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Stat5 Regulates the Phosphatidylinositol 3-Kinase/Akt1 Pathway during Mammary Gland Development and Tumorigenesis

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Stat5 (signal transducer and activator of transcription 5) is an essential mediator of cytokine receptor signaling and plays important roles in the proliferation of alveolar progenitors and the survival of functionally differentiated epithelial cells in the mammary gland. A deregulated expression and activation of Stat5 leads to precocious alveolar development in the absence of pregnancy hormones, impaired mammary gland remodeling following the cessation of lactation, and mammary tumor formation. We reported previously that Stat5 induces the transcription of the *Akt1* gene from a novel promoter. In this report, we provide experimental evidence that Akt1 is an essential mediator for the biological function of Stat5 as a survival factor. Additionally, Stat5 controls the expression of the regulatory and catalytic subunits of the phosphatidylinositol 3-kinase (PI3K) (p85α and p110α), thereby greatly augmenting signaling through the prosurvival PI3K/Akt pathway. In agreement with this model, we observed that the constitutive activation of Stat5 cooperates with the loss of function of the tumor suppressor PTEN by accelerating the formation of preneoplastic lesions and mammary tumors. The mammary gland-specific ablation of Stat5 is sufficient to prevent mammary carcinogenesis in a genuine mouse model for Cowden syndrome. Therefore, targeting the Jak2/Stat5 pathway might be a suitable strategy to prevent breast cancer in patients that carry a mutant *PTEN* allele.

Stat5a (signal transducer and activator of transcription 5a) was first identified as a prolactin (PRL)-responsive DNA binding protein in mammary epithelial cells (1, 2). Shortly after the discovery of Stat5a, Liu and colleagues (3) isolated another highly conserved Stat5 transcript from the mouse mammary gland, which they named Stat5b. Although both Stat5 proteins share an overall 96% amino acid similarity and are activated upon PRL stimulation, gene deletion studies in knockout mice revealed that only Stat5a is essential for the development of secretory mammary epithelial cells during pregnancy and lactation (4–6). The predominant role of Stat5a in this tissue is likely a consequence of the dissimilar expression of the two Stat5 proteins. This notion is supported by the fact that a compensatory upregulation of Stat5b can partially restore normal alveolar development in Stat5a knockout females (7). The two Stat5 proteins have therefore redundant functions, and a lack of both genes results in more severe developmental defects that affect the genesis of luminal epithelial progenitors (8). These abnormalities phenocopy a deficiency in the Janus kinase 2 (Jak2), which is required for the PRL-mediated activation of Stat5a and Stat5b (9, 10). In addition to their essential functions for the formation of luminal progenitors, Jak2 and Stat5 are equally important for the survival of functionally differentiated epithelial cells during late pregnancy and lactation (9, 10).

Within only a few days following the cessation of lactation and weaning of the offspring, the mammary gland undergoes a rapid remodeling process, which is characterized by the removal of the secretory epithelium. This process requires the inactivation of Stat5 in addition to the phosphorylation of another member of the Stat protein family, Stat3, which is activated by the leukemia inhibitory factor (LIF) and oncostatin M (OSM) (11–13). Using transgenic mice that overexpress hyperactive Stat5 or Jak2 in the mammary epithelium, we and others have shown that sustained Jak2/Stat5 signaling causes precocious alveolar development and extended survival of functionally differentiated epithelial cells (14–17). We recently reported that the serine/threonine protein kinase Akt1 is a transcriptional target of Stat5 and may mediate the phenotypic abnormalities associated with a persistent activation of this Stat protein (15). Stat5 binds to two or more consensus sequences within the *Akt1* locus in a growth factor-dependent manner, and it enhances the transcription of a unique *Akt1* mRNA (*Akt1m*) from a distinct promoter in mice. Expression and activation of both Stat5 and Akt1 decline during postlactational remodeling in the normal mammary gland, but identical to the Stat5 overexpression models, the continuous upregulation of Akt1 in transgenic mice promotes the survival of terminally differentiated epithelial cells and delays involution (18–20). Interestingly, a gain of function of Stat5 and Akt1 appears to be sufficient to override the proapoptotic role of active Stat3 (15, 18, 20). It has been suggested that Stat3 negatively regulates the activity of the phosphatidylinositol 3-kinase (PI3K)/Akt1 pathway through modulation of the ratio in the expression of the long form (p85α) and short forms (p50α/p55α) of the PI3K-regulatory subunits (21). We observed that the overexpression of Stat5 had no effect on the Stat3-mediated upregulation of p50α/p55α (15). Despite the increase in the expression of the shorter PI3K-regulatory subunits, the gain of function of Stat5 causes a sustained upregulation as well as activa-

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tion of Akt1. It is therefore apparent that active Stat5 plays a role in the expression and activation of the p110 catalytic and p85 regulatory subunits of the PI3K. Through interaction with p85 via the Gab2 scaffolding adapter, active Stat5 may indirectly promote the activity of the PI3K in hematopoietic cells (22). We also observed an association between Stat5 and p85α in growth factor-treated mammary epithelial cells and in functionally differentiated mammary tissues (23). Nonetheless, the level of the p85α protein declines sharply during the onset of involution (21), and therefore we postulate that active Stat5 may play a more direct role in regulating the expression of the PI3K in the mammary gland.

In this new report, we provide experimental evidence that the PI3K/Akt1 pathway is an important downstream effector of Jak2/Stat5 signaling. While the prosurvival function of Akt1 occurs independently of Stat5 activation, this serine/threonine kinase is essential for mediating evasion of cell death in response to a sustained activation of Stat5. We demonstrated in cell culture models and in transgenic mice that besides regulating the levels of Akt1, Jak2/Stat5 signaling controls the expression of the catalytic (p110α) and regulatory subunit (p85α) of the PI3K. These results provide an underlying mechanism by which active Stat5 is able to override the proposed proapoptotic function of Stat3 and its downstream targets p50α and p55α, thereby promoting a prolonged survival of mammary epithelial cells. The biologic significance of this signaling network was further tested in a mouse model for Cowden syndrome (CS). The sustained activation of Stat5 increased the rate of tumor formation, and a tissue-specific knockout of Stat5 prevented mammary carcinogenesis in mice that expressed mutant PTEN. Collectively, our findings suggest that inhibiting Jak2/Stat5 signaling might avert the development of premalignant lesions and breast cancer in CS patients.

MATERIALS AND METHODS

Mouse models. Besides FVB/N wild-type mice, we used nine genetically engineered mouse lines with the following genotypes for this study: *MMTV-myrAkt1* (18), *Wap-rtTA* (24), *TetO-Stat5*^{S710F} (15, 25), *Akt1*$^{fl/fl}$ (26), *Stat5a/b*$^{fl/fl}$ (10), *Pten*^{G129E} (27), *TetO-Cre* (28), *MMTV-Cre* (line A) (29), and *MMTV-tTA* (30). *Stat5a/b*$^{fl/fl}$ mice were kindly provided by Lothar Hennighausen (NIH), and *TetO-Cre* mice (MGI:2679524) were purchased from the Jackson Laboratory. All transgenes and targeted alleles were maintained in an FVB/N genetic background. For this purpose, we backcrossed the targeted *Akt1* and *Stat5* alleles into this particular background for 8 to 10 generations. All animals used in this study were treated humanely and in accordance with guidelines of the University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee (IACUC).

Doxycycline treatment and *in vivo* bioluminescence imaging. *Wap-rtTA TetO-Stat5*^{S710F} double-transgenic females and their single-transgenic controls were treated with 2 mg/ml doxycycline (Dox; Sigma, St. Louis, MO) in their drinking water starting on lactation day 7. The expression and activity of the luciferase reporter gene was monitored using an *in vivo* bioluminescence imaging machine (IVIS200; Caliper Life Sciences, Alameda, CA) as described previously (24). At day 10 of lactation, the left mammary glands were sealed to induce milk stasis as described previously (15). All mammary glands were collected 1, 2, 3, and 5 days later.

Histology and immunostaining. Mammary gland tissues were collected and procured for whole-mount staining with carmine alum or for histological examination of hematoxylin-and-eosin (H&E)-stained sections as described previously (31). Detailed protocols for the preparation of histologic sections and immunostaining can be found elsewhere (9). The following primary antibodies were used in this study: anti-pAkt (Ser473), anti-pStat3 (Tyr705), anti-pStat5 (Tyr694/699) from Cell Signaling, Inc., anti-Akt1 from Epitomics, anti-E-cadherin from BD Transduction Laboratories, and anti-Ki-67 from Dako. Corresponding secondary antibodies conjugated to Alexa Fluor dyes 488 and 594 (Invitrogen) were used for visualization of the specific targets. Alternatively, the detection of biotinylated secondary antibodies was performed using Vectastain Elite ABC kits from Vector Laboratories. Stained slides were examined with a LSM5 Pascal confocal microscope or an Axio Imager microscope (Carl Zeiss) equipped with a SPOT Flex camera (Diagnostic Instruments, Inc.). Ki-67-positive nuclei were counted in three representative areas (i.e., 1,000 nuclei in each area) of each specimen, and a paired Student *t* test using Prism (GraphPad Software, La Jolla, CA) was performed to assess statistically significant differences in cellular proliferation.

