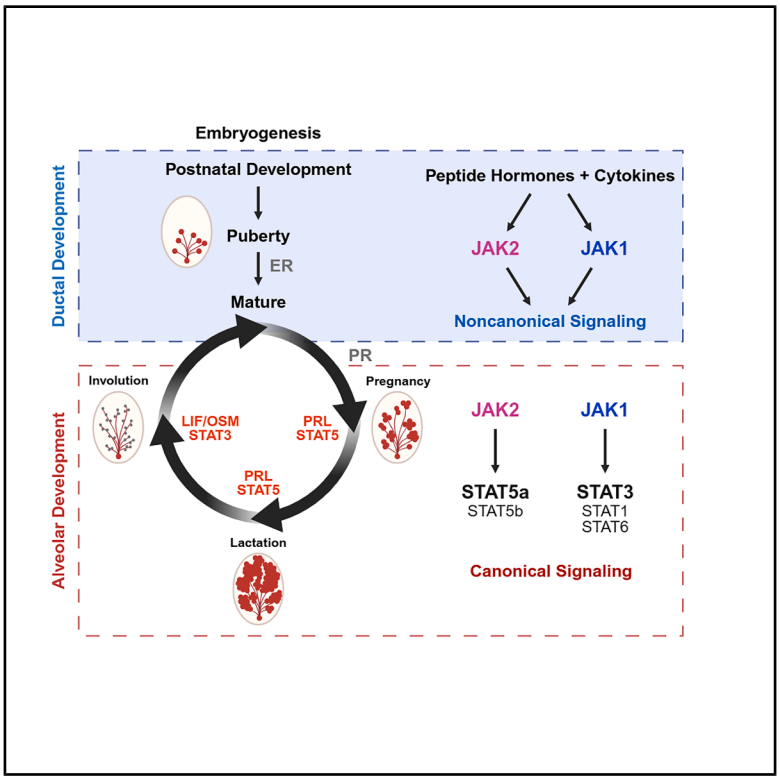
Cell Reports

Article

STAT-independent functions of Janus kinases 1 and 2 are obligatory for the postnatal development of mammary epithelial ducts

Graphical abstract



Highlights

- JAK1 and JAK2 are essential for the formation of mammary epithelial ducts
- Lack of STAT3 and STAT5 causes a compensatory upregulation of active STAT1
- The development of epithelial ducts does not require an activation of STAT proteins
- The expression of STAT proteins is required for the autophosphorylation of JAKs

Authors

Rayane Dennaoui, Madison N. Wicker, Carson Moen, ..., Lukas Kenner, Emilio Casanova, Kay-Uwe Wagner

Correspondence

kuwagner@wayne.edu

In brief

Dennaoui et al. demonstrate that cooperative functions of JAK1 and JAK2 are obligatory for the post-natal development of mammary epithelial ducts. They provide experimental evidence that these functions are independent of STAT proteins, suggesting that the early development of the mammary gland is governed by noncanonical cytokine signaling through JAKs.

Dennaoui et al., 2026, Cell Reports *45*, 116703
January 27, 2026
https://doi.org/10.1016/j.celrep.2025.116703

CellPress

Cell Reports

CellPress
OPEN ACCESS

Article

STAT-independent functions of Janus kinases 1 and 2 are obligatory for the postnatal development of mammary epithelial ducts

Rayane Dennaoui,[1] Madison N. Wicker,[1] Carson Moen,[1] Michaela Schlederer,[2] Kerry Vistisen,[1] Aleata A. Triplett,[3] Thomas Rülicke,[4,5] Hallgeir Rui,[6] Lukas Kenner,[2,7,8] Emilio Casanova,[5,9] and Kay-Uwe Wagner[1,10,*]

[1]Department of Oncology and Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Barbara Ann Karmanos Cancer Institute, Detroit, MI 48201, USA
[2]Department of Pathology, Medical University of Vienna, Vienna, Austria
[3]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198, USA
[4]Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Vienna, Austria
[5]Ludwig Boltzmann Institute for Hematology and Oncology, Vienna, Austria
[6]Department of Pharmacology, Physiology & Cancer Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA
[7]Unit of Laboratory Animal Pathology, University of Veterinary Medicine Vienna, Vienna, Austria
[8]Department of Molecular Biology, Umeå University, Umeå, Sweden
[9]Institute of Pharmacology, Center of Physiology and Pharmacology & Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria
[10]Lead contact
*Correspondence: kuwagner@wayne.edu
https://doi.org/10.1016/j.celrep.2025.116703

SUMMARY

Janus kinases 1 and 2 and STAT transcription factors are critical signaling nodes for numerous growth factors. In the mammary gland, JAK2 and STAT5a/b are essential for alveolar cell differentiation and lactation, but little is known about the cooperative roles of JAKs and STATs before pregnancy. We examined female mice conditionally deficient in JAK1/2 and discovered that both kinases jointly regulate epithelial cell proliferation and ductal morphogenesis. To assess the role of downstream STATs, we generated genetic models co-deficient in STAT3/5a/5b with or without STAT1 or JAK1. Although loss of STAT3/5a/5b leads to a JAK1-dependent upregulation of STAT1, the formation of mammary ducts is unaffected by the lack of expression and activation of all seven STAT proteins. Additionally, STAT deficiency impairs the cytokine-induced autophosphorylation of JAK1/2. These findings suggest that mammary duct development is orchestrated by STAT-independent signaling mechanisms of JAK1 and JAK2, potentially beyond their roles as tyrosine kinases.

INTRODUCTION

The postnatal development of the mammary gland is dependent on a multitude of cellular programs that are orchestrated by growth factor signaling networks.[1–4] Rudimentary mammary ducts without a defined lumen and distinct epithelial bilayer are present at birth. After a short period of allometric growth, the mammary gland undergoes a significant growth spurt at puberty under the influence of steroid and protein hormones, in particular estrogen and insulin-like growth factor.[5] This major phase of postnatal mammogenesis is characterized by the bifurcation and extension of epithelial ducts that eventually fill the entire mammary fat pad. Progesterone and prolactin (PRL) are essential for the formation of terminal ducts with lobuloalveolar units in mature glands of virgin females, as well as the proliferation and differentiation of milk-producing alveolar cells during pregnancy and lactation.[6–9] Locally produced cytokines such as IL-4 and IL-13 support the development of alveolar cells,[10] whereas essential functions of IL-6-class inflammatory cytokines (IL-6, LIF, and oncostatin M [OSM]) are restricted to the postlactational remodeling of the gland after the weaning of the offspring.[11]

More than 50 protein hormones and cytokines activate a variety of receptor complexes that share one or more of the four Janus tyrosine kinases (JAKs) and downstream signal transducers and activators of transcription (STATs). JAK1 and JAK2 are expressed ubiquitously and have pleiotropic functions, and the deficiency in either JAK1 or JAK2 causes embryonic or postnatal lethality.[12–15] While it had been proposed that STATs can be phosphorylated by receptor tyrosine kinases, such as the epidermal growth factor receptor,[16,17] molecular studies using conditional knockout mice and transplantation models demonstrated that JAK1 and JAK2 are essential for STAT activation, and more importantly, they show a remarkable specificity in the tyrosine phosphorylation of individual STAT proteins in particular cell types.[14,15,18,19,20] Of the seven mammalian STATs, five (STAT1, 3, 5a, 5b, and 6) have been reported to be activated

in the epithelium during mammary gland development.[21–23] JAK2 is the pivotal kinase that couples PRL and GH signaling to STAT5a and STAT5b.[14,18,19,24] PRL, the PRL receptor (PRLR), JAK2, and STAT5a/b are obligatory for the proliferation, differentiation, and survival of alveolar cells during pregnancy and lactation.[8,9,18,19,24] On the other hand, the activation of STAT1, STAT3, and STAT6 is primarily facilitated by JAK1.[15,20] Despite the broader impact of JAK1 deficiency on STAT activation, biologically relevant functions of this JAK are limited to postlactational remodeling. The loss of JAK1 uncouples LIF and OSM signaling from its downstream effector STAT3 and causes a delay in the removal of differentiated alveolar cells.[20,25] STAT1 is not required for alveolar morphogenesis, and STAT6 was reported to play a minor role in the functional differentiation of the gland during mid-pregnancy.[10,26,27]

The collective results from studies using genetically engineered models deficient in individual JAKs and STATs suggest that only three of these signal transducers (i.e., JAK2 and STAT5a/b) are essential for mammogenesis. However, their functions are limited to morphological changes during pregnancy and lactation. Females that lack JAK1, JAK2, or individual STAT proteins develop a mammary epithelial ductal tree. This is also the case for transplanted recipient mice with an epithelial cell-specific deletion of the PRLR or the growth hormone receptor (GHR).[28,29] Aside from established functions of individual growth factor pathways, little is known about the cooperative, epithelial cell-intrinsic roles of protein hormone and cytokine signaling in pre- and postpuberal mammogenesis prior to pregnancy. In this study, we generated females with an epithelial-specific double knockout of JAK1 and JAK2 and discovered that both tyrosine kinases jointly control the postnatal development of mammary ducts. Depending on the inhibition of individual JAK/STAT cascades, we observed that STAT proteins exhibit compensatory activation profiles. Most notably, STAT1 is significantly upregulated in the mammary epithelium of STAT3/5a/5b triple-knockout mice. Using quadruple-knockout mouse models that are deficient in STAT3, STAT5a/b, along with STAT1 or its upstream activator JAK1, we demonstrate that the growth of the mammary ductal epithelium does not require the expression and activation of all seven mammalian STAT proteins. Therefore, the development of the postnatal mammary gland is governed by STAT-independent functions of JAK1 and JAK2. We also discovered that STAT deficiency diminishes or blocks the cytokine-induced autophosphorylation of JAK1 and JAK2, which is a prerequisite for their function as tyrosine kinases. Based on these collective findings, we propose that the post-natal development of the mammary gland is mediated by noncanonical signaling mechanisms of JAK1 and JAK2 that may not exclusively rely on their kinase functions.

RESULTS

Cooperative signaling through JAK1 and JAK2 is essential for postnatal mammary gland development

We generated female mice with a mammary-epithelial specific knockout of JAK1 and JAK2 (MMTV-Cre *Jak1*$^{fl/fl}$ *Jak2*$^{fl/fl}$) to investigate whether protein hormones and cytokines that signal through these kinases have synergistic or compensatory roles in mammary gland development. While the formation of a mammary epithelial ductal tree was completed by 12–14 weeks of age in wild-type control animals and single-knockout mice that lack JAK1 or JAK2 (Figures 1A, I–III), adult females conditionally deficient in both JAKs exhibited a severe impairment of ductal development (Figures 1A, IV). Interestingly, several age-matched JAK1/2 double-deficient mice showed rudimentary ducts that were similar to newborn or prepubescent pups (Figures 1A, V). MMTV-Cre *Jak1*$^{fl/fl}$ *Jak2*$^{fl/fl}$ females were fertile and, on average, gave birth to the same number of pups per litter as *Jak1*$^{fl/fl}$ *Jak2*$^{fl/fl}$ breeder animals (7.4 ± 0.7 N = 15 and 7.39 ± 0.39 N = 43, respectively). None of the postpartum JAK1/2 double-knockout dams were lactating and able to support their offspring, despite some ductal growth after multiple full-term pregnancies (Figures 1A, VI).

The comparison of the mammary glands from single-knockout females confirmed our previous observation that, despite the complete development of the epithelial compartment, the ductal tree in mice lacking JAK2 had fewer tertiary side branches with alveolar buds,[19] whereas glands from nulliparous JAK1 knockout females were indistinguishable from age-matched controls (Figures 1A, I–III). To assess potential differences in the contribution of the two JAKs to ductal development, we analyzed conditional knockout mice that express only one copy of either JAK1 or JAK2 in the mammary epithelium (Figure 1B). In the complete absence of JAK1, one copy of JAK2 was sufficient to complete the development of the entire ductal tree. In contrast, haploinsufficiency of JAK1 in the conditional JAK2 knockout led to a significant impairment of epithelial growth. Approximately 70% of the glands (17 of 24) collected from 12- to 19-week-old nulliparous MMTV-Cre *Jak1*$^{fl/wt}$ *Jak2*$^{fl/fl}$ females exhibited an incomplete filling of their mammary fat pads (75% or less epithelial ducts), and 5 glands were severely underdeveloped with less than 30% ductal development. The collective findings of the phenotypic analysis of females conditionally deficient in JAK1 and JAK2 suggested that cooperative signaling of cytokines through JAKs plays essential roles in postnatal development. JAK2 appears to be the pivotal driver for ductal morphogenesis, and one wild-type allele of this kinase was sufficient to propel the elongation of ducts. While two copies of *Jak1* may compensate for the loss of JAK2, the absence of just one *Jak1* allele in the context of a JAK2 knockout impaired mammary gland development.

