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Making Connections: Guidance Cues and Receptors at Nonneural Cell–Cell Junctions

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The field of axon guidance was revolutionized over the past three decades by the identification of highly conserved families of guidance cues and receptors. These proteins are essential for normal neural development and function, directing cell and axon migration, neuron–glial interactions, and synapse formation and plasticity. Many of these genes are also expressed outside the nervous system in which they influence cell migration, adhesion and proliferation. Because the nervous system develops from neural epithelium, it is perhaps not surprising that these guidance cues have significant nonneural roles in governing the specialized junctional connections between cells in polarized epithelia. The following review addresses roles for ephrins, semaphorins, netrins, slits and their receptors in regulating adherens, tight, and gap junctions in nonneural epithelia and endothelia.

Guidance receptors on axonal growth cones respond to extracellular cues to steer axons to appropriate synaptic targets. Their activation engages dynamic cytoskeletal regulation, as well as the making and breaking of cell–matrix and cell–cell adhesive contacts, to navigate the extracellular milieu. In recent years, it has become clear that these guidance cues also influence the formation, maintenance and remodeling of cell–cell junctions outside the nervous system. Across a wide array of epithelia, as well as lymphatic and vascular endothelia, the ephrin, semaphorin, netrin, and slit families of guidance proteins and their receptors profoundly influence the formation, maintenance, and remodeling of classic adherens, tight and gap, cell–cell junctions.

Adherens Junctions

Adherens junctions (AJs) are essential for the organization and maintenance of tissue architecture and integrity, linking the actin cytoskeleton of two adjacent cells. The link is mediated by extracellular, calcium-dependent, homophilic interactions between classical cadherins. The cadherin cytoplasmic tail binds to β-catenin and p120. β-catenin can bind α-catenin to mediate binding to the actin cytoskeleton (Pokutta and Weis 2007). Cadherins are expressed in all

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epithelia and contribute to establishing and maintaining apico-basal polarity via signaling pathways that mediate the organization of endothelial adherens and tight junctions (TJs) (Taddei et al. 2008; Walsh et al. 2011). For a detailed review of AJs, see Mège and Ishiyama (2017).

Tight Junctions

Tight junctions (TJs) form a paracellular barrier at the apical-most portion of lateral membranes that establishes tissue boundaries by restricting permeability to ions and proteins. Bridging the paracellular space are tetraspan claudin family proteins (26 in humans and 27 in mice) that form hetero- or homotypic interactions between cells (Furuse et al. 1999; Morita et al. 1999; Tsukita and Furuse 1999). Many other transmembrane components, such as the integral membrane protein occludin, also contribute to the molecular architecture of TJs (Zihni et al. 2016). The cytosolic portion of TJs comprises a junctional plaque, bridging the junctional proteins with the cytoskeleton. Plaque components include adaptor proteins such as zonula occludens-1 (ZO-1) (Stevenson et al. 1986), as well as many downstream signaling components common to axon guidance receptors such as protein kinases, phosphatases, and GTPases (Guillemot et al. 2008). For a detailed review of TJs see Buckley and Turner (2017).

Gap Junctions

Gap junctions couple signaling molecules and metabolites between neighboring cells. In the vasculature, for example, they are essential for continuous and rapid modulation of the vascular network (Figueroa and Duling 2009). Gap junctions are composed of connexins (Cx) in chordates, and innexins in precordates (Goodenough and Paul 2009). These integral proteins combine to form hexamers, which bridge the intercellular gap to form a gated hydrophilic channel between cells (Goodenough and Paul 2009). Assembly into junctions, trafficking, and channel gating and turnover is regulated through phosphorylation by kinases, such as Src and protein kinase C (PKC) (Lampe and Lau 2000). Endothelial-specific connexins include Cx-43, Cx-40, and Cx-37 (Bruzzone et al. 1993; Reed et al. 1993; Little et al. 1995; Haefliger et al. 2004). A growing number of interacting partners for Cx-43 have been identified and include AJ proteins as well as components of the cytoskeleton (Xu et al. 2001b; Govindarajan et al. 2002). For a detailed review of gap junctions, see Delmar et al. (2017).

EPHRINS AND CELL–CELL JUNCTIONS

Substantial insight into the signaling mechanisms that regulate cell–cell junctions comes from the field of cancer cell biology, in which disruption of cell–cell adhesions is an early, critical step in progression toward a metastatic state. It is not surprising, then, that several of the guidance receptors that regulate the formation and maintenance of these adhesive contacts were first identified because of their dysregulated expression in cancer cells. The Eph receptor family, for example, was named because of its overexpression in an erythropoietin producing hepatocellular (EPH) carcinoma cell line (Eph Nomenclature 1997). Eph receptors and their ephrin ligands have well described roles in both healthy and diseased states, including cell–substrate adhesion, cancer, tissue boundary formation, and morphogenesis.

Eph-Ephrin Signaling

Eph proteins are the largest subfamily of receptor tyrosine kinases and mediate short-range cell–cell signaling (Fig. 1A). They are classified based on sequence similarity and ligand selectivity (Gale et al. 1996). EphAs (A1–A8, A10) preferentially bind the five glycosylphosphatidylinisotol (GPI)-anchored ephrin-A ligands (A1–A5), whereas the EphBs (B1–B4, B6) preferentially bind the three transmembrane ephrin-B ligands (B1–3), with a small number of exceptions. Ligand–receptor interactions are relatively promiscuous within the same subclass. All Eph receptors contain a globular ligand-binding extracellular domain, a Cys-rich domain with sushi and epidermal growth factor (EGF)-like motifs, and fibronectin domains. The trans-

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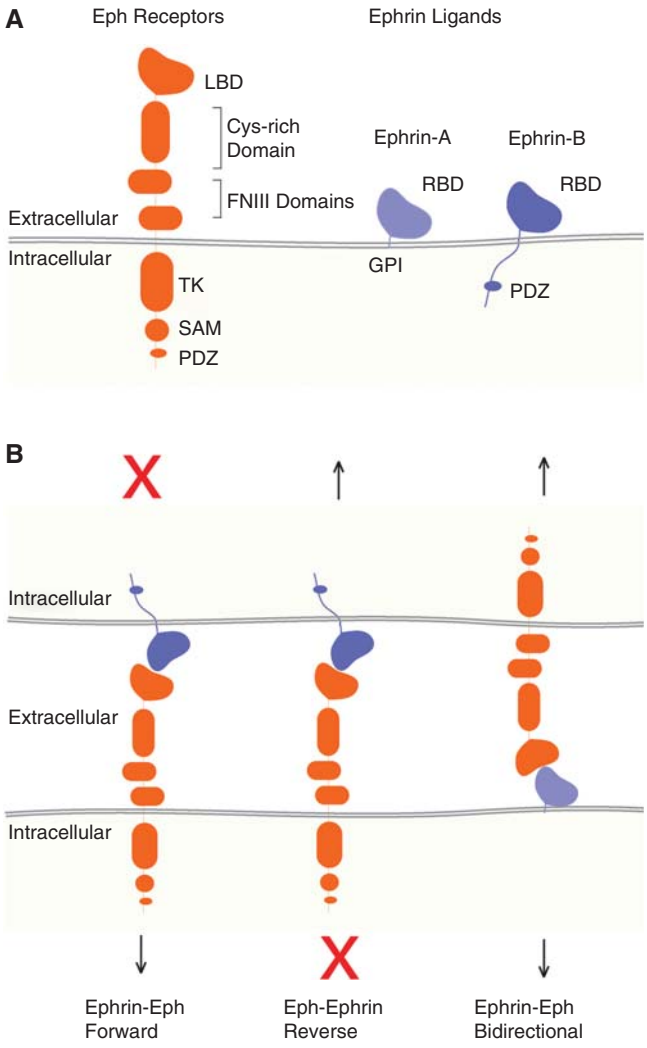

Figure 1. Schematic of Eph-ephrin structure and signaling at cell–cell junctions. (*Figure continues on following page.*)

membrane domain is followed by an intracellular tyrosine kinase domain, a SAM (sterile alpha motif) domain and a PDZ (postsynaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and ZO-1) domain. All ephrins are composed of a globular extracellular ligand-binding domain followed by either a transmembrane domain and an intracellular PDZ domain (ephrin-Bs) or a GPI-linkage to the cell membrane (ephrin-As).

Signaling can occur bidirectionally, through the "receptor" or the "ligand" (Fig. 1B) (Kania and Klein 2016). Forward signaling results when ephrins act in *trans* as ligands to activate Eph receptor tyrosine kinase activity. Eph receptors can also act as a ligand in *trans* with an ephrin, leading to the activation of Src family kinase (SFK)-dependent intracellular "reverse signaling." Simultaneous activation of pathways downstream from both Eph receptors and ephrins is termed bidirectional signaling. When Eph receptors and ephrin ligands are both expressed in two interacting cells, parallel bidirectional signaling, within the same cell, and antiparallel bidirectional signaling, in opposing cells, can occur. These multiple signaling modes con-

