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SANTA CRUZ

REGULATION OF MAMMARY STEM CELL SELF-RENEWAL BY THE
SLIT2/ROBO1 SIGNALING AXIS

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MOLECULAR, CELL AND DEVELOPMENTAL BIOLOGY

by

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June 2015

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ABSTRACT

The SLIT2/ROBO1 signaling axis regulates ductal development and stem cell self-renewal by controlling Insuteable through the SNAIL transcription factor

by

MIMMI SEWERIN BALLARD

The breast, or mammary gland in mice, is an epithelial bilayered ductal system that produces milk during pregnancy. It is composed of two main cell types, basal and luminal epithelial cells, and develops postnatally, primarily during puberty. In the mouse, this occurs between 3-6 weeks of age and is driven by stem cells in the end buds, located at the tips of the primary and secondary ductal branches. SLIT2, signaling through ROBO1 expressed on the surface of basal cells during development, regulates many aspects of mammary gland morphogenesis, including basal cell proliferation and branching, as well tumor cell transformation. Here, I first summarize how SLIT2/ROBO1 signaling drives, and in some cases, counteracts tumor progression, and then investigate if this signaling axis plays a role in the self-renewal of end bud stem cells. We show that mammary stem cells self-renew via asymmetric cell divisions (ACDs) and that SLIT2/ROBO1 prevents precocious stem cell expansion, thus maintaining the stem cell population, by controlling the expression of the ACD regulator Insuteable. Immunostaining, immunoblotting and quantitative real-time PCR (RT-qPCR) experiments demonstrate increased expression
of Inscurteable in Robo1-/- animals, while SLIT2 treatment of basal WT cells leads to decreased Inscurteable levels. Furthermore, we demonstrate that this regulatory relationship is mediated by the SNAIL (SNAI1) transcription factor, which is present at higher levels in the Robo1-/- tissue. Through luciferase promoter assays, we find that SNAI1 enhances Inscurteable expression by binding to a non-canonical DNA binding site (TCACA) in the Inscurteable promoter. To investigate the consequences of elevated Inscurteable levels during ductal development, we characterized mammary gland morphology in an Inscurteable over-expressing mouse model (mInsc KI/KI) and found enhanced ductal outgrowth, without increased cell proliferation, in the transgenic, compared to wild type, mice. Using in vivo immunostaining and an in vitro live-cell labeling assay, we found an increased frequency of symmetric cell divisions, and limiting dilution assays revealed an expanded stem cell population in the mInsc KI/KI compared to wild type mouse. The consequence of this stem cell expansion in puberty is decreased alveogenesis during pregnancy. Taken together, these results suggest that SLIT2/ROBO1 regulates the self-renewal of mammary stem cells during mammary gland development, and that this in turn influences ductal outgrowth, stem cell numbers and ultimately alveogenesis.
DEDICATION

I dedicate this dissertation to my husband, Blaine. Your endless love and support has given me strength and perseverance. I dedicate this to my daughter, Hanna Linnea, who instantly lit up my life and gave me clarity and purpose. Finally, I dedicate this to my mom, dad and brother who have inspired me to never settle, and have instilled in me the belief that I can become something great.
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Chapter 1

Abstract:

The Slit family of secreted proteins and their transmembrane receptor, Robo, were originally identified in the nervous system where they function as axon guidance cues and branching factors during development. Since their discovery, a great number of additional roles have been attributed to Slit/Robo signaling, including regulating the critical processes of cell proliferation and cell motility in a variety of cell and tissue types. These processes are often deregulated in cancer progression, allowing tumor cells to bypass safeguarding mechanisms in the cell and the environment in order to grow and escape to new tissues. In the last decade, it has been shown that Slit and Robo expression are often altered in a wide variety of cancer types, identifying them as targets during cancer progression. Further, studies have demonstrated Slits and Robos have dual roles in cancer, acting as both oncogenes and tumor suppressors, consistent with their bifunctional roles in axon guidance as both attractant and repellents for neuronal migration. These findings make this signaling axis challenging to define, yet attractive as a target for anti-cancer therapeutics. Here, we summarize our current understanding of the dual roles that Slit/Robo signaling play in development, epithelial tumor progression and tumor angiogenesis.
Introduction:

The existence of axon guidance molecules was postulated by Ramon y Cajal in the late 1800s, but 100 years elapsed before their molecular identification. As their name suggests, these cues act to instruct the migration of neurons and their axons in the developing nervous system, establishing the initial pattern of axonal projections that is subsequently refined by activity-dependent mechanisms. The cues are secreted by both target and non-target cells and are bi-functional, acting as both attractants and repellents in this patterning of the nervous system. Consequently, the response of a cell to these cues is specified by receptors expressed on that cell. These receptors are responsible for translating directional information to the cytoskeleton, which generates the movement and, in the case of axons, directional outgrowth required for pathfinding.

In addition to their role as axon guidance cues, these molecules also function outside the nervous system to regulate the development of other organs, including the immune and vascular systems, and epithelial organs and glands. In these contexts, “axon guidance” cues do not simply function as instructional signals, but rather act more broadly to control the growth, branching, adhesion and position of cells in complex tissues. Like many molecules that play key roles in development, “axon guidance” cues are often deregulated in disease processes, especially cancer, with many of these cues acting to either promote or suppress tumor growth and progression.
Among the many families of “axon guidance” molecules, Slits, signaling through their Roundabout (Robo) receptors, constitute a relatively small group of factors. They were originally identified as chemorepellents that play a crucial role in preventing developing commissural neurons from inappropriately re-crossing the midline. Once believed to have only restricted functions as guidance cues and branching factors in the developing nervous system (Brose et al., 1999; Wang et al., 1999), members of this family of secreted cues are now identified as key regulators of many cellular processes in multiple tissue types, including the mammary gland, heart, lung and kidney (Greenberg et al., 2004; Hinck, 2004; Medioni et al., 2010; Piper et al., 2000). In addition, they have also been implicated in multiple human pathologies including cancer and inflammation (Legg et al., 2008; London and Li, 2011; London et al., 2010; Wu et al., 2001).

As we learn more about the mechanisms of cancer progression, it is becoming clear that tumor cells hijack normal cellular processes to survive and metastasize to secondary tissues. Specifically, a cell’s proliferative, adhesive and migratory properties are often altered in the process of tumor cell transformation, allowing rapid proliferation and tumor growth, detachment from the surrounding tissue, and invasion into the vasculature leading to metastasis. Over the last decade, many studies have implicated Slit/Robo signaling in the regulation of cell proliferation, cell adhesion, and cell migration, raising the possibility that this pathway represents a key target for alteration in cells undergoing tumor cell transformation. In fact, Slit and Robo expression levels are altered in a majority of human cancers. However, recent work
suggests that the role of Slit/Robo in tumor progression is anything but simple. Emerging evidence postulates that Slits and Robos function both as oncogenes and tumor suppressors, often in the same tissue. In this review, we summarize how Slit/Robo signaling confers both tumor suppressive and oncogenic effects on the progression of various types of cancers, focusing most of our discussion on vertebrate systems, though invertebrate studies will be touched upon when relevant.

The Slit/Robo Signaling Interaction is Well Characterized

Slit is a Large Secreted Factor

Slits are secreted extracellular matrix proteins expressed in many cell types and tissues. While invertebrates express only one Slit molecule, vertebrates express three (Slit1, Slit2, and Slit3) that share a high degree of structural conservation (Dickson and Gilestro, 2006). Slits are large multidomain proteins with a unique tandem of four Leucine-rich repeats (LRRs, D1-D4), each connected via disulfide bonds, near the N terminus. These LRRs are followed by seven to nine epidermal growth factor (EGF)-like domains, and a laminin G-like domain capped by a C-terminal cysteine-rich module. Structural studies revealed that the LRR domains each contain a conserved motif that creates a concave shape that might be important for modulating the interaction of Slits with their cognate receptors (Howitt et al., 2004; Morlot et al., 2007a; Morlot et al., 2007b). Further structural studies showed that
Slits undergo post-translational modifications (Figure 1.1A). Slit proteins are proteolytically cleaved within the fifth EGF region to release an N-terminal fragment that binds Robo receptors and mediates all assayed cell guidance functions of Slit/Robo signaling (Nguyen Ba-Charvet et al., 2001).

The most commonly studied member of the Slit family of proteins is Slit2. It is known to regulate many aspects of tissue morphogenesis and cell function, including cell migration, proliferation, adhesion and death. While the function of Slit1 remains largely unknown, recent studies are beginning to ascribe functions to Slit3 in embryonic angiogenesis. Studies showed that Slit3 may function as an angiogenic factor involved in regulating endothelial cell proliferation and motility, in addition to regulating formation of vascular networks (Zhang et al., 2009). Thus, while our current knowledge confirms that the Slit family of axon guidance molecules plays important roles in many aspects of development, it is also clear that there are likely many unknown functions yet to be discovered.

Robo is a Highly Conserved Transmembrane Receptor

The Robo family of receptors is highly conserved, though the number of Robo genes differs between invertebrates and vertebrates. Whereas C. elegans has only one Robo (Sax-3) receptor, Drosophila, chick and zebrafish have three (Robo1–3), and mammals have four (Robo1/Dutt1, Robo2, Robo3/Rig-1, and Robo4/Magic Roundabout) (Figure 1.1B) (Challa et al., 2001; Hohenester, 2008; Huminiecki et al.,
While Robo1–3 contain a high degree of similarity, Robo4 seems to function distinctly from the other Robo family members and is uniquely expressed by endothelial cells (Park et al., 2003), and so will be discussed separately. Robo1–3 are single-pass transmembrane receptors that belong to the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) and are conserved between vertebrates and invertebrates (Hohenester, 2008). The ectodomain of mammalian Robo receptors contains five Ig domains followed by three fibronectin type 3 (FN3) repeats (Figure 1.1B). The Robo intracellular domain has no inherent catalytic activity, but confers a downstream signal by recruiting various factors to cytoplasmic conserved proline-rich domains, referred to as CC0-CC4, and a number of phosphorylatable sites. It was recently demonstrated that the different functions of Robo receptors in neuronal development are due to both gene expression levels and to specific characteristics of the different Robo receptors (Spitzweck et al., 2010). For example, by expressing each of the three Robo receptors from each Robo gene locus, the authors found that lateral positioning of longitudinal axon pathways depends on gene expression, not on the type of Robo expressed, whereas commissure formation depends on the specific combinations of Robos expressed. This suggests that the receptors have some structural differences that confer specific signaling responses, such as prevention of midline crossing by Robo1, and promotion of midline crossing by Robo2 (Spitzweck et al., 2010).

It has long been believed that the different signaling responses induced by each receptor in response to Slit binding are primarily due to variations in the number
and combinations of CC motifs in their intracellular domains. However, recent studies revealed that both intracellular and extracellular domains dictate the resulting signaling response induced by Slit binding. In *Drosophila* neuronal development, Robo2 has both lateral positioning and promidline crossing activities. In a 2010 study, Evans and Bashaw demonstrated that Ig1 and Ig3 are required for lateral positioning, whereas promidline crossing seems to be dictated by Ig2. The authors performed studies in which the cytoplasmic domains of Robo2 and Robo3 were replaced with that of Robo1 and found that stimulation with Slit conferred wild-type activities, strongly suggesting that the specificity lies in the ectodomains. However, complete loss of the cytoplasmic domains ablated all receptor activity, indicating that these are required for signaling. Furthermore, the authors demonstrated that the differences in signaling response are not simply due to differences in Slit2 binding to the ectodomain, but rather are due to differences in multimerization and receptor/ligand stoichiometry dictated by the Ig domains (Evans and Bashaw, 2010). These results suggest that while the cytoplasmic domain is important for downstream signaling, the specificity of the signaling response might be dictated by the ectodomains. As Slits and Robos are known to regulate multiple types of signaling responses, including cell motility and cell proliferation, and to play critical roles in the development of many vital organs including the kidney and breast, it will be interesting to learn if the Ig domains of Robo ectodomains play an equally important role in conferring Slit signaling in these diverse systems.
Cleavage of Robo Reveals Additional Regulatory Complexities

Current efforts examining post-translational modifications of Robo suggest that regulation of this receptor, and consequently the signaling pathways it mediates, is more complex than initially proposed. This was demonstrated using the Alexander hepatoma cell line, PLC/PRF/5, in which the intracellular domain of Robo is successively cleaved by metalloproteases and γ-secretases, yielding two distinct intracellular Robo1 fragments (Robo1-CTF1 and Robo1-CTF2) (Seki et al., 2010). The identification of several nuclear localization signals (NLSs) within these intracellular fragments of Robo1 suggests a potential transcriptional role for the receptor, at least in cancer cells. Biochemical fractionation of MG-132-treated PLC/PRF/5 cells show Robo1-CTF2 exclusively located in the nucleus, whereas Robo1-CTF1 was found in each of the membrane, cytosolic and nuclear fractions (Seki et al., 2010). This suggests that the successive cleavage of the Robo1-CTF is critical for proper localization within the cell and may play a regulatory role. However, removal of all three potential NLSs did not abolish nuclear localization, suggesting that perhaps nuclear localization of Robo1-CTF relies on other currently unidentified effector molecules (Seki et al., 2010). It is clear that further studies of NLS-containing Robo1-CTF binding partners are needed to elucidate the full mechanism of transcriptional regulation by Robo1-CTFs.

In addition to this intracellular cleavage, a number of axon guidance receptors, including Robo, undergo extracellular cleavages, generating protein products that
regulate a number of cellular functions, including migration. Recently, studies in *Drosophila* aimed at elucidating the exact mechanism of Robo activation following Slit-binding revealed a potential role for the metalloprotease-disintegrin Kuzbanian (ADAM 10 in mammals) in generating a free ectodomain by extracellular cleavage. Although Kuz/ADAM10 is expressed in both neurons and midline glia, only neuronal expression was required for Slit/Robo repulsion, suggesting that the protease acts on Robo-expressing neurons and not the glia (Coleman et al., 2010). Furthermore, it appears that extracellular cleavage of Robo is required for receptor activation following Slit stimulation, as expression of an uncleavable form of Robo was unable to rescue a Robo mutant phenotype. It was also found that cleavage of Robo by Kuz/ADAM10 is necessary for the recruitment of Son of sevenless (Sos) and other factors required for Slit/Robo-mediated repulsion at the midline (Coleman et al., 2010). Taken together, these data suggest that cleavage is an important mechanism that regulates the activation of Robo and its signal transduction, and it is likely that subsequent studies will reveal how cleavage regulates the many different functions of Slit/Robo signaling.

**Structural Studies Have Defined the Interaction Between Slit and Robo**

The interactions between Slit and Robo molecules are evolutionarily conserved, as evidenced by studies that showed that human Slit2 is able to bind *Drosophila* robo1 with similar affinity as its mammalian receptor, and vice versa, that
*Drosophila* slit successfully binds rat Robo1 and Robo2 (Brose et al., 1999). Biochemical studies showed that the interaction between this receptor/ligand pair involves the highly conserved second LRR domain (D2) of Slit and the Ig1 domain of Robo, while Ig2-Ig5 and all FN3 domains of Robo1 appear to be dispensable for binding (Figure 1.1C) (Chen et al., 2001; Fukuhaara et al., 2008; Howitt et al., 2004; Liu et al., 2004; Morlot et al., 2007b). Although no strong biochemical evidence for binding between Slits and Robo4 exists to date, *in vivo* studies showed a functional interaction (Jones et al., 2009; Marlow et al., 2010), suggesting that the interaction between ligand and receptor may involve an, as yet, unidentified co-receptor. Thus, with the exception of Robo4, the binding between Slits and Robos is highly conserved and structurally well defined.

Recent insight into the structural requirements for binding between Slits and Robo1 has revealed that the complex can be stabilized by heparan sulfate glycosaminoglycans (GAGs), which are required for functional Slit/Robo signaling in both *Drosophila* and vertebrate neurological development (Figure 1.1C) (Fukuhaara et al., 2008; Hu, 2001; Hussain et al., 2006; Inatani et al., 2003; Ogata-Iwao et al., 2011; Plump et al., 2002; Schulz et al., 2011; Smart et al., 2011). There appears to be dual functions for these GAGs: first, they bind to Slit in the extracellular matrix and stabilize their interaction with Robo, and second, they act on target cells to mediate Slit/Robo signaling by serving as co-receptors.

There are numerous studies demonstrating the importance of GAGs in facilitating the functional interaction between Slits and Robos. Structural studies by
Hussain and colleagues showed that heparin, a highly sulfated variant of heparan sulfate, binds to Slit and forms a ternary complex with Robo, resulting in a 10-fold increase in the affinity between Slit and Robo. Mutational studies demonstrated a key role for the second LRR of Slit (termed D2) in binding heparin via a conserved basic patch, and binding Robo via the adjacent concave face (Fukuhara et al., 2008; Hussain et al., 2006). Further crystallographic studies revealed a contiguous HS/heparin binding surface, extending across the Slit-Robo interface and consistent with at least five HS disaccharide units, as required to support Slit/Robo signaling (Fukuhara et al., 2008). These biochemical analyses are further supported by functional studies in this and others papers, which demonstrated that enzymatic removal of heparin sulfate from neurons using heparanases resulted in a loss of responsiveness to Slit (Hu, 2001; Hussain et al., 2006; Piper et al., 2006). In a different strategy, mutation of exostosin, an enzyme required for heparan sulfate synthesis, resulted in patterning defects at the mouse optic chiasm that phenocopy those observed in the Slit1-/-;Slit2-/- knock-out (Inatani et al., 2003; Plump et al., 2002). A similar study performed in zebrafish found that this loss of heparan sulfate synthesis phenocopied or even enhanced the guidance defects observed in Robo2/astray mutants (Kastenhuber et al., 2009; Lee et al., 2004). Taken together, these studies show that heparan sulfates mediate the formation of stable Slit/Robo signaling complexes and are critical for their signaling function. However, it is unclear whether or not these GAGs constitute membrane bound proteoglycans. Genetic studies in Drosophila suggest that this may be the case, as the heparan sulfate
proteoglycan, glypican, was shown to interact with slit (Liang et al., 1999; Ronca et al., 2001; Zhang et al., 2004) and regulate its distribution (Smart et al., 2011). Thus, heparan sulfate GAGs, either cell associated or present as free sugars in the extracellular matrix, concentrate and localize Slits, shaping the signaling environment by regulating their concentration and accessibility.

On the target cells, the heparan sulfate proteoglycan, syndecan (Sdc), plays a key role as a co-receptor for Robo (Johnson et al., 2004; Rhiner et al., 2005; Steigemann et al., 2004). In the Drosophila embryo, sdc is co-expressed with robo on axons and is absent in slit-secreting midline cells. Mutations in mammalian Sdc enhanced the muscle and axonal patterning phenotypes observed in loss-of-function Slit and Robo animals (Johnson et al., 2004; Steigemann et al., 2004). Moreover, cell type-specific rescue experiments in Sdc mutants revealed that axon guidance defects of the Sdc mutant were entirely rescued by Sdc expression in neurons, while there was no rescuing activity in response to Sdc expression in midline cells (Johnson et al., 2004; Steigemann et al., 2004). These findings indicate that Sdc activity does not participate in the production and/or secretion of Slit, but rather is required for the reception and/or the transmission of Slit signals in Robo-expressing target cells.

Further studies in neural development in C. elegans corroborate this important role for syndecan in regulating Slit/Robo mediated axon guidance. Mutational analyses demonstrated that only the extracellular domain of Sdc is required for Slit/Robo signaling (Chanana et al., 2009; Schulz et al., 2011), and that the chondroitin sulfate modification of Sdc is necessary for its co-receptor function on target cells (Chanana
et al., 2009).

Together, functional and structural evidence supports a model in which heparan sulfate proteoglycans enhance the relatively low-affinity interaction between Slits and Robos by acting as secondary receptors. Furthermore, studies in Drosophila suggest that there are different cellular requirements for proteoglycans, with syndecans acting on target cells, and glypicans acting on the slit-expressing cells. Glypicans are thought to sequester Slit and present it to the syndecan/Robo1 pair, thereby regulating the formation of the ternary signaling complex. Together, these collective interactions help to localize and fine-tune Slit/Robo signaling. While current data suggests that the interaction between Slit, Robo and GAGs represent a significant regulatory relationship, whether these heparan sulfate co-receptors are required for Slit/Robo signaling in all cell and tissues types in higher organisms and whether they play a role in disease processes will require more in-depth studies in mammalian models.

**Slit/Robo: Roles in Epithelial Tumorigenesis**

The role of axon guidance molecules in cancer progression has been studied for over a decade, yet their exact function remains elusive. By their nature, axon guidance molecules are bifunctional, acting as both attractant and repellents for migrating axons and cells. As such, Slits also have this dual role; in one example, this bifunctionality is displayed in a single trajectory of mesodermal cells in the
Drosophila embryo. These cells move away from the ventral midline, repelled by slit, and then migrate toward target muscles, attracted by slit (Kramer et al., 2001). Consistent with this dual role as both positive and negative cues, Slits are also capable of acting as both “friend and foe” in the progression of tumor cells; Slits have been shown to both promote and prevent tumor metastasis by suppressing or enhancing cellular attachments and migration depending on the cellular context (Tseng et al., 2010; Zhou et al., 2011b). This duality of Slit function is also observed in the regulation of tumor cell proliferation and survival where they promote proliferation and angiogenesis in some contexts (Bedell et al., 2005; Dunaway et al., 2011; Kaur et al., 2006; Kaur et al., 2008; Rhee et al., 2007; Rhee et al., 2002; Suchting et al., 2005; Wang et al., 2003; Wang et al., 2008; Yang et al., 2010; Zhou et al., 2011b), and prevent these same processes in others (Brantley-Sieders et al., 2011; Han and Zhang, 2010; Jones et al., 2009; Liu et al., 2006; Macias et al., 2011; Ning et al., 2011; Prasad et al., 2008; Tseng et al., 2010). Thus, current evidence renders it impossible to label Slits and Robos as either tumor suppressors or oncogenes, but, acting in either role, it is clear that they play important functions during tumor progression. This makes them attractive targets for cancer therapeutics and potential candidates for diagnostic purposes.

