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Roles of Slit Ligands and Their Roundabout (Robo) Family of Receptors in Bone Remodeling

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Abstract

Slit guidance ligands (Slits) and their roundabout (Robo) family of receptors are well-known axon guidance molecules that were originally identified in *Drosophila* mutants with commissural axon pathfinding defects. However, Slit-Robo signaling has been shown to be involved in not only neurogenesis, but also the development of other organs such as the kidney and heart. Recently, it was also revealed that Slit-Robo signaling plays an important role in bone metabolism. For example, osteoclast-derived Slit3 plays an osteoprotective role by synchronously stimulating bone formation by osteoblasts and suppressing bone resorption by osteoclasts through Robo receptors expressed on osteoblastic and osteoclastic cell lineages, making it a potential therapeutic target for metabolic bone disorders. Furthermore, osteoblast-derived Slit3 promotes bone formation indirectly as a proangiogenic factor. This review summarizes the recent progress on defining the roles of the Slit-Robo signaling in bone metabolism, and discusses the possible roles of the interaction between Robo and neural epidermal growth factor-like (NEL)-like (NELL) proteins that are novel ligands for Robo receptors.

Keywords

Clastokine · Coupling factor · NELL1 · Osteoblast · Osteoclast

Abbreviations

BMP	bone morphogenetic protein
EGFL	epidermal growth factor-like
FNIII	fibronectin type III
GTPase	guanosine triphosphatase
Ig	immunoglobulin
NELL	neural EGFL (NEL)-like
RANK	receptor activator of nuclear factor-κB
RANKL	RANK ligand
Robo	roundabout
Sema	semaphorin
Slit	Slit guidance ligand

1 Introduction

Bone locally repeats ossification and bone resorption for a lifetime to build skeletal structures without changing its morphology and serves as a calcium reservoir to maintain calcium homeostasis. These turnover processes are called bone remodeling (Zaidi 2007). Bone remodeling is

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carried out by temporary structures known as the basic multicellular units (BMUs) that are composed of two main cell types: osteoclasts and osteoblasts (Sims and Martin 2015, 2020; Kenkre and Bassett 2018). The bone remodeling cycle begins with the activation of osteoclastic bone resorption. After the resorption phase is completed, osteoclasts disappear and osteoblast precursors are recruited to the bone surface, and this phase is called the reversal phase. The formation phase follows with osteoblasts laying down bone matrix until the resorbed bone is completely replaced by new bone. The same amount of bone that was resorbed by osteoclasts must be newly formed to maintain the balance between osteoclastic bone resorption and osteoblastic bone formation. A local coupling factor linking bone resorption to subsequent bone formation has long been proposed to explain the coordination of bone resorption and formation in BMUs (Sims and Martin 2020; Martin and Sims 2005; Kim and Koh 2019). Several coupling factors have been identified, including those derived from the bone matrix, such as transforming growth factor-β1 (TGF-β1) (Tang et al. 2009) and insulin-like growth factor 1 (IGF-1) (Xian et al. 2012), and those secreted by osteoclasts, which act on osteoblasts, such as cardiotrophin-1 (Walker et al. 2008), sphingosine-1-phosphate (S1P) (Ryu et al. 2006), collagen triple helix repeat containing 1 (CTHRC1) (Takeshita et al. 2013) and complement factor 3a (Matsuoka et al. 2014).

In contrast to these coupling factors that act on osteoblasts, semaphorin (Sema) 3A, which belongs to the semaphorin family of axon guidance molecules, is known as a dual-acting factor that acts on both osteoblasts and osteoclasts to control their differentiation (Hayashi et al. 2012). Sema3A is a secreted protein that binds to plexin A/neuropilin-1 receptor complexes to suppress osteoclast differentiation and promote osteoblast differentiation synchronously. Although Sema3A plays an osteoprotective role, it is not produced by osteoclasts, but by osteoblasts and nerves. Interestingly, axon guidance molecules other than Sema3A have also been shown to be involved in bone remodeling. Sema4D, which is a transmembrane-type semaphorin family member, is produced by osteoclasts to suppress osteoblast maturation by binding to the plexin B1 receptor on osteoblasts (i.e., a "negative coupling factor") (Negishi-Koga et al. 2011). Netrin-1 has been reported to both promote and inhibit osteoclastogenesis through binding to the UNC5B receptor on osteoclasts (Mediero et al. 2015; Maruyama et al. 2016). These findings raise the question of whether Slit-Robo signaling is also involved in bone remodeling.