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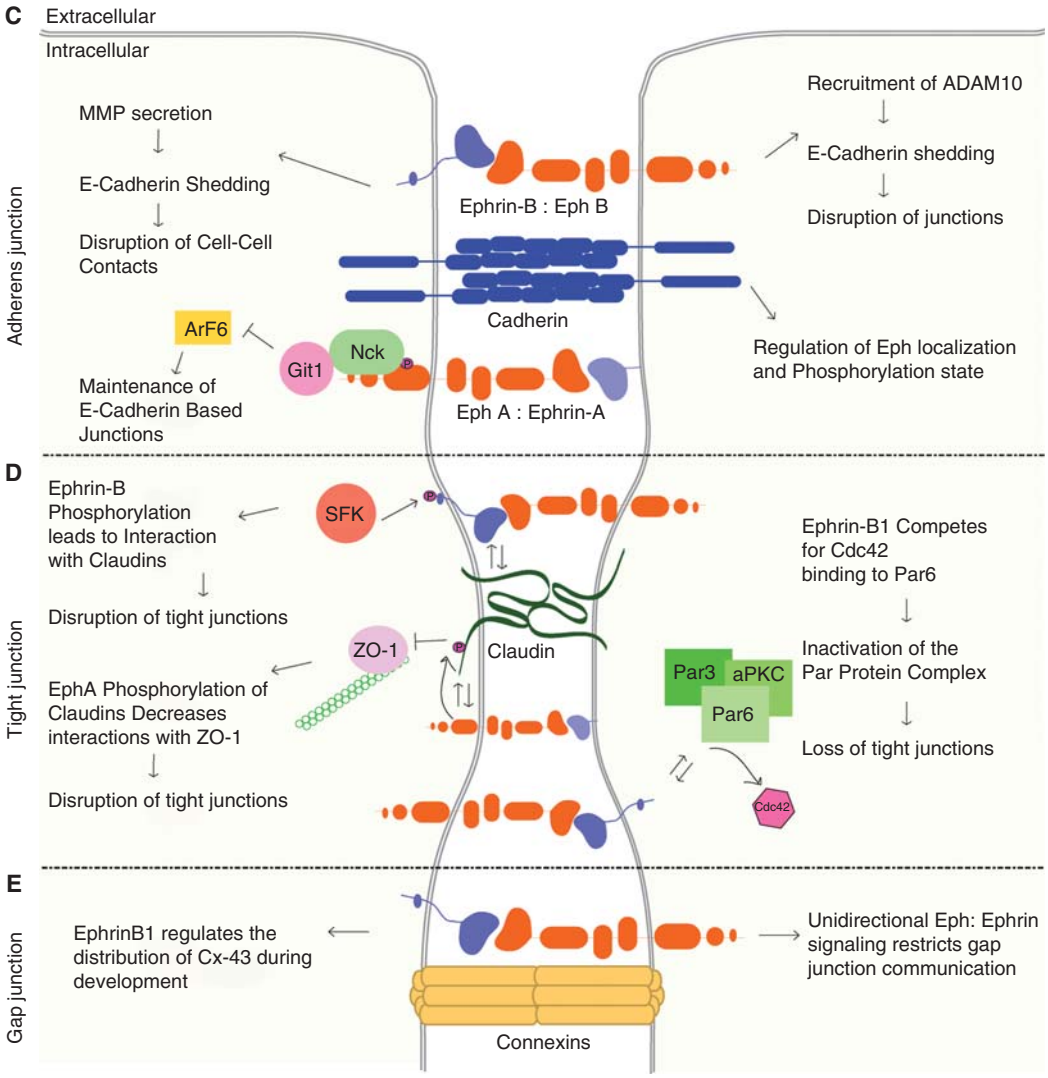

Figure 1. (*Continued*)

tribute to substantial versatility of Eph-ephrin signaling.

Ephrins binding to Eph receptors engage intracellular signaling proteins such as noncatalytic region of Tyr kinase adaptor protein 1 (Nck1) and Nck2, phosphoinositide 3-kinase (PI3K), and SFKs (Lisabeth et al. 2013). These then regulate small Rho GTPases like Rac1 and RhoA that can remodel the actin cytoskeleton. Both A and B class ephrins engage SFKs on activation by Eph receptors (Lisabeth et al. 2013). Specific effectors include Ret and p75 for A class ephrins, and the Grb4-Pak1-Dock180 complex for B class ephrins (Cowan and Henkemeyer 2001; Lim et al. 2008; Xu and Henkemeyer 2009; Bonanomi et al. 2012).

Eph-Ephrin Regulation of Adherens Junctions

Multiple studies have reported roles for ephrins and Eph receptors regulating tissue development and maintenance via modulation of cadherin-based cell–cell junctions. For example, during epithelial cell sorting in the developmen-

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tal establishment of tissue boundaries, EphBs regulate E-cadherin-based adhesions (Fig. 1C). In vitro studies using Madin-Darby canine kidney (MDCK) epithelial cells showed that EphB signaling recruits E-cadherin and ADAM10 (a disintegrin and metalloprotease 10) to sites of intercellular adhesion leading to local E-cadherin shedding. This causes asymmetric E-cadherin localization that results in different binding affinities between the two cell populations and the establishment of cellular boundaries (Solanas et al. 2011). Interestingly, the expression of a dominant-negative form of ADAM10 in Paneth cells in the small intestine phenocopies the inappropriate positioning of these epithelial cells along intestinal crypts found in EphB3-null mice, suggesting that EphB3 modulates ADAM10 function and cadherin distribution in the plasma membrane (Solanas et al. 2011). In turn, E-cadherin also regulates the differential expression of Ephs and ephrins (Orsulic and Kemler 2000), influencing their phosphorylation and subcellular localization (Zantek et al. 1999), showing a close relationship between E-cadherin and Eph function in epithelial cells.

Depending on expression levels and cell context, EphAs mediate opposing effects on the integrity of AJs as compared to EphBs (Fig. 1C). EphA2 expression in MDCK epithelial cells, for example, appears to regulate a positive feedback loop at E-cadherin-based cell–cell junctions to maintain intercellular adhesion (Miura et al. 2009). Ephrin-A1 ligand-dependent phosphorylation of EphA2 recruits G protein-coupled receptor kinase-interacting protein (Git) 1 and Nck1 to suppress the activity of ADP-ribosylation factor (Arf)6, a negative regulator of E-cadherin-based cell–cell contacts (Palacios et al. 2001, 2002; Miura et al. 2009). This role of EphA2 in E-cadherin regulation was then confirmed through EphA2 knockout (KO) studies. *EphA2*$^{-/-}$ mice have disrupted lens cell organization in the developing eye caused by a lack of EphA2-mediated SFK and cortactin phosphorylation, which together contribute to appropriate E-cadherin localization during lens morphogenesis (Cheng et al. 2013a). Altered E-cadherin based cell–cell junctions then result in altered lens function and cataracts (Cheng et al. 2013a). Indeed, mutations in EphA2 in humans are linked to congenital cataracts (Zhang et al. 2009b; Park et al. 2012). In the lens of the developing eye, EphA5 colocalizes with N-cadherin at sites of cell–cell adhesion, maintains N-cadherin at the surface of lens cells and increases the association of N-cadherin with β-catenin (Cooper et al. 2008). Activation of EphA5 by ephrinA5 regulates the organization of lens fiber cells and ephrinA5 null mice also develop an opaque lens and cataracts caused by improper regulation of AJs during lens morphogenesis (Cooper et al. 2008; Biswas et al. 2016).

The importance of Eph/ephrin regulation of AJs is also highlighted by their oncogenic effects across a variety of epithelial cells. Overexpression of EphA2 is found in multiple solid tumors; breast, colon, and prostate, as well as high ectopic expression in metastatic melanocytes (Kinch and Carles-Kinch 2003). EphA2 overexpression by cancer cells destabilizes AJs and promotes a metastatic phenotype through RhoA overactivation (Fang et al. 2008). Extracellular EphA2 cleavage by the membrane-anchored, membrane type-1 matrix metalloproteinase (MT1-MMP) has been implicated in the disassembly of cell–cell contacts and cancer cell invasion (Sugiyama et al. 2013). Further, ephrinB1 reverse signaling can promote tumor cell invasion by inducing MMP8 secretion (Tanaka et al. 2007a,b; Jiang et al. 2008). These findings suggest a central role for Eph/ephrin in the progression of epithelial cells toward a metastatic state by regulating cell–cell and cell–matrix adhesion.

Eph receptors and ephrins also mediate cell–cell adhesion in kidney glomeruli at the slit diaphragm, a specialized structure that mediates convective fluid flow between the interdigitating foot processes of podocytes (Pavenstädt et al. 2003; Hashimoto et al. 2007). The slit diaphragm is a modified AJ (Reiser et al. 2000) with a synapse-like organization that engages cadherins, Eph/ephrins, neurexins, and immunoglobulin (Ig)-like adhesion molecules (Grahammer et al. 2013). Interestingly, EphB4 signaling in podocytes promotes recovery following glomerular injury, raising the possibility that Eph signaling may regulate cadherin expression and function at this specialized cell–cell junction. Progressive

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renal diseases frequently show disruption of the Slit diaphragm (Grahammer et al. 2013) and Eph signaling may be a promising target for therapeutic intervention.

Eph-Ephrin Regulation of Tight Junctions

Recent studies have highlighted a role for Ephs (Wnuk et al. 2012) and ephrins in the phosphorylation state and localization of TJ proteins in epithelial cells. Ephrin-B1 and EphA2 negatively regulate the maintenance of cell–cell contacts through interactions with the claudin family of TJ proteins (Fig. 1D). Cell–cell contact leads to SFK-dependent, Eph receptor-independent, tyrosine phosphorylation of the ephrin-B1 cytoplasmic domain (Tanaka et al. 2005b) following ephrin-B1 binding in *cis* to claudin-1 or claudin-4 via their extracellular domains. This phosphorylation of ephrin-B1 then increases paracellular permeability and reduces the efficacy of intercellular adhesion (Tanaka et al. 2005b). In this way, TJ proteins themselves regulate the phosphorylation state of ephrin-B1 to regulate junctional integrity.

EphA tyrosine kinase activity can also alter the phosphorylation of the cytoplasmic tails of claudins, disrupting integration into junctions. For example, following activation by ephrin-A1, EphA2 regulates the permeability of TJs through *cis* interactions with claudin-4 (Tanaka et al. 2005a). This results in claudin-4 phosphorylation, attenuating its association with ZO-1 and inhibiting claudin-4 integration into TJs (Tanaka et al. 2005a). These findings show that Eph receptor kinase activity can regulate TJ integrity by altering claudin function.

