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REVIEW ARTICLE

Diverse roles for axon guidance pathways in adult tissue architecture and function

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Abstract

Classical axon guidance ligands and their neuronal receptors were first identified due to their fundamental roles in regulating connectivity in the developing nervous system. Since their initial discovery, it has become clear that these signaling molecules play important roles in the development of a broad array of tissue and organ systems across phylogeny. In addition to these diverse developmental roles, there is a growing appreciation that guidance signaling pathways have important functions in adult organisms, including the regulation of tissue integrity and homeostasis. These roles in adult organisms include both tissue-intrinsic activities of guidance molecules, as well as systemic effects on tissue maintenance and function mediated by the nervous and vascular systems. While many of these adult functions depend on mechanisms that mirror developmental activities, such as regulating adhesion and cell motility, there are also examples of adult roles that may reflect signaling activities that are distinct from known developmental mechanisms, including the contributions of guidance signaling pathways to lineage commitment in the intestinal epithelium and bone remodeling in vertebrates. In this review, we highlight studies of guidance receptors and their ligands in adult tissues outside of the nervous system, focusing on in vivo experimental contexts. Together, these studies lay the groundwork for future investigation into the conserved and tissue-specific mechanisms of guidance receptor signaling in adult tissues.

Key Points:

1. Axon guidance ligand and receptor expression often persist into adulthood in neuronal and non-neuronal tissues alike.
2. Recent work in genetic model organisms highlights the diverse roles of guidance factors in adult tissues.
3. Guidance factors are required intrinsically in a variety of adult tissues but can also regulate tissue function indirectly via functions in the nervous and vascular systems.

4. Studies outside of the nervous system are likely to enhance our understanding of these diverse siganling molecules and could suggest novel signaling modalities in the nervous system.

KEYWORDS
axon guidance, homeostasis, blood-brain barrier, mammary gland, pancreas, bone, stem cell niche, tissue-resident stem cell, organismal physiology

INTRODUCTION

During nervous system development, newly differentiated neurons project axons must navigate a dense signaling environment to reach their synaptic targets and form functional circuits. The trajectory of an axon is controlled by the suite of guidance receptors on its motile tip, or growth cone, which initiate changes in axon shape in response to extracellular cues. Historically, guidance signaling axes have been categorized as "attractive" or "repulsive" based on the response of the growth cone to ligand–receptor binding: the ligand–receptor pairs slit-Roundabout, ephrin-Eph, and semaphorin-Plexin stimulate repulsion; netrin similarly facilitates repulsion through its Uncoordinated-5 (Unc5) family of receptors, but also promotes attraction via Deleted in colorectal cancer (Dcc) and Neogenin receptors (Box 1). In the years since their first description, our understanding of guidance signaling axes has deepened. For example, it has become clear that attractive and repulsive outputs can be modified by combinations of receptors on the growth cone and that the signaling cascades initiated simultaneously by different ligand–receptor pairs can act both in parallel and synergistically to coordinate axon responses.[1] The mechanisms that regulate ligand–receptor interactions and control the cytoskeletal growth cone response to guidance cues remain robust areas of investigation.[2]

During development, both neurons and other cells must acquire specific fates through changes in their location, morphology, and gene expression. A growing number of studies describe non-canonical functions of axon guidance signaling pathways, including activities that control guidance through the regulation of gene transcription and translation[3,4] and those that affect other aspects of neuron behavior entirely, including cell proliferation, adhesion, survival, and migration.[5–8] Given the range of activities of guidance receptor signaling in nervous system development, it is perhaps unsurprising that many of the genes required to wire the nervous system are put to broad developmental use. Axon guidance pathways are essential for the morphogenesis of both vertebrate and invertebrate vascular systems[9–12] and the formation of stem cell niches[13,14]; furthermore, they control the development of a wide range of tissues including the pancreas, kidney, heart, and the skin.[15–20]

Axon guidance ligand and receptor expression often persist into adulthood in neuronal and non-neuronal tissues alike. The functions of these genes in adult tissues, however, are less clear. Indeed, the wide-ranging roles for guidance molecules during development have historically precluded genetic analysis of later stage tissues using global knockout approaches. In the past several decades, however, the advent of conditional knockout technologies and innovative paradigms for spatiotemporal control of gene expression have allowed researchers to circumvent developmental lethality to study the role of these genes specifically in adult tissues, often with single cell or even subcellular resolution. Furthermore, the rise of single cell sequencing technologies and the efforts to make these results accessible to other researchers are likely to generate new hypotheses regarding the function of guidance receptors and their ligands in adult tissues.

Here, we discuss recent work describing roles for axon guidance signals in adult tissues, highlighting in vivo studies in genetic model organisms. In adults, several roles for guidance factors, including adhesion, proliferation, and cell migration, mirror their developmental roles. The function of guidance receptors in adults also extends to regulating additional processes, including lineage commitment in tissue-resident stem cells. Moreover, guidance factors are required intrinsically in a variety of adult tissues but can also regulate tissue function indirectly via functions in the nervous and vascular systems. Together, studies in these alternative contexts will enhance our understanding of the diverse signaling mechanisms of these molecules, including whether they change over the course of development, and might even suggest novel signaling modalities in the nervous system.

AXON GUIDANCE FACTORS MAINTAIN TISSUE ARCHITECTURE

The mechanisms that control a tissue's homeostasis depend on the cell types it contains and its role in communicating and responding to the physiological status of the organism. Many tissues in adult organisms consist largely of postmitotic cells, and they do not grow significantly in adulthood. For example, in the human nervous system, structural plasticity arises primarily from synaptic remodeling of existing cells.[21,22] Other tissues, like the mammalian mammary gland, are highly dynamic, proliferating, and growing in response to episodic hormonal cues in the adult animal.[23] Furthermore, while limited in neurons themselves, the maintenance of most tissues also requires that dying or damaged cells be eliminated and replaced. Guidance factors play roles across organs with vastly different requirements for homeostasis. Below, we consider the role of guidance factors in the maintenance of tissues with disparate morphologies and homeostatic mechanisms: the branched vascular system and mammary gland, the pancreatic islet, and the bone.

Box 1. CLASSICAL AXON GUIDANCE RECEPTORS AND THEIR LIGANDS

Since their identification in regulating nervous system development, axon guidance receptors and their ligands have been the subjects of extensive genetic and biochemical investigation. Here, we provide an overview of the ligand–receptor pairs that feature in this review. For in-depth discussions of ligand–receptor structures and signaling mechanisms, we direct the reader to recent reviews.[2]

Slit-Roundabout

The Roundabout (Robo) receptors are single-pass transmembrane proteins that usually bind Slit ligands. Although initially studied for their roles in axon guidance, where Slit-Robo signaling is repulsive, the ligand–receptor pair is broadly expressed and has been implicated in variety of developmental and disease processes.[204] Robo receptors are present throughout phylogeny, although they have acquired distinct activities over time.[205,206] Notably, vertebrate Robo2-4 likely arose from a tandem gene duplication event, and despite naming conventions, all vertebrate Robos are most closely related to *Drosophila* Robo1.[207] Robo4 is an endothelial cell-specific paralog of the Robo family of receptors. While neither vertebrate Robo3 nor Robo4 bind Slits,[205,208] they each interact with other guidance receptors to regulate cellular responses to guidance cues: in neurons, Robo3 binds Dcc to potentiate Netrin signaling,[205] and in blood vessels, Robo4 binds Unc5B to promote vascular integrity.[58]

Netrin and its receptors

Netrins are secreted ligands that signal via several transmembrane receptors: Deleted in colorectal cancer (Dcc; Frazzled in *Drosophila*), Neogenin, and Unc5. During axon guidance, signaling through Dcc and Neogenin is usually attractive, and signaling through Unc5 is repulsive.[209] In addition to their Netrin-induced activities, Netrin receptors act as "dependence receptors" in some cellular contexts, stimulating apoptosis in the absence of Netrin.[93] Furthermore, in the *Drosophila* nervous system and ovarian germline, Frazzled has Netrin-independent transcriptional activity.[94,210,211]

Semaphorin-Plexin

Semaphorins (Semas) are a large and diverse family of signaling ligands that share a common extracellular domain.[212] This domain, the sema domain, mediates their interaction with their most common receptor class, the Plexins. Neuropilins also act as co-receptors for some Semas. Notably, the Sema family includes both secreted and transmembrane members, and transmembrane members can themselves act as receptors for Plexins. This phenomenon, "reverse signaling," allows for bidirectional signaling between closely associated cells. Semas are expressed broadly across phylogeny and, in addition to the roles described here, have been studied extensively in development,[213] the immune system,[128,214] and in cancer.[215]

Ephrin-Eph

Ephs are a large class of receptor tyrosine kinases encompassing two subclasses: EphA and EphB.[15] Their membrane-associate ligands, the ephrins, occupy two structurally distinct subclasses: ephrin-A and ephrin-B. Ephrin-A family members are associated with the membrane by a glycosylphosphatidylinositol tail, and ephrin-B family members are transmembrane proteins with short cytoplasmic domains. Generally, ephrin ligands bind promiscuously to their cognate receptor class (i.e., ephrin-A ligands tend to preferentially bind EphA receptors). Like semaphorin-plexin signaling, ephrin-Eph signaling is bidirectional, and intracellular signaling cascades can occur in cells expressing either the ligand or receptor. Furthermore, because both ephrins and Ephs are present at the cell membrane, signaling depends on close cellular contact. In addition to the functions described in this review, ephrin-Eph signaling has been studied extensively in development and in cancer.[15,20,216]

Guidance factors regulate adhesion and tissue integrity in the vascular system

Like axons and dendrites in the nervous system, endothelial vasculature is highly articulated, and a large body of literature underscores the two systems' shared developmental mechanisms.[24] In contrast to neurons, where single cells extend processes that branch and fasciculate, in the vascular system, proliferation is tightly linked to branching morphogenesis of cooperating groups of cells. During angiogenesis, endothelial tip cells, which share many of the morphological characteristics of neuronal growth cones, respond to ligands in the extracellular environment to direct vascular patterning.[25] Moreover, the vascular system requires axon guidance signals for appropriate branching during development, although reports conflict as to whether the signals promote or inhibit angiogenesis. For example, exogenously applied Netrin-1 can stimulate vascular sprouting in chick embryos,[26] but its intra-ocular injection into young postnatal mice reduces branching in retinal vasculature.[27] Mice and zebrafish with disrupted *Unc5b* exhibit ectopic blood vessel branching and tip cell filopodia extension during embryogenesis. The same phenotype presents in zebrafish with mutations in *netrin-1a*. Importantly, the effects of exogenous netrin application in mice depend on the presence of the Unc5b receptor, providing a clear link between ligand and receptor in this developmental context. It is tempting to speculate that the opposing effects of netrin-1 in vascular development may be mediated by

different receptors; this is the case in the nervous system. Indeed, Unc5b is broadly expressed in both chick and mouse vasculature during development[27–29]; Dcc and Neogenin, however, are not detected by in situ hybridization in embryonic mouse vasculature.[27] Thus, the question of how netrin-1 plays opposing roles during vascular development remains unanswered. Furthermore, during adulthood, sprouting angiogenesis is limited, occurring mainly under pathological or wound-healing conditions. Is the continuous expression of guidance receptors required to suppress neovascularization in adult animals?

