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SHORT REPORT

Scribble is required for pregnancy-induced alveologenesis in the adult mammary gland

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ABSTRACT

The cell polarity protein scribble (SCRIB) is a crucial regulator of polarization, cell migration and tumorigenesis. Whereas SCRIB is known to regulate early stages of mouse mammary gland development, its function in the adult gland is not known. Using an inducible RNA interference (RNAi) mouse model for downregulating SCRIB expression, we report an unexpected role for SCRIB as a positive regulator of cell proliferation during pregnancy-associated mammary alveologenesis. SCRIB was required in the epithelial cell compartment of the mammary gland. Lack of SCRIB attenuated prolactin-induced activation of the JAK2–STAT5 signaling pathway. In addition, loss of SCRIB resulted in the downregulation of prolactin receptor (PRLR) at cell surface and its accumulation in intracellular structures that express markers of the Golgi complex and the recycling endosome. Unlike its role in virgin gland as a negative regulator cell proliferation, SCRIB is a positive regulator of mammary epithelial cell proliferation during pregnancy.

KEY WORDS: Scribble, Mammary alveologenesis, Proliferation

INTRODUCTION

Scribble (SCRIB) is a scaffolding protein that contains 16 N-terminal leucine-rich repeats and four PSD95, Disc Large, ZO-1 (PDZ) domains. In *Drosophila,* SCRIB regulates apical-basal polarity and morphogenesis in epithelial cells (Bilder and Perrimon, 2000; Legouis et al., 2003), and spindle polarity in dividing neuroblasts (Albertson and Doe, 2003). In mammals, SCRIB regulates apical-basal polarity in epithelial cells (Qin et al., 2005; Godde et al., 2014; Pearson et al., 2011), front-back polarity in migrating astrocytes, T cells, fibroblasts and epithelial cells (Dow et al., 2007; Ludford-Menting et al., 2005; Nola et al., 2008; Osmani et al., 2006) and planar cell polarity of the stereociliary bundle in the inner ear cochlea (Montcouquiol et al., 2003).

Loss of SCRIB in mammals leads to perinatal lethality due to severe neural tube closure defects (Murdoch et al., 2003). Conditional loss studies have identified SCRIB as a regulator of biology in both lung and corneal epithelial cells (Yates et al., 2013; Yamben et al., 2013). In the mammary gland, loss of SCRIB activates RAS–MAPK signaling, and induces multilayering of the luminal epithelium and hyperbranching of ductal structures (Godde et al., 2014).

Apart from in early development, during pregnancy and in response to prolactin (PRL) and progesterone mammary epithelial cells undergo a large increase in cell number to develop lobuloalveolar structures that are used for production of milk (Oakes et al., 2006; Macias and Hinck, 2012). Luminal epithelial cells in these structures have well-established apical-basal polarity to facilitate vectoral secretion of milk into the lumen and mature tight junctions that create a permeability barrier to hold milk within the luminal space (Barcellos-Hoff et al., 1989; Nguyen and Neville, 1998). It is not known whether polarity proteins, such as SCRIB, have a role to play during alveologenesis. Conditional knockout approaches, where the SCRIB gene is inactivated during early stages of mammary gland development, are not well suited for investigating adult tissues.

To enable controlled inactivation of SCRIB in adult tissues, we have developed an inducible RNA interference (RNAi) mouse model to knockdown expression of SCRIB in adult mice. Using this model, we knockdown SCRIB expression in 12-week-old mice and report an unexpected role for SCRIB as a positive regulator of cell proliferation during alveologenesis and a regulator of prolactin receptor (PRLR) trafficking to the cell surface.

RESULTS AND DISCUSSION

Generation and characterization of an inducible knockdown mouse model for SCRIB

Mice expressing an inducible short hairpin RNA (shRNA) targeting SCRIB (ishSCRIB) (Fig. 1A; Fig. S1A) were generated by subcloning the SCRIB shRNA into a vector that contained a minimal tetracycline-responsive promoter to drive expression of the shRNA and green fluorescent protein (EGFP) (Fig. 1A, Fig. S1A) and that was targeted to the collagen A1 (ColA1) locus in mouse chromosome 11 in KH2 mouse embryonic stem cells (ESCs) (Fig. S1A). KH2 ESCs, derived from a C57BL/6-129/sv hybrid genetic background, contain a reverse tetracycline transactivator (rtTA) knocked-in to the ubiquitously expressed Rosa26 locus (Fig. 1A, Fig. S1A). Adult (12-week-old) transgenic mice were treated with doxycycline (dox) for 8 weeks and mammary glands were isolated. Immunoblots of lysates revealed a dox-induced expression of EGFP and a decrease in SCRIB levels (Fig. 1B). Dox treatment resulted in green tails (Fig. S1C) and loss of SCRIB expression in the mammary epithelial cells (Fig. 1C). In the control mammary gland, SCRIB expression was observed primarily in the E-cadherin-positive luminal epithelial cell population, but not in the cytokeratin (KRT) 14- or KRT5-positive basal epithelial cells of ducts and terminal end buds (Fig. S1D,E). SCRIB expression was undetectable in the luminal epithelial cells of dox-treated