Immunoprecipitation and Western blot analysis. Detailed experimental procedures for immunoprecipitation (IP) and Western blot analysis were described elsewhere (23). The following antibodies were used for immunoblotting: anti-β-actin (I-19), anti-Jak1 (Sc-7228), and anti-Stat5 (Sc-836) from Santa Cruz Biotechnology; anti-pTyr (05-321) and anti-p50/55/85α (06-195) from Millipore; anti-p110α (4249S), anti-Akt (9272), anti-pAkt (Ser473) (9271), anti-Stat3 (9139S), anti-pStat3 (Tyr705) (9145S), anti-pGsk3b (Ser9) (9336S), anti-pFoxo3a (Ser318/321) (9465S), and anti-pStat5 (Tyr694/699) (9351S) from Cell Signaling; anti-pStat5 (Tyr694/699) (AX1) from Advantex BioReagents; and anti-p85α (1675-1) and anti-Akt1 (1081-1) from Epitomics.

Chromatin immunoprecipitation assay (ChIP). Chromatin immunoprecipitation was performed according to a protocol by LeBaron et al. (32). Immunoprecipitation was performed using antibodies directed against Stat5a (a kind gift from Lothar Hennighausen, NIH) or an isotype-matched control IgG. Input and bound chromatin was detected by quantitative reverse transcription-PCR (qRT-PCR) using primer sets surrounding high-affinity Stat5-binding sites (TTCYNRGAA) in the murine *Pik3r1* and *Pik3ca* loci. Sequences of the specific primer sets for putative Stat5-binding sites will be provided upon request. Assay background was detected using primers directed against a nonpromoter site in *Pik3r1* (33) and was used for normalization in addition to comparison against a serial dilution of genomic DNA.

mRNA expression analyses using quantitative real-time PCR. Total RNA was extracted from flash-frozen tissues and cell pellets using standard guanidinium thiocyanate-phenol-chloroform extraction or the RNeasy minikit (Qiagen). The Super-Script II kit from Invitrogen with oligo(dT) primers was used to perform first-strand synthesis according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using mRNA-specific primer sets for *Pik3r1* (5′-CTG AAG CTG ACA CGG AGC AGC-3′ and 5′-GAC GTG TAC GTC GAT CAT CTC-3′) and *Pik3ca* (5′-GTG TGT GGC TGT GAC GAA TAC-3′ and 5′-CTA TCA ATC GGC AGC TGA GAG-3′) and iQ SYBR green Supermix (Bio-Rad, Hercules, CA). In this study, we also used qPCR primer sets to assess mRNA expression of β-*casein* (5′-CGA CTG TGT CAT GAC ATG TAC-3′ and 5′-TTG AAA GCA TTA TGT TCT CTC TGG-3′) and *Wap* (5′-CAT CCT CGC CTG CCT TGT GGC-3′ and 5′-CCA TGA GAT TCA CCT TCT GAA G-3′). The qPCRs were carried out in triplicate in a CFX96 real-time PCR detection system (Bio-Rad). The expression values obtained were normalized against *Gapdh* as described previously (15).

Mammary epithelial cell cultures. HC11 cells and primary mammary epithelial cells (MECs) were cultured as described previously (23). Details about the cloning of retroviral vectors expressing the constitutively active prolactin receptor (caPRLR) or the Flag-tagged Stat5a can be found elsewhere (15). HC11 cells were treated with 10% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor (EGF), and 10 μg/ml insulin as growth factors (GF). Inhibition of Jak/Stat signaling was achieved by treating the cells with 50 μM AG490 in dimethyl sulfoxide (DMSO). Immortalized mammary epithelial cells with two *Stat5a/b* conditional knockout alleles were isolated from midpregnancy female mice that carry two *Cdkn2a* null alleles in addition to the targeted Stat5 loci (*Stat5a/b*$^{fl/fl}$ *Cdkn2a*$^{-/-}$). The Cre-mediated deletion of the *Stat5* conditional knockout alleles was

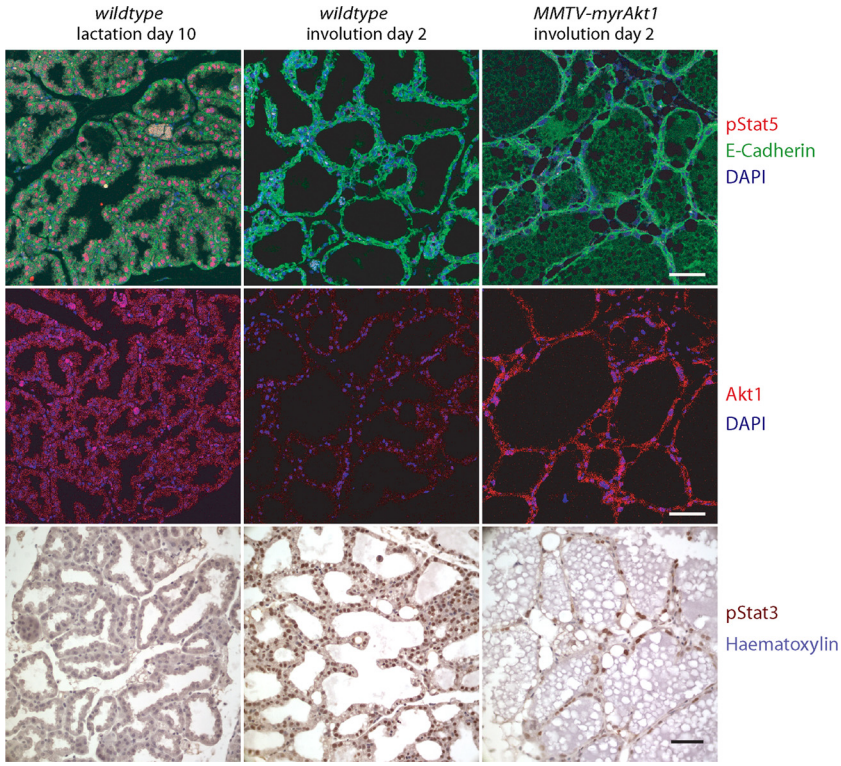

FIG 1 The gain of function of Akt1 causes impaired mammary gland involution in a Stat5-independent manner. Immunostaining of active Stat5, Akt1, and phosphorylated Stat3 in mammary glands of wild-type and *MMTV-myrAkt1* transgenic females at the second day of involution is shown. Tissues of wild-type mice at day 10 of lactation were used as controls, since they exhibit the highest expression levels of pStat5 and Akt1 but typically lack expression of pStat3. Slides were counterstained with DAPI (4′,6′-diamidino-2-phenylindole) (top and middle) or hematoxylin (bottom). Bars, 50 μm.

achieved by infecting MECs with green fluorescent protein (GFP)-conjugated AdCre adenoviral particles and fluorescence-activated cell sorting as previously described (23). PTEN-mutant primary mammary tumor cells that express exogenous Stat5 were explanted from *MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E} triple-transgenic females and cultured in Dulbecco's modified Eagle medium (DMEM)–F-12 medium supplemented with 2% FBS, EGF, hydrocortisone, and insulin (23). To assess the effects of PI3K inhibition on normal and neoplastic epithelial cell growth, 10,000 HC11 cells or 20,000 viable mammary cancer cells from PTEN-mutant females were seeded in triplicate into 6-well dishes and counted 24 and 48 h or 2, 4, and 6 days following treatment with 10 μM LY294002 in DMSO. Cell viability was determined using a trypan blue exclusion assay.

Orthotopic mammary tumor transplantation. Mammary tumors were excised under sterile conditions, dissociated into small fragments using scissors, washed multiple times with 1× PBS, and viably stored using recovery cell culture freezing medium (Gibco, Life Technologies) at −80°C prior to transplantation. Orthotopic transplantations were performed by surgically implanting small tumor fragments into the no. 4 mammary fat pads of athymic nude females (NCr strain). Tumor volumes were measured at defined time intervals using a caliper. To estimate the tumor volume, we used the following equation: volume = (length × width^2)/2.

RESULTS

Akt1 plays an important role in the extended survival of mammary epithelial cells expressing hyperactive Stat5. We previously identified Akt1 as a transcriptional target of Stat5 in the lactating mammary epithelium. We anticipated that as a downstream effector of Jak2/Stat5 signaling, a sustained activation of Akt1 is sufficient to promote the survival of functionally differentiated epithelial cells without concomitant activation of Stat5. To experimentally address this assumption, we performed immunofluorescence staining of tyrosine-phosphorylated Stat5 on histologic sections of mammary tissues from mice that express myristoylated Akt1 under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (Fig. 1). The highest levels of active Stat5 and Akt1 were present in the secretory epithelium of lactating mice (Fig. 1, left). The phosphorylation of Stat5 and expression of Akt1 declined rapidly within hours after weaning of the offspring, and active Stat5 was virtually absent during the second day of involution prior to extensive remodeling of the mammary gland (Fig. 1, middle). During a normal involution process, the decrease in the level of phosphorylated Stat5 is typically accompanied by an increase in the nuclear accumulation of active Stat3 (Fig. 1, bottom). The analysis of mammary tissues from *MMTV-myrAkt1* transgenic females revealed that expression of exogenous, hyperactive Akt1 had no effect on the activation status of Stat5. Identical to wild-type mammary tissues, Stat5 was swiftly dephosphorylated following the cessation of lactation in transgenic mice (Fig. 1, right). Mammary epithelial cells expressing myristoylated Akt1 had an extended survival of almost 1 week (18), and it is therefore evident that this process is not mediated by a sustained expression and activation of Stat5. This clearly confirms the notion that overexpression and activation of Akt1 are sufficient to prolong mammary epithelial cell survival independently of lactogenic hormone signaling and Stat5 activation during involution. Identical to a gain of function of Stat5 (15), overexpression of Akt1 impaired normal mammary gland