Eph-ephrin signaling can also indirectly affect the formation of TJs through interactions with junctional regulators. Ephrin-B1, for example, regulates the Par protein complex, which is a master regulator of epithelial cell polarization and TJ formation (Shin et al. 2006). Ephrin-B1 competes with Cdc42 binding to Par6, inactivating the Par protein complex and resulting in loss of TJs (Lee et al. 2008). Phosphorylation of ephrin-B1 disrupts the Par6 interaction and restores junctions, with phosphorylated ephrin-B1 remaining enriched at TJs (Lee et al. 2008).

Ephs and ephrins also regulate angiogenesis (Cheng et al. 2002) and intercellular adhesion between endothelial cells critically regulates vascular permeability (Corada et al. 1999; Bazzoni 2006). EphA2 stimulation leads to increased vascular permeability in the lung, which underlies increased permeability in models of lung injury (Larson et al. 2008; Cercone et al. 2009). In contrast, EphB4 positively regulates vascular barrier integrity in a pathway downstream from another angiogenic factor, sphingosine 1-phosphate (S1P), and vascular endothelial (VE)-cadherin (McVerry and Garcia 2005; Tobia et al. 2012).

Eph-Ephrin Regulation of Gap Junctions

Gap junction communication (GJC) plays a central role in tissue patterning. Initial insights into the influence of ephrin-Eph signaling on gap junction function came from in vitro studies of segmental patterning during embryogenesis, highlighting the importance of directional signaling from this receptor–ligand pair (Fig. 1E). Unidirectional signaling results in restricted GJC, whereas bidirectional signaling limits the intermingling of adjacent cell populations (Mellitzer et al. 1999). During development, ephrin-B1 also interacts with Cx-43 and regulates its distribution. Ephrin-B1 is X-linked, and mosaic ephrin-B1 expression following X-inactivation in ephrin-B1 heterozygous mice results in the formation of ectopic Eph/ephrin boundaries. The inhibited GJC results in a neural crest cell phenotype associated with cell sorting defects that underlie craniofrontonasal syndrome (Davy et al. 2006).

SEMAPHORINS AND CELL–CELL JUNCTIONS

Semaphorins are a large family of secreted and integral membrane proteins that were first identified for their capacity to collapse nerve growth cones (Luo et al. 1993). Multiple receptors and coreceptors are engaged by semaphorins, mediating downstream signaling that regulates neuronal migration (Chen et al. 2008a), synapse formation (Leslie et al. 2011), and synaptic

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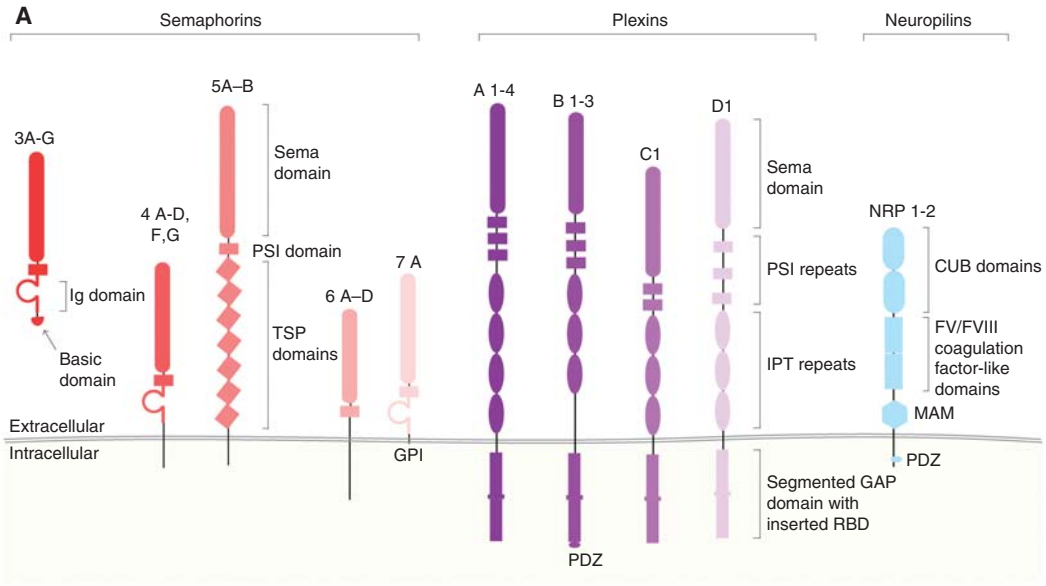

Figure 2. Schematic of semaphorin–plexin–neuropilin structure and signaling at cell–cell junctions. (*Figure continues on following page.*)

transmission (Sahay et al. 2005). Semaphorins are also key regulators of angiogenesis, the immune system, cancer progression and tumorigenesis, and cytoskeletal organization in a wide range of cell types (Kruger et al. 2005; Yazdani and Terman 2006). Known in the guidance field for directing F-actin organization and cell–matrix contacts in migrating growth cones, novel roles for semaphorins in the control of cell–cell junctions in nonneural tissues suggest they are critical regulators of junctional dynamics; however, mechanistic insights into how semaphorins control intercellular junctions remain limited.

Semaphorins and Their Receptors

The semaphorin gene family is defined by the presence of an extracellular ~500 amino acid sema-PSI domain that mediates receptor binding (Fig. 2A) (Antipenko et al. 2003; Love et al. 2003; Janssen et al. 2010). There are eight classes, with vertebrate semaphorins constituting class 3 through 7. The secreted class 3 semaphorins contain Ig-like domains and a carboxy-terminal domain that is rich in basic residues (Goodman et al. 1999). Classes 4, 5, and 6 comprise transmembrane proteins that can elicit bidirectional responses during axon guidance. Class 7 semaphorins include an intracellular PDZ domain whereas class 5 proteins contain extracellular thrombospondin repeats. Sema7A contains an Ig-like domain and is tethered to the membrane via a GPI anchor (Goodman et al. 1999). The best characterized signaling downstream from semaphorins is mediated by high-affinity plexin receptors and neuropilin coreceptors.

Plexins are a family of transmembrane proteins, with nine vertebrate plexins divided into four distinct classes: four class A (A1–4), three class B (B1–3), PlexinC1, and PlexinD1 (Fig. 2A) (Nogi et al. 2010). All plexins are composed of an extracellular sema domain, followed by two or three PSI and IPT (immunoglobulin domains shared by plexins and transcription factors) repeats. Plexin cytoplasmic domains are all very similar, containing a Rho and Ras-family-specific GTPase-activating protein (GAP) domain with an inserted Rho GTPase-binding domain (RBD) (Tong et al. 2009). Semaphorin-dependent receptor dimerization relieves autoinhibition to promote GAP activity (Oinuma

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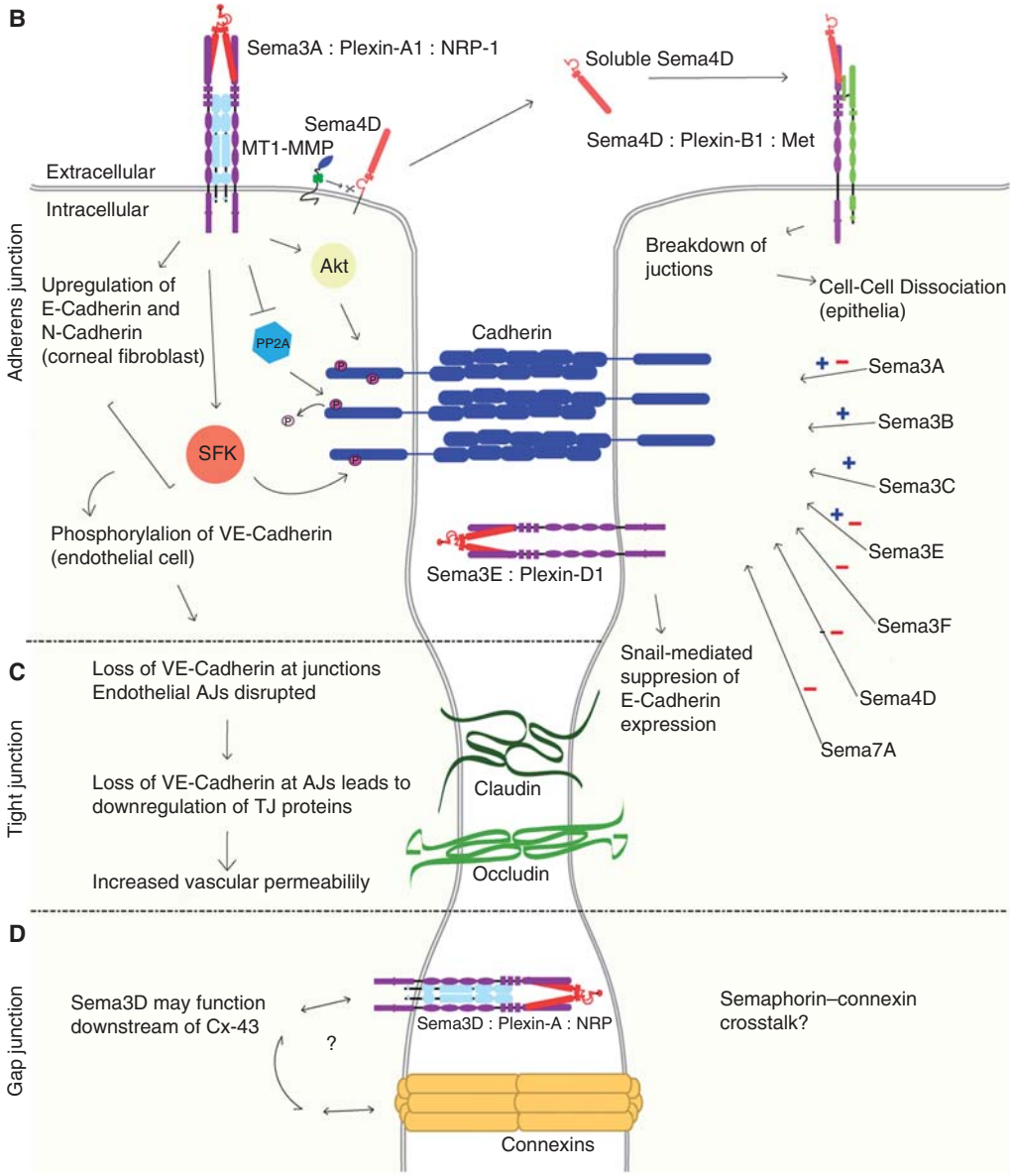

Figure 2. (*Continued*)

et al. 2004; Wang et al. 2012). Class A plexins bind the secreted class 3 semaphorins, with PlexinA1 also mediating signaling downstream from transmembrane Sema6A. Class B Plexins contain a carboxy-terminal PDZ motif and are receptors for class 4 and 5 semaphorins. PlexinC1 binds to Sema7A and PlexinD1, the latter of which is specifically expressed by endothelial cells and signals downstream from the secreted semaphorin class 3 ligands (Tamagnone et al. 1999; Van Der Zwaag et al. 2002; Torres-Vázquez et al. 2004). Plexin signaling downstream from secreted semaphorins requires a neuropilin coreceptor to form a holoreceptor complex.