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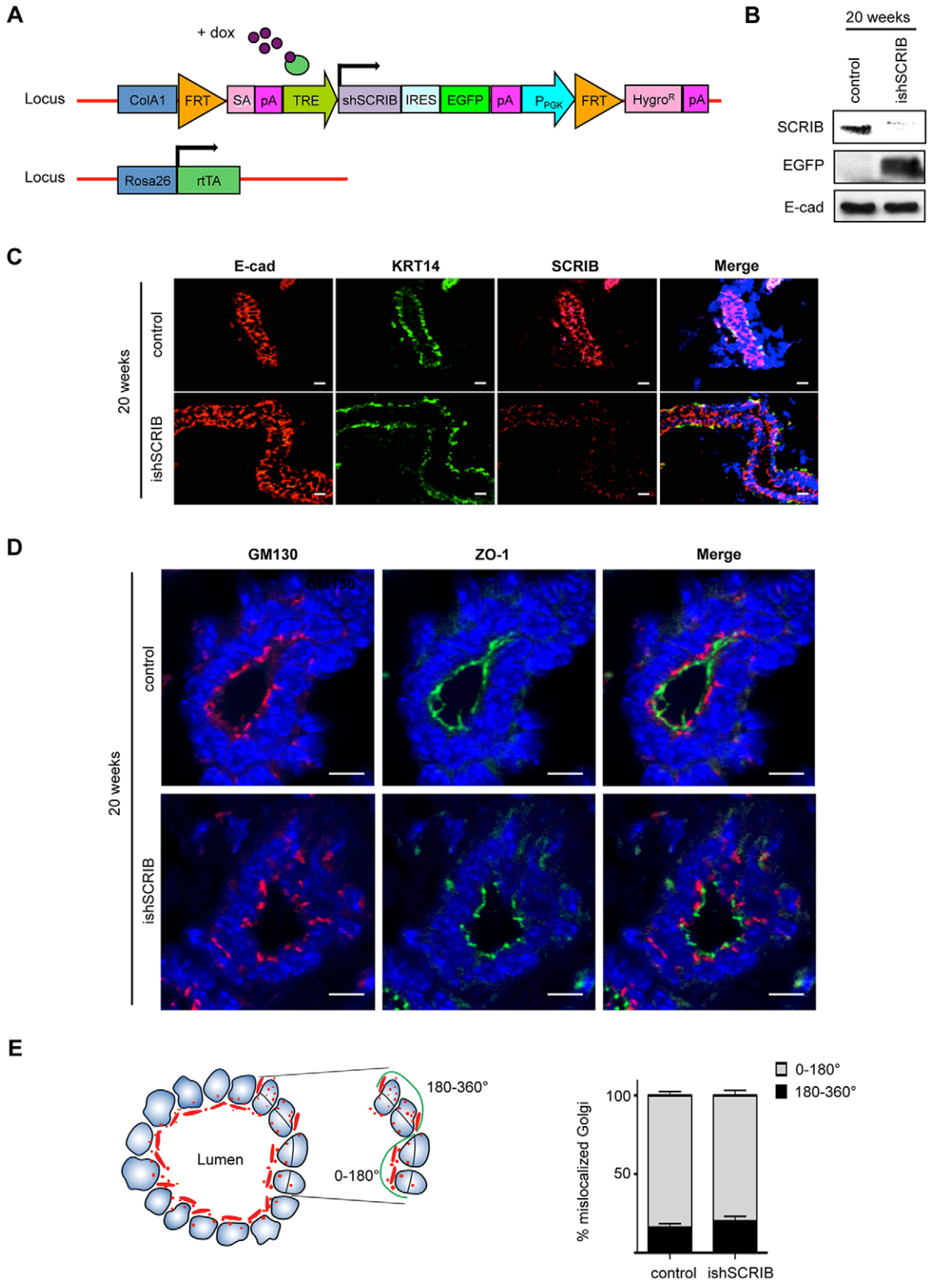

Fig. 1. Generation and characterization of an inducible knockdown mouse model for SCRIB. (A) Schematic of the collagen 1 (ColA1) and Rosa26 locus in KH2 ESCs for generation of ishSCRIB mouse. TRE, tetracycline response element. (B) Cell lysates from mammary glands isolated from control and ishSCRIB mice treated with dox for 8 weeks analyzed for expression of SCRIB, EGFP and E-cadherin (E-Cad). (C) Expression of E-cad, KRT14 and SCRIB analyzed by immunohistochemistry in mammary glands isolated from control and ishSCRIB mice treated with dox for 8 weeks. Scale bars: 50 µm. (D) Immunohistochemical staining of GM130 (red) and ZO-1 (green) in mammary glands isolated from control and ishSCRIB mice treated with dox for 8 weeks. Scale bars: 30 µm. (E) Schematic diagram of the method used to quantify changes in Golgi localization (see Materials and Methods for details). The graph on the right represents percentage of mislocalized Golgi (mean±s.e.m.; n=50 in five ducts). P>0.05 by Student's t-test.

mice, whereas, the expression patterns of E-cadherin and KRT14 remained unaffected (Fig. 1C). Moreover, loss of SCRIB did not alter the expression of the epithelial or the mesenchymal markers (Fig. S1F), or alter localization of the apical polarity markers ZO-1 (also known as TJP1) and GM130 (also known as GOLGA2) (Fig. 1D,E; Fig. S1G). Thus, we have generated an inducible knockdown mouse model of SCRIB and demonstrated that SCRIB is dispensable for maintaining differentiation and polarization of adult mammary epithelial cells.

SCRIB is required for epithelial expansion during pregnancy-induced alveologenesis

To determine whether SCRIB plays a role during pregnancy-induced alveologenesis, 12-week-old virgin mice were treated with dox and mated at 20 weeks of age. Their mammary glands were subjected to whole-mount analysis during early (day 7.5), mid (day 13.5) and late (day 17.5) pregnancy (Fig. 2A). Dox-treated mice displayed an unexpected decrease in the number of alveolar buds and overall epithelial content during early, mid and late pregnancy as compared to untreated mice (Fig. 2B). However, ishSCRIB mice did not show defects in litter size with litter numbers ranging from 7–11 in untreated mice and 6–10 in dox-treated mice (n=10). ishSCRIB mice were able to lactate, as monitored by lipid droplets in mammary glands collected from day 1 of lactation, and observation of milk spots in 1-day-old pups (Fig. S2A,B). The ability of mammary glands to recover from early defects is consistent with several studies that show phenotypic recovery (Jones and Stern, 1999; Mailleux et al., 2007). The recovery in alveologenesis might be due to the restoration of SCRIB levels by late pregnancy (Fig. S2C).

Epithelia in dox-treated ishSCRIB glands had a significantly lower rate of cell proliferation compared to control mice during early and mid pregnancy (Fig. 2C). However, neither the apical-basal polarity nor the bilayered epithelial organization surrounding a central lumen was affected by SCRIB loss (Fig. S2D,E). Thus, SCRIB is required for pregnancy-induced alveolar proliferation and not epithelial differentiation or polarization.

Previous studies using an MMTV-Cre-driven knockout mouse have demonstrated that loss of SCRIB induces hyperproliferation and disrupted ductal morphogenesis due to elevated levels of MAPK signaling (Godde et al., 2014). Consistent with that study, we observed increased ERK1 and ERK2 (ERK1/2, also known as MAPK3 and MAPK1, respectively) phosphorylation upon SCRIB loss in pregnant glands (Fig. S2F), suggesting that the alveologenesis phenotype reported here might not be related to SCRIB-mediated regulation of MAPK signaling.

We validated the SCRIB-induced alveolar defect using a non-tumorigenic mouse luminal epithelial cell line, EpH4, which undergoes alveolar morphogenesis in three-dimensional (3D) culture. EpH4 cells respond to lactogenic hormones (insulin, hydrocortisone and prolactin) by forming alveoli-like structures that secrete milk proteins (Barcellos-Hoff et al., 1989). Knockdown of SCRIB did not affect the epithelial differentiation state of EpH4 cells (Fig. S3A–C), but significantly inhibited formation of alveoli-like structures in response to lactogenic hormones (Fig. S3D,E), with a 7.5-fold decrease in β-casein mRNA levels compared to control cells (Fig. S3F). The defect in proliferation was specific to differentiation conditions and not observed in monolayers under serum stimulation (Fig. S3G). The data suggests that SCRIB is required for both alveolar morphogenesis and differentiation of EpH4 acini.