The cooperative functions of JAK1 and JAK2 in mammary gland development are epithelial cell intrinsic

The MMTV-Cre is expressed in basal and luminal epithelial cells but not in the mammary stroma. This transgene is also known to exhibit a mosaic expression pattern in several other tissues, including skin, salivary gland, thymus, and spleen.[30,31] The mammary gland is a derivative of the ectoderm, and variations in the severity of the mammary gland phenotype resulting from the deletion of JAK1/2 (Figures 1A, IV and V) were likely a consequence of differences in the timing and uniformity of Cre expression that starts in the embryonic ectoderm and increases significantly during the first week of postnatal development. To exclude the possibility that the defect in mammary

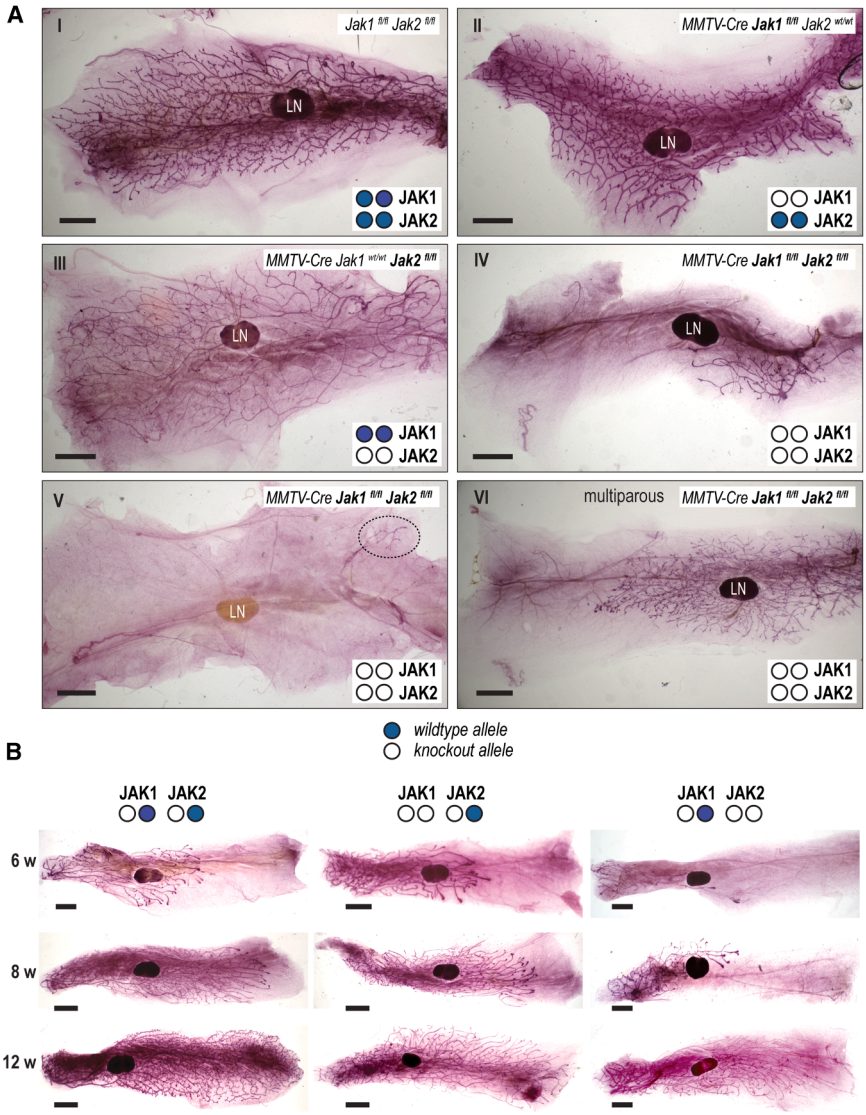


Figure 1. Combined signaling through JAK1 and JAK2 is essential for postnatal mammary gland development

(A) Carmine alum-stained mammary glands from 14-week-old nulliparous (I–V) and nonpregnant multiparous (VI) female mice with a mammary epithelial cell-specific knockout of JAK1 (II), JAK2 (III), or both Janus kinases (IV–VI) in comparison to a wild-type control (I); LN, lymph node; bars, 2 mm. The dotted oval in panel V marks the location of the rudimentary JAK1/2-deficient mammary epithelium. The circles within each panel illustrate the combination of wild-type (blue) and knockout (white) alleles.

(B) Mammary glands of 6-, 8-, and 12-week-old mice that are haploinsufficient in JAK2 with a JAK1 conditional knockout (middle; MMTV-Cre $Jak1^{fl/fl}$ $Jak2^{fl/wt}$) and vice versa (right; MMTV-Cre $Jak1^{fl/wt}$ $Jak2^{fl/fl}$), as well as JAK1/2 heterozygous knockout controls (left; MMTV-Cre $Jak1^{fl/wt}$ $Jak2^{fl/wt}$); bars, 2 mm. The images are representative of 6–12 females per genotype and time point.

gland development in the conditional double-knockout females is a secondary consequence of the loss of JAK1/2 in other organs, we performed a transplantation experiment of GFP-labeled epithelial cells of mutant mice and controls into epithelial-free fat pads of the contralateral #4 (inguinal) mammary glands of six wild-type recipient females (Figure 2A). 6–8 weeks after transplantation, we confirmed the successful engraftment of the transplanted epithelia. We observed a significant delay in the elongation of JAK1/2-deficient ducts compared to JAK1/2 haploinsufficient controls (Figure 2B). Two transplanted recipients were bred, and the examination of the glands immediately following the birth of the offspring revealed a major difference in epithelial growth (Figure 2C). In contrast to the strongly GFP-positive contralateral control gland, the JAK1/2 double-knockout transplant lacked the expression of GFP and exhibited alveolar development. This observation suggested that pregnancy hormones fueled a selective growth and differentiation of epithelial cells that did not express the MMTV-Cre transgene.

Similar to the findings from the transplantation experiment, we also observed a strong negative selection of cells with a co-deletion of JAK1 and JAK2 when primary mammary epithelial cells were explanted and immortalized through the expression of the SV40 large T antigen. None of the resulting cell cultures exhibited a loss in the expression of JAKs as determined by immunoblot (Figure 2D, upper panel; Figure S1A). The flow cytometric analysis showed that, in contrast to the controls, the vast majority of expanding cells from the JAK1/2 double-knockout mice were GFP-negative (Figure 2E), and both JAKs were still expressed in cultures derived from sorted GFP-positive clones (Figure 2D, lower panel; Figure S1B).

Deficiency in STAT3 and STAT5a/b does not phenocopy the impaired ductal development in JAK1/2 double-knockout females

Given the phenotypic resemblance of conditional knockout mice lacking JAK2 and STAT5a/b, as well as JAK1 and STAT3,[19,20,24,25] it was rational to assume that the block in postnatal mammary gland development in JAK1/2 double-deficient females was due to the lack of tyrosine phosphorylation of STAT5a/b and STAT3. This notion would imply that STAT proteins may function in a compensatory manner during early postnatal mammary gland development. To examine the activation of STAT3 and STAT5 in the glands of newborn mice, we employed the MMTV-tTA TetO-H2B-GFP double transgenic reporter to determine the sex of newborn pups using GFP visualization goggles (Figure S2A).

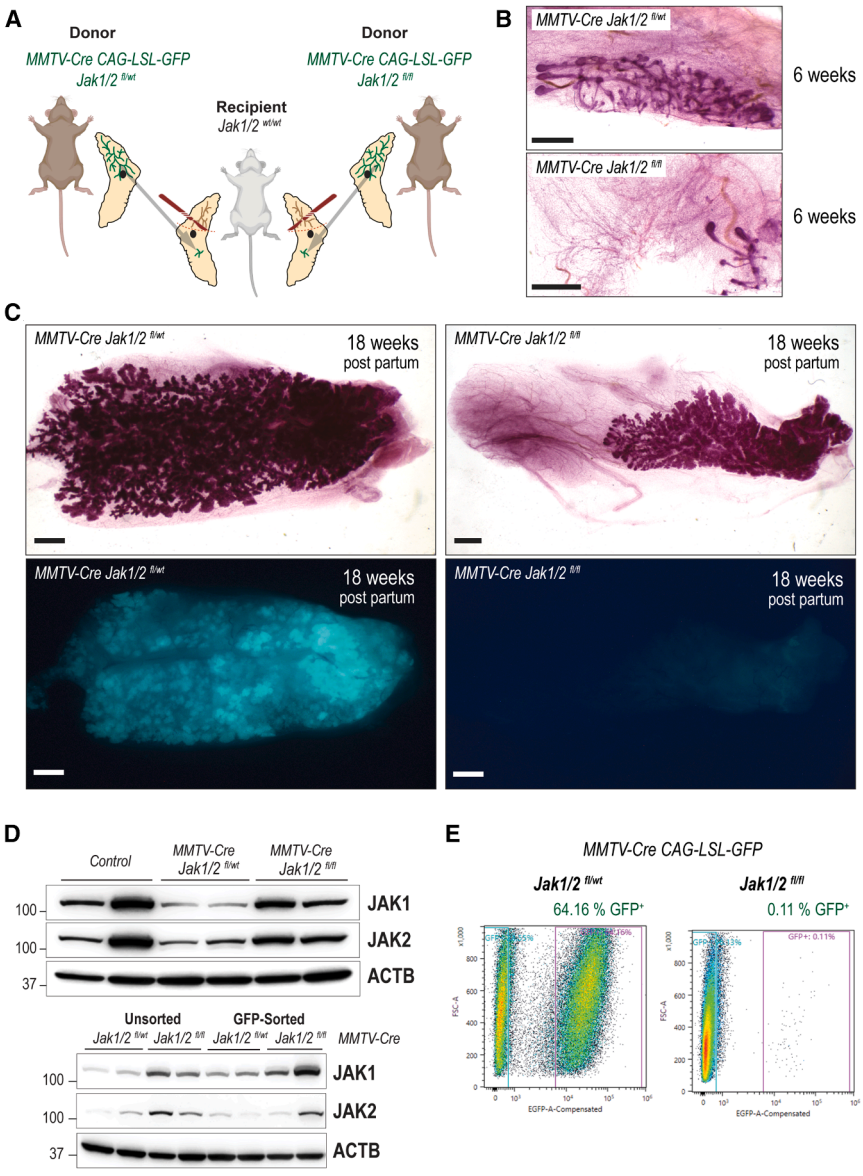


Figure 2. Epithelial cell-intrinsic functions of JAK1 and JAK2 are essential for the growth of mammary ducts

(A) Schematic outline of the transplantation of GFP-labeled mammary epithelium from JAK1/2 conditional knockout females (MMTV-Cre *Jak1*$^{fl/fl}$ *Jak2*$^{fl/fl}$ CAG-LSL-GFP) and heterozygous JAK1/2 knockout controls (MMTV-Cre *Jak1*$^{fl/wt}$ *Jak2*$^{fl/wt}$ CAG-LSL-GFP) into the contralateral epithelial-free fat pads of 3-week-old wild-type recipient females (N = 6).

(B) Carmine alum-stained mammary gland wholemounts of a recipient female 6 weeks after transplantation; bars, 1 mm.

(C) Mammary gland wholemounts of a postpartum recipient female 18 weeks after transplantation; carmine alum-stained (upper panel) and corresponding GFP fluorescent image (lower panel) prior to fixation and staining; bars, 1 mm.

(D) Immunoblot analysis of JAK1 and JAK2 expression in immortalized explanted mammary epithelial cells from heterozygous and homozygous JAK1/2 conditional knockout mice and wild-type control (upper panel), as well as unsorted and GFP-sorted immortalized epithelial cells from the mammary glands of MMTV-Cre *Jak1*$^{fl/fl}$ *Jak2*$^{fl/fl}$ CAG-LSL-GFP females in addition to MMTV-Cre *Jak1*$^{fl/wt}$ *Jak2*$^{fl/wt}$ CAG-LSL-GFP heterozygous control females (lower panel; two biological repeats per genotype). Beta-actin (ACTB) was used as a loading control. The densitometry results of immunoblots from 3 technical repeats of the 2 biological repeats are shown in Figures S1A and S1B.

(E) Fluorescence-activated sorting and enrichment scores of GFP-labeled mammary epithelial cells from JAK1/2 conditional double-knockout females and heterozygous knockout controls shown in (D).

The specific GFP signal was used to microdissect the mammary glands of 3-day-old pups under a fluorescent stereoscope (Figure S2B). The histological analysis of immunostained sections of these glands revealed that STAT3 was highly active in immature mammary ducts (Figure S2C). The strongest nuclear accumulation of STAT3 was observed in the outer epithelial cells adjacent to the stroma, and the staining was gradually weaker toward the center of the ducts, where most of the GFP-positive cells resided. Since the knockout of JAK1 and the consequential lack of STAT3 activation were not essential for ductal development,[20] we postulated that their functions could be compensated by JAK2/STAT5 signaling (Figure S2D). To address this notion, we examined mammary gland sections of prepubescent JAK1 conditional knockout females (MMTV-Cre *Jak1*$^{fl/fl}$). As anticipated, active STAT3 was absent in the JAK1-deficient mammary epithelium (Figure S2E, upper panel). Although we observed pSTAT3 in the epithelium of wild-type controls, the immunofluorescent signal was less intense compared to newborn females, but more importantly, the nuclear expression of STAT5 was markedly higher in the JAK1-deficient epithelium compared to wild-type controls (Figure S2E, lower panels).

To determine the biological relevance of the observed compensatory activation of STAT5 when JAK1/STAT3 signaling is inhibited, we utilized a conditional knockout mouse model where the three evolutionarily related *Stat3*, *Stat5a*, and *Stat5b* genes can be deleted in a single recombination event (Figure S3A). The lack of the encoded STAT proteins was validated in *Stat3/5*$^{fl/fl}$ embryonic fibroblast cultures expressing Cre recombinase (Figure S3B). Male and female conditional triple-knockout mice (MMTV-Cre *Stat3/5*$^{fl/fl}$) were fertile and similar in size to *Stat3/5*$^{fl/fl}$ littermates, and an off-target deletion of STAT3 and STAT5 was not detected in immune cells (Figure S3C). Contrary to the notion that a combined deficiency in STAT3 and STAT5a/b may phenocopy the critical cooperative functions of their upstream JAK1 and JAK2 in early mammogenesis, the mammary

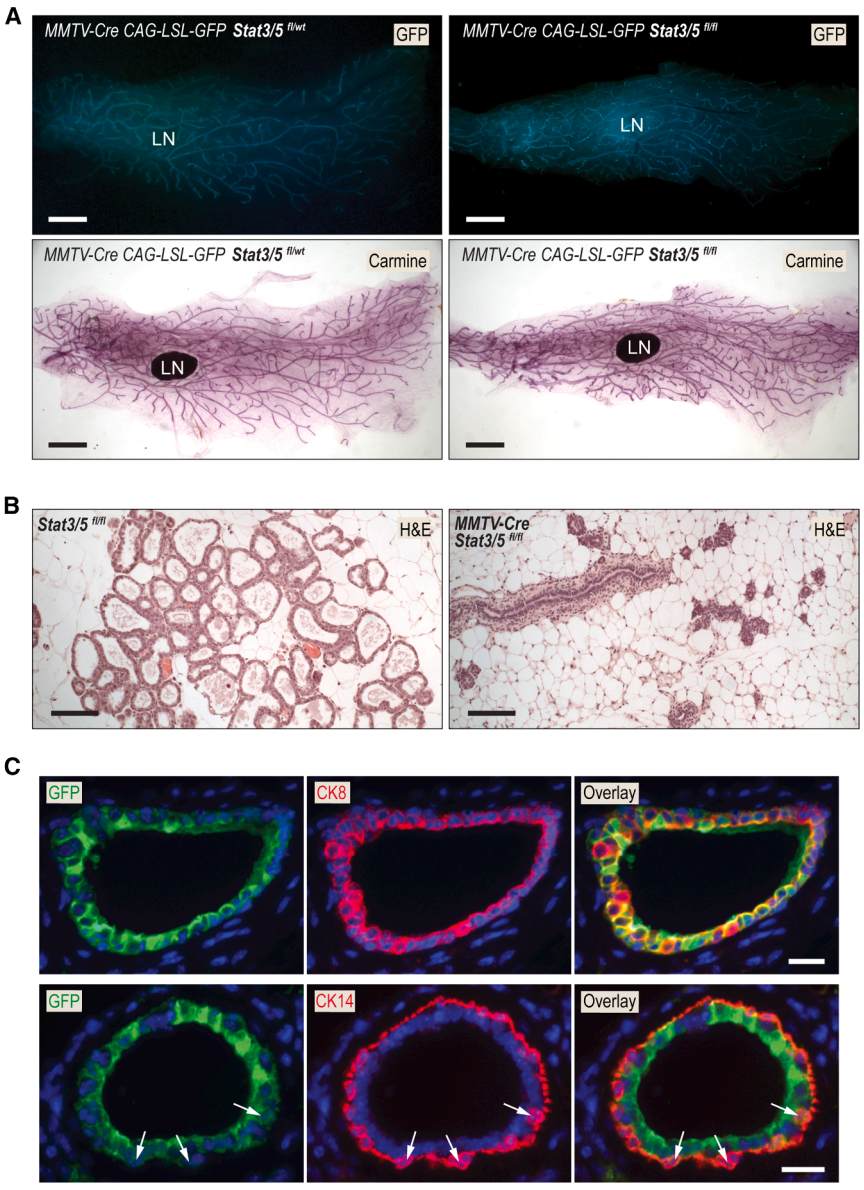


Figure 3. Mammary gland-specific STAT3 and STAT5a/b triple-knockout females develop mammary ducts but exhibit impaired alveologenesis

(A) GFP fluorescence and carmine alum-stained mammary gland wholemounts of a STAT3- and STAT5a/b conditional knockout female (MMTV-Cre *Stat3/5*$^{fl/fl}$ CAG-LSL-GFP) and a haploinsufficient control (MMTV-Cre *Stat3/5*$^{fl/wt}$ CAG-LSL-GFP); LN, lymph node; bars, 2 mm.