Netrin receptors are likely to remain active in adult tissues, although their precise expression patterns and functions remains unclear. Unc5b's continuous expression in adult vasculature, in addition to its localization throughout the endothelium, positions it as a potential suppressor of adult neovascularization,[27] although this hypothesis remains untested. Evidence of other receptors' expression in the adult vascular system is either absent or conflicting. For example, Neogenin is expressed in vascular smooth muscle cell culture[26] and localizes to the filopodia of human umbilical artery endothelial cells,[30] but its adult expression has not been described in vivo. Further, reports of Dcc expression in endothelial cell lines conflict, with some studies suggesting it is expressed[31] and others failing to detect it by polymerase chain reaction.[26,32] Nevertheless, the responsiveness of adult tissues to netrin application suggests that netrin receptors are present in the adult blood and lymphatic vasculature. Aortic discs from adult mice cultured ex vivo in the presence of netrin-1 have increased cell outgrowth.[31] Because outgrowth is driven by cell division in the vascular endothelium, this suggests that netrin-1 can promote cell proliferation. This effect is concurrent with nitric oxide production and is abrogated in the presence of a nitric oxide scavenger. Given that nitric oxide has many roles in the vascular endothelium, including regulating vascular tone,[33] netrin-dependent signaling may be important for general vascular function.

Which netrin receptor mediates its requirement in blood vessels? Dcc is required for netrin-1-induced cell proliferation in vitro, and cells treated with a Dcc function-blocking antibody do not respond to netrin-1 application.[31] However, there are currently no in vivo reports of Dcc expression in adult blood vessels. Nevertheless, viral transduction of netrin-1 in the adult mouse brain also increases the size of blood vessels,[34] indicating that while their identity is not known, netrin receptors are likely to be active in adult vasculature. Moreover, these blood vessels incorporate BrdU, indicating that their change in size is likely due to increased proliferation. Netrin application also induces endothelial cell proliferation in several in vitro contexts.[26,34,35] Specifically, in cultured lymphatic dermal human microvascular endothelial cells, netrin-4 induces activation of several signaling pathways implicated in proliferation, including Akt, PI3K, and Erk.[35] Furthermore, simultaneous pharmacological inhibition of these pathways blocks netrin-induced proliferation, suggesting that they promote cell division downstream of netrin signaling. Another intriguing possibility is that netrin-induced proliferation is a context-dependent output of focal adhesion kinase (FAK) signaling and/or Src signaling, both of which act downstream of netrin-4 in vitro[35] and have been implicated in axon guidance.[36,37] In addition to their roles in axon outgrowth, both FAK and Src have broad roles promoting cell proliferation.[38] Thus, the precise mechanism by which netrin signaling controls cell proliferation may be separable or comparable to that by which it controls axon outgrowth.

While these studies suggest that netrin receptors are active in the adult vasculature, they do not directly examine the role of endogenous netrins in adult animals. Indeed, netrin-1 is expressed in the adult rat and mouse brains,[39–41] although its importance to brain vasculature has not been addressed through adult-specific loss of function studies. Similarly, netrin-4 is present in adult tissues, but it has been primarily studied in the context of overexpression. Netrin-4 is detected by antibody in lymphatic vessels in the adult mouse intestine, lymph nodes, and the skin, and overexpression of netrin-4 in adult mouse keratinocytes increases lymphatic vasculature leakage.[35] Among netrins, only netrin-4 has been shown to interact directly with laminins in the basement membrane.[42] Indeed, netrin-4 is also expressed in the basement membrane of vasculature in the adult mouse eye.[43] While *netrin-4* global knockout does not affect basement membrane integrity,[43] *netrin-4* knockout mice have increased blood vessel tortuosity and leakage in the retina.[44] It remains unclear if this is because of a developmental requirement for *netrin-4* in blood vessel maturation or if this reflects an adult-specific requirement.

Indeed, the role of guidance receptors in adult vasculature can closely mirror the roles of genes during development. For example, ephrin-Eph signaling is required during angiogenesis and is a determinant of arterial/venous characteristics in adults. Veins and arteries diverge significantly over the course of development in ways that reflect their distinctive roles in the vasculature; for example, arteries have significantly thicker walls than veins. While these changes may reflect adaptations to the physical stress accommodating their different physiological roles, there are genetic differences in the vertebrate vasculature before blood flow begins.[45,46] Notably, in the developing circulatory system, ephrin-B2 is exclusively expressed in arteries, and EphB4, one of its receptors, is expressed only in veins. *Ephrin-B2* and *EphB4* knockout mice each have dramatic defects in angiogenesis, including widespread blood vessel fusion and truncation, and die in utero.[47–50] The striking similarity of these mutant phenotypes strongly suggests that ephrin-B2 is the predominant ligand for EphB4 in vivo.[48] Although the cellular mechanism of this activity has yet to be determined, the bidirectional nature of ephrin-Eph signaling means that each could act to intrinsically determine venous and arterial identity during development. While adult-specific knockout experiments have not been performed, venous graft experiments have shed light on the importance of ephrin-Eph signaling in adult tissues. In both mice and humans, surgically transplanting EphB4-positive veins to an arterial environment, where surrounding arteries are EphB4 negative, leads to loss of EphB4 expression in blood vessel endothelium.[51] Interestingly, while the veins do not begin to express ephrin-B2, they adopt other arterial characteristics: they become thicker and accumulate α-actinin. Injecting mice with ephrin-B2/Fc before surgery, which should bind and induce signaling via EphB4, limits this effect, suggesting that active EphB4 signaling suppresses arterial characteristics. In support of this model, veins transplanted from *EphB4* heterozygous mice grow

significantly thicker than veins from wild-type mice. While this is a property of the vein itself, the authors concede that this may also reflect a contribution of EphB4 activity from smooth muscle cells. Indeed, in vitro experiments with primary culture of human adult venous smooth muscle cells indicate that Eph-B4 is present and active in this cell population.[52] Stimulation of primary smooth muscle cells with ephrin-B2/Fc also leads to accumulation of α-actinin. It will be interesting to see whether selective deletion of *EphB4* in adult endothelium or smooth muscle cells can also lead to "arterialization" in the absence of a physiological environment that promotes such a transformation, and if veins lacking EphB4 in adults have any other defects.

Other guidance receptors and their ligands have been implicated in endothelial integrity in the vascular system, and mice lacking guidance receptors have compromised postnatal vascular integrity. For example, Robo4, the endothelium-specific form of the Roundabout receptor, is required for blood vessel integrity, and dye injection experiments demonstrate that 8–10 weeks old *Robo4* null mice have leaky retinal endothelia.[53] This effect is rescued by pharmacological inhibition of Src family of non-receptor tyrosine kinase (SFK) signaling, suggesting that SFK is usually suppressed by Robo4 to promote vascular integrity. In the *Robo4* global knockout mouse, however, it is unclear if these adult effects are due to defects in development or a continuous role for Robo4 in adult vasculature. Intriguingly, Robo4 vascular leakage phenotypes can be rescued by expression of a Robo4 variant lacking its cytoplasmic domain, suggesting that downstream signaling is likely to reflect additional interactors rather than the direct action of Robo4 itself.[54] Similarly, during *Drosophila* nervous system development, the cytoplasmic domain of Robo2 is dispensable for its ability to promote axon growth across the midline.[55] This is consistent with a model in which Robo2 promotes crossing not by binding to slit directly, but by binding to Robo1 in *trans*, thus preventing slit-Robo1 interaction. In the retinal vasculature, it is unlikely that Robo4 directly responds to a slit ligand: while slit2 co-injection suppresses vascular leakage phenotypes in models of vascular permeability, Robo4 lacks the conserved slit-binding domains present in Robo1 and Robo2.[56,57] However, Robo4 physically interacts with Unc5,[58] and it may physically interact with other Robo receptors to influence their interaction with slit2. Indeed, Robo1 and Robo2 are both expressed and required in the postnatal mouse vasculature. *Robo1;2* double knockout mice have reduced blood vessel outgrowth in the postnatal retina, as do *slit1;2* double knockout mice.[59] It remains to be seen whether interactions between Robo4 and other Robo receptors may explain its apparent interaction with Slit2 to regulate vascular permeability.

In contrast to slit-Roundabout signaling, where mutant phenotypes may be attributed to developmental contributions, semaphorins and their receptors clearly regulate vascular integrity in adult animals, where they interact with vascular endothelial growth factor (VEGF). VEGF promotes angiogenesis under normal and disease conditions and acts via its receptor (VEGFR) and co-receptors, including Neuropilins, which also act as semaphorin co-receptors.[60–63] In endothelial cells, however, sema3A and VEGF165 (an isoform of VEGF) bind distinct, nonoverlapping sites on Neuropilin-1.[64] Sema3A, but not sema3B or sema3F, injected into wild-type mice causes a dose-dependent increase in vascular permeability, demonstrating that sema3A controls this process after development.[65] A similar effect occurs when sema3A is injected into the eye.[66] In vitro experiments suggest sema3A activity is mediated by the downstream phosphorylation of VE-cadherin, which causes destabilization of the adherens junctions between endothelial cells, therefore increasing blood vessel permeability.[65,66] Furthermore, endothelial-cell-specific conditional knockout of Neuropilin-1 in adult mice abrogates this permeability defect, providing a functional link between ligand and receptor in this process.[65] What is the relevance of ectopically administered sema3A to physiological conditions? Sema3A levels become elevated in animal models of diabetes and in diabetic patients. Sema3A levels are high in vitreous fluid from the eyes of humans suffering from diabetic macular edema, and mice with pharmacologically induced type 1 diabetes mellitus have increased sema3A in retinal ganglion cells.[66] Lentiviral-induced knockdown of sema3A in neurons abrogates the permeability phenotype, indicating that while sema3A is expressed in several cell types, its neuronal production drives the phenotype in this context. Conditional knockout of *Neuropilin-1* in the circulatory system also protects mice from permeability defects, supporting a model in which sema3A is secreted from neurons to act on its receptor in the vasculature.

Interestingly, although sema3A is also expressed and required in the retinal vasculature during development for filopodia formation in tip cells, endothelium-specific conditional knockout in adult mice demonstrates that it is dispensable for blood vessel integrity later in life.[67] Likewise, semaphorin-Plexin signaling appears to be required during lymphatic development but is dispensable in adult tissues. Sema3G is expressed in developing arteries[68] and acts on PlexinD1 in lymphatic endothelial cells during development to regulate the morphogenesis of the lymphatic system and repel it from the blood vasculature.[69] In *PlexinD1* lymphatic endothelial-cell-specific knockout mice and *sema3G* null mice alike, the lymphatic and blood vasculature remain closely aligned, indicating a failure in repulsion during developmental guidance. Interestingly, this is a transient effect, and adult *sema3G* mutant mice correct the alignment disparity. Thus, in contrast to compromised slit-Robo signaling, which has lifelong implications, semaphorin signaling may be supplanted by other signals postnatally to regulate vascular integrity.