Slit ligands and their Robo family of receptors were originally identified as axon guidance molecules in *Drosophila* mutants that show abnormal projections of commissure neurons in the central nervous system (Rothberg et al. 1988; Seeger et al. 1993). However, Slit-Robo-mediated signaling is not limited to neural development, but is also involved in the development of various organs such as the kidney and heart (Lu et al. 2007; Wainwright et al. 2015; Zhao and Mommersteeg 2018). In addition, Slit-Robo signaling is involved in the regulation of angiogenesis, cancer development, cell migration, and cell proliferation (Dai et al. 2019; Gara et al. 2015; Blockus and Chedotal 2016). Recently, it was revealed that Slit-Robo signaling plays an important role in bone metabolism (Kim et al. 2018a; Xu et al. 2018). In this review, I discuss the roles of Slit-Robo signaling in bone remodeling and future research directions.

2 Slit Ligands and Robo Receptors: Structure and Interactions

In mammals, three *Slit* genes (*Slit1–3*) have been identified (Fig. 1) (Li et al. 1999). All *Slit* genes encode large (~200 kDa) secreted glycoproteins that comprise, from their N- to C-terminus, four leucine-rich repeats (LRRs), six EGFL repeats, a laminin G (LG) domain, a further three EGFL repeats, and a C-terminal cysteine knot-like domain. Slit proteins can be cleaved into large N-terminal (~140 kDa; Slit-N) and short C-terminal (~60 kDa; Slit-C) fragments at the putative proteolytic site between the fifth and sixth EGFL repeats (Brose et al. 1999). Both the

