could have a direct effect on the pathway of STAT3 activation, it would be predictable that activated STAT5 has a regulatory role in this pathway. This hypothesis is in agreement with a previous report demonstrating that overexpression or forced-activation of STAT5 in transgenic mice promotes a lower level of STAT3 activation than that observed in WT mice during involution [31].

STAT3 activation, hardly detectable during lactation, is strongly induced by leukemia inhibitory factor (LIF) at the onset of involution [13, 14]. Therefore, we have examined LIF mRNA levels by real-time PCR in lactating and 24h weaned glands, when STAT3 activation is maximal, in NOS-2^{-/-} and WT mice. As shown in figure 4C, LIF was substantially upregulated in involution compared with lactation in WT mice. With regard to KO mice, LIF was also statistically increased when compared with control lactating but the fold-increase was smaller than in WT littermates at the same time of weaning.

In order to determine the physiological relevance of a lower STAT3 activity in NOS-2^{-/-} mice, we analyzed the expression pattern of Bcl-3 in our experimental model. Bcl-3 is a member of the NFκB/IκB family of proteins known to be a target of the STAT3 pathway [6]. Although the functional role of this protein in mammary gland development is still unclear, it has been reported that its expression is strongly induced in mice mammary gland after 24h of forced weaning [13]. Indeed, Bcl-3 expression was induced after 24 weaning in both WT and KO mice (Figure 4D). Nevertheless, Bcl-3 mRNA levels increased significantly more in WT than in KO animals at 24h of weaning (6.5±0.4 fold increase in WT vs. 3.3±0.1 fold increase in KO mice), reaching the same steady-state mRNA levels after 72h weaning. We would like to highlight that the magnitude of Bcl-3 up-regulation at 24h after weaning is the same as that found for STAT3 activity in both animal types at that time point, suggesting that STAT3 could be involved in the modulation of Bcl-3 expression at 24h weaning. However, the pattern of Bcl-3 expression at 48 and 72h weaning, does not correlate with STAT3 activation. Therefore, from our results we cannot discern the particular contribution of STAT3 to Bcl-3 expression along the whole time course of the experiment. Our data clearly show that at least at 24h after weaning STAT3 is phosphorylated, its DNA binding activity is increased and Bcl-3, one of its target genes, is up-regulated to a higher level in WT compared with KO mice. Therefore, we can

conclude that NO seems to play a role in the modulation of those signals leading to STAT3 molecular and functional activation in mammary tissue after 24h of involution.

Modulation of NF κ B activity by NO during mammary gland involution.

NF κ B is a key transcription factor in the modulation of different stages of mammary gland development and has been suggested to act as a “checkpoint” modulator of apoptosis [38]. NF κ B is activated during pregnancy, almost completely suppressed during lactation and re-activated in early involution [8, 16]. In different experimental models, NO has been shown to indirectly up-regulate NF κ B binding activity, via I κ B α degradation [39] or nitration [40]. We investigated whether this factor could be modulated by NO and responsible, at least in part, for the delayed involution observed in NOS-2 KO mice. The differences found in NF κ B binding activity during lactation and early involution, are known to be caused by the modulation of its nuclear translocation [34] and do not affect the p65/p50 protein levels. Thus, we analyzed by western blot the decrease in the cytoplasmic levels of I κ B α protein, indicative of I κ B α degradation and indirectly, of NF κ B activation. As shown in figure 5A, I κ B α degradation while maximal at 24 and 48h after weaning in WT mice, it was delayed to 48 and 72h in KO mice. As expected, I κ B β protein levels did not change during weaning. These results strongly suggest that NF κ B is differentially activated in WT and KO mice.

This observation was confirmed by the analysis of NF κ B DNA binding activity at lactation and during weaning (Figure 5B). NF κ B binding activity was increased 24h after litter removal reaching a maximum at 72h of involution in WT mice. NF κ B activity also increased in the NOS-2 KO animals during weaning, but to a lesser extent (Fig. 5B), in agreement with the delayed degradation of I κ B α that was observed above. Surprisingly, in WT mice we found that I κ B α is re-synthesized at 72h weaning while p65 is still activated. It has been long accepted that NF κ B modulates I κ B α expression as a negative feed-back regulation in order to control the extent of the NF κ B-dependent response. Once I κ B α is again accumulated, it would interact with p65 within the nucleus and modulate its nuclear export back to the cytoplasm. In our experimental model, our results at 72h weaning most probably reflect the final step of this NF κ B signaling pathway, where NF κ B is involved in the I κ B α gene expression and therefore it is still active. However, it is interesting to remark that this process is known to be more complicated than a simple stoichiometric-dependent regulation. It has been demonstrated that binding of p65-I κ B α and its subsequent nuclear export to the cytoplasm is modulated by deacetylases/ acetyltransferases activated by different stimulus. In this way, acetylation of p65 would block its interaction with I κ B α and therefore it would be still active within the nuclear compartment [41, 42].

Role of NO in the modulation of NF κ B-associated genes during weaning

The mammary gland involution is the consequence of a multifactorial regulation that results in the apoptosis of the mammary tissue and the preparation for a new cycle of pregnancy/lactation. The process of involution must be finely coordinated, and NF κ B has been largely considered a key modulator of converging signaling pathways that ultimately would lead to the apoptosis and remodeling of the gland. Based on the result that NF κ B activation during weaning was delayed in NOS-2^{-/-} mice, we investigated the extent of this effect on those pathways known to be associated to NF κ B response. Therefore, we analyzed the expression of 84 genes associated with NF κ B using superarray technology. In WT mice the expression pattern found after 48h of pup removal was similar to the values described previously by microarray studies [6, 7]. Our results showed that 30 genes were up-regulated meanwhile 14 were down-regulated, when compared with gene expression at

the peak of lactation. We next examined this pathway in lactating and 48h weaned NOS-2 KO mice, and the expression pattern was similar to that observed in WT mice, but the number of genes up-regulated was reduced to twenty-one. Taking into account these results, statistical analysis was performed between weaned-WT and weaned-KO, showing that twenty-three genes were significantly down-regulated in weaned NOS-2 KO mice compared to WT (Table II) and none was up-regulated.

The detailed study of data shown in table II provides additional and important information on the role of NO in the regulation of mammary gland involution: i) the fact that all genes found to be differentially regulated in KO animals versus WT were down-regulated suggests that the NF κ B signaling pathway during weaning is delayed in KO-mice and thus NO could be an important signal involved in the mechanisms that trigger the NF κ B response. Since none of the genes were up-regulated we should conclude that NO do not participate in the silencing of any NF κ B-associated gene. ii) Some of the down-regulated genes, such as caspases, Bcl-3, CSFs, Elk-1 or TNF are largely considered pro-apoptotic [43, 44]. For instance, an 80% decrease of FasL expression in NOS-2^{-/-} would suggest a protection from apoptosis in KO mice. In fact no apoptotic cells were detected during the first 3 days of involution in FasL-deficient mice [45]. Moreover, the authors of this study suggest a defect during the first phase of involution that does not affect to the second phase. iii) Some other genes are known to be antiapoptotic, and one would expect them to be up-regulated in a process with a less severe apoptosis as in NOS-2^{-/-} mice. Strikingly, those genes are also down-regulated. This is the case of ICAM-1, which is reduced a 55% in NOS-2 KO compared to WT mice. It has been suggested that the response of adjacent cells to localized apoptosis, is to induce the expression of cell adhesion molecules such as ICAM-1 to maintain survival. Nevertheless, it has been shown a correlation between the extent of apoptosis and the expression of ICAM-1 [38]. Therefore, it would be reasonable to think that a less severe apoptosis during the first phase of involution would induce a poorer response in terms of ICAM-1 expression. The definition of the first stage of involution can explain this apparent functional contradiction: On the whole we will find the addition of both pro- and anti-apoptotic signals that upon persistence of forced weaning will unbalance the response towards the irreversible phase of apoptosis and remodeling of the gland. Finally, our data suggest that although NF κ B is in some way involved in the modulation of all the listed genes, there is not a solely specific pathway that can be affected by the absence of NOS-2 expression at 48h weaning. This observation suggests that although NO targets are at the very early onset of involution, including the prolactin receptor/JAK/STAT pathway, the nature of the mammary gland allows the amplification of the signal through the modulation of several pathways.