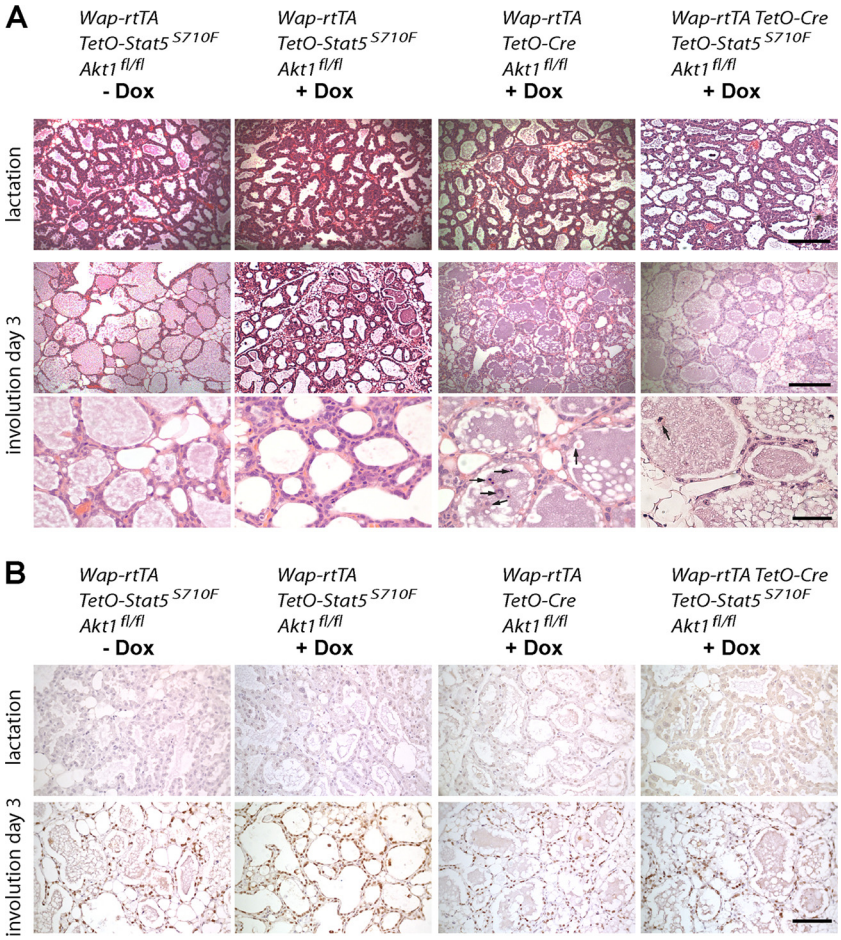

FIG 2 Akt1 is an essential downstream mediator for the prosurvival function of active Stat5. (A) Hematoxylin and eosin (H&E)-stained sections of lactating and involuting mammary tissues (day 3) from transgenic females that expressed hyperactive Stat5 in an Akt1 conditional knockout background (*Wap-rtTA TetO-Stat5*^{S710F} *TetO-Cre Akt1*$^{fl/fl}$). Mice that conditionally expressed Stat5 in a Dox-controlled manner (*Wap-rtTA TetO-Stat5*^{S710F} *Akt1*$^{fl/fl}$; +Dox and −Dox) or that were deficient only in Akt1 (*Wap-rtTA TetO-Cre Akt1*$^{fl/fl}$; +Dox) were used as controls. Arrows mark the locations of apoptotic cells. Bars (top two rows), 200 μm. The bottom row shows closeups of the involuting tissues. Bar, 50 μm. (B) Immunohistochemical staining of active Stat3 on the lactating and involuting tissue sections shown in panel A. Bar, 100 μm.

involution despite activation of Stat3 (Fig. 1, bottom). This clearly suggests that active Stat5 and its downstream target Akt1 are sufficient to override the proapoptotic role of Stat3 in functionally differentiated mammary epithelial cells, which consequently causes a delay in mammary gland remodeling.

To assess whether Akt1 mediates the extended survival of alveolar cells expressing hyperactive Stat5, we generated a complex genetic model that permits a doxycycline (Dox)-induced deletion of the *Akt1* gene in mammary epithelial cells that also express exogenous Stat5 (*Wap-rtTA TetO-Stat5*^{S710F} *TetO-Cre Akt1*$^{fl/fl}$). In female mice, the Dox-regulatable transactivator protein (rtTA, Tet-ON) is expressed under the endogenous whey acidic protein gene (*Wap*) promoter and therefore targeted exclusively to secretory mammary epithelial cells. Administration of Dox induces the simultaneous, ligand-controlled expression of hyperactive Stat5 (Stat5^{S710F}) and luciferase as well as Cre recombinase from two TetO-driven responder transgenes. Expression of Cre then leads to the excision of the *Akt1* conditional knockout alleles. The upregulation of hyperactive Stat5, the expression of Cre recombinase, and the knockout of *Akt1* did not have any discernible effects on the secretory epithelium following administration of Dox starting at day 10 of lactation (Fig. 2A, top). A few animals also carried a CAG-Lox-Stop-Lox-GFP reporter transgene, which allowed us to label individual cells that express Cre recombinase. The presence of GFP in the mammary gland following 5 days of Dox administration suggested that there was no selective elimination of Akt1-deficient cells prior to involution (data not shown). Pups were removed on day 15 of lactation (i.e., the fifth day of Dox treatment) to induce milk stasis and to initiate postlactational remodeling. The histological examination of mammary tissues revealed that upregulation of Stat5 in the Dox-treated controls expressing Akt1 (*Wap-rtTA TetO-Stat5*^{S710F} *Akt1*$^{fl/fl}$) caused a sustained survival without the typical signs of remodeling (Fig. 2A, middle and bottom). Control females that were deficient in Akt1 and that did not express exogenous Stat5 (*Wap-rtTA TetO-Cre Akt1*$^{fl/fl}$) exhibited extensive shedding of apoptotic cells (Fig. 2A, bottom [arrows]). This was indicative of precocious remodeling and confirms that a lack of Akt1 leads to accelerated involution (34). Similar to Akt1-deficient controls, experimental animals that express hyperactive Stat5 in an Akt1 conditional knockout

background (*Wap-rtTA TetO-Stat5*^{S710F} *TetO-Cre Akt1*$^{fl/fl}$) showed accelerated remodeling. Hence, the mammary glands of these mice did not phenocopy the sustained presence of secretory epithelial cells in Stat5-overexpressing females that carry *Akt1* wild-type alleles (*Wap-rtTA TetO-Stat5*^{S710F}). These findings clearly indicate that Akt1 is essential for the extended survival of functionally differentiated mammary epithelial cells in response to sustained Jak2/Stat5 signaling. The conditional deletion of *Akt1* or overexpression of hyperactive Stat5 in an Akt1 wild-type or Akt1-deficient background had no noticeable effect on the nuclear accumulation of phosphorylated Stat3 during postlactational remodeling (Fig. 2B).

Involution is associated with a reduction in the mRNA and protein levels of the PI3K subunits p85α and p110α. We demonstrated previously that the hyperactivation of Stat5 leads to a sustained expression of both total and phosphorylated Akt1 despite an increase in Stat3 activity and upregulation of its transcriptional targets p50α and p55α (15). It therefore seems improbable that the involution process in the mammary gland is primarily driven by an increased expression of these shorter regulatory PI3K subunits (p50α/p55α) that were suggested to inhibit the catalytic p85α/p110α complex (33). To shed light on this conundrum, we assessed the expression and activation of signal transducers in the Jak/Stat and PI3K/Akt1 pathways during the first phase of involution prior to the actual remodeling process. For this study, we used lactating wild-type mice and sealed lateral glands 3, 4, and 5 to induce milk stasis and involution (Fig. 3). This strategy provided an internal control for lactating (open) and involuting (closed) tissue samples at identical time points (i.e., involution days 1, 2, and 3) from the same animals. Besides the expected decrease in the phosphorylation of Stat5 and increase in the activity of Stat3, the immunoblot analysis confirmed a reduced expression of p85α and an upregulation of the shorter PI3K subunits p50α/p55α in involuting tissues (Fig. 3A). In contrast to previous reports, however, we also observed a reduction in the levels of the p110α catalytic subunit of PI3K (total and p85 bound), suggesting that the decline in the observed pAkt1 is a result of lower PI3K expression and not only a shift in the association with particular regulatory subunits. Using quantitative RT-PCR, we found that the reductions in p85α and p110α expression were a consequence of a decline in the transcriptional activation of these genes (Fig. 3B).

Upon close examination of the DNA sequences, we found at least three high-probability Stat5-binding sites (TTCYNRGAA) in the 5′ regions of *Pik3r1* and *Pik3ca*, which encode p85α and p110α (Fig. 3C). To determine whether active Stat5 is capable of binding to any of these sites *in vivo*, we performed a chromatin immunoprecipitation (ChIP) assay combined with real-time PCR on lactating tissues and samples from day 2 of involution (Fig. 3C). While the first two consensus sites within the *Pik3r1* locus showed no association, the third site was significantly enriched for Stat5 in the lactating samples. The binding to this particular site was greatly reduced by the second day of involution when Stat5 is no longer active. Similarly, Stat5 is capable of binding two consensus sites within *Pik3ca* with different affinities. In particular, the binding of Stat5 to the second site was greatly enriched in the lactating tissue but was absent in the involuting mammary gland. Using the UCSC Genome Browser, we performed an *in silico* analysis of the Stat5 ChIP-sequencing data sets on mammary tissues published by Yamaji et al. (35). Peaks representing a high degree of enrichment for Stat5 within *Pik3r1* and *Pik3ca* aligned perfectly with the preferred Stat5-binding sites (i.e., gamma interferon activation sites [GAS] 3 and 2, respectively) that we identified in our experiments (data not shown). Collectively, these results show that Stat5 associates in lactating tissues with at least one Stat5 recognition site in both genes encoding p85α and p110α. Besides these high-probability Stat5-binding sites, both loci contain a number of additional, but less stringent, consensus sites (TTCNNNGAA) that might mediate binding of Stat5 as homodimers as well as tetramers.