A cell-autonomous role for SCRIB in the epithelial compartment

Given that the Rosa26 promoter is ubiquitously expressed, we investigated whether the alveologenesis defect was autonomous to the epithelial compartment or involved the stroma. Control and ishSCRIB cells were isolated from 10-week-old mice and injected into a cleared fat pad of a 3-week-old ishSCRIB or control mouse, respectively. Mice were then treated with dox, mated six weeks following transplantation, and mammary glands were isolated at early pregnancy (day 7.5). ishSCRIB cells injected into control mouse mammary fat pads displayed fewer alveolar buds compared to the control cells injected into the ishSCRIB knockdown mammary fat pads (Fig. 2D). Thus the alveologenesis defect in ishSCRIB mice was cell autonomous to the epithelial cells.

SCRIB knockdown impairs PRL signaling *in vivo*

Mice containing deletions in PRL, its receptor, PRLR, or the downstream signaling pathway effectors JAK2 or STAT5 display impaired lobuloalveolar development in the mouse mammary gland (Ormandy et al., 1997; Gallego et al., 2001; Horseman et al., 1997; Miyoshi et al., 2001; Shillingford et al., 2002; Liu et al., 1997). These studies demonstrate that the PRL–PRLR–JAK2–STAT5 signaling axis is a dominant regulator of alveologenesis. We found that levels of phosphorylated STAT5 were decreased during early pregnancy (day 7.5) (Fig. 2E) in mammary glands from ishSCRIB mice compared to the control. In addition, we did not observe any changes in the total levels of PRLR in ishSCRIB mice. This suggests that decreased alveologenesis in ishSCRIB glands is likely due to impaired prolactin signaling.

Loss of SCRIB attenuates PRL-induced JAK2–STAT5 signaling

To gain mechanistic insight, we decided to use mouse and human cell culture models. Mouse EpH4 cells do not respond to PRL stimulation in monolayer cultures, but respond when held in suspension. Consistent with the *in vivo* data, EpH4 cells expressing shRNA against SCRIB (shSCRIB) showed a reduction in STAT5 phosphorylation upon PRL stimulation in suspension (Fig. S3H). Unlike EpH4 cells, T-47D, a human breast-cancer-derived cell line, is commonly used for investigating PRL-induced signaling and biology in monolayer conditions (Johnson et al., 2010; Gallego et al., 2001). T-47D cells stably expressing shSCRIB displayed a marked decrease in STAT5 phosphorylation starting at 15 min of PRL stimulation compared to control cells (Fig. 3A). To rule out off-target effects of the shRNA, we rescued shSCRIB cells lines with full-length, shRNA-resistant, SCRIB cDNA. Re-expression of SCRIB rescued STAT5 phosphorylation to control levels (Fig. 3B). Upon binding to PRL, PRLR undergoes dimerization and induces tyrosine phosphorylation of JAK2, PRLR and STAT5 (Bole-Feysot et al., 1998). T-47D cells expressing shSCRIB showed a two-fold decrease in prolactin-induced tyrosine phosphorylation of PRLR and JAK2 (Fig. 3C–E). SCRIB loss neither affected the half-life of PRLR, as determined by cycloheximide assay (Fig. 3F), nor the formation of the PRLR–JAK2 complex, as determined by co-immunoprecipitation analysis (Fig. S4A), demonstrating that SCRIB neither affects the stability of PRLR nor functions as a scaffolding molecule to assemble the signaling complex.

SCRIB regulates steady state levels of PRLR at the cell surface

We next investigated whether SCRIB affects localization of PRLR. In T-47D shSCRIB cells, there was less PRLR receptor at the