Proteinase activity in wild-type and NOS-2 knockout mice

Activation of NF κ B induces apoptosis, with concomitant loss of extracellular matrix contacts [46]. The rise in NF κ B binding activity shown (Fig. 5B) coincided with an increased rate of apoptosis in the mammary gland (Fig. 1C). The extent of apoptosis has been reported to correlate with an increase in MMP activity at the onset of major remodeling of the gland during the second phase of involution [3, 38]. In fact, MMPs are synthesized as latent zymogens, which have to be biologically activated by cleavage of their proenzyme peptide. Accumulating evidence suggests that the activation of NF κ B is one of the most important signaling events required for the inducible expression of MMP-9 in response to different stimuli [47]. Consequently, we explored the functional consequences of delayed NF κ B activation in NOS-2^{-/-} mice during the second phase of involution. Real time RT-PCR analysis was performed in WT and NOS-2^{-/-} mice at the peak of lactation and weaned animals. The steady-state mRNA levels of MMP-2 and

MMP-9 (Fig. 6A) were significantly higher after 48h weaning than those mice at the peak of lactation. The maximal induction of both MMP-2 and -9 was observed after 72h weaning. It is noteworthy that there is no difference in MMP-2 mRNA up-regulation between WT and KO mice in all the conditions studied (Fig. 6A, upper panel). However, MMP-9 induction is much more pronounced during weaning in WT than in KO mice (Fig. 6A, lower panel). Furthermore, NF κ B activation is delayed in NOS-2 KO during involution and MMP-9 mRNA is reduced in these mice, suggesting that the activation of MMP-9 could be dependent on NF κ B DNA binding. In order to verify this possibility, we performed an analysis of NF κ B binding to MMP-9 promoter by ChIP assay. As shown in figure 6B, while p65/p50 was bound to MMP-9 promoter at 48h weaning in WT mice, no DNA binding was observed in NOS-2^{-/-} mice at the same time of litter removal.

MMPs activation was observed by zymography studies; MMP-9 proteinase activity (105 kDa) appeared faintly after 48h weaning, increasing at 72h weaning in WT mice. In other hand, activation of pro-MMP-2 (70 kDa band) to an active form occurred at 72h weaning (Fig. 6C left panel). This increased enzyme activity correlated with mRNA levels in the weaned mammary tissue. When analyzing these MMPs in NOS-2 KO animals, the proteinase activity seemed to be lower than in WT mice at the same times of weaning (Fig. 6C right panel). Indeed, the intensity of the bands for MMP-2 and -9 is lower at 72h weaning in KO when compared to WT mammary glands and no band for MMP-9 activity (105 kDa) is observed at 48h weaning in KO mice.

MMPs are essential in the ECM remodeling that takes place during the involution of the mammary gland [3]. In fact, MMP-9 is a key proteinase governing the degradation of basement membrane collagen as well as different types of gelatin [5]. The results presented here suggest that the effect of NO in the regulation of mammary gland involution is not limited to the first phase of involution. Therefore, it seems that by the modulation of either, prolactin signaling and transcription factors activities, and/or their interactions, the signal generated by NO during the first phase of involution is amplified throughout the cascade of events triggered during the second phase. In an attempt to shed light into this point, we have performed microarray analysis to compare the genetic profile between NOS-2^{-/-} and WT during lactation and at 48h of involution (unpublished data). A list with 11 extracellular matrix related-genes which are differentially expressed during weaning in KO and WT mammary glands is shown in Table III. As observed, tissue inhibitors of MMPs (TIMP1 and -4) are increased in KO mice when compared to WT at 48h weaning., which could explain the lower activity of MMP-2 and -9 observed in those animals (Fig. 6C). In addition, MMP-3, one of the most highly induced MMP genes during mammary gland involution [7], which is expressed in the stromal cells of mammary tissue [3], was also decreased in 48h weaned KO mammary gland. With regard to the other genes that appear down-regulated in KO mice, they are involved either in collagen metabolism (Col3a1, Col5a1, Col14a1, Lox, Lum and Adamts2) or profibrogenic factors such as Ctgf and Postn, suggesting that the remodeling of the mammary stroma is also affected in those transgenic mice. This adds further support to our belief that NO plays a significant physiological role as an inducer of apoptosis modulating not only the initial signaling pathways, but also the ECM signaling cascade.

Physiological implications

The involution of the mammary gland that takes place at the end of lactation or after litter removal involves apoptosis of secretory epithelial cells and tissue regression, resulting in a return to a virgin-like state. Several signaling pathways have been involved in this physiological event, although the molecular mechanisms regulating them are not well understood. The role of NO in the regulation of gene expression, enzyme activity or

transcription factors activation has been extensively documented in numerous experimental models other than mammary gland [17, 22, 26]. NO might be an early potential signal triggering involution; indeed, using chromatin immunoprecipitation (ChIP) assays we already demonstrated that NF κ B induces the expression of NOS-2 during weaning, resulting in an increase in NO levels and, subsequently, increased protein nitration [8, 21].

We have shown that NOS-2 KO mice have increased serum prolactin levels at the onset of lactation, suggesting an inhibitory role for NO in prolactin secretion as it has been previously described [29]. The differences in the concentration of this lactogenic hormone between WT and KO mice could explain the increased milk production or even the delayed phospho-STAT5 inactivation and β -casein down-regulation observed in transgenic mice. However, the phenotype observed in NOS-2^{-/-} mice cannot only be explained by the modulation of this prolactin-dependent pathway; indeed blood prolactin levels were reduced in WT and KO mice after 24h weaning (data not shown). From these results, one could argue that the differences observed along the second phase of involution are not due to a maintained prolactin level but instead we postulate that NO might be involved in STAT5 inactivation by other still unknown mechanisms. In support of this notion, it has been described that NO reduces both JAK and STAT5 phosphorylation in T cells [33] and how increased NO levels affect STAT5 DNA-binding activity in the macrophage cell line Raw 264.7 [32].

In this paper, we have explored the pattern of those early factors known to be crucial for mammary gland involution such as STAT3 and NF κ B and their target genes, and have demonstrated that their activation is clearly delayed in NOS-2 KO mice. Interestingly, the most remarkable differences were observed at 48h weaning where both, STAT3 and NF κ B might play a prominent role, affecting also the ECM remodeling that takes place during the second phase of involution. This paper emphasizes the importance of NOS-2 up-regulation in the fine tuning of the coordinated changes that occur during mammary gland regression, since remodeling is delayed but not impaired in mice lacking inducible nitric oxide synthase. Nevertheless, it seems that maintaining the correct levels of NO, together with other regulatory factors in the mammary gland, is crucial for the modulation of transcription factors activities and/or interactions that lead to the involution of the mammary tissue.

ACKNOWLEDGEMENTS AND FUNDINGS

This work was supported in part by grants from Ministerio de Educación y Ciencia [BFU2007-62036 to J.R.V.; CIT-010000-2007-51 to L.T. and J.R.V. and Programa Ramón y Cajal to E.R.G-T] and Consellería de Sanidad [AP-071/09 to R.Z.]. A.B holds a Rio Hortega fellowship from Instituto de Salud Carlos III and R.Z and J.S are fellows from Ministerio de Ciencia e Innovación (Programa Juan de la Cierva).

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Table I. Comparison of nitrite concentrations in mammary gland of WT and NOS-2 KO mice. Nitrites from WT or NOS-2 KO mice at the peak of lactation (control) or after 24 h weaning. Values are presented as means \pm SEM (n=5). Significance of differences was determined by Student's t-test. *P < 0.05, compared with lactating control; †P < 0.05, significantly different from wild-type littermates.