The neuropilin receptors NRP-1 and NRP-2 are exclusively expressed by vertebrates and, in the context of semaphorin-plexin signaling, function as coreceptors for the class 3 sema-

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phorins (Fig. 2A) (He and Tessier-Lavigne 1997; Takahashi et al. 1999). Extracellularly, these transmembrane receptors contain two complement-like (CUB) domains, two FV/FVIII coagulation factor-like domains, which constitute the semaphorin-binding region and one meprin/A5-protein/PTPmu (MAM) domain (Janssen et al. 2012). Sema3E is an exception and can signal through PlexinD1 in endothelial cells in the absence of neuropilins (Gu et al. 2005). Neuropilins contain a short cytoplasmic tail that includes a PDZ-binding motif that presumably mediates intracellular protein–protein interactions.

Secreted semaphorins signal via plexin/neuropilin complexes (Fig. 2B). Transmembrane semaphorins, however, signal through multiple mechanisms. While at the cell surface, these ligands mediate short-range, cell–cell interactions via "forward" signaling through plexins (Hota and Buck 2012). Shedding and release of the extracellular domain of a transmembrane semaphorin by metalloproteases, such as MT1-MMP-mediated cleavage of Sema4D, can generate long-range paracrine signaling via plexins on target cells (Basile et al. 2007). Semaphorins can also induce plexin-mediated, *trans*-activation of plexin-associated receptor tyrosine kinases (Winberg et al. 2001). Similar to Eph/ephrin signaling, transmembrane semaphorins can activate "reverse" signaling pathways, to transduce a signal via their own cytoplasmic tails, either cell-autonomously (Cafferty et al. 2006; Komiyama et al. 2007), or in a ligand-dependent manner (Toyofuku et al. 2004). Moreover, transmembrane semaphorins can bind to plexins in *cis*, effectively blocking responses to secreted semaphorins (Haklai-Topper et al. 2010; Matsuoka et al. 2011; Sun et al. 2013).

Semaphorin Regulation of Adherens Junctions

Emerging evidence suggests that semaphorins contribute to the maintenance of epithelial barrier integrity (Fig. 2B). Semaphorins influence the subcellular localization of proteins that make up cell–cell junctions. The application of exogenous Sema3F to a breast cancer cell line resulted in NRP-1 dependent mislocalization of E-cadherin and β-catenin (Nasarre et al. 2005). Further, Sema3A released by corneal fibroblasts in the eye up-regulates levels of both E-cadherin and N-cadherin in adjacent corneal epithelium. Together with increased Sema3A and NRP-1 found in corneal epithelia during wound healing, this suggests that Sema3A regulates junctional dynamics in the cornea in healthy and pathological conditions.

Sema3E has been implicated in cancer progression in many cancer types; however, the mechanism of Sema3E action on the regulation of intercellular junctions remains underexplored. Perhaps the best example is highlighted by the role of Sema3E signaling through PlexinD1 in ovarian endometrioid tumors. In this context, Sema3E mediates epithelial–mesenchymal transition (EMT) and increases cancer cell migration (Fig. 2B) (Tseng et al. 2011). This is achieved by activation of PI3K, and is dependent on nuclear localization of Snail1 (SNAI1) (Tseng et al. 2011), a zinc finger transcription factor that promotes EMT by suppressing E-cadherin expression and decreasing intercellular adhesion (Batlle et al. 2000; Domínguez et al. 2003; Shook and Keller 2003).

Other classes of semaphorins also contribute to regulating cell–cell junction dynamics. Sema4D stimulation of normal and tumor epithelial liver cells triggers cell–cell dissociation, promoting invasive cell growth (Giordano et al. 2002). This is mediated by activation of Met, a receptor tyrosine kinase and proto-oncogene that associates with the Sema4D receptor PlexinB1 and is activated by Sema4D–PlexinB1 binding (Fig. 2B) (Giordano et al. 2002; Cagnoni and Tamagnone 2014). GPI-linked Sem7A also influences invasive phenotypes, in this case by regulating EMT (Allegra et al. 2012). Acting via the Ets-2-repressor, Sema7A is required for transforming growth factor (TGF)-β-induced EMT through the down-regulation of E-cadherin (Allegra et al. 2012).

Semaphorins and Tight Junctions

Functions for class 3 semaphorins have emerged in vascular remodeling, paracellular permeabil-

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ity and endothelial barrier integrity, suggesting a role regulating TJs. Most evidence to date has focused on the regulation of VE-cadherin and its effects on vascular permeability (Fig. 2C). VE-cadherin signaling at AJs up-regulates the TJ gene encoding claudin-5 and the loss of this up-regulation is thought to underlie the increased permeability across endothelial cells when VE-cadherin is inhibited (Taddei et al. 2008).

Early studies showed that autocrine secretion of class 3 semaphorins by endothelial cells regulates integrin function to remodel the vasculature (Serini et al. 2003). More recently, an anti-angiogenic and provascular permeability role has been described for several of the secreted semaphorins (Kessler et al. 2004; Varshavsky et al. 2008; Maione et al. 2009; Sakurai et al. 2010; Mishra et al. 2015; Yang et al. 2015). For example, Sema3A signaling through NRP-1 and PlexinA1 receptors destabilizes endothelial AJs and increases vascular permeability by regulating VE-cadherin (Fig. 2B, C) (Acevedo et al. 2008; Le Guelte et al. 2012). In peripheral endothelial cells, Sema3A-mediated NRP-1 activation leads to PI3Kγ/Akt pathway activation and subsequent VE-cadherin phosphorylation and junctional dysregulation, independent of Src activation (Acevedo et al. 2008). In brain endothelia, however, tumor-derived Sema3A increases vascular permeability through phosphorylation-dependent internalization of VE-cadherin in a Src-dependent manner (Le Guelte et al. 2012). In this model, Sema3A activates Src, which phosphorylates the serine-threonine protein phosphatase 2A (PP2A), releasing it from the VE-cadherin cytoplasmic domain. This promotes VE-cadherin serine phosphorylation and its subsequent internalization, thereby increasing vascular permeability (Le Guelte et al. 2012). These findings identify Sema3A to be a potent regulator of vascular integrity by destabilizing VE-cadherin-based interendothelial junctions.

Semaphorins and Gap Junctions

During development, semaphorins influence cell migration by governing gap junction function (Fig. 2D). For example, cell–cell interactions, mediated by Cx-43-containing gap junctions, couple the activity of neural crest cells as they migrate to the heart, a process dependent on semaphorins (Brown et al. 2001; Feiner et al. 2001). Sema3A limits neural crest cell motility by antagonizing integrin signaling (Xu et al. 2006); however, the processes of Cx-43α1 null neural crest cells fail to retract in response to Sema3A. Although neural crest cell motility does not appear to require intercellular communication (Xu et al. 2001a, 2006), these findings suggest that Cx-43 may somehow mediate cross-talk between semaphorins and integrins that underlies cell migration. Connexins are associated with dynamic regulation of the actin cytoskeleton through interactions with proteins usually considered as AJ components, such as vinculin, catenins, and α-actinin, during gap junction assembly (Xu et al. 2001a; Govindarajan et al. 2002). Such interactions could contribute to semaphorin-connexin cross-talk during neural crest cell migration. Perhaps consistent with this, Sema3D also appears to function downstream from Cx-43 during fin regeneration in zebrafish (Ton and Iovine 2012). Although these findings are intriguing, clearly more research is required to decipher the mechanism underlying the convergence of semaphorin–connexin function.

NETRINS AND CELL–CELL JUNCTIONS

Netrins are a small family of highly conserved, secreted, laminin-related, extracellular proteins, first identified as chemotropic guidance cues that direct cell and axon migration in the developing nervous system (Ishii et al. 1992; Kennedy et al. 1994; Serafini et al. 1994). Subsequent studies identified roles for netrins in the development and organization of the vasculature, lung, pancreas, muscle, mammary gland, and in tumorigenesis.

Netrins and Their Receptors

Mammals express four secreted netrins: netrin-1, -3, -4, and -5 (Fig. 3A) (Lai Wing Sun et al. 2011). All netrins are ∼600 amino acids in length, composed of amino-terminal sequences

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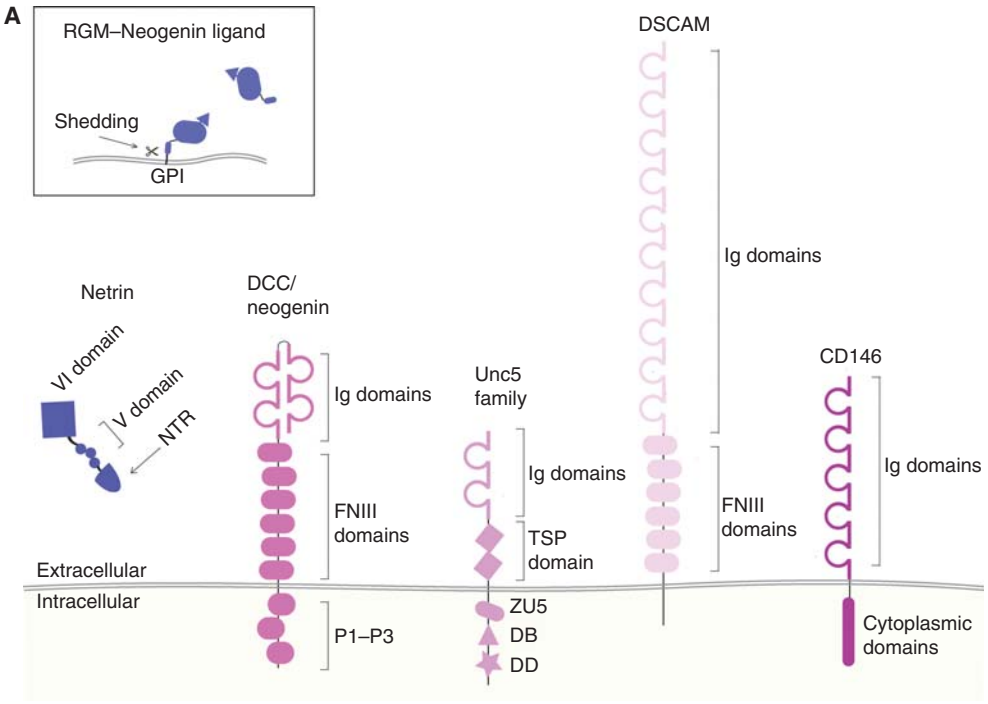

Figure 3. Schematic of netrins and their receptors' structure and signaling at cell–cell junctions. (*Figure continues on following page.*)

homologous to domains VI and V at the amino termini of laminins (Yurchenco and Wadsworth 2004). These domains are followed by a carboxy-terminal domain named "domain C" or the netrin-like (NTR) module. Domain C is rich in basic amino acids, binds heparin, and shows limited sequence similarity to tissue inhibitors of metalloproteinases (TIMPS) (Bányai and Patthy 1999; Kappler et al. 2000; de Wit and Verhaagen 2007).

Secreted netrins are bifunctional, acting as either chemoattractant or chemorepellent migratory cues, depending on the receptors expressed by the responsive cell (Fig. 3A). Canonical receptors for netrin-1 in mammals include deleted in colorectal cancer (DCC), the DCC paralog neogenin, and the four UNC5 homologs, UNC5A–D, all of which are Ig superfamily, cell adhesion molecule (CAM)-like, type 1 transmembrane proteins (Lai Wing Sun et al. 2011). In addition to secreted netrins, draxin and cerebellins are also ligands for DCC (Ahmed et al. 2011; Wei et al. 2012; Haddick et al. 2014), and UNC5 homologs bind members of the fibronectin and leucine-rich transmembrane (FLRT) protein families (Yamagishi et al. 2011). Additional ligands for neogenin include bone morphogenic proteins (BMPs) and repulsive guidance molecules (RGMs) (Rajagopalan et al. 2004; Hagihara et al. 2011).

The molecular mechanisms underlying netrin-1 signaling via DCC during axonal chemoattraction have been relatively well studied, while less is known regarding netrin chemorepellent responses (Lai Wing Sun et al. 2011). DCC activation directs the reorganization of F-actin (Shekarabi and Kennedy 2002; Dent et al. 2004). In neurons responding to netrin-1 as a chemoattractant, DCC constitutively binds the adaptor Nck1 and FAK (Li et al. 2002, 2004). On netrin-1 binding, FAK activation recruits SFKs (Li et al. 2004; Ren et al. 2004, 2008), leading to Nck1-dependent recruitment of the kinase PAK1 and the Rho GTPases Cdc42 and Rac (Shekarabi and Kennedy 2002; Shekarabi et al. 2005), both of which regulate F-actin. Netrin-1

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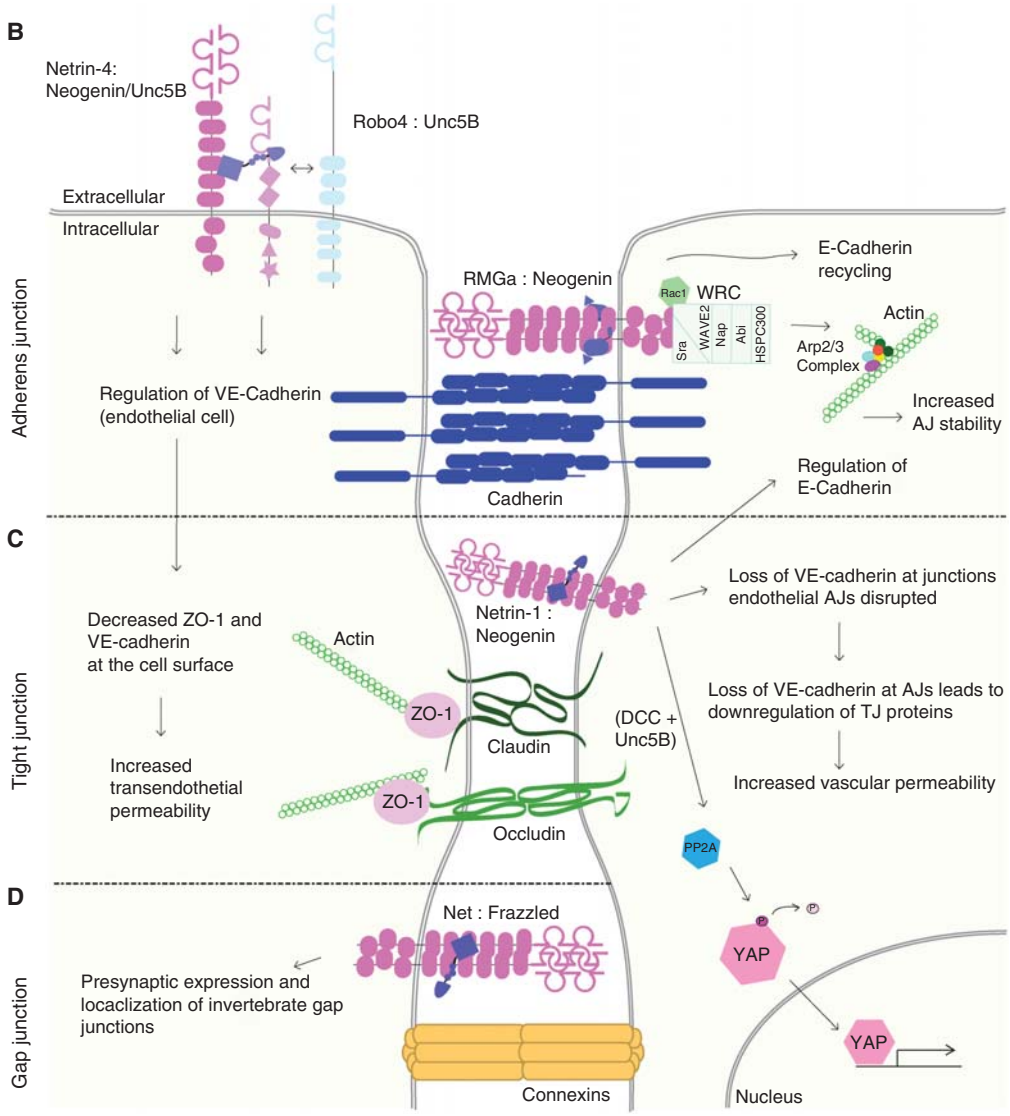

Figure 3. (*Continued*)

binding to DCC also activates mitogen-activated protein kinase (MAPK) (Forcet et al. 2002), PI3K (Ming et al. 1999), inactivates RhoA (Moore et al. 2008), and stimulates local protein synthesis (Campbell and Holt 2001; Leung et al. 2006; Tsai et al. 2006, 2007; Tcherkezian et al. 2010). Additional receptors implicated in netrin function include Down syndrome cell adhesion molecule (DSCAM) (Andrews et al. 2008; Ly et al. 2008), CD146 on endothelial cells (Tu et al. 2015), and several integrins, as described below.

Netrins and Adherens Junctions

An initial understanding of the role of netrins and their receptors in regulating cell–cell junctions outside the nervous system was the discovery that a netrin-1/neogenin interaction is essential for the proper morphogenesis of the mammary gland (breast). In this capacity, netrin-1 provides spatial and temporal information that guides tissue and organ development, similar to how it directs migrating ax-

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ons within the nervous system (Srinivasan et al. 2003).

The enlarged termini of developing mammary gland ducts, called terminal end buds (TEBs), invade mammary fat pads and enlarge the nascent mammary tree through a process of ductal elongation and branching (Williams and Daniel 1983), which is achieved by proliferating cap cells and their underlying prelumenal epithelium (Williams and Daniel 1983). Cap cells adhere to each other through P-cadherin containing junctions, whereas prelumenal cells depend on E-cadherin intercellular junctions (Daniel et al. 1995). Surprisingly, the close apposition of the cap cell layer to the prelumenal cell layer depended on a netrin-1/neogenin interaction, with netrin-1 expressed by the prelumenal cells, and neogenin by the cap cells (Srinivasan et al. 2003). This provided the first clear example of netrin-1 and its receptors mediating an adhesive, rather than a guidance function, during organogenesis. It remains to be elucidated what anchors the secreted netrin-1 to the prelumenal cells. Netrin-1 binds heparan and chondroitin sulfates (de Wit and Verhaagen 2007), and a likely possibility is that a chondroitin or heparan sulfate proteoglycan, such as a glypican or syndecan, may bind netrin-1 on the cell surface and mediate this cell–cell adhesion.

Although there is no evidence that netrins function as chemotactic cues to guide TEB outgrowth, they may play a role in regulating branching morphogenesis. Branch initiation appears to be dependent on asymmetric duct geometry coupled with a contraction of ductal cells that transmits force to adjacent cells through AJs (Nelson et al. 2005; Gjorevski and Nelson 2010). This mechanical stress then initiates sites of de novo branch formation by regulating FAK signaling and MMP production (Gjorevski and Nelson 2010). Netrin-1 regulates E-cadherin expression in mammary epithelial cells, and has also been shown to induce MMP-dependent degradation of E-cadherin in mesenchymal stem cells, (Strizzi et al. 2005; Lee et al. 2014). These findings suggest that netrin-1 may regulate AJs to direct branch initiation of mammary ducts in much the same way that it directs cell–matrix adhesions during axonal pathfinding.

Junctional stability at AJs depends on F-actin nucleation machinery to maintain the apical actin ring for actomyosin contractility linked to E-cadherin-mediated, cell–cell adhesion (Gumbiner 2005; Behrndt et al. 2012; Maître et al. 2012). Recent findings have identified neogenin as a key regulatory component of AJs within epithelial cells. siRNA-mediated depletion of neogenin in epithelial cells did not impede the assembly of AJs; however, it altered cadherin recycling on the plasma membrane, thereby influencing AJ stability (Fig. 3B). Neogenin also recruits the WAVE regulatory complex (WRC) and activates the actin related protein 2/3 complex (Arp2/3) (Lee et al. 2016). The WRC comprises five subunits organized into the Sra/Nap and WAVE/Abi/HSPC300 subcomplexes (Chen et al. 2010; Verma et al. 2012). Neogenin directly interacts with, and recruits, the WRC to AJs via a WRC interacting receptor sequence (WIRS) present in its cytoplasmic domain (Lee et al. 2016). This interaction requires Rac activation downstream from RGMa-mediated engagement of neogenin and leads to Arp2/3 activation at AJs that modulates tension through peri-junctional nucleation of F-actin (Lee et al. 2016). These findings identify neogenin as a critical factor that regulates the maintenance, integrity, and stability of AJs. As WIRS domains have also been identified in the netrin receptors DCC and UNC5D, and the Slit receptor Roundabout (Robo), this suggests a common mechanism downstream from receptors for guidance cues in AJ regulation (Chen et al. 2014).

Netrin-4 is a component of vascular basal lamina and inhibits angiogenesis through coordinated signaling via neogenin and Unc5B (Larrivee et al. 2007; Lejmi et al. 2008). Unc5B, expressed on endothelial tip cells, restricts sprouting and Unc5B null mice die during embryogenesis owing to vascular defects (Lu et al. 2004). Interestingly, Unc5B can also be activated by the slit family receptor Robo4 to inhibit angiogenesis (Fig. 3A) (Koch et al. 2011). Robo4 acts to stabilize vasculature through the regulation of VE-cadherin presentation at the

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cell surface (see slit section below) (Jones et al. 2009; London and Li 2011). These data suggest cross-talk between netrin and slit guidance pathways to influence vascular physiology by regulating endothelial cadherin-based junctions.

In *Drosophila melanogaster*, the netrin homolog NetA promotes the endocytosis and degradation of the DCC homolog frazzled to enhance epithelial dissociation during *Drosophila* wing eversion. Frazzled inhibits eversion via the ERM-family protein moesin. During eversion, peripodial epithelial cells of the wing lose AJs and apico-basal polarity, taking on an invasive, migratory phenotype, similar to EMT. Loss of netrin expression inhibits AJ dissolution and results in the formation of holes in the peripodial epithelium (Manhire-Heath et al. 2013). Loss of frazzled also leads to an invasive, migratory phenotype of eye-antennal disc cells associated with increased levels of phospho-ERK and MMP-1 as well as changes in cadherin expression (VanZomeren-Dohm et al. 2011). These findings suggest that frazzled maintains AJs and cell polarity to ensure epithelial junctional stability.

Netrins and Tight Junctions

Emerging roles for netrins regulating TJs comes from studies of the vascular and lymphatic systems in which these junctions are at the crux of physiology and disease (Fig. 3C). For example, netrins have been implicated in the regulation of blood–brain barrier (BBB) integrity by up-regulating endothelial junctional proteins (Podjaski et al. 2015). The BBB regulates the passage of molecules and cells between the blood and the central nervous system and may become compromised during cases of inflammatory brain pathology such as multiple sclerosis and stroke (Unterberg et al. 2004; Kebir et al. 2007). Astrocytic end-feet wrap the basolateral surface of the brain endothelium providing factors that promote barrier integrity, such as angiotensin and sonic hedgehog (SHH) (Wosik et al. 2007; Alvarez et al. 2011a,b). Netrin-1 and netrin-4 appear to reduce pathology through actions on endothelial proliferation, vascular branching, and vessel density (Wilson et al. 2006; Hoang et al. 2009). Targeted deletion of the SHH receptor SMO from endothelial cells decreases netrin-1 expression, and in this context, netrin-1, through its receptor neogenin, mediates SHH-dependent signaling to maintain barrier integrity (Podjaski et al. 2015). In fact, netrin-1 levels in serum were more than twofold higher in patients with multiple sclerosis compared to controls, suggesting that netrin-1 expression increases with neuroinflammation. Netrin-1 acts on endothelial cells to reduce diffusion across the BBB by up-regulating TJ proteins F11R, occludin, claudin-5, TJP1, CTNND1, and α-catenin. Furthermore, these proteins were recruited to lipid raft microdomains within human brain-derived endothelial cells, suggesting that netrin-1 contributes to the proper targeting of these key junctional constituents (Podjaski et al. 2015). Together, these data identify netrin-1 as a potent regulator of endothelial cell–cell adhesion that may be acting through neogenin-dependent mechanisms similar to those engaged in epithelia.

Netrin-4 also contributes to the maintenance of TJs. In the lymphatic system, netrin-4 increases lymphatic transendothelial permeability by down-regulating ZO-1 and VE-cadherin at the surface of endothelial cells (Larrieu-Lahargue et al. 2010). Initial siRNA suppression studies suggested that this might be independent of UNC5B and neogenin, both netrin-4 receptors that are implicated in vascular maintenance (Larrieu-Lahargue et al. 2010). The same group later identified α6β1 integrin as a key receptor for netrin-4 in lymphatic endothelium (Larrieu-Lahargue et al. 2011). These data suggest a role for netrins in the regulation of TJs that govern endothelial cell–cell permeability and integrity within the lymphatic system.

Netrins and Gap Junctions

A role for netrins and their receptors in the regulation of gap junctions has yet to be identified outside of the nervous system. However, a recent study in *Drosophila* showed a crucial role for netrin/frazzled signaling in the presynaptic expression and localization of the invertebrate gap junction proteins, innexins (Fig. 3D) (Orr et al. 2014). Both netrin and frazzled loss-of-function

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mutants displayed defects in synaptic transmission, dye coupling, and gap junction localization at a giant interneuron: the "jump" motor neuron synaptic connection (Orr et al. 2014). This raises the possibility that netrins and their receptors may regulate gap junctions in the mammalian nervous system and in other organs. Indeed, microtubules, tethered at AJs via a mechanism dependent on microtubule plus-end-tracking protein EB1, its interactor p150 (Glued), and cadherin–catenin complex interactions, regulate the targeting of vesicles containing connexin 43 for gap junction assembly (Shaw et al. 2007). It is therefore plausible that netrin receptor regulation of cadherins may have consequences for connexin hemichannel targeting to sites of cell–cell adhesion.

Future Directions: Netrins, Junctions, and the Hippo-YAP Pathway

The Hippo-YAP pathway controls cell proliferation through contact inhibition and regulates cell–cell junctions by establishing apical cell polarity (Genevet and Tapon 2011; Gumbiner and Kim 2014). In turn, intercellular junctions act as signaling hubs that sense the physical organization of cells and modulate the activity of the Hippo-YAP pathway to control organ size and cancer development (Gumbiner and Kim 2014). For example, the AJ component α-catenin tethers phosphorylated-Yes-associated protein (YAP), a transcriptional coactivator, to cell–cell contacts through the adaptor protein 14-3-3 (Schlegelmilch et al. 2011). Recently, the oncogenic effects of netrin-1 have been attributed to YAP regulation. In prostate cancer cells, hypoxia increases netrin-1 expression, leading to dephosphorylation and nuclear accumulation of YAP (Chen et al. 2016b). Netrin-1 treatment in other carcinoma cell lines also resulted in YAP dephosphorylation, a process found to be mediated by DCC and UNC5B recruitment of protein phosphatase 1A (PP1A) (Qi et al. 2015). As netrins and their receptors modulate many aspects of intercellular junctions, it will be interesting to see to what extent netrin signaling at cell–cell junctions influences Hippo-YAP pathway activity, and vice versa.

SLITS AND CELL–CELL JUNCTIONS

Like other guidance cues, Slits and their receptors, the Robo family of proteins, function in many cellular processes outside of the nervous system (Kidd et al. 1998). Diverse roles for Slits and Robos have now been established in a variety of developmental processes, including vascularization and organogenesis of the kidney, mammary gland, lung, and heart (Grieshammer et al. 2004; Hinck 2004; Medioni et al. 2010; Ye et al. 2010; Macias et al. 2011). Slit-Robo interactions have also been identified in numerous pathologies, including inflammatory responses, epithelial tumorigenesis, and tumor angiogenesis (Wu et al. 2001; Xian et al. 2001; Wang et al. 2003; London et al. 2010; Ye et al. 2010; London and Li 2011). In these different contexts, definitive roles for these proteins in the regulation of cell–cell junctions have begun to be elucidated; however, as there is little data to support a role for slits in the regulation of gap junctions, only adherens and TJs will be discussed.