Jak2/Stat5 signaling regulates PI3K expression and enhances Akt1 activation *in vitro* and *in vivo*. To determine whether a highly active Jak2/Stat5 signaling pathway in mammary epithelial cells is capable of elevating the levels of p85α and p110α, we generated a series of HC11 mouse mammary epithelial cell (MEC) lines that overexpress wild-type Stat5, a constitutively active prolactin receptor (PRLR), or both. The quantitative RT-PCR data and Western blotting results show that expression of exogenous Stat5 and its constitutive activation by the PRLR were sufficient to significantly increase the transcriptional activation and protein expression of the regulatory and catalytic subunits of the PI3K (Fig. 4A and B). Identical to our previous observations (15), elevated levels of active Stat5 greatly increased the expression of total and phosphorylated Akt1 as well as phosphorylation of downstream mediators of PI3K/Akt signaling (i.e., pGSK3b and pFoxo3a) in a growth factor-independent manner (Fig. 4B).

To assess whether inhibiting Jak2/Stat5 signaling results in the reduced expression of p85α and p110α, we performed two sets of experiments; one study employed the pharmacological inhibition of the Janus kinase, and a second line of investigation was performed using the Cre/Lox-mediated deletion of *Stat5a* and *Stat5b* in primary MECs. For the first experiment, we treated HC11 cells with AG490 or DMSO as the control and determined the expression of the PI3K subunits by real-time PCR and Western blot analysis. Treatment with the Jak inhibitor led to a significant reduction in the transcriptional activation of both PI3K subunits (Fig. 4C). While chronic inhibition of Jak for 24 and 48 h demonstrated a significant reduction in p110α protein levels, the effect on p85α levels was less dramatic, albeit noticeable when corrected for the actin loading control (Fig. 4D). As many small-molecule inhibitors, including AG490, have off-target effects, we also determined the expression of PI3K subunits in primary MECs that lack the *Stat5* locus. For this purpose, we isolated MECs from midpregnancy females that carry two conditional *Stat5a/b* alleles. By deleting the entire *Stat5* locus in immortal MECs, we generated isogenic pairs of cell lines with or without expression of Stat5a and Stat5b. The quantitative RT-PCR results showed that a complete lack of Stat5 led to a statistically significant reduction in both p85α and Akt1 (Fig. 4E). Although the lower expression of p110α was insignificant on the mRNA level, there was a more noticeable reduction of the p110α protein (Fig. 4F).

The collective results from the cell culture experiments suggest that active Jak2/Stat5 signaling increases the expression of both p85α and p110α. Inhibiting Jak2 or deleting Stat5, on the other hand, leads to a reduced expression of PI3K subunits to various degrees depending on the cellular model. To determine whether these results are biologically significant under *in vivo* conditions, we examined the transcriptional activation and protein expression of p85α and p110α in lactating and involuting mammary tissues of females that conditionally express hyperactive Stat5 in a

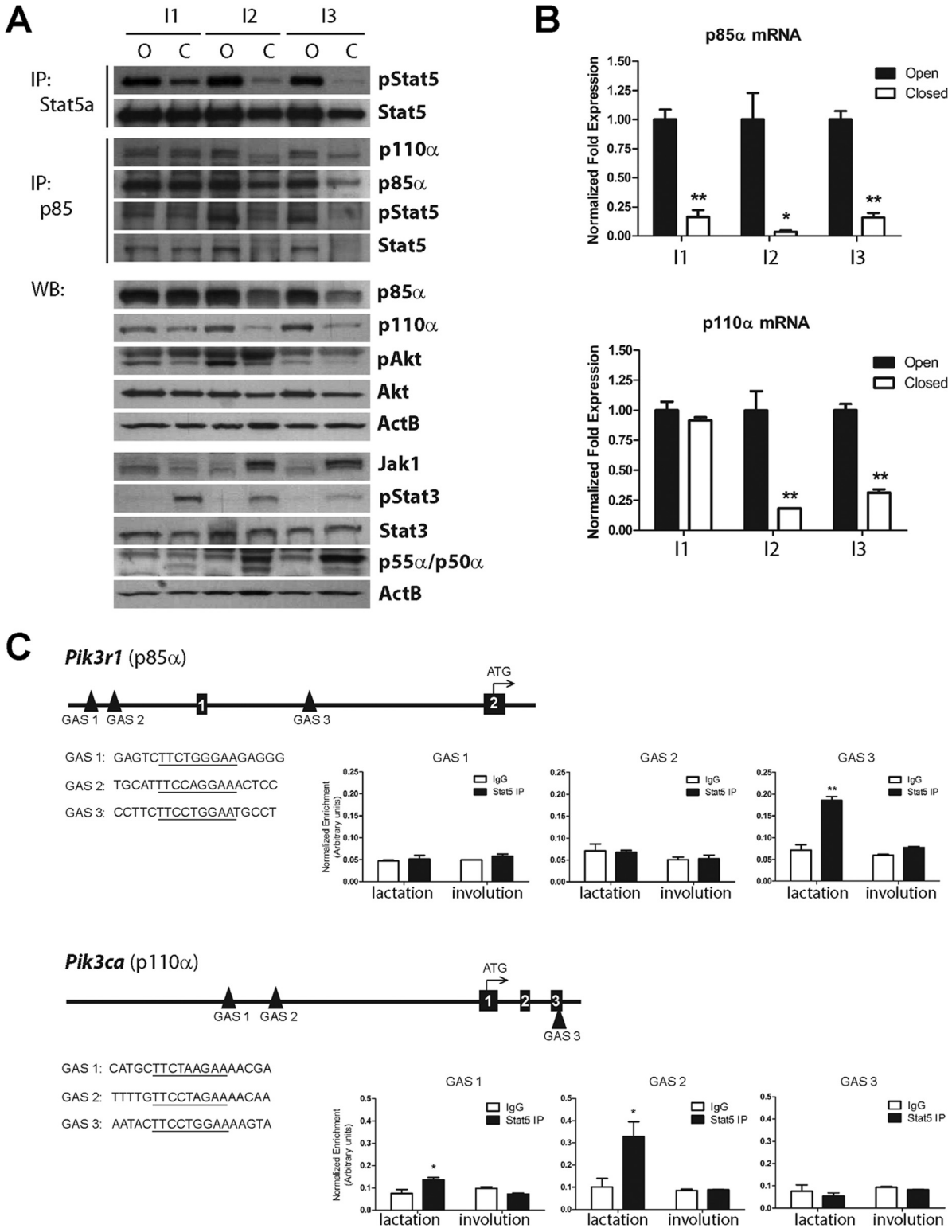

FIG 3 A reduction in the mRNA and protein levels of the PI3K subunits p85α and p110α precedes the postlactational regression of the mammary gland. (A) Immunoblot analysis of selected signal transducers within Jak/Stat and PI3K/Akt pathways in lactating (open [O]) tissues and collateral sealed (closed [C]) mammary glands of wild-type mice. The sealed mammary glands correspond to days 1, 2, and 3 of forced involution due to milk stasis. Beta-actin (ActB) was used as a loading control. (B) Quantitative real-time RT-PCR analysis to assess the expression of *p85*α and *p110*α mRNA transcripts in the corresponding lactating and involuting tissues. *, $P < 0.05$; **, $P < 0.01$. (C) Schematic outlines of the 5′ region and location of three high-probability Stat5-binding sites (i.e., gamma interferon activation sites [GAS]; TTCYNRGAA) within the genes encoding the p85α and p110α PI3K subunits (*Pik3r1* and *Pik3ca*). Insets show the results from quantitative real-time PCR analyses of DNA isolated by chromatin immunoprecipitation (ChIP) from lactating (day 10) or involuting (day 2) mammary tissues with an antibody against Stat5a or an IgG control. The PCR assays were performed using specific primer sets surrounding the Stat5-binding sites located within the 5′ regions of *Pik3r1* and *Pik3ca*. Values were normalized against background DNA by using primer sets for nonconsensus binding sites. Error bars represent standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$.