Slit and Robo Expressions are Altered in Cancer:
The progressive transformation of normal cells into malignant progeny involves the accumulation of genetic changes, such as the loss or silencing of tumor suppressor genes and the induction of oncogenes. Studies show that Slit and Robo expression is altered in a long list of cancers, including prostate, brain, renal, ovarian, cervical, breast and colorectal cancers (Astuti et al., 2004; Dallol et al., 2002; Dallol et al., 2003a; Dallol et al., 2003b; Dickinson et al., 2004; Narayan et al., 2006; Qiu et al., 2011; Shivapurkar et al., 1999a; Shivapurkar et al., 1999b; Singh et al., 2007; Werbowetski-Ogilvie et al., 2006; Xu et al., 2010; Yu et al., 2010). In examining the literature, it is interesting to note that there are examples of both up and down-regulation of these genes, suggesting that the Slit/Robo pathway can function in both promoting and suppressing tumor cell survival, proliferation and migration. Currently it is unclear whether these genes are differentially regulated based on tumor type or stage, but mounting evidence suggests that changes in the expression of these genes play important roles in regulating tumor progression.

*Slit Expression is Altered in Epithelial Tumor Progression*

The most frequently observed alteration of *Slit* expression is downregulation. This is evidenced by allelotyping studies of 44 breast carcinoma samples that show loss of heterozygosity (LOH) at several regions on chromosome 4, one of which has been identified as the *Slit2* gene locus (4q25-26) (Shivapurkar et al., 1999a; Shivapurkar et al., 1999b; Singh et al., 2007). These studies reported allelic deletion...
in 63% of breast carcinomas, 35% of cervical carcinoma and in >60% of small cell lung carcinoma and mesothelioma (Shivapurkar et al., 1999a; Shivapurkar et al., 1999b; Singh et al., 2007). Thus, it seems that a common method for alteration of Slit/Robo signaling in cancer is via Slit gene silencing. In addition to gene loss by deletion, several other mechanisms of gene silencing occur at the Slit gene locus. Of these, the most commonly encountered mechanism is hypermethylation of the promoter region. Numerous studies have shown that regions frequently hypermethylated in cancers contain the genes for Slit1, Slit2, Slit3, Robo1 and Robo3 (Dallol et al., 2005). For example, Slit2 is silenced through hypermethylation in the majority of samples from numerous tumor types including: breast, non-small cell lung cancer, ovarian, gliomas, hepatocellular, colorectal and lymphocytic leukemia (Dallol et al., 2003a; Dallol et al., 2003b; Dunwell et al., 2009; Jin et al., 2009; Qiu et al., 2011; Sharma et al., 2007). In further support of a role for Slits in suppressing tumor growth and, consequently, being silenced during tumor progression, re-expression of Slit2 greatly inhibited the proliferation of transformed cell lines derived from many of these tumor types (Dallol et al., 2002; Dallol et al., 2003b; Jin et al., 2009; Qiu et al., 2011). Although Slit2 is the most frequently studied of the Slit proteins expressed in mammals, similar expression studies reveal silencing of Slit3 via hypermethylation in breast (41%), colorectal (33%) and glioma (29%) tumor cell lines, with similar frequencies of Slit3 and Slit1 promoter hypermethylation reported in these types of primary tumors (Dickinson et al., 2004).

One additional consequence of the epigenetic silencing of Slit2 and Slit3 is
downregulation of microRNA (miR)-218-1 and miR-218-2, which are located within intron 15 of human Slit2 and intron 14 of human Slit3, respectively (Angeloni et al., 2006; Tie et al., 2010). This miR was shown to negatively regulate the expression of Robo1 in gastric, head and nasopharyngeal cancers (Alajez et al., 2011; Tie et al., 2010) providing one explanation for the observation that Robo1 is only infrequently silenced in tumor samples (Grone et al., 2006; Ito et al., 2006; Xu et al., 2010).

Interestingly, it is possible that loss of this negative feedback loop contributes to tumor progression because the inappropriate upregulation of Robo1 in tumor cells could allow them to migrate in response to Slits that are provided by non-tumor cells in the surrounding environment (Alajez et al., 2011), or by tumor cells that have only partially silenced Slit expression (Tie et al., 2010). Thus, hypermethylation of the Slit gene loci in solid tumors may contribute to tumor progression by switching Slit/Robo1 signaling from autocrine to paracrine, facilitating the metastasis of tumor cells that are responding to this deregulated pathway. Given the frequency of Slit hypermethylation in human tumors and its effect on miR-218 expression, this family of genes represents attractive candidates for therapeutic strategies that reverse epigenetic silencing or re-establish miR-218 expression.

Recently, a second mechanism of Slit gene silencing was observed in human cancer samples. A genome-wide location analysis of human prostate cancer samples identified Slit2 as a target of epigenetic repression via the polycomb group (PcG) member EZH2 (Yu et al., 2010). Polycomb group (PcG) proteins are transcriptional repressors that function through multimeric chromatin-associated
polycomb repressive complexes to epigenetically silence gene expression by catalyzing the methylation of specific histone residues. In prostate cancer samples, low Slit2 expression correlated with not only high EZH2 expression level, but also with the aggressiveness of the cancer and the degree of metastasis. Furthermore, treatment with either methylation inhibitors or EZH2-suppressing compounds decreased metastasis and increased Slit2 expression (Yu et al., 2010). Taken together, these studies introduce a novel mode of Slit silencing that had previously not been recognized in cancer samples.

In contrast to downregulation of Slits, which is well documented in the literature, there are relatively few papers that identify the upregulation of Slits in cancer as occurs, for example, in human ductal carcinoma samples and prostate and nitrofen-hypoplastic lung cancers (Brantley-Sieders et al., 2011; Doi et al., 2009; Latil et al., 2003). However, data mining also reveals their upregulation in lobular breast cancers, a type of breast carcinoma that has been ascribed only a few unique molecular characteristics (Christgen et al., 2009; Ma et al., 2004). These data suggest that at least some types of tumors are associated with Slit overexpression, but how it contributes to tumor development in these circumstances is currently unknown.

Robo Expression is Altered in Epithelial Tumor Progression

Robo1 was discovered in Drosophila as a gene required for proper midline crossing of commissural axons during development (Kidd et al., 1999). It was also
found to be deleted in the small-cell lung cancer cell line U2020, hence the name Deleted in U twenty twenty, or Dutt1 (Xian et al., 2001). Robo1 and Dutt1 genes are derived from alternative promoters of the same gene and appear to have differential spatial and temporal patterns of transcriptional activity, with the Dutt1 form expressed ubiquitously, and the Robo1 form restricted primarily to embryogenesis (Clark et al., 2002). In addition to being silenced by deletion, Dutt1 is also hypermethylated in subsets of primary tumor samples, such as primary invasive breast cancer (19%), primary clear cell renal cell cancer (18%) and in primary non-small cell lung cancer (4%). Of those tumors, 80% of breast and 75% of primary clear cell renal cell carcinomas also contain allelic losses in the genomic region containing Dutt1, an observation supporting a role for Dutt1 as a tumor suppressor that obeys Knudson's two hit hypothesis (Dallol et al., 2002). More recently, hypermethylation at the Robo1 and Robo3 gene loci was reported in early dysplastic lesions of head and neck (Ghosh et al., 2009), as well as in cervical cancer (Narayan et al., 2006). While these data point to a tumor suppressor function for Robos, in fact, the percentage of tumors displaying reduced or silenced Robo expression is much less than that seen for Slit genes. Indeed, the opposite is true, with Robo1 expression elevated in numerous cancers, including human hepatocellular carcinoma, colorectal cancer, non-small cell lung cancer, and glioma samples (Gorn et al., 2005; Grone et al., 2006; Ito et al., 2006; Mertsch et al., 2008; Xu et al., 2010). In one of these tumor samples, there was coordinate regulation of Slit and Robo, with decreased Slit concomitant with increased Robo1 expression, as predicted by recent reports on miR-
218 regulation of the Slit/Robo signaling axis (Xu, 2010). Thus, this finding of a negative regulatory loop that upregulates Robo when Slit is silenced adds a layer of complexity to the study of Slit/Robo1 in cancer cells. While it was simple to label the pathway as tumor suppressive when Slits are found to be silenced, this new finding introduces the possibility that, under these circumstances, Slit/Robo signaling can function oncogenically due to the upregulation of Robo in a tumor setting.

In sum, since the first hint that Slit and Robo could play a role in tumor biology, there have been numerous studies documenting changes in their expression level in tumor samples. Recent insights demonstrate the complexity of this regulation, as evidenced by the negative feedback loop for Robo expression under the control of non-coding RNAs encoded intronically in Slit2 and Slit3. The fact that Slits can regulate the expression of Robo proposes the possibility that the opposite might also occur. This regulatory relationship suggests that the level of both ligand and receptor must be assessed when drawing conclusions about the overall effect of Slit/Robo signaling on tumor progression. Moreover, because the Slit/Robo pathway regulates many common signaling pathways that are often deregulated in cancers, such as those mediated by the Rho family of small GTPases and β-catenin, it is becoming clear that changes in Slit and Robo expression have effects that extend beyond the roles originally identified for these proteins as instructive cues for cell migration. In fact, a growing body of literature shows that Slit/Robo signaling affects other aspects of tumor cell behavior, including the survival and growth.
Slits and Robos Mediate Tumor Cell Survival and Proliferation

Once the process of transformation has been initiated by driver mutations, the expansion and progression of premalignant cells to metastatic carcinomas depends on a multi-step process involving the evasion of pro-apoptotic signals and reception of pro-survival and pro-proliferative signals. Thus, the early stages of cancer development involve the response of nascent tumor cells to cues such as Slits in their surrounding environment. While there are only some suggestions in the literature that Slits may regulate cell survival, our understanding of its role in regulating cell proliferation is growing. Slits appear to control cell proliferation through β-catenin, a signaling target that has overlapping functions at the plasma membrane, where it mediates cell-cell communication in association with E-cadherin, and in the nucleus, where it regulates cell proliferation in association with Lef/Tcf transcription factors. Consequently, through this one downstream target, Slits influence two aspects of tumor cell transformation: cell proliferation and adhesion.

Slits and Robos: Inhibitors of Tumor Cell Death

A critical step of tumor cell transformation is achieving immortality. Cells employ several mechanisms to execute apoptosis in order to prevent the survival of rogue cells. Apoptosis can be triggered intrinsically, for example via p53, leading to the release of cytochrome c from the mitochondria and activation of caspases via the apoptosome (Fulda and Debatin, 2006). Apoptosis can also be initiated extrinsically
by signaling through the Death Receptors (DRs), also culminating in the activation of the caspases (Fulda and Debatin, 2006). An alternative extrinsic cell death pathway is mediated by so-called “dependence receptors” that require or “depend on” their ligand to prevent their own constitutive pro-apoptotic signaling, which occurs when their ligand falls below a critical concentration. While Robos have not been identified as dependence receptors, the receptors for the Netrin family of axon guidance cues, DCCs and UNC5s, do fit into this category of receptors. Consequently, in addition to mediating axon guidance through these receptors, Netrin also acts as a survival factor for both axons during normal development and also for cancer cells during tumor progression (Delloye-Bourgeois et al., 2009; Fitamant et al., 2008; Furne et al., 2008). Intriguingly, Slits have been found to regulate Netrins by binding and sequestering them (Stein and Tessier-Lavigne, 2001). Consequently, Slits may act as pro-apoptotic factors by allowing the concentration of Netrin to fall below threshold levels for survival. Although there is no direct evidence to support a link between the regulation of Netrin by Slit, and the loss of apoptosis in cancer, this regulatory loop does suggest a possible mechanism by which Slit might exert a tumor suppressive function by promoting the pro-apoptotic signaling of dependence receptors. The silencing of *Slits*, which occurs in many types of cancer, could, therefore, contribute to the immortality of tumor cells by disabling one of the pathways that culls rogue cells that have tumor forming potential.

*Slits and Robos: Regulators of Tumor Cell Proliferation*
Following survival of a few transformed cells, cancer progression requires proliferation of these cells to generate tumor mass. Investigation into the role of Slit/Robo signaling in regulating cell proliferation revealed both positive and negative effects, as predicted by its bifunctionality as an attractant and repellent guidance cue. In support of a role for Slit in suppressing cell proliferation, numerous studies suggest that signaling through Robo1 regulates the subcellular localization of β-catenin, inhibiting its transcriptional function in the nucleus by promoting its localization at the membrane. In a recent study, we showed that during murine breast (mammary gland) development, Slit/Robo signaling restricted the proliferation of the outer layer of basal cells by increasing the cytoplasmic and membrane pools of β-catenin at the expense of its nuclear pool (Macias et al., 2011). This loss of growth control during early postnatal mammary gland development generated an overabundance of myoepithelial cells that produced an excess of growth factors, leading to an overall increase in cell proliferation and excessive branching morphogenesis. Eventually, these surplus myoepithelial cells invaded the luminal population and disrupted cell adhesion (Strickland et al., 2006), and, along with other changes that occur, such as upregulation of CXCR4 and SDF1, spurred the development of hyperplastic lesions with basal characteristics (Marlow et al., 2008).

The imbalance in growth control during early mammary gland development observed in Robo1-/- tissue provides a gratifying developmental correlate for the role of Slits in suppressing growth in models of breast and non-small cell lung cancer (Prasad et al., 2008; Tseng et al., 2010). In breast cancer cell lines, overexpression of
Slits inhibited the transcriptional activity of β-catenin by activating glycogen synthase kinase (GSK)-3β through the Phosphoinositol-3-kinase (PI3K)/Akt signaling pathway. These overexpressing cells displayed enhanced intercellular adhesions and greater co-localization of β-catenin with the homotypic cell adhesion protein, E-cadherin (Figure 1.2A). Further, studies performed in xenograft models of breast cancer showed that tumors generated from Slit-overexpressing cells are significantly smaller compared to control tumors (Marlow et al., 2008; Prasad et al., 2008). A comparable but converse experiment was performed in a cell line derived from non-small cell lung cancer in which knock-down of Slit increased the metastatic potential of the cells by inhibiting GSK-3β activity, again via the PI3K/Akt pathway (Tseng et al., 2010). This, in turn, increased the levels of nuclear β-catenin and increased the expression of Snail, a crucial regulator of epithelial-mesenchymal transitions, resulting in decreased cadherin expression, reduced cell adhesion and increased cell motility (Figure 1.2A) (Tseng et al., 2010). Taken together, these studies show that, at least in breast and lung, Slits act as tumor suppressors promoting the adhesive role of β-catenin at the membrane at the expense of its proliferative role in the nucleus.

Reminiscent of the context-dependent roles that Slit plays as both attractant and repellent in cell migration (Kramer et al., 2001), the opposite role for Slit as inducer of proliferation has also been documented. In cell lines derived from colorectal carcinoma, Slit/Robo1 signaling enhanced tumor growth and metastasis by regulating cadherin degradation and, thereby, increasing cell proliferation and
migration (Zhou et al., 2011b). Overexpression of either Slit2 or Robo1 or recombinant Slit2 treatment of Robo1-expressing colorectal epithelial carcinoma cells resulted in recruitment of the ubiquitin ligase, Hakai, to E-cadherin and its subsequent ubiquitination and lysosomal degradation (Figure 1.2B). The downregulation of E-cadherin in these cells was accompanied by an epithelial-mesenchymal transition (EMT), increased proliferation and increased migration, and in a xenograft model, this corresponded to increased tumor growth and incidence of liver metastasis.

Clinical data corroborated these observations, showing an increase in Slit and Robo1 expression in metastatic, compared to non-metastatic, human colorectal carcinoma samples. This increase inversely correlated with the overall survival of patients, supporting the idea that in some tumor contexts, Slit/Robo signaling can function oncogenically to promote cell growth and migration (Zhou et al., 2011b). In addition, studies on embryonic chick neural retinal cells have identified a second mechanism for down-regulating cadherin through Slit/Robo. In this setting, Slit/Robo signaling induces the recruitment of Cables to the Abelson tyrosine kinase (Abl), which is bound by Robo (Rhee et al., 2007). This causes Cables, in turn, to bind to β-catenin and form a complex with N-cadherin at the plasma membrane, which brings Abl into position to phosphorylate β-catenin on Y489. This triggers the dissociation of β-catenin from N-cadherin, compromising cell-cell adhesion and allowing translocation of Y489-phosphorylated β-catenin to the nucleus where it activates Tcf/Lef-mediated transcription (Figure 1.2C) (Rhee et al., 2007). Although a correlation between this
Slit/Robo-mediated increase in nuclear β-catenin activity and enhanced cell proliferation was not reported in this study, an increase in proliferation in a different cell type from the retina, retinal pigment epithelial cells, has been observed in response to recombinant Slit2 treatment (Zhou et al., 2011a). Thus, several signaling pathways have been identified that support an oncogenic role for Slit in reducing cell adhesion and enhancing cell proliferation.

Taken together, these studies suggest that Slit/Robo1 signaling regulates cellular proliferation by targeting both cadherins and β-catenin in order to regulate the transcriptional activity of β-catenin. In events that suppress tumor growth, Slit/Robo1 directs the subcellular localization of β-catenin through the PI3K/Akt pathway, an effect that has been documented in both non-small cell lung and breast cancer models, as well as during normal breast development (Macias et al., 2011; Prasad et al., 2008; Tseng et al., 2010). In contrast, two different mechanisms have been identified that achieve oncogenic outcomes downstream of Slit/Robo signaling. In both examples, the cadherin/β-catenin complex is disrupted, releasing β-catenin. However, in one mechanism this occurs through Slit/Robo-induced cadherin lysosomal degradation and in the other, through targeted phosphorylation of β-catenin by an Abl/Robo complex. In conclusion, additional studies are required to determine the extent to which these pro-proliferative, pro-migratory mechanisms regulate Slit signaling in normal and disease settings.
Slits and Robos Mediate Tumor Cell Motility and Metastasis

Tumor cell metastasis requires multiple steps including: weakening the associations between the tumor cell and neighboring cells or the environment, rearranging the actin cytoskeleton to drive formation of actin protrusions and other structures necessary for cell motility, and sensitizing the cell to attractant signaling gradients. These changes occur while the cell is simultaneously desensitized to repellent signaling molecules in the environment, thus allowing cell migration. Slits and Robos have been implicated in each of these steps, and not surprisingly, given their ability to mediate both chemoattraction and chemorepulsion (Kramer et al., 2001), they have been found to act as oncogenes and tumor suppressors that both enhance and inhibit tumor cell invasion, depending on the cellular context.

Slits and Robos: Regulators of Cell-Cell Adhesions

Cadherins are expressed in all epithelial cells and play a key role in establishing contact between a cell and its environment. Cadherin expression is often misregulated in cancer cells, which leads to decreased cell attachment and a more metastatic phenotype (Blanco et al., 2004). This allows tumor cells to migrate and invade the vasculature, leading to cancer metastasis. Slit/Robo signaling has been shown to regulate this first step towards metastasis by influencing cell adhesion through its action on cadherins and β-catenin. As discussed above, one consequence of this regulation is altered subcellular distribution of β-catenin, which increases
proliferation with its translocation to the nucleus (Prasad et al., 2008; Rhee et al., 2007; Rhee et al., 2002; Tseng et al., 2010; Zhou et al., 2011b). There is additional evidence, mostly genetic and collected in developmental settings, that further demonstrates a role for Slit and Robo in regulating cadherin-mediated cell-cell adhesion. Again, unsurprisingly, given Slits function as attractant and repellent in axon guidance, both an increase and decrease in cell adhesion have been attributed to Slit/Robo signaling, dependent on the biological context. For example, a positive role for Slit/Robo in enhancing cell-cell adhesion has been observed during chick cranial trigeminal gangliogenesis when cells derived from neural crest and ectodermal placodes interact to generate ganglionic structures (Shiau and Bronner-Fraser, 2009). Trigeminal placode cells express N-cadherin and Robo1, while the intermingling neural crest cells express Slit1. Loss of either N-cadherin or Robo1 resulted in dispersed and disorganized placodal neurons within the trigeminal ganglion, suggesting that N-cadherin and Robo1 might function in collaboration to mediate the proper coalescence of placode-derived neurons (Shiau and Bronner-Fraser, 2009). In concordant studies, overexpression of either Slit or Robo resulted in both the post-translational upregulation of N-cadherin and its redistribution to the placodal cell membrane, again leading to a model in which Slit/Robo signaling stabilizes sites of cell-cell contact by influencing the subcellular localization of cadherin (Shiau and Bronner-Fraser, 2009). It is important to note, however, that in this study no changes in the level or distribution of β-catenin were reported. In a second example of Slit mediating increased cell adhesion in collaboration with a cadherin, P-cadherin was
shown to co-localize with Slit in the basal cell layers of normal oral mucosa, with this expression down-regulated in oral squamous cell carcinoma (OSCC) (Bauer et al., 2011). In an OSCC cell line that overexpresses P-cadherin, a complex of P-cadherin and Robo3 was detected, and treatment of these cells with Slit resulted in a dose-dependent down-regulation of cell migration that could be relieved using a small interfering RNA that reduced Robo3 expression (Bauer et al., 2011). Taken together, these studies support a tumor suppressive role for the Slit/Robo signaling axis in maintaining cell-cell adhesion and, consequently, a non-invasive cellular state by enhancing cadherin function.