(B) Hematoxylin and eosin (H&E)-stained sections of mammary gland tissues from a postpartum STAT3- and STAT5a/b triple-knockout female and control without the MMTV-Cre transgene 3 h after the birth of their offspring; bars, 100 μm.

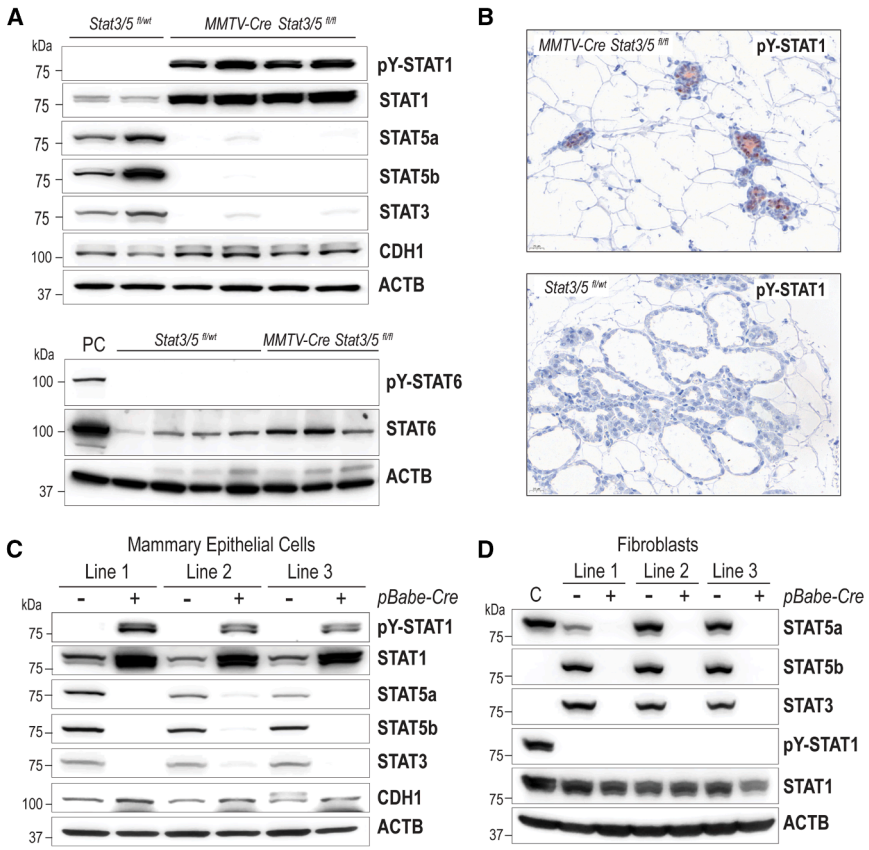
(C) Immunofluorescent co-staining of GFP along with cytokeratin 8 (CK8) or cytokeratin 14 (CK14) on histologic sections of mammary glands from a nulliparous MMTV-Cre *Stat3/5*$^{fl/fl}$ CAG-LSL-GFP female; bars, 20 μm. Arrows point to examples of CK14-positive basal epithelial cells expressing GFP.

epithelial-specific knockout of the entire *Stat3/5* locus did not cause any developmental abnormalities in the formation of the epithelial ductal tree (Figure 3A). However, postpartum females were unable to nurse their offspring due to the complete absence of secretory alveolar cells that are known to depend on the expression and activation of JAK2 and STAT5 (Figures 3B and S3D). The strong and uniform fluorescence of GFP from the CAG-LSL-GFP reporter (Figure 3A) and the complete absence of alveologenesis during pregnancy were indicative that there was no negative selection of MMTV-Cre-expressing ductal epithelial progenitors and their descendants within luminal and basal epithelial cell lineages, as validated by immunostaining (Figure 3C). In conclusion, the impaired development of mammary epithelial ducts in JAK1/2 double-knockout mice was not a direct consequence of a deficiency in the activation of STAT3 and STAT5a/b.

A mammary epithelial cell-specific knockout of STAT3 and STAT5 causes a sustained upregulation of STAT1 in ductal and functionally differentiated alveolar cells

The absence of STAT3, STAT5a, and STAT5b in the mammary epithelium of conditional triple-knockout females was validated by immunoblot, supporting the notion that these three STAT proteins are not essential for the postnatal development of the epithelial ductal tree (Figure 4A). However, the lack of STAT3/5 resulted in significantly elevated levels of total and tyrosine phosphorylated STAT1 but not STAT6 (Figure 4A). The compensatory upregulation of STAT1 was specific to the mammary epithelium (Figure 4B) and independent of the reproductive state (Figure S4). Interestingly, a sustained increase in STAT1 was also observed when STAT3 and STAT5a/b were deleted in cultured mammary epithelial cells (Figures 4C and S5), suggesting that the molecular determinants for the elevated levels of STAT1 are not dependent on the mammary gland stroma. The compensatory expression of STAT1 was specific to mammary epithelial cells and not observed in cultured fibroblasts that lack STAT3 and STAT5a/b (Figure 4D).

Next, we generated and analyzed mammary epithelial-specific knockout females with a targeted deletion of STAT3 and STAT5 in developing alveolar cells during late pregnancy and lactation (WAP-Cre *Stat3/5*$^{fl/fl}$). The transcriptional activation of the whey acidic protein (*Wap*) gene promoter, which targets a high expression of Cre recombinase to functionally differentiated alveolar cells, is dependent on PRL signaling and the JAK2-mediated phosphorylation of STAT5 (Figure S6A). Similar to JAK2 and



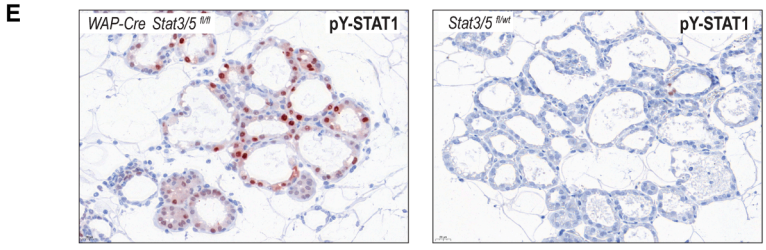


Figure 4. Upregulation and tyrosine phosphorylation of STAT1 in mammary epithelial cells lacking STAT3 and STAT5a/b

(A) Immunoblot analysis of the expression and activation of selected STAT proteins in mammary epithelial cells from STAT3 and STAT5a/b conditional triple-knockout mice (MMTV-Cre *Stat3/5*$^{fl/fl}$; $N = 4$) and age-matched *Stat3/5*$^{fl/wt}$ controls ($N = 2$ and 4, respectively). E-cadherin (CDH1) and beta-actin (ACTB) were used as loading controls. PC, wild-type control epithelial cells treated with IL-4 served as a positive control for tyrosine-phosphorylated STAT6 (pY-STAT6).

(B) Immunohistochemistry of tyrosine-phosphorylated STAT1 (pY-STAT1) on histologic sections of mammary glands from postpartum STAT3/5 triple-knockout and control females; bars, 20 μm.

(C and D) Immunoblot analysis of active and total levels of STAT1 before and after the retroviral-based expression of Cre recombinase in three immortalized mammary epithelial cell lines (C) and three mouse embryonic fibroblast lines (D). Wild-type mammary epithelial cells and interferon gamma-treated wild-type mouse fibroblasts served as positive controls (C) for STAT5a and active STAT1, respectively. The densitometry results of the STAT proteins in (C) from 3 technical repeats of the 3 biological repeats are shown in Figure S5.

(E) Immunohistochemistry of active STAT1 (pY-STAT1) in alveolar cells of mammary glands of a postpartum WAP-Cre *Stat3/5*$^{fl/fl}$ female and an age-matched control; bars, 20 μm.

STAT5a/b conditional knockouts, the combined deficiency in STAT5 and STAT3 resulted in the loss of secretory alveolar cells and impaired milk production and secretion (Figures S6B and S6C). While these findings confirm that the survival of secretory epithelial cells is contingent on STAT5, they also indicate that the loss of STAT5-deficient epithelial cells did not require the cell death-promoting functions of STAT3. More importantly, the triple knockout of STAT3 and STAT5a/b was accompanied by a strong upregulation of pY-STAT1 (Figure 4E), suggesting that the acute compensatory activation of this STAT protein is not specific to the ductal epithelium and also occurs in differentiated luminal alveolar cells that express the late milk protein WAP.

The compensatory expression and activation of STAT1 in the absence of STAT3 and STAT5 are not essential for postnatal mammary gland development

To determine the biological significance of the observed consequential activation of STAT1 when STAT3 and STAT5a/b were deleted from the mammary epithelium, we generated conditional knockout mice that lack STAT3 and STAT5a/b on a STAT1-deficient background (MMTV-Cre *Stat3/5*$^{fl/fl}$ *Stat1*$^{-/-}$; Figure 5A). As we and others reported previously,[26,27] a knockout of STAT1 did not have any noticeable effect on the growth and functional differentiation of the mammary gland. Remarkably, the combined deficiency in STAT3, STAT5a/b, and STAT1 in the epithelium did not result in a developmental arrest of mammary ducts before or after puberty. The mammary glands of mature nulliparous quadruple-knockout mice were indistinguishable from age-matched littermate controls expressing STAT3 and STAT5 (Figure 5B). The targeted deletion of STAT3 and STAT5a/b in STAT1-deficient mammary epithelial cells of MMTV-Cre *Stat3/5*$^{fl/fl}$ *Stat1*$^{-/-}$ females was validated by immunoblot (Figure 5C, upper panel). Moreover, we confirmed the absence of STAT4, whose expression is restricted to immune cells. The STAT2 protein, typically absent in the mammary epithelium, was co-upregulated along with STAT1 when STAT3 and STAT5 were deleted (Figure 5C, middle panel). STAT2 was not detected in the STAT1/3/5a/5b quadruple-knockout epithelial cells, supporting the notion that the atypical expression of STAT2 was dependent on STAT1. Out of the seven known STAT proteins, only STAT6 was expressed in the

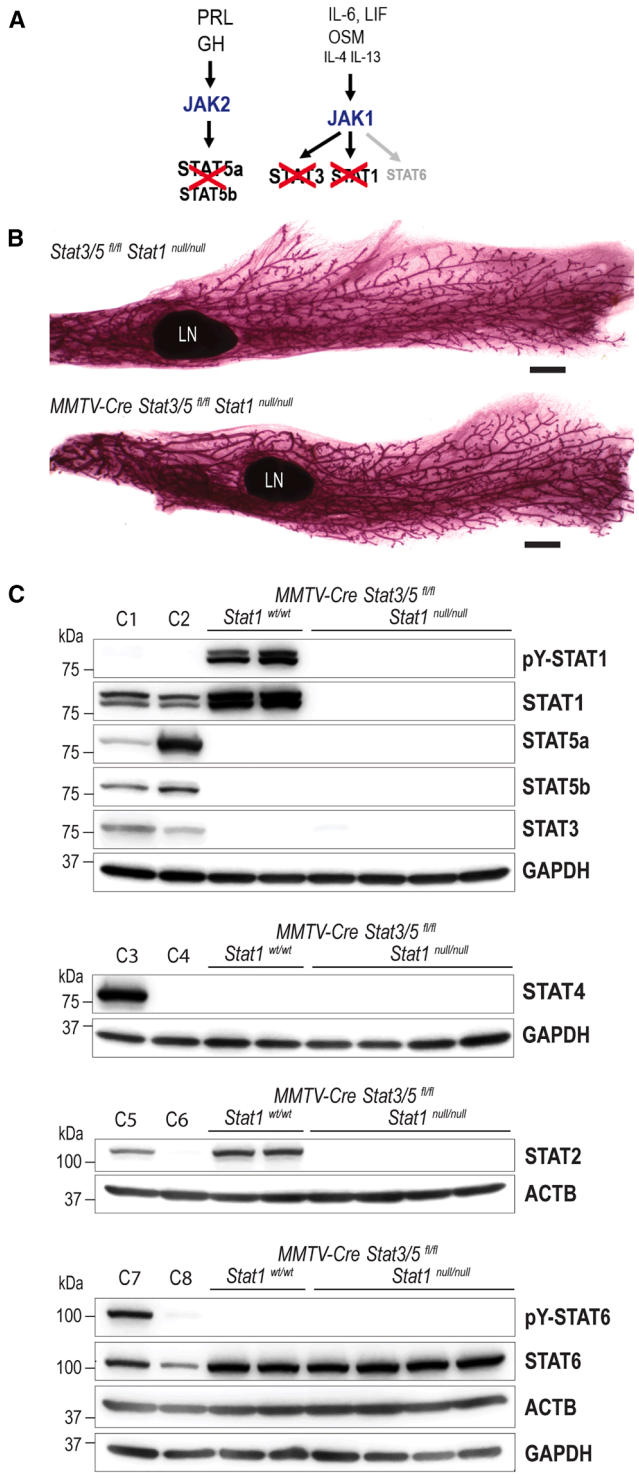


Figure 5. The mammary epithelial-specific expression and compensatory activation of STAT proteins are not required for the formation of the mammary ductal tree

(A) Schematic of the knockout of STAT1, STAT3, STAT5a, and STAT5b in canonical JAK/STAT signaling cascades of the mammary gland.