<i>Nitrite concentration (μM)</i>	<i>Wild Type</i>	<i>NOS-2 Knock Out</i>
Control	0.98 \pm 0.02	0.79 \pm 0.06 [†]
24 h Weaning	1.5 \pm 0.03*	1.06 \pm 0.05* [†]

Table II. Fold down-regulation in genes associated with NF κ B signaling pathway during weaning in NOS-2 KO versus WT mice. mRNA levels in 48h weaned mammary glands from NOS-2 KO and WT mice were compared by RT² profiler PCR array (PAMM-025; SuperArray Bioscience). Those genes that were differentially expressed (P < 0.05) are listed below:

GenBank	Symbol	Description	Fold
NM_033601	Bcl3	B-cell leukemia/lymphoma 3	0.70
NM_009778	C3	Complement component 3	0.47
NM_130859	Card10	Caspase recruitment domain family, member 10	0.43
NM_009807	Casp1	Caspase 1	0.61
NM_001025432	Crebbp	CREB binding protein	0.64
NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	0.20
NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	0.39
NM_007922	Elk1	ELK1, member of ETS oncogene family	0.53
NM_010169	F2r	Coagulation factor II (thrombin) receptor	0.56
NM_010177	FasL	Fas ligand (TNF superfamily, member 6)	0.21
NM_008311	Htr2b	5-hydroxytryptamine (serotonin) receptor 2B	0.56
NM_010493	Icam1	Intercellular adhesion molecule	0.45
NM_008337	Ifng	Interferon gamma	0.3
NM_019777	Ikbke	Inhibitor of kappaB kinase epsilon	0.45
NM_008362	Il1r1	Interleukin 1 receptor, type I	0.42
NM_008390	Irf1	Interferon regulatory factor 1	0.61
NM_010735	Lta	Lymphotoxin A	0.47
NM_011952	Mapk3	Mitogen activated protein kinase 3	0.59
NM_019408	Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	0.52
NM_029780	Raf1	V-raf-leukemia viral oncogene 1	0.62
NM_013693	Tnf	Tumor necrosis factor	0.65
NM_009397	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	0.62
NM_011610	Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	0.67
NM_009539	Zap70	Zeta-chain (TCR) associated protein kinase	0.39

Table III. Fold-change of extracellular matrix-related genes at 48h postweaning in NOS-2 KO versus WT mice. Microarray experiments were performed to determine the gene expression profile in 48h weaned mammary glands from NOS-2 KO compared to WT mice. Fold down- or up-regulation of selected genes is shown (P<0.05)

GenBank	Symbol	Description	Fold
NM_080639	Timp4	Tissue inhibitor of metalloproteinase 4	1.88
NM_001044384	Timp1	Tissue inhibitor of metalloproteinase 1	1.52
NM_175643	Adams2	A disintegrin-like and metallopeptidase	-1.53
NM_015734	Col5a1	Collagen, type V, alpha 1	-1.71
NM_010809	Mmp3	Matrix metallopeptidase 3	-1.74
NM_181277	Col14a1	Collagen, type XIV, alpha 1	-2.00
NM_010217	Ctgf	Connective tissue growth factor	-2.02
NM_015784	Postn	Periostin, osteoblast specific factor	-2.15
NM_009930	Col3a1	Collagen, type III, alpha 1	-2.18
NM_010728	Lox	Lysyl oxidase	-2.20
NM_008524	Lum	Lumican	-2.22

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FIGURE LEGENDS.

Figure 1. Involution is delayed in mammary glands from NOS-2^{-/-} mice. **A)** Hematoxylin and eosin-stained sections show mammary gland morphology from wild type (i, iii, v, vii, ix) and NOS-2 knock out (ii, iv, vi, viii, x) mice at the peak of lactation (i, ii) and 6h (iii, iv), 24h (v, vi), 48h (vii, viii) and 72h (ix, x) after induced involution as described in Experimental section. Magnification 100 x. **B)** Ratio between inguinal mammary gland wet weight and total body weight in wild type (WT) and NOS-2 knock out (KO) mice at the peak of lactation and after different times of litter removal. Values presented in the graph are means \pm SEM (n=6). **C)** Caspase 3 activity in mammary tissue at different times of involution. Values are means \pm SEM of three different experiments performed in triplicate. *p<0.05 when comparing WT and KO mice at the same time point.

Figure 2. Milk production in NOS-2 knock out mice. Graphs demonstrating the milk production of NOS-2^{-/-} mice compared to WT mice. Milk yield was calculated according to the equation from Sampson and Jansen that takes into account pup weight and weight gain. Litters were normalized to 7-8 pups. The start of the graph represents day 1 of lactation (first day post-partum). Means \pm SEM are represented for each day of lactation (n=6). Statistical differences between WT and KO at each time point are shown, *p<0.05.

Figure 3. Activation of STAT5 signaling pathway. Analysis were carried out in WT and NOS-2 KO mice at different times after weaning: at the peak of lactation (0) and at 6, 24, 48 and 72h post-involution **A)** Levels of phospho-Tyr⁶⁹⁴ STAT5 and STAT 5a were evaluated by western blot in the mammary tissue. **B)** Real-time PCR for β -casein gene expression. Total RNA was isolated from the inguinal mammary gland of wild type (WT) and NOS-2 knock out (KO). Four mice were used per genotype and experimental condition. β -casein mRNA levels were normalized to 18s ribosomal for each sample and the graph represents the mean \pm SEM relative amount of the replicate tissue samples. Statistical differences between WT and KO at each time point are shown, *p<0.05.

Figure 4. STAT3 activity in mammary gland involution. **A)** Western blot analysis of phosphor-Tyr⁷⁰⁵ STAT3 and STAT3 in lysates derived from wild type (WT) and NOS-2 knock out (KO) mammary glands at day 10 of lactation (0h) and 6, 24, 48 and 72h of involution. **B)** STAT3 DNA-binding activity at each experimental point was measured using the TransAM STAT family kit (n=4 for each genotype and time point). **C)** LIF expression in the mammary gland of control and 24h weaned mice determined by real-time PCR. **D)** Bcl-3 gene expression was assessed by real-time PCR in the mammary gland of WT and KO mice at different times after weaning. For C) and D), four mice were used per genotype and experimental condition. LIF and Bcl-3 mRNA levels were normalized to 18s ribosomal for each sample and the graphs represent the mean \pm SEM relative amount of the replicate tissue samples. *p<0.05 when comparing WT and KO mice at the same time point.

Figure 5. Activation of NF κ B in mouse mammary tissue. **A)** Western blot analysis for I κ B α and I κ B β was performed in mammary extracts obtained from WT and NOS-2 KO mice at day 10 of lactation (0) and 6, 24, 48 and 72h of involution. Three separate mice were used per genotype and developmental stage. **B)** Nuclear extracts from the same experimental conditions mice were assayed for NF κ B p65 activation (n=4 for each genotype and time point). *p<0.05 when comparing WT and KO mice at the same time point.

Figure 6. Activation of MMP-9 but not MMP-2 is delayed in NOS-2^{-/-} mice during involution. **A)** Real time PCR was performed on both gelatinase genes, MMP-2 and -9. Expression levels were compared at each time after weaning (6, 24, 48 and 72h) between wild type (WT) and NOS-2^{-/-} (KO) mice. Each PCR was normalized against the housekeeping gene 18s ribosomal and the graph represents the mean \pm SEM relative amount in at least three experiments. * $p < 0.05$ when comparing WT and KO mice at the same time point. **B)** *In vivo* association of p65/p50 with the MMP-9 promoter during weaning. Results of a representative ChIP experiment, performed in samples from control lactating mammary gland, and 48h weaned from WT and NOS-2 KO mice are shown. Chromatin was immunoprecipitated with anti-p65 and anti-p50 antibodies. An aliquot of the input chromatin is also shown. Primers specific to the promoter region for MMP-9 gene were used to amplify the DNA isolated from the ChIP assay. **C)** Protein extracts from lactating (0) and weaned glands (48 and 72h) of both WT and NOS-2 KO genotypes were used to measure MMP-2 and -9 activation by gelatin zymography.

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Figure 1.