Conversely, slit/robo signaling has also been shown to inhibit cadherin function at the membrane during Drosophila heart tube formation, thus decreasing cell-cell adhesion (Santiago-Martinez et al., 2008). Again, genetic evidence suggests that robo and e-cadherin/shotgun (shg) function together in modulating cardioblast adhesion, but in this biological context, their actions oppose one another. This is evidenced by the observation that robo loss-of-function phenocopied e-cadherin/shg gain-of-function, generating embryos with no lumen due to enhanced cardioblast adhesion (Santiago-Martinez et al., 2008). Similarly robo gain-of-function phenocopied e-cadherin/shg loss-of-function, but in this circumstance lumen formation was blocked due to insufficient cardioblast adhesion. These studies support a role for Robo antagonizing E-cadherin/Shg function, with Robo mediating a repulsive or anti-adhesive signal that functions in opposition of the pro-adhesive actions of E-cadherin/Shg.
By targeting cadherins and the cadherin/β-catenin complex, Slit/Robo signaling regulates two of the crucial steps in tumor progression: cell proliferation and cell adhesion. A challenge for researchers is to understand the circumstances that determine whether this signaling pathway acts positively to enable cell contacts, or negatively to deter them. For other guidance families, different complexes of receptors specify attraction versus repulsion. For example, attraction via Netrin is mediated by DCC in a complex with DSCAM, whereas repulsion requires an UNC5 receptor that acts either together with DCC or alone (Moore et al., 2007). In contrast, no co-receptors have been identified that specifically regulate the attractant or repellent functions of Robo, although perhaps its interaction with cadherin, albeit indirect, serves this role. Regardless of these events at the plasma membrane, a central requirement for either the positive or negative response of a cell to Slit is the interaction of Robo with the actin cytoskeleton, a topic that is discussed in the next section.

*Slits and Robos: Regulators of the Actin Cytoskeleton*

Following detachment of cells from the surrounding tissue, tumor progression requires enhanced cell motility, which is accompanied by increased actin polymerization and the enhanced activity of proteins that optimize its turnover. Developmental studies show that Slit/Robo signaling affects cell motility by controlling the activity of several proteins involved in reorganizing the actin
cytoskeleton, including the small GTPases comprising the Rho-family (Rac, Cdc42, and RhoA), and other key regulators of the actin cytoskeleton, such as the non-receptor tyrosine kinase, Abl, and Ena/Vasp proteins.

*Rho GTPases:* Many studies have shown that Rho GTPases play an important role in modulating the downstream action of Slit/Robo1 signaling. These proteins switch between active and inactive states, and are regulated by GEFs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins): the former stimulates, and the latter inhibits GTPase function. During *Drosophila* neural development, slit/robomediated repulsion of commissural neurons at the midline requires the activation of rac (Fan et al., 2003), and recruitment of both a rac GEF, called son of sevenless (sos), and a rac GAP, called vilse/crGAP (Hu et al., 2005; Lundstrom et al., 2004; Yang and Bashaw, 2006). Sos binds to robo through the adaptor protein dock (Nck in mammals) (Fan et al., 2003; Yang and Bashaw, 2006), whereas vilse/crGAP interacts directly with robo (Lundstrom et al., 2004 Hu, 2005 #798) (Figure 1.3A). Studies show that the activities of both this GAP and GEF support slit/robomediated repulsion, as both sos and vilse/crGAP mutants displayed mild defects in midline repulsion that can be significantly enhanced through loss of one copy of either *slit* or *robo* (Figure 1.3A) (Hu et al., 2005; Yang and Bashaw, 2006). This raises the question; how does both the activation and inhibition of Rac lead to axonal repulsion? One possibility is that these GAPs and GEFs function in distinct steps, with each required for different molecular actions that support repulsion. Alternatively, it could
be that Rac cycling alone is sufficient for repulsion, which may not depend on the maintenance of a specific level of Rac-GTP. In any case, these studies demonstrate the importance of GAPs and GEFs in regulating Slit/Robo signaling by controlling the activity state of small GTPases.

With their central role in regulating the actin cytoskeleton, Rho GTPases are in the unique position to regulate cell motility in response to Slit. Accordingly, a number of studies have demonstrated the importance of Cdc42 in mediating the aggressive spread of tumor cells and the role Slit/Robo1 signaling plays in inhibiting this invasion by attenuating Cdc42 activation. For example, a study on medulloblastoma revealed expression of Slit and Robo in a variety of tumor samples and cell lines, but no evidence that Slit is silenced by methylation (Werbowetski-Ogilvie et al., 2006). Treatment of cells derived from these tumors with Slit2 inhibited their invasiveness, without affecting the direction of migration or their proliferation. The authors suggest that these affects are due to a reduction in the activity of Cdc42 (Werbowetski-Ogilvie et al., 2006). This result is in contrast to data from studies performed on gliomas. Two studies on glioma cell lines and tumor samples reported reduced expression of Slit in primary human glioma specimens and invasive glioma cells, compared to normal brain cells and astrocytes (Parsons et al., 2008; Yiin et al., 2009). Furthermore, treatment of glioma cells with recombinant Slit2 or its overexpression in these cell lines inhibited cell migration and reduced invasion by decreasing Cdc42 activity, an effect that was prevented by siRNA-mediated reduction of either Slit or Robo1 expression. This inhibition of cell
invasion is further confirmed in xenograft studies that demonstrated decreased infiltration of Slit2-expressing glioma cells implanted into the brains of mice. Interestingly, there is no evidence that these effects are mediated through the regulation of β-catenin by Slit as neither the level of β-catenin, its phosphorylation status nor its association with N-cadherin was altered in Slit-expressing glioma cells (Yiin et al., 2009). Taken together these studies on two different types of neural tumors demonstrate that one downstream target of Slit/Robo signaling is the small GTPase Cdc42, and depending on tumor type, its activity is either up or downregulated.

Slit/Robo (s/r) GAP is a strong candidate for the GAP that inhibits the Cdc42 activity downstream of Slit and Robo. s/rGAP has been shown to reduce the activation of both Cdc42 and Rho, but not Rac (Wong et al., 2001). Studies demonstrated that a dominant negative form of this GAP blocked the inactivation of Cdc42 by Slit and also the migration of cells in response to Slit (Wong et al., 2001). Furthermore, a study in tumor and nontransformed cultured cells showed that Slit2/Robo1 counteracts Hepatocyte Growth Factor (HGF)-induced migration by directly targeting and inhibiting Cdc42 and, as a consequence, actin-based protrusive forces (Figure 1.3A) (Stella et al., 2009). Taken together, these data show that Slits inhibit the motility of tumor cells by negatively regulating the Cdc42 Rho GTPase, and that one of the consequences of losing Slit expression during tumor progression is inappropriate cellular migration due to this deregulation.
**Abl and Ena:** In addition to the Rho family of small GTPases, Slit binding to Robo also leads to the recruitment of at least one kinase that regulates both actin cytoskeletal rearrangements and the activity of Robo itself. Structural and genetic studies showed that the Abelson tyrosine kinase (Abl) and its substrate Enabled (Mena in mammals) interact directly with the cytoplasmic domains of Robo (Figure 1.3B) (Bashaw et al., 2000). Genetic studies in *Drosophila* demonstrated opposing roles for ena and abl in robo-mediated axonal repulsion, whereby abl antagonized repulsive robo signaling and ena enhanced it (Bashaw et al., 2000). In a series of studies, it was shown that reducing the level of abl suppressed robo loss-of-function phenotypes, while its overexpression inhibited robo function. The opposite is true for ena, as reducing its levels enhanced robo loss-of-function phenotypes and suppressed robo gain-of-function. Abl phosphorylates robo to inhibit its function because a Y-F mutation in a conserved tyrosine that is targeted by abl generated a hyperactive robo receptor. In contrast, deleting the cytoplasmic domain of robo that binds ena reduced the ability of that robo mutant to rescue robo loss-of-function phenotypes (Bashaw et al., 2000).

While these studies demonstrate the consequences of Abl and Ena interactions with Robo, the molecular mechanism by which these proteins mediate their effect on the directional outgrowth of an axon through Robo is still poorly understood. For Abl, one possibility is that it binds to, and phosphorylates unliganded Robo, inhibiting the ability of signaling proteins to interact with their docking sites on the Robo cytoplasmic domain, It is possible that this inhibition could be relieved by Slit.
Abl is known to phosphorylate Ena, but the significance of this phosphorylation is poorly understood. The Ena proteins (Mena, Vasp and EVL in vertebrates) contain N and C terminal Ena/Vasp homology domains that flank a proline-rich central region. They are generally thought of as positive regulators of actin assembly that function in promoting the growth of long, sparsely branched actin filament networks. Consequently, it is still unclear how a protein that enhances actin polymerization and filopodial/lamellipodial protrusion plays a role in repulsive axon guidance downstream of Robo (Bear and Gertler, 2009), except that it may direct the growth of the cell away from Slit by promoting assembly at sites distal to high ligand concentration. Moreover, few studies have been published on how Ena and Abl contribute to the migration of tumor cells in response to Slit. Transformation of cells with the Src oncogene activated Abl, which was shown to stabilize Robo1 at the plasma membrane, leading to the activation of both Cdc42 and Rac, as well as Slit-independent cell migration (Khusial et al., 2010). Thus, under these circumstances, activation of Robo positively regulates downstream Rho GTPases and induces migration, whereas during central nervous system development and in other tumor types, the opposite effect is observed. Taken together, these studies demonstrate that Slit/Robo signaling communicates with the actin cytoskeleton to regulate cell motility, although the nature of the response depends upon the developmental and disease context. It is clear from many studies that Rho GTPases, Abl and Ena all play important roles in promoting tumor cell metastasis (Allington and Schiemann, 2011; Gertler and Condeelis, 2011; Hall, 2009). However, additional research is required to
determine how Slits regulate these cytoskeletal effectors in tumor cells and whether Slits could be potential therapeutic targets for hindering tumor cell motility by interfering with these signaling routines.

*Slits and Robos: Regulators of Cell Chemotaxis*

Cells must decipher and integrate a complex set of signals in order to migrate toward targets. Indeed, even the metastatic migration of tumor cells is not a random walk and many types of cancers preferentially target specific organs. While Slit is one of the cues cells respond to in the extracellular environment, there are many others, such as chemokines. Compared to large, extracellular matrix-associated Slits, chemokines are small (8-10 kD) soluble factors, first identified in the immune system, but now with documented roles in regulating the migration of many cell types, including tumor cells. A number of studies have examined how Slits affect the motility of immune and tumor cells in response to other extracellular factors, notably chemokines, but also growth factors such as HGF and Platelet-Derived Growth Factor (PDGF). Although the preponderance of data support a role for Slits in inhibiting the migration of cells responding to stimulant, there are two examples of Slit augmenting cell migration in response to cues (Schmid et al., 2007; Ye et al., 2010).

The first of these studies was published over a decade ago. Standard Transwell assays were used to evaluate the effects of Slit on leukocyte migration from the upper to lower chamber in response the chemokine CXCL12 (SDF1) or
bacterial chemotactic factor, \( N \)-formyl peptide f-Met-Leu-Phe (fMLP). It was found that Slit reduced the chemotactic migration of leukocytes when added to either the upper, lower or both chambers, indicating that Slit reduces the overall motility of cells, rather than acting as a repulsive cue that guides their migration (Wu et al., 2001). Further studies have refined our understanding of the underlying molecular mechanisms. Videomicroscopic live cell tracking demonstrates that Slit2 selectively impairs chemotaxis, defined as the directional migration of cells, but not chemokinesis, which is the random movement of neutrophils in response to stimulant (Tole et al., 2009). Slit2 achieves this effect by suppressing the activation of Cdc42 and Rac2 that would normally occur in response to stimulation, with consequent disruption of actin free barbed end formation. A similar inhibition of migration and downregulation of Rac activity was observed in vascular smooth muscle cells in response to platelet derived growth factor (Liu et al., 2006).

These observations were translated in vivo using a mouse model of chemical irritant peritonitis. Preadministration of Slit2 either intraperitoneally or by tail vein injection significantly reduced the recruitment of neutrophils to the site of inflammation (Tole et al., 2009). These data suggest that localized or systemic delivery of Slit2 reduces leukocyte recruitment and, consequently, the tissue damage associated with inflammation. This finding is in accordance with observations from other inflammation models, including glomerulonephritis-associated kidney injury, global cerebral ischemia, and skin sensitization to allergin (Altay et al., 2007; Guan et al., 2003; Kanellis et al., 2004), in which Slit similarly functions in an anti-
inflammatory manner. However, a recently published study using two different models of allergic airway inflammation suggests that Slits have a more complex role in the immune system. In the first model of endotoxin-induced lung inflammation, Slits acted bifunctionally to suppress neutrophil chemotaxis, while in the second model of ovalbumin (OVA) airway inflammation, Slits appeared to enhance eosinophil chemotaxis (Ye et al., 2010). Eosinophils and neutrophils both express Robo1, while Clara cells in the bronchial epithelium secrete Slit2. Aerosol challenge of wildtype mice with OVA triggered leukocyte infiltration, primarily eosinophils, and this infiltration was significantly enhanced in Slit2 transgenic (Slit2-Tg) mice, which express nonselective overexpression of Slit2 under the control of the cPMV promoter (Yang et al., 2010). Similarly, aerosol challenge of wildtype mice with an endotoxin also triggered leukocyte infiltration, but in contrast, primarily neutrophils were mobilized. Moreover, in this case, significantly fewer neutrophils were observed in the lungs of Slit2-Tg mice, an effect that was reversed by the application of a function-blocking antibody directed against the extracellular domain of Robo1. Together with in vitro studies that demonstrate an enhancement in eotaxin-induced eosinophil migration by Slit, but a reduction in CXCL12 mediated neutrophil migration, these data suggest that, depending on the circumstance, Slits can have differential effects on leukocytes. The molecular basis for these distinct responses can be traced to levels of s/rGAP expression, with eosinophils containing significantly lower levels of this Slit/Robo effector compared to neutrophils. This results in the activation of Cdc42 in eosinophils, rather than inhibition, which occurs
in neutrophils when s/rGAP is present (Yang et al., 2010). Thus, even though almost a decade of work has pointed to a single role for Slit as an inhibitory factor in the immune system, with this recent finding, it appears that this is not the case and that, once again, depending on the cellular context, Slit has a dual role as activator and inhibitor of cellular response.

The role of Slit as an inhibitor of inflammation has potentially far-reaching implications in terms of its role in cancer biology as a tumor suppressor. In normal tissue during wound healing, removing the irritant or completing the repair limits the inflammatory response. In contrast, tumors become essentially unhealed wounds, characterized by chronic inflammation, which promotes rather than suppresses tumor growth by releasing growth and survival factors, creating genomic instability, promoting angiogenesis and remodeling the extracellular matrix to facilitate invasion. That Slits inhibit the infiltration of not only leukocytes, but also dendritic cells (Guan et al., 2003) T lymphocytes and monocytes (Prasad et al., 2007), could be harnessed therapeutically to normalize the inflammatory network and restrict infiltrating cells with tumor-promoting properties, while attracting those cells with tumor-suppressing properties.

Another way that Slit could function as a therapeutic agent in the war on cancer is by inhibiting metastasizing cells. This has been evidenced by a number of studies using breast cancer models that demonstrated the ability of Slit/Robo signaling to counter the pro-migratory, pro-metastatic consequences of the CXCL12/CXCR4 chemokine axis. A study by Muller and colleagues a decade ago showed
that the pattern of breast cancer metastases is governed, at least in part, by this chemokine axis (Muller et al., 2001). CXCR4 is upregulated in breast cancer cells (Salvucci et al., 2006), and, upon metastasis, guides these cells to organ sites with high CXCL12 levels: lung, liver and bone. The involvement of CXCR4 in metastasis is not confined to breast cancer, as it is also expressed in other tumor cell lines that respond to CXCL12, such as astrogliomas, prostate carcinomas, B-cell lymphomas and chronic lymphocytic leukemias (Moore, 2001). Using Transwell filters, two studies have demonstrated that Slit2 has the capacity to counteract CXCL12-induced chemotaxis of breast cancer cell lines that express both Robo1 and CXCR4 (Prasad et al., 2004; Schmid et al., 2007). By signaling through Robo1, Slit inhibited a number of downstream effectors that are activated by CXCR4, such as the focal adhesion components RAFTK/Pyk2, focal adhesion kinase, paxillin, PI3K, p44/42 MAP kinase, and metalloproteases 2 and 9 (Prasad et al., 2004). In the absence of Slit, which is downregulated in over 50% of sampled breast tumors, the expression of both CXCL12 and CXCR4 was upregulated. This contributed to the development of hyperplastic lesions in Slit and Robo1 knockout mammary glands (Marlow et al., 2010). Another study on the role of Slit/Robo signaling in the inhibition of breast cancer cell migration in response to CXCL12 implicates ubiquitin-specific protease 33 (USP33), a deubiquitinating enzyme. The authors provide evidence that Slit stalls the chemotaxis of breast cancer cells by inducing the redistribution of Robo to the plasma membrane, a process that is dependent on USP33 (Yuasa-Kawada et al., 2009). Taken together, these data once again raise the possibility that Slits could...
function therapeutically, in this case to combat tumor metastasis by inhibiting tumor cell migration in response to CXCL12.

Slit/Robo: Roles in Tumor Angiogenesis

Angiogenesis describes the process of new vessel growth from mature, pre-existing vessels. This process occurs naturally throughout development and later in the adult during both wound healing and pregnancy, both times of increased tissue remodeling. Angiogenesis involves several steps: first, endothelial cells migrate towards a pro-angiogenic stimulus, such as Vascular Endothelial Growth Factor (VEGF), released extracellularly into the environment, and second, endothelial cells become migratory and congregate at the source of the angiogenic stimulus to form loops, and later vessels, as more and more cells arrive (Potente et al., 2011). Tumor cells often hijack several aspects of this process in order to enable additional tumor growth and metastasis, both of which require an increased supply of oxygen and other nutrients. Without increased angiogenesis, tumors are limited in their growth potential and become necrotic. Thus, tumor cells must induce angiogenesis to transition from a small group of cells to a large, malignant tumor, and to ultimately metastasize to other tissues. These processes require intimate communication between tumor cells and endothelial cells, and studies from the last decade provide strong support for the Slit/Robo pathway in mediating this crosstalk. However, how Slit/Robo signaling affects tumor angiogenesis remains unclear, as reports
demonstrate both pro- and anti-angiogenic functions in pathological systems. What is clear, however, is that Slits and Robos play key roles in regulating the process of tumor angiogenesis.

**Vascular Expression of Slits and Robos**

Of the three Slit proteins expressed in vertebrates, multiple studies have reported Slit2 and Slit3 in the vasculature in multiple cell and tissue types (Han and Zhang, 2010; Humiecki and Bicknell, 2000; Humiecki et al., 2002; Marlow et al., 2010; Park et al., 2003; Wu et al., 2001). On the one hand, only one study reported detection of Slit1 in the vasculature, suggesting that it may not play such a key role in developmental and tumor angiogenesis, although future studies may change this view (Abdollahi et al., 2007). In the vasculature, Slit2 and Slit3 signal via Robo4, which is expressed on the surface of endothelial cells, and to a lesser extent, Robo1, which may also be expressed on at least some types of blood vessels (Huminiecki et al., 2002; Legg et al., 2008; Park et al., 2003; Sheldon et al., 2009; Verissimo et al., 2009). Robo4 was the last member of the Roundabout family of receptors identified due, in part, to its apparent lack of structural homology with the other Robo receptors. In contrast to the five Ig domains and three FN3 domains of Robo1-3, Robo4 contains only two Ig and two FN3 domains in its extracellular domain, and only two CC domains, CC0 and CC2, in its intracellular domain (Huminiecki and Bicknell, 2000; Huminiecki et al., 2002). Moreover, while strong evidence for an interaction between
the extracellular domain of Robo1-3 and the D2 domain of Slits exist, only limited evidence exists to support binding between Slits and Robo4 (Hohenester et al., 2006). Nevertheless, in vitro and in vivo functional studies demonstrate Robo4-dependent Slit signaling in the vasculature, supporting a role for Robo4 as at a component of a Slit receptor complex (Huminiecki and Bicknell, 2000; Huminiecki et al., 2002; Jones et al., 2009; Kaur et al., 2006; Marlow et al., 2010; Park et al., 2003).