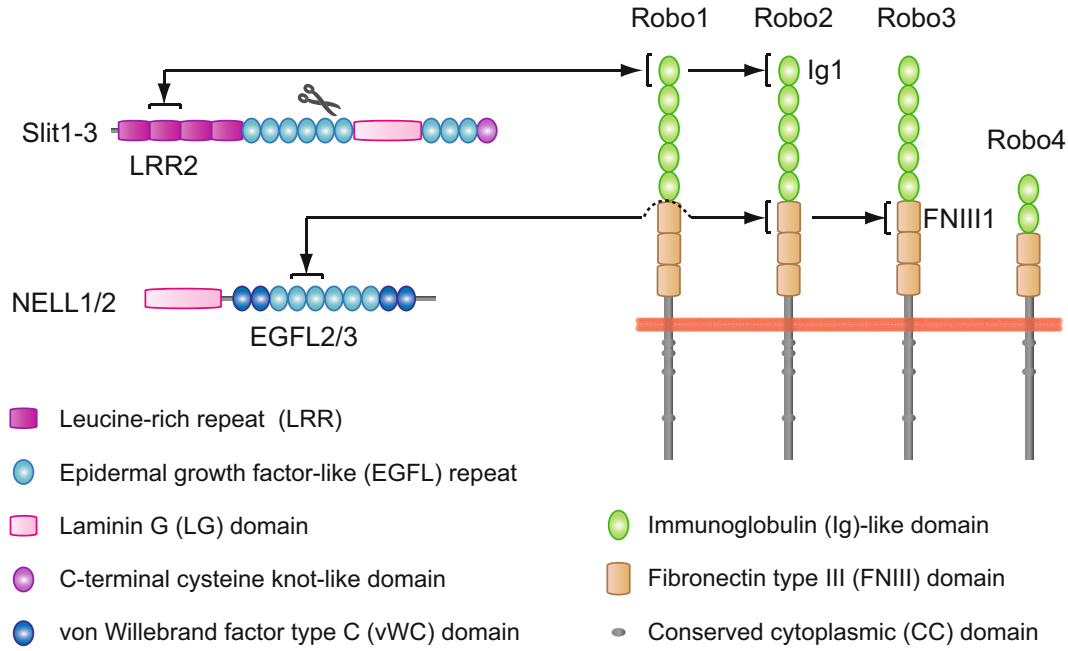

Fig. 1 Structures of the Slit, Robo, and NELL protein families. Slit ligands bind to the Robo1/2 Ig1 domain through their LRR2 domain, whereas NELL1/2 bind to the Robo2/3 FNIII1 domain through their EGFL2/3 domains. Slit proteins are proteolytically cleaved between EGFL5 and EGFL6 domains to create N- (Slit-N) and C-terminal (Slit-C) fragments. Both fragments bind to different receptors. A direct interaction between Slit/NELL ligands and Robo4 has not been demonstrated. The intracellular domains of Robo have several short conserved motifs that are important for Robo signaling

uncleaved and cleaved Slit proteins induce chemorepulsion by binding to Robo receptors through their second LRR within Slit-N or by binding to plexin A1 receptor for Slit-C (Delloye-Bourgeois et al. 2015).

The first *Robo* gene was named after the phenotype of the *Drosophila* mutant in which commissural axons inappropriately cross the midline many times in the manner of a roundabout (Seeger et al. 1993). *Robo* was found to encode a protein that is a single-pass transmembrane receptor for Slit ligands (Brose et al. 1999). Four *Robo* genes (*Robo1–4*) have been identified in mammals (Fig. 1) (Kidd et al. 1998; Huminiecki et al. 2002). The extracellular domains of Robo1–3 have five immunoglobulin (Ig)-like domains and three fibronectin type III (FNIII) domains, whereas Robo4, an endothelial-specific member of the Robo family, has only two Ig domains and two FNIII domains in its extracellular domain. The intracellular domain of Robo is poorly conserved, but there are four short conserved cytoplasmic (CC) motifs (CC0–3). Because the intracellular domain has no catalytic activity, various intracellular signaling molecules that bind to these CC motifs mediate the signal. Both Robo1 and Robo2 contain the binding site for Slit proteins in their first Ig domain (Morlot et al. 2007). However, Robo3 has lost the ability to bind Slit proteins because of a few amino acid changes in its first Ig domain (Zelina et al. 2014). Instead, Robo3 interacts with neural EGFL (NEL)-like protein 2 (NELL2) to induce chemorepulsion (Jaworski et al. 2015). Robo4 has been suggested to transduce Slit2/3 signaling in endothelial cells, but a direct interaction between Robo4 and Slit2/3 has not been demonstrated so far (Dai et al. 2019). Transmembrane heparan sulfate proteoglycans (HSPGs), such as syndecan and glypican, bind to both Slit and Robo via the heparan sulfate moiety, forming a ternary complex and stabilizing their interaction