Journal of Cell Science (2016) 129, 2307-2315 doi:10.1242/jcs.185413

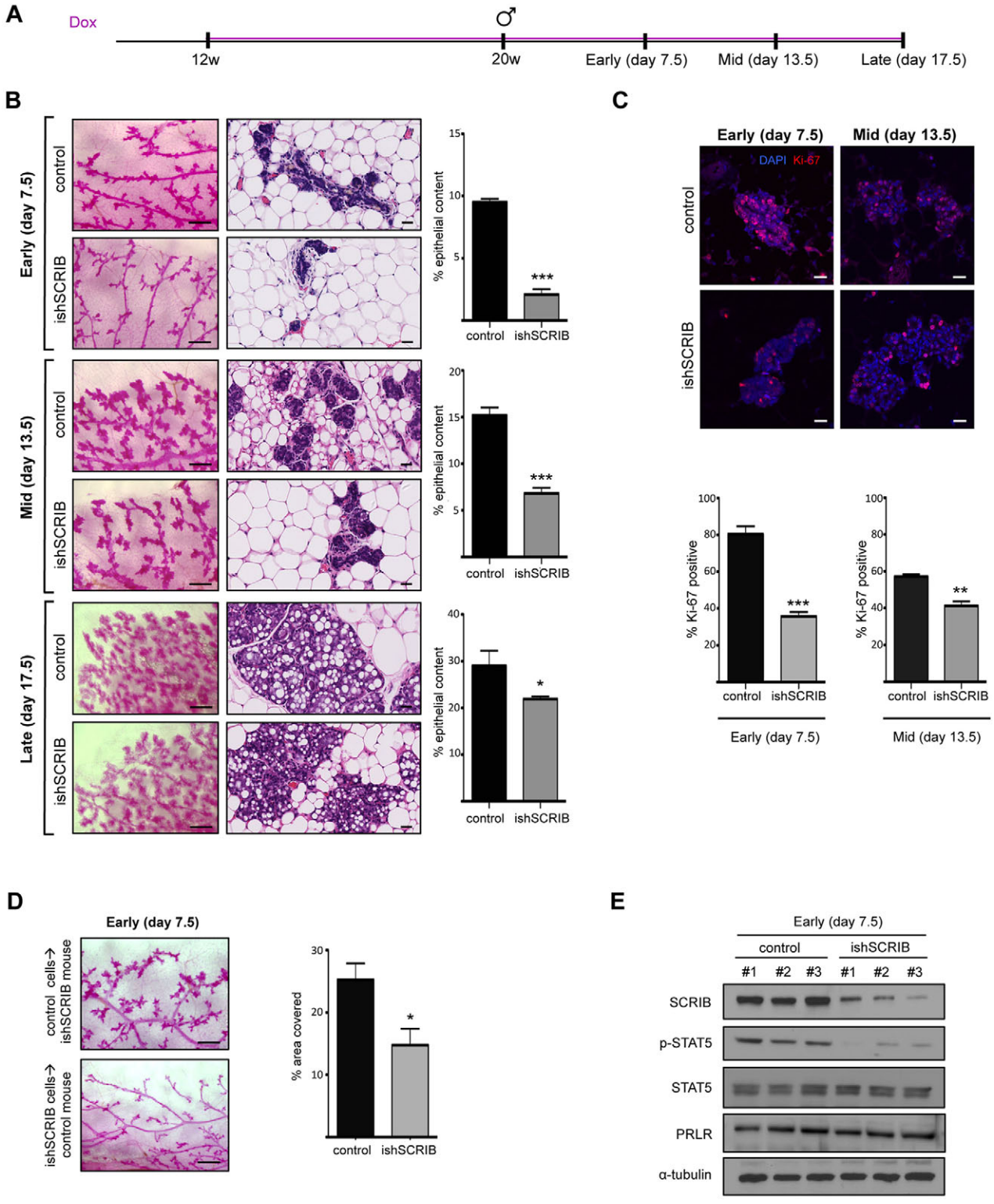

Fig. 2. SCRIB is required for expansion of epithelial cells during pregnancy-induced alveologenesis. (A) Schematic of experimental timeline. (B) Whole-mount analysis (left) and a representative hematoxylin and eosin (H&E) image (middle) of mammary glands collected from control and ishSCRIB mice with or without (control) dox treatment at the indicated time points. Scale bars: 50 µm (left-hand panels); 20 µm (middle panels). The graphs (right) show quantification of epithelial content calculated from H&E sections of three pairs of mice for each time point (mean±s.e.m.; n=3). ***P<0.0001, *P<0.05 by Student's t-test. (C) Ki-67 staining of alveolar structures from control and ishSCRIB mice with or without (control) dox treatment during early (day 7.5) and mid (day 13.5) pregnancy. Scale bars: 20 µm. Quantification of Ki-67-positive nuclei (below) in three pairs of glands (mean±s.e.m.). ***P<0.0001. **P<0.001 by Student's t-test. (D) Whole-mount analysis of mammary glands isolated from mice during early (day 7.5) pregnancy following cleared fat pad transplantation. Scale bars: 50 µm. The graph (right) shows the percentage of area covered (mean±s.e.m.; n=3). *P<0.05 by Student's t-test. (E) Cell lysates from three different mammary glands (#1, #2, #3) isolated from control and ishSCRIB mice during early pregnancy (day 7.5) and analyzed for expression of SCRIB, phosphorylated (p)-STAT5 and PRLR.

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Journal of Cell Science (2016) 129, 2307-2315 doi:10.1242/jcs.185413

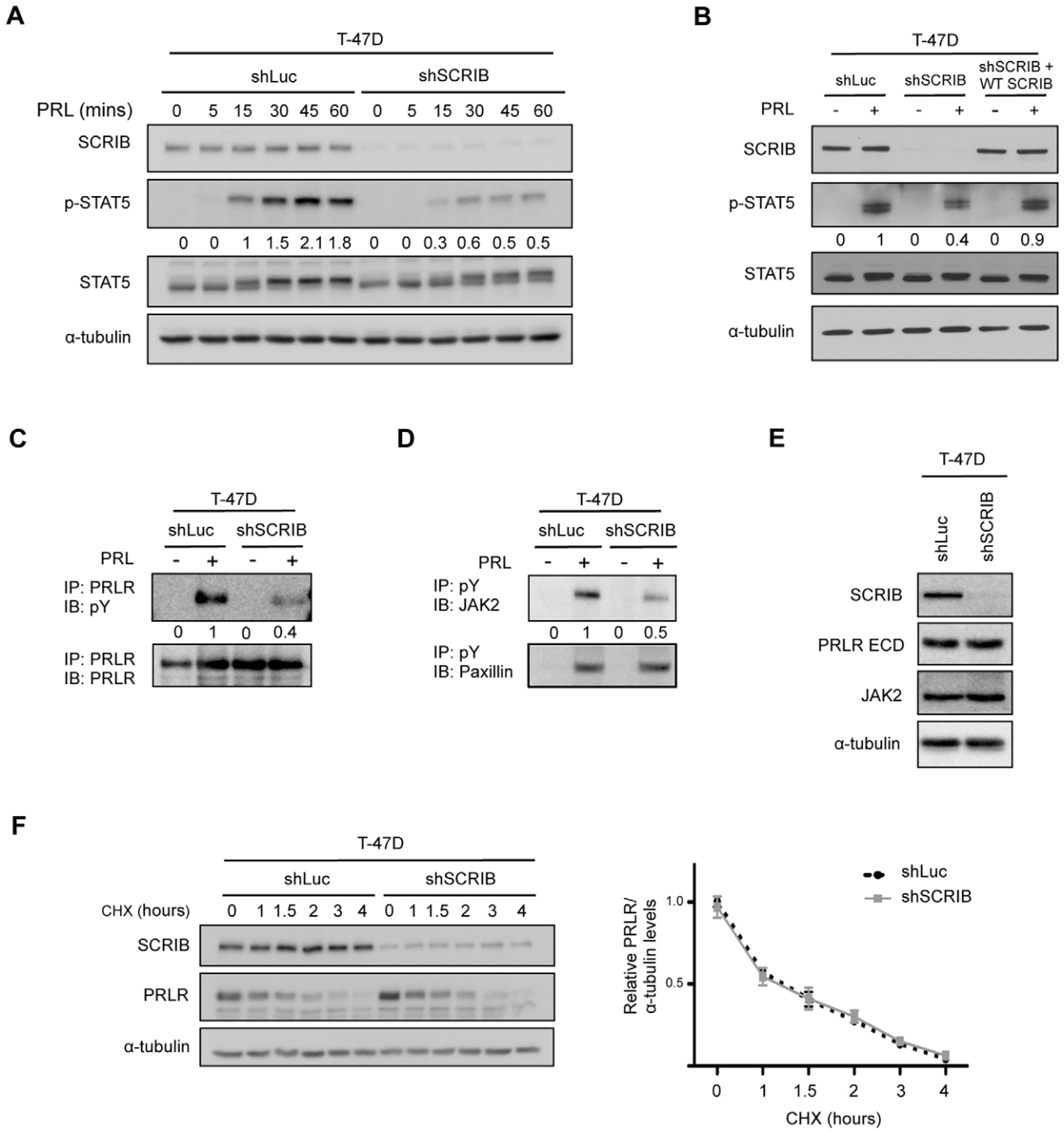

Fig. 3. Loss of SCRIB attenuates prolactin-induced JAK2–STAT5 signaling. (A) T-47D shLuc and shSCRIB cells were serum starved overnight and stimulated with prolactin (20 ng ml^{-1}) for the indicated times. Protein lysates were analyzed for phosphorylation of STAT5 (p-STAT5). (B) T-47D shLuc, shSCRIB and shSCRIB cells expressing shRNA-resistant SCRIB cDNA were serum starved and stimulated with prolactin (20 ng ml^{-1}) for 15 min. Protein lysates were analyzed for phosphorylation of STAT5. (C) T-47D shLuc and shSCRIB cells were serum starved and stimulated with prolactin (20 ng ml^{-1}) for 15 min. PRLR was immunoprecipitated (IP) and immunoblotted (IB) with anti-phosphotyrosine (pY) and PRLR antibodies. (D) Stimulation of T-47D shLuc and shSCRIB cells were performed as in C. Tyrosine-phosphorylated proteins were immunoprecipitated using phosphotyrosine agarose beads and immunoblotted with anti-JAK2 and anti-paxillin (control) antibodies. (E) Protein from T-47D shLuc and shSCRIB cells were analyzed for total levels of PRLR and JAK2. (F) T-47D shLuc and shSCRIB cells were serum starved overnight and treated with 50 µgml^{-1} cycloheximide (CHX) for the indicated times. Cell lysates were analyzed for PRLR. Receptor levels were normalized to loading control (α-tubulin) and these normalized values were used to determine fold change in levels compared to the receptor levels in shLuc cells at time zero. Results represent mean±s.e.m. (n=3). P>0.05 by Student's t-test.

membrane but more in an intracellular compartment compared to the shLuc control cells (Fig. 4A). In spite of our efforts, we were unable to determine PRLR localization by immunohistochemistry in the mouse mammary gland (see Fig. S4B for details). In contrast to PRLR, surface levels of E-cadherin were not changed in T-47D shSCRIB cells (Fig. 4A). Further analysis showed that in control cells, PRLR colocalized with the cell adhesion molecule E-cadherin at the cell membrane (Fig. 4A; Fig. S4C), in contrast, shSCRIB

Journal of Cell Science (2016) 129, 2307-2315 doi:10.1242/jcs.185413

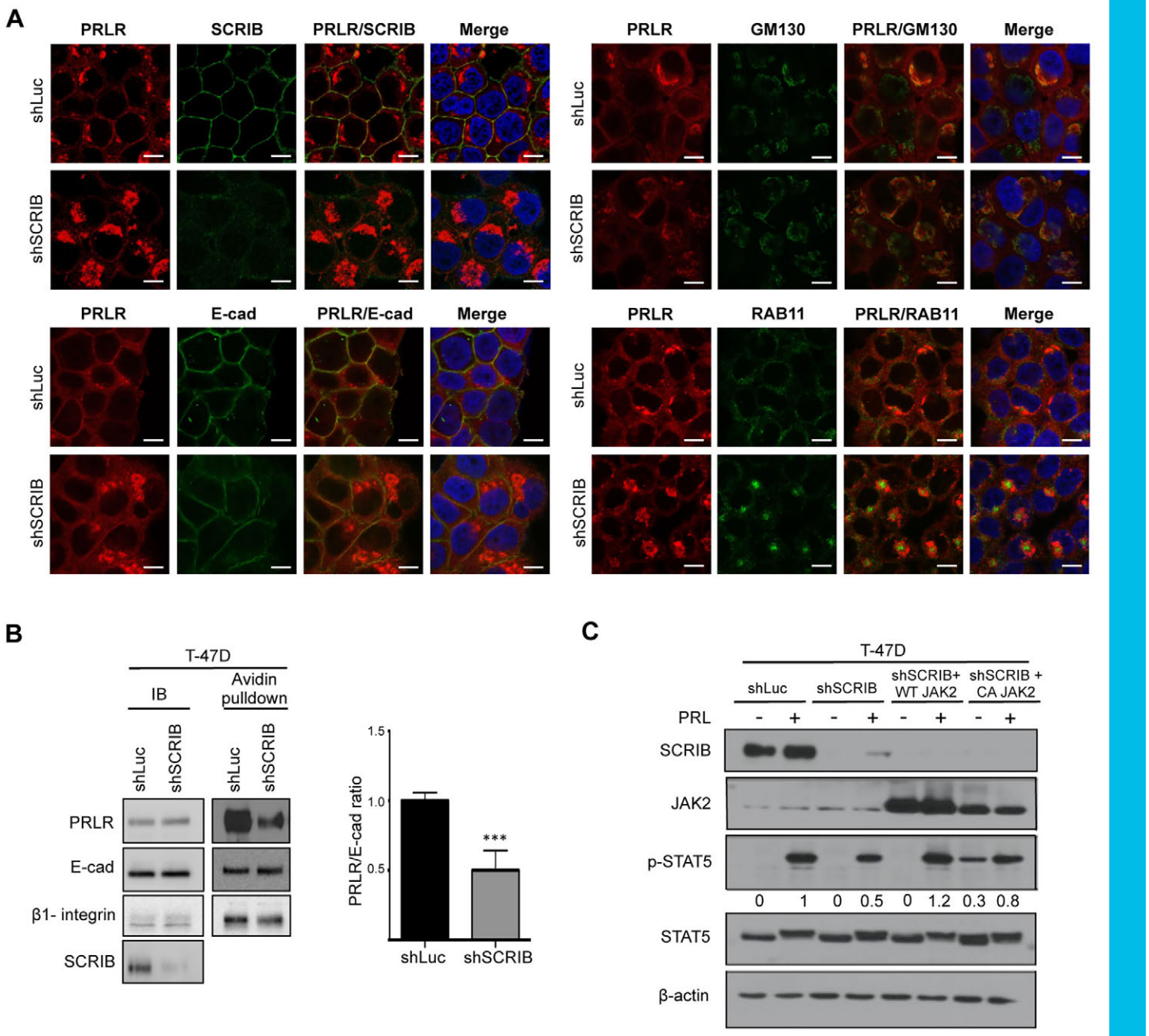

Fig. 4. SCRIB regulates the steady-state levels of PRLR at the cell surface. (A) T-47D shLuc and shSCRIB cells were immunostained for DAPI (blue), PRLR (red) and SCRIB, E-cad, GM130 or RAB11 (green). Scale bars: 10 µm. (B) T-47D shLuc and shSCRIB cells were surface labeled with biotin for 10 min. Cells were lysed and surface proteins were pulled down using avidin beads (Avidin pulldown). Changes in surface levels of PRLR, E-cad and β1-integrin were analyzed by immunoblotting (IB). The graph on the right represents quantification of surface levels of PRLR normalized to E-cad. Results represent mean±s.e.m. (n=4). ***P<0.0001 by Student's t-test. (C) T-47D shLuc, shSCRIB and shSCRIB cells stably overexpressing JAK2 (WT JAK2) and a constitutively active JAK2 (CA JAK2) were stimulated with prolactin (20 ngml^{-1}) for 15 min. Cell lysates were analyzed for changes in STAT5 phosphorylation (p-STAT5).

cells, PRLR partially colocalized with the cis-Golgi protein GM130 and the recycling endosome associated protein RAB11 (Fig. 4A, Fig. S4C). No colocalization was observed with the endoplasmic reticulum protein calnexin (CANX) in either shLuc or shSCRIB cells (Fig. S4D).