Function of Slits and Robos in the Vasculature

Robo4 was initially identified as a regulator of angiogenesis in a study aimed at identifying genes whose expression is perturbed in an Activin receptor-like (Alk) mutant mouse model (Park et al., 2003). Alk is a member of the TGF-β superfamily of receptors and is involved in the normal development of the vasculature (Johnson et al., 1996). Mice lacking Alk expression developed abnormal connections between arterial and venous vascular beds and died at midgestation (Urness et al., 2000). In this study, Robo4 was identified as a gene whose expression is perturbed in these Alk mutants, suggesting that it might play a role in regulating normal vascular development. Subsequent studies showed that Robo4 is highly expressed during embryogenesis in developing blood vessels (Park et al., 2003), and in the adult at sites of active angiogenesis (Huminiecki et al., 2002). Studies in zebrafish support a developmental role for robo4, as either its morpholino knockdown or overexpression resulted in stunted or absent intersomitic vessels, although normal patterning of axial
vessels were seen, suggesting both primary and redundant roles for \textit{robo4} in this system (Bedell et al., 2005). Furthermore, numerous studies have shown that Slits affect the migration of endothelial cells, acting as both a chemoattractant (Howitt et al., 2004; Kaur et al., 2006; Wang et al., 2003; Wang et al., 2008) and chemorepellent (Marlow et al., 2010; Park et al., 2003; Seth et al., 2005; Zhang et al., 2009). It is therefore surprising that \textit{Robo4-/-} mice displayed normal vessel patterning in a variety of contexts: intersomitic and cephalic vessels during early embryogenesis, stereotypical nerve-artery alignment in embryonic limb skin and normal vascularization of the mammary gland during postnatal organogenesis (Jones et al., 2008; Marlow et al., 2010). These data raise the question of whether or not Robo4 has an alternate function in the mammalian endothelium.

Although a comprehensive understanding of the function of Robo4 in the vasculature has been hindered by contradictory findings, there is currently a growing consensus that Robo4 signaling maintains the barrier function of the mature vascular network and restrains angiogenesis during pathological neovascular processes (Han and Zhang, 2010; Huang et al., 2009a; Jones et al., 2008; Jones et al., 2009; Koch et al., 2011; London and Li, 2011; Mulik et al., 2011), as well as during normal periods of robust sprouting angiogenesis such as occurs in pregnancy (Marlow et al., 2010). Again analysis of the \textit{Robo4-/-} mouse provided the first insight into this function by showing that in wildtype, but not knockout animals, Slit2 suppressed VEGF-induced hyperpermeability of retinal endothelium (Jones et al., 2009). Further experiments employed two models of vascular disease, oxygen-induced retinopathy and laser-
induced choroidal neovascularization, both of which resulted in pathological angiogenesis (Jones et al., 2009). In both cases, intravitreal administration of Slit2 reduced angiogenesis in wildtype mice, but not *Robo4*-/- mice. However, the opposite effect has been observed in concordant experiments looking at breast development and cancer, where loss of Slit/Robo4 signaling resulted in excessive angiogenesis in response to VEGF, which was elevated during pregnancy and preneoplasia (Marlow et al., 2010). Again, there was no phenotype in the absence of pro-angiogenic stimulation (Marlow et al., 2010). In all these examples, Slit/Robo4 regulates angiogenesis by inhibiting signaling downstream of VEGF/VEGFR, including the activation of Src, Rac and FAK (Jones et al., 2008; Marlow et al., 2010). Further analysis of the pathway showed that Robo4 interacts directly with paxillin and the ArfGAP (ADP-ribosylation factor- directed GTPase activating proteins), GIT1, to block the activation of Arf6 in response to VEGF and fibronectin (Figure 1.4A) (Jones et al., 2009).

Additional studies on disease models support the role of Slit/Robo4 signaling in inhibiting angiogenesis and enhancing vascular stability. One study used infection with herpes simplex virus (HSV) to generate chronic inflammatory lesions, called stromal keratitis, in the cornea, a condition of pathogical angiogenesis that is driven by VEGF (Suryawanshi et al., 2011) (Mulik et al., 2011). Following HSV infection, *Robo4* was upregulated in endothelial cells of the corneal tissues. However, a corresponding increase in *Slit2* was not observed, suggesting that the Slit/Robo4 signaling axis is unable to control angiogenesis as a result of this infection due to
limited ligand supply. To investigate, Slit2 was subconjunctivally administered with the result of reduced neovascularization, which was not observed upon knockdown of Slit expression using shRNA. The researchers also examined the activity status of Arf6 and Rac, which were found to be reduced after Slit2 treatment, supporting previous studies that Slit/Robo signaling opposes VEGF/VEGFR signaling by modulating downstream signaling pathways. In other studies, an inflammatory reaction was triggered by the administration of lipopolysaccharide (LPS), which induced endothelial hyperpermeability (London et al., 2010). In this context, Slit2 treatment reduced LPS-induced vascular permeability by increasing the localization of V/E cadherin to cell/cell contacts and stabilizing its interaction with p120-catenin. Taken together, these studies have paved the way to a new view on the function of Robo4 in which it functions as a guardian of blood vessel stability by countering pro-angiogenic and pro-inflammatory signals.

There are, nevertheless, several aspects to this model of Robo4 function that remain unresolved. First, is the question concerning the role of Robo1, which is (1) expressed on at least some endothelial cells, (2) heterodimerizes with Robo4 (Kaur et al., 2006; Kaur et al., 2008; Sheldon et al., 2009), and (3) is required for some (Sheldon et al., 2009), but not all (Marlow et al., 2010), of Robo4 functions. The papers summarized above describing the anti-angiogenic and anti-inflammatory roles for Robo4 do not address the function of Robo1 in these processes. However, there is some evidence that Robo1 binds Robo4 and may be required to transduce the Slit signal. Because Robo4 lacks many of amino acids critical for Slit2 binding (Morlot
et al., 2007b), it is considered unlikely that it binds Slit directly. Instead, Robo1 may serve as a co-receptor for Robo4, and in this circumstance, the heterodimer may function to promote endothelial cell migration and angiogenesis (Figure 1.4B) (Kaur et al., 2006; Kaur et al., 2008; Sheldon et al., 2009). The notion that Robo1 plays a functional role in angiogenesis, either alone or in complex with Robo4, is supported by a number of studies, including one on tumor angiogenesis (Wang et al., 2003). In addition, the analysis of gene expression levels during retinal development reveals fluctuations in Robo1 levels that coincide with times of active retinal vascular development, and further that loss of Robo1 in monkey choroidal retinal endothelial cells perturbs tube formation, in addition to lowering cell proliferation and migration (Huang et al., 2009b). An assay to elucidate human genes whose expression correlates with either increased or decreased angiogenesis identified Robo1 as a putative proangiogenic gene (Abdollahi et al., 2007). Moreover, elevated levels of Robo1 are seen in several pathological animal models, such as retinopathy of prematurity and neovascularized corneas (Han and Zhang, 2010; Huang et al., 2009a). Taken together these data suggest that Robo1 functions to restrict angiogenesis, either on its own or in a complex with Robo4, but additional studies are required to fully elucidate its role during developmental and pathological angiogenesis.

A second issue concerning Robo4 and its role in angiogenesis is the uncertain status of Slit as ligand. Since it is unlikely that Slit binds Robo4, Koch and colleagues undertook a protein-protein interaction screen to identify candidate Robo4
binding partners using its extracellular domain fused to the Fc portion of IgG as bait. They identified a single binding partner, UNC5B, which is a chemorepellent receptor for Netrin. Further characterization of this interaction resulted in the surprising finding that Robo4 acts as the ligand, not the receptor, in this relationship, and that Robo4/UNC5B signaling counters the activation of Src kinase by VEGF/VEGFR (Figure 1.4C) (Koch et al., 2011). Moreover, soluble Robo4 protein rescues vessel hyperpermeability in the Robo4-/- mice, all data that support an interconnected role for these proteins in regulating VEGF signaling. There is, however, no evidence that the vascular phenotypes of Robo4-/- and Unc5b-/- mice are similar, a result that may be expected for proteins in a ligand/receptor relationship. Altogether, these data suggest that Robo4 signals through UNC5B to block signaling pathways downstream of VEGF/VEGFR and, consequently, to inhibit VEGF-induced changes in blood vessels. This is the same mechanism of action that had previously been proposed for Slit/Robo4 signaling (Figure 1.4A), suggesting that there are elaborate mechanisms regulating VEGF signaling in endothelial cells.

Slit and Robo Expression is Altered in Tumor Angiogenesis

Like other members of the Robo family of receptors, there is documentation that the level of Robo4 expression is altered in samples from tumor and other diseases. Increased Robo4 expression on endothelial cells has been reported in a number of pathologies, including tumor samples that display increased angiogenesis.
Conversely, decreased Robo4 expression has also been observed in hepatocellular carcinoma samples analyzed by quantitative RT-PCR (Avci et al., 2008), and datasets from microarray analyses on human breast tumor samples show decreased Robo4 expression in human breast cancer (Richardson et al., 2006). These expression data suggest that one way a pro-angiogenic tumor environment reduces Slit/Robo4 signaling and, as a result, releases the brake on VEGF/VEGFR signaling, could by downregulating Robo4 expression. Thus, while it is possible that Robo4 is both up- and down-regulated in disease models, at this point the data suggest that it is most often increased, suggesting a link between Slit/Robo4 signaling and new vessel formation, at least in a tumorigenic setting. There is some documentation of Robo1 expression on endothelial cells in tumors and other tissues undergoing neovascularization (Han and Zhang, 2010; Wang et al., 2003), although whether Robo1 is expressed and how it functions in blood vessels remains controversial. Of the Slit proteins, Slit2 and Slit3 have been detected in normal tissues in vasculature smooth muscle cells/pericytes that encircle blood vessels (Jones et al., 2009; Liu et al., 2006; Marlow et al., 2010). Their regulation in tissues surrounding blood vessels has already been discussed, with examples of both up and down regulation of Slit expression in tumors and other tissues undergoing neovascularization (Han and Zhang, 2010; Wang et al., 2008; Yang et al., 2010). There is also some data suggesting that Slit expression is regulated by EphA2 tyrosine kinase, and that loss of EphA2 expression in a tumor setting elevates Slit expression, which acts in a pro-
angiogenic manner (Brantley-Sieders et al., 2011).

**Slits and Robos: Regulators of Tumor Angiogenesis**

Tumor growth and metastasis requires increased supplies of oxygen and nutrients, and this need can be met by the induction of enhanced angiogenesis in the surrounding vasculature (Potente et al., 2011). In order to achieve increased angiogenesis, tumors must eliminate anti-angiogenic signals within endothelial cells and then secrete proangiogenic cues to attract endothelial cells (Kerbel, 2008). Currently there are no studies that directly examine the functional role of Robo4 in tumor angiogenesis, although in a preneoplastic setting in breast, loss of Robo4 resulted in enhanced angiogenesis in response to epithelial-derived VEGF and CXCL12 (Marlow et al., 2010). These data are in line with the documented anti-angiogenic role of Robo4 in stabilizing the vasculature during pathological angiogenesis as described above.

In contrast, Geng and colleagues have shown that Robo1 is expressed on tumor endothelial cells and plays a role in new vessel formation in tumors that upregulate Slit expression (Wang et al., 2003; Wang et al., 2008). In these studies, a function-blocking antibody to Robo1, called R5, or the extracellular domain of Robo1, inhibited both the chemoattractive migration of endothelial cells and their tube formation in response to Slit2, as well as tumor angiogenesis in xenograft models, suggesting that Slit2 functions pro-angiogenically in tumors that overexpress
it, and further, that its signal is transduced via Robo1, and not Robo4 (Wang et al., 2003; Wang et al., 2008). Furthermore, overexpression of Slit2 in a non-metastatic pancreatic islet cell model of carcinogenesis (RIP1-Tag2) was reported by the same group to promote tumor lymphangiogenesis and lymphatic metastasis by signaling through Robo1, expressed on the lymphatic endothelial cells. Again, administration of the R5 function-blocking antibody reversed the enhanced angiogenesis and decreased tumor formation (Yang et al., 2010).

A possible explanation for the apparent pro- versus anti-angiogenic functions of Slit2/Robo signaling comes from a study by Dunaway and colleagues, in which Slit2 promotes angiogenesis on its own, but inhibits it in the presence of ephrin-A1 (Dunaway et al., 2011). Ephrin-A1 is the primary glycosylphosphatidylinositol (GPI)-linked ligand for the EphA2 receptor, whose expression was shown by these researchers in a previous paper to inversely correlate with Slit expression (Brantley-Sieders et al., 2011). The present study, however, shows that Slit2 stimulates angiogenesis through the activation of Akt and Rac GTPase, an effect that is inhibited in the presence of ephrin-A1. Thus, this study echoes the common theme that, because Slit/Robo signaling targets common signaling pathways, the context in which it signals determines the outcome. Clearly, additional studies are required to understand the complex and intertwined signaling that regulates neovascularization during disease processes. The current data are incomplete and conflicting, but there is one take home message from all these studies: Slit plays a central role in endothelial cell biology. As such, it holds tremendous promise as a therapeutic agent.
that could be used to treat pathologies and cancers that are made worse by enhanced angiogenesis.
Figures:

Figure 1.1. Structural Representation of Slits, Robos and Their Interaction. (A) At their N-terminus, vertebrate and invertebrate Slits consist of four Leucine-Rich Repeats (LRRs), termed D1 – D4. These LRRs are followed by seven-nine epidermal growth factor (EGF)-like domains, a laminin G-like domain (ALPS), and a C-terminal cysteine-rich knot. Slits are proteolytically cleaved between two EGF-like domains. (B) Vertebrates have four Robos (Robo1-4), while fly, chick and zebrafish have three (Robo1-3). Robo1, 2 and 3 contain five immunoglobulin (Ig) domains and 3 fibronectin type 3 (FN3) domains. Robo4 contains only two Ig domains and two FN3 domains. Zebrafish Robo4 is unique in that it contains three Ig domains instead of two. In their cytoplasmic tail, Robos contain between two and four conserved proline-rich domains (CC0-CC3). (C) The Slit/Robo signaling pair can be stabilized via heparan sulfate glycosaminoglycans (GAGs) that are present either in the extracellular matrix or attached to membrane-associated proteins such as the heparan sulfate proteoglycan (HSPG) syndecan.
Figure 1.1 (A-C)
Figure 1.2: Slit/Robo Signaling Regulates Cell Proliferation and Cell-Cell Contacts by Controlling the Localization of β-catenin In the Cell. (A) As illustrated by red arrows, binding of Slit to Robo inhibits phosphatidylinositol kinase (PI3K)-induced Akt activity. Glycogen synthase kinase-3beta (GSK3β) is consequently left in its non-phosphorylated, active form, and targets β-catenin for phosphorylation, excluding it from the nucleus and thus preventing its transcriptional activity. Cytoplasmic β-catenin either becomes ubiquitinated through the GSK3β-adenomatous polyposis coli (APC)-Axin complex or transferred to the membrane where it interacts with E-cadherin, stabilizing cell-cell contacts and preventing cell migration. As illustrated by green lines, Slit2/Robo1 signaling blocks Snail expression by preventing the translocation of β-catenin to the nucleus, thus relieving the repression of E-cadherin expression and enhancing cell-cell contacts.

Slit/Robo signaling can also function to decrease cell-cell contacts and increase proliferation (B, C). (B) Slit/Robo signaling recruits the Abelson tyrosine kinase (Abl), which binds to the adaptor protein Cables. Cables links the Robo/Abl complex to the N-Cadherin/β-catenin complex, thus enabling Abl to phosphorylate β-catenin on Y489, causing its translocation to the nucleus where it activates transcription of cell proliferation genes. (C) In another context, Slit/Robo signaling drives cell migration by recruiting the ubiquitin ligase Hakai to E-cadherin and of β-catenin. This results proteasomal degradation of β-catenin and Hakai-mediated lysosomal
degradation of E-cadherin, causing decreased cell-cell contacts and enhanced cell migration.
Figure 1.2 (A-C)
Figure 1.3: Slit/Robo Signaling Regulates Cell Migration by Controlling the Activation State of Actin Cytoskeleton Modulators.  (A) Slit/Robo signaling regulates actin polymerization, and thus cell migration, by controlling the activity level of Rho GTPases. Slit/Robo signaling prevents cell migration by recruiting Slit/Robo (s/r)GAPs to the CC1 and CC2 domains, which inactivate the small Rho GTPases RhoA and Cdc42, and Vilse/CrGAP to the CC0 domain, which exchanges RacGTP for RacGDP. In other contexts, Slit/Robo signaling drives actin polymerization by recruiting Dock, which in turn recruits son of sevenless (SoS) GEF and PAK p21-activated kinase. SoS GEF activates the small Rho GTPase Rac by exchanging GDP for GTP, leading to actin polymerization. RacGTP in turn activates PAK, which also drives actin polymerization.  (B) The anti-migratory function of Slit/Robo signaling is regulated by the Abl, which attenuates Robo signaling via phosphorylation of Robo at Y1073 near CC0, possibly preventing substrate binding, and by directly targeting the Robo effector protein Enabled (Ena). In the absence of Abl, Ena binds to CC0 and functions to inhibit cell migration by preventing actin polymerization.
Figure 1.3 (A, B)
Figure 1.4: Slit/Robo Signaling Regulates the Process of Tumor Angiogenesis.

(A) Slit2 binds to Robo4/proteoglycan complex and signals to block pro-angiogenic signaling downstream of VEGF/VEGFR. (B) Slit promotes angiogenesis by binding to a Robo1/Robo4 heterodimer and driving endothelial migration. (C) Robo4 binds UNC5B, and signals to block pro-angiogenic signaling downstream of VEGF/VEGFR.

Figure 1.4 (A-C)
Conclusion:

Since their early discovery as key regulators of axon migration across the midline in the developing nervous system, the Slit/Robo signaling pair has been implicated in a wide variety of key developmental and pathological processes. More specifically, Slit/Robo signaling has been found to have a significant impact on a cell’s behavior, from regulating cell migration to controlling cell growth, both processes critical in tumor cell progression. When activated aberrantly, these signaling events can promote tumor cell growth and migration, contributing to tumor metastasis and poor patient prognosis. As such, Slits and Robos are ideal candidates for anti-cancer therapeutics. For example, in its pro-migratory role, Slit-induced Robo signaling causes decreased cell-cell attachments and initiates pro-migratory pathways. In this context, treatment with RoboN, the ectodomain of Robo1, causes sequestration of Slit in the environment, thus stabilizing cell-cell attachments and preventing metastasis. Alternatively, Slit/Robo signaling has been shown to enhance cell-cell contacts by increasing E-cadherin stability and downregulating transcriptional programs that promote proliferation. In this case, sequestering Slit by RoboN treatment would potentially weaken cell-cell contacts, enabling, rather than inhibiting, pro-migratory signals and tumor cell metastasis. Thus, since the downstream effects of Slit/Robo signaling vary greatly depending on both the extracellular and intracellular milieu, development of effective therapeutics will require a better understanding of their disparate signaling consequences in different tissue and cell types. Furthermore, it will be necessary to design therapeutics with elaborate delivery mechanisms that
ensure delivery of the drug to specific tissues, to prevent adverse effects caused by eliminating normal Slit/Robo signaling in nearby tissues, as loss of Slit/Robo signaling in normal tissues and cells could cause deleterious effects. Despite these cautions, it is increasingly clear that Slits and Robos are key regulators of a wide variety of developmental and adult processes, from the epithelium to the vasculature, and that they hold great potential as therapeutic targets in the fight against cancer and other diseases.
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Chapter 2

Self renewal of mammary stem cells is regulated by Slit2/Robo1 signaling through Snail and Insuteable

Abstract

Tissue homeostasis requires that somatic stem cells maintain a balance between self-renewal and expansion; however mechanisms regulating this during organogenesis are not well understood. Here, we demonstrate that SLIT2/ROBO1 signaling regulates the choice between self-renewing asymmetric cell divisions (ACDs) and expansive symmetric cell divisions (SCDs) by governing the expression of a key member of the spindle orientation machinery, Insuteable (mINSC), through the transcription factor Snail (SNAI1). Loss of SLIT2/ROBO1 signaling increases SNAI1 levels in the nucleus, where it targets the mINsc promoter and functions as a transcriptional activator. This increase in mINsc expression does not change overall cell proliferation in the mammary gland, but instead results in more cap cells dividing perpendicular to the basal lamina, producing larger mammary outgrowths. Using colony passaging and limiting dilution assays, we show that mINsc KI/KI mammary glands contain more stem cells. Dye labeling studies demonstrate that this surplus of stem cells is due to an increased proportion of SCD at the expense of ACD. Furthermore, we identify ACD in both body and cap cells of the end bud, suggesting the presence of stem cells in both compartments, and show that
excess mINSC reduces ACD frequency \textit{in vivo}. Together, our studies provide insight into the identity of mammary stem cells and how their number is regulated by the extracellular cue, SLIT2.