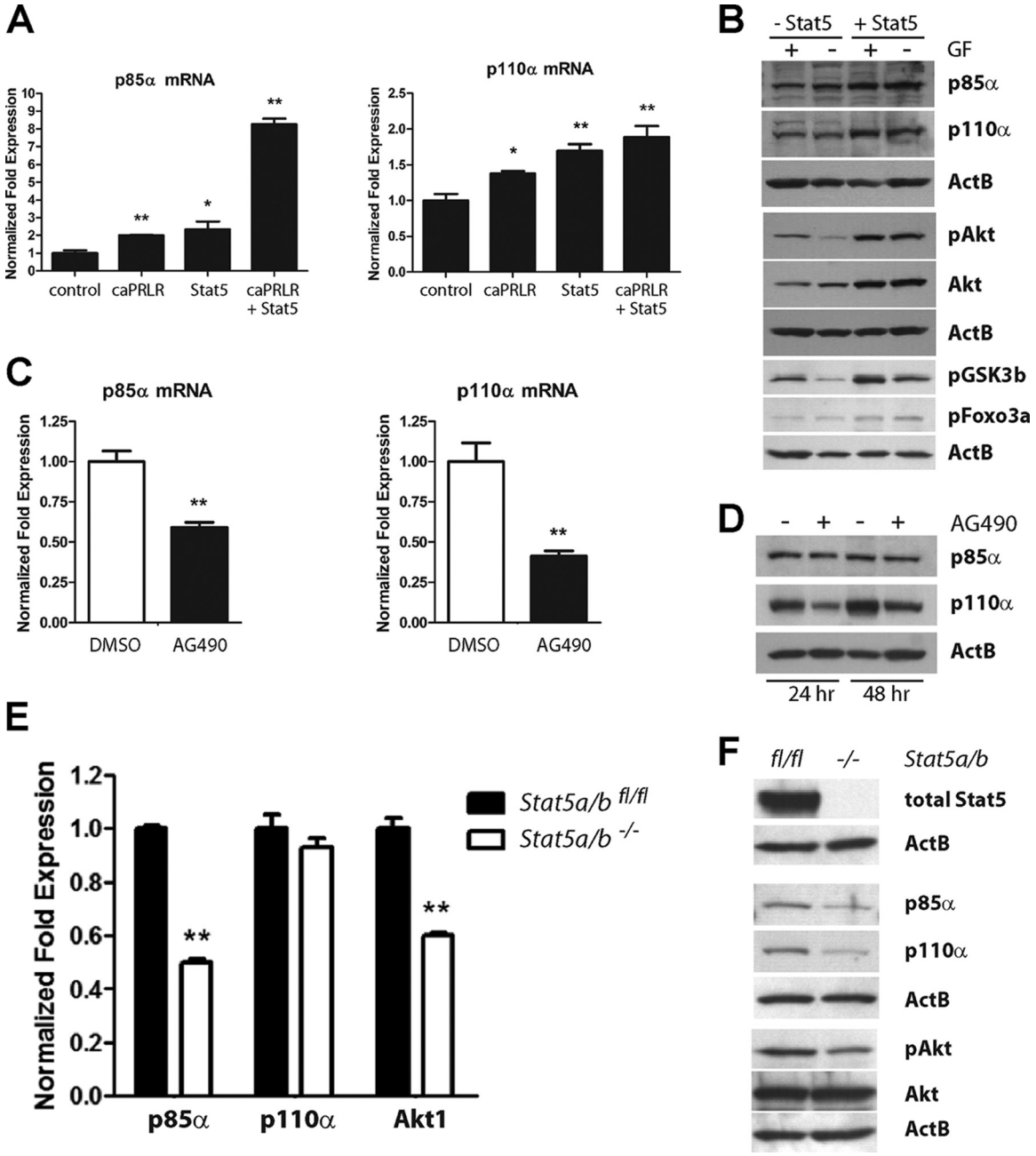

FIG 4 Jak2/Stat5 signaling regulates the mRNA and protein expression of p85α, p110α, and Akt1 in established and primary mammary epithelial cell lines. (A) Quantitative real-time RT-PCR analysis to assess the transcriptional activation of *p85α* and *p110α* in an immortal mouse mammary epithelial cell line (HC11) expressing the constitutively active prolactin receptor (caPRLR), wild-type Stat5, or both. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$. (B) Western blot analysis to determine the levels of p85α and p110α as well as total and phosphorylated Akt and selected downstream effectors (pGSK3b and pFoxo3a) in HC11 cells expressing caPRLR in the absence or presence of exogenous Stat5. Cells were maintained in the presence or absence of growth factors (GF; insulin, EGF, and FBS). Beta-actin (ActB) was used as a loading control. (C and D) qRT-PCR and immunoblot analyses to determine the mRNA and protein expression of p85α and p110α in HC11 cells that were treated with AG490 to inhibit Jak signaling and their DMSO-treated controls. (E and F) mRNA and protein expression of p85α, p110α, and Akt in primary mammary epithelial cells from Stat5a/b conditional knockout females.

Dox-inducible manner (*Wap-rtTA TetO-Stat5*^{S710F}). Experimental mice were treated with Dox for 5 days starting at lactation day 10. The lateral mammary glands were then sealed, and tissues were collected 2 days later. Using immunohistochemical staining, we confirmed that upregulation of hyperactive Stat5 in Dox-treated mice led to a persistent expression of active Akt1 despite nuclear accumulation of phosphorylated Stat3 in epithelial cells of the sealed glands (Fig. 5A). In comparison to normal involuting tissues of untreated *Wap-rtTA TetO-Stat5*^{S710F} females, expression of hyperactive Stat5 in Dox-treated double-transgenic animals resulted in significantly higher expression of p85α and p110α mRNA and protein (Fig. 5B and C). The sustained upregulation of

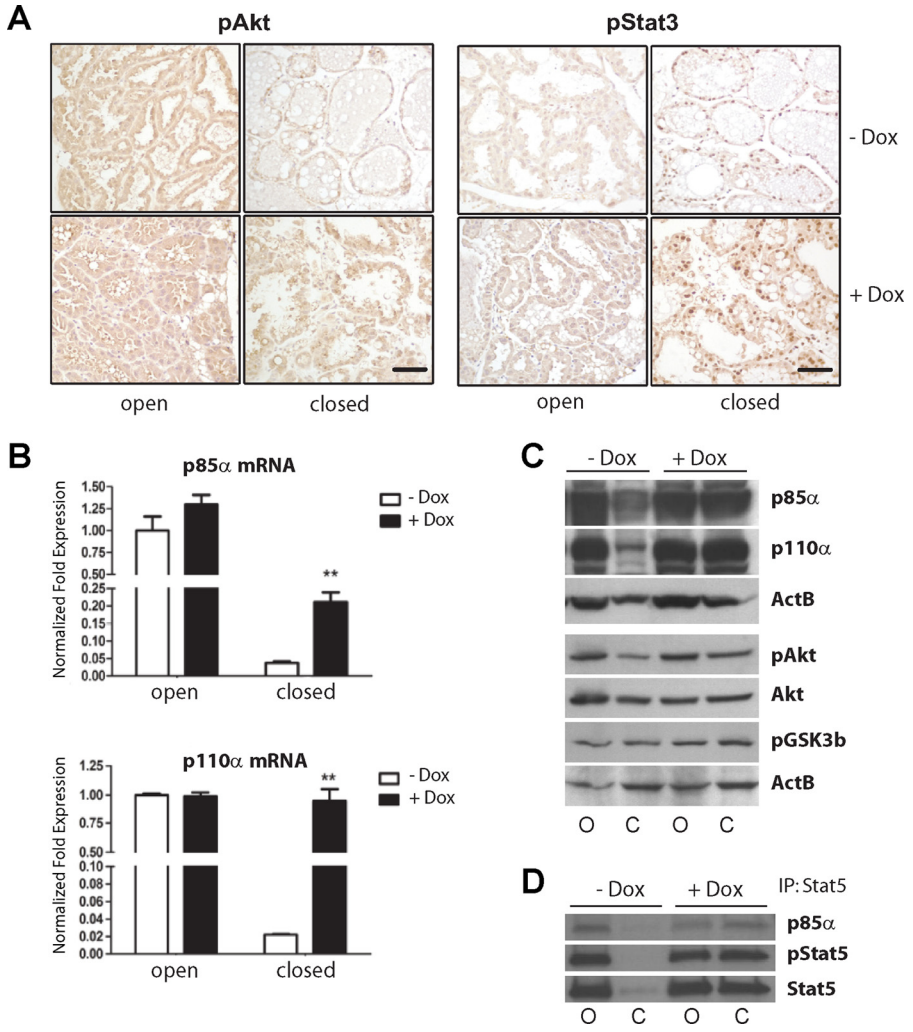

FIG 5 Inducible expression of hyperactive Stat5 in postlactational mammary glands of transgenic mice leads to persistent upregulation of p85α, p110α, and pAkt despite nuclear accumulation of pStat3. (A) Immunohistochemical staining of mammary tissue sections to assess the expression of pAkt(Ser473) and pStat3(Tyr705) in sealed glands (closed; involution day 2) of Wap-rtTA/TetO-Stat5(S710F) double-transgenic females that had been treated with Dox along with their untreated controls. The lactating (open) glands of the same animals served as intraindividual controls. Bars, 50 μm. (B) Quantitative real-time RT-PCR analysis to assess the transcriptional activation of *p85α* and *p110α* in lactating (open) and involuting (closed) tissues of double-transgenic females that express hyperactive Stat5 in a Dox-inducible manner (+Dox, Tet-ON). Untreated double-transgenic mice (−Dox) that lack expression of exogenous Stat5 served as controls. **, $P < 0.01$. (C) Immunoblot analysis to verify the sustained upregulation of p85α, p110α, pAkt, and pGSK3b. Beta-actin (ActB) was used as a loading control. (D) IP and Western blotting to assess the interaction of the SH2 domain-containing proteins Stat5 and p85α. O, open; C, closed.

these PI3K subunits in involuting tissues expressing active Stat5 was accompanied by a noticeable elevation in the levels of pAkt and pGSK3b. As shown in Fig. 2A, active Stat5 and p85α are capable of interacting in the normal lactating mammary gland, and this association is preserved in involuting tissues expressing hyperactive Stat5 (Fig. 5D), which is indicative of a high activity of the PI3K/Akt1 pathway.