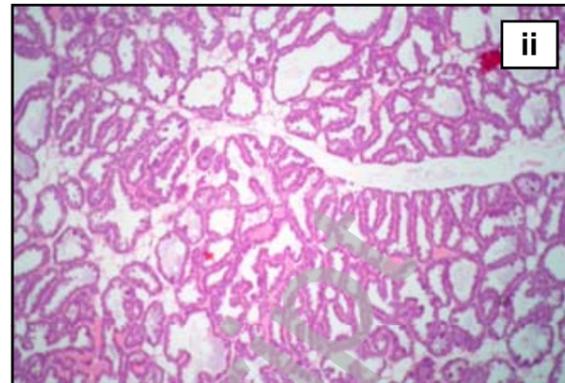
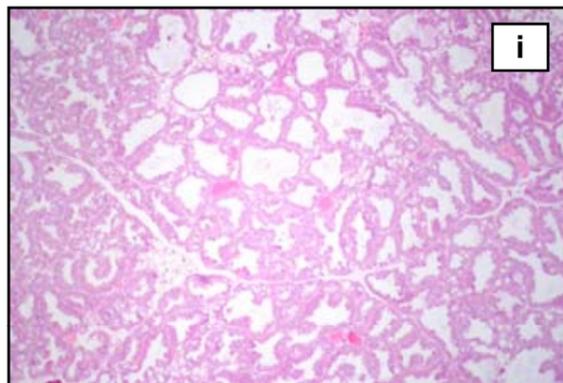
A)

Time after
Weaning (h)

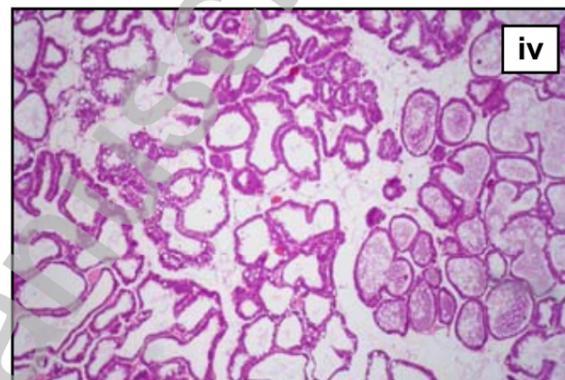
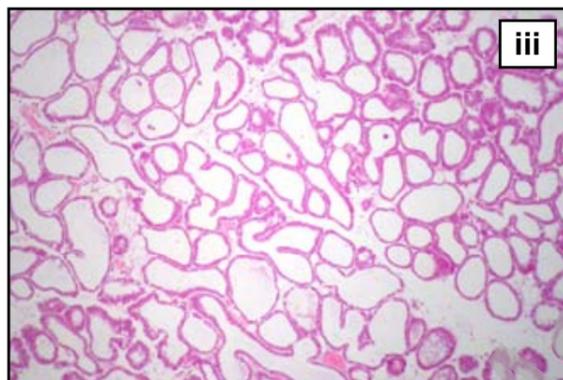
WT

KO

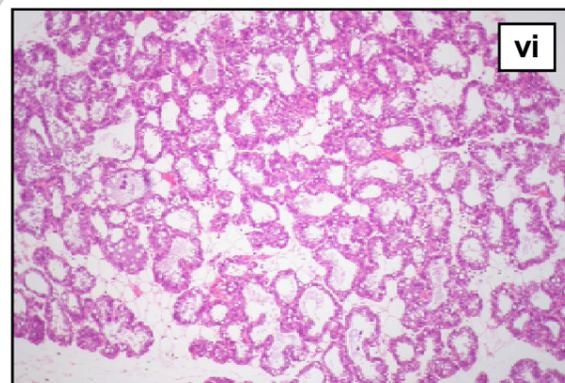
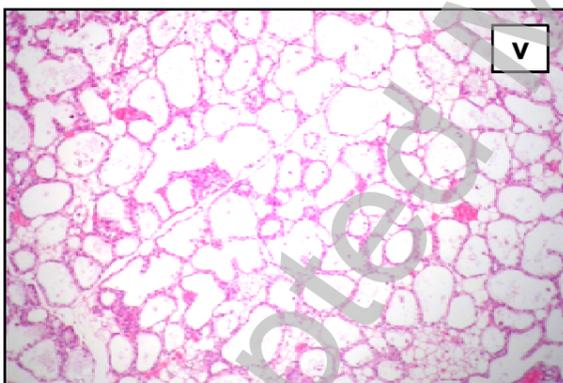
0



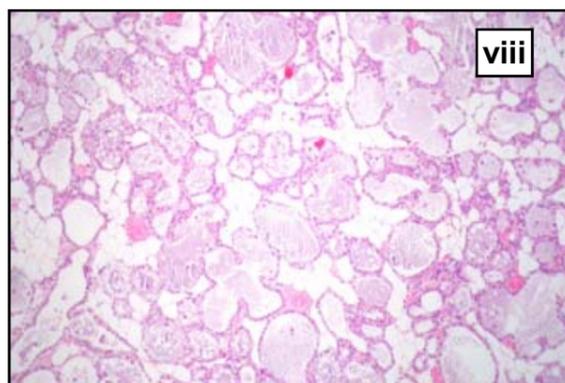
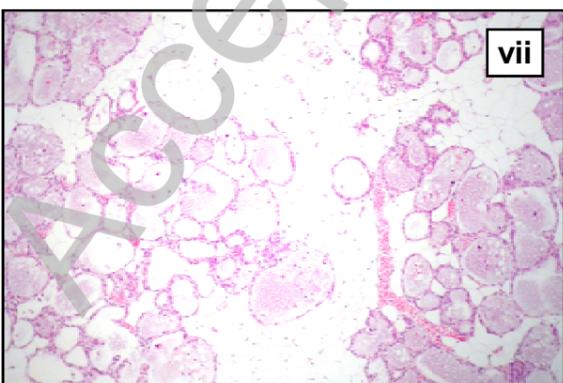
6



24



48



72

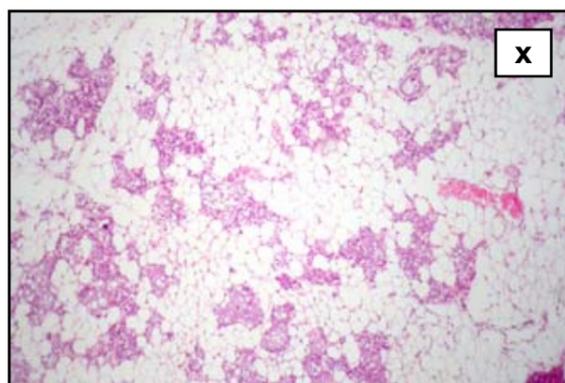
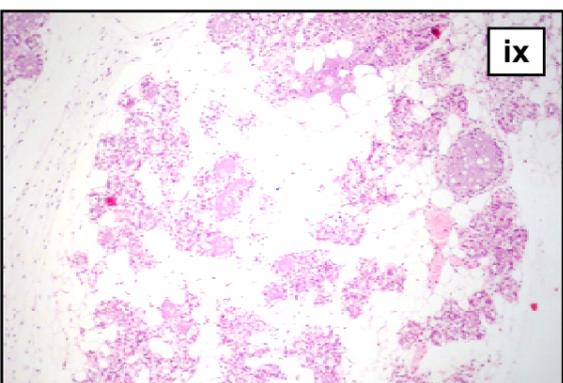
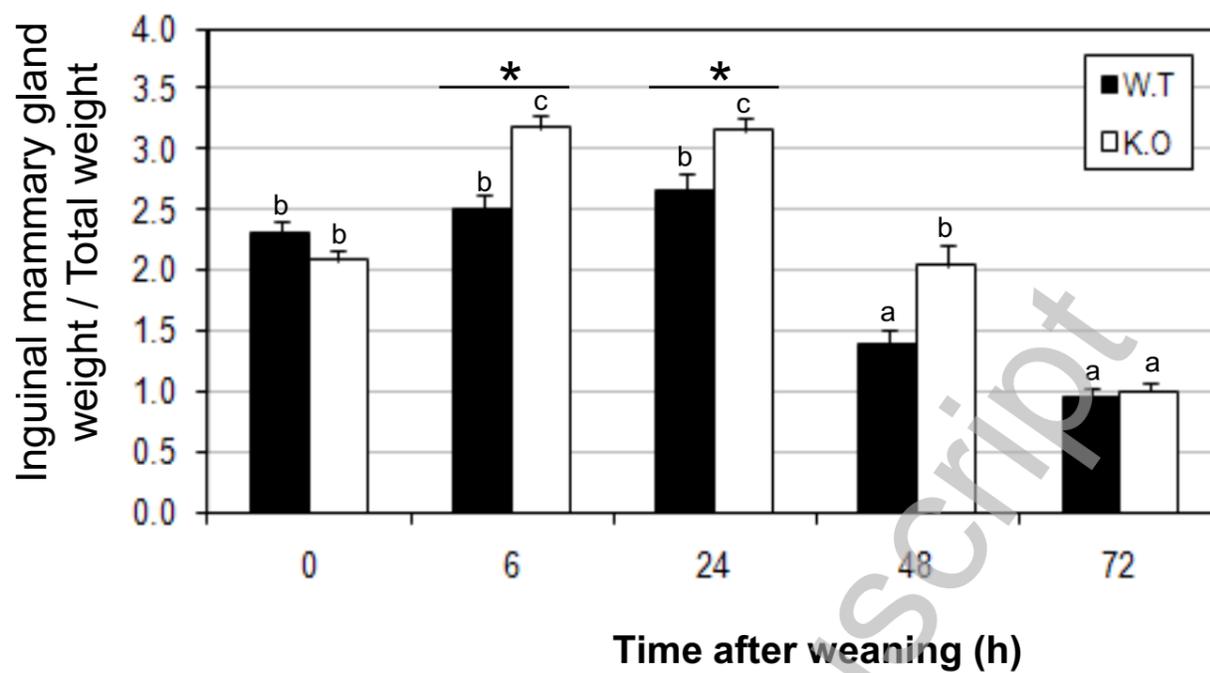