(B) Carmine alum-stained mammary gland wholemounts of nulliparous female mice with a targeted deletion of four *Stat* genes in the mammary epithelium (MMTV-Cre *Stat3/5*$^{fl/fl}$ *Stat1*$^{-/-}$) and a STAT1 single-knockout littermate control; bars, 1 mm.

(C) Immunoblot analysis of the seven known mammalian STAT proteins in mammary epithelial cells (MECs) from STAT1/3/5a/5b quadruple-knockout females ($N = 4$) in comparison to age-matched STAT3/5a/5b triple-knockout mice ($N = 2$). Other controls: C1 and C2, positive controls for active STAT3 and STAT5, mammary gland tissues from wild-type mice on day 1 of involution and lactation, respectively; C3, spleen as a positive control for STAT4; C4, wild-type MECs as a negative control for STAT4; C5 and C6 interferon-treated and untreated MECs as positive and negative controls for STAT2; C7 and C8, IL-4-treated and untreated wild-type MECs as positive and negative controls for active STAT6. GAPDH and ACTB served as loading controls.

mammary epithelium of the quadruple-knockout mice. However, this signal transducer was neither upregulated nor tyrosine phosphorylated (Figure 5C, lower panel), suggesting that STAT6 did not compensate for the loss of all other members of the STAT family.

A conditional knockout of JAK2 in mouse mammary epithelial cells had no effect on the PRL-induced activation of the mitogen-activated protein kinase pathway, c-SRC, and the focal adhesion kinase. However, the targeted deletion of JAK2 resulted in reduced PI3K/AKT signaling.[32] In developing alveolar cells during late pregnancy, PRL signaling through JAK2 and STAT5 enhances the transcriptional expression and activation of AKT1 as well as the regulatory and catalytic subunits of the PI3K (p85 and p110a).[33,34] A weaker activation of AKT was observed when JAK2-expressing mammary epithelial cells from non-pregnant females were treated with growth hormone (Figure S7A). Accompanied by the absence of phosphorylated STAT5, a knockout of JAK2 in these cells suppressed the activation of AKT, but JAK2-deficient cells still exhibited a robust activation of AKT in response to cytokine signaling through JAK1 and STAT1/3 (Figure S7B). Since these results confirmed the previously established roles of JAKs for the cytokine-induced activation of PI3K/AKT signaling, we investigated whether the development of mammary epithelial ducts in the absence of STAT protein expression in the presence of JAK1/2 was a consequence of a compensatory increase in PI3K/AKT signaling (Figure S8). Compared to the previously observed high expression of AKT in functionally differentiating alveolar cells of pregnant mice, the activation of PI3K/AKT signaling in the normal mammary epithelium of non-pregnant females was very low. More importantly, we did not observe a compensatory upregulation of active AKT, and we found that epithelial cells from STAT1/3/5a/5b quadruple-knockout females completely lacked the expression of the smaller regulatory PI3 kinase subunits, confirming that p50 and p55 encoded by the *Pik3r1* locus are direct targets of JAK1/STAT3 signaling.[20,35]

Noncanonical, STAT-independent functions of JAK1 and JAK2 govern the development of the postnatal mammary gland

The combined knockout of STAT1, STAT3, STAT5a, and STAT5b in the mammary epithelium resulted in a complete deficiency in the expression or activation of all seven STAT proteins. This suggested that, unlike the upstream kinases JAK1 and JAK2, STAT proteins and their canonical activation by JAKs may not be required for the postnatal development of mammary epithelial

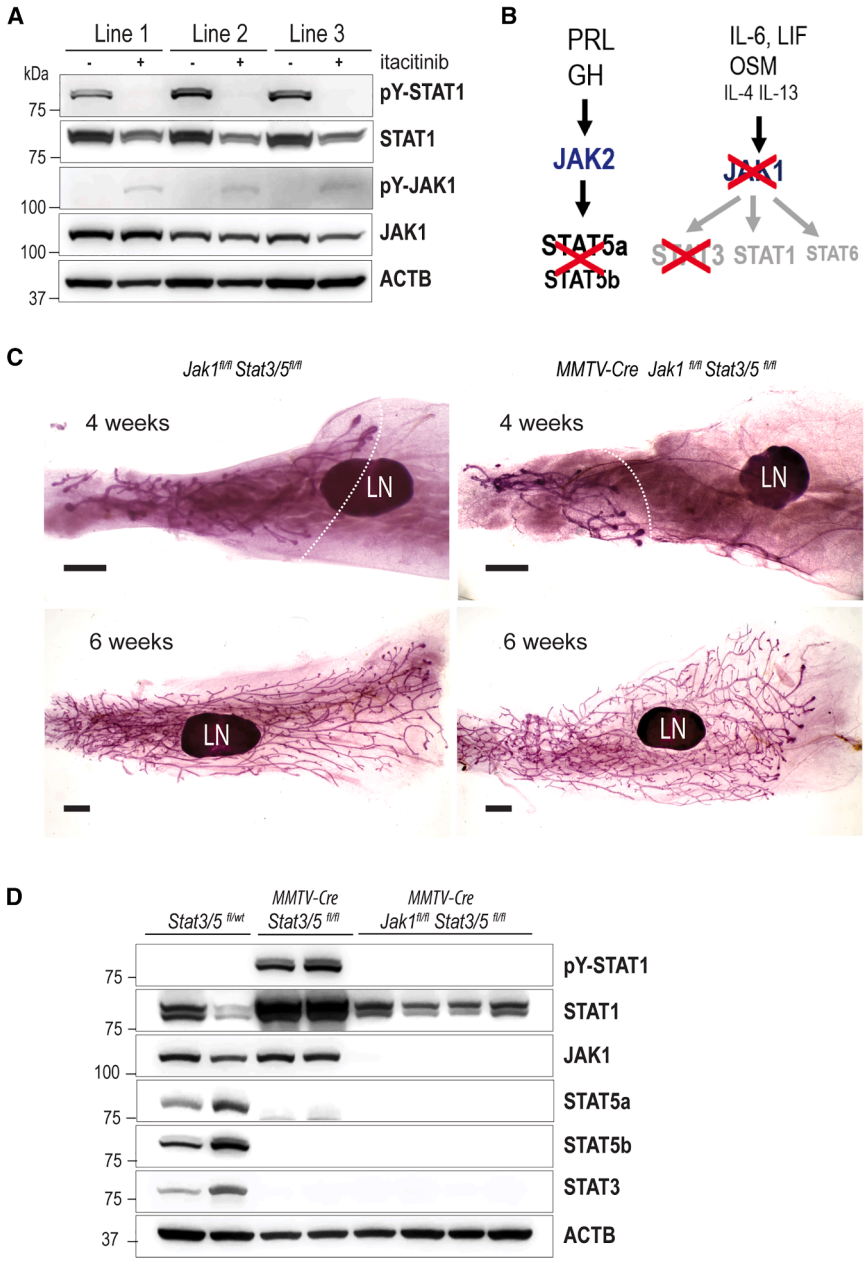


Figure 6. Differential control of postnatal mammary gland development by JAKs and STATs

(A) Immunoblot analysis of STAT1 and JAK1 expression and activation in response to the pharmacological inhibition of JAK1 using 1 μM itacitinib in three mammary epithelial cell lines co-deficient in STAT3, STAT5a, and STAT5b. Beta-actin (ACTB) was used as a loading control. The densitometry results of immunoblots from 2 technical repeats of the 3 biological repeats are shown in Figure S9A.

(B) Schematic of a quadruple knockout of STAT3, STAT5a, and STAT5b along with JAK1 to genetically ablate the compensatory activation of STAT1 in the STAT3/5a/5b triple-knockout mammary epithelium and to determine the STAT-independent contribution of JAK2 to mammary gland development.

(C) Carmine alum-stained mammary gland whole-mounts of 4- and 6-week-old nulliparous females that are conditionally deficient in STAT3, STAT5a/b, and JAK1 (MMTV-Cre *Stat3/5*$^{fl/fl}$ *Jak1*$^{fl/fl}$) and littermate controls without the MMTV-Cre transgene; bars, 1 mm. Dotted lines mark the invasive fronts of the terminal ends of ducts.

(D) Immunoblot analysis of STAT1 expression and activation in response to the knockout of JAK1 in the mammary epithelium of STAT3/5a/5b triple-knockout females; 4 biological repeats of quadruple-knockout females compared to 2 wild-type controls and 2 STAT3/5a/5b triple-knockout mice. Beta-actin (ACTB) served as a loading control. The densitometry results of immunoblots from the 4 biological and 2 technical repeats are shown in Figure S9B.

ducts. To validate this important finding, we first investigated whether the compensatory upregulation of active STAT1 in response to the deletion of STAT3 and STAT5a/b was dependent on JAK1. For this purpose, we treated three STAT3/5 triple-knockout epithelial cell lines expressing high levels of active STAT1 (Figure 4C) with the JAK1-selective inhibitor itacitinib. The administration of this pharmacological agent led to the complete inhibition of the tyrosine phosphorylation of STAT1 and a consequential decrease in total STAT1 (Figures 6A and S9A). The paradoxical increase in pY-JAK1 expression that we observed in itacitinib-treated cells suggested that the mode of JAK1 inhibition by this drug was similar to ruxolitinib, which traps JAKs in a tyrosine phosphorylated, yet functionally inhibited, conformation that prevented its dephosphorylation.[36]

After determining the JAK1-dependent upregulation of active STAT1, we generated a conditional quadruple knockout of JAK1, STAT3, and STAT5a/b to block the activation of STAT1 and the remaining STATs. The main goal of this line of investigation was to study the development of the postnatal mammary gland when only JAK2 was present in epithelial cells that were devoid of JAK1 and the expression or compensatory activation of STAT proteins, in particular, STAT5a and STAT5b, which are the main effectors of JAK2 (Figure 6B). Unlike the genetic ablation of the STATs, the deletion of JAK1 in addition to STAT3/5a/5b led to a minor but noticeable delay in ductal elongation before and after the onset of puberty (Figure 6C). In contrast to the terminal ends of epithelial ducts that reached the lymph nodes at 4 weeks of age in wild-type control females, the JAK1/STAT3/5a/5b-deficient epithelium in age-matched quadruple knockouts exhibited a delay in ductal growth. The decelerated formation of the ductal tree and incomplete filling of the mammary fat pad were still noticeable at 6 weeks

(Figure 6C, lower panel), but the knockout glands were morphologically indistinguishable from those in wild-type controls at 12 weeks of age. The immunoblot analysis of isolated mammary epithelial cells from mature virgin quadruple-knockout females showed that the knockout of JAK1 was sufficient to block the high compensatory activation of STAT1 in the glands of STAT3/5a/5b-deficient females (Figures 6D and S9B). The collective data from the JAK1/STAT3/5a/5b quadruple-knockout model reaffirmed that the expression and compensatory activation of STAT proteins are not essential, suggesting that STAT-independent functions of JAK1 and JAK2 govern the early postnatal development of the mammary gland.

The analysis of mammary glands that are haploinsufficient in either JAK1 or JAK2 on the background of a bi-allelic knockout of the JAK1 or JAK2 counterparts revealed that JAK2 was likely the pivotal driver for the postnatal growth spurt of the mammary epithelial ducts (Figure 1B). This finding was confirmed by the direct comparison of the fully developed mammary glands of JAK2 conditional knockout females with age-matched glands from the two quadruple-knockout models that are deficient in the expression and activation of STATs alone and in combination with the knockout of JAK1 (Figure S10). In comparison to JAK1/2 double knockouts (Figure S10, II), JAK2-deficient mammary epithelial cells expressing JAK1 in aging MMTV-Cre *Jak2*$^{fl/fl}$ nulliparous females (Figure S10, IV) were capable of filling the fat pad. However, the JAK2-deficient ducts were narrower and had fewer side branches compared to littermate controls expressing JAK1/2 (Figure S10, I and III) and females that were conditionally deficient in STAT1/3/5a/5b (Figure S10, VIII) or JAK1/STAT3/5a/5b (Figure S10, VI). This suggested that the pivotal biological functions of JAK2 in postnatal mammary gland development are independent of the canonical activation of its downstream targets STAT5a/b and the other STATs controlled by JAK1. The main discriminating feature between the STAT-deficient glands and wild-type controls that bore some similarity to the JAK2 knockout was the lack of alveolar units at the terminal ends of ducts, confirming the primary role of canonical JAK2/STAT5 signaling in alveolar progenitors.[19,24]

The cytokine-induced tyrosine phosphorylation of JAK1 and JAK2 depends on the expression of STAT proteins

Since STAT proteins were not essential for the pivotal role of JAK2 and the cooperating function of JAK1, we postulated that both JAKs engage noncanonical signaling mechanisms to promote the postnatal development of the mammary gland. Insulin receptor substrates 1 and 2 (IRS-1 and IRS-2, respectively) are suggested phosphorylation targets of JAK2 and JAK1 in response to PRL and GH signaling in the liver, as well as IL4/IL-13 stimulation in cultured mammary epithelial cells derived from mid-pregnant females.[37,38] We conducted a series of immunoblot analyses and found that IRS-1 and, particularly, IRS-2 protein levels were very low in isolated mammary epithelial cells from non-pregnant females (Figure S8). While insulin and insulin-like growth factor (IGF-1) treatment resulted in the expected tyrosine phosphorylation of IRS-1 in the epithelial cells of wild-type mice, signaling through their corresponding receptor tyrosine kinase receptors did not lead to a cross-activation of JAK1 and JAK2 (Figure S11). More importantly, autoactivated JAK1 (Figures S11A and S11C) and JAK2 (Figure S11B) that were capable of phosphorylating STAT6, STAT3, and STAT5 did not result in the tyrosine phosphorylation of IRS-1. Based on these results, as well as the low expression of the insulin receptor substrates in the glands of non-pregnant females, IRS-1 may not be a biologically important candidate for noncanonical signaling of JAKs in mammary ducts. These results correspond to a previous report demonstrating that both IRS-1 and IRS-2 are specifically upregulated in secretory alveolar cells during lactation, where IRS-1 plays a role in optimal milk production.[39]

The functionality of JAK1 and JAK2 as tyrosine kinases that phosphorylate canonical STAT proteins and noncanonical substrates such as IRS-1 is dependent on their autophosphorylation.[40,41] To examine the cytokine-induced autophosphorylation of JAK1/2 in the absence of STAT proteins, we derived primary mammary epithelial cells from quadruple STAT1/3/5a/5b mammary gland-specific knockout females and wild-type littermate controls and treated these cells with OSM, human growth hormone (hGH), and IL-4. In sharp contrast to the controls, where the administration of the growth factors stimulated the autophosphorylation of JAK1 (OSM) and JAK2 (hGH), the tyrosine phosphorylation of both JAKs was not detected in mammary epithelial cells that were deficient in STAT protein expression (Figure 7A). This was also the case when the STAT1/3/5a/5b-knockout cells were simultaneously treated with both growth factors (Figure 7B), excluding the possibility of a JAK1/2 transphosphorylation as part of heterodimeric receptor complexes. This phenomenon was also observed when the quadruple-knockout cells were treated with IL-4 (Figure 7C). Compared to OSM stimulation, the tyrosine phosphorylation of JAK1 was weaker in IL-4-treated wild-type epithelial cells, but more importantly, the JAK1 phosphorylation was largely absent in the quadruple STAT knockout cells. This suggested that none of the other three JAKs were engaged in the transphosphorylation of JAK1 as part of IL-4 heterodimeric receptor complexes. As a functional consequence, the tyrosine phosphorylation of STAT6, which is dependent on JAK1 in normal or neoplastic epithelial cell types,[20,42,43] was lower or absent in the quadruple STAT knockout cells that were stimulated with supraphysiological levels of IL-4 (Figure 7C). This may explain why the only expressed STAT protein (i.e., STAT6) in the STAT1/3/5a/5b quadruple knockout was not phosphorylated or upregulated in a compensatory manner (Figure 5C). If, as generally accepted, the autophosphorylation of JAKs upon cytokine receptor stimulation is critical for their role as tyrosine kinases on downstream effectors, the collective results of this line of investigation may suggest that growth factor signaling networks rely on noncanonical and perhaps kinase-independent functions of JAK1 and JAK2 (Figure 7D).