Active Stat5 promotes extensive alveolar hyperplasia and mammary tumor formation in a PTEN mutant mouse model for Cowden syndrome. The phosphatase and tensin homolog (PTEN) is a negative regulator of the PI3K/Akt signaling pathway and functions as a tumor suppressor. Mutations in *PTEN* are responsible for inherited predispositions to cancer such as Cowden syndrome (CS). One of the most frequently diagnosed malignancies in CS patients is breast cancer, and introducing CS-related *PTEN* mutations into the germ line of mice predisposes females to mammary cancer after a long latency (27). We anticipated that in their role as positive and negative regulators of the PI3K/Akt1 pathway, Stat5 and PTEN might synergistically promote mammary epithelial growth and, perhaps, neoplastic transformation. To experimentally address this notion, we generated females that constitutively express hyperactive Stat5 in the presence of the G129E mutant *Pten* allele that is found in CS patients (*MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E}). The MMTV-tTA-mediated (Tet-OFF), mammary-specific activation of the Stat5 transgene, which coexpresses luciferase from the same locus (25), was monitored by bioluminescence imaging throughout the study (Fig. 6A). While a single mutation in *Pten* or overexpression of Stat5 alone did not result in any obvious changes in the mammary gland of 14-week-old nulliparous mice, we consistently observed extensive alveolar budding in young, age-matched *MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E} triple-transgenic females (Fig. 6B). Over a period of 15

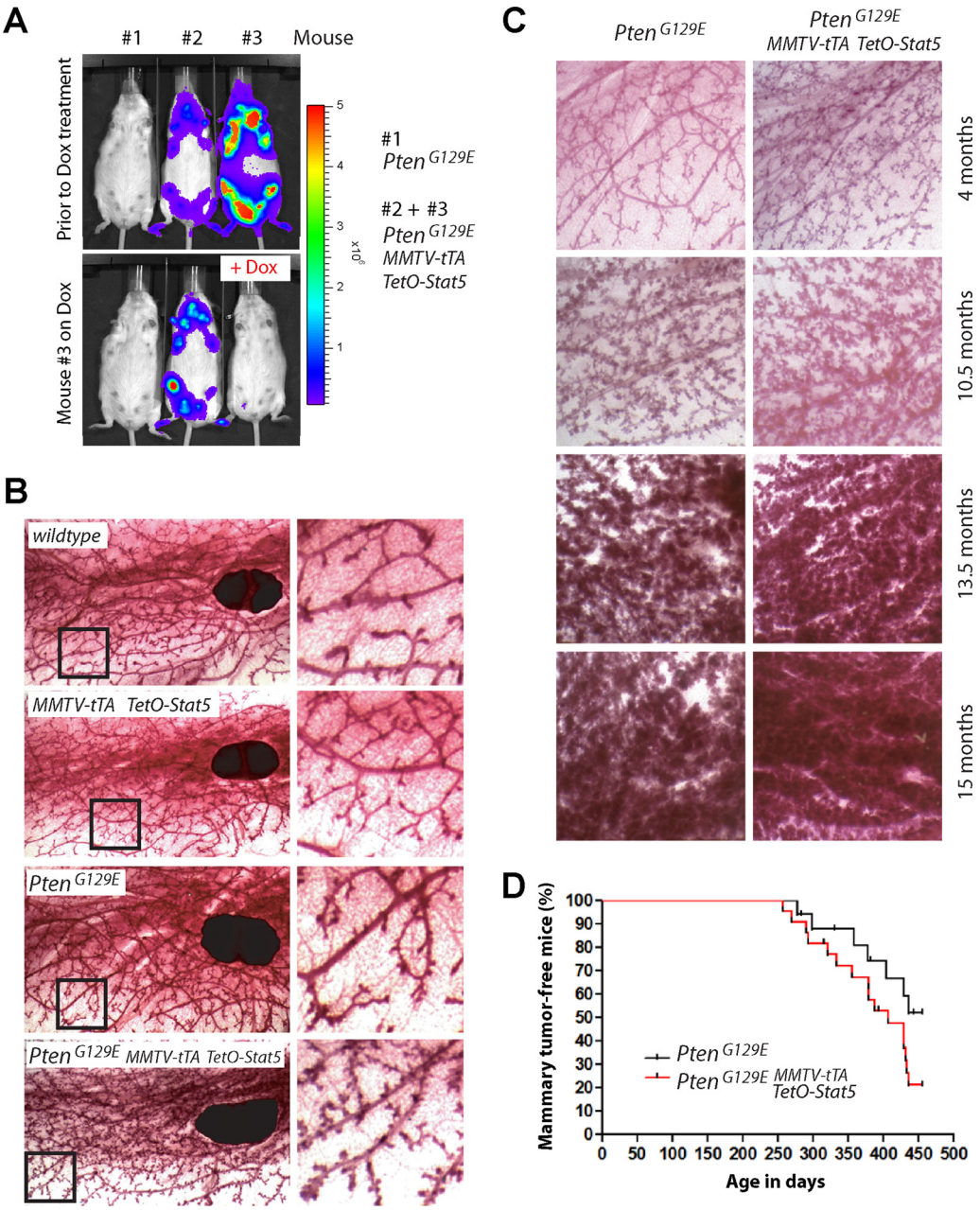

FIG 6 Active Stat5 promotes alveolar hyperplasia and mammary tumor formation in mice that carry a $PTEN^{G129E}$ mutant allele. (A) *In vivo* bioluminescence imaging on *MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E} triple-transgenic mice to assess the ligand-regulatable, mammary-specific activity of the *TetO-Stat5*^{S710F} transgene (Tet-OFF), which coexpresses luciferase from an internal ribosomal entry site (IRES) following the Stat5 coding sequence. Images were taken prior to and 1 week following Dox treatment of mouse 3 (Tet-OFF). (B and C) Carmine-stained mammary gland whole mounts from triple-transgenic females expressing mutant PTEN and exogenous Stat5 as well as their wild-type, PTEN single-mutant, and Stat5-expressing controls at 14 weeks (B) and up to 15 months of age (C). Bar, 1 mm. (D) Kaplan-Meier curve illustrating the mammary-tumor-free survival of mice expressing mutant PTEN in the absence or presence of exogenous Stat5.

months, the loss of function of PTEN was sufficient to promote alveolar hyperplasia, and this process was clearly accelerated in PTEN mutant females that express hyperactive Stat5 (Fig. 6C). Many of the triple-transgenic females developed extended secretory alveoli that resemble late-pregnancy or early lactating mammary glands. Similar to the report by Wang and colleagues (27), 48% of the mice carrying the *Pten*^{G129E} allele developed palpable mammary tumors by 15 months (Fig. 6D). While expression of hyperactive Stat5 in the mutant PTEN background did not dramatically accelerate tumor onset, the incidence of mammary cancer formation increased to 79% in triple-transgenic females within the same time period. All mammary tumors were histopathologically similar to those reported previously (27). Collectively, the examination of *MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E} triple-transgenic mice and their controls revealed that the gain of function of Stat5 promotes precocious alveolar development in

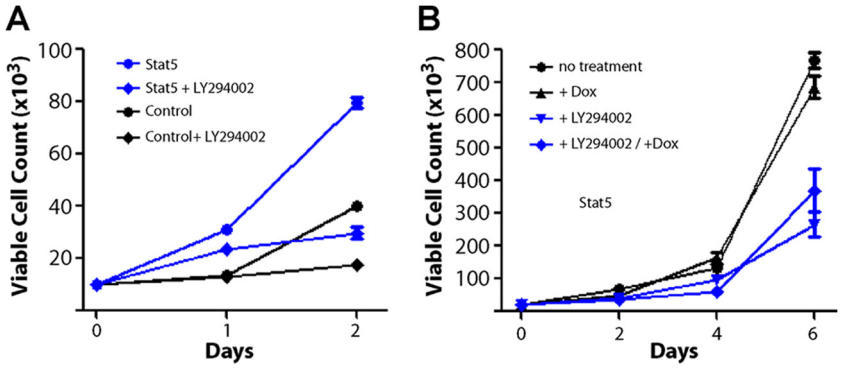

FIG 7 Inhibition of PI3K affects the growth of normal mammary epithelial cells expressing exogenous Stat5 and PTEN-associated mammary tumor cells in culture. (A) Viable-cell counts of untransformed mammary epithelial cells (HC11) with or without expression of exogenous, active Stat5. Both cell lines were treated with a 10 μM concentration of the PI3K inhibitor LY294002 or DMSO as control. (B) Growth rates of cultured mammary tumor cells from *MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E} triple-transgenic females in the presence of 10 μM LY294002 or DMSO. Additionally, cells were treated with doxycycline (+Dox) to repress the expression of exogenous Stat5.

mice expressing mutant PTEN. This synergistic action culminated in an increased predisposition to mammary tumor formation in experimental mice compared to age-matched controls. In addition to mammary tumorigenesis, we observed that the vast majority of mice carrying the mutant *Pten* allele developed lymphoma regardless of the expression of Stat5, which prevented us from extending the study beyond the 15-month experimental endpoint.

Targeting Stat5 prevents alveolar hyperplasia and tumorigenesis in females that carry a mutant *Pten* allele. Similar to our findings that a constitutive activation of Stat5 promotes the numeric expansion of alveolar cells *in vivo*, we observed an accelerated proliferation of normal, luminal-type mammary epithelial cells (HC11) overexpressing Stat5 in culture (Fig. 7A). A short-term treatment of these cells with a potent inhibitor of the PI3K (LY294002) was sufficient to significantly slow their growth ($P < 0.0001$), suggesting that the PI3K/Akt pathway plays a critical role in the proliferative boost mediated by active Stat5. Following neoplastic transformation *in vivo*, a subset of fast-growing mammary tumors in *MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E} triple-transgenic females exhibited a strong activation of the tTA-driven Stat5 transgene in the absence of Dox (Tet-OFF). We explanted and cultured primary mammary cancer cells from these animals and treated them with LY294002 to examine whether their proliferation was dependent on the activation of PI3K (Fig. 7B). While the numeric expansion of the tumor cells that were selected under culture conditions was not strictly dependent on the levels of exogenous Stat5 (+Dox), the pharmacological inhibition of the PI3K still led to a significant decrease in cancer cell proliferation ($P < 0.001$). These results clearly underline the importance of the PI3K/Akt pathway in PTEN mutant mammary epithelial cells that were transformed in the presence of hyperactive Stat5. To assess whether a constitutive expression of exogenous Stat5 contributes to mammary tumor cell proliferation *in vivo*, we transplanted small mammary tumor fragments that had never been cultured orthotopically into the no. 4 mammary glands of six athymic nude mice. The engraftment and continuous expression of the MMTV-tTA-driven TetO-Stat5 transgene was monitored using bioluminescence imaging (Fig. 8A, left). When palpable tumors reached a size of approximately 16 mm^3, the mice were randomized, and half of the recipients were treated with Dox to suppress the expression of Stat5 (Fig. 8A, right). All tumors were measured periodically over 10 weeks. Given the fact that PTEN-associated mammary tumors typically express high levels of endogenous Stat5, which may lead to transgene independence in culture, we were surprised that the downregulation of exogenous Stat5 had a noticeable impact on the growth of transplanted cancer cells in the recipient mice (Fig. 8B). The slower growth of Dox-treated tumors was associated with reduced cell proliferation, as determined by Ki-67 staining (Fig. 8C).