Guidance molecules regulate vascular integrity at the blood–brain barrier

Blood flow to the brain is tightly regulated by the blood–brain barrier, a multicellular vascular assembly that is structurally distinct from other endothelial vessels in several ways.[70,71] For example, blood–brain barrier-specific transporter and receptor proteins regulate transcellular transport, and tight junctions limit the movement between adjacent cells. These structural differences between endothelial cells in the central nervous system do not appear to be the consequence of intrinsic properties of the vasculature; rather, they arise from interactions with cells in the nervous system itself, including astrocytes.[72–75] This

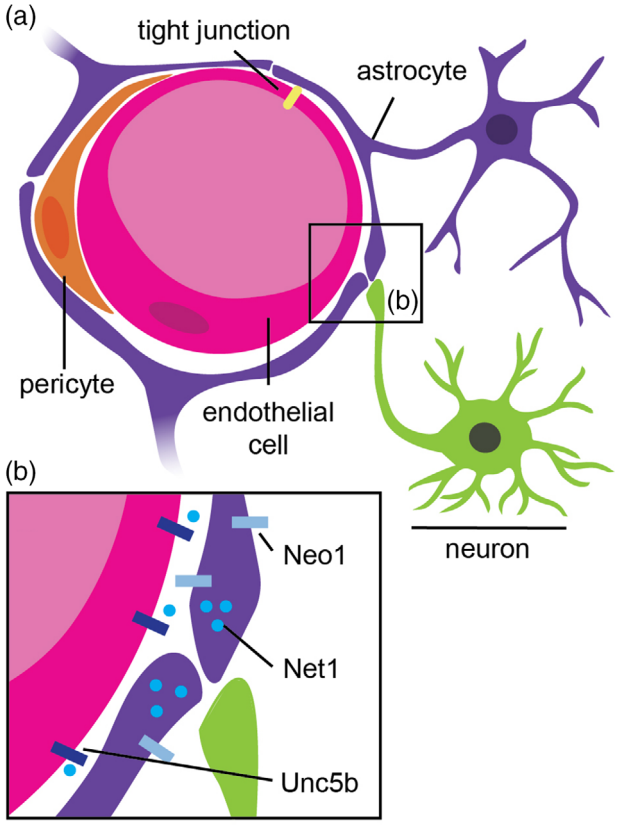

FIGURE 1 Netrin-1 and its receptors regulate the architecture of the blood–brain barrier. (a) The specialized endothelial cells at the blood–brain barrier (pink) are joined by tight junctions (yellow). Astrocytes extend their feet (purple) to tile across blood vessels and communicate with both endothelial cells and with neurons (green). Pericytes (orange) also regulate neurovascular function, although the role of guidance receptors there has not been described. (b) Netrin-1 and its receptors (blue) are expressed and required in the neurovascular unit. At the blood–brain barrier, Neogenin1 is expressed in astrocytes and cell-autonomously regulates netrin-1 expression. Netrin-1 may act to regulate vascular integrity via endothelial cell Unc5b, which is required for blood–brain barrier integrity.

"neurovascular unit" protects and maintains neuronal functionality (Figure 1a).

In the tight associations of the neurovascular unit, guidance molecules act in the vasculature and the nervous system to maintain the blood–brain barrier (Figure 1b). Both Unc5b and Neogenin1 are expressed in the neurovascular unit. Neogenin1 expression in astrocytes is critical for blood vessel integrity in the brain.[76] Astrocytes form processes that closely associate with blood vessels, supporting their remodeling and maintaining and regulating the blood–brain barrier once it has formed.[77] In mice, adult, astrocyte-specific conditional knockout of *Neogenin1* ("*Neo1* conditional knockout mice") leads to sporadic distribution of astrocytes along blood vessels in the somatosensory cortex.[76] While *Neo1* conditional knockout mice contain more blood vessels in their somatosensory cortices, these blood vessels leak and contain more proliferating endothelial cells than controls. Intriguingly, netrin-1 RNA levels are reduced in *Neo1* conditional knockout mouse astrocytes, and virally encoded netrin-1 ameliorates the *Neo1* conditional knockout phenotype. These observations suggest that netrin-1 is likely signaling through another receptor to maintain the blood–brain barrier. One candidate receptor is Unc5b. In mice, Unc5b is highly and broadly expressed in the vasculature during development,[27] but the expression of an Unc5b reporter decreases once angiogenesis is complete.[29] Nevertheless, adult-specific conditional knockout of *Unc5b* in endothelial cells leads to brain blood vessel leakage.[78] This leakage phenotype is restricted to the brain vasculature, suggesting that Unc5b is not broadly expressed throughout the vasculature or else that it has unique interactions in the neurovascular unit. Does brain-derived netrin normally act on Unc5b to prevent vascular leakage? *Netrin-1* knockout mice have reduced levels of the tight junction proteins occludin and JAM-A in their brains and increased blood vessel leakage,[79] although this may indicate developmental and/or adult requirements for netrin-1. Indeed, in other systems, netrins are present and required in the adult nervous system. For example, netrin-1 is expressed in the adult rat brain,[40] and Netrin is continuously required for nervous system architecture in the planarian flatworm *Schmidtea mediterranea*. Global netrin knockdown in *Schmidtea* adults leads to a disorganized and defasciculated nervous system, a phenotype that is recapitulated when the lone *Schmidtea* netrin receptor is knocked down in adult animals.[80] Together, these data suggest that netrins may regulate cell adhesion after development.

Guidance pathways regulate mammary gland remodeling

Like the vascular and lymphatic systems, mammary epithelia are highly branched. In contrast to those systems, mammary glands undergo significant expansion and remodeling in the life of mammals. Mammary epithelia are bilayered, consisting of an outer layer of basal myoepithelial cells (MECs; also called basal cells) and inner luminal epithelial cells (LECs)[23,81] (Figure 2a). Growth and elaboration are guided by terminal end buds, a heterogeneous cell population that arises during puberty.[82] The mammary epithelia are situated in fat pads containing adipocytes and fibroblasts and are exposed to systemic signals from the lymph system and vasculature.[83] Mammary gland development occurs in three stages: during embryogenesis, tubes are established and reticulate into their primary structure; during puberty, they elongate and form secondary branches; during pregnancy, they form tertiary branches and the luminal epithelium divides to give rise to the secretory alveoli. Mammary glands can undergo multiple cycles of growth and involution in the lifetime of a female. This happens in response to specific hormonal cues, which act on progenitor cells to control the size and branching of the mammary epithelium.[23] Lineage tracing studies indicate that under physiological conditions, adult MECs and LECs are maintained by unipotent progenitor cells.[84–86] However, single cells sorted from the MECs of adult mice can reconstitute functional mammary gland in cleared adult fat pads,[84,87,88] suggesting that the fat pad

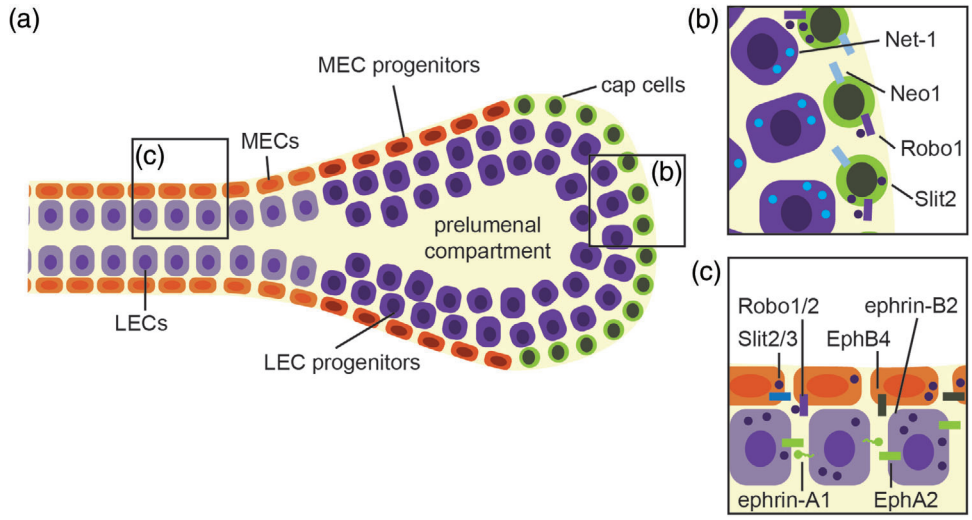

FIGURE 2 Axon guidance factors control mammary gland remodeling. (a) The mammary gland terminal end bud guides gland growth and elaboration within the fat pad. Proliferative cap cells at the bud end (green) are thought to contain a bipotent stem cell population[217] that supports mammary regeneration throughout life. In the prelumenal compartment, cap cells primarily give rise to progenitor cells (dark orange) fated to become basal myoepithelial cells (MECs; also called "basal cells"; light orange). Luminal epithelial cells (LECs; light purple) are primarily derived from body cells in the inner mass of the terminal end bud (dark purple). (b) Members of the netrin-Neogenin (blue) and slit-Robo pathway (purple) are expressed in the prelumenal compartment and in cap cells at the terminal end bud. Ephrin-B2 expression, not pictured, also extends to the prelumenal compartment. (c) Members of the slit-Robo pathway (blue) and ephrin-Eph pathway (green) are expressed in LECs and MECs.

is capable of reprogramming cells under transplantation conditions to increase their potency.[89,90] This discrepancy in cellular behavior under physiological versus transplantation conditions creates a significant caveat in the interpretation of transplantation experiments.

During puberty, terminal end buds are highly mitotic and facilitate ductal morphogenesis, and several axon guidance signals are important for this process. Growth is primarily driven by cap cells, which are a single layer of cells at the end of the terminal bud (Figure 2b). Cap cells proliferate to give rise to MECs, which contribute to the elongation and morphogenesis of the duct.[81] In the terminal end buds of pubertal mice, netrin-1 and Neogenin1 are expressed in complementary patterns: netrin-1 expression is present throughout the prelumenal compartment, while Neogenin1 expression is limited primarily to cap cells.[91] Mammary glands transplanted from *neogenin1* or *netrin-1* mutant mice into wild-type cleared fat pads have disorganized terminal end buds, suggesting a role for ligand–receptor signaling in cell adhesion. While cap cells remain adhered to other cap cells in *neogenin1* and *netrin-1* mutant mammary glands, they become detached from their usual location at the end of the terminal end bud. Rather, groups of cap cells are commonly found in the prelumenal space, where they occasionally undergo apoptosis.

The presence of apoptotic cells in *netrin-1* mutant mammary glands could indicate that Neogenin1 acts as a "dependence receptor" at terminal end buds. Dcc initiates apoptosis in cells that are not exposed to netrin in various cellular contexts, including specific contexts in the vertebrate nervous system, cancer cell lines, and some mouse cancer models.[41,92,93] However, mammary glands from *neogenin1* mutant mice also undergo low levels of apoptosis at terminal end buds, indicating that Neogenin1 itself is not required to drive apoptosis in the mammary gland.[91] Guidance receptors have been implicated in cell survival in other processes that are not consistent with the dependence receptor model. For example, in the *Drosophila* ovary, germlines lacking the insect Dcc homolog, Frazzled, fail to complete oogenesis, and egg chambers undergo apoptosis.[94] In contrast, netrin is dispensable for cell survival, and global *netrin-AB* mutants have apparently wild-type egg chambers.[94,95] While this pattern of cell death does not fit the dependence receptor model, it raises the possibility that Frazzled may interact with cell death machinery by an alternate mechanism. Similarly, netrin-Neogenin signaling in the mammary gland may impinge on the cell death machinery by an as-yet-unknown mechanism.