\textbf{Introduction}

Stem cells use spindle pole positioning to regulate the balance between symmetric cell division (SCD) and asymmetric cell division (ACD). The former allows them to expand their population, whereas the latter allows them to self-renew while producing daughters with different cell fates. ACDs are a distinguishing characteristic of stem cells, and in the strictest sense, they are defined by the asymmetric inheritance of cell fate determinants through mitotic spindle repositioning along the apico-basal axis (Williams and Fuchs 2013). The molecular machinery directing spindle pole orientation during ACDs has been identified largely through studies in invertebrate systems, where Inscreteable (mInsc in vertebrates) serves as a link between two complexes: the apically-localized Baz (PAR3)/PAR6/apical protein kinase C (aPKC) complex and the microtubule-associated Mud (nuclear mitotic apparatus, NuMA)/Pins (LGN)/Go\(i\) complex. Early studies suggested a simple model in which mINSC, localized to the apical cortex through its interaction with the PAR complex, recruits NuMA/LGN/Go\(i\), leading to reorientation of the mitotic spindle along the apical/basal. This model, however, does not incorporate recent biochemical data.
revealing that mINSC and NuMA bind LGN in a mutually exclusive manner, with preferential binding of mINSC (Mapelli and Gonzalez 2012). These data identify a central role for mINSC, acting as a molecular baton, handing off LGN to NuMA and potentially governing the orientation of the spindle in the cell. Further support of a central role for mINSC in regulating vertebrate ACD is that mINSC has only one clearly defined mammalian homolog, unlike the other proteins of the core spindle orienting machinery. Moreover, in several vertebrate systems both the up and down-regulation of mINSC changes spindle axis orientation (Zigman et al. 2005; Postiglione et al. 2011). Thus, due to its influence on spindle orientation, mINSC has the potential to be a very specific regulatory target, capable of governing the balance between ACD and SCD. There is much to be learned, however, not only about how mlnsc is regulated, but also about the consequences of this regulation, especially in vertebrate tissue.

The mammary gland, one tissue in which ACD may play a role in establishing a stem cell hierarchy, is composed of two distinct layers of epithelia: an outer, basal layer of myoepithelial cells (MECs) and an inner layer of luminal epithelial cells (LECs). These layers form a bi-layered tube that branches into a ductal epithelial tree that extends throughout an adipocyte-rich stromal matrix (Macias and Hinck 2012). Mammary epithelia have tremendous regenerative capacity, as evidenced by the sequence of profound expansion and subsequent involution that occurs with each pregnancy. Lineage tracing studies have
provided evidence for both unipotent and bipotent stem cells that presumably have the capacity to undergo SCD, allowing them to expand under appropriate circumstances, as well as ACD, allowing them to self-renew and produce progenitors (Van Keymeulen et al. 2011; de Visser et al. 2012; van Amerongen et al. 2012; Rios et al. 2014; Wang et al. 2015). This collective evidence supports the presence of stem cells throughout the gland at regular intervals from nipple to ductal termini. Terminal end buds (TEBs) at the tips of growing ducts traverse the fat pad during development and therefore may be responsible for disseminating stem cells along the ducts. TEBs are composed of inner layers of LECs, called body cells, and an outer layer of basal cells, called cap cells. These cap cells give rise to a specialized subpopulation of “drop down” cap cells (Williams and Daniel 1983; Srinivasan et al. 2003), possibly through a rotation of the mitotic spindle that places one daughter cell in the body cell compartment, resulting in an oriented SCD or ACD (Regan et al. 2013; Rios et al. 2014).

However, many questions concerning the nature of postnatal mammary stem cells remain unresolved, including how their division type is regulated.

The Snail family of transcription factors plays a central role in tissue morphogenesis. While SNAI2 displays the highest levels of expression in the mammary gland, SNAI1 is also expressed in both LECs and MECs, albeit at low levels, whereas SNAI3 is only expressed at very low levels in basal cells (Nassour et al. 2012). Through its role as a transcriptional repressor of genes such as E-
cadherin, SNAI1 plays a key role in breast tumorigenesis by enhancing a tumor cell’s commitment to undergo the epithelial-to-mesenchymal transition (EMT). This role of SNAI1 as a transcriptional repressor has been extensively studied; nevertheless, there is growing evidence that it can also act as a transcriptional activator by collaborating with a number of different factors at numerous sites (Hu et al. 2010) (Rembold et al. 2014). In Drosophila, loss of SNAI1 results in decreased Inscuteable expression, interfering with ACD in neuroblasts (Ashraf and Ip 2001) (Cai et al. 2001), but whether an analogous role for a SNAI1/mInsc axis occurs during vertebrate tissue morphogenesis has not been explored.

Studies from Drosophila suggest that SLITs may influence SCD/ACD division type in stem/progenitor cells. Slit was shown to govern terminal ACD of neural precursor cells by indirectly regulating asymmetric cellular localization of Inscuteable (Mehta and Bhat 2001). SLITs are a highly conserved family of extracellular proteins that play an essential role as axon guidance cues during embryogenesis, guiding neurons to their targets by regulating cytoskeletal dynamics. In the developing mammary gland, SLIT2 is expressed in both body and cap cells of the end bud, whereas expression of its receptor, ROBO1, is restricted to cap cells (Strickland et al. 2006). Here we hypothesize that SLIT2/ROBO1 signaling governs the balance between ACD and SCD during mammary gland morphogenesis. Our study identifies a role for SLIT2 as an extracellular regulator of mammary stem cell number by signaling through
SNAI1 to regulate the abundance of mINSC and consequently the balance between ACD and SCD.

**Materials and Methods**

**Mouse strains**

Robo1-/- mice were generated as described {Long, 2004 #548}, as were mINsc KI/+ and mINsc KI/KI mice {Postiglione, 2011 #907}. This research conformed to guidelines set by the University of California, Santa Cruz animal care committee (IACUC).

**Western blotting**

Transformed cell, MEC, LEC and whole tissue protein lysates were prepared and analyzed by western blot as described {Marlow, 2010 #852; Macias, 2011 #856}. Cell fractionation was performed using the Qproteome Cell Compartment kit (Qiagen). Antibodies used: Ck α-GFP (Aves Lab, 1:5000), Rb α-mINsc (Knoblich lab, 1:200), Rb α-LGN (ProteinTech, 1:100), Rb α-GAPDH (SCBT, 1:1000) Ms α-SNAI1 (Cell Signaling, 1:2000), Ms α-CYCLIND1 (Cell Signaling, 1:1000), Ms α-β-Tubulin, (Sigma, 1:20,000), Rb α-HSP70-HRP (SCBT, 1:1000), Rb α-Histone H1 (SCBT, 1:400).
**Cell culture and chemical treatments**

HEK293 cells were obtained from ATCC and grown in growth medium (DMEM supplemented with 10% fetal bovine serum (FBS), 1x Pen/Strep). MDA-MB-231 (gift from Dr. Bissell, Lawrence Berkeley National Laboratory) and MDA-MB-231 cell lines stably expressing SLIT2-HA were grown as described {Marlow, 2010 #852}. Normal murine mammary gland (NMuMG) cells were a gift from Dr. Keely (University of Wisconsin) and grown in growth medium supplemented with 10 µg/mL Insulin. MDA-MB-231 cells were treated with 2 µM CHIR99021 (Cayman Chemical) for 4 hours, then lysed and analyzed by immunoblot. Purified SLIT2-myc was prepared as described {Brose, 1999 #414} and used at 2 µg/mL for 24 hours (NMuMGs) or at 1 µg/mL for 7 days, treating every 3 days (primary colonies).

**Dual-luciferase reporter assay**

To construct pGL4-CP-TK-TCACA, an 128-bp sequence of human genomic DNA containing the proposed SNAI1 binding site (TCACA) was PCR-amplified and passaged through pCRII-TOPO-TA vector (Life Technologies) and into linearized pGL4-CP-TK. To assess promoter activity, the pGL4CP-TK and pGL4CP-TK-TCACA constructs were co-transfected into HEK293 cells with pRL-SV40 (Promega) using Lipofectamine 2000 (Sigma). After treatments, cells were harvested and assayed for Renilla and Firefly luciferase activities using a dual-
luciferase reporter assay system, as described in the protocol (Promega), using Victor Light 1420 Luminescence Counter and software (PerkinElmer). Firefly luciferase activity was normalized to that of Renilla to generate promoter activity. Cells were treated with 40 mM MG132 (a gift from Dr. Sullivan, University of California, Santa Cruz) and 10 µm Lithium Chloride (Thermo Fisher Scientific) at the indicated time points before being lysed and analyzed for promoter activity. Each experiment was performed three times, in duplicate.

**Immunostaining and ACD analysis**

Immunostaining was performed as described {Marlow, 2010 #852} {Harburg, 2014 #900}. Antibodies used: α-SMA (Sigma, 1:1000), Gt α-NuMA (SCBT, 1:250), Rb α-LGN (ProteinTech, 1:100), Gt α-GFP (SCBT, 1:100) Rb α-mINSC (Abgent, 1:100), Hoechst 33342 (Life Technologies, 1:5000), Ms α-SNAI1 (Cell Signaling, 1:500), Ms α-Ki67 (1:500). Mitotic cells were identified based on chromatin condensation and quantified as a percentage of total cells. Only cells in metaphase and anaphase were analyzed in the NuMA ACD assay.

**Mammary cell preparation, FACS analysis, colony formation and PKH26 assays**

Whole tissue, LEC and MEC cell fractions were prepared from mammary glands and lysed to obtain purified cell fractions {Macias, 2011 #856}. For preparation
of single-cell suspensions for fluorescence-activated cell sorting (FACS), thoracic and inguinal mammary glands were harvested and mammary epithelial single-cell suspensions were prepared as previously described {Harburg, 2014 #900}. All cells for the limiting dilution analysis and basal colony 3-D Matrigel cultures were generated from FACS-purified Lin-CD24+CD29hi (basal) cells. To stain colonies, FACS-purified basal cells were resuspended in 100% Matrigel (Corning) and 5000 cells were plated per 20 μL Matrigel in 8-well chamber slides (Labtek). Colony media (DMEM-F12, 1% FCS, 0.5 μg/mL Hydrocortisone, 1 μg/mL Insulin, 10 ng/mL EGF, 20 ng/mL Cholera toxin, 1% Pen/Strep) was added after Matrigel had solidified, and colonies grown at 37°C in hypoxic conditions. After 7 days in culture, colonies were either immunostained, or imaged, counted, harvested from Matrigel using BD Recovery Solution (BD), dissociated using 0.05% Trypsin-EDTA, counted and re-plated (for PKH26 and passaging assays) {Harburg, 2014 #900}. For the PKH26 assay, cells were stained with PKH26 per manufacturer’s protocol (Sigma, 1:750) then cultured as described above. After 7 days, colonies were imaged, counted, dissociated, stained with 7AAD (Life Technologies, 1:250) to select live cells, and FACS analyzed for PKH26 fluorescence intensity and viability. Colony counts and diameter measurements were performed using Fiji.

*In vivo* limiting dilution assay and ductal outgrowth analysis
Robo1-/− and mInsc KI/KI cells sorted by flow cytometry were manually counted and transplanted contralaterally with wild-type control cells at limiting dilution (or 2000 cells) into mice that had been pre-cleared of endogenous epithelium {Strickland, 2006 #569; Harburg, 2014 #900}. Outgrowths were harvested 8 weeks post-transplant and imaged for GFP fluorescence (mInsc KI/KI tissue) and subjected carmine alum staining for whole mount outgrowth analysis. Limiting dilution analysis was performed using extreme limiting dilution analysis (ELDA) program (http://bioinf.wehi.edu.au/software/elda/index.html) {Hu, 2009 #921}.

**RNA Extraction and RT-qPCR**

Total RNA was isolated from MEC and LEC primary cell fractions (separated as described {Macias, 2011 #856}) or from FACS-purified basal (Lin−CD24+CD29hi) and luminal (Lin−CD24lowCD29low) cells using TRIzol reagent (Invitrogen) and prepared as previously described {Macias, 2011 #856}. cDNA was prepared from 1 µg RNA using an iScript cDNA synthesis kit (Bio-Rad). RT-qPCR was performed in triplicate using LightCycler 480 SYBR Green I Master (Roche) and quantified using either Rotor Gene 6000 real-time PCR machine and software, or Bio-Rad CFX Connect Real-Time System and CFX Manager software (Bio-Rad). Quantification of gene expression was carried out using the method of Livak and Schmittgen {Livak, 2001 #922}. Results were normalized to that of Gapdh or
Actin.

**Primer sequences**

<table>
<thead>
<tr>
<th>NAME</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh-1*</td>
<td>F: CATGGCCCTCCGTGTTCCCTA</td>
</tr>
<tr>
<td></td>
<td>R: CCTGCTTCACACCTTTCTCTTGTAT</td>
</tr>
<tr>
<td>mlInc</td>
<td>F: CCAGCACCTCAGCTACCTT</td>
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<tr>
<td></td>
<td>R: CCACTGCTGCTTGTGACCA</td>
</tr>
<tr>
<td>LGN</td>
<td>F: TGCCCAGTGGGTGATAATAG</td>
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<td></td>
<td>R: AAGCCGACTTCAAGTGGAAAA</td>
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<tr>
<td>NuMA</td>
<td>F: GTCAAGGCCCTTGGAGACT</td>
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<tr>
<td></td>
<td>R: AGCGGGCCAGAGACTGAGT</td>
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<tr>
<td>Actin**</td>
<td>F: CTGAACCTAAGGCCAACCC</td>
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<tr>
<td></td>
<td>R: CCAGAGGCATACAAGGACAG</td>
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<tr>
<td>Snai1***</td>
<td>F: AAGATGCACATGCCAAGCACC</td>
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<tr>
<td></td>
<td>R: CTCATTGCTGTGTGAGGACA</td>
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<tr>
<td>CyclinD1</td>
<td>F: AGTGCGTGCAGAAGGAGATT</td>
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<tr>
<td></td>
<td>R: CCAACCTTCGCGCAGTCAA</td>
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<tr>
<td>Axin2</td>
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<td></td>
<td>R: TGCCACACAGTGGCTACA</td>
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<tr>
<td>mlInc4****</td>
<td>F: TTTCATCAGCTGTTGGGTGGGTGGTT</td>
</tr>
<tr>
<td></td>
<td>R: AGGAGGCTGAGGAGCTGACACAA</td>
</tr>
</tbody>
</table>

* used as control for mlInc, LGN and NuMA RT-qPCR quantification
** used as control for CyclinD1, Snai1 RT-qPCR quantification.
*** from {Guo, 2012 #899}
**** used to amplify SNAIL binding motif for luciferase assay.

**Microscope information**

Fluorescence or brightfield images were acquired using a Biorevo BZ-9000 Digital Microscope (Keyence), Leica Spot Scanning Confocal SP5 microscope, Leica Wide-field DM5500 or Volocity Spinning Disk Confocal Microscope (PerkinElmer). Images were processed using Volocity 3D image analysis software (PerkinElmer), Fiji {Schindelin, 2012 #923} software, and Adobe Photoshop CS5.
Statistics

Statistics were performed using Prism software (GraphPad). Two-tailed unpaired student's t-test was used in all Figures except in Figure 2.2.4C: one-way Anova and Chi-square test. Significance is indicated by *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001, ns: p > 0.05. Graph columns represent the mean and error bars represent the standard error of the mean (SEM).

SLIT2/ROBO1 regulates \textit{mInsC} expression

To investigate asymmetric cell division during mammary gland development, we focused on a core component of the spindle machinery, the evolutionarily conserved \textit{mInsC}. We separated mammary epithelial cells into luminal (LEC) and basal, myoepithelial (MEC) cell fractions and by western blotting observed \textit{mInsC} in both fractions, with higher expression in LECs (1A). Since ROBO1 expression is restricted to the basal cell compartment (Strickland et al. 2006), we generated colonies from fluorescently activated cell sorted (FACS)-purified, single, basal (Lin-CD24+CD29hi) cells, grew them in Matrigel and treated with SLIT2. We found that SLIT2 treatment resulted in a 6.1-fold decrease in \textit{mInsC} expression (Figure 2.1B), suggesting that the regulation is transcriptional. To determine if this change in expression also occurs in mammary cell lines, we
examined mINSC protein levels in both normal murine mammary gland (NMuMG) cells treated with purified SLIT2, and three different lines of basal-like, MDA-MB-231 breast cancer cells stably expressing SLIT2-HA (Marlow et al. 2008). In both contexts, the presence of SLIT2 resulted in reduced mINSC (2.2 and 1.9-fold, respectively) (Figure 2.1C, Supplementary Figure 2.1A). Since mINSC functions in a complex with LGN and NuMA, we examined the expression of these core components of the spindle orientation machinery. First, we performed RT-qPCR on Robo1/-/- and WT MEC fractions and observed a 5.1-fold increase in mInsc in Robo1/-/- cells, but no change in NuMA and Lgn levels (Figure 2.1D). Similarly, at the protein level we observed a 2.7-fold increase in mINSC in the Robo1/-/- MEC fraction and no changes, compared to WT, in NuMA and LGN MEC fractions (Figure 2.1E). Examining Robo1/-/- LEC fractions, we saw no difference in the levels of these three proteins, compared to WT LEC fractions (Supplementary Figure 2.1B), which is consistent with the basal expression of ROBO1 in glands (Strickland et al. 2006). We also assessed the expression of mINSC by immunohistochemistry in end buds of the mammary gland, and observed diffuse localization throughout cells (Figure 2.1F), with higher levels seen in the basal cap cells of Robo1/-/- end buds (Figure 2.1G). Taken together, these data indicate that SLIT2/ROBO1 signaling regulates the level of mINSC and, therefore, may influence spindle pole orientation and division type.
SLIT2/ROBO1 regulates \textit{mInsc} via SNAI1

In searching for possible transcriptional regulators of \textit{mInsc} that may be targeted by SLIT2, we identified Snail (SNAI1) as a candidate because it has been implicated in the activation of \textit{Inscuteable} expression in \textit{Drosophila} (Ashraf and Ip 2001; Cai et al. 2001). To investigate, we performed RT-qPCR and western analysis on lysates from primary WT LECs and MECs, harvested during development (5.5 weeks), and found \textit{Snai1} expression in both compartments (Figure 2.2A, B). Previously, we showed that \textit{mInsc} mRNA is upregulated in \textit{Robo1/-} tissue (Figure 2.1D). If SNAI1 regulates \textit{mInsc} in this tissue, we might expect an increase in its expression in \textit{Robo1/-} lysates. Western analysis on these lysates revealed increased SNAI1 levels in the \textit{Robo1/-} MEC, but not LEC, fraction (Figure 2.2C), which is again consistent with the restricted expression of ROBO1 in the basal compartment (Strickland et al. 2006). It is well established that SNAI1 binds to E-box sequences when functioning as a transcriptional repressor. Although less well understood, SNAI1 also acts as a transcriptional activator, possibly through sites other than the E-box (Hu et al. 2010). We interrogated the \textit{mInsc} promoter region for potential SNAI1 binding sites, and found such a site, which includes a consensus sequence (TCACA) that is flanked by binding sites for stimulatory protein 1 (SP1) and early growth response gene 1 (EGR1). These sites were previously shown to enhance the transcriptional
activating function of SNAI1 (Hu et al. 2010). To determine if this site was capable of inducing *mlnsc* transcription, a 224 basepair DNA fragment containing this region was placed in front of the luciferase gene in a construct containing the thymidine kinase (TK) minimal promoter. We expressed this construct in HEK293 cells and observed a 2.0-fold increase in promoter activity (Figure 2.2D). This increase is relatively modest, which could be due to the labile nature of SNAI1, which is rapidly degraded by the proteasome. Thus, to increase its endogenous levels, we treated cells with the GSK3beta inhibitor, LiCl, and the proteasome inhibitor, MG132 (Zhou et al. 2004). We observed an increase in SNAI1 in pGL4CP-TK-TCACA-expressing cells upon treatment with these inhibitors (Figure 2.2E), and also saw a concomitant increase in promoter activity (Figure 2.2F). To examine the relationship between SLIT2, SNAI1 and *mlnsc*, we transfected NMuMGs with *Snai1* and found a significant increase in mINSC that was inhibited upon treatment with SLIT2 (Figure 2.2G). Taken together, these data suggest that SLIT2 regulates *mlnsc* through SNAI1, which acts as an enhancer of *mlnsc* expression by binding to a TCACA sequence in the *mlnsc* promoter.

Next we addressed the mechanism by which SLIT2 regulates SNAI1. To determine if SLIT2/ROBO1 signaling regulates SNAI1 at the level of transcription, we performed RT-qPCR analysis on cDNA from *Robo1/-/-* MECs, which contain increased mINSC (Figure 2.1D) and increased SNAI1 (Figure
2.2C), but found no difference between Robo1/- and WT Snai1 mRNA levels (Supplementary Figure 2.2A). These results suggest the regulation occurs post-transcriptionally, and, indeed, studies have shown that the nuclear localization and degradation of SNAI1 is regulated through the GSK3beta pathway (Zhou et al. 2004). We examined SNAI1 levels in 3 lines of MDA-MB-231 cells, stably overexpressing SLIT2, and in NMuMGs treated with purified SLIT2. In both cases, we observed a significant decrease in SNAI1 levels (Figure 2.2H, Supplementary Figure 2.2B), demonstrating an effect of SLIT2 on SNAI1 protein.