Figure 1.

B)



C)

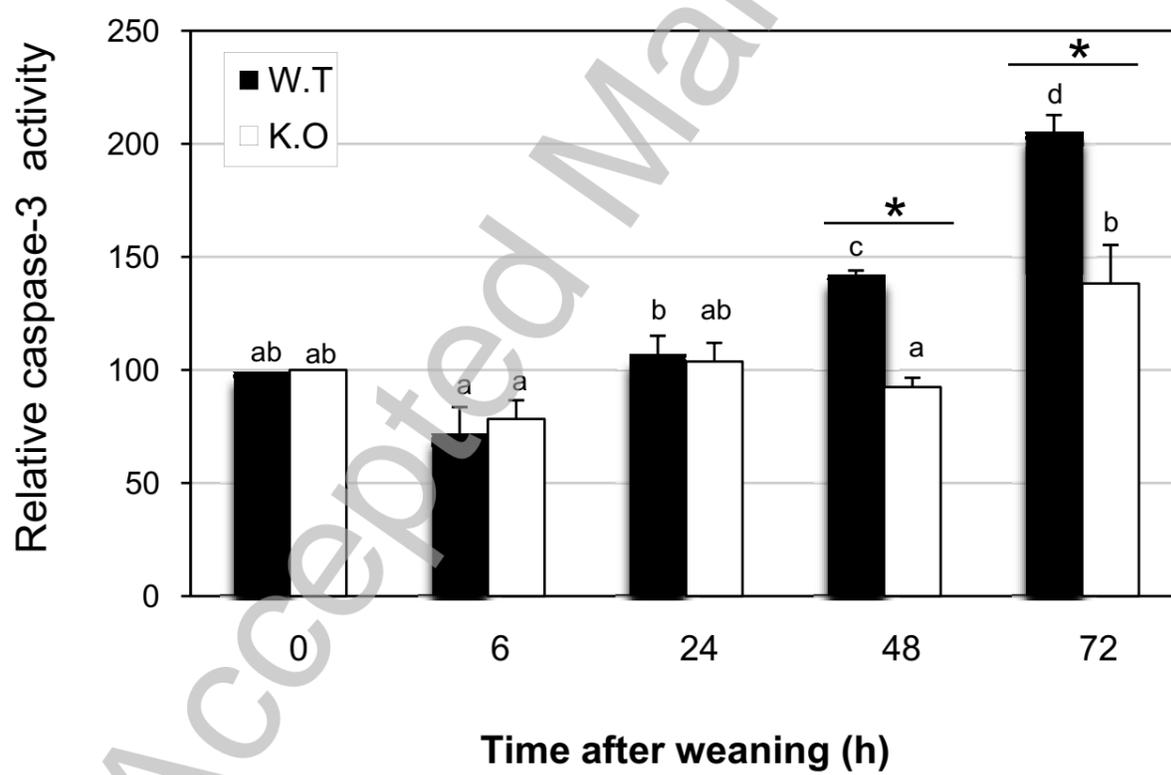
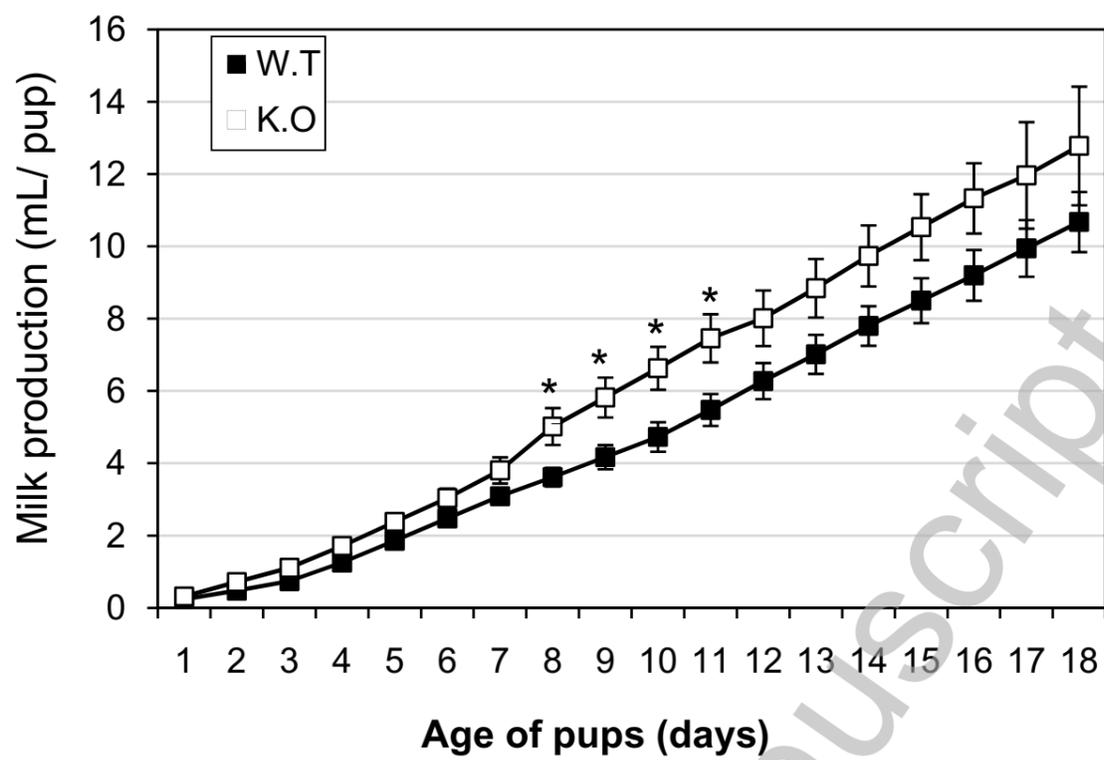


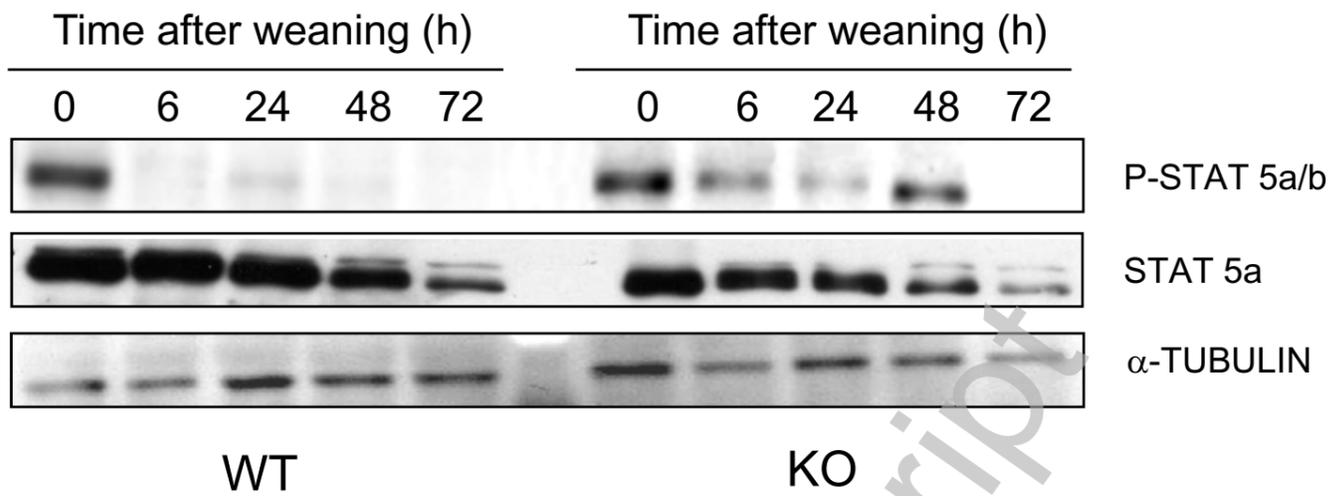
Figure 2.