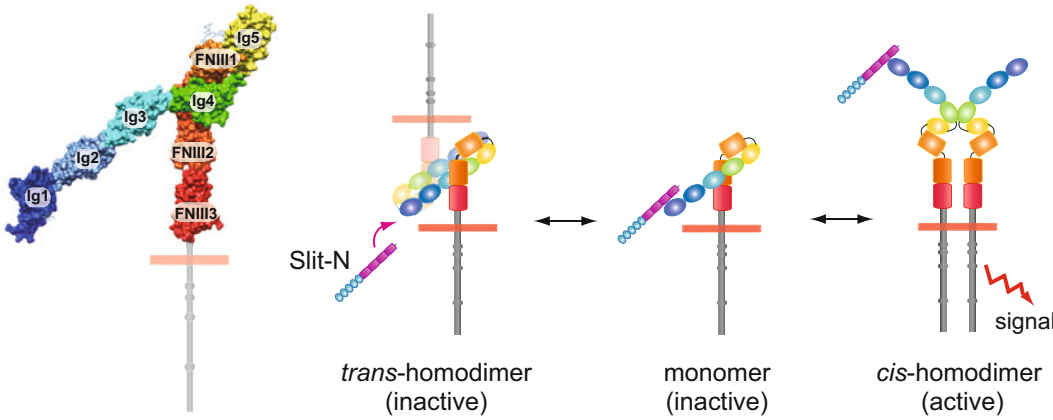

Fig. 2 Hairpin-like architecture of the Robo2 ectodomain and a model of Robo2 activation. (*Left*) Crystal structure of the human Robo2 ectodomain in surface representation (PDB ID: 6IAA). The nomenclature of Robo extracellular Ig and FNIII domains is shown on each domain structure. (*Right*) Model of Slit-induced Robo2 activation. *Trans*-interacting Robo2 homodimers are tightly auto-inhibited. Both *trans*-interacting Robo2 homodimers and monomers are inactive. Slit-N binding to the Robo2 Ig1 domain induces dissociation from *trans*-homodimers to monomers and facilitates formation of Ig4-mediated *cis*-homodimers following activation of the intracellular signals

at cell surfaces (Hussain et al. 2006). Robo receptors interact with other membrane receptors, such as deleted in colorectal carcinoma (DCC) and C-X-C motif chemokine receptor 4 (Cxcr4), to elicit their functions via shared downstream partners, including small guanosine triphosphatases (GTPases), cytoplasmic kinases, and adaptor molecules (Stein and Tessier-Lavigne 2001; Wu et al. 2001).

Robo1/2 form homo- and heterodimers in *cis*- and *trans*-positions (Fig. 2) (Hivert et al. 2002; Zakrys et al. 2014). Crystal structure and biochemical analyses have revealed that *cis*-homodimers form via the fourth Ig domain (Ig4) of Robo (Seiradake et al. 2009; Yom-Tov et al. 2017), whereas *trans*-homodimers are formed by head-to-head interactions between Ig1 and Ig5 of Robo on adjacent cell surfaces (Barak et al. 2019). Barak et al. recently demonstrated that the extracellular domain of Robo2 has a hairpin-like architecture as a monomer, which is unable to form *cis*-homodimers, but *trans*-homodimers because the Ig4-mediated dimerization interface is intramolecularly blocked by the FNIII domains (Barak et al. 2019). Because *cis*-dimerization is essential for Robo receptor activation, a hairpin-like architecture enables an auto-inhibited conformation of Robo receptors. Furthermore, *trans*-homodimer formation reinforces the auto-inhibition state strongly. It has been suggested that Slit binding to Robo reverses Robo auto-inhibition following by *cis*-dimerization and activation.

3 Roles of Slit-Robo Signaling in Bone Remodeling

Slit1 is minimally expressed in bone cells, whereas *Slit2* is expressed in osteoblastic cell lineages (Sun et al. 2009). *Slit3* is expressed in vascular endothelial cells other than the nervous system and has angiogenic functions (Zhang et al. 2009). In bone, *Slit3* is expressed in both osteoclastic and osteoblastic cell lineages (Kim et al. 2018a; Xu et al. 2018), although the expression levels may vary depending on the status of the cell (discussed later). *Robo1* and *Robo3* are expressed in osteoclastic cell lineages, while *Robo1* and *Robo2* are expressed in osteoblastic cell lineages (Kim et al. 2018a). *Robo4* is hardly expressed in bone cells (Xu et al. 2018). In a previous study, Slit2 was shown to inhibit osteoblast differentiation *in vitro* (Sun et al. 2009).