Slit-Robo signaling also regulates terminal end bud morphology in mouse mammary glands. *Slit2* and *slit3* transcriptional reporters both indicate ligand expression in MECs and LECs along the mammary duct, but *slit2* alone is expressed in cap cells at terminal end buds during ductal outgrowth[96] (Figure 2b,c). Furthermore, mammary glands from *slit2* null, but not *slit3* null, mice have disorganized terminal end buds. Ductal adhesion defects in glands from *slit2* mutant mice are recapitulated in *Robo1* null mice, indicating that slit2 could signal through Robo1 to control terminal end bud morphology. Indeed, Robo1 is expressed in both MECs and cap cells of the terminal end bud. While the terminal end bud phenotype in *slit-Robo* mutant mammary glands closely resembles that in *netrin-1* and *Neogenin1* mutant mice, duct adhesion defects in glands from *slit2* mutant mice are made worse by removal of *netrin-1*, suggesting that the two pathways act in parallel. In contrast to *netrin-1* mutant mammary glands, where cell adhesion appears to be largely responsible for the mutant phenotype,[91] *Robo1* normally suppresses cap cell proliferation. Cap cells in *Robo1* mutant mice incorporate EdU twice as frequently as those in control mice, and ducts from these mice contain

additional cells.[97] Taken together, these data support a model in which slit2 signals through Robo1 to suppress terminal end bud proliferation in mammary gland cap cells.

Robo2 is expressed in MECs, where it suppresses cell proliferation in the mammary stem cell population[98] (Figure 2c). Mammary gland fragments from *Robo2* or *slit2;3* mutant mice are capable of twice as many generations of serial transplantation as those from control and *Robo1* mutant mice. Moreover, FACS of the tissue indicates that MECs in *Robo2* null serial transplants remain proliferative for more generations. While Robo2 clearly plays a role in suppressing cell division under transplantation conditions, the relevance of this phenotype to the physiology of the mammary gland is controversial. Transplantation experiments theoretically facilitate the investigation of a mutant tissue in an otherwise wild-type animal, but given the capacity of the fat pad to reprogram cells under transplantation conditions, the transplanted tissue may not behave as it would endogenously.[89,90] Thus, further experimentation, including evaluation of endogenous mutant tissue, will be necessary to delineate the role of Robo2 in vivo.

The role of Roundabout receptors in suppressing proliferation in the mammary epithelium is reminiscent of that in the developing mouse cortex. Here, slit-Robo signaling suppresses neuronal proliferation in intermediate progenitor cells, although the precise signaling events at play are disputed.[99,100] Double *slit1;2* mutants have increased numbers of intermediate progenitor cells in the ventricular zone, yet these progenitors fail to develop into mature neurons. Reports diverge as to the likely receptors mediating the effects of slits in this context, with one study suggesting that it acts primarily through Robo2[99] and another suggesting that Robo1 is the critical receptor.[100] A likely explanation for this discrepancy is the differences in genetic backgrounds in single and double Roundabout mutant mouse strains used by the different groups.[3,100] Intriguingly, intermediate progenitor proliferation can be rescued by overexpression of the Notch target Hes1 in the cortex.[99] Furthermore, in vitro experiments suggest that Robo2 signaling may directly control Hes1 transcription. As Notch signaling is also integral to the proliferative dynamics of the mammary gland,[101] it will be interesting to see whether it also interacts with slit-Robo signaling in this context.

During axon guidance, slit-Robo signaling often enhances neuronal branching, although this process is not linked to proliferation.[102] In contrast, in the mammary gland, branching requires cell proliferation, and the role of slit-Robo in suppressing proliferation would also suppress branching.[103] Terminal end bud proliferation provides the cells necessary for lateral branching in the pubertal mammary gland.[104] Consequently, while mammary epithelia from *Robo1* or *slit2;3* null mice transplanted into donor mice grow to the same length as those transplanted from wild-type mice, they are significantly more branched.[97] Furthermore, exogenous slit2 introduced into the fat pad limits ductal branching. How does slit-Robo1 signaling inhibit branching morphogenesis under normal conditions? One possibility is that it interacts with the Wnt signaling pathway, a positive regulator of MEC proliferation in the mammary gland.[105] Indeed, exogenous slit2 application to mammary glands reduces the expression of Axin2, a β catenin target.[97,106] Furthermore, cultured cells treated with slit2 have increased β catenin intensity at the plasma membrane, suggesting that slit2 can inhibit nuclear accumulation of β catenin.[97] These data are consistent with a model in which Slit2-Robo1 signaling prevents β catenin target gene expression and, ultimately, proliferation.

Like the slit-Robo pathway, ephrin is required for mammary gland homeostasis. Mammary-specific conditional knockout of *ephrin-B2* also perturbs gland architecture and inhibits nuclear accumulation of β catenin.[107] In contrast to the role of Robo1 in branching morphogenesis, however, ephrin-B2 plays a critical role in the maintenance of mammary gland epithelia. Expression of ephrin-B2 and its receptor, EphB4, both fluctuate during the mouse estrus cycle, with highest levels of expression during proliferative stages of the cycle, and are expressed in LECs and MECs, respectively[108] (Figure 2c). At the point of lactation, mammary glands are generally terminally differentiated and have little cell death or proliferation.[23] Mammary glands from *ephrin-B2* conditional knockout mice, however, have high levels of both cell death and proliferation.[107] While the increase in cell death could indicate premature gland involution, proliferation is not a hallmark of involution, suggesting a general requirement for ephrin-B2 in mammary cell survival. Additionally, ephrin-Eph signaling may also regulate mammary gland proliferation and branching morphogenesis in earlier gland development.[109] EphA2 is expressed in LECs, and *EphA2* mutant mice have rudimentary mammary epithelia with limited proliferation and branching. Furthermore, mammary gland transplantation from mutant to wild-type animals often leads to engraftment failure, suggesting an intrinsic requirement for EphA2 in proliferation. While an EphA2 ligand, ephrin-A1, is also expressed in LECs,[109] it remains to be seen if it is similarly required for mammary gland morphogenesis.

Guidance receptors regulate the architecture and function of the pancreas

The relationship between tissue organization and function is well-illustrated in the pancreas: while pancreatic architecture is usually fixed in adult organisms, its structure is disrupted in both rodents and humans with diabetes.[110,111] Approximately 95% of the pancreas is a tubular exocrine organ that secretes digestive enzymes. The remaining 5%, the "endocrine pancreas," comprises spheroid islets, micro-organs responsible for regulating glucose homeostasis through a network of endocrine, paracrine, and autocrine signaling. Islets in both humans and mice consist primarily of insulin-secreting β cells, which form clusters and are electrically coupled via gap junctions[112] (Figure 3a). Pancreatic islets also contain additional endocrine cells, including α cells and δ cells, which secrete glucagon and somatostatin, respectively.

Several recent studies highlight the importance of guidance receptors in maintaining pancreatic islet architecture and in controlling interactions between islet endocrine cells. For example, the selective deletion of *Robo2* in the mature β cells of postnatal *Robo1* mutant mice ("*Robo1/2* conditional knockout mice") significantly disrupts islet architecture.[113] Specifically, islets in *Robo1/2* conditional knockout mice have reduced "circularity," although innervation and vascularization of the islet remains intact.[114] This phenotype is reminiscent of

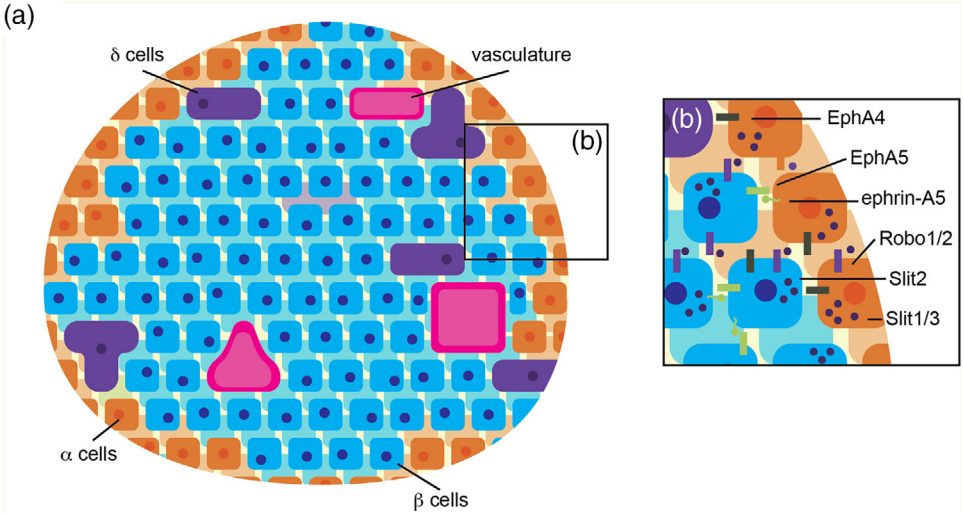

FIGURE 3 Axon guidance factors regulate pancreatic architecture and function. (a) In the mouse endocrine pancreas, each islet comprises a mantle of glucagon-producing α cells (orange) surrounding a core of insulin-producing β cells (blue). Somatostatin-producing δ cells (purple) make up a small percentage of the islet and are also enriched at its periphery. Pancreatic islets are vascularized (pink) and innervated (not shown). Other cells not relevant to this review, including ε cells and pancreatic polypeptide cells, have been omitted for simplicity. Data from mice should be interpreted with an understanding that islet architecture is different between humans and mice.[218] (b) Members of the slit-Robo pathway (purple) and ephrin-Eph pathway (green) are expressed in adult endocrine cells. Slit2 is highly expressed in β cells, whereas Slit1 and Slit3 are present in α and β cells. Robo1 and Robo2 are present in endocrine cells in the pancreas, including β cells. Ephrin-A4 is expressed in α cells and, to a lesser extent, in β cells. While bidirectional signaling via its EphA5 receptor on β cells regulates glucose-stimulated insulin secretion, it is possible that ephrin-Eph signaling also regulates paracrine signaling.

the loss of compaction seen in the posterior signaling center (PSC) of *Drosophila robo2* mutant larvae.[14] In this context, Robo2 expressed in the PSC responds to its Slit ligand to guide its constituent cells to the correct location and to facilitate their clustering. In contrast, in the adult mouse pancreatic islet, Robo1/2 appear to regulate cellular interactions. In addition to their altered shape, islets from *Robo1/2* conditional knockout mice have significant changes in islet organization.[113] For example, α cells, normally restricted to the periphery in the mouse islet, are distributed in its internal core in *Robo1/2* conditional knockout mice. Consequently, β cells in *Robo1/2* conditional knockout mice are less likely to contact other β cells than they are in islets from wild-type mice.[114] Is this phenotype slit dependent? While slit1-3 are expressed in the adult mouse islet[115] (Figure 3b), their in vivo functions remain untested. In in vitro cultured mouse islet cells, siRNA-mediated slit1-3 knockdown reduces β cell survival,[115] suggesting that the ligands may promote cell survival in vivo. *Robo1/2* conditional knockout mice, however, have normal β cell survival,[113] raising the possibility that Robo acts as a dependence receptor in the pancreatic islet. In this model, Robo signaling promotes cell survival in the presence of its Slit ligands, and upon their removal, initiates cell death. While this signaling modality has been described in multiple contexts for netrin receptors, including in the nervous system,[92,93] there is no evidence that Robo can act as a dependence receptor. While technically challenging, genetic removal of Slit ligands in vivo will establish whether Robo receptors act as dependence receptors in the pancreatic islet.