Despite our observation that exogenous Stat5 may accelerate the numeric expansion of cancer cells in at least a subset of tumors, it is evident that endogenous Stat5 is sufficient to mediate mammary tumorigenesis in mutant PTEN control mice that lack expression of hyperactive Stat5. To determine whether Stat5 is essential for mammary tumor initiation in the mouse model for Cowden syndrome, we generated mutant PTEN females with a mammary-specific knockout of both *Stat5* genes (*Pten*^{G129E} *MMTV-Cre Stat5*$^{fl/fl}$). Compared to wild-type mice, the deletion of *Stat5* resulted in reduced terminal branching and alveolar bud formation (Fig. 9A, left two columns). More importantly, the precocious alveolar hyperplasia that we observed in mutant PTEN mice was completely inhibited by the conditional knockout of Stat5 (Fig. 9A, right two columns). It should be noted that expression of Cre recombinase alone had no effect on alveolar bud formation or the development of alveolar hyperplasia in aging, 9-month-old *MMTV-Cre Pten*^{G129E} control mice. Six out of 13 (46%) *Pten*^{G129E} littermate controls with or without *MMTV-Cre* in the presence of one *Stat5* floxed allele developed palpable mammary tumors within 1 year. In contrast, none of the *Pten*^{G129E} *MMTV-Cre Stat5*$^{fl/fl}$ experimental females developed mammary cancer, suggesting that deficiency in Stat5 protects against the initiation of mammary neoplasms in this Cowden syndrome model. Alveolar cells seem to be highly abundant in females carrying the *Pten*^{G129E} allele. To determine the cellular basis for the protective effect of Stat5 deficiency in this cancer model, we used real-time RT-PCR to quantify the expression of β-*casein* and *Wap* mRNA. Using this assay, we confirmed that deficiency in PTEN causes primarily hyperplasia and neoplastic transformation in the alveolar compartments of the mammary glands of *Pten*^{G129E} *Stat5*$^{fl/fl}$ females (Fig. 9B). This particular epithelial lineage is strictly dependent on the expression and activation of Stat5 and virtually

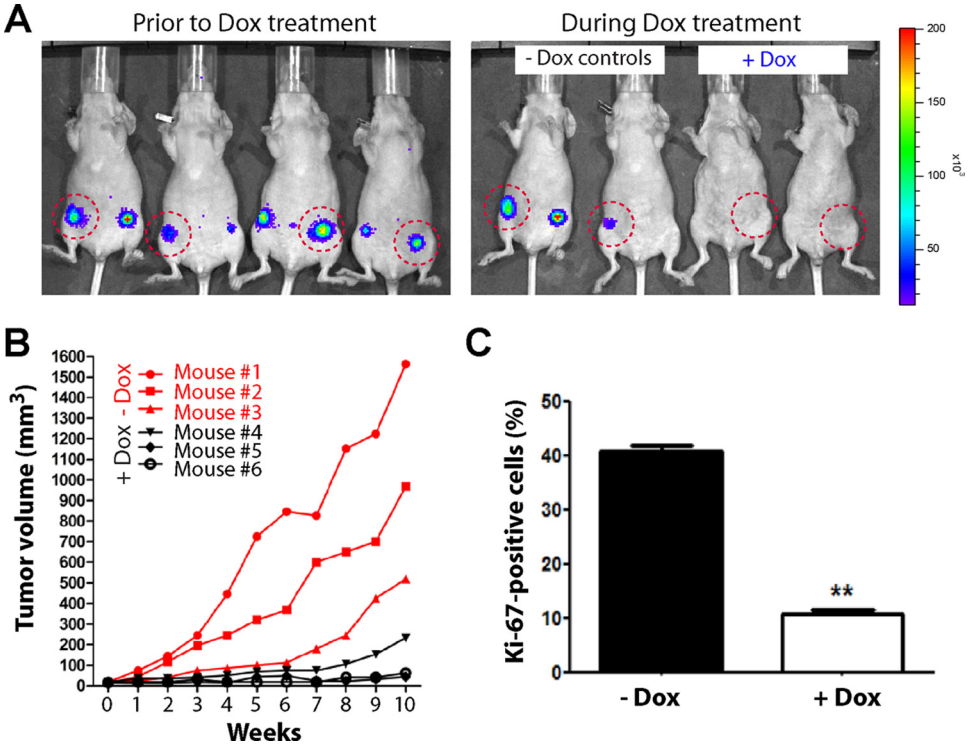

FIG 8 Downregulation of exogenous, hyperactive Stat5 slows the growth of PTEN-mutant mammary tumors *in vivo*. (A) *In vivo* bioluminescence imaging on immunocompromised recipient mice that were orthotopically transplanted with mammary tumor cells from *MMTV-tTA TetO-Stat5*^{S710F} *Pten*^{G129E} triple-transgenic donors. The ligand-regulatable expression of the TetO-driven Stat5 transgene (Tet-OFF) was monitored prior to and 1 week after treatment with Dox. (B) Growth curves of mammary cancers in untreated and Dox-treated recipients (i.e., with or without exogenous Stat5). (C) Relative number of proliferating cells as determined by Ki-67 immunostaining. **, $P < 0.01$.

absent in tumor-free *Pten*^{G129E} *MMTV-Cre Stat5*$^{fl/fl}$ mice, as indicated by the lack of expression of β-*casein* and *Wap*. These observations strongly suggest that the cells of origin for neoplastic transformation in the mouse model for Cowden syndrome are alveolar progenitors and possibly their more differentiated descendants.

DISCUSSION

The postnatal development of the mammary gland is orchestrated by a number of peptide growth factors and their corresponding receptors (see the recent comprehensive review by Hynes and Watson [36]). It is evident that signal transduction through these growth factor receptors has common downstream signaling mediators, and this might explain why an abnormal activation of different growth factor pathways can cause similar phenotypic abnormalities that may ultimately result in the formation of mammary tumors. Specifically, the constitutive expression and activation of cytokine receptors and receptor tyrosine kinases that bind to ligands such as PRL, growth hormone, insulin-like growth factor 1 (IGF-1), and transforming growth factor α (TGF-α) cause precocious alveologenesis, a prolonged survival of differentiated alveolar cells, and a significant delay in postlactational remodeling of the mammary gland (37–40). The same developmental defects were observed in mice that express hyperactive variants of their downstream signal transducers, in particular Jak2, Stat5, PI3K (p110), and Akt1 (14–20, 41–43), as well as females with a mammary-specific deletion of the *Pten* tumor suppressor gene (44). Our previous observations (15, 23) and the collective findings described in this report demonstrate that active Stat5 increases the expression of Akt1 as well as the PI3K (i.e., p85α and p110α). Therefore, a sustained activation of Stat5 greatly augments signaling through the PI3K/Akt pathway, and the model illustrated in Fig. 10 might explain, on a mechanistic level, why Stat5 is an important survival factor for mammary epithelial cells. It has been reported that lack of Akt1 alone or in combination with reduced expression of Akt2 resulted in lower levels of Stat5 activation (34, 45), giving rise to the notion that Akt1 acts upstream of the tyrosine-phosphorylated Stat5, possibly through an Akt-induced increase in autocrine PRL signaling (46). Our study, however, shows that an extended survival of mammary epithelial cells in transgenic mice overexpressing Akt1 does not require a sustained activation of Stat5. Moreover, we can demonstrate using Akt1 conditional knockout mice that this serine/threonine-protein kinase is essential for executing Stat5's function as a survival factor. Collectively, the analysis of both genetic models suggests that the PI3K/Akt1 pathway is a downstream effector of Jak2/Stat5 signaling, and this notion is supported by our findings that active Stat5 directly enhances the transcriptional activation of the PI3K and Akt1. By upregulating PI3K and its effector Akt1, the Jak2/Stat5 signaling cascade might set the stage for a more effective activation of the PI3K through additional signals from cytokine receptors and receptor tyrosine kinases (RTKs) (Fig. 10). Additionally, Stat5 can bind to the p85 regulatory subunit of PI3K via the Gab2 scaffolding adapter and thereby augment downstream signaling (22). Regardless of the various signaling modes that are capable of inducing PI3K activation, the canonical function of Stat5 as a tran-