To quantify the differences in surface PRLR levels, cells were labeled with membrane-impermeable biotin and labeled proteins were affinity purified using strepavidin–Sepharose beads. T47D cells lacking SCRIB had a more than two-fold decrease in the levels of PRLR at the cell surface (Fig. 4B) with no detectable effect on surface levels of E-cadherin. In addition, EpH4 cells lacking SCRIB also showed a decrease in PRLR at cell surface (Fig. S4E), demonstrating that regulation of cell surface levels of PRLR is observed in both human and mouse cells. Loss of β1 integrin impairs alveologenesis and lactation (Naylor et al., 2005) and activation of RAC1 downstream of integrins is required for PRL-induced JAK2–STAT5 activation in mammary epithelial cells (Akhtar and Streuli, 2006). However, surface levels of β1 integrin did not decrease in shSCRIB cells (Fig. 4B), demonstrating that the SCRIB effects are specific of modulation of PRLR surface levels among the regulators of PRL signaling.

Evidence for the role of SCRIB in trafficking was first demonstrated in neuroendocrine cells, where loss of SCRIB impaired Ca^{2+}-dependent exocytosis (Audebert et al., 2004). In *Drosophila*, SCRIB modulates retromer-dependent sorting events that can return internalized cargo to the cell surface (de Vreede et al., 2014). In mammals, SCRIB is required for ligand-stimulation-induced recycling of thyroid stimulating hormone receptor, N-methyl-D-asparate receptor and integrin α5 back to the plasma membrane (Lahuna et al., 2005; Piguel et al., 2014; Michaelis et al., 2013). Our results demonstrate a role for SCRIB in unstimulated cells, thus identifying a new role for SCRIB as regulator of steady-state surface levels of a membrane receptor.

SCRIB regulates PRL signaling upstream of JAK2

To determine whether the defect in PRLR surface levels can be rescued by activation of downstream molecules, we expressed either full-length wild-type JAK2 (WT JAK2), or a constitutively active JAK2 construct (V617F, CA-JAK2) (Fig. 4C) in shSCRIB cells. Expression of WT JAK2 restored the prolactin-induced increase in STAT5 phosphorylation levels. As expected, expression of CA-JAK2 induced phosphorylation of STAT5 in the absence of PRL stimulation, which was not enhanced upon PRL stimulation. Thus, the defect in PRLR signaling observed in shSCRIB cells can be rescued by increasing the level of JAK2 to compensate for decrease in PRLR at the cell surface. This demonstrated that the PRL signaling defect in shSCRIB cells is specific to the limiting levels of activation of JAK2 activation.

In summary, we identified an unexpected role for the polarity protein SCRIB as a positive regulator of cell proliferation during early stages of alveologenesis in the adult mammary gland. Mechanistically, SCRIB was required for PRL-induced JAK–STAT5 signaling by regulating cell surface levels of PRLR. In SCRIB-knockdown cells, PRLR was partially colocalized with the Golgi and the recycling-endosome-associated protein RAB11. Therefore, SCRIB regulates steady-state surface levels of PRLR and plays an important role in regulating cell proliferation during pregnancy-induced alveologenesis.

MATERIALS AND METHODS

Reagents and antibodies

Antibodies against the following proteins were purchased from commercial sources: SCRIB (C-20), PRLR (H-300) and β1-integrin (M-106) (Santa Cruz Biotechnology); β-actin, α-tubulin (Sigma); EGFP (Zymed); E-cadherin, STAT5, GM130, RAB11 (BD Bioscience); KRT5, KRT14 (Covance); KRT18 (TROMA-1, Developmental Studies Hybridoma Bank); phosphorylated (p)-STAT5, ZO-1, PRLR ECD (Life Technologies); Ki-67, calnexin (Thermo Scientific); p-ERK1/2, ERK1/2, RAB7, EEA1, JAK2, paxillin, cleaved caspase 3 (Cell Signaling); AP1 and AP2, LAMP1 (Abcam); p-Tyrosine (4G10, Millipore). Details of catalog numbers and dilutions can be found in Table S1. Alexa-Fluor- and horseradish peroxidase (HRP)-conjugated secondary antibodies (Life Technologies) and secondary antibodies were used (GE Healthcare Life Science). Additional reagents, unless otherwise stated, were obtained from Sigma.

Plasmids

Control and SCRIB shRNA vectors used were previously described (Zhan et al., 2008; Aranda et al., 2006). The WT SCRIB rescue construct was generated by cloning out the 5′ and 3′ UTR region of the full-length SCRIB construct (Zhan et al., 2008) using the following PCR primers: forward, 5′-ATGATGTCTAGAATGCTCAAGTGCATCCCGCTGT-3′ and reverse, 5′ATGATGGAATTCCTAGGAGGGCACAGGGCCCAG-3′. The PCR product was subcloned into the pMSCV puro vector (Addgene) using the XhoI and EcoRI restriction sites. pMSCV WT JAK2 and V617F JAK2 constructs were a kind gift from Dwayne Barber (Department of Medical Biophysics, Princess Margaret Cancer Centre, Canada).

Generation of the ESC-derived inducible SCRIB RNAi mouse model

SCRIB shRNA (Zhan et al., 2008) was subcloned into the ColA1 targeting vector (cTGM) using the restriction enzymes EcoRI and XhoI. Mice were generated as previously described (Dow et al., 2012). All animal experiments were performed according to approved guidelines.

Cell culture

EpH4 cells were cultured in DMEM with F-12 (Life Technologies) supplemented with 2% fetal bovine serum (FBS; Life Technologies), gentamycin (5 mg ml^{-1}) and insulin (5 mg ml^{-1}). T-47D cells were cultured in Phenol-Red-free RPMI-1640 (Life Technologies) supplemented with 10% FBS and insulin (5 mg ml^{-1}). Stable cell lines were generated by retroviral infection (Debnath et al., 2003; Ory et al., 1996).

Immunobloting

Mouse mammary glands were lysed and analyzed as previously described (Zhan et al., 2008). Western blots were quantified using CareStream MI Software.

Golgi localization quantification

Mouse mammary tissue sections were stained with the Golgi marker GM130, and 0.4-µm *z*-stack images were acquired using a Nikon™ C2 plus si microscope using a 60× (NA 1.3) oil objective. A cell was divided along the apical-basal axis and this division was used to gauge the position of the GM130 staining; apical localization was taken as 0–180°, and basal or mislocalized as 180–360°. A minimum of five ducts were imaged through the *z*-plane spanning the entire tissue section and was used to determine the orientation of the Golgi. From each ductal structure, ten cells were quantified, starting at an arbitrary position and continued in direct succession.

Mammary fat pad transplantation

Single-cell mammary gland suspensions were generated from freshly isolated 4th and 9th inguinal glands of 10–12-week-old control and ishSCRIB mice by enzymatic digestion as previously described (Joshi et al., 2010). Cells were resuspended in Hanks buffer plus 2% FBS and 20% Matrigel at a concentration of 7500 cells $µl^{-1}$. 10 µl volumes were injected using a 10-µl 22-gauge Hamilton syringe into the inguinal glands of 3-week-old ishSCRIB and control mice that have been cleared of endogenous epithelium. All reagents for enzymatic digestion were purchased from StemCell™ Technologies.

EpH4 differentiation assay

50 µl matrigel was spread evenly on each well of a four-well chamber slide (BD Bioscience) and allowed to solidify for 30 min at 37°C. $2×10^4$ EpH4 cells were plated in 350-µl differentiation medium plus serum per chamber (DMEM/F12, 10% FBS, 1 µg ml^{-1} hydrocortisone, 5 µg ml^{-1} insulin, 3 µg ml^{-1} prolactin). At 24 h after plating, medium was changed to differentiation medium without serum. Medium was changed every day until day 6.

Quantification of number of cells per acinus

Acini at day 6 of culture were fixed in methanol at −20°C for 5 min and stained with 1 µg ml^{-1} DAPI (Life Technologies) with 3% BSA for 1 h at room temperature. *z*-stacks of 0.3 µm optical thickness were obtained using a Nikon™ C2 plus si microscope with a 20× (NA 0.4) objective, reconstituted using the Imaris software and manually counted.

Quantitative polymerase chain reaction

RNA was isolated from EpH4 cultures using Trizol (Life Technologies) and reverse transcribed as previously described (Zhan et al., 2008). β-casein primers were as follows: forward, 5′-CATATGCTCAGGCTCAAACCAT-CTCT-3′ and reverse, 5′-GTACTGCAGAAGGTCTTGGACAGAC-3′.

Immunofluorescence

shLuc and shSCRIB cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for

5 min. Cells were blocked with 3% BSA for 1 h and incubated in primary antibody overnight, and then incubated with fluorochrome-conjugated secondary antibody and 1 µg ml^{-1} DAPI (Life Technologies) for 1 h at room temperature. Cells stained with AP1/2, EEA1, RAB7 and LAMP1 were fixed with methanol and remainder of the procedure was performed as described above. Images were acquired using a Nikon™ C2 plus si imaging platform and images were processed and quantified using ImageJ.

Biotin labeling and strepavidin immunoprecipitation

T-47D cells were labeled with 400 µM EZ-link-sulfo-NHS-SS-Biotin (in PBS) for 30 min at 4°C. The reaction was quenched with 150 mM glycine. The cells were lysed in 1% Triton X-100 lysis buffer [50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 5 mM NaF, plus protease inhibitor and phosphoSTOP tablets (Roche)]. 500 µg of protein lysate was incubated with 20 µl of strepavidin–Sepharose high-performance beads and rotated for 3 h at 4°C. The immunoprecipitated proteins were further analyzed by SDS-PAGE and immunoblotting.

Statistical analysis

The specific statistical tests used are indicated in the figures alongside the *P* values.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.K.M. and L.B. conceived the study; L.B. performed the majority of the experiments and co-wrote the manuscript with S.K.M. M.B. performed Golgi localization studies. F.A. and T.K. assisted with surface protein analysis. M.F. and A.Z. we involved in generation of the inducible RNAi mice and S.L. shared his then unpublished technology to generate inducible RNAi mice.

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Supplementary information

Supplementary information available online at
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References

Akhtar, N. and Streuli, C. H. (2006). Rac1 links integrin-mediated adhesion to the control of lactational differentiation in mammary epithelia. *J. Cell. Biol.* **173**, 781-793.

Albertson, R. and Doe, C. Q. (2003). Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. *Nat. Cell Biol.* **5**, 166-170.

Aranda, V., Haire, T., Nolan, M. E., Calarco, J. P., Rosenberg, A. Z., Fawcett, J. P., Pawson, T. and Muthuswamy, S. K. (2006). Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. *Nat. Cell Biol.* **8**, 1235-1245.

Audebert, S., Navarro, C., Nourry, C., Chasserot-Golaz, S., Lécine, P., Bellaiche, Y., Dupont, J.-L., Premont, R. T., Sempere, C., Strub, J. M. et al. (2004). Mammalian Scribble forms a tight complex with the betaPIX exchange factor. *Curr. Biol.* **14**, 987-995.

Barcellos-Hoff, M. H., Aggeler, J., Ram, T. G. and Bissell, M. J. (1989). Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* **105**, 223-235.

Bilder, D. and Perrimon, N. (2000). Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**, 676-680.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N. and Kelly, P. A. (1998). Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr. Rev.* **19**, 225-268.

De Vreede, G., Schoenfeld, J. D., Windler, S. L., Morrison, H., Lu, H. and Bilder, D. (2014). The Scribble module regulates retromer-dependent endocytic trafficking during epithelial polarization. *Development* **141**, 2796-2802.

Debnath, J., Muthuswamy, S. K. and Brugge, J. S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* **30**, 256-268.

Dow, L. E., Kauffman, J. S., Caddy, J., Zarbalis, K., Peterson, A. S., Jane, S. M., Russell, S. M. and Humbert, P. O. (2007). The tumour-suppressor Scribble dictates cell polarity during directed epithelial migration: regulation of Rho GTPase recruitment to the leading edge. *Oncogene* **26**, 2272-2282.

Dow, L. E., Premsrirut, P. K., Zuber, J., Fellmann, C., McJunkin, K., Miething, C., Park, Y., Dickins, R. A., Hannon, G. J. and Lowe, S. W. (2012). A pipeline for the generation of shRNA transgenic mice. *Nat. Protoc.* **7**, 374-393.

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.

Godde, N. J., Sheridan, J. M., Smith, L. K., Pearson, H. B., Britt, K. L., Galea, R. C., Yates, L. L., Visvader, J. E. and Humbert, P. O. (2014). Scribble modulates the MAPK/Fra1 pathway to disrupt luminal and ductal integrity and suppress tumour formation in the mammary gland. *PLoS Genet.* **10**, e1004323.