The perturbed islet architecture of *Robo1/2* conditional knockout mice affects pancreas function. Robust, pulsatile insulin secretion is required for glucose homeostasis and depends on synchronous Ca^{2+} oscillations coordinated by β cell clusters.[114] Elegant intravital Ca^{2+} imaging experiments demonstrate that in vivo, *Robo1/2* conditional knockout mice have impaired synchronicity in β cell clusters.[114] Gap junctions between β cells are intact in these mice, indicating that electrical coupling is intact between adjacent cells. However, in the aberrant islet structure, β cells are less likely to interact with other β cells than in control mice. While impaired synchronicity could be the result of a loss of these homotypic interactions, an increase in β cell interactions with α cells and δ cells is also likely to influence autocrine and paracrine signaling within the islet.

Indeed, guidance receptor signaling has also been proposed to modulate paracrine signaling within the pancreatic islet. Transcriptomic approaches indicate that EphA4 is highly expressed in α cells and, to a lesser extent, in β cells (Figure 3b).[116] As insulin is secreted from β cells to lower blood glucose, α cell glucagon secretion is inhibited.[117] Under fasting conditions, when blood glucose levels fall, α cells secrete glucagon to stimulate glycogen catabolism in the liver. *EphA4* mutant mice have low blood glucagon levels after fasting, raising the possibility that ephrin-Eph signaling is important for glucagon secretion from α cells.[118] In support of this model, *EphA4* mutant mice have high blood insulin levels under both glucose challenge and normal feeding conditions. The ligand responsible for this effect is currently unknown.

Ephrin-eph signaling also regulates insulin secretion from β cells. In both healthy and insulin-resistant mice, a pan-EphA antagonist increases plasma insulin levels and improves glucose tolerance.[119] Indeed, ephrin-A5 and EphA5 proteins are both expressed in adult mouse islets, including β cells; this pattern is mirrored in the human

pancreas.[120] Global *EphA5* mutant mice have impaired glucose tolerance, which could indicate a role for EphA5 in many cell types in the pancreas. Ephrin-A5 knockdown in MIN6 cells, a commonly used in vitro model of β cells, leads to reduced glucose-stimulated insulin secretion. However, since MIN6 cells contain other pancreatic endocrine populations,[121] it remains possible that this reflects a requirement outside of β cells. Nevertheless, further ex vivo and in vitro evidence substantiates the link between ephrin-Eph signaling and glucose-stimulated insulin secretion. Mouse and human pancreatic islets cultured ex vivo with peptides to enhance reverse ephrin-Eph signaling or inhibitors of forward signaling have increased glucose-stimulated insulin secretion.[120,122] In contrast, treating ex vivo islet cultures with a peptide enhancing forward ephrin-Eph signaling reduces glucose-stimulated insulin secretion. These changes correlate with changes in activity of the small GTPase Rac1 and F-actin organization in vitro.[120] In cultured β cells, Rac1 translocates from the cytosol to the cell membrane in response to glucose stimulation, and a dominant negative Rac1 inhibits glucose-stimulated insulin secretion.[123] While ephrin-Eph signaling is also mediated by Rac1 in the nervous system, where it controls the shape of the cytoskeleton and regulates receptor transendocytosis,[124] insulin exocytosis represents a distinct signaling output. Taken together, these data support a model in which ephrin-Eph signaling coordinates the activity of neighboring β cells in response to glucose stimulation. As the broad changes in islet architecture in *Robo1/2* conditional knockout mice are not present in *eph5a* global knockout mice, mutation of *Robo1/2* likely disrupts an earlier process in homeostasis.[114,120] Because both ephrins and their Eph receptors are membrane bound, only apposed cells can signal to one another in *trans*; thus, a disruption of cell—cell contacts, such as that seen in *Robo1/2* mutant mice, would alter ephrin-Eph signaling. Is the loss of ephrin-Eph signaling contributing to the *Robo1/2* phenotype? While EphA2 and Robo1 can heterodimerize in some cancer cell lines, direct interactions under physiological conditions have not been reported.[125] The relationship between the two guidance signaling pathways in the pancreas will be better understood with more selective genetic manipulations as well as by identifying downstream effectors of each respective pathway.

Guidance receptors control bone homeostasis

In vertebrates, bone remodeling occurs asynchronously across the skeleton throughout life.[126] Remodeling is initiated by osteoclasts, monocyte—macrophage-derived cells that break down and resorb existing bone (Figure 4a). Following resorption, mesenchymal-derived osteoblasts, are recruited to the site, ultimately undergoing a series of differentiation events to facilitate new bone formation. Osteoblasts and osteoclasts are spatially segregated by a quiescent region of the bone, but the close coupling of their activities is required to maintain bone integrity throughout the cycle. Disrupted bone remodeling underlies several pathological conditions, including osteoporosis, where bone density is lost, and osteopetrosis, where bone density increases.[127] Thus, communication between osteoclasts and osteoblasts is imperative for bone homeostasis and for organismal health.

Guidance receptors and their ligands are expressed within the bone marrow and have been implicated in immune[128] and hematopoietic stem cell (HSC) function (see below). Furthermore, reciprocal expression of guidance receptors and their ligands suggests they may participate in the bone remodeling cycle. For example, sema4D transcript is present at high levels in osteoclasts, but not osteoblasts[129] (Figure 4b,c). Global *sema4D* mutant mice have abnormally thickened bone,[129,130] although the mechanism underlying this phenotype is disputed. One possibility is that *sema4D* mutant mice have an increased number of osteoblasts, ultimately leading to more ossification of bone.[129] In support of this model, osteoclastic bone resorption is unaffected in *sema4D* mice. How does osteoclast-derived sema4D influence osteoblast activity? Soluble sema4D may act on its receptors in osteoblasts. Indeed, global mutation of *Plexin-B1*, a sema4D receptor, generates phenotypes that closely mirror those observed in *sema4D* mutants. Furthermore, Plex-B1 expression is induced in cultured osteoblasts during differentiation, suggesting it may receive sema4D signal on osteoblasts to result bone density. Interestingly, in *sema4D* and *Plex-B1* mutant mice alike, osteoblasts and osteoclasts are closer together on the bone. As these cells mediate different aspects of the bone remodeling cycle, their location in the bone is tightly coordinated to maintain proper bone density. The proximity of osteoblasts and osteoclasts in *sema4D* and *Plex-B1* mutant mice raises the possibility that sema-Plexin signaling may regulate their localization. Interestingly, in vitro experiments demonstrate that recombinant sema4D accelerates osteoblast motility, possibly by regulating adhesion via Cadherin-11. Does sema4D signal exclusively via Plex-B1 in the bone? Since Plexin-B2 is also expressed in the bone, sema4D may act on multiple receptors in this context. Indeed, global double mutants for *sema4D* and *Plex-B1* have higher bone density than *Plex-B1* mutants alone, suggesting osteoclast-derived sema4D acts on multiple receptors to control bone density.

If sema4D is required specifically in osteoclasts to regulate bone homeostasis, bone marrow transplantation experiments should lead to donor-dependent bone density phenotypes. However, reports conflict as to whether aberrant bone density in *sema4D* mice depends on bone marrow alone. In one study, bone marrow transplantation from *sema4D* global mutants to wild-type mice confers increased bone density, consistent with a model in which sema4D is secreted from osteoclasts to regulate bone density.[129] This conflicts, however, with another report indicating that in mouse vertebrae, the *sema4D* mutant phenotype is sexually dimorphic, with female, but not male, mice manifesting the bone density phenotype[130]; the authors do not evaluate osteoblast function. Moreover, the phenotype is reverse in ovariectomized mice, suggesting a hormonal contribution. Because ovariectomized mice have lower bone density and are used as a model of osteoporosis,[131] this effect could be due to compensatory effects in a different pathway rather than ovary-directed secretion of sema4D. Nevertheless, conflicting results from bone marrow transplantation studies leave the question of where sema4D is required to regulate bone homeostasis unanswered.[129,130] To reconcile these studies, it will be necessary to

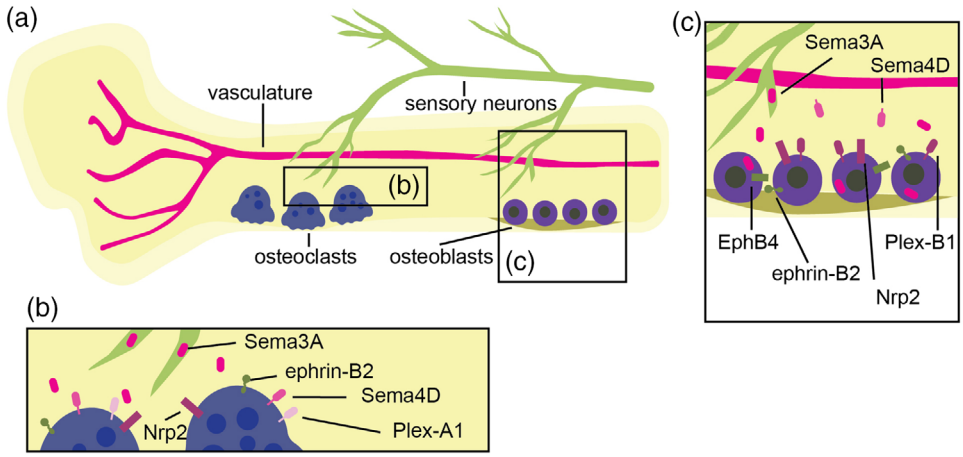

FIGURE 4 Axon guidance factors control bone homeostasis. (a) Bones are highly vascularized (pink) and innervated (green). Osteoclasts (blue) initiate the bone remodeling cycle by absorbing existing bone. Later, osteoblasts (purple) deposit new bone. (b and c) Sema-Plex (pink) and ephrin-Eph (green) signaling regulate osteoclast and osteoblast function during bone remodeling. While Sema3A is expressed in multiple cells present in the bone marrow, it is specifically required in sensory neurons to regulate bone homeostasis. Plex-A1 and Plex-A4 are also broadly expressed in the bone marrow, raising the possibility that they act as Sema receptors to regulate bone density through their activity in other cell types. Ephrin-Eph signaling also appears to be broadly required in the bone marrow for bone homeostasis.

generate conditional knockout mice lacking osteoclast sema4D or to perform cell-specific rescue experiments in *sema4D* global knockout mice.