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Figure 3.

A)



B)

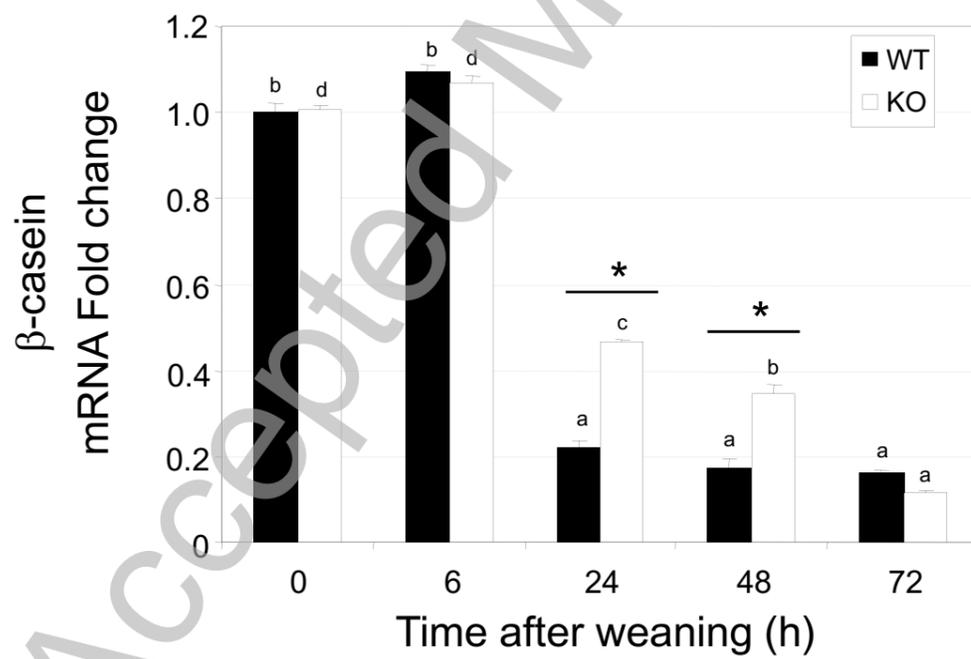
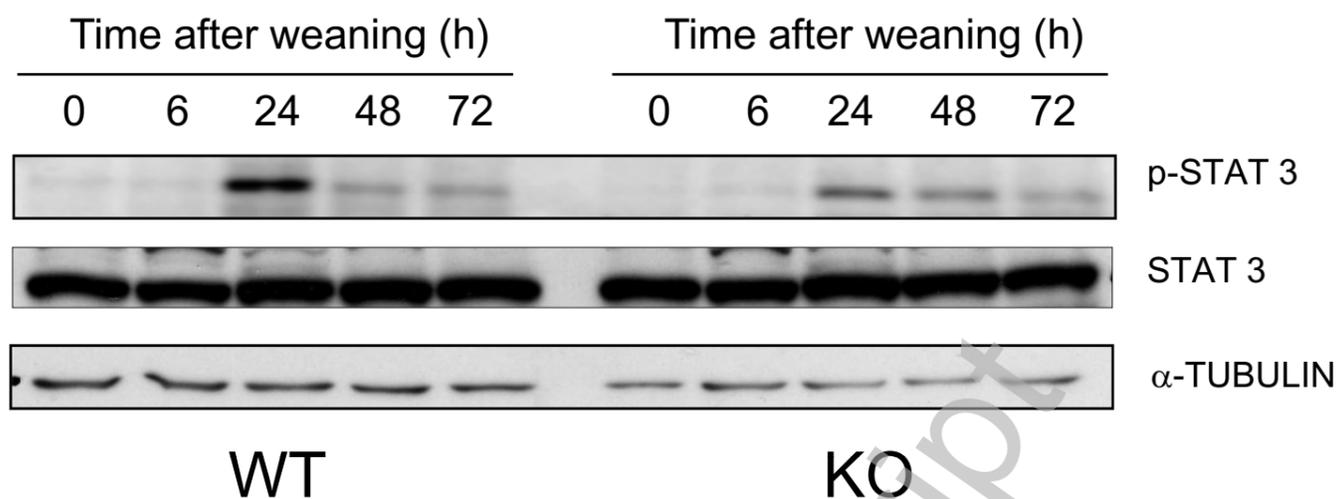
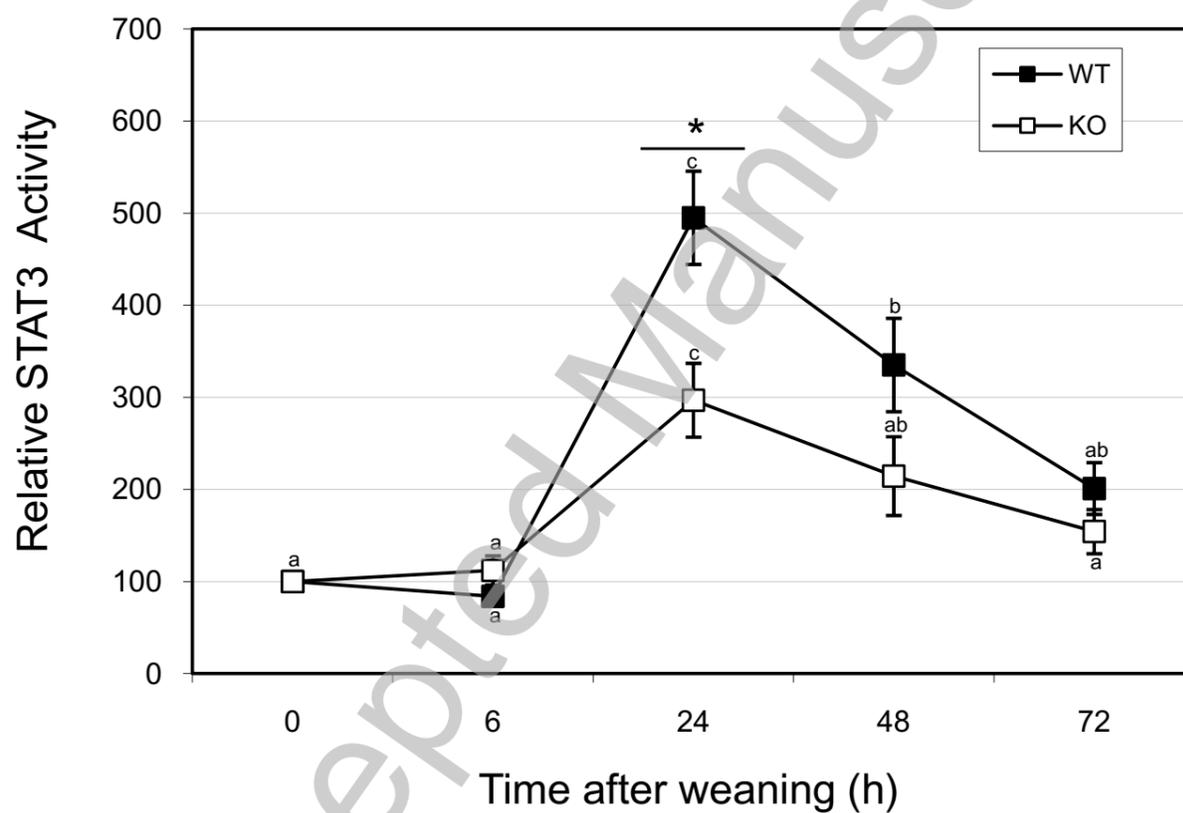


Figure 4.