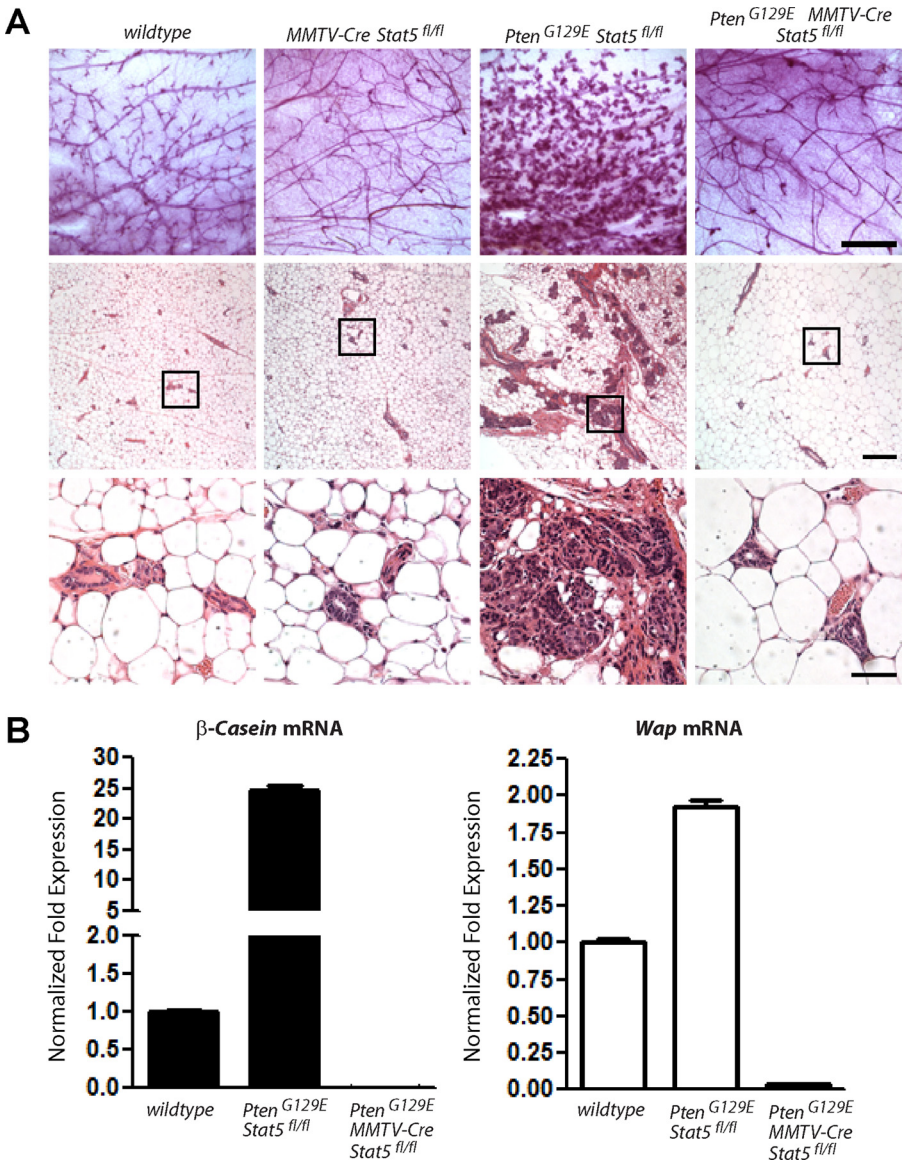

FIG 9 Stat5 deficiency prevents the formation of preneoplastic lesions and genesis of mammary cancer in females that carry the *Pten*^{G129E} allele. (A) Carmine-stained mammary gland whole mounts (top) and H&E-stained histologic sections (middle and bottom) of mammary glands from wild-type females and Stat5 mammary-specific knockout mice (*MMTV-Cre Stat5*$^{fl/fl}$) in the absence or presence of the mutant *Pten*^{G129E} allele. Only *Pten*^{G129E} *Stat5*$^{fl/fl}$ control mice that expressed mutant PTEN in the presence of wild-type Stat5 developed alveolar hyperplasia. The bottom panel shows a closeup of the areas marked in the middle panel. Bars, 1 mm (top), 250 μm (middle), and 50 μm (bottom). (B) Quantitative real-time RT-PCR to assess the expression of β-*casein* and *Wap* mRNA in mammary tissues from nonpregnant wild-type as well as *Pten*^{G129E} mutant females that expressed endogenous Stat5 or lacked expression of all four *Stat5a* and *Stat5b* alleles in the mammary epithelium (*Pten*^{G129E} *Stat5*$^{fl/fl}$ and *Pten*^{G129E} *MMTV-Cre Stat5*$^{fl/fl}$, respectively).

scription factor for both *Pik3r1* and *Pik3ca* encoding p85α and p110α is a key requirement for optimal Akt1 signaling to facilitate the survival of functionally differentiated mammary epithelial cells until the onset of involution.

Within a short period after the cessation of lactation, the mammary gland undergoes a dramatic remodeling process, which is preceded by a series of molecular events that involve the down-regulation of survival factors and the activation of proapoptotic signals. The initiation of apoptosis of alveolar cells is induced by milk stasis and requires a swift dephosphorylation of Stat5 and concurrent activation of Stat3. Similar to a gain of function of Stat5, deficiency in Stat3 causes a delayed remodeling of the alveolar compartment (47, 48). Based on their observation that the involution process is associated with a reduced expression of p85α and Stat3-mediated upregulation of p50α and p55α, Abell and coworkers (33) proposed that the initiation of programmed cell death is controlled by a switch in the expression of the regulatory subunits of the PI3K. Besides the previously reported decrease in the expression of p85α, we also observed a sharp decline in the levels of the p110α catalytic PI3K subunit during normal involution. We demonstrated in cell culture and *in vivo* that, in addition to the levels of Akt1, expression of p110α and p85α is controlled by active Stat5. This may now provide the underlying mechanism by which active Stat5 is able to override the proposed proapoptotic

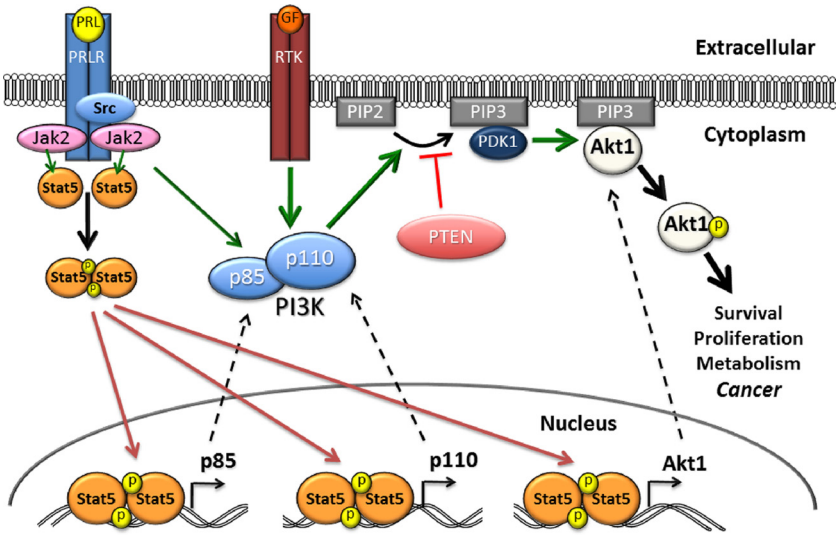

FIG 10 The PI3K/Akt1 pathway is a downstream effector of Jak2/Stat5 signaling in luminal mammary epithelial cells.

function of Stat3 and p50α/p55α, thereby promoting a prolonged survival of mammary epithelial cells. Collectively, the findings of this study and our previous report (15) suggest that a normal involution process of the mammary gland is primarily driven by a reduced expression of p85α and the p110α regulatory subunit of the PI3K in response to the loss of Stat5 activation. This notion is supported by the observation that the gain of function of p110α is sufficient to delay mammary gland remodeling (42). In addition to the downregulation of the p85α/p110α during normal involution, it is possible that a rapid inactivation of the PI3K is augmented through the Stat3-mediated switch in the expression of the shorter regulatory subunits. However, it still needs to be verified using a suitable genetic model that a deregulated expression of p50α and p55α may cause any alterations in the activity of p110α and associated phenotypic changes in the mammary gland during alveologenesis and postlactational remodeling.

Studies on several genetically engineered mouse models that overexpress Stat5 demonstrated that a gain of function of this signal transducer mediates evasion from apoptosis and self-sufficiency in growth signals, which are two hallmarks for carcinogenesis (49). Nuclear Stat5 was observed in a significant subset of human breast cancers (50, 51), and we have demonstrated recently that the Jak2-mediated activation of Stat5 is a prerequisite for mammary tumor formation in genetic models that overexpress ErbB2 and PRL (52, 53). Based on the long latency of mammary carcinogenesis and the absence of metastatic disease in transgenic mice overexpressing Stat5, it is evident that this transcription factor by itself has only very weak oncogenic properties. Following our observation that Jak2/Stat5 signaling can enhance the PI3K/Akt1 pathway, we experimentally tested the idea that Stat5 can synergistically promote preneoplastic changes and tumor onset in a mouse model for Cowden syndrome (CS). Like human CS patients, these mice carry one mutant allele of the *Pten* tumor suppressor gene, which is known to negatively regulate the PI3K/Akt pathway (27). The results presented in this study showed that overexpression of hyperactive Stat5 accelerated the development of premalignant changes (i.e., mostly alveolar hyperplasia) in the mammary glands of PTEN mutant females, and consequently, these mice exhibited a higher incidence of mammary cancer formation. Stat5 deficiency, on the other hand, was sufficient to completely prevent the genesis of premalignant lesions and PTEN-associated mammary carcinogenesis. Moreover, downregulation of Stat5 in established mammary tumors led to a reduced proliferation of cancer cells. Collectively, these observations may suggest that targeting the Jak2/Stat5 pathway might be a suitable strategy to prevent disease onset in CS patients, and treatment with clinically available JAK inhibitors might be beneficial to breast cancer patients with Stat5-positive tumors.

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