However, a recent study could not detect the inhibitory effect of Slit2 on osteoblast differentiation, probably because of the difference in methodology using recombinant Slit2 protein instead of conditioned medium containing Slit2 (Park et al. 2019). Accordingly, Slit2 may have no activity in osteoblasts but suppresses osteoclast differentiation through the Robo1 receptor by reducing the migration and fusion of preosteoclasts (Fig. 3a) (Park et al. 2019). Slit2 has been shown to interact with Gremlin, an antagonist of bone morphogenetic proteins (BMPs), to constitute a negative feedback loop where Slit2 blocks Gremlin inhibition of BMP2 and canonical BMP2 signaling downregulates Slit2 expression, although the role in bone remodeling is not presently known (Tumelty et al. 2018).

Recently, two different groups identified Slit3 as a bone anabolic factor. Kim et al. identified Slit3 in conditioned medium of osteoclasts as a coupling factor that coordinates bone resorption and formation (Kim et al. 2018a). They demonstrated that osteoclast-derived Slit3 acts on Robo1/2 receptors expressed by osteoblasts to promote osteoblast migration and proliferation, and also acts on Robo1 expressed by osteoclasts in an autocrine manner to suppress osteoclast differentiation (Fig. 3a). Mice lacking Slit3 specifically in osteoclasts have a low bone mass due to decreased bone formation and increased bone resorption, whereas mice with either neuron-specific Slit3 deletion or osteoblast-specific Slit3 deletion have a normal bone mass. These results indicate that Slit3 is a "clastokine", an osteoclast-derived osteoblast differentiation factor. They

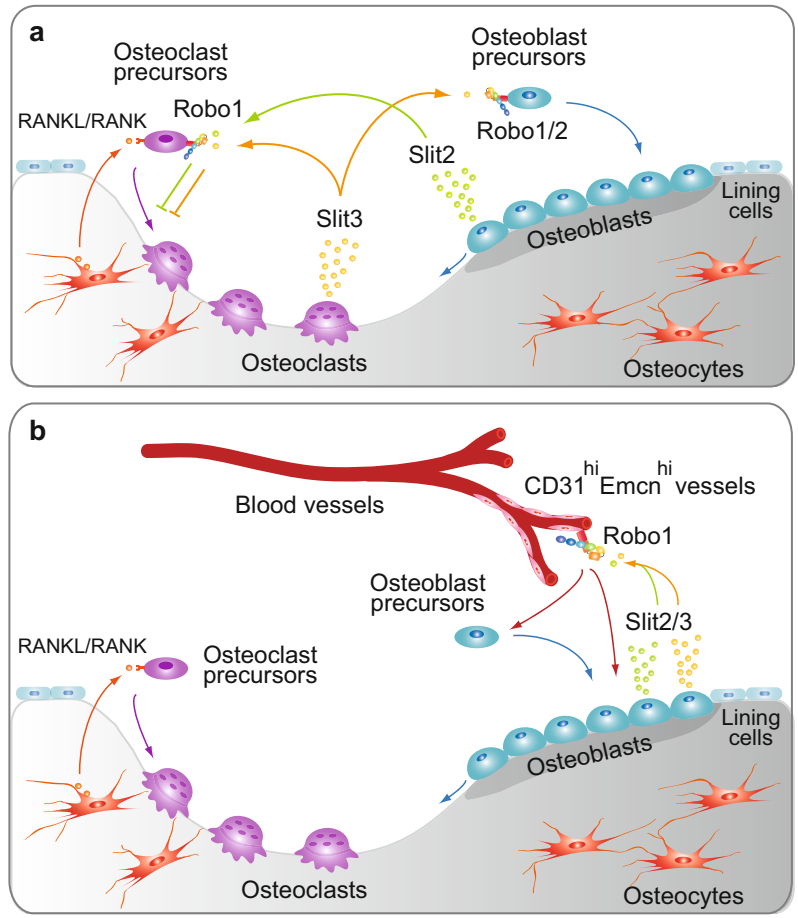

Fig. 3 Roles of Slit-Robo signaling in bone remodeling. (**a**) Slit3 secreted by osteoclasts and Slit2 secreted by osteoblasts suppress osteoclastogenesis through Robo1 binding. In addition, Slit3 promotes osteoblast migration and proliferation through Robo1/2 binding. (**b**) Slit3 secreted by osteoblasts induces $CD31^{hi}Emcn^{hi}$ blood vessels through Robo1 binding to promote osteoblast differentiation indirectly. Slit2 has a similar effect. The signaling protein RANKL secreted by osteocytes activates the RANK receptor on osteoclast precursors to stimulate osteoclastogenesis