We have previously shown that SLIT2/ROBO1 signaling activates GSK3beta by inhibiting AKT in basal cells of the developing gland (Macias et al. 2011). This regulation of GSK3beta has also been shown to occur in a lung cancer cell line, resulting in increased SNAI1 expression (Tseng et al. 2010). To investigate if this mechanism is regulating SNAI1 signaling downstream of SLIT2/ROBO1 in breast cells, we inhibited GSK3beta signaling by treating MDA-MB-231 clonal lines with the GSK3beta inhibitor CHIR99021. We found that this treatment rescued SNAI1 levels (Figure 2.2H), suggesting that SLIT2 regulates the overall levels of SNAI1 through GSK3beta. Next, we examined if SLIT2 also regulates the subcellular localization of SNAI1 by immunostaining MDA-MB-231 clonal lines. We observed a striking reduction in punctate, nuclear SNAI1 staining, along with a reduced overall level of SNAI1 in the SLIT2-expressing lines (Figure 2.2I, J). These changes in the subcellular localization of SNAI1 were confirmed by biochemical
fractionation of lysates generated from these SLIT2-expressing lines (Supplementary Figure 2.2C). To address if this regulation also occurs in primary cells, we immunostained colonies derived from FACS-purified, single basal cells that were grown in 3-D Matrigel. This analysis revealed increased overall SNAI1 expression in Robo1−/−, compared to WT, colonies, indicating that the SLIT2/SNAI1 signaling axis also occurs in primary mammary cells (Figure 2.2K,L). Taken together, these data support a model in which SLIT2/ROBO1 signaling activates GSK3β to inhibit the activity of SNAI1 by regulating both its subcellular localization and overall level of expression.

**Overexpression of mInsc enhances ductal outgrowth**

Our data show that the level of mInsc is regulated by SLIT2/ROBO1 signaling, with loss of Robo1 leading to elevated mInsc levels. To investigate the effects of excess mINSC, we analyzed transgenic mice that ubiquitously express mInsc-GFP from the Rosa26 locus (R26mInsc::GFPmInsc KI/KI) (Postiglione et al. 2011). Immunostaining for GFP confirmed the expression of the transgene in both cap and body cells of end buds (Supplementary Figure 2.3A). Similarly, immunostaining for mINSC showed a low level of diffuse expression in both body and cap cells of WT (mInsc +/+) TEBs, and, as expected, higher expression in the same pattern in TEBs from mInsc KI/KI glands (Figure 2.3A). To quantify,
we performed western blots on WT and mInsc KI/KI glands and found a 3-fold increase in mINSC expression (Figure 2.3B) that is similar to the increase observed in Robo1-/- glands (Figure 2.1E).

Next we investigated the morphological consequences of this increased mINSC expression. Given our observation that Robo1-/- and mInsc KI/KI glands contained a similar excess of mINSC (Figure 2.1F, 3B), one possibility is that mInsc KI/KI tissue resembles Robo1-/- tissue. During development, there is enhanced MEC proliferation in Robo1-/- glands as a consequence of elevated beta-catenin signaling, leading to increased ductal branching and disorganized tissue structure (Macias et al. 2011). There was not, however, increased beta-catenin signaling in mInsc KI/KI tissue as measured by RT-qPCR of target gene Axin2 (Figure 2.3C), nor did we observe the same morphological phenotypes (Figure 2.3D and Supplementary Figure 2.3B). Instead, we found an expansion of the ductal network as evidenced by a significant increase in the percentage of the fat pad filled with mInsc KI/KI epithelium at 5.5 weeks (Figure 2.3D). To confirm this enhanced growth phenotype, we injected 2000 WT or mInsc KI/KI cells into pre-cleared fat pads, and analyzed the outgrowths after 8 weeks. Again, we observed a significant increase in the percentage of the fat pad filled with epithelia generated from mInsc KI/KI cells (Figure 2.3E). This enhanced growth cannot be explained by either increased cell proliferation or decreased cell death because we observed no changes in either CyclinD1, both at the transcript and
protein levels (Figure 2.3F, G), or cell viability (Supplementary Figure 2.3C). Thus, overexpression of mInsc enhanced the expansion of the epithelial compartment, without altering cell growth or death. These results point to a change in the behavior of mammary stem cells, which are responsible for generating the progenitor cells required for post-natal ductal morphogenesis. Studying the consequence of mInsc overexpression in Robo1-/- tissue is difficult, however, due to an overall decrease in GSK3beta activity (Macias et al. 2011), resulting in a number of downstream signaling effects, among which are the upregulation of SNAIL1 and mInsc (Figure 2.1B-G). Therefore, in order to investigate the specific consequences of increased mINSC on stem cells of the mammary gland, we focused our studies on mInsc KI/KI tissue.

**Surplus mINSC expands the mammary stem cell population**

One way to investigate stem cell activity is to serially passage FACS-purified MECs in 3-D Matrigel. WT MECs possess a limited capacity for self-renewal with colonies becoming progressively smaller until they senesce at passage 4-5. Overexpression of mInsc led to a two-passage extension, accompanied by increased colony number and colony size at each passage, suggesting an expansion of the stem cell fraction (Figure 2.4A-C). Since the MEC population is not pure, but only enriched for basal stem cells, we also performed limiting
dilution assays using FACS purified MECs harvested from WT or mINSC KI/KI glands the frequency of mammary repopulating units (MRUs). We found a significant increase in MRU frequency from 1/304 (95% CI 1/189-1/487) in mINsc +/+ to 1/97 (95% CI 1/53-1/178) in mINsc KI/KI tissue (Figure 2.4C). Not only do these data suggest there are more stem cells in the mINsc KI/KI tissue, but the cells appeared to be more robust because injection of only 10 cells produced more frequent and larger outgrowths (Figure 2.4C, D) and produce larger outgrowths as measured by percentage of fat pad filling (Figure 2.4E). Taken together, our results suggest that the level of mINSC regulates stem cell number in the mammary gland.

**Increased levels of mINSC lead to more perpendicular divisions**

One mechanism by which stem cells regulate their number is to reorient the position of their spindle poles during division, thereby shifting the balance between ACD and SCD division types. Because mINSC is a core component of the spindle orientation machinery, we examined its role in orienting spindles in ROBO1-expressing, highly proliferative, basal cap cells of the TEB. By staining for NuMA, which localizes to the mitotic spindle poles during mitosis and thus indicates the orientation of the division, we find both lateral and oblique/perpendicular cell divisions in the TEB (Figure 2.5A). TEBs are
globular; consequently it can be difficult to obtain a precise division angle measurement along the curved surface of this structure using a 2-D approach. To obtain accurate measurements, we used 3-D image reconstruction and computational analysis to determine mitotic spindle angle in relation to the basal plane of the adjoining lamina (Figure 2.5B) (Juschke et al. 2014). This method restricted our analysis to the outer layer of cap cells because they reside adjacent to the basal lamina. Measuring spindle orientation, we found that the proportion of cap cells dividing laterally (0°-30°) decreased from 82% in WT to 43% in mInsc KI/KI. Concordantly, the proportion of oblique and perpendicular (30°-90°) divisions increased from 18% in WT to 57% in mInsc KI/KI outer cap cells (Figure 2.5C). This change in spindle orientation in mInsc KI/KI tissue represents a significant increase in the mean mitotic spindle angle that is supported by mathematical modeling showing a horizontal enrichment of spindle angles in WT outer cap cells and perpendicular enrichment in mInsc KI/KI cells (Figure 2.5D-F). One consequence of having an overabundance of oblique and perpendicular cell divisions in the mInsc KI/KI tissue is that we found more “drop down” cap cells in the luminal compartment (Figure 2.5G). Thus, this change in spindle orientation in mInsc KI/KI cap cells could lead to increased ductal outgrowth, if as our data suggest (Figure 2.4), these reoriented divisions lead to an expansion of stem cells by SCD.
Excess mINSC converts ACD to SCD

To investigate if the observed change in spindle orientation in mINSC KI/KI tissue results in increased stem cell number by switching ACD to SCD, we employed an in vitro assay to determine division type frequency (Figure 2.6A). FACS-purified basal cells (Lin-CD24+CD29+) were labeled with the fluorescent membrane dye, PKH26, and grown in Matrigel. PKH26 binds to cell membranes and thus is distributed to daughter cells upon division. ACDs generate one quiescent stem cell that maintains fluorescence and another cell that continues to divide, diluting the dye and diminishing fluorescence. After seven days in culture, the resulting colonies were distinguished by the presence of a single, PKH26-labeled cell. In contrast, SCDs result in dye dilution and the resulting colonies were composed of unlabeled cells. In addition, a fraction of plated cells remain single and were PKH26-positive (Figure 2.6A, B). We performed this assay on primary cells from WT and mINSC KI/KI tissue. After 7 days in culture, imaging revealed that 24.5% of WT colonies (> 3 cells) contained a single PKH26 positive cell, indicating these colonies arose from an ACD (Figure 2.6C). The rest of the colonies (75.5%) contained no PKH26 positive cells, indicating SCD (Supplementary Figure 2.4). Performing this labeling on mINSC KI/KI cells, we observed a shift in colony frequency with fewer colonies containing one PKH26 positive cell (8%) (Figure 2.6C), suggesting that more colonies were generated
via a stem cell undergoing SCD, and not ACD. To further quantify this assay, we assessed the frequency of single PKH26-positive cells that were not incorporated into colonies and found no significant difference between genotypes (Figure 2.6D). Colonies were then dissociated and analyzed by FACS for viability and fluorescence intensity. We detected no significant difference in viability after dissociation (Figure 2.6E). However, we again observed a significant decrease in the number of PKH26-positive *mInsc* KI/KI cells compared to WT (Figure 2.6F), further support for the notion that fewer ACDs occurred in *mInsc* KI/KI cells. Taken together, these data suggest that elevated *mInsc* results in increased stem cell number by favoring SCDs at the expense of ACDs.

**mINSC regulates ACD in cap and body cells of end buds**

To investigate if dividing cells undergo ACD in mammary TEBs, we examined the developing gland during puberty by generating serial, longitudinal sections of flattened WT glands and immunostaining them for the machinery associated with ACD: NuMA and LGN. We observed enrichment of both proteins in a crescent-like structure above one spindle pole in a subpopulation of dividing end bud cells (Figure 2.7A). Of the over 700 mitotic cells observed along the ducts, not a single one contained a NuMA/LGN crescent (data not shown). In end buds, however, we determined that 11% of all mitotic cap cells undergo ACD (Figure
2.7B), with 69% residing in the outer cap cell layer (Figure 2.7B, C) and 31% residing in the luminal compartment as “drop down” cap cells (Figure 2.7B, D). During this analysis we also identified ACDs in 8% of mitotic body cells (Figure 2.7E). These data suggest that stem cells in each compartment of the postnatal gland undergo self-renewal via ACD (Figure 2.7B-F). Next, we examined the frequency of ACD in end buds of mInsc KI/KI tissue and observed a significant decrease, compared to WT (Figure 2.7G,H), in both the body and cap cell populations. These data support our in vitro analysis (Figure 2.6), and strongly indicate that excess mINSC inhibits the frequency of classic ACD. We previously showed that SLIT2/ROBO1 signaling, which occurs exclusively in the basal population during development, regulates mInsc levels. Therefore, we assessed the frequency of ACD in Robo1-/- end buds and found a significant decrease in cap cells, but not body cells (Figure 2.7 G, H). Taken together, our data support a model in which SLIT2/ROBO1 signaling regulates stem cell frequency in cap cells of the mammary TEB by governing stem cell division type through a SNAI1/mInsc axis.
Figures:

Figure 2.1. SLIT2/ROBO1 signaling regulates mInsc expression in the mammary gland. (A) Immunoblot of wild type (WT) LEC and MEC lysates from 5.5-week tissue. (B) Quantification of mInsc expression in SLIT2-treated and control basal colonies grown for 7 days in Matrigel. (C) Representative immunoblot and quantification of total mINSC in SLIT2-treated and control normal murine mammary gland cells (NMuMGs). (D) RT-qPCR analysis of mInsc, Lgn and Numa mRNA levels in Robo1+/+ and Robo1-/- MECs. Dashed line represents normalized Robo1+/+ level. (E) Quantification and representative immunoblots of mINSC, LGN and NuMA protein levels in Robo1-/- and Robo1+/+ basal cells. (F, G) Immunostaining of cryosections of Robo1 +/+ (F) and Robo1 -/- (G) end buds shows increased mINSC expression in the basal (inset, smooth muscle actin, SMA+), but not luminal (arrow, SMA-) cells. Insets show magnified views. Scale bars represent 25 µm and 10 µm (insets). Data are represented as mean ± SEM. n = 3 independent experiments.
Figure 2.1

A

B

C

D

E

F

G

Ballard_Fig.1
Figure 2.2. SLIT2/ROBO1 regulates mInsc by controlling the activity of SNAI1. RT-qPCR. (A) and immunoblotting analyses (B) on WT MEC/LEC fractions harvested at 5.5 week. (C) Quantification and representative immunoblots show SNAI1 expression in Robo1 -/- MECs and LECs harvested at 5.5 weeks. (D-G) SNAI1 enhances mInsc expression by binding to the TCACA enhancer sequence in the mInsc promoter. (D) Quantification of promoter activity in cells expressing either pGL4CP-TK or pGL4CP-TK-TCACA together with pRL-SV40. Immunoblot analysis shows SNAI1 expression (E) and promoter activity (F) in cells treated with 10 mm MG132 and 40 mM LiCl at the indicated times post-transfection. (G) Quantification and representative immunoblots of mINSC expression in NMuMGs transfected with pcDNA3-Snai1-HA, treated with 2 mg/mL SLIT2-myc, or both, for 24 hours. (H) Quantification of SNAI1 levels in 3 clones of MDA-MB-231 cells stably expressing pSecTagB or pSecTagB-Slit2-HA (SLIT2-HA), and treated with CHIR99021 or DMSO for 4H. (I) Representative images of SNAI1 in SLIT2-HA and MDA-MB-231-SLIT2 stable cell lines. (J) Quantification of the % of total cells with nuclear vs. cytoplasmic localization of SNAI1 by immunostaining with SNAI1 and Hoechst. (K) Immunostaining of Robo1 +/- and -/- and SLIT2-treated (1 mg/mL) colonies grown for 7 days in Matrigel from single FACS-purifed basal cells with SNAI1 and Hoechst. (L) Quantification of the average pixel intensity of SNAI1 immunofluorescence in >5 colonies/condition. Scale bar represents 10 µm. Data are represented as mean ±
SEM. n = 3 biological replicates (A-C, M, N), n = 3 independent experiments (D, F, G) and n = 3 separate clones expressing each construct (H-J).

Figure 2.2
Figure 2.3. Overexpression of mInsc in mammary tissue enhances ductal outgrowth. (A) Immunostaining tissue from 5.5 week-old mice for mINSC reveals increased protein levels in mInsc KI/KI (KI) glands compared to mInsc +/+ (WT) glands. Small panels are magnified views of boxed inset. Arrow in (A) points to mINSC accumulation in basal cytoplasmic region of cap cells. (B) Quantification and representative blot of mINSC expression in whole-gland lysates from 5.5 week old WT and KI mice. (C) RT-qPCR analysis of Axin2 mRNA expression in WT and KI FACS-purified basal (Lin-CD24+CD29hi) cells. (D,E) Representative images and quantification of ductal outgrowth in carmine-stained WT and KI glands at 5.5 weeks of age (D), and after injection into pre-cleared fat pads of 2000 WT or KI cells (E). (F, G) WT and KI glands do not exhibit changes in cell proliferation. (F) RT-qPCR analysis of CyclinD1 mRNA levels in FACS-purified basal cells from WT and KI adult mice. (G) Representative immunoblot and quantification of CYCLIND1 expression in whole-gland lysates from WT and KI glands at 5.5 weeks of age. Scale bars (A) and 1.2 mm (E, F). Data are represented as mean ± SEM. n = 3 biological replicates.
Figure 2.3

**Panel A**

SMA, mINSC, Hoechst

**Panel B**

Relative mINSC Expression

**Panel C**

Relative Axin2 Expression

**Panel D**

Outgrowths at 5.5 weeks of Age

**Panel E**

2000 FACS-Purified Basal Cells

**Panel F**

Relative CyclinD1 Expression

**Panel G**

Relative CYCLIN D1 Levels
**Figure 2.4. Increased mINSC leads to stem cell expansion.** Quantification of the total number of colonies (A) and colony diameter (B) obtained from an initial 20,000 FACS-purified *mlnc* +/- (WT) and *mlnc* KI/KI (KI) basal cells plated in Matrigel over serial passages. (C) Representative images of colonies at specified passages. Insets are magnified views of cells in black boxes. (D) Estimate of stem cell frequency in WT and KI tissue as determined by Extreme Limiting Dilution Analysis (ELDA) with upper and lower limits shown in parentheses. (E) Bar graph showing the frequency (%) of glands with a positive outgrowth, (>5% of fat pad filled) in cleared fat pads injected with 10 WT or KI FACS-purified single basal cells. n indicates the number of glands analyzed. (F) Representative images and quantification of % of fat pad filled with epithelia in glands with positive outgrowths after injection with 10 WT or KI FACS-purified single basal cells. Scale bars represent 0.5 mm (black bar) and 125 µm (white bars) (C) and 1.2 mm (E, F) Data are represented as mean ± SEM. n = 3 independent experiments (A, B) and as indicated in (D-F).
Figure 2.4

A. Total Number of Colonies

B. Average Colony Diameter (μm)

C. Passage 2 vs. Passage 5

D. Number of Outgrowths

<table>
<thead>
<tr>
<th>Cells per Injection</th>
<th>Number of Outgrowths (Number/Total)</th>
<th>Stem Cell Frequency (Upper and Lower Limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2000</td>
<td>7/7 (1/1087 to 1/189)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>9/15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4/12</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6/21</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3/16</td>
</tr>
<tr>
<td>KI</td>
<td>2000</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7/8 (1/178 to 1/53)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3/6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6/9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6/8</td>
</tr>
</tbody>
</table>

E. Frequency of Outgrowth after a 10-cell Injection

F. % of Field Unfilled

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Figure 2.5. Excess mINSC alters spindle orientation in outer cap cells of the mammary gland. (A) Representative image of an end bud with an outer cap cell dividing via non-classic ACD perpendicular (spindle angle between 60-90°, orange box) or lateral (spindle angle 0-30°, grey box) with respect to the basal lamina (marked by the white line). (B) Cartoon demonstrating the method for determining the mitotic spindle angle between a vector drawn through the spindles (blue dots) and the basal plane formed by 5 points along the basal lamina (green). (C) Distribution of spindle angles in mInsc +/+ (WT) and mInsc KI/KI (KI) end buds as a percent of total mitotic outer cap cells. (D) Quantification of the mean mitotic spindle angle of dividing outer cap cells in WT and KI end buds. (E, F) Graphical representation of mitotic spindle angles in WT (E) and KI (F) outer cap cells. Blue line indicates random angle distribution. Red line represents the line of best fit and the 95% confidence interval is shaded in pink. λ_P = Perpendicular enrichment score, λ_L = Lateral enrichment score, where a score of 0 indicates a random spindle orientation, and positive values for each are interpreted as active enrichment in direction. (G) Quantification of the number of drop down cap cells in WT and KI end buds. Data are represented as mean ± SEM. n = 49 WT and n = 31 KI cells (C – F) and n = 33 WT and 102 KI cells (G) from at least 3 animals per genotype. Scale bars indicate 32 µm (left) and 4 µm (right).
Figure 2.5

A

Hoechst
NuMA SMA

0 15 30 45 60 75 90

0

20

40

60

80

100

WT Outer Cap Cells

Lateral (0-30°)
Oblique (30-60°)
Vertical (60-90°)

B

C

% of Total Mitotic Outer Cap Cells

WT
KI

5%
13%
82%

34%
57%

D

Mean Mitotic Spindle Angle (Degrees)

WT
KI

0 10 20 30 40 50

E

WT Outer Cap Cells

Fraction (%)

0 20 40 60 80 100

Angle α (degree)

λ_P = 0.32
λ_L = 0.97

F

KI Outer Cap Cells

Fraction (%)

0 20 40 60 80 100

Angle α (degree)

λ_P = 0
λ_L = 0.97

G

Average # of Drop Down cap cells

WT
KI

0 2 4 6 8 10

****
**Figure 2.6. mINSC regulates the frequency of ACD *in vitro.* (A) Cartoon depicting the method of ACD quantification by PKH26 assay, in which FACS-purified basal cells are fluorescently labeled with PKH26 and grown in Matrigel for 7 days. (B) Representative images of a colony with a single PKH26+ cell (left, ACD), a colony with no PKH26+ cell (middle, SCD) and a single PKH26+ cell (right). (C) Quantification of the number of colonies containing a PKH26+ cell in mINsc +/- (WT), mINsc KI/KI (KI) cultures from FACS-purified enriched basal stem cells. n = 1139 WT and n = 330 KI colonies. (D) Frequency of single PKH26+ cells as a percent of total number of cells and colonies counted after 7 days in culture. n = 3133 (WT) and n = 751 (KI) cells and colonies. (E) Quantification of viability in dissociated cultures stained with 7AAD and analyzed by FACS. (F) Quantification of the PKH26 fluorescence intensity in dissociated colonies, as analyzed by FACS. Scale bars represent 12.5 µm (B). Data are represented as mean ± SEM. n = 3 independent experiments (C – E), n = 4 independent experiments (F).
Figure 2.6