In contrast to sema4D, sema3A is expressed primarily in the osteoblast lineage, although it is also broadly expressed outside of the bone itself.[132] *Sema3A* global mutant mice have a severe low bone mass phenotype, and in vitro studies indicate compromised osteoblast differentiation in cells from these mice. Its co-receptor, *Neuropilin1*, is also broadly expressed in the bone marrow, although in vivo data regarding its cellular expression is lacking.[133] Mice carrying a version of *Neuropilin1* that cannot bind semaphorins ($Nrp1^{sema-}$) have a similar low bone mass phenotype to the *sema3A* knockout mice, suggesting Nrp1 may be mediating the response to sema3A.[132] How do sema3A and Nrp1 work together to promote bone homeostasis? One possibility is that repulsive signaling between osteoblasts, which produce sema3A, and osteoclasts, which do not, would prevent osteoclasts from destroying osteoblast-synthesized bone. Indeed, in vitro experiments indicate that sema3A regulates the migration of cells in the bone marrow to facilitate bone homeostasis and that migration is impeded in bone marrow from $Nrp1^{sema-}$ mice. Moreover, injection of male mice with recombinant sema3A increases bone volume. These mice have decreased osteoclast number but increased osteoblast surface, suggesting that sema3A can act both by reducing bone resorption (through suppression of osteoclast activity) and increasing deposition (by increasing osteoblast activity). This makes sema3A an interesting candidate to act at the transition point of bone remodeling.

While it is expressed in the osteoblast lineage, elegant conditional knockout experiments demonstrate that sema3A is also required specifically in sensory neurons.[134] *Sema3A* knockout specifically in osteoclasts does not recapitulate global knockout phenotypes.[134] Rather, *sema3A* knockout in neurons leads to mice with low bone density. Sensory neurons form close associations with bones, and while not required for bone formation, their ablation perturbs bone density.[135] For example, capsaicin treatment, which causes cell death of unmyelinated sensory neurons innervating the bone, leads to reduced bone density.[136] Intriguingly, capsaicin treatment does not enhance bone density defects in adult *sema3A* neuronal knockout mice, indicating that the mutant neurons remain dysfunctional into adulthood. Furthermore, bone formed in response to ablation in these mice is not properly innervated and does not reach wild-type density.

What receptors bind sema3A to induce signaling in the bone? *Plexin-A4* global mutant mice also have similar bone innervation patterns, suggesting that the receptor may mediate Sema3A signaling.[134] Given the broad expression patterns of many plexins in bone, it remains possible that other receptors bind sema3A. For example, Plexin-A1 is expressed in primary osteoclast cultures.[137] *Plexin-A1* global mutant mice also have osteopetrosis phenotypes, with defects in differentiation of osteoclasts.[137] Furthermore, Neuropilin1 and Neuropilin2 are both expressed in bone marrow, although they are found in both the osteoclastic and osteoblastic lineages of adult mice,[133,137] positioning them as possible co-receptors. Additional members of the sema-Plexin pathway may participate in bone homeostasis: mice with bone-specific overexpression of human sema3B have smaller bones with more osteoclasts.[138] A full accounting of the endogenous expression of each member of the semaphorin pathway, as well as tissue-specific mutant analysis, will provide clarity on the role of sema-Plexin signaling in bone homeostasis.

Like sema-Plexin signaling, ephrin-Eph signaling is important for bone remodeling, although its precise role is less clear. Mice overexpressing EphB4 in osteoblasts throughout development have both increased bone mass and an increased rate of bone formation compared to control mice, suggesting that EphB4 promotes bone deposition.[139] Moreover, these mice have fewer osteoclasts, and reduced bone resorption generally, indicating a reduction in osteoclast

function. The authors hypothesize that osteoblast EphB4 interacts directly with ephrin-B2 on osteoclasts to antagonize bone resorption. However, during in vivo bone remodeling, osteoblasts and osteoclasts rarely interact in the tissue. Furthermore, ephrin-B2 is broadly expressed, and osteoblast-specific ablation of ephrin-B2 in mice leads to increased cell death,[140] suggesting that ephrin-B2 is also required in the osteoblast lineage. Thus, while ephrin-Eph signaling appears to modulate bone homeostasis, additional experiments, including cell-type-specific ablation, will be necessary to determine its mode of action in this context.

AXON GUIDANCE PATHWAYS REGULATE TISSUE-RESIDENT STEM CELLS AND THEIR PROGENY

Stem cells, which can self-renew and give rise to daughter cells with distinct fates, provide a source of replacement cells over an organism's lifespan, thus allowing adult organisms to regulate whole-body physiology as well as respond to injury and disease. Several reproductive tissues are also supported by stem cell populations, enabling the production of high-quality gametes throughout life.[141] To support tissue homeostasis, stem cells must balance maintenance of the stem cell pool with proliferation to give rise to daughters. Many morphogens controlling neuronal development, including hedgehog and bone morphogenic protein, have well-documented roles in stem cell populations.[142,143] Furthermore, classical axon guidance factors have been implicated in stem cell maintenance, proliferation, and lineage commitment. In this section, we describe the role of guidance receptors and their ligands in stem cell/niche interactions and in lineage commitment.

Guidance factors mediate stem cell/niche interactions

Stem cell maintenance and activity are regulated by the coordinated actions of intrinsic factors and local and systemic extrinsic factors. This microenvironment, or "niche," consists of cells and extracellular matrix that send short-range signals to stem cells to balance their proliferation and maintenance and may also interact with systemic factors, including hormones, delivered through the circulatory system.[144–146] In the early stages of development, axon guidance factors regulate the formation of niches. For example, during *Drosophila* embryogenesis, Robo1 is required for development of the gonad,[13] and in *Drosophila* larvae, Slit secreted from the vasculature acts on Robo receptors to promote clustering, proliferation, and function of the cells in the PSC, which regulates hematopoiesis in developing animals.[14] In adult organisms, axon guidance factors have been detected at a broad range of stem cell niches.[147–149] Several studies support a role for axon guidance factors in niche adhesion. In this section, we describe recent studies addressing the functional relevance of axon guidance factors at the stem cell niche, including the well-established niches of the *Drosophila* germ lines, the HSC niche, and the niches that support the vertebrate and invertebrate intestines.

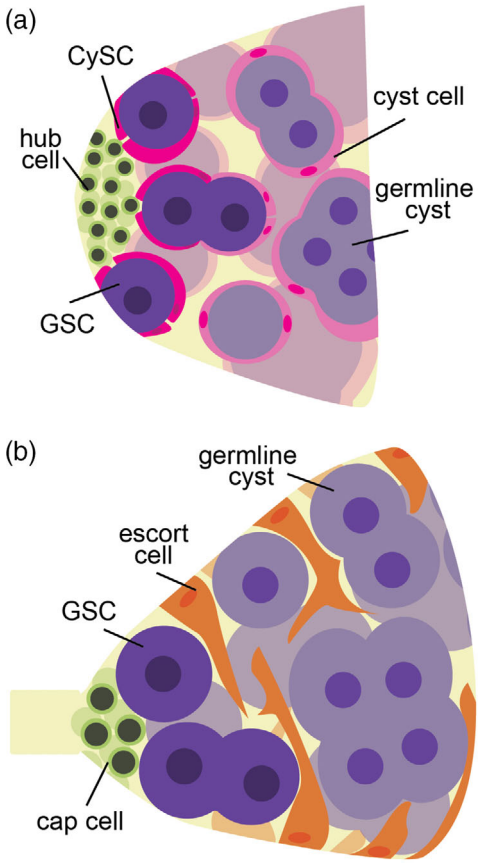

FIGURE 5 Axon guidance factors are expressed and required at germline stem cell (GSC) niches. (a) At the apex of the *Drosophila* testis, postmitotic hub cells (green) and cyst stem cells (CySCs) form a niche to support a GSC population (dark purple). As GSCs and CySCs divide, their progeny (germline cysts and cyst cells, respectively) remain in close association. Robo2 in CySCs mediates niche adhesion to ensure continuous spermatogenesis. (b) At the anterior tip of the *Drosophila* ovariole, GSCs (dark purple) are housed in the germarium, which contains a somatic niche composed primarily of cap cells (green) and anterior-most escort cells (orange) GSCs divide asymmetrically to give rise to the germline lineage (light purple). Net-A is expressed in anterior-most escort cells, and its knockdown leads to GSC loss by an unknown mechanism.

Many ligands secreted from the niche signal at short range, which limits the size of the stem cell maintenance compartment. As such, many stem cells require physical adhesion to their niches for continuous niche occupancy.[150] For example, at the apex of the *Drosophila* testis, a population of closely associated germline and somatic stem cells (cyst stem cells, CySCs) are anchored to somatic hub cells by E-cadherin[151] (Figure 5a). The hub secretes unpaired to activate JAK/STAT signaling in GSCs and regulate their adhesion to the hub.[152] This observation raises the possibility that while stem cell adhesion to the niche clearly regulates its physical ability to respond to niche-derived signals, adhesion itself may *also* be controlled by those signals. For example, in the *Drosophia* ovary, GSCs are also retained in a somatic niche by E-cadherin (Figure 5b). Loss of E-cadherin in cap cells leads to GSC loss from the niche,[153] and germ cells overexpressing E-cadherin

conversely increase their contact with cap cells.[154] Taken together, these studies demonstrate a striking pattern of systems using adhesion molecules to regulate stem cell populations.

In the Drosophila testis, Robo2 regulates niche adhesion to regulate stem cell competition at the niche. Loss of Robo2 in adult CySCs leads them to be outcompeted by neighboring CySCs for hub occupancy.[147] Strikingly, while mutant clones of *robo2* are lost rapidly from the niche, RNAi-mediated knockdown of Robo2 in all CySCs has no effect on their maintenance, suggesting that Robo2 regulates the ability of CySCs to compete for niche occupancy. In contrast to *robo2* null CySCs, *ableson kinase* (*abl*) mutant CySCs are maintained at the hub better than wild-type CySCs, hinting that Robo2 may act upstream of Abl to inhibit its function. Indeed, knockdown of Abl in *robo2* mutant CySCs leads to the rescue of the *robo2* mutant phenotype to wild-type maintenance.[147] Furthermore, and in line with both genes acting to mediate niche adhesion, *abl* mutant CySC retention requires E-cadherin, and somatic cell overexpression of E-cadherin rescues the Robo2 loss of function phenotype. What is the nature of the Abl/Robo2 interaction? While Robo1 physically interacts with Abl, Robo2 lacks the conserved domains required to do so.[155] Nevertheless, *Robo2* genetically interacts with *Abl* to regulate axon guidance in *Drosophila*,[156] suggesting additional players may link Robo2 to Abl. Slit and Robo1 are possible candidates as they are both expressed at the *Drosophila* hub; additional analysis will be necessary to understand their interplay with Robo2 and Abl in this context of niche adhesion.