also demonstrated that a genetic variant of the *SLIT3* gene was associated with bone parameters in postmenopausal women, and that high circulating SLIT3 levels in plasma are associated with high bone mineral density values. These observations suggest that SLIT3 is a potential biomarker to predict bone health in humans. Finally, administration of a truncated recombinant Slit3 to ovariectomized (OVX) mice significantly rescues bone loss by promoting osteoblastic bone formation and inhibiting osteoclastic bone resorption, indicating that Slit3 can be used as a therapeutic target for osteoporosis.

Shortly after this work, Xu et al. identified Slit3 as a proangiogenic factor that increases $CD31^{hi}endomucin^{hi}$ ($CD31^{hi}Emcn^{hi}$) vascular endothelium (type H vessel) levels, which positively regulate bone formation (Fig. 3b) (Xu et al. 2018). Mice lacking adaptor protein Schnurri-3 (SHN3) specifically in osteoblasts have markedly elevated bone formation with increased levels of Slit3 and $CD31^{hi}Emcn^{hi}$ endothelium, suggesting that Slit3 is an SHN3-controlled and osteoblast-derived regulator of bone formation by coupling between osteoblasts and $CD31^{hi}Emcn^{hi}$ endothelium. However, there are some differences in the results between the two groups, probably due in part to the differences in experimental methods and design. For example, Xu et al. observed robust *Slit3* expression in osteoblasts, but not in osteoclasts (Xu et al. 2018). They did not observe a direct effect of Slit3 on osteoblasts, but observed osteopenic phenotypes in osteoblast-specific *Slit3*-deficient mice with three different cre-deleter strains (Xu et al. 2018). They also demonstrated that Slit3 treatment had only a modest effect on osteoclastogenesis *in vitro* and osteoclast-specific *Slit3*-deficient mice showed normal bone mass (Li et al. 2020). These results indicate that osteoblasts are the major source of skeletal Slit3. However, another group has shown recently that osteoclast-derived Slit3 promotes osteoblast differentiation (Shin et al. 2020). The discrepancy between the two groups might be due in part to differences in tissue-specific cre-deleter strains utilized for generating conditional knockout mice. Although the primary source of skeletal Slit3 and the direct effect on bone cells continue to be debated, it seems certain that Slit3 acts as a potent angiogenic factor through Robo2 binding on endothelial cells to evoke osteoanabolic responses because Kim et al. also observed a decrease in type H vessels in global *Slit3*-deficient mice (Kim et al. 2018a; Peng et al. 2020). Further studies are required to better understand the role of Slit3-Robo signaling in bone remodeling.

4 Downstream of Slit-Robo Signaling in Bone Remodeling

The major signaling molecules downstream of Robo receptors are cytoplasmic kinases. Abelson (Abl) tyrosine kinase associates with the intracellular domain of the Robo receptor (Rhee et al. 2007). Upon binding to Slit ligands, Robo forms a multimolecular complex containing Abl kinase, adaptor protein Cables (Cdk5 and Abl enzyme substrate), N-cadherin, and β-catenin. Abl kinase subsequently phosphorylates β-catenin on tyrosine 489, resulting in the release of cadherin-associated β-catenin and the loss of N-cadherin-mediated cell adhesion. Slit3 has been shown to promote chondrocyte differentiation through the Robo2 receptor by suppression of β-catenin activity, thereby regulating endochondral ossification (Kim et al. 2018b). Interestingly, this is the opposite of the effect on osteoblasts in which Slit3 activates β-catenin to stimulate migration and proliferation (Kim et al. 2018a), indicating that the effect of Slit3 on β-catenin activity depends on the cell type.