DISCUSSION

Estrogen and epithelial cell-intrinsic signaling mechanisms of its receptor (ERα) play pivotal roles in the elongation of epithelial ducts following the onset of puberty.[44,45] Other growth factors, including protein hormones such as GH and PRL, and multiple cytokines (e.g., IL-4 and IL-17) contribute directly and indirectly to the growth, differentiation, and survival of mammary epithelial

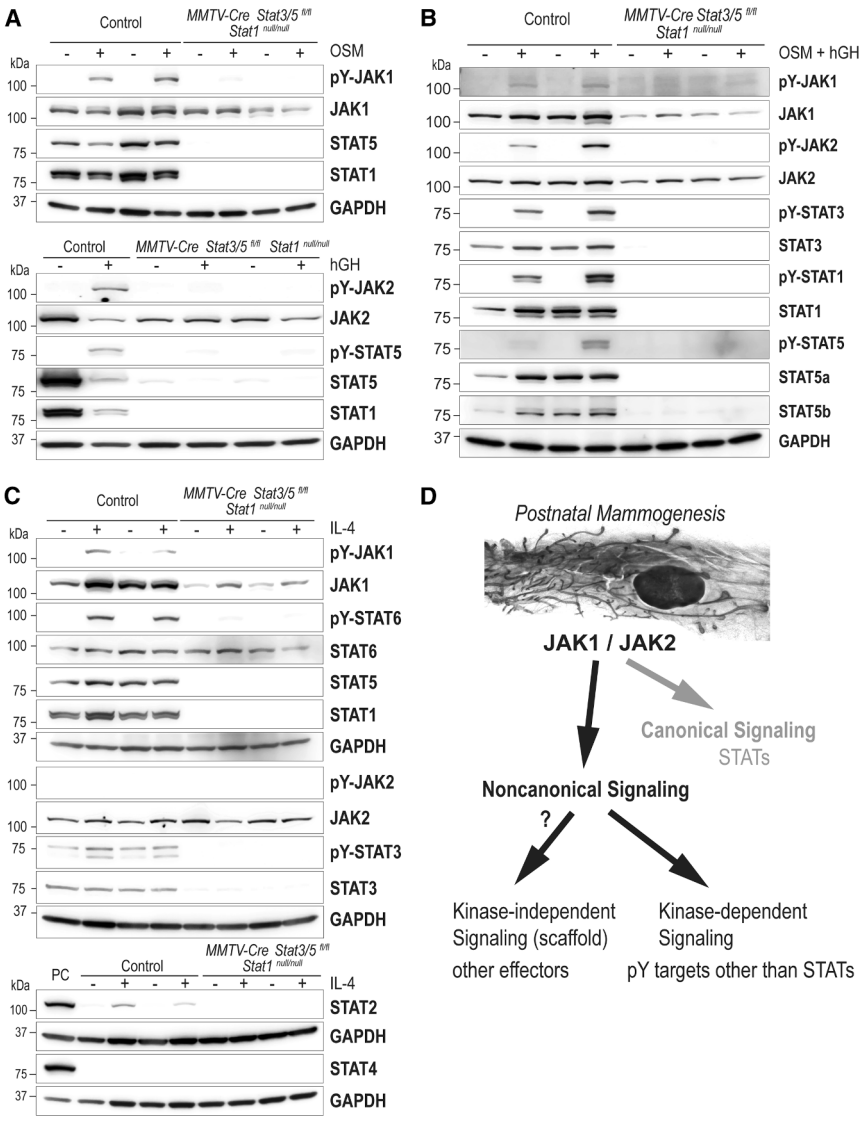


Figure 7. Lack of STAT protein expression and activation impairs the autophosphorylation of JAK1 and JAK2—A case for important noncanonical functions of JAKs?

(A and B) Immunoblot analysis of tyrosine-phosphorylated JAK1 and JAK2 and expression of selected STAT proteins in mammary epithelial cells from quadruple STAT1/3/5a/5b conditional knockout mice and wild-type controls that were treated with either oncostatin M (OSM) alone and human growth hormone (hGH) alone (A) or a combination of both (B); 2 biological repeats of experimental and control animals. GAPDH served as a loading control.

(C) Immunoblot analysis to assess the activation of STAT6 and tyrosine phosphorylation of JAK1 in STAT1/3/5a/5b-deficient quadruple-knockout cells and controls following stimulation with IL-4. GAPDH was used as a loading control. PC, splenocytes as positive controls for the validated absence of STAT2 and STAT4 in the quadruple-knockout epithelial cells.

(D) Summary of canonical and noncanonical signaling mechanisms by which JAK2, in cooperation with JAK1, drives the postnatal development of the mammary gland.

cells. Previous studies have elucidated that the nonredundant functions of essential hormones like PRL are limited to morphological changes in the adult mammary gland during pregnancy and lactation.[3,46] Their potential contributions to mammary epithelial cell lineage diversification and proliferation during earlier stages of postnatal development are not well defined. This might be due to the functional redundancy among protein hormones and cytokines that share the same intracellular signaling mediators, such as the receptor-associated JAK1 and JAK2. JAK1 and JAK2 are ubiquitously expressed and execute a variety of functions in many cell types, but our previous studies have demonstrated that both JAKs play distinctly different roles and are only essential for mammary gland development during the gestation cycle.[18–20] Using a mammary epithelial-specific double-knockout of JAK1 and JAK2, we investigated, in this study, the potential cooperative roles of growth factor signaling through these two receptor-bound JAKs and their downstream STATs. We discovered that both tyrosine kinases jointly control the postnatal development of mammary ducts. Considering that JAK1/2 conditional double-knockout females are fertile, the impaired development and, in some cases, absence of mammary epithelial ducts in adult females suggest that the concerted functions of cytokines that engage JAK/STAT signaling cascades precede the actions of estrogen.

Multiple laboratories, including ours, have established that individual STAT proteins are specifically activated by either JAK1 (STAT1/3/6) or JAK2 (STAT5a/b) in mammary epithelial cells.[19,20,24,25] It was therefore rational to investigate putative compensatory roles of STAT proteins as mediators of the cooperative functions of the two JAKs in early postnatal mammary gland development. Interestingly, we observed that STAT3 was tyrosine phosphorylated in mammary gland tissues of newborns. Contrary to the cell death-promoting functions of STAT3 during postlactational involution, the functional consequences of active STAT3 in neonatal mice are likely different and more similar to breast cancer cells, which exhibit a persistent activation of STAT3.[47,48] The loss of STAT3 activation in JAK1-deficient females led to a prolonged increase in the expression of pY-STAT5. An analogous compensatory activation of STAT3 has been previously observed in STAT5-deficient erythroid cells,[49] but both STAT proteins have opposing roles in activating specific transcriptional targets such as *Foxp3* and *Bcl6*.[50–52] The generation and phenotypic examination of triple-knockout mice that we conducted in this study show

that the compensatory roles of STAT3 and STAT5a/b are not essential for epithelial cell lineage determination and ductal growth. However, a deficiency in these three STAT proteins resulted in an unusually high expression and activation of STAT1, independent of the developmental stage and functional differentiation of the mammary gland epithelium. Our findings are similar to liver-specific STAT5a/b knockout mice, which show a compensatory activation of STAT1 and STAT3.[53] Given that STAT1 has tumor-suppressive functions,[27,54] it is interesting to note that the very high expression and activation of STAT1 in the STAT3/5-deficient mammary gland epithelium does not have any major impact on the proliferation of ductal epithelial cells. Moreover, STAT1 did not rescue the survival of STAT5-deficient alveolar cells that lack STAT3 and its proapoptotic role in this epithelial subtype.[25,55]

The generation and analysis of mice with a conditional knockout of STAT3/5a/5b on either a STAT1-deficient background or with a co-deletion of JAK1 demonstrated that the expression and activation of STAT proteins are not essential for the postnatal development of the mammary ductal tree. Most notably, epithelial cells in MMTV-Cre *Stat3/5*$^{fl/fl}$ *Stat1*$^{-/-}$ females lack the genes or mRNA expression of five and the protein expression of six of the seven mammalian STATs. Moreover, these cells do not exhibit a compensatory upregulation or tyrosine phosphorylation of STAT6. Therefore, the cooperative roles of JAK1 and JAK2 in early mammogenesis are likely mediated by noncanonical mechanisms that are STAT independent. Consequently, noncanonical functions of unphosphorylated STAT proteins (U-STATs) and their transcriptional complexes, as reviewed by Yang and Stark,[56] seem to have no significant roles in the development of mammary ducts.

Previous reports and confirmatory experiments conducted in this study demonstrated that peptide hormones and cytokines that rely on functional JAK/STAT signaling can co-activate the PI3K/AKT pathway in mammary epithelial cells (for a comprehensive review and relevant citations, please refer to Rädler et al.[57]). It is important to note that most signaling modalities between JAK/STAT and PI3K/AKT that have been elucidated previously are dependent on active STAT proteins and play exclusive roles in secretory mammary epithelial cells. Therefore, the transcriptional expression of the PI3 kinase regulatory subunits p50/p55 by STAT3[58] (Figure S8) and the reported role of STAT5 for the upregulation of PI3K and AKT1[33,34] can be excluded as critical signaling modalities of JAK1/2 for the formation of mammary ducts. This notion is supported by our observation that the postnatal development of mammary glands in the absence of STAT protein expression and activation is not accompanied by a compensatory upregulation of PI3K signaling. Moreover, our extended investigations did not establish a role for IRS-1 as a noncanonical substrate for the catalytic functions of JAK1/2, as reported previously.[37,38] Despite the validated activation of the corresponding STAT proteins and autophosphorylation of JAK1 and JAK2 in response to stimulation with IL-4, OSM, and GH, we did not detect a simultaneous increase in the tyrosine phosphorylation of IRS-1. Our findings support a study by Hadsell and co-workers[39] who reported that IRS-1 is not required for the development of mammary ducts. Its expression is significantly elevated during lactation, where IRS-1 plays a role in milk production.

Future investigations may identify novel noncanonical substrates of catalytically active JAK1 and JAK2 in mammary epithelial cells. However, we made the surprising observation that a deficiency in the expression of STAT proteins impaired the autophosphorylation of JAK1 and JAK2, which is widely accepted as a prerequisite for the functions of JAKs as tyrosine kinases.[40,41] It is, therefore, possible that biologically relevant functions of JAKs in mammogenesis are not solely mediated by kinase-dependent but also by kinase-independent functions, such as those discussed for TYK2 and JAK2 by Majoros et al.[59] Kinase-inactive mutants of JAK1 can mediate the expression of interferon (IFN)-gamma transcriptional targets.[60] More recently, Keil et al.[61] made the interesting observation that a catalytically inactive JAK2 protein (JAK2$^{YY1007/1008FF}$) facilitates the formation of the heteromeric IFN-gamma receptor complex with wild-type JAK1 that, in turn, phosphorylates STAT1. We did not detect any evidence for a transphosphorylation of JAKs in OSM-, GH-, and IL-4-stimulated STAT-deficient epithelial cells. Based on this finding and the observation that STATs are dispensable for mammary epithelial duct formation, the mechanism(s) by which JAK2, in cooperation with JAK1, controls the development of the gland is likely distinctly different from any of the previously known functions of JAKs as signaling scaffolds. The notion that biologically significant functions of JAK1/2 extend beyond their roles as tyrosine kinases for the phosphorylation of STATs and other noncanonical phosphorylation targets might be supported by recent observations in a study by Chang et al.,[62] who reported that compared to ruxolitinib, a ruxolitinib-based PROTAC for the targeted degradation of the JAK1 and JAK2 proteins significantly improved the therapeutic outcome in a model for acute lymphocytic leukemia. Since both pharmacological agents blocked the JAK1/2 kinase functions, the results might imply that noncanonical functions of JAKs outside their roles as tyrosine kinases are critical for the growth and survival of malignant cells.

Limitations of the study

While the collective results provide experimental evidence for STAT-independent biological roles of JAK1 and JAK2 in mammary duct formation, the proposed noncanonical functions of JAKs during postnatal mammogenesis remain to be elucidated. In particular, the kinase-independent roles of JAK1/2, which were inferred from the impaired autophosphorylation of JAKs in STAT-deficient epithelial cells, need experimental validation using genetic models that express receptor-associated JAKs capable of functioning as signaling scaffolds without catalytic tyrosine kinase activity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Kay-Uwe Wagner (kuwagner@wayne.org).

Materials availability

JAK1 and JAK2 conditional knockout mice will be distributed by the Mutant Mouse Resource & Research Centers (MMRRC ID: 71383 and ID: 71382). Mouse tumor-derived cell lines and plasmids created as part of this study

are available upon request. A completed Material Transfer Agreement may be required.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

The authors thank Bridget Reno as well as members of the Animal Model and Therapeutics Evaluation Core (AMTEC) at the Karmanos Cancer Institute (KCI) for the preparation of histologic sections. The Microscopic Imaging and Cytometry Resources (MICR) Core Facility assisted with the flow cytometric sorting of GFP-labeled mammary epithelial cells. The KCI core facilities are supported by the Public Health Service grant CA022453.