A. Lin^+CD24^+CD29^+ PKH26+ cell
- ACD Colony
- SCD Colony
- Single Cell
- Cell Division: 0, 1, 2, 3

B. ACD  SCD
Single PKH26-Stained Cell

C. Frequency of Colonies with 1 PKH26+ Cell
- WT  KI

D. Frequency of Single PKH26+ Cells
- WT  KI

E. % Viability
- WT  KI

F. Relative PKH26 Fluorescent Intensity
- WT  KI

Ballard_Fig.6
Figure 2.7. mINSC regulates the frequency of ACD in body and cap cells. (A) Representative image of a dividing cell obtained by immunostaining for Hoechst, NuMA, and LGN to mark the ACD machinery at the apical pole during an ACD. Magnified views show the apical LGN (top) and NuMA (bottom) crescents (arrowhead). (B) Quantification of ACD frequency in cap cells (n = 122 cells in metaphase through telophase from 3 mice). Right bar shows distribution of classic ACDs in outer, versus drop down, cap cells. (C, D) Representative images of SMA-positive outer (C) and drop-down cap cells (D) (white boxes), undergoing ACD. Arrowheads point to NuMA crescent. (E) Quantification of classic ACD frequency in body cells (n = 308 cells in metaphase through telophase from 3, 5.5 week-old mice). (F) Representative image of a body cell (white box) undergoing classic ACD with arrowhead pointing to NuMA crescent. (G) Quantification of ACDs in cap cells in +/+ (n = 146), Robo1-/- (n = 106 cells), and mINsc KI/KI (n = 39 cells) end buds from 3, 5.5 week-old mice/genotype. (H) Quantification of ACDs in body cells in WT (n = 292 cells), Robo1-/- (n = 106 cells), and mINsc KI/KI (n = 90 cells) end buds from 3, 5.5 week-old mice/genotype. Scale bars represent 12 µm (A, C, D, F). Data are represented as mean ± SEM (B, E, G, H).
Figure 2.7

**Figure 2.7**

A. Hoechst, NuMA, LGN

B. Mitotic Cap Cells
- 11% (Mitotic Cap Cells)
  - 69% (outer cap cells)
  - 31% (drop down cap cells)
- 89%
  - ACD
  - SCD

C. Hoechst, NuMA, SMA

D. Hoechst, NuMA, SMA

E. Mitotic Body Cells
- 8%
  - ACD
  - SCD
- 92%

G. Relative Frequency of Classic ACD in Cap Cells

H. Relative Frequency of Classic ACD in Body Cells

Ballard_Fig.7

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Supplemental Figure 2.1. (A) Representative immunoblot and quantification of total mINSC in 3 independent lines of MDA-MB-231 cells stably expressing either pSecTagB or pSecTagB-Slit2-HA (SLIT2-HA). (B) RT-qPCR analysis of mInsc, Lgn and Numa mRNA levels in Robo1+/+ and Robo1/-/- LECs. Dashed line represents normalized Robo1+/+ level. Data are represented as mean ± SEM. n = 3 independent experiments.
Supplemental Figure 2.2. (A) RT-qPCR analysis of Snai1 mRNA levels in Robo1 +/+ and Robo1 -/- basal cells. ns: not significant. (B) Quantification and representative immunoblots of SNAI1 expression in NMuMGs treated with purified SLIT2-myc at 2 mg/mL for 24 hours. (C) Representative immunoblots and quantification of SNAI1 expression in nuclear and cytoplasmic fractions of MDA-MB-231 cells stably expressing pSecTagB or pSecTagB-SLIT-HA. Data are represented as mean ± SEM. n = 3 biological replicates (A), n = 3 independent experiments (B) and n = 3 separate clones expressing each construct (C).
Ballard_Supplemental Fig. 2

A

Relative Snai1 mRNA Levels (MEC)

Robo1+/+ vs. Robo1-/-

B

Relative SNAI1 Expression (Pixel Intensity)

SLIT2 myc

MDA-MB-231

C

SNAIL Expression (Pixel Intensity)

Cytoplasmic vs. Nuclear

MDA-MB-231

pSecTagB

SLIT2-HA
Supplemental Figure 2.3. (A) Representative images of mInsc +/+ (WT, left) and mInsc KI/+ (right) adult tissue immunostained for Hoechst and mINSC-GFP using a GFP antibody. Immunostaining reveals mINSC-GFP expression in both the outer cap (arrow) and body (arrowhead) cell compartments. (B) Quantification of primary and secondary ductal branching, and tertiary branch points in WT and mInsc KI/KI (KI) glands at 8 weeks of age. (C) Quantification of cell viability in dissociated primary WT and KI cells at 8 weeks of age, stained with 7AAD and analyzed by FACS. Scale bars represent 40 µm and 20 µm (insets). Data are represented as mean ± SEM. n = 2 (B) or 3 (C, D) biological replicates.
Supplemental Figure 2.4. (A) Quantification of the number of colonies that do not contain a PKH26+ cell in mInsc +/+ (WT) and mInsc KI/KI (KI) cultures from FACS-purified enriched basal stem cells. n = 3 independent experiments.
Discussion

Somatic stem cells are essential for tissue growth during development, homeostasis in the adult animal and repair after injury. However, our understanding of how somatic stem cell hierarchies provide the necessary progenitors required to achieve these processes is incomplete, particularly in actively cycling, solid tissues such as mammary epithelium. Research in model organisms suggests that ACD is required for stem cell self-renewal and concomitant generation of progenitors, but how such divisions are regulated, especially by extracellular factors, is largely unknown. Here, we show through the immunolocalization of NuMA and LGN, stem cells undergoing ACD \textit{in situ}, in both luminal and basal compartments of the mammary end bud. We identify an extracellular cue, SLIT2, signaling through its ROBO1 receptor, that targets the expression of a key member of the spindle orientation machinery, $mInsc$, by regulating the subcellular localization of the transcription factor SNAI1. We identify a SNAI1 target sequence in the promoter of $mInsc$ that drives its expression \textit{in vitro}. This relationship between SNAI1 and $mInsc$ was further supported by a search of the Geo Profiles database that revealed an upregulation of $mInsc$ in breast cancer cell lines that overexpress SNAI1 (Edgar et al. 2002)\cite{Edgar2002}(accessions GSE58252, GSE50889, GSE52593). Increased levels of of $mInsc$ in the mammary gland, which also occurs in the absence of SLIT2/ROBO1.
signaling, enhances ductal outgrowth due to an overabundance of stem cells that are generated through a switch in stem cell division type from ACD to SCD. This study elucidates a new mechanism for regulating stem cell division type during tissue morphogenesis.

The role for SNAI1 as an EMT inducer is well established, and its reactivation during many types of tumorigenesis, including breast cancer, promotes metastasis and negatively correlates with survival. Recently, there is growing appreciation that activation of EMT is associated with the acquisition of stem cell traits by normal and tumor cells; SNAI1, which is a transcription factor with many targets, is a prime candidate for a protein that bridges these programs. In numerous cancer models (Lim et al. 2013; Hwang et al. 2014; Zhou et al. 2014) and in our studies on normal development, SNAI1 expression is associated with enhanced stem cell properties such as increased colony formation. Here, we show one mechanism generating enhanced stemness is a SNAI1-mediated switch in the division mode of cells from ACD to SCD through target mInsc. Such an influence of SNAI1 on division type was also observed in a colorectal cancer model, though through a different mechanism (Hwang et al. 2014). In this context, the delineated pathway involved SNAI1 promoting the nuclear accumulation of beta-catenin. This, in turn, led to the activation of miR-146a and repression of Numb, increasing SCD. There is also an increase in nuclear beta-catenin in the absence of SLIT2/ROBO1 signaling (Tseng et al.
2010; Macias et al. 2011). While this could mean that miR-146a/Numb signaling is contributing to SCD in mammary epithelial cells, we show that mINsc overexpression, alone, produces the same results as loss of Robo1: data that support our model in which mINSC plays the key role in regulating a switch between ACD and SCD during mammary morphogenesis. Indeed, novel targets of SNAI1 are increasingly being identified as researchers probe the full consequences of its activation in different biological contexts. For example, SNAI1 was recently identified as a factor that enhances fibroblast reprogramming (Gingold et al. 2014), possibly by binding and down-regulating let-7 miR (Unternaehrer et al. 2014) and promoting a dedifferentiated state. The current challenge is elucidating how and when SNAI1 is deployed, with our work establishing SLIT2 as an extracellular cue that regulates the subcellular localization of SNAI1 through GSK3beta during mammary morphogenesis.

Results from numerous studies have suggested that the level and timing of mINSC expression in cells during development is a key determinant in governing whether a cell undergoes SCD versus ACD. For example, in studies in the developing epidermis, a burst of mINsc overexpression reorients spindles from parallel to perpendicular, driving ACD (Lechler and Fuchs 2005; Poulson and Lechler 2010; Williams et al. 2011), whereas longer term overexpression does not (Poulson and Lechler 2010). Similarly in the mammary TEB, we find that the overexpression of mINsc reoriented spindles from parallel to
perpendicular, although in this context a switch from ACD to SCD occurred. This difference in outcome may be due to the tissue specific ways that stem cells are governed. In the mammary TEB, cap cells not only differentiate into myoepithelial cells, but a subpopulation also “drops down” into the basal compartment. We show that one consequence of having more perpendicularly oriented cells in \textit{mlnsc KI/KI} end buds is having more “drop down” cap cells. Currently, the fate of these “drop down” cells is unknown, but we speculate they may become stem cells that are seeded along the ducts (Williams and Daniel 1983; Srinivasan et al. 2003). As such they could be responsible for the enhanced outgrowth and increased stem cell number observed in this tissue. In studies on the developing cortex, one of the authors (J. A. K.) and coworkers have shown using this same mouse model of constitutive \textit{mlnsc} overexpression that more neural stem cells, termed radial glia, adopt an oblique or perpendicular spindle orientation. This results in a switch in ACD type to indirect neurogenesis, in which radial glia give rise to more intermediate progenitors that, in turn, divide symmetrically into two differentiating neurons (Postiglione et al. 2011; Juschke et al. 2014). Thus, both the cell type and tissue in which mINSC levels are regulated influence the biological outcome.

Recent biochemical studies offer a mechanistic explanation for the observed effects of excess mINSC by showing that mINSC and NuMA bind to the same site on LGN, with mINSC having a higher affinity for this site (Mapelli and
Gonzalez 2012). This structural insight suggests that the levels of mINSC can have profound effects on spindle orientation by controlling how the spindle orientation machinery is assembled. In mammary epithelial cells, our data support a model in which excess mINSC effectively titrates all the binding sites on LGN. This prevents NuMA and associated astral microtubules from tethering to LGN, thereby obstructing self-renewal via ACD, even as the spindle reorients and positions the daughter cell into a different environment. In this way, mINSC shifts the balance from ACD toward SCD, as evidenced by enhanced mammary gland growth and an increase in mammary stem cells in mInsc KI/KI tissue.

A burst of mInsc may be more analogous to the type of regulation of mINSC occurring during normal development, when changes in mINSC level at specific times could regulate whether a cell undergoes ACD. We discovered a defect in ACD by examining the Robo1−/− and mInsc KI/KI mammary glands, which display chronic overexpression of mInsc. During normal development, however, we speculate that finely tuned regulation of SLIT2/ROBO1 signaling may have the capacity to govern ACDs by regulating the amount of mInsc in cells. The extracellular availability of SLIT and its association with ROBOs is regulated by a number of extracellular matrix components, including heparin sulfate proteoglycans and collagen types XV and XVIII (Ballard and Hinck 2012). Indeed, studies in the nervous systems show that different heparin sulfotransferases play distinct roles in modifying the axon guidance functions of SLITs (Conway et
al. 2011), suggesting that similar to the glycosaminoglycan codes regulating WNT and FGF signaling (Zhang 2010), extracellular mechanisms also modify SLIT action. These modifications have the capacity to specifically orient the mitotic spindle and induce an ACD by temporally and spatially restricting the presentation of ligands to cells, as recently demonstrated by immobilizing WNT3A on a bead and delivering it to a single embryonic stem cell (Habib et al. 2013).

Here, we demonstrate that the extracellular SLIT2/ROBO1 signaling pathway has the capacity to influence the balance between ACD and SCD in the breast through mINSC, and thus plays a role in determining the number of stem cells. This finding may have implications for tumor biology because SLIT/ROBO signaling is altered in 40.7% of basal breast tumors, a subtype associated with EMT and stem-like characteristics (Cancer Genome Atlas 2012). In basal tumors, the switch from ACD to SCD that we observed in the absence of Robo1 or in the presence of excess mINSC may occur in cancer stem cells and facilitate tumor growth. Thus, we propose that one of the ways SLIT2/ROBO1 signaling keeps cellular proliferation in check is to specify division mode by modulating the levels of mINsc through regulating the subcellular localization of SNAI1.
References:


Chapter 3

Future Directions: Inscuteable regulates mammary cell fate specification and mammary gland morphology.

Abstract

The development of a functional mammary gland depends on proper cell lineage specification during ductal outgrowth. During puberty, infiltration of the mammary fat pad by the ductal tree is driven by proliferation of cells in the end buds. Located at the tips of the ducts, end buds contain both cap and body cells that self-renew while also differentiating into myoepithelial and luminal progenitor cells, respectively. Here, we investigate how spindle orientation regulates the identity of daughter cells, and how changes in Inscuteable (mInsc) levels affect lineage specification and ductal morphology and function. We have previously shown that increased mInsc reorients the mitotic spindle in outer cap cells surrounding the end bud, causing them to place one daughter cell in the body cell compartment. However, it is unclear if these daughter cells maintain a cap cell fate, and either migrate back into the cap cell layer or remain in the body cell compartment. Alternatively, external cues may cause them to take on a body cell fate. Our preliminary data shows that outer cap cells can give rise to drop down cap cells, which express basal markers but reside in the body cell compartment, and to body cells, which express luminal markers. Here, we will use a lineage tracing mouse model to label cap cells, and will use immunostaining techniques to investigate the identity and fate of their daughter cells. We will also investigate the long-term effects of increased mNSC, and therefore increased stem
cell numbers, during development on the functionality of the mammary gland during development. These studies will elucidate the importance of mechanisms regulating stem cell self-renewal during ductal development for the function of the gland in the adult mouse. Taken together, the studies proposed in this chapter will explore the possibility of a stem cell niche in the mammary end bud, where both intrinsic cell fate determinants and external signaling cues dictate the fate of the daughter cells.

**Introduction**

**The mammary gland**

The breast is composed of a fat pad filled with a ductal network of epithelial cells and is responsible for first producing, and then delivering milk to the nipples. The development of the mouse mammary gland closely resembles that of the human breast, making it an ideal model system for study of this organ. Furthermore, the mammary gland is an ideal model system because it is amenable to external surgical and chemical manipulations (Hinck and Silberstein, 2005).

During embryogenesis, a rudimentary ductal system made of epithelial cells surrounded by stroma emerges (Macias and Hinck 2012). Throughout early development, the ductal system remains dormant, expanding only to keep pace with the overall growth of the animal. At the onset of puberty, however, expansive proliferation occurs, and the ductal network begins to fill the fat pad. This outgrowth is driven in large part by the proliferation of cells in the end buds (EBs). EBs are large, club-like structures composed of an outer layer of basal cap cells surrounding
an inner mass of body cells. The majority of body cells are luminal, but there also exists a small population of basal body cells called “drop-down” cap cells (Figure 3.1A). As proliferation pushes the end buds through the fat pad, the body and cap cells differentiate to become luminal epithelial and myoepithelial cells, and form a hollow bi-layered tube through which the milk is transported (Macias and Hinck 2012).

**Mammary stem cells and cell specification**

The mammary stem cell lineage has been under intense study for many years, and as discussed in the previous chapter, the existence of mammary stem cells is well established. However, we still do not have reliable markers to identify and sort these cells, and do not fully understand if they are uni- or multipotent. The use of transgenic mouse models that allow cell-type specific labeling has been widely used in other systems (de Visser et al., 2012). Until recently, the mammary gland field was hesitant to adopt this method to investigate the mammary stem cell hierarchy because the most widely used transgenic mouse models require the use of Tamoxifen to induce the expression of, for example, RFP in the targeted cell population. Tamoxifen is an antagonist of the estrogen receptor, which is highly expressed in the mammary gland. Therefore, it was believed that lineage-tracing studies using Tamoxifen treatment would interfere with normal mammary gland development. Recently, however, a study by Rios and colleagues demonstrated that at low
concentrations, ample induction of the CreER promoter could be achieved without affecting the normal development of the gland (Rios et al. 2014).

While many recent lineage-tracing studies demonstrate evidence for an embryonic multipotent mammary stem cell, there are conflicting views regarding the existence of multipotent adult mammary stem cells. For instance, using K5, K14, K8 and K18-driven lineage tracing systems, van Keymeulen and colleagues show that by puberty, the basal and luminal cell lineages are propagated by distinct lineage-restricted stem cells (Van Keymeulen et al. 2011). However, using these and other lineage-tracing models, others have demonstrated the presence of multipotent stem cells throughout adult development and even during pregnancy (Rios et al. 2014). Thus, there is still much to be learned about the identity and nature of mammary stem cells.

One hallmark of stem cells is their ability to undergo an asymmetric cell division (ACD) (Williams et al. 2013). As described in the previous chapter, both basal (cap) and luminal (body) cells undergo ACDs during ductal outgrowth, suggesting the presence of stem cells in both cell populations. To investigate if these stem cells are uni- or multipotent, I will use a lineage tracing mouse model to induce tdTomato (ai9) expression in K5-expressing cap cells and immunostain for basal markers (Smooth muscle actin, SMA, and Cytokeratin 5, K5) and luminal markers (Cytokeratin 8, K8) to assess the identity and fate of the progeny during ductal outgrowth.
It is currently believed that drop down cap cells originate from outer cap cells, yet whether they migrate into the body cell compartment after cell division, or are placed there during cell division is not known. In the previous chapter, I describe a mechanism whereby SLIT2/ROBO1 signaling regulates the expression of the spindle orientation component, Insuteable (mInsc in vertebrates), thus controlling how basal cells divide during ductal outgrowth. The molecular machinery directing spindle pole orientation during ACDs has been defined largely through studies in invertebrate systems, where Insuteable serves as a link between two complexes: the apically-localized Baz (Par3)/Par6/apical protein kinase C (aPKC) complex and the microtubule-associated Mud (nuclear mitotic apparatus, NuMA)/Pins (LGN)/Gαi complex. Early studies suggested a simple model in which mINSC, localized to the apical cortex through its interaction with the Par complex, recruits NuMA/LGN/Gαi, leading to reorientation of the mitotic spindle along the apical/basal axis. This model, however, does not incorporate recent data that shows that mINSC and NuMA bind to LGN in a mutually exclusive manner, with preferential binding of mINSC (Mapelli and Gonzalez 2012). These data identify a central role for mINSC, acting as a molecular baton, handing off LGN to NuMA and potentially governing the orientation of the spindle in the cell. Further support of such a central role in regulating vertebrate ACD is that mINSC has only one clearly defined mammalian homolog, unlike the other proteins comprising the core spindle orienting machinery, and in several vertebrate systems both the up and down-regulation of mINSC changes
spindle axis orientation {Zigman et al., 2005, Postiglione et al., 2011}. Thus, mINSC has the potential to be a very specific regulatory target for governing spindle orientation in mammalian tissues. Indeed, I have shown that overexpression of mInsc either through loss of ROBO1 (Robo1-/-), or through a transgenic mouse model (R26-mInsc-GFP) is sufficient to change the frequency of ACDs and alter the spindle orientation in vivo (see chapter 2).

In the previous chapter, I show that while the majority of outer cap cells divide laterally, placing their daughter cells in the same compartment, a small portion of these cells divide obliquely, placing one daughter cell in the body cell compartment. One possibility is that the body cell compartment serves as a niche, conferring a luminal fate to the cap daughter cell that is placed there. Another possibility is that intracellular signals determine the fate of cap and body cells, and that mere placement of a cap daughter cell in the body cell compartment is not sufficient to change its fate. In this case, an outer cap cell dividing perpendicularly to the basal lamina could generate drop down cap cells (Williams and Daniel 1983; Srinivasan et al. 2003). To investigate, I will use lineage tracing and immunostaining methods to track the location and fate of dividing outer cap cells, and will assess the possible consequences of increased perpendicular divisions in the cap cell population on end bud morphology and cell type composition. These studies will elucidate a mechanism involving mitotic spindle orientation for the generation of drop down cap cells and will investigate the possibility that body and cap cell fate is specified by
both intracellular and external fate determinants, and will assign a novel role for SLIT2/ROBO1 signaling in regulating the mammary cell hierarchy.

Basal cap cells give rise to basal progenitors and differentiated myoepithelial cells, while luminal body cells give rise to luminal progenitors and differentiated luminal cells. There are several classes of luminal cells, and their subtype can be identified based on their expression of, or lack of expression of various hormone receptors, including estrogen (ER) and progesterone (PR). These cells are considered the origin of various types of breast cancer, and the expression signature can predict patient outcome. However, it is not clear how these different subtypes arise. To investigate if all luminal cell types arise from a common luminal progenitor, I propose to use a lineage tracing mouse model to induce ai9 expression in K8-expressing luminal cells and immunostain the clones for ER and PR. This approach will investigate the existence of a common luminal progenitor capable of giving rise to multiple types of differentiated luminal cells.