Stem cell–niche adhesion at the HSC niche is also modulated by axon guidance factors. In mammals, HSCs—the rare, immature cells that give rise to multipotent progenitors and restricted hematopoietic progenitors—sustain blood count and immune function throughout life.[157] In adults, most HSCs reside in bone marrow, where they are maintained by signaling from mesenchymal stromal cells, endothelial cells, and circulating systemic factors.[158,159] While HSCs are a heterogeneous population, they commonly express a variety of proteins, including integrins, that facilitate their adhesion to the niche and retain them in the niche environment.[160,161]

Robo4 is highly expressed in a subpopulation of HSCs capable of long-term multilineage reconstitution upon transplantation.[162] In competitive engraftment experiments, HSCs from *Robo4* null mice initially promote blood formation at similar levels to wild-type, but cannot sustain it, suggesting a failure to engraft into bone marrow.[149] This could be explained by observations that HSCs from *Robo4* null mice are not able to home to the bone marrow of irradiated recipients[163]; however, whether *Robo4* global mutant mice have lower levels of HSCs in bone marrow than their wild-type counterparts[149] or not[163] remains unresolved. Nevertheless, these results implicate Robo4 in HSC niche occupancy. This model is consistent with another study demonstrating that cells with high levels of Robo4, but not those with low levels, are capable of long-term engraftment and multilineage differentiation.[164] Interestingly, this effect appears to be specific to the bone marrow niche; spleen colony-forming assays are not affected by loss of *Robo4* function.[149]

How does Robo4 signal in HSCs? *Robo4* mutant HSCs have increased levels of the G-protein-coupled chemokine receptor CXCR4, which controls adhesion and retention of HSCs in the adult bone marrow.[149,165] Whether Robo4 and CXCR4 bind directly or interact in some other capacity has not been described. Interestingly, CXCR4 is also transiently expressed by vertebrate ventrally projecting motor neurons, and *CXCR4* knockout mice mis-project ventral motor neurons within the spinal cord and in sensory ganglia.[166] Although the precise mechanism of its activity remains unknown, CXCR4 interacts with Robo1 in leukocytes to promote their migration in vitro,[167] indicating that it is capable of interacting with slit-Robo signaling.[168] Critically, Robo4 is unlikely to bind slit in the bone marrow. While initially reported to be a slit receptor,[169] Robo4 lacks the slit-binding domains of Robo1 and Robo2, and its ability to bind slit ligands remains contested (for further discussion, see Box 1). Slit2 is expressed in some cells in the bone marrow,[164,170] but whether local slit signaling is controlling Robo4 activity in this context is untested. One possibility is that Robo4 interacts with additional receptors to fine-tune their own signaling responses. Indeed, Neogenin is also expressed in HSCs, although its role in this population remains to be determined.[162] Intriguingly, *slit2* expression is positively correlated with HSC number in inbred mouse strains, and ectopic slit2 improves HSC engraftment in transplantation experiments.[171] While the relevance of cadherin-mediated adhesion at the HSC niche is contested,[150] this model of a competitive advantage conferred by slit-Robo signaling is reminiscent of the role of Robo2 in CySCs in the *Drosophila* testis[147] (see above). Furthermore, as with Robo2 in the testis, Robo4 levels are downregulated in differentiating cells in the HSC lineage.[149]

In addition to its importance to stem cell maintenance, HSC niche adhesion is likely to have implications for bone marrow transplantation as mobilizing HSCs to peripheral blood requires that they exit the niche. Ephrin-B2 and its receptor, EphB4, are expressed in complementary patterns in the mouse bone marrow, with ephrin-B2 present in cells including HSCs and EphB4 in the sinusoidal tissue that comprises the niche.[172] Blocking eph-Ephrin signaling by injecting a specific blocking peptide reduces the mobilization of HSCs to peripheral blood under chemical induction and physiological conditions, and mice studied weeks after blood transplantation have no donor cells in their bone marrow. In contrast, bone marrow engraftment is successful, indicating that mobilization and engraftment are not always necessarily interdependent. The relationship between the two molecules at the niche could position their signaling as an important "sensor" of the need to mobilize.

Other guidance molecules expressed at niches have not been directly implicated in niche adhesion. For example, in the *Drosophila* ovary, GSC number may depend on expression of netrin-A (netA) in neighboring somatic cells.[173] As in the testis, in the ovary, E-cadherin-mediated adhesion maintains germline stem cells (GSCs) in close apposition to postmitotic cap niche cells (Figure 5b). Cap cells and closely associated escort cells produce ligands, including hedgehog and decapentaplegic, that suppress GSC differentiation.[174,175] Escort cells are highly dynamic and extend processes to envelop developing germline cysts. While escort cells appear morphologically similar throughout the germarium, recent single cell RNA-seq experiments revealed that escort cells at different locations in the germarium

differentially express multiple genes, possibly creating distinct "differentiation compartments."[148,176] Net-A, one of two netrin ligands in *Drosophila*, is expressed in the anterior-most escort cells, leading to speculation that its expression is required for GSC maintenance. Indeed, knockdown of netrin-A in a subset of adult cells that includes escort cells leads to a reduction in GSC number.[148] When considered over the many ovarioles in each ovary, the net-A driven loss of GSCs has significant implications for fertility. While it remains possible that this reflects a requirement for net-A in other tissues,[177] it is nevertheless tempting to speculate that local net-A signaling promotes a niche environment. Is netrin signaling instructive for maintenance, or does it prevent differentiation? What signaling pathways does it work with? Global *netrin-AB* mutants lay fewer eggs than control counterparts,[95] although it is unclear whether that reflects tissue-intrinsic or neuronal roles for net-A. Interestingly, netrin-independent Frazzled signaling is required cell-autonomously by germ cells later in oogenesis,[94] but its role in early oogenesis has not been explored. Future experiments should test germline and niche requirements for Netrin receptors Unc5 and Frazzled in GSC maintenance.

Systemic effects of guidance signaling may regulate stem cells in vascularized niches

The stem cell populations that reside in vascularized niches, including neural and spermatogonial stem cells, are subject to systemic signaling. As guidance factors regulate the formation and maintenance of the vasculature (see Section 1), they may play roles in governing interorgan communication. For example, in addition to its cell-autonomous role in HSCs, Robo4 regulates the hematopoietic lineage through its role in vascular development. Under transplantation conditions, *Robo4* mutant mice fail to localize wild-type HSCs to the bone marrow, likely because blood vascular leakage prevents their efficient trafficking.[178] Guidance receptor signaling may thus regulate organismal physiology indirectly by controlling the vasculature. On the other hand, guidance cues derived specifically from the vasculature can influence the development of neighboring organs, as is observed during the development of the PSC of the *Drosophila* larval lymph gland. Flies with reduced Robo2 levels in the PSC are specified correctly, but over-proliferate and disperse at the second larval instar.[14] Cardiac-tube-specific knockdown of slit recapitulates these phenotypes, implicating slit ligand in this process. Interestingly, clustering and proliferation are differentially controlled. Clustering defects can be rescued by overexpressing a dominant negative form of the small RhoGTPase Cdc42 and overexpression of DE-cadherin. In contrast, proliferation defects in Robo2 knockdown flies are rescued by overexpressing dMyc. Thus, the vasculature provides both a route for trafficking in the body and a source of signals to guide organ development, and axon guidance cues have been implicated in both processes.

In addition to being regulated by signals from a vascularized niche, guidance cues may influence production of systemic signals such as hormones. For example, in mammals, spermatogonial stem cells are maintained in a highly vascularized niche that includes somatic Sertoli cells and testosterone-producing Leydig cells.[179] Robo1 is expressed in the Leydig cells of adult male mice; however, despite reduced intratesticular testosterone in global Robo1 mutants, adult mice have normal fertility,[180] indicating that any changes to hormone levels are not sufficient to have detectable phenotypic consequences. Testosterone injection into wild-type mice also leads to an increase in Robo1, slit1, and slit3 RNA levels, indicating that slit-Robo signaling could be directly or indirectly hormonally regulated. This is reminiscent of the regulation of ephrin-Eph signaling by estrogen in the mouse mammary gland. EphB4 and ephrin-B2, normally expressed in the mammary gland epithelium (Figure 2), are not present in ovariectomized mice.[108] Expression is rescued by injection of mice with estradiol, whereupon it resumes its stereotyped pattern. Thus, guidance cues in several adult tissues appear to be sensitive to hormonal cues, allowing them to respond to organismal physiology.

Axon guidance pathways regulate lineage commitment in the intestine

During embryonic development and organogenesis, axon guidance factors play integral roles in lineage commitment.[181] In adult tissues containing multipotent stem and progenitor cells, lineage commitment regulates tissue integrity, and improper specification of stem cell daughters can disrupt tissue homeostasis. Guidance receptor signaling also regulates lineage commitment in adult tissue contexts. In some cases, these mechanisms mirror well-understood roles for guidance signaling axes in nervous system development. For example, in the mammalian intestine, guidance cues regulate the migration of daughter cells, allowing them to adopt location-specific fates. In other cases, however, the relationship between the guidance function of these molecules is unclear, raising the possibility of distinct signaling modalities.

In both the vertebrate and invertebrate gut/intestine, axon guidance signaling has been implicated in lineage commitment and daughter cell function. In the mammalian small intestine, a series of crypts and villi increase the surface area and facilitate efficient nutrient uptake during digestion (Figure 6a). The colon, by contrast, contains crypts without villi. High cell turnover in the intestine and colon is supported by a stem cell population of mitotically active crypt base columnar cells (CBCs) that express leucine-rich repeat-containing G-protein-coupled receptor (LGR5).[182,183] Paneth cells, which contain distinctive granules of antimicrobial peptides, are interspersed with CBCs and form part of the stem cell niche.[184] Intestinal epithelial cells migrate from their origin at the crypt base to maintain the spatial organization of the organ.[185]

Ephrin-B and its Eph receptors have striking reciprocal expression patterns in the mouse small intestine, and these patterns have implications for tissue organization during rapid cell turnover (Figure 6b,c). In wild-type tissue, ephrin-B1 is enriched at the crypt–villus junction, while EphB2 and B3 are both detected in the proliferative compartment of the crypt.[186] Notably, EphB3 is strikingly restricted to the proximity of the putative stem cell population, present in both CBCs