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.

Johnson, K. J., Peck, A. R., Liu, C., Tran, T. H., Utama, F. E., Sjolund, A. B., Schaber, J. D., Witkiewicz, A. K. and Rui, H. (2010). PTP1B suppresses prolactin activation of Stat5 in breast cancer cells. *Am. J. Pathol.* **177**, 2971-2983.

Jones, F. E. and Stern, D. F. (1999). Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation. *Oncogene* **18**, 3481-3490.

Joshi, P. A., Jackson, H. W., Beristain, A. G., Di Grappa, M. A., Mote, P. A., Clarke, C. L., Stingl, J., Waterhouse, P. D. and Khokha, R. (2010). Progesterone induces adult mammary stem cell expansion. *Nature* **465**, 803-807.

Lahuna, O., Quellari, M., Achard, C., Nola, S., Méduri, G., Navarro, C., Vitale, N., Borg, J.-P. and Misrahi, M. (2005). Thyrotropin receptor trafficking relies on the hScrib-betaPIX-GIT1-ARF6 pathway. *EMBO J.* **24**, 1364-1374.

Legouis, R., Jaulin-Bastard, F., Schott, S., Navarro, C., Borg, J.-P. and Labouesse, M. (2003). Basolateral targeting by leucine-rich repeat domains in epithelial cells. *EMBO Rep.* **4**, 1096-1100.

Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A. and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes. Dev.* **11**, 179-186.

Ludford-Menting, M. J., Oliaro, J., Sacirbegovic, F., Cheah, E. T.-Y., Pedersen, N., Thomas, S. J., Pasam, A., Iazzolino, R., Dow, L. E., Waterhouse, N. J. et al. (2005). A network of PDZ-containing proteins regulates T cell polarity and morphology during migration and immunological synapse formation. *Immunity* **22**, 737-748.

Macias, H. and Hinck, L. (2012). Mammary gland development. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 533-557.

Mailleux, A. A., Overholtzer, M., Schmelzle, T., Bouillet, P., Strasser, A. and Brugge, J. S. (2007). BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. *Dev. Cell* **12**, 221-234.

Michaelis, U. R., Chavakis, E., Kruse, C., Jungblut, B., Kaluza, D., Wandzioch, K., Manavski, Y., Heide, H., Santoni, M.-J., Potente, M. et al. (2013). The polarity protein Scrib is essential for directed endothelial cell migration. *Circ. Res.* **112**, 924-934.

Miyoshi, K., Shillingford, J. M., Smith, G. H., Grimm, S. L., Wagner, K.-U., Oka, T., Rosen, J. M., Robinson, G. W. and Hennighausen, L. (2001). Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. *J. Cell Biol.* **155**, 531-542.

Montcouquiol, M., Rachel, R. A., Lanford, P. J., Copeland, N. G., Jenkins, N. A. and Kelley, M. W. (2003). Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. *Nature* **423**, 173-177.

Murdoch, J. N., Henderson, D. J., Doudney, K., Gaston-Massuet, C., Phillips, H. M., Paternotte, C., Arkell, R., Stanier, P. and Copp, A. J. (2003). Disruption of scribble (Scrb1) causes severe neural tube defects in the circletail mouse. *Hum. Mol. Genet.* **12**, 87-98.

Naylor, M. J., Li, N., Cheung, J., Lowe, E. T., Lambert, E., Marlow, R., Wang, P., Schatzmann, F., Wintermantel, T., Schüetz, G. et al. (2005). Ablation of beta1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *J. Cell Biol.* **171**, 717-728.

Nguyen, D.-A. D. and Neville, M. C. (1998). Tight junction regulation in the mammary gland. *J. Mammary Gland Biol. Neoplasia* **3**, 233-246.

Nola, S., Sebbagh, M., Marchetto, S., Osmani, N., Nourry, C., Audebert, S., Navarro, C., Rachel, R., Montcouquiol, M., Sans, N. et al. (2008). Scrib regulates PAK activity during the cell migration process. *Hum. Mol. Genet.* **17**, 3552-3565.

Oakes, S. R., Hilton, H. N. and Ormandy, C. J. (2006). The alveolar switch: coordinating the proliferative cues and cell fate decisions that drive the formation of lobuloalveoli from ductal epithelium. *Breast Cancer Res.* **8**, 207.

Ormandy, C. J., Binart, N. and Kelly, P. A. (1997). Mammary gland development in prolactin receptor knockout mice. *J. Mammary Gland Biol. Neoplasia* **2**, 355-364.

Ory, D. S., Neugeboren, B. A. and Mulligan, R. C. (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA* **93**, 11400-11406.

Osmani, N., Vitale, N., Borg, J.-P. and Etienne-Manneville, S. (2006). Scrib controls Cdc42 localization and activity to promote cell polarization during astrocyte migration. *Curr. Biol.* **16**, 2395-2405.

Pearson, H. B., Perez-Mancera, P. A., Dow, L. E., Ryan, A., Tennstedt, P., Bogani, D., Elsum, I., Greenfield, A., Tuveson, D. A., Simon, R. et al. (2011). SCRIB expression is deregulated in human prostate cancer, and its deficiency in mice promotes prostate neoplasia. *J. Clin. Invest.* **121**, 4257-4267.

Piguel, N. H., Fievre, S., Blanc, J.-M., Carta, M., Moreau, M. M., Moutin, E., Pinheiro, V. L., Medina, C., Ezan, J., Lasvaux, L. et al. (2014). Scribble1/AP2 complex coordinates NMDA receptor endocytic recycling. *Cell Rep.* **9**, 712-727.

Qin, Y., Capaldo, C., Gumbiner, B. M. and Macara, I. G. (2005). The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. *J. Cell Biol.* **171**, 1061-1071.

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.

Yamben, I. F., Rachel, R. A., Shatadal, S., Copeland, N. G., Jenkins, N. A., Warming, S. and Griep, A. E. (2013). Scrib is required for epithelial cell identity and prevents epithelial to mesenchymal transition in the mouse. *Dev. Biol.* **384**, 41-52.

Yates, L. L., Schnatwinkel, C., Hazelwood, L., Chessum, L., Paudyal, A., Hilton, H., Romero, M. R., Wilde, J., Bogani, D., Sanderson, J. et al. (2013). Scribble is required for normal epithelial cell-cell contacts and lumen morphogenesis in the mammalian lung. *Dev. Biol.* **373**, 267-280.

Zhan, L., Rosenberg, A., Bergami, K. C., Yu, M., Xuan, Z., Jaffe, A. B., Allred, C. and Muthuswamy, S. K. (2008). Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell* **135**, 865-878.