A)



B)



C)

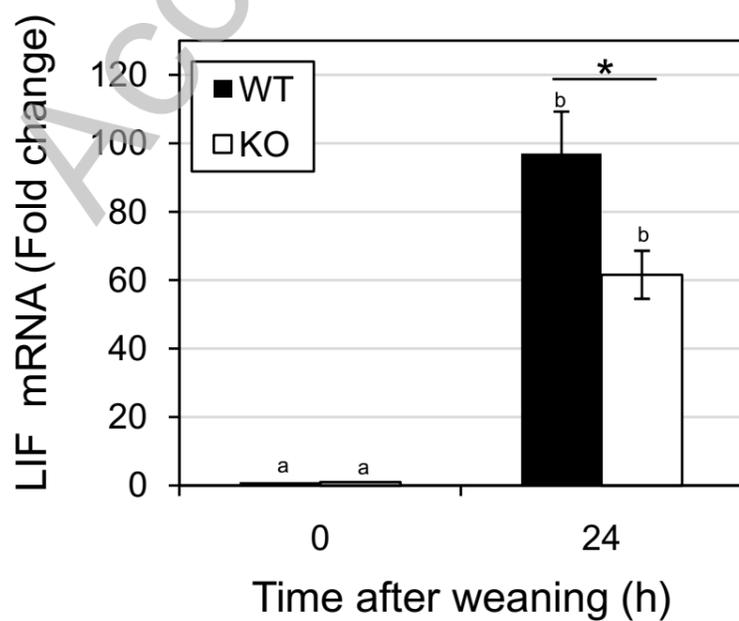
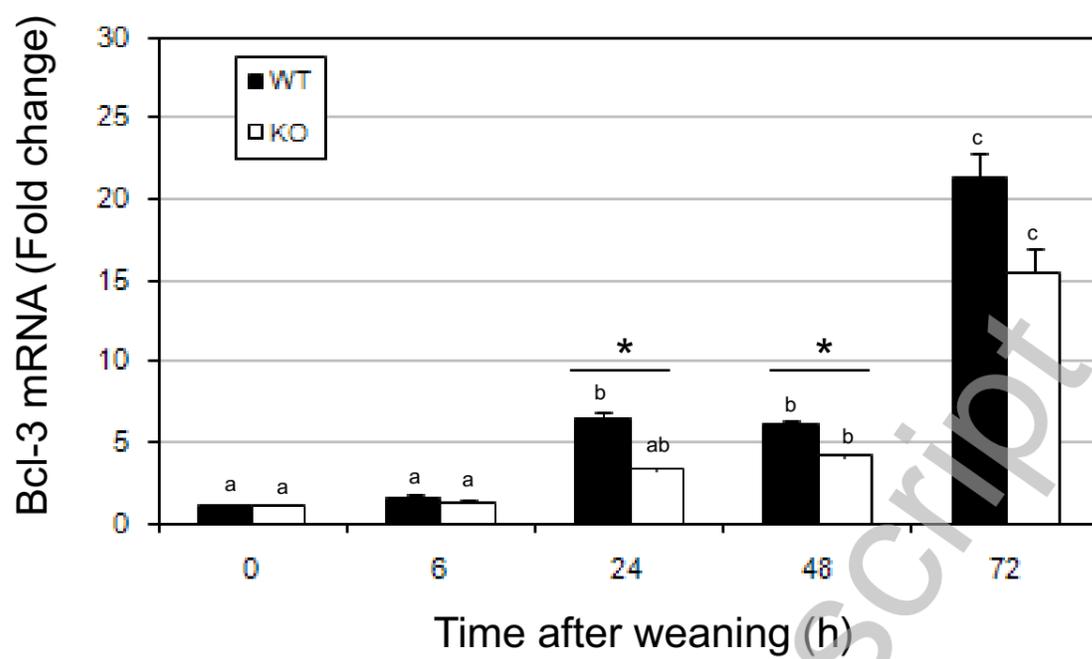


Figure 4.

D)



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Figure 5.

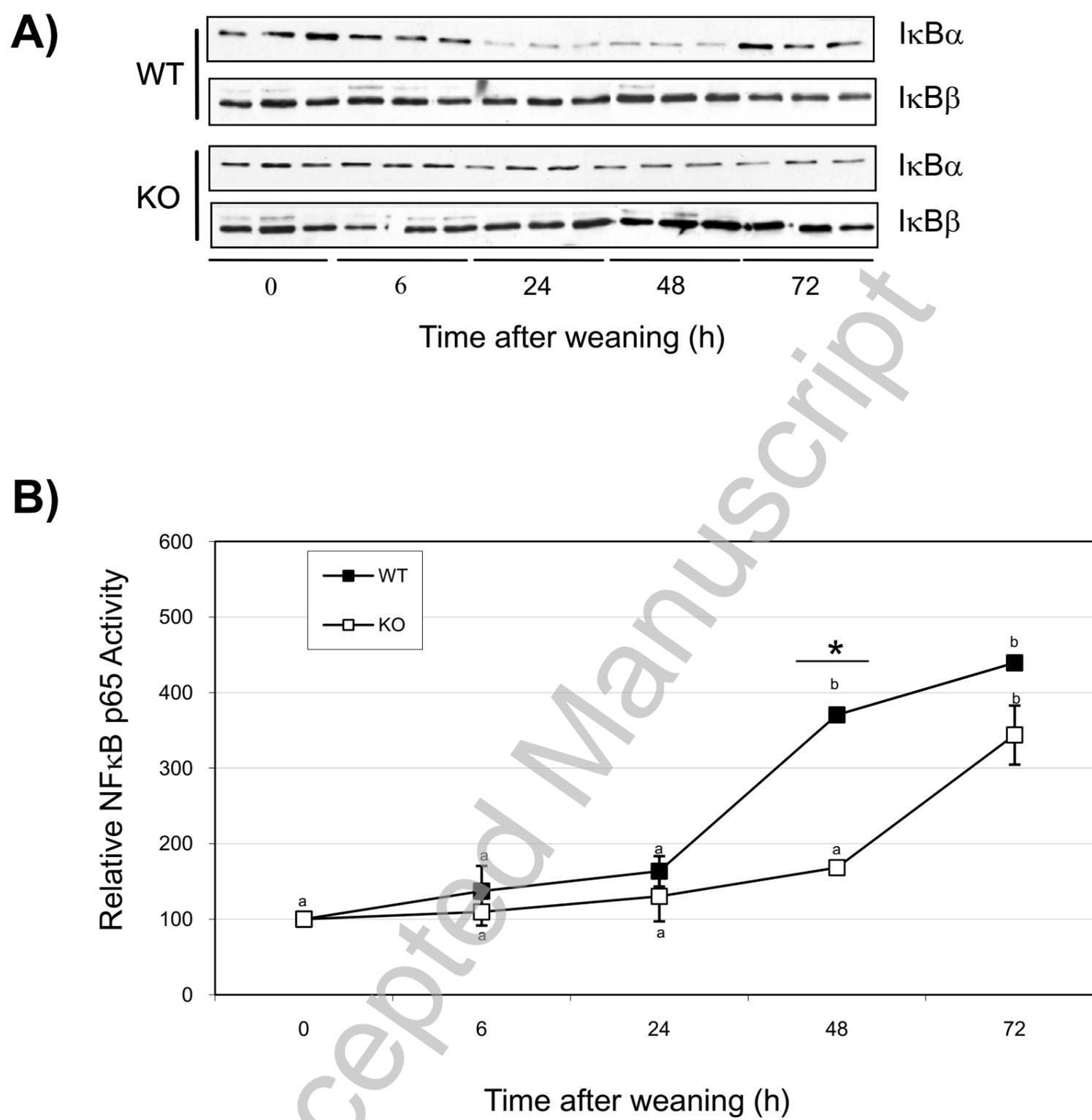
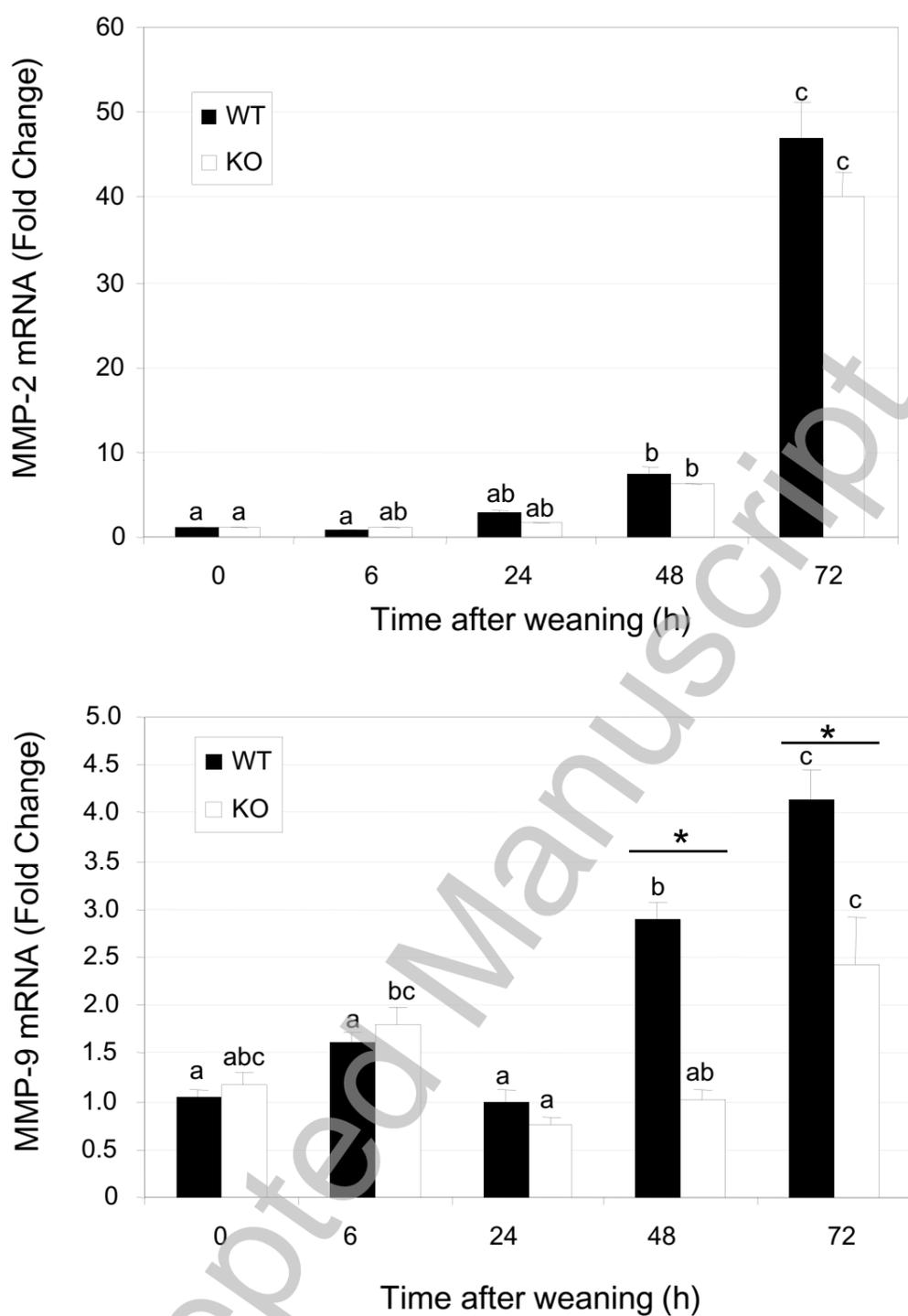
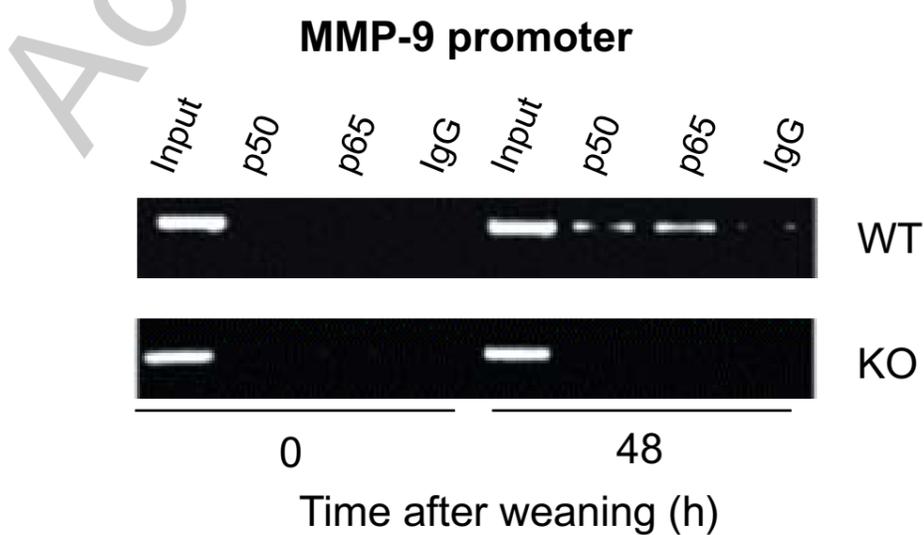


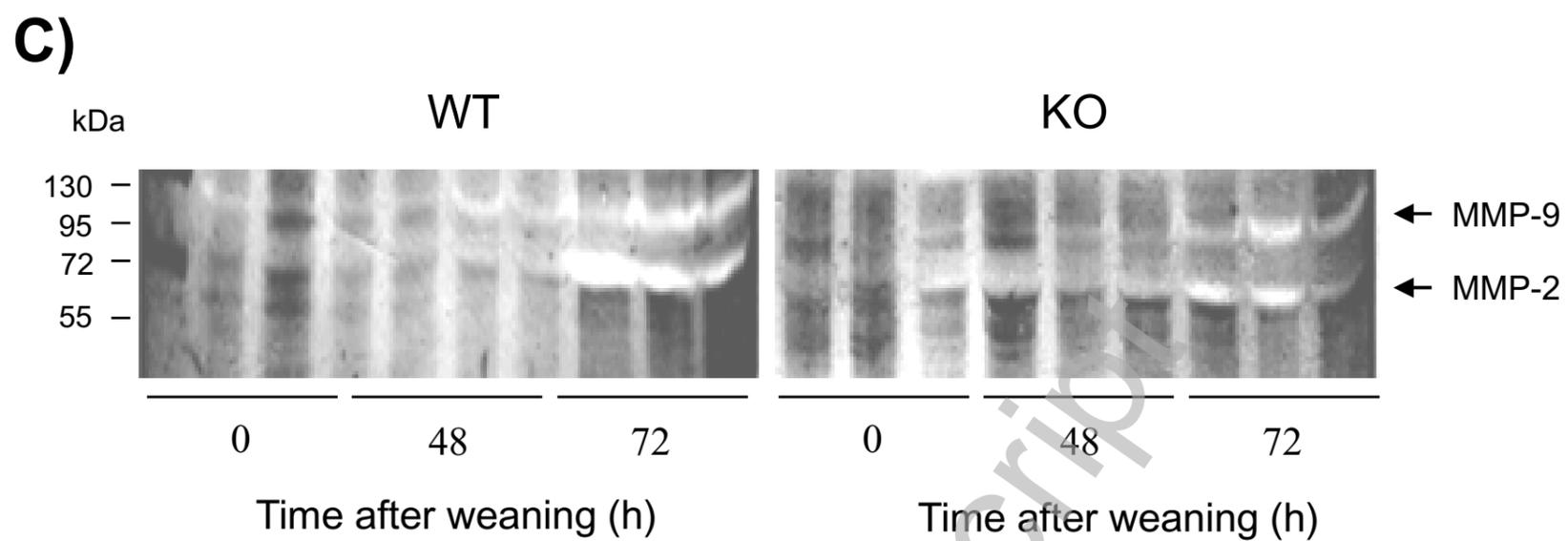
Figure 6.

A)



B)





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