Financial support by Public Health Service grants CA117930 and CA202917 (to K.-U.W.) was imperative to maintain and study selected mouse strains used in this study. K.-U.W. is the Lloyd and Marilyn Smith Endowed Professor for Breast Cancer Research. A pilot grant from the Karmanos Cancer Institute (KCI-SIRG-2025) and additional institutional support were provided by Wayne State University. R.D. and M.N.W. were supported by the Ruth L. Kirschstein National Research Service Award T32 (CA009531). E.C. was supported by the Austrian Science Fund (FWF grants 10.55776/P36728, 10.55776/P33430, 10.55776/P32900, and 10.55776/DOC59) and by the City of Vienna Fund for Innovative, Interdisciplinary Cancer Research. The funders had no role in the study design, data collection, analysis, the decision to publish, or the preparation of the manuscript.

AUTHOR CONTRIBUTIONS

K.-U.W. formulated the overarching research goals and aims, supervised the research, and wrote the manuscript. R.D. established all intercrosses of the various experimental mouse models, conducted the morphological analysis of their mammary glands, completed all immunoblot and most immunofluorescent staining experiments, and performed statistical analyses. M.N.W. and C.M. assisted in the execution of immunostaining protocols and the maintenance and selection of cell lines and provided control specimens for immunoblots. M.S. and L.K. conducted the immunohistochemistry of STAT1. K.V. and A.A.T. drafted the institutional animal study protocols at Wayne State University and UNMC, developed genotyping protocols, and were responsible for the collection of tissue specimens. K.V. also assisted in transplantation experiments and the establishment of mammary epithelial cell lines. E.C. generated the mice with the conditional STAT3/5a/5b-knockout allele and provided genotyping protocols and technical expertise. T.R. was instrumental in the cryopreservation and shipment of this knockout strain. H.R. provided conceptual and technical expertise in cytokine signaling and immunoblotting.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2025.116703.

Received: May 22, 2025
Revised: September 22, 2025
Accepted: November 19, 2025
Published: December 16, 2025

REFERENCES

1. Hennighausen, L., and Robinson, G.W. (2005). Information networks in the mammary gland. Nat. Rev. Mol. Cell Biol. *6*, 715–725.
2. Robinson, G.W. (2007). Cooperation of signalling pathways in embryonic mammary gland development. Nat. Rev. Genet. *8*, 963–972. https://doi.org/10.1038/nrg2227.
3. Brisken, C., and O'Malley, B. (2010). Hormone action in the mammary gland. Cold Spring Harb. Perspect. Biol. *2*, a003178. https://doi.org/10.1101/cshperspect.a003178.
4. Pellacani, D., Tan, S., Lefort, S., and Eaves, C.J. (2019). Transcriptional regulation of normal human mammary cell heterogeneity and its perturbation in breast cancer. Embo J. *38*, e100330. https://doi.org/10.15252/embj.2018100330.
5. Macias, H., and Hinck, L. (2012). Mammary gland development. Wiley interdisciplinary reviews. Develop. Biol. *1*, 533–557. https://doi.org/10.1002/wdev.35.
6. Grimm, S.L., Seagroves, T.N., Kabotyanski, E.B., Hovey, R.C., Vonderhaar, B.K., Lydon, J.P., Miyoshi, K., Hennighausen, L., Ormandy, C.J., Lee, A.V., et al. (2002). Disruption of steroid and prolactin receptor patterning in the mammary gland correlates with a block in lobuloalveolar development. Mol. Endocrinol. *16*, 2675–2691. https://doi.org/10.1210/me.2002-0239.
7. Brisken, C., Park, S., Vass, T., Lydon, J.P., O'Malley, B.W., and Weinberg, R.A. (1998). A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc. Natl. Acad. Sci. USA *95*, 5076–5081.
8. Horseman, N.D., Zhao, W., Montecino-Rodriguez, E., Tanaka, M., Nakashima, K., Engle, S.J., Smith, F., Markoff, E., and Dorshkind, K. (1997). Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J. *16*, 6926–6935.
9. Ormandy, C.J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., and Kelly, P.A. (1997). Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev. *11*, 167–178.
10. Khaled, W.T., Read, E.K.C., Nicholson, S.E., Baxter, F.O., Brennan, A.J., Came, P.J., Sprigg, N., McKenzie, A.N.J., and Watson, C.J. (2007). The IL-4/IL-13/Stat6 signalling pathway promotes luminal mammary epithelial cell development. Development *134*, 2739–2750.
11. Li, M., Liu, X., Robinson, G., Bar-Peled, U., Wagner, K.U., Young, W.S., Hennighausen, L., and Furth, P.A. (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc. Natl. Acad. Sci. USA *94*, 3425–3430.
12. Rodig, S.J., Meraz, M.A., White, J.M., Lampe, P.A., Riley, J.K., Arthur, C.D., King, K.L., Sheehan, K.C., Yin, L., Pennica, D., et al. (1998). Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell *93*, 373–383.
13. Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M.,

et al. (1998). Jak2 is essential for signaling through a variety of cytokine receptors. Cell *93*, 385–395.

14. Krempler, A., Qi, Y., Triplett, A.A., Zhu, J., Rui, H., and Wagner, K.U. (2004). Generation of a conditional knockout allele for the Janus kinase 2 (Jak2) gene in mice. Genesis *40*, 52–57.
15. Sakamoto, K., Wehde, B.L., Rädler, P.D., Triplett, A.A., and Wagner, K.U. (2016). Generation of Janus kinase 1 (JAK1) conditional knockout mice. Genesis *54*, 582–588. https://doi.org/10.1002/dvg.22982.
16. Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C.W. (2002). Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene *285*, 1–24.
17. Olayioye, M.A., Beuvink, I., Horsch, K., Daly, J.M., and Hynes, N.E. (1999). ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J. Biol. Chem. *274*, 17209–17218.
18. Shillingford, J.M., Miyoshi, K., Robinson, G.W., Grimm, S.L., Rosen, J.M., Neubauer, H., Pfeffer, K., and Hennighausen, L. (2002). Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium. Mol. Endocrinol. *16*, 563–570.
19. Wagner, K.U., Krempler, A., Triplett, A.A., Qi, Y., George, N.M., Zhu, J., and Rui, H. (2004). Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice. Mol. Cell Biol. *24*, 5510–5520.
20. Sakamoto, K., Wehde, B.L., Yoo, K.H., Kim, T., Rajbhandari, N., Shin, H.Y., Triplett, A.A., Rädler, P.D., Schuler, F., Villunger, A., et al. (2016). Janus Kinase 1 Is Essential for Inflammatory Cytokine Signaling and Mammary Gland Remodeling. Mol. Cell Biol. *36*, 1673–1690. https://doi.org/10.1128/mcb.00999-15.
21. Watson, C.J., and Neoh, K. (2008). The Stat family of transcription factors have diverse roles in mammary gland development. Semin. Cell Dev. Biol. *19*, 401–406.
22. Wagner, K.U., and Rui, H. (2008). Jak2/Stat5 signaling in mammogenesis, breast cancer initiation and progression. J. Mammary Gland Biol. Neoplasia *13*, 93–103.
23. Wagner, K.U., and Schmidt, J.W. (2011). The two faces of Janus kinases and their respective STATs in mammary gland development and cancer. J. Carcinog. *10*, 32. https://doi.org/10.4103/1477-3163.90677.
24. Cui, Y., Riedlinger, G., Miyoshi, K., Tang, W., Li, C., Deng, C.X., Robinson, G.W., and Hennighausen, L. (2004). Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol. Cell Biol. *24*, 8037–8047.
25. Chapman, R.S., Lourenco, P.C., Tonner, E., Flint, D.J., Selbert, S., Takeda, K., Akira, S., Clarke, A.R., and Watson, C.J. (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev. *13*, 2604–2616.
26. Klover, P.J., Muller, W.J., Robinson, G.W., Pfeiffer, R.M., Yamaji, D., and Hennighausen, L. (2010). Loss of STAT1 from mouse mammary epithelium results in an increased Neu-induced tumor burden. Neoplasia *12*, 899–905.
27. Schneckenleithner, C., Bago-Horvath, Z., Dolznig, H., Neugebauer, N., Kollmann, K., Kolbe, T., Decker, T., Kerjaschki, D., Wagner, K.U., Müller, M., et al. (2011). Putting the brakes on mammary tumorigenesis: loss of STAT1 predisposes to intraepithelial neoplasias. Oncotarget *2*, 1043–1054.
28. Brisken, C., Kaur, S., Chavarria, T.E., Binart, N., Sutherland, R.L., Weinberg, R.A., Kelly, P.A., and Ormandy, C.J. (1999). Prolactin controls mammary gland development via direct and indirect mechanisms. Dev. Biol. *210*, 96–106.
29. Gallego, M.I., Binart, N., Robinson, G.W., Okagaki, R., Coschigano, K.T., Perry, J., Kopchick, J.J., Oka, T., Kelly, P.A., and Hennighausen, L. (2001). Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. Dev. Biol. *229*, 163–175.
30. Wagner, K.U., Wall, R.J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P.A., and Hennighausen, L. (1997). Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. *25*, 4323–4330.
31. Wagner, K.U., McAllister, K., Ward, T., Davis, B., Wiseman, R., and Hennighausen, L. (2001). Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res. *10*, 545–553.
32. Sakamoto, K., Creamer, B.A., Triplett, A.A., and Wagner, K.U. (2007). The Janus kinase 2 is required for expression and nuclear accumulation of cyclin D1 in proliferating mammary epithelial cells. Mol. Endocrinol. *21*, 1877–1892.
33. Creamer, B.A., Sakamoto, K., Schmidt, J.W., Triplett, A.A., Moriggl, R., and Wagner, K.U. (2010). Stat5 promotes survival of mammary epithelial cells through transcriptional activation of a distinct promoter in Akt1. Mol. Cell Biol. *30*, 2957–2970.
34. Schmidt, J.W., Wehde, B.L., Sakamoto, K., Triplett, A.A., Anderson, S.M., Tsichlis, P.N., Leone, G., and Wagner, K.U. (2014). Stat5 regulates the phosphatidylinositol 3-kinase/Akt1 pathway during mammary gland development and tumorigenesis. Mol. Cell Biol. *34*, 1363–1377. https://doi.org/10.1128/MCB.01220-13.
35. Pensa, S., Neoh, K., Resemann, H.K., Kreuzaler, P.A., Abell, K., Clarke, N.J., Reinheckel, T., Kahn, C.R., and Watson, C.J. (2014). The PI3K regulatory subunits p55alpha and p50alpha regulate cell death in vivo. Cell Death Differ. *21*, 1442–1450. https://doi.org/10.1038/cdd.2014.59.
36. Tvorogov, D., Thomas, D., Liau, N.P.D., Dottore, M., Barry, E.F., Lathi, M., Kan, W.L., Hercus, T.R., Stomski, F., Hughes, T.P., et al. (2018). Accumulation of JAK activation loop phosphorylation is linked to type I JAK inhibitor withdrawal syndrome in myelofibrosis. Sci. Adv. *4*, eaat3834. https://doi.org/10.1126/sciadv.aat3834.
37. Yamauchi, T., Kaburagi, Y., Ueki, K., Tsuji, Y., Stark, G.R., Kerr, I.M., Tsushima, T., Akanuma, Y., Komuro, I., Tobe, K., et al. (1998). Growth hormone and prolactin stimulate tyrosine phosphorylation of insulin receptor substrate-1, -2, and -3, their association with p85 phosphatidylinositol 3-kinase (PI3-kinase), and concomitantly PI3-kinase activation via JAK2 kinase. J. Biol. Chem. *273*, 15719–15726. https://doi.org/10.1074/jbc.273.25.15719.
38. Wu, W.J., Wang, S.H., Wu, C.C., Su, Y.A., Chiang, C.Y., Lai, C.H., Wang, T.H., Cheng, T.L., Kuo, J.Y., Hsu, T.C., et al. (2021). IL-4 and IL-13 Promote Proliferation of Mammary Epithelial Cells through STAT6 and IRS-1. Int. J. Mol. Sci. *22*, 12008. https://doi.org/10.3390/ijms222112008.
39. Hadsell, D.L., Olea, W., Lawrence, N., George, J., Torres, D., Kadowaki, T., and Lee, A.V. (2007). Decreased lactation capacity and altered milk composition in insulin receptor substrate null mice is associated with decreased maternal body mass and reduced insulin-dependent phosphorylation of mammary Akt. J. Endocrinol. *194*, 327–336. https://doi.org/10.1677/joe-07-0160.
40. Feng, J., Witthuhn, B.A., Matsuda, T., Kohlhuber, F., Kerr, I.M., and Ihle, J.N. (1997). Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol. Cell Biol. *17*, 2497–2501.
41. Babon, J.J., Lucet, I.S., Murphy, J.M., Nicola, N.A., and Varghese, L.N. (2014). The molecular regulation of Janus kinase (JAK) activation. Biochem. J. *462*, 1–13. https://doi.org/10.1042/bj20140712.
42. Wehde, B.L., Radler, P.D., Shrestha, H., Johnson, S.J., Triplett, A.A., and Wagner, K.U. (2018). Janus Kinase 1 Plays a Critical Role in Mammary Cancer Progression. Cell Rep. *25*, 2192–2207.e2195. https://doi.org/10.1016/j.celrep.2018.10.063.
43. Shrestha, H., Rädler, P.D., Dennaoui, R., Wicker, M.N., Rajbhandari, N., Sun, Y., Peck, A.R., Vistisen, K., Triplett, A.A., Beydoun, R., et al. (2024). The Janus kinase 1 is critical for pancreatic cancer initiation and progression. Cell Rep. *43*, 114202. https://doi.org/10.1016/j.celrep.2024.114202.
44. Mallepell, S., Krust, A., Chambon, P., and Brisken, C. (2006). Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland. Proc. Natl. Acad. Sci. USA *103*, 2196–2201.
45. Feng, Y., Manka, D., Wagner, K.U., and Khan, S.A. (2007). Estrogen receptor-alpha expression in the mammary epithelium is required for ductal and

alveolar morphogenesis in mice. Proc. Natl. Acad. Sci. USA *104*, 14718–14723.

46. Berryhill, G.E., Trott, J.F., and Hovey, R.C. (2016). Mammary gland development–It's not just about estrogen. J. Dairy Sci. *99*, 875–883. https://doi.org/10.3168/jds.2015-10105.
47. Garcia, R., Bowman, T.L., Niu, G., Yu, H., Minton, S., Muro-Cacho, C.A., Cox, C.E., Falcone, R., Fairclough, R., Parsons, S., et al. (2001). Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene *20*, 2499–2513. https://doi.org/10.1038/sj.onc.1204349.
48. Berishaj, M., Gao, S.P., Ahmed, S., Leslie, K., Al Ahmadie, H., Gerald, W.L., Bornmann, W., and Bromberg, J.F. (2007). Stat3 is tyrosine-phosphorylated through the interleukin-6/glycoprotein 130/Janus kinase pathway in breast cancer. Breast Cancer Res. *9*, R32.
49. Kerenyi, M.A., Grebien, F., Gehart, H., Schifrer, M., Artaker, M., Kovacic, B., Beug, H., Moriggl, R., and Müllner, E.W. (2008). Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1. Blood *112*, 3878–3888. https://doi.org/10.1182/blood-2008-02-138339.
50. Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E.M., Moriggl, R., et al. (2007). Nonredundant roles for Stat5a/b in directly regulating Foxp3. Blood *109*, 4368–4375. https://doi.org/10.1182/blood-2006-11-055756.
51. Tran, T.H., Utama, F.E., Lin, J., Yang, N., Sjolund, A.B., Ryder, A., Johnson, K.J., Neilson, L.M., Liu, C., Brill, K.L., et al. (2010). Prolactin inhibits BCL6 expression in breast cancer through a Stat5a-dependent mechanism. Cancer Res. *70*, 1711–1721. https://doi.org/10.1158/0008-5472.can-09-2314.
52. Walker, S.R., Nelson, E.A., Yeh, J.E., Pinello, L., Yuan, G.C., and Frank, D.A. (2013). STAT5 outcompetes STAT3 to regulate the expression of the oncogenic transcriptional modulator BCL6. Mol. Cell Biol. *33*, 2879–2890. https://doi.org/10.1128/mcb.01620-12.
53. Cui, Y., Hosui, A., Sun, R., Shen, K., Gavrilova, O., Chen, W., Cam, M.C., Gao, B., Robinson, G.W., and Hennighausen, L. (2007). Loss of signal transducer and activator of transcription 5 leads to hepatosteatosis and impaired liver regeneration. Hepatology *46*, 504–513. https://doi.org/10.1002/hep.21713.
54. Chan, S.R., Vermi, W., Luo, J., Lucini, L., Rickert, C., Fowler, A.M., Lonardi, S., Arthur, C., Young, L.J., Levy, D.E., et al. (2012). STAT1-deficient mice spontaneously develop estrogen receptor alpha-positive luminal mammary carcinomas. Breast Cancer Res. *14*, R16. https://doi.org/10.1186/bcr3100.
55. Humphreys, R.C., Bierie, B., Zhao, L., Raz, R., Levy, D., and Hennighausen, L. (2002). Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli. Endocrinology *143*, 3641–3650.
56. Yang, J., and Stark, G.R. (2008). Roles of unphosphorylated STATs in signaling. Cell Res. *18*, 443–451. https://doi.org/10.1038/cr.2008.41.
57. Radler, P.D., Wehde, B.L., and Wagner, K.U. (2017). Crosstalk between STAT5 activation and PI3K/AKT functions in normal and transformed mammary epithelial cells. Mol. Cell. Endocrinol. *451*, 31–39. https://doi.org/10.1016/j.mce.2017.04.025.
58. Abell, K., Bilancio, A., Clarkson, R.W.E., Tiffen, P.G., Altaparmakov, A.I., Burdon, T.G., Asano, T., Vanhaesebroeck, B., and Watson, C.J. (2005). Stat3-induced apoptosis requires a molecular switch in PI(3)K subunit composition. Nat. Cell Biol. *7*, 392–398.
59. Majoros, A., Platanitis, E., Kernbauer-Hölzl, E., Rosebrock, F., Müller, M., and Decker, T. (2017). Canonical and Non-Canonical Aspects of JAK-STAT Signaling: Lessons from Interferons for Cytokine Responses. Front. Immunol. *8*, 29. https://doi.org/10.3389/fimmu.2017.00029.
60. Briscoe, J., Rogers, N.C., Witthuhn, B.A., Watling, D., Harpur, A.G., Wilks, A.F., Stark, G.R., Ihle, J.N., and Kerr, I.M. (1996). Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. Embo J *15*, 799–809.
61. Keil, E., Finkenstädt, D., Wufka, C., Trilling, M., Liebfried, P., Strobl, B., Müller, M., and Pfeffer, K. (2014). Important scaffold function of the Janus kinase 2 uncovered by a novel mouse model harboring a Jak2 activation-loop mutation. Blood *123*, 520–529. https://doi.org/10.1182/blood-2013-03-492157.
62. Chang, Y., Min, J., Jarusiewicz, J.A., Actis, M., Yu-Chen Bradford, S., Mayasundari, A., Yang, L., Chepyala, D., Alcock, L.J., Roberts, K.G., et al. (2021). Degradation of Janus kinases in CRLF2-rearranged acute lymphoblastic leukemia. Blood *138*, 2313–2326. https://doi.org/10.1182/blood.2020006846.
63. Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell *85*, 27–37.
64. Sakamoto, K., Schmidt, J.W., and Wagner, K.U. (2012). Generation of a novel MMTV-tTA transgenic mouse strain for the targeted expression of genes in the embryonic and postnatal mammary gland. PLoS One *7*, e43778. https://doi.org/10.1371/journal.pone.0043778.
65. Moll, H.P., Mohrherr, J., Blaas, L., Musteanu, M., Stiedl, P., Grabner, B., Zboray, K., König, M., Stoiber, D., Rülicke, T., et al. (2019). A Mouse Model to Assess STAT3 and STAT5A/B Combined Inhibition in Health and Disease Conditions. Cancers *11*, 1226. https://doi.org/10.3390/cancers11091226.
66. Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K., and Miyazaki, J. (2000). A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. FEBS Lett. *470*, 263–268.
67. Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W.E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. Science *303*, 359–363.
68. Durbin, J.E., Hackenmiller, R., Simon, M.C., and Levy, D.E. (1996). Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell *84*, 443–450. https://doi.org/10.1016/s0092-8674(00)81289-1.
69. DeOme, K.B., Faulkin, L.J., Bern, H.A., and Blair, P.E. (1959). Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res. *19*, 515–520.
70. Krempler, A., Henry, M.D., Triplett, A.A., and Wagner, K.U. (2002). Targeted deletion of the Tsg101 gene results in cell cycle arrest at G1/S and p53-independent cell death. J. Biol. Chem. *277*, 43216–43223.
71. Zhao, J.J., Gjoerup, O.V., Subramanian, R.R., Cheng, Y., Chen, W., Roberts, T.M., and Hahn, W.C. (2003). Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell *3*, 483–495. https://doi.org/10.1016/s1535-6108(03)00088-6.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal, pY-JAK1 (Y1034/1035)	Cell Signaling	Cat#74129; RRID:AB_2799851
Mouse monoclonal, JAK1	Cell Signaling	Cat#50996; RRID:AB_2716281
Rabbit monoclonal, pY-JAK2 (Y1007/1008)	Cell Signaling	Cat#3776; RRID:AB_ 2617123
Rabbit monoclonal, JAK2	Cell Signaling	Cat#3230; RRID:AB_2128522
Rabbit polyclonal, pY-STAT1 (Y701)	Origene	Cat#TA309955
Rabbit monoclonal, pY-STAT1 (Y701)	Cell Signaling	Cat#9167; RRID:AB_561284
Rabbit polyclonal, STAT1	Santa Cruz	Cat#sc-592; RRID:AB_632434
Rabbit monoclonal, pY-STAT3 (Y705)	Cell Signaling	Cat#9145; RRID:AB_2491009
Mouse monoclonal, STAT3	Cell Signaling	Cat#9139; RRID:AB_331757
Rabbit polyclonal, pY-STAT5 (Y694)	Cell Signaling	Cat#9351; RRID:AB_2315225
Rabbit polyclonal, STAT5	Santa Cruz	Cat#sc-836; RRID:AB_632445
Rabbit monoclonal, STAT5a	Abcam	Cat#ab32043; RRID:AB_778107
Mouse monoclonal, STAT5b	Santa Cruz	Cat#sc-1656; RRID:AB_2197067
Rabbit polyclonal, pY-STAT6 (Y641)	Cell Signaling	Cat#9361; RRID:AB_331595
Rabbit polyclonal, STAT6	Santa Cruz	Cat#sc-981; RRID:AB_632450
Rabbit monoclonal, STAT2	Cell Signaling	Cat#72604; RRID:AB_2799824
Rabbit monoclonal, STAT4	Cell Signaling	Cat#2653; RRID:AB_ 2255156
Chicken polyclonal, GFP	Avēs Labs	Cat#GFP-1020; RRID:AB_10000240
Rat monoclonal, CK8/18	Developmental Studies Hybridoma Bank	Cat# TROMA-I; RRID:AB_531826
Rabbit polyclonal, CK14	Covance	Cat# PRB-155P; RRID:AB_292096
Rabbit monoclonal, pT308-AKT	Cell Signaling	Cat#4056; RRID:AB_331163
Rabbit polyclonal, pS473-AKT	Cell Signaling	Cat#9271; RRID:AB_329825
Rabbit polyclonal, AKT	Cell Signaling	Cat#9272; RRID:AB_329827
Rabbit monoclonal, EpCAM	Cell Signaling	Cat#93790; RRID:AB_2800214
Rabbit monoclonal, E-cadherin	Cell Signaling	Cat#3195; RRID:AB_2291471
Rabbit monoclonal, p110a	Cell Signaling	Cat#4249; RRID:AB_2165248
Rabbit polyclonal, p85 (p50/p55)	Upstate	Cat#06–195; RRID:AB_310069
Rabbit polyclonal, pFoxO3a (S318/321)	Cell Signaling	Cat# 9465; RRID:AB_2106498
Rabbit polyclonal, FoxO3a	Cell Signaling	Cat# 9467; RRID:AB_2106672
Rabbit polyclonal, IRS-1	Cell Signaling	Cat# 2382; RRID:AB_330333
Rabbit polyclonal, IRS-2	Cell Signaling	Cat# 3089; RRID:AB_2125771
Mouse monoclonal, phosphotyrosine	Sigma-Aldrich	Cat# 05–321; RRID:AB_2891016
Rabbit monoclonal, pIGF-I Receptor β (Y1135)	Cell Signaling	Cat# 3918; RRID:AB_10548764
Rabbit polyclonal, IGF-I Receptor β	Cell Signaling	Cat# 3027; RRID:AB_2122378
Mouse monoclonal, ACTB	Santa Cruz	Cat#sc-47778; RRID:AB_626632
Rabbit monoclonal, GAPDH	Cell Signaling	Cat#5174S; RRID:AB_10622025
Digital anti-Mouse-HRP	Kindle Biosciences	Cat# R1005; RRID:AB_2800463
Digital anti-Rabbit-HRP	Kindle Biosciences	Cat# R1006; RRID:AB_2800464
Alexa Fluor 488 goat anti-chicken	Invitrogen	Cat# A11039; RRID:AB_2534096
Alexa Fluor 594 donkey anti-rat	Invitrogen	Cat# A-21209; RRID:AB_2535795
Alexa Fluor 594 donkey anti-rabbit	Invitrogen	Cat# A21207; RRID:AB_141637

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
One Shot™ Stbl3™ Chemically Competent E. coli	Thermo Fisher	Cat#C737303
Biological samples		
Mouse: Mammary glands: MMTV-Cre Jak1 fl/fl	This paper	N/A
Mouse: Mammary glands: Jak1 fl/fl Jak2 fl/fl	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Jak2 fl/fl	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Jak1 fl/fl Jak2 fl/fl	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Jak1 fl/+ Jak2 fl/+	This paper	N/A
Mouse: Mammary glands: wildtype FVB control	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Jak1 fl/fl Jak2 fl/+	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Jak1 fl/+ Jak2 fl/fl	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Stat3/5 fL/fL	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Stat3/5 fL/+	This paper	N/A
Mouse: Mammary glands: Stat3/5 fL/wt	This paper	N/A
Mouse: Mammary glands: Stat3/5 fL/fL	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Stat3/5 fL/fL Stat1 −/−	This paper	N/A
Mouse: Mammary glands: Stat3/5 fL/fL Stat1 −/−	This paper	N/A
Mouse: Mammary glands: MMTV-Cre Stat3/5 fL/fL Jak1 fl/fl	This paper	N/A
Mouse: Mammary glands: WAP-Cre Stat3/5 fL/fL	This paper	N/A
Mouse: Spleen	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Puromycin dihydrochloride	Sigma	Cat#P7255-100G
Human growth hormone	Invitrogen	Cat#RP-10928
Recombinant human IGF-I	Invitrogen	Cat#PHG0071
Mouse mammalian IFN-β	Pbl Assay sciences	Cat#12405
Recombinant mouse IL-4	BD Pharmingen	Cat#550067
Recombinant mouse interferon gamma	Millipore	Cat#IF005
Recombinant mouse Oncostatin M (OSM)	R&D Systems	Cat#495-MO
Critical commercial assays		
KwikQuant Western Blot Detection Kit	Kindle BioSciences	Cat#R1002
Dynabeads™ Protein A	Invitrogen	Ref #10001D
SensiTek HRP Anti-Polyvalent Staining System	ScyTek Laboratories	Cat#AFG-600
BD Pharmingen™ AEC Substrate Kit	BD Pharmingen	Cat#551015
Experimental models: Cell lines		
Mouse: Immortalized mammary epithelial cell line Wildtype FVB (Cell line 1)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line Wildtype FVB (Cell line 2)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line MMTV-Cre CAG-GFP Jak1 fl/+ Jak2 fl/+ (Cell line 1)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line MMTV-Cre CAG-GFP Jak1 fl/+ Jak2 fl/+ (Cell line 2)	This paper	N/A
Mouse: Immortalized Mammary epithelial cell line MMTV-Cre CAG-GFP Jak1 fl/fl Jak2 fl/fl (Cell line 1)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line MMTV-Cre CAG-GFP Jak1 fl/fl Jak2 fl/fl (Cell line 2)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line Stat3/5 fL/fL (Line 1)	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: Immortalized mammary epithelial cell line Stat3/5 fL/fL (Line 2)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line Stat3/5 fL/fL (Line 3)	This paper	N/A
Mouse: Immortalized Embryonic Fibroblasts Stat3/5 fL/fL (Line 1)	This paper	N/A
Mouse: Immortalized Embryonic Fibroblasts Stat3/5 fL/fL (Line 2)	This paper	N/A
Mouse: Immortalized Embryonic Fibroblasts Stat3/5 fL/fL (Line 3)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line MMTV-Cre CAG-GFP Stat3/5 fL/fL Stat1$^{-/-}$ (Cell line 1)	This paper	N/A
Mouse: Immortalized mammary epithelial cell line MMTV-Cre CAG-GFP Stat3/5 fL/fL Stat1$^{-/-}$ (Cell line 2)	This paper	N/A
Experimental models: Organisms/strains		
Mouse: *Jak1fl*, [Jak1^{tm1Kuw}]	Sakamoto et al., 2016a[15]	MGI:5688302
Mouse: *Jak2fl*, [Jak2^{tm1Kuw}]	Krempler et al., 2004[14]	MGI:3045140
Mouse: MMTV-Cre line A, [Tg(MMTV-cre)1Mam]	Wagner et al., 1997	MGI:2176165
Mouse: CAG-LSL-GFP, [Tg(CAG-cat,-EGFP)39Miya]	Provided by Dr. Miyazaki (Osaka University)	Kawamoto et al., 2000
Mouse: WAP-Cre, [Tg(Wap-cre)11738Mam]	Wagner et al., 1997	MGI:2176167
Mouse: MMTV-tTA, [Tg(MMTV-tTA)25754Kuw]	Sakamoto et al., 2012	MGI:5300558
Mouse: TetO-H2B-GFP, [Tg(tetO-HIST1H2BJ/GFP)47Efu/J]	Jackson Laboratory	Strain #: 005104
Mouse: *Stat3/5*$^{fL/fL}$	Provided by Dr. Casanova	Moll et al., 2019
Mouse: *Stat1*$^{null/null}$,[B6.129S(Cg)-Stat1tm1Dlv/J]	Jackson Laboratory	Strain #: 012606
Mouse: *cdkn2a,* [Cdkn2atm1Rdp]	Serrano et al., 1996[63]	MGI:1857942
Mouse: FVB/N	Charles River	Strain Code: 207
Mouse: Athymic, NCr$^{nu/nu}$	Charles River	Strain Code: 553
Oligonucleotides		
See Table S1 for Genotyping PCR primer sequences	This paper	N/A
Recombinant DNA		
pBabe-puro-Cre	This Lab	Krempler et al., 2002
pBabe-puro SV40-LT	Addgene	Plasmid #: 13970
Software and algorithms		
Prism v6 (v6.07)	GraphPad Software	RRID:SCR_002798
ImageJ	URL: https://imagej.net/ij/	RRID:SCR_003070
Fiji	URL: https://fiji.sc/	RRID:SCR_002285
KwikQuant Image Analyzer 5.9 for Mac OS	Kindle Biosciences	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Genetically modified mouse strains

Our team generated genetically engineered mice with conditional knockout alleles for JAK1 (*Jak1*^{fl}, *Jak1*^{tm1Kuw})[15] and JAK2 (*Jak2*^{fl}, *Jak2*^{tm1Kuw})[14] as well as MMTV-Cre line A [Tg(MMTV-cre)1Mam], WAP-Cre [Tg(Wap-cre)11738Mam], and MMTV-tTA [Tg(MMTV-tTA)25754Kuw] transgenic lines.[30,64] The generation of the STAT3/5a/5b conditional triple-knockout mouse model was recently described.[65] The CAG-LSL-GFP reporter strain [Tg(CAG-cat,-EGFP)39Miya] was a gift from Dr. Miyazaki at Osaka University.[66] The TetO-H2B-GFP [Tg(tetO-HIST1H2BJ/GFP)47Efu/J] reporter line[67] and the STAT1 knockout strain [B6.129S(Cg)-*Stat1*^{tm1Dlv}/J][68] were purchased from the Jackson Laboratory. *Cdkn2a* knockout mice [*Cdkn2atm1Rdp*][63] were obtained from the Mouse Model for Human Cancer Consortium (MMHCC) at the National Cancer Institute in Frederick, MD. Individual strains with targeted alleles and transgenes were maintained on an FVB/N background. To investigate the roles of JAKs and STATs in the development of the

mammary gland, only female experimental mice and age-mateched controls were used for all experiments. Depending on the study, the nulliparous mice were between 3 days and 12 to 19 weeks old. The genotypes of the mice for all transgenes and targeted endogenous alleles were determined by PCR as described in the method details section. The PCR primers for genotyping all transgenic constructs and alleles are provided in Table S1. Animals were maintained under pathogen-free conditions in micro-isolator cages on a 12h/12h light/dark cycle. The care of animals followed the ARRIVE guidelines and was in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal study protocols were approved by the Institutional Animal Care and Use Committees at the University of Nebraska Medical Center and Wayne State University.

METHOD DETAILS

Genotyping

For PCR-based genotyping of mice, genomic DNA was extracted from digested tail biopsies of mice (10 mg/mL proteinase K in 1% SDS, 50nM Tris/HCl pH8, 100nM NaCl, and 50 mM EDTA) using an AutoGenprep2000. The PCR primer sets listed in Table S1 were used to genotype the genetically engineered alleles and transgenes.

Mammary epithelial transplantation

The surgical procedures to clear the endogenous mammary epithelium from the #4 inguinal fat pads of 3-week-old female recipient mice and the transplantation of tissue fragments from donor mice were established by DeOme et al.[69] Briefly, Athymic Nude female mice (CrTac:NCr-*Foxn1*^{nu}) were anesthetized, and the epithelia from the #4 inguinal mammary glands were excised. Mammary gland tissues containing GFP-positive ducts from controls and JAK1/2 double-knockout donor females were cut into fragments of 1–2 mm in size under a fluorescent stereoscope. Next, GFP-positive fragments were placed inside the cleared mammary fat pads of six recipient females. Four transplanted recipients were maintained as nulliparous females for 6–8 weeks and analyzed. Two recipients were bred, and the mammary glands were examined immediately following the birth of the offspring.

Histological analysis and immunostaining

For wholemount preparation and analysis, mammary glands were spread on glass slides and examined with a Discovery.V8 fluorescence stereoscope (Carl Zeiss, Inc.) for GFP expression. Next, the glands were fixed in Carnoy's fixative, rehydrated, stained with Carmine Alum for 3 days, dehydrated, and covered with Permount mounting medium and a coverslip. For histological examination, mammary gland tissues were fixed in 10% buffered formalin overnight at room temperature and stored in 70% ethanol before paraffin embedding and sectioning. For routine histology, slides were stained with hematoxylin and eosin (H&E) and scanned on a brightfield slide scanner from Leica Microsystems, Inc. For immunostaining, histologic sections were deparaffinized three times in 100% Histo-Clear (National Diagnostics) for 10 min each, rehydrated in decreasing concentrations of ethanol (100%, 95%, 90%, 70%, 50%, and 30% for 3 min each), and washed for 5 min in 1x PBS. Tissue sections were pressure-cooked in ImmunoRetriever with Citrate (Bio SB #BSB0022) using a Bio SB TintoRetriever pressure cooker, which was set to 116°C–121°C for 1 min for antigen retrieval. Once the slides had returned to room temperature, they were washed for 5 min in 1x PBS and blocked with 3% BSA blocking buffer for 1h. The primary antibodies were added based on the recommended dilutions listed in Table S2. Slides were incubated with primary antibodies overnight at 4°C in a wet chamber and then washed three times in 1x PBS for 5 min. Fluorophore-conjugated secondary antibodies were added, and slides were incubated in the dark for 1h at room temperature. After washing the slides twice with distilled water, Vectashield DAPI mounting media (Vector, H-1200) and coverslips were applied. For the immunohistochemical staining of pY-STAT1, slides were deparaffinized, rehydrated, and an antigen heat retrieval was performed in a Citrate-based solution, pH = 6 (DAKO, S2369). After cooling the slides down to room temperature, the endogenous peroxidase was inhibited with 3% H_2O_2 in PBS for 10 min. A blocking step was performed before the slides were incubated with the primary pY-STAT1 antibody (Cell Signaling, 9167) 1:100 overnight at 4°C. Next, the detection was carried out using the SensiTek HRP staining system (ScyTek Laboratories, AFG-600), and the signal was visualized with AEC (BD Pharmingen AEC Substrate Kit, 551015) followed by counterstaining with hematoxylin. Stained slides were examined with an Axio Imager microscope (Carl Zeiss) equipped with an SPOT Flex camera.

Generation of primary and immortalized cell cultures

Mouse embryonic fibroblasts (MEFs) were derived from 12.5-day-old embryos and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, nonessential amino acids, gentamicin, and penicillin-streptomycin as described.[70] Primary mammary epithelial cells (MECs) were derived from enzymatically dissociated mammary glands of 12 to 16-week-old female mice and maintained in DMEM/F12 medium supplemented with 5% FBS, insulin, epithelial growth factor, linoleic acid complex, fungizone, pen/strep, hydrocortisone, bovine serum albumin, gentamicin, and beta-mercaptoethanol. To establish isogenic immortalized fibroblasts and mammary epithelial cell lines with and without expression of STAT3 and STAT5a/b, we isolated primary MEFs and MECs from 16-week-old *Stat3/5*$^{fL/fL}$ females that were bred on a CDKN2A knockout background (*Cdkn2a*$^{-/-}$). Using retroviral gene transfer to express Cre recombinase and delete both *Stat3/5a/5b* alleles, cells were infected with pBabe-Cre-puro and pBabe-puro as a control and selected with increasing concentrations of puromycin (1, 3, and 7 μg/mL) as described previously.[70] To establish immortalized MEC cultures from 12 to 16-week-old MMTV-Cre *Jak1*$^{fl/fl}$ *Jak2*$^{fl/fl}$ females and *MMTV-Cre Jak1*$^{fl/wt}$ *Jak2*$^{fl/wt}$ controls, primary mammary epithelial cells were infected with pBabe-puro retroviral particles

expressing the SV40 large T antigen (Addgene #13970)[71] and selected with puromycin. All parental primary cell lines and derived subcultures were authenticated by PCR and Western blots using the primers and antibodies in Tables S1–S3. Cell lines were routinely tested for mycoplasma contamination.

Kinase inhibition and cytokine treatment

To inhibit JAK1 and the tyrosine phosphorylation of STAT1, immortalized STAT3/5a/5b triple-knockout MEC cultures were treated for 48 h with 1 μM itacitinib (INCB039110). Before cytokine treatment, MECs were maintained in serum-free DMEM/F12 medium for 16 h and treated for 15 min with a single cytokine or a combination of the following growth factors: 500 ng/mL hGH (Invitrogen, RP-10928), 25 ng/mL recombinant mouse OSM (R&D Systems, Cat. # 495-MO), 50 ng/mL recombinant mouse IL-4 (BD Pharmingen, 550067), 200 IU/mL mouse IFN-β (PbI Assay sciences, Cat. #12405), 10 ng/mL IFN-γ (Millipore, IF005), and 100 nM human recombinant IGF-I (Invitrogen, Cat. #PHG0071). Cells were harvested, flash-frozen on dry ice, and stored at −80°C before being used in immunoblotting analyses.

Fluorescence-activated cell sorting

Mammary epithelial cells that were heterogeneous for the expression of GFP were prepared for FACS sorting by detaching cells from culture plates using 0.05% trypsin. Trypsin was quenched by FACS buffer (1% FBS in DMEM/F12), centrifuged at 900 rpm for 5 min, resuspended with 4 mL of FACS buffer, and then filtered through 40 μm nylon mesh filters (Fisher Scientific) to remove cell aggregates. Cell count and viability were determined using an automated cell counter (CellDrop, Denovix) or by manual counting on a hemacytometer through the Trypan Blue exclusion method. Eight x 10^6–10^7 cell suspensions were then prepared for sorting along with appropriate positive and negative controls. Fluorescence-activated sorting of GFP-labelled cells was performed using a 130 μm nozzle on the SH800 cell sorter (Sony Biotechnology). Sorted GFP-positive and GFP-negative cells were plated and maintained in DMEM/F12 with 5% FBS.

Immunoprecipitation and western blot analysis

Cell pellets or homogenized tissues were sonicated for 4 s in complete lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.4 units/mL aprotinin, 1 mM NaF, leupeptin, and 0.1 mM sodium orthovanadate. Alternatively, one tablet of the complete Mini Protease Inhibitor Cocktail (Roche, 11836153001) was dissolved in 10 mL of 1% NP-40 to formulate a lysis buffer. Lysed protein samples were kept on ice for 30 min. Whole-cell extracts were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Invitrogen). The membranes were blocked for 1 h at room temperature in 5% dry milk in 1x TBST (Tris-buffered saline with 0.05% Tween 20) buffer or 5% Bovine Serum Albumin (BSA) in 1x TBST for phosphotyrosine-specific antibodies. Next, membranes were incubated overnight with primary antibodies in a blocking buffer at 4°C. Table S3 lists the source of primary and secondary antibodies with recommended dilutions that were used for immunoblotting. Next, membranes were washed three times for 5 min in 1x TBST and incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary antibodies [Digital anti-Mouse-HRP (R1005) or Digital anti-Rabbit-HRP (R1006) from KwikQuant] in blocking buffer. Membranes were washed three times for 10 min with 1x TBST and then two times for 10 min in 1x TBS (Tris-buffered saline without Tween 20) and finally for 5 min in ultrapure water. Protein bands were detected using the ECL chemiluminescence kit for immunoblot analysis [KwikQuant Ultra Digital-ECLTM Substrate Solution (Cat. #R1002)] according to the manufacturer's instructions (Kindle Biosciences, LCC). Chemiluminescence and brightfield images of the blots with size markers were taken using a D1001 KwikQuant Imager (Kindle Biosciences, LLC). The protein band intensity of selected immunoblots was quantified using KwikQuant Image Analyzer 5.9 (Kindle Biosciences, LLC). Membranes were stripped using a mild glycine stripping buffer (Abcam protocol) for consecutive detection of various proteins.

For immunoprecipitation experiments, cell pellets were lysed in complete lysis buffer as described above without the sonication step and were kept on ice for 1 h. Clarified lysates corresponding to 500 μg to 1 mg of total protein were immunoprecipitated with an antibody against IRS-1 (1:50 dilution) and Dynabeads Protein A according to the manufacturer's instructions (Invitrogen Cat. #10001D). The immunoprecipitated proteins were then analyzed by western blotting as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

Graphic illustrations and statistics were performed with GraphPad Prism (v6.07; GraphPad Software, La Jolla, CA). All reported measurements were taken from distinct samples. Unless otherwise indicated in the figure legends, experimental data are shown as mean values ± SEM or mean ± SD. For statistical analysis, the data were assessed for normality followed by unpaired Student's t-tests, and p-values < 0.05 (*), <0.01 (**), <0.001 (***), and <0.0001 (****) were considered statistically significant.

ADDITIONAL RESOURCES

N/A.