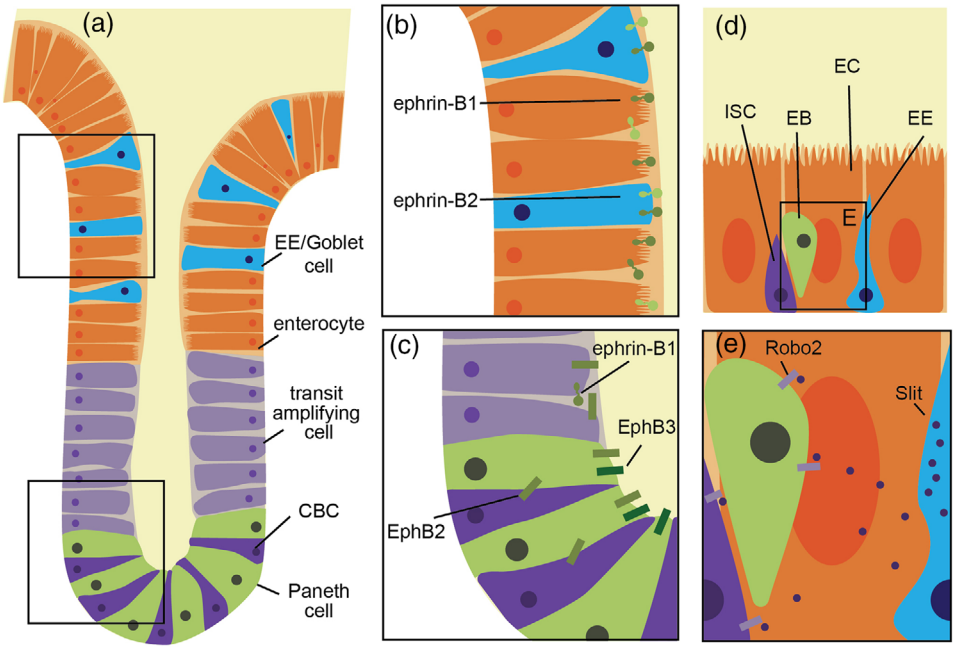

FIGURE 6 Axon guidance factors regulate lineage commitment in the vertebrate and invertebrate intestine. (a) The mammalian intestine is composed of crypts and villi. High cell turnover in the intestine is sustained by a proliferative population of crypt base columnar cells (CBCs, purple), which are directly opposed to postmitotic Paneth cells (green). CBCs divide to give rise to a transit-amplifying population (light purple), which migrate out of the crypt and adopt various cell fates that sustain intestinal homeostasis, including absorptive enterocytes (ECs, orange) and secretory enteroendocrine cells (EEs) and goblet cells (blue). (b and c) Ephrin-Eph signaling (green) regulates lineage commitment in the intestine. Ephrin ligand levels are highest near the crypt–villus junction (b), but low levels of ephrin-B1 are present at the crypt base (c). EphB2 is expressed throughout the crypt base, but EphB3 expression is restricted to Paneth cells. (d) In the *Drosophila* midgut, intestinal stem cells (ISCs, purple) support tissue homeostasis throughout life. ISC division can give rise to two types of daughter cells: enteroblasts (EBs, green), which differentiate into polyploid, absorptive ECs (orange), or EEs (blue), which secrete peptide hormones. (e) Slit-Robo signaling (purple) regulates lineage commitment of ISC daughter cells. Slit is produced by EEs and binds to Robo2 on EBs and ISCs to gate, but not to instruct, EE lineage commitment.

and Paneth cells. EphB2 is absent from Paneth cells. In *EphB2;B3* global double mutants, newborn mice have wild-type ephrin-B expression. However, the ephrin-B gradient becomes disrupted as mice age, suggesting that EphB2 and EphB3 are required to maintain, but not to establish, the ephrin-B gradient. As *EphB2/B3* mice age, the ephrin-B expression domain expands to cells throughout crypts, encompassing cells at the crypt–villus junction that normally lack ephrin-B expression.[186,187] As a result, cells are disorganized along the crypt–villus axis. Similarly, overexpression of a dominant negative EphB2 receptor throughout intestinal epithelium disrupts precursor cell localization: rather than following the ephrin-B gradient, precursors align randomly along the crypts. Together, these data indicate that ephrin-Eph signaling instructs the localization of cells in the adult intestine.

Unlike other CBC daughter cells, which migrate out of the crypt as they differentiate, Paneth cells migrate to the base of the crypt, where they form an important part of the CBC niche (Figure 6a). Indeed, genetic models that do not have Paneth cells ultimately lose Lgr5+ stem cells.[188] What causes Paneth cells to migrate in the opposite direction of other stem cell daughters? While Paneth cells do not express EphB2, they express high levels of EphB3, suggesting they could be repelled by the increasing gradient of ephrin-B ligands on the sides of the crypt. In *EphB3* global mutant mice, Paneth cells are randomly scattered throughout the crypt,[186,187] supporting a model in which ephrin-Eph signaling regulates Paneth cell migration. Acute inhibition of EphB signaling in wild-type mice by injecting mice with unclustered monomeric ephrin-B2 ectodomains recapitulates this phenotype, indicating that ephrin-Eph signaling is required in adult mice, where it actively instructs daughter cell migration.[187] Cells in the villi are actively extruded and then could be passively replaced by a proliferating population, but this repellent system indicates there is an active effort to replace them.

In addition to its importance in positioning ephrin-B expressing precursors, EphB3 may be required for normal Paneth cell differentiation. Paneth cells in *EphB3* global mutant mice lack hallmarks of mature cells, including antimicrobial granules.[186] This could reflect the coupling of differentiation and migration or indicate that EphB3 has multiple roles in cell fate in the gut. Could this requirement be mirrored in other organs? Interestingly, ephrin-B1, EphA4, and EphB4 mRNA are detected in a stem cell population in the hair follicle bulge in mice (among other cells).[189] Although their functional role there has not been described, this raises the possibility that ephrin-Eph

signaling plays roles in the spatial organization of multiple stem cell compartments.

Like the mammalian digestive tract, the adult *Drosophila* midgut is maintained by a population of intestinal stem cell (ISCs)[190] (Figure 6d). ISCs undergo both symmetric and asymmetric division. When ISCs undergo asymmetric division, daughter cells can become enteroblasts (EB), which mature into polyploid, absorptive enterocytes (EC), or pre-enteroendocrine cells (pre-EE), which mature into diploid, neurosecretory enteroendocrine cells (EE).[191,192] The presence of these two transitional cells was recently delineated, leading to the revision of an earlier model wherein all cells moved through the EB stage.[191,192]

In the *Drosophila* midgut, slit-Robo signaling regulates lineage commitment of ISC daughters[190] (Figure 6e). Slit is transcribed in EEs and secreted to bind to Robo2 on ISCs and EBs in the midgut.[191,192] ISCs in *robo2* clones proliferate and self-renew normally but double their proportion of Prospero-positive EEs. Ubiquitous knockdown of Slit recapitulates this lineage commitment shift, as does EE-specific slit knockdown (albeit to a lesser extent). Could slit-Robo2 signaling allow ISCs to specify daughter cell fate according to tissue composition? Overexpression of either ligand or receptor does not suppress EE commitment, suggesting that EE specification is gated, not instructed, by slit-Robo2 signaling. This is reminiscent of the role of Prospero in EE commitment. Prospero was initially identified as a marker for EE cells[193] and is necessary, but not sufficient, for EE fate.[192] Indeed, the Robo2 knockdown phenotype in ISCs and EBs can be rescued by simultaneously knocking down Prospero, suggesting that Robo2 may act upstream of Prospero in ISCs to regulate EE commitment.[191] Intriguingly, Scute, a transcription factor whose ISC-specific overexpression *does* lead to ectopic Prospero-positive cells,[192,194] is required for the Robo2 knockdown phenotype.[195] ISC-specific knockdown of Scute suppresses the Robo2 RNAi phenotype, although it remains unclear whether this reflects a genetic interaction between the signaling pathways or the operation of parallel pathways controlling EE fate specification. Irrespective of the mechanism, by regulating lineage commitment to the EE fate, slit-Robo2 signaling regulates tissue integrity and function; midguts lacking EE cells have disrupted endocrine-related processes, included insulin signaling.[196] It will be interesting to consider how signaling outputs in this case of lineage commitment may diverge from those commonly understood in the context of axon guidance. In the developing *Drosophila* embryo, for example, Robo2 directs growth cone repulsion and lateral positioning via cell-autonomous mechanisms and binds in *trans* to Robo1 to inhibit repulsion at an earlier stage of development.[55] Robo1 is not detected in the fly midgut,[191] and while Robo2 appears to physically interact with Slit protein, the midgut phenotypes do not indicate a role for slit-Robo2 in repulsion. Furthermore, the functional importance of locally-secreted slit to lineage commitment is uncertain based on the absence of phenotypes in *slit* mutant clones.[197] Finally, as Robo2 loss of function phenotypes become more severe with age,[191,192] it will be interesting to explore how slit-Robo signaling itself changes with age or interacts with pathways that change with age, including JNK and Notch signaling.[198]

PERSPECTIVES

During development, organisms build functional, physiologically integrated tissues by responding to intrinsic and extrinsic cues that balance cell specification, migration, and proliferation. Once constructed, the fitness of that organism requires it to maintain the structure and function of these tissues. Lacking the context of developmental cues, how is the integrity of established tissues maintained? Furthermore, how do tissues adapt to changing organismal physiology to perform their roles? Many of the genes that drive development are repurposed in later life to regulate tissue homeostasis, which poses an interesting question: do developmental signals regulate development and homeostasis differently? Again, we must consider various aspects of the tissue: its homeostasis, its response to injury, and its response to organismal physiology and aging. Studies in cell culture continue to provide significant mechanistic insight into the regulation and downstream effectors of these pathways. Translating these mechanistic studies to a physiological context is technically challenging, but critical to our understanding of the endogenous functions of guidance receptors and their ligands. The advent of genetic tools that allow spatiotemporal gene manipulation, as well as novel techniques to precisely generate molecularly defined mutations in animals, will allow us to address these questions. Additionally, a growing number of single cell sequencing data sets will doubtless serve as a resource for developing new hypotheses.

In addition to acting locally in tissue homeostasis, axon guidance signaling may regulate organismal physiology. Guidance factors are integral to the development of the vascular system, which shares many similarities with nervous system development.[9,199] For example, in the developing pancreas, guidance receptors regulate the organ's innervation and vascularization, both of which are integral to its function.[200,201] However, under conditions of adult neovascularization, the physiological environment is significantly different from that during development.[202] It is tempting to speculate that guidance receptors and their ligands may coordinate neurogenesis with angiogenesis in contexts where they occur simultaneously, including the adult songbird brain.[203] Moreover, guidance receptors may provide important links between the brain and peripheral tissues, either by regulating tissue innervation or controlling the formation of circuits that respond to and regulate organismal physiology. Tissue- and cell-type-specific manipulations will be necessary to continue to delineate the varied contributions of guidance signaling in vivo.

Few studies definitively link ligand and receptor signaling outside of the nervous system. In the absence of a functional readout of signaling activity, it can be difficult to demonstrate a connection between receptor and ligand. Even within the nervous system, where neurons are bathed in ligand, receptors have ligand-independent activities. Notably, in the *Drosophila* nervous system, Frazzled acts independently of its canonical ligand to regulate axon guidance via a transcriptional mechanism. Moreover, some guidance receptors have roles that only occur in the absence of ligand, such as the dependence receptor activity of Dcc.[93] Thus, the presence of both receptor and ligand expression in

a particular tissue does not preclude ligand-independent singling, and future studies should take care to consider the possibility of ligand-independent activity. The advent of more complex genetic technology should permit additional specific manipulations to pinpoint the precise requirement of guidance cues, hopefully elucidating their downstream effectors.

AUTHOR CONTRIBUTIONS

Writing (original draft, review, and editing) and visualization: Kaitlin M. Laws. *Writing (review and editing) and supervision*: Greg J. Bashaw.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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