**Overexpression of Inscuteable affects alveogenesis**

I have previously shown that increased mInsc expression, as seen in the mInsc KI/KI mouse, causes enhanced ductal outgrowth (chapter 2). However, by 8 weeks of age, the WT and mInsc KI/KI glands have the same morphology. It is therefore unclear if this enhanced ductal outgrowth during puberty, caused by the increased stem cell number observed in the mInsc KI/KI mouse, has any long-term effects. Stem cells self-renew during ductal outgrowth to support pregnancy, when alveolar
progenitors differentiate and form large alveoli at the tips of ducts that produce milk. To investigate if the changes observed in the mInsc KI/KI mouse during puberty affect the process of alveogenesis, I will analyze the morphology and function of mammary glands in 17.5 days pregnant (dp) mice. This study will help to elucidate if changes that occur during ductal development can have long-lasting effects on the function of the mammary gland.

Taken together, the studies I have proposed in this section will shed light on the importance of cell division orientation, and the signaling molecules that regulate this process, on both cell lineage specification during ductal outgrowth, as well as the function of the gland during pregnancy.

Methods and Materials:

Mouse strains: mInsc KI/+ and mInsc KI/KI mice were generated as described (Postiglione et al., 2011). This research conformed to guidelines set by the University of California, Santa Cruz animal care committee (IACUC).

Western blotting: Transformed cell, MEC, LEC and whole tissue protein lysates were prepared and analyzed by western blot as described (Marlow et al., 2010, Macias et al., 2011). Briefly, samples were separated by SDS-PAGE, blocked with 5% milk overnight, incubated with primary antibody diluted in either 4% Bovine Serum Albumin (Rb α-mINSC) or 5% milk and proteins were detected using clarity ECL substrate (Pierce). Antibodies: Ms α-CYCLIND1 (Cell Signaling, 2926,
1:1000), Ms α-β-Tubulin, (Sigma, T7816, 1:20,000), Rb α-GAPDH (SCBT G29, 1:1000). Secondary antibodies used were hybridized to HRP.

**RNA Extraction and RT-qPCR**

Total RNA was isolated from FACS-purified basal (Lin CD24^+CD29^{hi}) and luminal (Lin^CD24^lowCD29^{low}) cells using TRIzol reagent (Invitrogen 15596-026), and prepared as previously described {Macias et al., 2011}. cDNA was prepared from 1 µg RNA using an iScript cDNA synthesis kit (Bio-Rad 170-8891). RT-qPCR was performed in triplicate using LightCycler 480 SYBR Green I Master (Roche) and quantified using either Rotor Gene 6000 real-time PCR machine and software, or Bio-Rad CFX Connect Real-Time System and CFX Manager software (Bio-Rad). Quantification of gene expression was carried out using the method of Livak and Schmittgen {Livak, 2001 #917}. Results were normalized to that of Gapdh or Actin.

**Primer sequences**

<table>
<thead>
<tr>
<th>NAME</th>
<th>Sequence (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>Actin</td>
<td>F: CTGAACCCTAAGGCCAACC</td>
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<tr>
<td></td>
<td>R: CCAGAGGCATACAGGGACAG</td>
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<td>Axin</td>
<td>F: TGACTCTCTTCCAGATCCCA</td>
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<tr>
<td></td>
<td>R: TGCCCCACACTAGGCTGACA</td>
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<tr>
<td>CyclinD1</td>
<td>F: AGTGCAGTCAGAAGGAGATT</td>
</tr>
<tr>
<td></td>
<td>R: CACAACTTTCGGCAGTCAA</td>
</tr>
</tbody>
</table>

**Mammary cell preparation and FACS analysis:** Whole tissue, LEC and MEC cell fractions were prepared from mammary glands and lysed to obtain purified cell fractions (Macias et al., 2011). For preparation of single-cell suspensions for
fluorescence-activated cell sorting (FACS), thoracic and inguinal mammary glands were harvested and mammary epithelial single-cell suspensions were prepared as previously described (Shackleton et al., 2006, Vaillant et al., 2008, Zeng et al., 2010).

**Lineage tracing:** For lineage tracing studies, animals were given 1-2 mg Tamoxifen dissolved in sunflower oil by intraperitoneal (IP) injection at 5 weeks of age, unless specified. 24 hours before gland harvest, mice were injected with Trypan Blue 0.4% (Life Technologies 15250-061). 3rd and 4th mammary glands were harvested and spread onto glass slides and fixed with paraformaldehyde (PFA) for 30 minutes, treated with ammonium chloride (NH₄Cl), washed, and then dissected to remove excess fat and stroma. Tissue was then blocked in blocking buffer (1% BSA, 5% donkey serum, 0.8% Triton X-100 in PBS) for 3 hours at room temperature, and incubated in the desired primary antibody over night at room temperature under agitation. Tissue was then washed and incubated in secondary antibody for 5 hours at room temperature, followed by Hoechst incubation for 30 minutes, several washes, and then mounting on a coverslip using Fluoromount. Clone size was determined based on the number of labeled cells adjacent to one another.

**Immunostaining:** Tissue was cryopreserved using Tissue-Tek Cryo-OCT (Fisher 140373-65) and 16 µm sections were fixed using 2% paraformaldehyde (PFA) for 8 minutes, and subjected to antigen retrieval (1% SDS), permeabilization (0.5% Triton X-100), and 0.2% glycine treatment followed by antibody block (10% Normal
Doneky Serum or Heat Inactivated Goat Serum in PBS) for one hour, primary antibody incubation overnight, secondary antibody incubation for 1 hour, and subsequently mounted using Fluoromount aqueous mounting medium (Sigma-Aldrich, F4680). MDA-MB-231 cells were fixed for 2 minutes using 10% Formalin, permeabilized using 0.5% Triton X-100, blocked using 10% Normal Donkey Serum in PBS for 30 minutes, incubated with primary antibody in 5% Normal Donkey Serum in PBS for 1.5 hours, incubated in secondary antibody in PBS for 1 hour, and mounted using Fluoromount. Antibodies used: X anti-Ki67, Rt anti-K8 (Troma-1, Developmental Studies Hybridoma Bank, Rb anti-K5 (Abcam, ab24647), Secondary antibodies used were AlexaFluor Donkey α-Ms 488, Donkey α-Rb 555, Donkey α-Goat 555, Donkey α-Rb 488 (Jackson Immuno Research Laboratories, 1:5000).

**Carmine whole-mount staining and ductal outgrowth analysis**

Whole glands were spread onto glass slides and fixed overnight in Carnoy’s (75% ethanol, 25% glacial acetic acid), briefly dehydrated in 70% ethanol, stained overnight in Carmine alum (0.2% carmine dye [wt/vol], 0.5% aluminum potassium sulfate [wt/vol], destained until background is minimal in destaining solution (2% HCl in 70% ethanol), dehydrated to 100% ethanol, incubated in toluene, mounted in Permount (Fisher, SP15-500), and imaged. Outgrowths were quantified based on percent of fat pad filled with epithelium.

**Alveogenesis quantification**
To quantify alveogenesis, 3rd and 4th mammary glands from 17.5 dp mice were harvested and stretched out onto glass slides, stained with Carmine alum, imaged, paraffin embedded as previously described (Marlow et al., 2008) and sectioned. Sections from the top, middle and bottom, of each region of the gland were then stained with Hematoxylin and Eosin (H&E) and imaged. The percent of each image that was filled by alveoli was quantified using Fiji (Image J), and the values from all images were combined to give the average percent alveogenesis for that gland.

**Spindle orientation measurements**

Mitotic cells were identified based on chromatin condensation and quantified as a percentage of total cells. Mitotic spindle angle was measured as described {Postiglione, 2011 #1358}. Briefly, 16 mm cryosections were stained with NuMA, SMA and LGN, and imaged using a Leica Spot scanning confocal SP5 microscope. Z stacks were rendered into a 3-D image using Volocity software, and points in the X, Y and Z planes were assigned to the mitotic spindle poles, and to 5 points along the basal plane of the dividing cell. The best-fitting plane was determined by orthogonal distance regression using the coordinates of the five points. The angle \( \phi \) between the vector connecting the two points marking the centrosomes and the normal vector of the regression plane was calculated, and 90° minus the angle \( \phi \) was used as the division angle \( \alpha \). All calculations were done using the R programming environment. For further details, and for a description of the determination of the upper limit of
error for the division angle calculation, see Postiglione et al.

Microscope information

Fluorescence or brightfield images were acquired using a Biorevo BZ-9000 Digital Microscope (Keyence), Leica Spot Scanning Confocal SP5 microscope (Leica), Leica Wide-field DM5500 (Leica) or Volocity Spinning Disk Confocal Microscope (PerkinElmer). Images were processed using Volocity 3D image analysis software (PerkinElmer), Fiji (Schindelin and Arganda-Carreras et al., 2012), GraphPad Prism (GraphPad Software), and Adobe Photoshop CS5.

Statistics

Statistics were performed using Prism software (GraphPad). Two-tailed unpaired student’s t-test was used in all Figures. Significance is indicated by *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001, ns: p > 0.05. Graph columns represent the mean and error bars represent the standard error of the mean (SEM).

Preliminary Results:

Outer cap cells give rise to both basal and luminal daughter cells. Divisions that are perpendicular with respect to a basal lamina are thought to result in differential cell fates due to the displacement of daughter progenitors to a different microenvironment. To investigate, we used smooth muscle actin (SMA) to distinguish cap (SMA+) from luminal body (SMA-) cells. We find that the majority
of these perpendicular divisions generate two SMA+ cells, possibly reflecting outer cap cells symmetrically renewing and giving rise to drop down cap cells that enter the luminal compartment (Figure 3.1B). We find that only a few (1%) of perpendicular divisions result in a SMA+ cap cell giving rise to a SMA- luminal cell (Figure 3.1C). In very rare cases, we have observed a SMA+ drop down cap cell giving rise to a SMA- body cell (Figure 3.1D), suggesting that cap cells divide asymmetrically, and can be considered multipotent during development. Taken together, these results suggest that while in the majority of cases, outer cap cells divide within the outer cap cell compartment, they can turn their spindle and give rise to both basal and luminal body cells.

To investigate the long-term identity and fate of clones placed in the body cell compartment, as well as to investigate the long-term identity of clones generated by drop down cap cells, we are currently optimizing a protocol to stain lineage-traced tissue with basal and luminal markers. To this end, mice expressing Tamoxifen-inducible K5-driven CreER were mated with mice expressing ai9 at the Rosa 26 (R26) locus and the resulting mice have been validated for use in the lineage tracing study, as we did not detect clones in untreated mice. After 24 hours, single K5-positive clones were detected in the mice induced with 1.5 mg Tamoxifen, but not in the untreated mice, demonstrating that the K5-CreER promoter is not leaky, and that the dose is appropriate for single-clone induction (data not shown). To investigate how increased levels of mINSC may affect cell specification and daughter cell placement during ductal outgrowth, we generate K5CreER; mInsc KI/ai9 mice, along
with the control K5CreER; +/ai9 (wild type, WT). At this time, we have observed a significant number of clones in K5CreER; mInsc KI/ai9 mice, but are still collecting data from wild-type mouse. Therefore, the data presented below is representative of the biology in mice overexpressing mInsc. After 36 hours, larger clones were observed in end buds, and 33% (11/33) of clones were drop down cap cells, and 67% (23/33) of clones were outer cap cells (Figure 3.2A). However, after 48 hours, the clones were observed in subtending ducts, but not in the end buds, suggesting that the cells in the end bud at the time of induction have been replaced by new, unlabeled daughter cells (data not shown). This limits our analysis of end bud cells to 24-36 hours post induction, and thus limits the size of the clones that we can analyze. At 36 hours post-induction, the majority of clones exist as single, double or triple clones (42% and 24%, 12% respectively). The rest of the clones (22%) contained 4 or more cells (Figure 3.2B). We further investigated the location of the cells in outer cap cell clones with 2 or more cells in K5CreER; mInsc KI/ai9 end buds and find several scenarios (Figures 3.2C, D). The majority of clones contained cells that remained in the outer cap cell layer (Figure 3.1Da, d, 57%, 8/14 outer cap cells), while some clones contained cells in the outer cap cell layer as well as the body cell compartment (Figure 3.1Db, c, 43%, 6/14). Taken together, the results from this study demonstrate a mechanism by which cap cells located in the outer cap cell compartment can generate both outer and drop down cap cells during ductal outgrowth.
Changes in Inscuteable expression affects cell fate acquisition. In the previous chapter, I demonstrated that increased Inscuteable in the \textit{mInsc KI/KI} leads to an increased frequency of outer cap cells dividing perpendicularly with respect to the basal lamina. In this mouse, we also observed an increased frequency of drop-down cap cells, suggesting that the cells placed in the body cell compartment remain there as cap cells, and do not migrate back up to the outer cap cell layer. To investigate the long-term fate of these drop-down cap cells, I will immunostain lineage-traced tissue over-expressing mInsc-GFP. Because both \textit{ai9} and \textit{mInsc-GFP} are expressed at the Rosa26 locus, we can only express one copy of \textit{mInsc-GFP}. In the \textit{mInsc KI/KI}, we saw a 3-fold increase in mINSC expression. By immunoblotting, we find that the \textit{mInsc KI/+} basal cells contain a 2.6-fold increase mINSC, and the luminal cells contain a 2.9-fold increase, compared to the respective WT populations (Figure 3.3A). To investigate if this increase in mINSC is sufficient to alter the spindle orientation in outer cap cells, we performed the spindle orientation assay in tissue from 5.5 week-old \textit{mInsc KI/+} mice, and found that the proportion of outer cap cells dividing laterally (0°-30°) decreased from 82% in WT, to 59% in \textit{mInsc KI/+} tissue. Concordantly, the proportion of oblique and oriented (30°-90°) divisions increased from 18% in WT to 41% in \textit{mInsc KI/+} outer cap cells (Figure 3.3B). As with \textit{Robo1-/-} tissue, these changes in spindle orientation in \textit{mInsc KI/+} represent a significant increase in the mean mitotic spindle angle (Figure 3.2C). This is supported by mathematical modeling that shows a horizontal enrichment of spindle angles in WT outer cap cells and random distribution in \textit{mInsc KI/+} cells (Figure 3.3D, E), which is
similar to that observed in Robo1/- cells. Taken together, these data strongly suggest that the changes in spindle orientation observed in Robo1/- tissue are due to increased expression of mINSC, which has previously been shown to regulate the spindle machinery. Thus, we have identified an extracellular cue that regulates the spindle machinery through one of its key components, mINSC.

To investigate possible consequences of randomized spindle orientations in the outer cap cell population during ductal outgrowth, we Carmine stained whole-mounted tissue from 5.5 week-old WT and mInsc KI/+ mice. We found that while there is no increase in ductal outgrowth, as we observed in the mInsc KI/KI, the end buds were significantly larger in the mInsc KI/+ compared to the WT (Figure 3.4A). To rule out the possibility that this phenotype is due to increased proliferation, we first quantified Cyclin D1 levels, both at the protein level in whole cell lysates and transcript levels in the basal fraction, and found no significant difference between the WT and mInsc KI/+ samples (Figure 3.4B, C). FACS analysis of dissociated cells from adult tissue revealed no difference in the proportion of basal and luminal cells between the two genotypes (Figure 3.4D). These results suggest that the larger end buds are not due to increased proliferation cells, but rather due to the placement of the daughter cells, with more daughter cells being placed in the body compartment, as observed in the mInsc KI/KI. Taken together, our preliminary results suggest a mechanism where SLIT2/ROBO1 signaling regulates mammary ductal development by controlling the orientation of the spindle in the outer cap cells.
**Increased Inscuteable affects alveogenesis**

Preliminary data in the lab has shown that alveogenesis is delayed in Robo1 -/- mice at 17.5 days pregnant (dp) (data not shown). Therefore, to investigate if this phenotype is due to changes in the frequency of ACD in the stem cell population during ductal outgrowth, we first assessed alveogenesis by quantifying the percent of fat pad filled with alveoli at 17.5 dp in WT, mInsc KI/+ and mInsc KI/KI mice. Indeed, we found decreased alveogenesis in both the mInsc KI/+ and mInsc KI/KI mice compared to WT at 17.5 dp (Figure 3.5A-D). These data suggest that mechanisms regulating stem cell behavior during development can have long-term effects on the morphology of the gland. To further investigate the effects of increased mINSC on the functionality of the gland, we are currently collecting tissue to assess the levels of milk production by measuring whey acidic protein (WAP) and beta-casein mRNA levels by qPCR in WT, mInsc KI/+ and mInsc KI/KI samples.

Taken together, our studies reveal a mechanism whereby the level of Inscuteable affects the specification of mammary cell lineages, end bud morphology, and ultimately alveogenesis. Breast cancer tumor cells express signature markers that suggest their cell of origin, and establishes a tumor cell hierarchy. Understanding the process of how breast cell lineages are established during development can help us understand how certain mutations can affect tumor cell identity and how basal-like tumor cells can in fact originate from luminal epithelial cells.
Figure 3.1 Mammary cap cells divide asymmetrically.

(A) The mammary gland is a bilayered ductal system that is composed of myoepithelial and luminal cells. During development, the growth of the ductal tree is driven by a highly proliferative structure called the end bud (eb). The mammary eb is composed of outer and drop down basal cap cells that differentiate into myoepithelial cells, and luminal body cells, that differentiate into luminal epithelial cells. (B, C) Representative images of an outer cap cells (white boxes), undergoing a perpendicular division generating two SMA+ daughter cells (B), or one SMA+ and one SMA- daughter cell (C). (D) Representative image of a drop down cap cell dividing to generate one SMA+ and one SMA- daughter cell. Arrowhead points to a NuMA crescent at one cortex, indicating an asymmetric cell division. Mammary tissue from 5.5 week-old mice was stained with Hoechst, NuMA, and smooth muscle actin (SMA) to mark DNA, mitotic spindle poles, and basal cells, respectively. Basal lamina is marked by solid white line. In magnified images, dividing cell is outlined with a dashed white line. Scale bars: 32 µm and 16 µm (B, C) and 12 µm in D).
Figure 3.1 (A-D)
Figure 3.2 Lineage tracing in cap cells of the mammary end bud.

(A, B) Quantification of the location of K5-driven ai9-expressing clones (A) and number of cells per clone (B) in K5CreER; mInsc KI/ai9 end buds after a 36 hour induciton with 1.5 mg Tamoxifen. (C) Cartoon model of the types of clones (labeled a-f) observed in K5CreER; mInsc KI/ai9 end bud. (D) Two cross sections of a K5CreER; mInsc KI/ai9 end bud stained with Hoechst to label nuclei, and imaged to show ai9 clones. The small letters (a-f) represent the clone types outlined in B. Dashed lines outline the cap cell layer. Yellow lines outline individual cells in a-c. n = 1 animal. Scale bars represent 30 µm.
Figure 3.2 (A-D)

A

B

C
d
c
d
f
e
b
a

D

Hoechst tdTomato (a19)
a
c
d
e

1 cell
2 cells
3 cells
4+ cells
Total=33

Drop Down Cap Cells
Outer Cap Cells
Total=33

1 cell
2 cells
3 cells
4+ cells
Total=33

155
Figure 3.3 mINSC regulates spindle orientation during ductal development.

(A) Representative immunoblot (left) and quantification (right) of total mINSC in $mlnsc$ $+/+$ and $mlnsc$ KI/+ MEC and luminal fractions from 5.5 week-old mice with total GAPDH as loading control and pCAGGS-HA-Insc as positive control. (B) Distribution of mitotic spindle angles in dividing outer cap cells in $mlnsc$ $+/+$ and $mlnsc$ KI/+ end buds. $n = 49$ and $n = 37$ cells, respectively from at least 3 animals per genotype. (C) Quantification of the mean mitotic spindle angle of dividing outer cap cells (from B) $mlnsc$ $+/+$ and $mlnsc$ KI/+ end buds. (D-E) Graphical representation of mitotic spindle angles in $mlnsc$ $+/+$ (D), and $mlnsc$ KI/+ (E) outer cap cells.
Figure 3.3 (A-E)

A

B

C

D

E

mInsc+/+ mInsc KI/+
Figure 3.4 Increased Insuteable leads to larger end buds

(A) Quantification of the end bud size in *mInsc +/+* and *mInsc KI/+* tissue at 5 weeks of age. *n* = 3 biological replicates. (B, C) *mInsc +/+* and *mInsc KI/+* glands do not exhibit changes in cell proliferation. (B) Representative immunoblot and quantification of CYCLIND1 expression in whole-gland lysates from *mInsc +/+* and *mInsc KI/+* glands at 5.5 weeks of age. (C) RT-qPCR analysis of *CyclinD1* mRNA levels in FACS-purified basal cells from *mInsc +/+* and *mInsc KI/+* adult mice. *n* = 3 biological replicates. (D) Quantification of the proportion of basal (Lin-CD24+CD29hi) and luminal (Lin-CD24+CD29low) cells as a % of total epithelial cells. *n* = 3 biological replicates.
Figure 3.4 (A-D)
Figure 3.5. Increased Inscuteable leads to decreased alveogenesis.

(A-C) Representative images of H&E-stained mammary tissue from 17.5 dp mice. (D) Quantification of alveogenesis in 17.5 dp mInsc +/+ , mInsc KI/+ and mInsc KI/KI mice as determined by the % of fat pad filled with alveoli. n = 2 mice per genotype.
Figure 3.5

A. mlnsc +/+

B. mlnsc KI/+  

C. mlnsc KI/KI

D. % Alveologenesis (% of total fat pad filled with alveoli)
References:


