SLIT/ROBO1 Signaling Suppresses Mammary Branching Morphogenesis by Limiting Basal Cell Number

Hector Macias,1 Angel Moran,1 Yazeed Samara,1 Melissa Moreno,1 Jennifer E. Compton,1 Gwyndolen Harburg,1 Phyllis Strickland,1 and Lindsay Hinck1,*
1Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz, CA 95064, USA
*Correspondence: hinck@biology.ucsc.edu
DOI 10.1016/j.devcel.2011.05.012

SUMMARY
In the field of breast biology, there is a growing appreciation for the “gatekeeping function” of basal cells during development and disease processes yet mechanisms regulating the generation of these cells are poorly understood. Here, we report that the proliferation of basal cells is controlled by SLIT/ROBO1 signaling and that production of these cells regulates outgrowth of mammary branches. We identify the negative regulator TGF-β1 upstream of Robo1 and show that it induces Robo1 expression specifically in the basal layer, functioning together with SLIT2 to restrict branch formation. Loss of SLIT/ROBO1 signaling in this layer alone results in precocious branching due to a surplus of basal cells. SLIT2 limits basal cell proliferation by inhibiting canonical WNT signaling, increasing the cytoplasmic and membrane pools of β-catenin at the expense of its nuclear pool. Together, our studies provide mechanistic insight into how specification of basal cell number influences branching morphogenesis.

INTRODUCTION
Like other glandular organs, the mammary gland (breast) contains a bilayered epithelial structure consisting of an outer layer of basal myoepithelial cells (MECs) encircling an inner layer of luminal epithelial cells (LECs) (Silberstein, 2001). Historically, the basal layer has been largely overlooked by researchers, who focused instead on LECs, considered the origin of most carcinomas. Recently, however, appreciation has grown for the importance of this basal layer as an “epithelial gatekeeper,” generating the boundary between epithelial and stromal compartments, organizing tissue structure, maintaining stem cells, and suppressing cancerous growth (Barsky and Karlin, 2006; Gudjonsson et al., 2005). Nevertheless, the mechanisms regulating the generation and proliferation of these cells are poorly understood.

During postnatal mammary morphogenesis, highly mitotic structures at the tips of growing ducts called “end buds” invade the fatty stroma and establish the mammary tree. Cap cells, composing the basal layer of the end bud, differentiate into MECs that fully ensheath the ducts (Williams and Daniel, 1983). During pregnancy, however, the LEC population greatly expands as alveoli develop, resulting in sparse MEC coverage as basal cells stretch to accommodate the increased volume. This discontinuous coverage of an expanding LEC population also occurs during tumorigenesis when uncontrolled growth of LECs breaks through the myoepithelial barrier, resulting in the transition from ductal carcinoma in situ to infiltrating ductal carcinoma. Thus, understanding the mechanisms that regulate basal cell proliferation promises insight into basic developmental processes such as tissue morphogenesis and disease processes such as tumor metastasis.

Branching morphogenesis is a developmental program that imparts functional complexity to many biological systems (Andrew and Ewald, 2010). End bud bifurcation generates the primary ductal architecture, but lateral outgrowth of secondary and tertiary ducts is required to achieve full arborization of the mammary tree (Silberstein, 2001). The branching pattern of the mammary gland is stochastic, with the major requirement being an open ductal architecture that allows pregnancy-induced alveolar infilling. Consequently, inhibitory signals are critical and TGF-β1 is a key negative regulator of this process (Ewan et al., 2002; Inman and Robertson, 2008; Nelson et al., 2006). It functions by inhibiting cellular proliferation, but how it restricts cell growth, especially in a cell type-specific manner, is not well defined. In LECs, noncanonical WNT5A acts downstream of TGF-β1 (Pavlovich et al., 2011; Roarty and Serra, 2007) and inhibits cell growth by antagonizing canonical WNT signaling (Roarty et al., 2009). In cap cells or MECs, no downstream mediators of TGF-β1 have been identified to date.

SLITs are a conserved family of secreted proteins that were originally discovered in the nervous system, where they signal through ROBO receptors to mediate axonal guidance and branching (Brose et al., 1999; Wang et al., 1999). Their guidance function is well conserved and involved in directing migration of many cell types, including neural crest, immune, and tumor cells (Ypsilanti et al., 2010). In contrast, the branching function of SLITs has been chiefly described in the vascular system (Jones et al., 2008; Marlow et al., 2010) and seldom in epithelial organs of vertebrate animals (Griesshammer et al., 2004), where instead a distinct role for SLITs and ROBOs as tumor suppressors has been identified (Dallol et al., 2005; Marlow et al., 2008; Prasad et al., 2008; Yang et al., 2010). Thus, SLIT/ROBO signaling is emerging as an important regulator of cellular interactions.
In the mammary gland during branching morphogenesis, SLITs are expressed by both LECs and MECs, whereas expression of ROBO1 is restricted to just basal cap cells and MECs (Strickland et al., 2006). In the current study, we investigate the mechanism by which loss of Slits or Robo1 results in a precocious branching phenotype characterized by an excess of disorganized MECs. We identify the negative regulator, TGF-β1, upstream of ROBO1 and show that it induces Robo1 specifically in the basal layer, functioning together with SLIT2 to control branch formation. We determine that basal cell number alone influences branch number and demonstrate that SLIT/ROBO1 signaling limits branch formation by antagonizing canonical WNT signaling and restricting basal cell proliferation.