Slits and Their Receptors

Slit was first identified in *Drosophila* nearly three decades ago, followed by the subsequent identification of homologs in species from *Caenorhabditis elegans* to human (Fig. 4A) (Nusslein-Volhard et al. 1984; Rothberg et al. 1988; Itoh et al. 1998; Hao et al. 2001). A single Slit homolog is expressed in worms and flies. Three *Slit* genes are present in mammals and expressed widely in neural and nonneural tissues (Dickson and Gilestro 2006). Slits are secreted, ~200 kDa extracellular matrix proteins, composed of four leucine-rich repeat (LRR) domains (D1–D4), seven to nine EGF repeats, an agrin-perlecan-laminin-slit (ALPS)/laminin-G-like domain, and a carboxy-terminal cysteine-rich module. Slits are proteolytically cleaved within the fifth EGF region to form an amino-terminal fragment that binds Robos and mediates all assayed cell guidance functions of Slit/Robo signaling (Nguyen Ba-Charvet et al. 2001). The carboxy-terminal fragment of Slit also functions in axon guidance, but its effects are linked to the sema-

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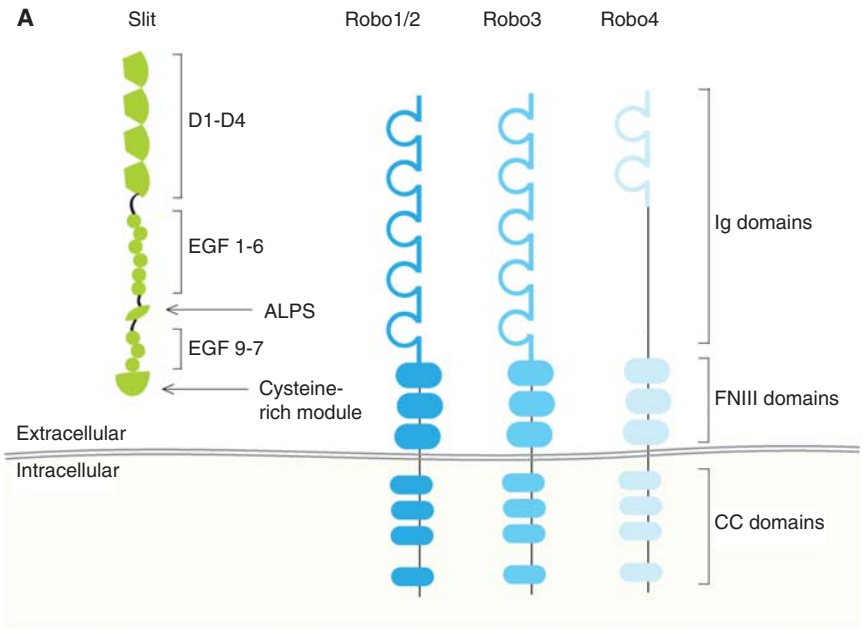

Figure 4. Schematic of slit-robo structure and signaling at cell–cell junctions. (*Figure continues on following page.*)

phorin receptor, PlexinA1 (Nguyen Ba-Charvet et al. 2001; Delloye-Bourgeois et al. 2015).

There is one member of the conserved Robo gene family in *C. elegans* (Sax-3), three in *Drosophila* and chicken (Robo1-3), and four in zebrafish and mammals (Robo1-4) (Brose et al. 1999; Park et al. 2003a). They are single-pass type 1 transmembrane proteins (Fig. 4A). The extracellular domains of Robos 1-3 are composed of five Ig-like domains, three fibronectin (FN) repeats, and a transmembrane domain followed by four "cytoplasmic conserved" (CC0-3) domains (Dickson and Gilestro 2006). The intracellular domain lacks intrinsic enzymatic activity, but recruits adaptor and signaling proteins to initiate intracellular signaling. Alternative splicing and ectodomain shedding further contribute to Robo protein diversity (Chen et al. 2008b; Coleman et al. 2010). Heparan and chondroitin sulfate proteoglycans concentrate and localize slits to regulate their signaling, with the proteoglycan syndecan considered as a Robo coreceptor (Johnson et al. 2004; Steigemann et al. 2004; Rhiner et al. 2005).

The unconventional Robo4 is required for angiogenesis and was thought to be specific to endothelial cells (Huminiecki et al. 2002; Bedell et al. 2005), until a recent finding outlined a role regulating neuronal migration in the developing neocortex (Zheng et al. 2012). Robo4 contains only two extracellular Ig and FN domains and two CC domains intracellularly (Huminiecki et al. 2002). It does not bind to Slits directly (Suchting et al. 2005; Morlot et al. 2007), but can be found in a complex with Slit2 and Robo1 (Park et al. 2003b; Sheldon et al. 2009; Zhang et al. 2009a). Slit2/Robo4 signaling restricts angiogenesis by down-regulating vascular endothelial growth factor (VEGF) signaling in the mature vasculature. This occurs during periods of robust sprouting angiogenesis, for example, when blood vessels encapsulate every alveolus during pregnancy in the mammary gland, and during pathological neovascular processes (Jones et al. 2008, 2009; Huang et al. 2009; Han and Zhang 2010; Marlow et al. 2010; Koch et al. 2011; London and Li 2011; Mulik et al. 2011). More recently, studies have shown that Robo4 can also function independently of its cytoplasmic domain, as a ligand for UNC5B, to inhibit angiogenesis and vessel permeability

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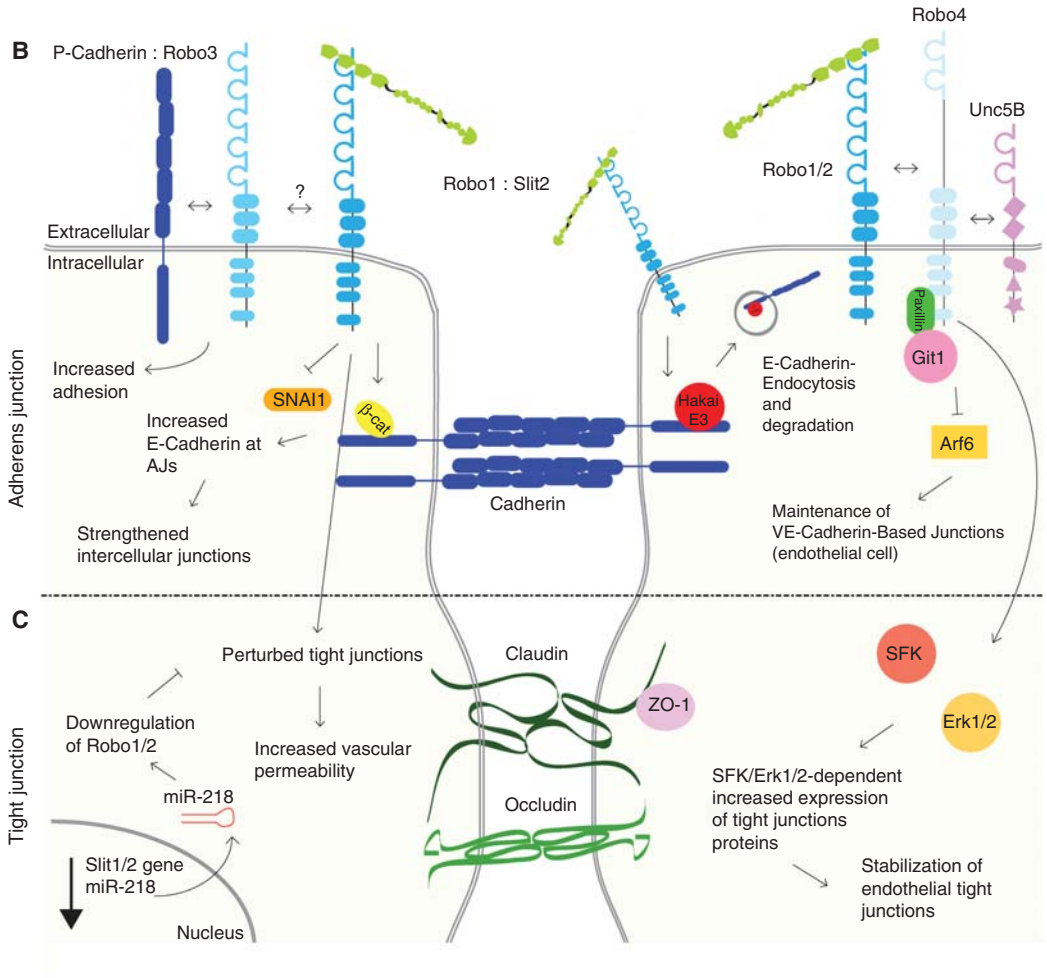

Figure 4. (*Continued*)

by reducing VEGF-R activation (Koch et al. 2011; Zhang et al. 2016).

Slit-Robo Regulation of Adherens Junctions

Like other axon guidance cues, Slits and Robos contribute to both homeostatic maintenance and pathological dysregulation of AJs. Again, much of our understanding of these processes comes from cancer cell biology, in which chromosomal mutations at regions encoding axon guidance proteins have been correlated with levels of metastasis and poor prognosis (Tseng et al. 2010). Hypermethylation or deletion of the *Slit2* gene, for example, occurs in a variety of cancers and this appears to contribute to cancer progression (Dallol et al. 2003; Astuti et al. 2004; Narayan et al. 2006). *Slit1* and *Slit3* epigenetic inactivation has also been reported in human cancers (Dickinson et al. 2004).