**RESULTS**

**ROBO1 Inhibits Branching Morphogenesis of Mammary Epithelium**

To investigate a role for SLIT/ROBO1 signaling in epithelial branching morphogenesis, we examined the Robo1 loss-of-function phenotype by transplanting Robo1−/− and wild-type (WT) littermate epithelium into contralateral fat pads of immunocompromised (Foxn1nu) mice that were precleared of their endogenous mammary epithelial buds prior to puberty (Strickland et al., 2006). For this initial analysis, we used transplanted epithelium to assess the outgrowth and branching of epithelia without potential secondary effects of the Robo1−/− mutation and to ensure that both Robo1−/− and WT tissues were subject to the same hormonal environment. We observed that Robo1−/− and WT ducts grew to similar lengths, but that Robo1−/− transplants displayed excessive side branching (Figure 1A). We quantified the phenotype and found a >2-fold increase in secondary branches and tertiary buds in Robo1−/− transplants (Figure 1B) but no significant difference in primary branch number (Figure 1C), indicating that increased lateral bud formation, rather than excessive end bud bifurcation, is responsible for the phenotype. We previously observed that transplanted knockout tissue contains a hyperplastic phenotype (Marlow et al., 2008; Strickland et al., 2006), and therefore we quantified branching in intact, unmanipulated Robo1−/− glands. Intact glands are similarly hyperbranched (H.M., unpublished data), but during this early stage of development they do not display the hyperplastic changes associated with transplanted tissue (see Figure S1A available online).

We also examined branching morphogenesis in an organotypic culture model generated from intact Robo1−/− glands in which aggregated cells (Figure 1D) or ductal fragments (Figure S1B) were grown in growth factor-reduced Matrigel (Ewald et al., 2008; Holliday et al., 2009). Robo1−/− organoids were devoid of hyperplastic changes, such as luminal infilling, and contained a bilayered epithelium (Figure 1D; Figure S1C). The majority of Robo1−/− organoids were branched, whereas WT organoids were unbranched hollow structures (Figure 1E). The few WT organoids containing branches had an average of three branches, whereas Robo1−/− organoids had twice as many branches (Figure 1F). Fragment organoids generated from Robo1−/− tissue also recapitulated the hyperbranched phenotype (Figures S1B and S1D). Together, these data demonstrate that under the same conditions, Robo1−/− epithelium generates more branches than WT epithelium.

**SLIT2 Is the ROBO1 Ligand that Inhibits Mammary Branching**

SLITs are ligands for ROBO1, and previous studies have shown that Slit2 and Slit3, but not Slit1, are expressed in the mammary gland (Strickland et al., 2006). To evaluate whether combined loss of Slit2 and Slit3 phenocopies the Robo1−/− hyperbranching defect, we transplanted Slit2−/−;Slit3−/− epithelium into precleared fat pads of Foxn1nu mice. Loss of Slits, similar to loss of Robo1, led to a significant increase in secondary branches and tertiary buds but no difference in primary duct number (Figures 2A and 2B).

Next, we examined whether exogenous SLIT inhibits branch formation. We implanted, at the forefront of WT mammary trees, Elvax slow-release pellets containing either recombinant SLIT2, observed by immunohistochemistry in a 5 mm radius around the pellet (H.M., unpublished data), or control BSA (Figure 2C). Elvax is a biologically compatible polymer that is used to deliver molecules, including functionally inert BSA (Silberstein and Daniel, 1987). SLIT2, rather than SLIT3, was implanted because it is highly expressed during branching morphogenesis (Strickland et al., 2006). After 7 days, secondary branching was suppressed in regions near SLIT2 pellets (Figure 2C, right, box), with the few branches in proximity containing small lateral buds, which frequently turned away from SLIT2 (Figure 2C, arrow). The distance between secondary branches, located within 5 mm of the pellets, was significantly longer in regions surrounding SLIT2 pellets (Figure 2D). There was also a preference for growth away from SLIT2, and this was quantified by counting the secondary branches extending toward (ipsilateral) or away from (contralateral) the pellets (Figure 2E). These data show that SLIT2 inhibits lateral branch formation but not the growth of primary ducts past the pellet.

We also examined the effects of SLIT2 on organoid branching. Because WT organoids are largely unbranched in the absence of growth factors (Figures 1D–1F), we induced branching by adding hepatocyte growth factor (HGF), and then challenged the cultures with SLIT2. There was an 80% reduction in the number of WT branched organoids, a reduction that did not occur with Robo1−/− organoids (Figures 2F–2H). Together, these studies strongly support the idea that SLIT2 and ROBO1 function in a ligand/receptor relationship to regulate lateral branching during mammary morphogenesis.

**ROBO1 Is a Downstream Effector of TGF-β1 in Myoepithelial Cells**

TGF-β1 is a key negative regulator of mammary ductal development and branching morphogenesis. One explanation for our data is that SLIT/ROBO1 signaling is downstream of TGF-β1 and, indeed, transcriptional profiling experiments identified Robo1 as a TGF-β1-upregulated transcript in mammary cell lines (Labbe et al., 2007). To investigate the biological significance of this result, we cultured primary mammary epithelial cells (ECs) with TGF-β1 along with inhibitors of both protein synthesis (cycloheximide) and the TGF-β1 receptor type 1 (SB431542). We found a TGF-β1–induced, ~2-fold increase in Robo1 mRNA and protein, with the change in mRNA prevented by the presence of either inhibitor (Figures 3A and 3B), suggesting that TGF-β1 signaling upregulates ROBO1 via a noncanonical
pathway, rather than Smad signaling, which does not depend on protein synthesis (Yue and Mulder, 2001).

We previously showed that Robo1 is specifically expressed on cap cells and MECs during branching morphogenesis (Strickland et al., 2006). To assess whether this pattern is recapitulated in organoids, we assayed for β-galactosidase (β-gal) activity, taking advantage of lacZ inserted downstream of the Robo1 promoter (Figures 3C–3E) (Long et al., 2004). As predicted by Robo1 expression in vivo, we observed positive β-gal staining on the surface of organoids that coimmunostained with an MEC marker (Figure 3C). In a typical Robo1−/− organoid, ~30% of MECs stain positive for β-gal, and we considered this the threshold for positivity. Organoids were treated with TGF-β1 for 24 hr, resulting in significantly more β-gal-positive organoids (Figures 3D and 3E). To investigate whether this ROBO1 upregulation contributes to branch inhibition, we used HGF to elicit branching of WT organoids, followed by treatment with TGF-β1, SLIT2, or both (Figure 3F). TGF-β1 or SLIT2

Figure 1. Loss of Robo1 in Mammary Epithelium Leads to Excess Branching Morphogenesis

(A) Contralaterally transplanted, hematoxylin-stained, virgin WT and Robo1−/− outgrowths. Insets represent magnified images.

(B and C) Branchpoint analysis (n = 5 animals).

(D) Representative images of WT and Robo1−/− organoids obtained with phase contrast (left) and immunofluorescence using CK-14 (MECs) and E-cadherin (LECs) (right).

(E) Quantification of total branched Robo1−/− and WT organoids (n = 4 experiments, >300 organoids/genotype).

(F) Quantification of branches per Robo1−/− and WT organoid (n = 3 experiments, >300 organoids/genotype).

Scale bars represent 3 mm (A) and 30 μm (D). Asterisks indicate significance in a Student’s t test (NS, not significant).
Figure 2. Loss of Slit2 Results in Excess Branching; Conversely, Exogenous SLIT2 Treatment Results in Decreased Branching

(A) Contralaterally transplanted, hematoxylin-stained, virgin WT and Slit2−/−:Slit3−/− outgrowths.
(B) Branchpoint analysis (n = 10 animals).
(C) Representative whole-mount images of carmine-stained glands contralaterally implanted with Elvax pellets containing either BSA or SLIT2. Black dashed lines outline pellets, white dashed boxes highlight areas near pellets, and the arrow points to an end bud turning away from SLIT2.
(D) Quantification of the distance between 2 branches (5 mm radius; n = 5 animals).
(E) Quantification of 2 branches ipsilateral or contralateral to the pellet (n = 5 animals).
(F) Representative phase-contrast images of WT or control Robo1−/− organoids induced to branch with HGF. After 24 hr, organoids were treated with HGF either alone or with SLIT2 and allowed to grow for 6 days.
(G and H) Quantification of the number of WT and Robo1−/− organoids in each condition that had three or more branches (n = 3 experiments, >100 organoids/treatment).

Scale bars represent 1 mm (A and C) and 75 μm (F). Asterisks indicate significance in a Student’s t test (NS, not significant).
Figure 3. TGF-β1 Upregulates Robo1, Leading to Enhanced Branch Inhibition in Response to SLIT2

(A) Robo1 levels after treatment with TGF-β1 alone or in combination with SB431542 or cycloheximide. Relative RT-qPCR analysis of ECs harvested from virgin mice (n = 3 independent RNA sets).

(B) ROBO1 protein levels after TGF-β1 treatment. Positive control is COS-7 cells expressing pSecTagBRobo1myc.

(C) Representative images of Robo1−/− organoids stained for β-gal (blue) (left) with a magnified image showing β-gal (upper) and coimmunostaining with CK-14 (green), E-cadherin (red), and nuclear marker Hoechst (blue) (lower).

(D and E) Representative phase-contrast images of β-gal-stained Robo1−/− organoids after mock or TGF-β1 treatment. The percentage of organoids containing ≥30% positive cells was quantified (n = 3 experiments, 100 organoids/treatment).

(F and G) WT and Robo1−/− organoids were stimulated to branch with HGF, treated with SLIT2, TGF-β1, or both, and imaged using bright-field microscopy (n = 3 experiments, >200 organoids/treatment).

(H and I) Quantification of WT and Robo1−/− organoids in each condition that had three or more branches (n = 3 experiments, >100 organoids/treatment).

Scale bars represent 30 μm (C, D, F, and G). Asterisks indicate significance in a Student’s t test (E) or ANOVA (A and H) (NS, not significant).
Figure 4. SLIT2/ROBO1 Signaling Inhibits the Proliferation of Basal Cap/Myoepithelial Cells

(A) Quantification of percentage of proliferating (EdU+) cells in 2D organoids (n = 3 experiments, >500 cells).

(B–E) RT-qPCR and western blot analysis of Cyclin D1 and Cyclin D1 levels, respectively, in WT and Robo1−/− MECs and LECs (RT-qPCR: n = 3 independent RNA sets; western blot: n = 3 experiments).
inhibited branching to a similar degree, but the effect was significantly enhanced upon treatment with both TGF-\(\beta\)1 and SLIT2 (Figures 3F and 3H). Moreover, Robo1\(^{-/-}\) tissue was refractory to TGF-\(\beta\)1 treatment (Figures 3G and 3I), as it was to SLIT2 treatment (Figures 2F and 2H). These data support the notion that up-regulation of ROBO1 in basal cells by TGF-\(\beta\)1 restricts branching by enhancing the inhibitory effects of SLIT.

**SLIT/ROBO1 Signaling Regulates Basal Cell Proliferation**

TGF-\(\beta\)1 inhibits mammary branching morphogenesis by reducing overall cellular proliferation (Ewan et al., 2002). To investigate whether SLIT/ROBO1 signaling similarly inhibits cell proliferation, but specifically in basal cells, we generated ductal fragments from WT glands and cultured them as 2D, bilayered, circular organoids (Figure S2A). SLIT2 treatment resulted in an \(-50\%\) reduction in MEC proliferation (Figure 4A; Figure S2B), similar to the reduction observed in a human MEC line, HM50 (Figures S2C and S2D), with no change in LEC proliferation (Figure 4A). These results suggest that only MECs are regulated by SLIT/ROBO1 signaling, consistent with the restricted expression of ROBO1 on these cells. However, LECs had a low basal index of proliferation, perhaps due to contact inhibition in the organoid center. To address this possibility, we separated WT and Robo1\(^{-/-}\) MECs from LECs using differential trypsinization (Figures S2E–S2H) (Darcy et al., 2000), and examined a regulator of cell-cycle entry, Cyclin D1. There was a significant increase in Cyclin D1 by RT-quantitative PCR (Figure 4B) and western blot (Figure 4D) in Robo1\(^{-/-}\) MEC-enriched fractions, whereas no differences between genotypes were observed in LEC-enriched fractions (Figures 4C and 4E).

We also assessed cell proliferation in vivo in mammary glands by intraperitoneal injections of 5-ethyl-2\(^{-}\)deoxyuridine (EdU) (Figure 4F). We initially focused on the mitotically active end buds and found an \(-2\times\) increase in cap cell proliferation in Robo1\(^{-/-}\) and glands with no significant change in LEC proliferation (Figures 4G and 4H), consistent with our data obtained in cell culture (Figures 4A–4E). Cap cell proliferation was also evaluated in glands containing SLIT2 and BSA Elvax pellets (Figures 4I and 4J), and a concordant \(-2\times\) decrease in cap cell proliferation was observed in end buds near SLIT2 pellets with, again, no significant difference in LEC proliferation.

We also examined subtending ducts to evaluate the consequences of having surplus cap cells, which differentiate into MECs. In agreement with previous studies (Bresciani, 1968), we found very few proliferating basal cells along WT or Robo1\(^{-/-}\) ducts, suggesting that, unlike cap cells, differentiated MECs are refractory to the proproliferative consequences of losing SLIT/ROBO1 signaling (H.M., unpublished data). Evaluation of ductal morphology, however, revealed an overabundance of MECs in Robo1\(^{-/-}\) ducts, suggesting that the consequence of exuberant cap cell proliferation is excess MECs (Figure 4K). We quantified both the number of MECs and the distance between them, and found that Robo1\(^{-/-}\) glands have significantly more cells that are closer together (Figures 4L and 4M). We also used fluorescence-activated cell sorting (FACS) to examine the relative levels of basal cells in WT and Robo1\(^{-/-}\) glands and found a \(-2\times\) increase in basal cells (Lin\(^{-}\)CD24\(^{-}\)CD29hi) in Robo1\(^{-/-}\) tissue (Figure 4N). Together, these data show that SLIT2/ROBO1 signaling constrains cap cell proliferation, and that in its absence there is an excess of disorganized MECs.

**The Number of Basal Cells Positively Influences the Number of Branches**

These studies raise the question as to whether basal cell number alone influences branching. To investigate, we analyzed organoids (\(-100\ \mu\)m diameter) that were either unbranched or contained one bud or branch. We observed MECs congregating at these bud/branch sites, with formation of a single bud/branch correlating with increased MEC number (Figures 5A and 5B; Figure S3A). To evaluate the consequences of MEC localization on bud growth, we generated and labeled WT organoids with EdU, and again analyzed similarly sized organoids containing a single bud (Figures 5C and 5D). Quantification of EdU\(^{+}\) cells in each quadrant revealed that bud-containing quadrants had \(-2\times\) more EdU\(^{+}\) cells (Figure 5E). Previous studies have shown that fibroblastic growth factor 2 (FGF2) is secreted from MECs and positively regulates mammary branching (Gomm et al., 1997). We evaluated FGF2 levels in WT and Robo1\(^{-/-}\) MECs and, while both populations express FGF2, Robo1\(^{-/-}\) cells express significantly higher levels (Figure 5F).

Our data suggest that MEC number regulates mammary branching by supplying growth factors. To address this role for MECs, we performed mixing experiments in which we manipulated the ratio of MECs to LECs. First, we ensured that organoids in these assays arose from cell aggregates, rather than a single stem/progenitor cell, by mixing MECs from β-actin-EGFP mice with unlabeled LECs and documenting the formation of mixed-labeled organoids (Figure S3B). Next, we removed HGF from the culture media and manipulated the proportion of MECs to LECs, generating organoids that contained either a normal (~1:3) or high (~3:1) ratio of cells (Darcy et al., 2000). These ratios were confirmed by immunoblotting the input mixtures with MEC (CK-14) or LEC (E-cadherin) markers (Figure 5G). After 7 days, we categorized them as either branched or unbranched (Figure 5H), and quantified the number in each category (Figure 5I). A high ratio of MECs to LECs produced significantly more branched structures compared to a low ratio, which produced more unbranched structures, consistent with basal cell number having a corresponding influence on branch number (Figures 1, 2, and 4). Together, these data support a model in which SLIT/ROBO1 restricts the number of MECs by limiting cap cell

---

(F-H) Individual channel images of Hoechst-stained, EdU-labeled, p63-immunostained WT and Robo1\(^{-/-}\) end buds (n = 3 animals).
(I and J) Quantification of MEC and LEC EdU\(^{+}\) nuclei in WT glands surrounding SLIT2 and BSA pellets (5 mm radius) (Figure 2C) (n = 3 animals).
(K) Individual and merged channel images of p63-immunostained and Hoechst-stained WT and Robo1\(^{-/-}\) ducts.
(L) Quantification of MECs in Robo1\(^{-/-}\) and WT ducts (n = 3 animals).
(M) Quantification of the distance between MECs in Robo1\(^{-/-}\) and WT ducts (n = 3 animals).
(N) FACS analysis of the relative level of basal (Lin\(^{-}\)CD24\(^{-}\)CD29hi) to total (Lin\(^{-}\)CD24\(^{+}\)) epithelial cells in Robo1\(^{-/-}\) and WT littermate glands.
Scale bars represent 20 \(\mu\)m (F and K). Asterisks indicate significance in a Student’s t test (NS, not significant).
Figure 5. Basal Cell Number Influences Organoid Branching State

(A) Merged channel images of unbranched, budded, or branched WT organoids stained with Hoechst, phalloidin, and MEC marker p63.

(B) Quantification of organoid diameter and MEC number in budded, branched, and unbranched organoids (n = 3 experiments, >50 organoids/branching state).

(C) Cartoon model of an EdU-labeled organoid divided into quadrants with a bud containing a quadrant designated Q1.

(D and E) Quantification of quadrants from organoids labeled with EdU (red) and Hoechst (blue) (n = 3 experiments, >50 organoids/quadrant).

(F) Relative RT-qPCR analysis of FGF2 levels in MECs harvested from WT and Robo1−/− glands (n = 3 independent RNA sets).

(G) Representative immunoblots from lysates of input cells at different MEC and LEC ratios: MEC marker, CK-14; LEC marker, E-cadherin; loading control, tubulin.

(H) Representative images of 1MEC:3LEC and 3MEC:1LEC organoids obtained with phase contrast (left) and immunofluorescence using p63 and phalloidin (right).

(I) Quantification of branched 3MEC:1LEC versus 1MEC:3LEC organoids (n = 3 experiments, >300 organoids/population).

Scale bars represent 30 μm (A, D, and H). Asterisks indicate significance in a Student’s t test (NS, not significant).
proliferation. In the absence of SLIT/ROBO1 signaling, a surplus of MECs is generated that positively regulates branching by providing growth factors, such as FGF2.

**SLIT/ROBO1 Signaling Regulates the Subcellular Localization of β-Catenin**

Overexpression of activated β-catenin in the basal compartment of mammary gland results in excess proliferation and hyperbranching (Teuliere et al., 2005), similar to the phenotype described in this study. It also produces basal-type hyperplasias similar to, but more severe than, phenotypes observed at later stages of development in Robo1−/− and Slit2−/−;Slit3−/− outgrowths (Marlow et al., 2008) (Figures 1A and 2A). To investigate whether β-catenin is downstream of SLIT/ROBO1 in basal cells, we treated HME50 cells with SLIT2 and, using biochemical fractionation, detected a shift in β-catenin from the nuclear to the cytosolic/membrane fractions (Figure 6A). We confirmed this change in subcellular localization of β-catenin with immunocytochemistry. Figure 6B shows that SLIT2 treatment enhances the staining of β-catenin and E-cadherin at the membrane, with no change in the levels of total protein as assayed by immunoblot (Figure 6C). β-catenin was also activated in these cells using lithium chloride (LiCl) following SLIT2 treatment and, again, there was increased β-catenin membrane staining in SLIT2-treated samples and significantly decreased nuclear translocation (Figure S4A). Together, these studies suggest that SLIT/ROBO1 signaling influences β-catenin’s subcellular localization. In cancer cells, this occurs through the Akt/PKB pathway (Prasad et al., 2008; Tseng et al., 2010), which negatively regulates glycogen synthase kinase 3-beta (GSK-3β) downstream of growth factor receptors (Cross et al., 1995). Similarly, we found that EGF and insulin (IF) treatment of primary MECs and LECs, as well as HME50 cells, increased the phosphorylation of Akt and GSK-3β (Figure 6D; Figure S4B). Pretreatment of cells with SLIT decreased this response in MECs and HME50 cells, but not in LECs. Decreased phosphorylation of GSK-3β activates it (Cross et al., 1995), favoring the accumulation of β-catenin in the cytosol and membrane of these cells (Figures 6A–6C).

Next, we probed whole MEC lysates with an antibody directed against active β-catenin (ABC) (Staal et al., 2002) and observed a decrease in this form upon SLIT2 treatment (Figure 6E). We used this antibody to examine the basal layer of WT organoids. In untreated organoids, there is modest positive staining in the nucleus. Treating cells with an activator of canonical WNT signaling dramatically increased the nuclear staining of unphosphorylated β-catenin, whereas treatment with SLIT2 reduced β-catenin’s nuclear staining while increasing its membrane staining (Figure 6F). These data indicate that SLIT2 inhibits nuclear translocation of β-catenin, likely decreasing its transcriptional functions. To investigate, we evaluated LEF/TCF transcriptional targets by RT-qPCR and found increased expression of Axin2, Cyclin D1, and Tcf1 mRNA in primary MECs harvested from Robo1−/−;Slit2−/−;Slit3−/− glands, and a concordant decrease in mRNA from WT MECs treated with SLIT2 (Figure 6G). One of these transcripts can also be monitored in vivo using Axin2lacZ/+ mice. These mice faithfully reflect β-catenin signaling by reporting Axin2 expression in multiple tissues (Lustig et al., 2002). During branching morphogenesis, there is robust β-gal staining in cap cells of the end bud and basal MECs of subtending ducts (Figure S4C) (Zeng and Nusse, 2010). We implanted SLIT2 and BSA pellets into Axin2lacZ/+ glands and observed significantly reduced β-gal staining in MECs with SLIT2 but not BSA (Figure 6H). These data indicate that SLIT2 inhibits the proliferation of ROBO1-expressing basal cells by opposing the activation of β-catenin. Taken together, our data suggest a mechanism for restricting mammary branching morphogenesis by controlling cell number, specifically in the basal layer of the bilayered mammary gland (Figure 7).

**DISCUSSION**

Our studies define a mechanism governing mammary branching morphogenesis whereby SLIT/ROBO1 signaling inhibits lateral branch formation by controlling the proliferation of the basal cell layer. Specificity of signaling is achieved by restricting the expression of ROBO1 to the basal layer and regulating it with TGF-β1. This mechanism of SLIT regulating branching is different from the mechanisms identified in the nervous system, where an extracellular source of SLIT signals to ROBO receptors expressed on growth cones or axon shafts, resulting in cytoskeletal reorganization that leads to growth cone bifurcation or lateral extension of membrane away from the axonal shaft (Ypsiloni et al., 2010). In contrast, in the vasculature, a mechanism has been identified that is potentially similar to the one observed in the mammary gland. Here, SLIT is expressed by pericytes and signals through endothelial ROBO4 receptor to restrain sprouting angiogenesis by downregulating pathways activated by VEGF/VEGFR (Jones et al., 2008, 2009). VEGF increases the nuclear localization of β-catenin in endothelial cells (Ilan et al., 2003). If this drives sprouting angiogenesis, then SLIT/ROBO4 signaling could inhibit this process by sequestering β-catenin in the cytoplasm, similar to the effects observed in the mammary gland (Figure 6). Thus, the mechanism of SLIT/ROBO action in the mammary gland, via restricting β-catenin-dependent cell proliferation, may apply to vessel sprouting as well.

These studies highlight the importance of MECs as key regulators of breast development. MECs are responsible for producing components of the basal lamina and mediating interactions between ductal LECs and the extracellular environment. During development, they synthesize and secrete many key growth factors, including WNTs and FGFs (Figure 5F) (Gomm et al., 1997; Kouros-Mehr and Werb, 2006), which act as branching factors during morphogenesis (Lindvall et al., 2006; Lu et al., 2008). FGF does not promote MEC proliferation directly, but instead functions in a paracrine fashion to induce LEC proliferation (Figures 5C–5F) (Gomm et al., 1997). This distinction between basal and luminal cells, however, may not exist in the end bud. Instead, in this context, loss of FGF receptor 2 in a subset of cells leads to decreased proliferation of cap and luminal body cells (Lu et al., 2008), in addition to a hypobranching phenotype that highlights the positive contribution of cell proliferation in the end bud to branch formation (Lu et al., 2008; Parsa et al., 2008). Changes in branching are also observed upon constitutive activation of canonical WNT signaling, as demonstrated by overexpression of an N-terminally truncated, activated form of β-catenin in the basal cell layer that results in excess basal cells and precocious lateral bud formation (Teuliere et al., 2005). Furthermore, the opposite phenotype, fewer
Figure 6. SLIT/ROBO1 Signaling Regulates the Subcellular Localization of β-Catenin

(A) Biochemical fractionation of HME50 cells treated with SLIT2. Top: representative immunoblots for β-catenin; nuclear loading control, histone H1; cytoplasmic loading control, GAPDH; membrane loading control, cadherin. Bottom: quantitative analysis of β-catenin (n = 3 experiments).

Developmental Cell
SLIT/ROBO1 Restricts Mammary Branch Formation

Figure 7. The SLIT/ROBO1 Signaling Axis Regulates Mammary Gland Branching Morphogenesis

Cartoon model of how the mammary basal layer promotes branching morphogenesis, and how this effect is countered by SLIT/ROBO1 signaling. From left to right, TGF-β1 elevates the expression of Robo1 in basal cells. ROBO1 then interacts with ligand SLIT2 to inhibit the nuclear accumulation of β-catenin by inhibiting Akt activation. Inhibiting Akt results in unphosphorylated, activated GSK-3β, which phosphorylates β-catenin and favors its degradation or accumulation at the membrane (not pictured), thereby inhibiting its translocation to the nucleus and subsequent activation of transcription. Thus, by curbing basal cell proliferation, SLIT/ROBO1 signaling inhibits mammary gland branching morphogenesis.

(B) Merged channel images of Hoechst-, β-catenin- (top) or E-cadherin- (bottom) stained HME50 cells. Plasma membrane signals were recorded as mean pixel intensities over 5 μm of the highest-staining membrane (n = 3 experiments, >50 cells/treatment).
(C) Representative immunoblots and quantification of E-cadherin and β-catenin after SLIT2 treatment of HME50 cells (n = 3 experiments).
(D) Representative immunoblots and quantification for p-Akt (left) and p-GSK-3 (right) in HME50 cells treated with SLIT2 alone or in combination with growth factors (total Akt and GSK-3β) as loading controls (n = 2 experiments).
(E) Representative immunoblots and quantification for activated β-catenin (top) in MECs treated with SLIT2 (total β-catenin [bottom] as loading control) (n = 2 experiments).
(F) Individual and merged channel images of 6-day-old organoids stained with p63, ABC, and Hoechst after mock, WNT3A, or SLIT2 treatment. White dashed lines highlight nuclear area. Nuclear ABC levels were recorded as mean pixel intensities of 254 μm of nuclear area (n = 3 experiments, >50 cells/treatment).
(G) Relative RT-qPCR analysis of β-catenin target genes Axin2, Cyclin D1, and Tcf1 in WT compared to Robo1−/− MECs (top), and WT compared to SLIT2-treated WT MECs (bottom) (n = 3 independent RNA sets).
(H) β-gal staining of Axin2+/− mice mammary tissue in regions near SLIT2 (right) and BSA (left) Elvax pellets. Top panels reveal ductal proximity to Elvax pellets; bottom panels are magnified images of highlighted (red boxes) ductal area. Percentage of β-gal-positive MECs (CK14+) was quantified in ducts within 5 mm of the pellet (n = 3 experiments).

Scale bars represent 10 μm (B and F) and 0.5 mm (H). Asterisks indicate significance in a Student’s t test (NS, not significant).
strong evidence for a developmental correlate of SLIT’s role as a suppressor of tumor cell growth by showing its function in opposing canonical WNT signaling and limiting basal cell proliferation during mammary branching morphogenesis.

Recently, the basal cell population has been shown to contain a subpopulation of mammary stem cells (MaSCs) (Shackleton et al., 2006; Stingl et al., 2006) whose regenerative capacity is regulated by canonical WNT signaling (Badders et al., 2009; Zeng and Nusse, 2010). Because MaSCs have the potential to generate the repertoire and number of new cells necessary for branching, it is tempting to speculate that they are required for branch formation. Alternatively, it is possible that bipotent progenitor cells, which may not have a basal phenotype, are the operative cell type. In either case, it raises the possibility that SLIT affects branching by regulating the production of stem/progenitor cells. Indeed, recent data show that progesterone, which is responsible for side branching, initiates a series of events whereby LECs spur the proliferation of MaSCs by providing growth factors such as WNT4 and RANKL (Asselin-Labat et al., 2010; Joshi et al., 2010). Branching was not evaluated in these studies, and currently there is no evidence that MaSCs contribute directly to branching, but our studies have not excluded an effect of SLIT in countering the effects of progesterone and restricting the proliferation of MaSCs.

In conclusion, this report shows that SLIT/ROBO1 signaling is a central agent within a pathway that controls branching morphogenesis. Our studies provide mechanistic insight into how ROBO1 levels are influenced by a negative regulator, TGF-β1, and how this, in turn, curtails basal cell production by regulating the subcellular localization of β-catenin and inhibiting canonical WNT signaling. We propose that specification of basal cell number is a critical component regulating branch formation, with SLIT/ROBO1 acting to check growth factor signaling by curbing basal cell proliferation.

EXPERIMENTAL PROCEDURES

Animals

The study conformed to guidelines set by the University of California, Santa Cruz animal care committee (IACUC). Mouse Slit2, Slit3, Robo1, and Axin2^-/-^- mice were generated and genotyped as described (Lustig et al., 2002; Strickland et al., 2006). The promoters for Robo1 and Axin2 drive the expression of lacZ and was assessed by β-gal staining (Strickland et al., 2006).

Mammary Fat Pad Clearing, Transplantation, and Branching Analysis

Mammary anlage were rescued from knockout embryos and transplanted into precleared fat pads of Foxn1^-/-^- mice (Strickland et al., 2006). Contralateral outgrowths were harvested 4 weeks posttransplant and subjected to whole-mount hematoxylin staining. Primary branches were defined as ducts extending from the nipple and terminating in an end bud. Secondary and tertiary branches were defined as bifurcating from primary ducts or secondary branches, respectively.

Primary Mouse Mammary Epithelial Cell Culture

Glands were digested with collagenase and dispase (Figures S2E–S2H) (Darcy et al., 2000). Differential trypsinization was performed to obtain purified MEC and LEC fractions (Darcy et al., 2000). For mammary cell sorting, single-cell suspensions from thoracic and inguinal mammary glands were prepared as previously described (Shackleton et al., 2006). FACS analysis was performed using a FACSAria (Becton Dickinson).

RNA Extraction and RT-PCR Analysis

RNA was extracted using a PureLink RNA Mini kit (Invitrogen). cDNA was prepared using an iScript cDNA synthesis kit (Bio-Rad). PCR was performed in triplicate and quantified using a Rotor Gene 6000 real-time PCR machine and software (Corbett Research) to assay SYBR green fluorescence (Bio-Rad) (Livak and Schmittgen, 2001). Results were normalized to that of GAPDH.

In Vitro Branching Morphogenesis Assays

Three-dimensional primary cultures were generated as previously described (Lee et al., 2007). Briefly, to generate organoids, we embedded 10,000 ECs in 100 μl of growth factor-reduced Matrigel (BD Biosciences)/0.7 cm². Fragment organoids were obtained by embedding purified epithelial fragments into Matrigel (Ewald et al., 2008), and stimulated with 2.5 nM bFGF (Sigma).

Elvax Slow-Release Pellet Preparation and Surgical Implantation

Elvax pellets containing 271 ng of SLIT2 and 0.45 mg of BSA or only 0.45 mg of BSA (control) were contralaterally implanted at the forefront of the growing ductal tree in wild-type CD1 mice and harvested after 7 days (Silverstein and Daniel, 1987).

Antibodies, Reagents, and Cell Lines

Antibodies used were as follows: CK-14 (Covance); E-cadherin (R&D Systems); p63 (Santa Cruz Biotechnology); ROBO1 (Abcam); Myc (9E10); tubulin (Sigma); GAPDH (Santa Cruz Biotechnology); (β-catenin (601154) (BD Biosciences); ABC (8E7) (Millipore); histone H1 (Santa Cruz Biotechnology); and Akt, p-Akt (Thr308), GSK-3β, and p-GSK-3β (Ser9) (Cell Signaling). Nonantibody markers used were: Alexa Fluor 546 phalloidin for filamentous actin (Invitrogen), Hoechst (Invitrogen) for nuclei, and EnUv (Invitrogen) to label proliferating cells. HME50 cells were cultured in DMEM-F12 supplemented with 100 x× mammary epithelial cell growth supplement (Cascade Biologics).

Western Blot and Cellular Fractionation

Tissue protein lysates were prepared and analyzed by western blot as described (Marlow et al., 2008). For cellular fractionation, HME50 cells were treated with SLIT2 for 4 hr and then fractionated using the Qproteome Cell Compartment kit (QIAGEN).

Proliferation Assays

In vitro cultures were treated with 10 μM EduU for 1 hr before detection. In vivo labeling was accomplished by intraperitoneal injections of EduU (25 ng/g of body weight) followed by harvest 2 hr postinjection. Samples were subjected to Click-IT chemistry (Invitrogen).

Statistical Analysis

Statistical tests and p values are indicated in the figure legends. Graph columns represent the mean and error bars represent the standard error of the mean.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.devcel.2011.05.012.

ACKNOWLEDGMENTS

We thank Marisela Marinez and Fay Davidson for technical assistance; Gary Silverstein (University of California, Santa Cruz), Mark Sternlicht (FibroGen), and Dr. Yi Arial Zeng (Shanghai Institutes for Biological Sciences), who also supplied WNT3, for thoughtful comments on the manuscript; Susan Strome for use of a confocal microscope (NIH grants GM46295 and GM43059); and Santa Cruz Biotechnology for their generous donation of antibodies. Slit3^-/-^- mice were kindly provided by Dr. David Ornitz (Washington University); Slit2^-/-^- and Robo1^-/-^- mice by Dr. Marc Tessier-Lavigne (Genentech); and Axin2^-/-^- mice by Dr. Walter Birchmeier (Max Delbrueck Center) and Roel Nusse (Stanford University). We acknowledge grants from the NIH (R01 CA-128902), Congressionally Directed Medical Research Program (W81XWH-08-1-0380), Santa Cruz Cancer Benefit Group, Initiative for Maximizing Student Diversity
Developmental Cell

SLIT/ROBO1 Restricts Mammary Branch Formation

(NIH GM058903)(H.M.), and Center for Biomolecular Science and Engineering (SP41HG002371-09) (H.M.) for funding this research.

Received: November 30, 2010
Revised: April 12, 2011
Accepted: May 16, 2011
Published: June 13, 2011

REFERENCES