Investigating mechanisms that contribute to tumor cell growth and metastasis, recent studies have found a link between Slit/Robo function and the regulation of cadherin-based junctions (Fig. 4B). Possibly acting via an autocrine mechanism, Slit2 signals through Robo1 to increase both β-catenin stability and E-cadherin expression via PI3-kinase. This enhances the colocalization of β-catenin and E-cadherin, strengthens intercellular adhesions, and increases junctional

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stability, reducing both the proliferation and migration of Slit2-overexpressing breast cancer cells (Prasad et al. 2008). A subsequent study on nonsmall cell lung cancer cells showed the opposite effect, with down-regulation of Slit2 boosting cancer progression by increasing nuclear β-catenin and enhancing the activity of the transcriptional repressor SNAI1 (Tseng et al. 2010), which regulates EMT in cancer by down-regulating E-cadherin expression (Yook et al. 2005). Interestingly, the Slit2-Robo1-β-catenin signaling axis plays a role in breast branching morphogenesis by regulating the proliferation of progenitor cells in the mammary end bud (Macias et al. 2011). The Slit2-Robo1-SNAI1 signaling axis also plays a role in breast development by governing somatic stem cell maintenance through inscuteable, a protein that controls spindle pole orientation (Ballard et al. 2015).

In a surprising twist to Slit-Robo signaling in AJ regulation, up-regulation of Slit2 in some cancers promotes malignant transformation through E-cadherin degradation (Zhou et al. 2011). In this context, Slit2 treatment of Robo1-expressing colorectal epithelial carcinoma cells recruits the ubiquitin ligase Hakai for E-cadherin ubiquitination and lysosomal degradation, promoting EMT, tumor growth, and metastasis (Fig. 4B) (Zhou et al. 2011). In flies, Slit/Robo signaling inhibits the formation of cadherin-mediated cell–cell junctions during heart development (Santiago-Martinez et al. 2008). An interesting recent study in *Drosophila* provides evidence that Slit/Robo repellent signaling through JNK extrudes tumorigenic cells from epithelia by disrupting E-cadherin function (Vaughen and Igaki 2016). The investigators found that overexpression of Slit/Robo2 results in cells being dislocated from the epithelial surface, resulting in tumor formation within the luminal space. In contrast, loss of Slit/Robo2 signaling prevents cell extrusion, leading to tumor growth within the epithelium (Vaughen and Igaki 2016). In this way, the investigators propose that both over- and underactivation of Slit/Robo2 signaling contributes to tumorigenesis and cancer progression.

Mediators of Slit/Robo signaling are implicated in the formation of AJs. Studies in *C. elegans* show a role for Slit/Robo GTPase-activating proteins (srGAPs) in the formation of adherens-like junctions during early embryonic morphogenesis (Wong et al. 2001; Zaidel-Bar et al. 2010). The only worm homolog of mammalian srGAPs, SRGP-1, contributes to membrane dynamics at nascent cell–cell contacts and the formation of new AJs during gastrulation. This localization to, and regulation of, new AJs occurs independently of the cadherin homolog, HMR-1 (Zaidel-Bar et al. 2010). This raises the possibility that Slit-Robo signaling through mammalian srGAPs may contribute to AJ formation during embryogenesis in a cadherin-independent manner.

During neural development, a well-characterized macromolecular complex containing Robo and N-cadherin integrates guidance and adhesion information (Rhee et al. 2007), suggesting that such a complex may also function in nonneural tissue. Indeed, in oral epithelial cells that can give rise to oral squamous cell carcinoma, the divergent Robo family member, Robo3, forms a Slit2-induced complex with P-cadherin to regulate migration and intercellular adhesion (Bauer et al. 2011). Function blocking antibodies against P-cadherin reduced the secretion of Slit2 by oral epithelia, as well as reduced P-cadherin/Robo3 complex formation (Bauer et al. 2011). Complicating matters, a recent study investigating the divergent roles of Robo3 compared to other Robo family members, revealed that Robo3 does not bind tightly to Slit proteins (Zelina et al. 2014). Instead, the secreted factor NELL2 is the high-affinity ligand for Robo3 (Jaworski et al. 2015). This suggests that perhaps other Robo family members expressed by oral epithelia contribute to P-cadherin-mediated secretion of Slit2, and to the modulation of Robo3/P-cadherin interactions, presenting an avenue for further studies of Robo cross-talk and regulation.

Slit-Robo signaling in endothelia is bifunctional. Robo1 and Robo2 have pro-angiogenic effects (Wang et al. 2003; Rama et al. 2015). On the other hand, Robo4 stabilizes the vasculature and decreases transendothelial permeability, and the molecular mechanisms underlying these effects have been well described

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(Fig. 4B) (Jones et al. 2008, 2009; London and Li 2011; Cai et al. 2015; Zhang et al. 2016). Slit enhances vascular barrier function by increasing the amount of cadherin presented on the endothelial cell surface (London and Li 2011). Slit activation of Robo4 results in a direct interaction between Robo4 and the intracellular adaptor paxillin (Jones et al. 2009). This Robo4-paxillin complex then recruits the Arf-GAP GIT1 to block activation of the small GTPase Arf6, which regulates the cell surface localization of cadherin (Jones et al. 2009; London and Li 2011). A Slit2/Robo4-paxilllin-GIT1 network, therefore, regulates cadherin presentation to inhibit the cellular protrusive activity that underlies neovascularization and vascular leak.

In line with its stabilizing effects on the vasculature, anti-angiogenic effects of Robo4 have also been shown in vitro and in vivo (Suchting et al. 2005). Interestingly, this effect was recapitulated using a soluble form of the extracellular portion of Robo4 (Robo4-fc) (Suchting et al. 2005). This suggests that Robo4 interacts with another cell surface molecule to regulate endothelial cell function, and there is evidence that Robo4 signals through the netrin receptor Unc5B (Koch et al. 2011; Zhang et al. 2016). A report that Robo1 and Robo4 can form a heterodimeric complex in vitro suggests that this binding partner could also be another Robo (Sheldon et al. 2009; Chen et al. 2016a). Robo4 function may also be influenced by endothelial expression of heparan sulfate proteoglycans such as a syndecan, which are established coreceptors for other Robos during axon guidance (Hu 2001; Steigemann et al. 2004). Indeed, inhibition of syndecan-2 on endothelial cells impairs angiogenesis (Noguer et al. 2009; Ballard and Hinck 2012).

Slit-Robo Regulation of Tight Junctions: A Focus on miR Regulatory Axes

Although direct evidence is scarce, a role for Slit signaling in TJ function is suggested by recent insights into micro-RNA (miR) regulation of angiogenesis. A bonafide target of numerous well-studied miRs, Slit/miR/Robo regulatory axes have been shown in a variety of contexts. During angiogenesis, a process that requires dynamic regulation of TJs and associated proteins to regulate permeability across endothelial walls, Slit/Robo interactions are subject to microRNA-based control. Slit2 enhances angiogenesis via Robo1 and Robo2 in postnatal mouse retina (Rama et al. 2015), by promoting endothelia cell migration (Rama et al. 2015). The highly conserved miR-218 was implicated in regulating Slit/Robo signaling in neovascularization, because of its previously identified role during nasopharyngeal cancer progression (Alajez et al. 2011) and during cardiac morphogenesis (Fig. 4C) (Fish et al. 2011). Encoded in the introns of *Slit1* and *Slit2*, miR-218 negatively regulates *Robo1* and *Robo2* expression (Fish et al. 2011). In a model of oxygen-induced retinal neovascularization, miR-218 expression down-regulated *Robo1* levels to inhibit retinal angiogenesis (Han et al. 2016). This Slit/miR-218/Robo axis has since been implicated in the inhibition of tumor angiogenesis in gastric cancer (Xiangyuan et al. 2017), and glioma cell tumorigenesis and proliferation (Gu et al. 2016).

Mice overexpressing human *Slit2* show increased endothelial permeability because of the disruption of TJs (Han and Geng 2011). Similar disruption of BBB permeability in Slit2-overexpressing mice suggests that the mechanisms used by Slit2 signaling through Robo1/2 to promote angiogenesis also affect the maintenance of TJs and endothelial cell *trans*-permeability (Li et al. 2015). How Slit2/Robo1/2 signaling may influence the localization and function of TJ proteins, however, remains to be determined.

In accordance with its role in vascular stability, Robo4 regulates proteins involved in the formation and maintenance of endothelial TJs. In a blood/tumor barrier model, as well as within the retina, Robo4 regulates the expression of TJ proteins ZO-1, occludin, and claudin-5 by endothelial cells (Fig. 4C) (Cheng et al. 2013b; Cai et al. 2015). This is dependent on SFK and ERK1/2 activation downstream fromRobo4 (Cai et al. 2015). Targeting Robo4 with short hairpin RNA also leads to increased MMP-9 activity to increase vascular permeability, whereas pretreatment with an MMP-9 inhibitor partially rescues the effect (Cai et al. 2015). Presumably,

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this is the result of MMP-9 degrading extracellular matrix components that support TJs (Fukuda et al. 2004), and degrading TJ proteins themselves (Vermeer et al. 2009). Interestingly, biomechanical studies indicate that changes in extracellular matrix stiffness down-regulate miR-203 in epithelial cells and increase *Robo1* expression (Le et al. 2016). It will be of interest to see whether similar feedback mechanisms may contribute to the control of endothelial TJs by Robo4 in a Robo4/MMP-9/miR regulatory axis that may sense changes in extracellular matrix (ECM) around TJs to feedback onto expression of Robo4 itself.

CONCLUSION

The guidance of axonal growth cones requires finely tuned cytoskeletal reorganization, dynamic regulation of adhesive contacts, and the formation of stable intercellular adhesions on reaching their synaptic partners. Indeed, guidance cues play integral roles in synaptogenesis and synaptic plasticity after connections have been established. Because the synapse can be thought of as a specialized AJ, it follows that these guidance cues are well positioned to influence cell–cell contacts outside of the nervous system. It is now clear that ligand–receptor pairs, critically involved in axon guidance, also direct the formation of many tissues in the body through actions on intercellular junctions. As the mechanisms that regulate both the physiology and pathophysiology of cell–cell junctions continue to be unraveled, a focus on the interplay of guidance cues regulating sites of cell–cell contact will provide key insight into the intricacies of these densely organized protein complexes.

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Ian V. Beamish, Lindsay Hinck and Timothy E. Kennedy

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