The Rho family of small GTPases (Rac, Cdc42, and RhoA) also plays an important role in regulating the downstream responses of Slit-Robo signaling. They are key regulators of cytoskeletal reorganization critical for cell motility. Activation of Slit-Robo signaling recruits Slit-Robo GTPase-activating proteins (srGAPs) to the intracellular domain of the Robo receptor and inactivates Rho GTPases, thereby inhibiting actin polymerization and stress fiber formation (Wong et al. 2001). In Slit2-suppressed osteoclastogenesis, inhibition of Cdc42 GTPase by Slit2 is critical (Park et al. 2019). In contrast, Slit3-suppressed osteoclastogenesis is mediated by

inhibition of Rac1 GTPase (Kim et al. 2018a). A recent study showed that female mice deficient for srGAP2 specifically in myeloid lineages have high levels of Rac1 activation and increased bone formation with enhanced Slit3 production (Shin et al. 2020). A feedback loop in which Rac1 activity stimulates expression of Slit3 has been suggested to exist, which in turn acts to limit Rac1 activation through Robo1 and srGAP2 in osteoclastic cells.

5 NELL Family Proteins: Novel Ligands of Robo2/3 Receptors

Slit ligands have been shown to interact with several receptor proteins other than Robo, such as plexin A1 and dystroglycan (Delloye-Bourgeois et al. 2015; Wright et al. 2012). However, Slit proteins were the only known ligands of Robo receptors until NELL2 was recently identified as a ligand for Robo3 to function as a repulsive axon guidance cue that contributes to commissural axon guidance to the midline (Jaworski et al. 2015). Although NELL1 also binds to Robo3, NELL1 has a weak effect on commissural axon guidance repulsion (Pak et al. 2020). NELL1 and NELL2 constitute a NELL family of large secretory glycoproteins that contain an LG domain, four von Willebrand factor type C (vWC) domains, and six EGFL repeats (Fig. 1) (Zhang et al. 2010). Both *NELL1* and *NELL2* genes are expressed primarily in the brain and have high similarity in their amino acid sequences. However, the biological functions of the proteins they encode are greatly different (Kuroda et al. 1999). NELL2 plays multiple roles in neuronal development, survival, and activity (Aihara et al. 2003), whereas NELL1 mainly functions as a regulator of craniofacial skeletal morphogenesis (Zhang et al. 2010; Li et al. 2019). Most recently, NELL2 was shown to function as a regulator of sperm maturation by binding to an orphan receptor tyrosine kinase, c-ros oncogene 1 (ROS1) (Kiyozumi et al. 2020). The *NELL1* gene was originally identified as a gene overexpressed in human sporadic coronal craniosynostosis characterized by premature cranial suture closure with bone over growth (Ting et al. 1999). Transgenic mice overexpressing NELL1 display a phenotype similar to human craniosynostosis patients (Zhang et al. 2002), whereas *NELL1*-deficient mice have cranial and vertebral skeletal defects (Desai et al. 2006; Zhang et al. 2012). Currently, NELL2 targets Robo3 and ROS1 receptors (Jaworski et al. 2015; Kiyozumi et al. 2020), whereas the receptors targeted by NELL1 include integrin β1, HSPGs and contactin-associated protein-like 4 (Cntnap4) (Shen et al. 2012; Takahashi et al. 2015; Li et al. 2018).

To gain more insight into NELL-Robo interactions, our group examined the binding activity between NELL1/2 and the Robo family of receptors, and found that Robo2 is a potential receptor for NELL1/2 (Yamamoto et al. 2019). When using a fragment of Robo FNIII domains, NELL1/2 bind to the first FNIII domain of Robo2 and Robo3 through their EGFL domains, but not to those of Robo1 and Robo4 (Fig. 1). However, NELL1/2 bind to the whole ectodomain of Robo3, but not to that of Robo2. We hypothesized that this is because the conformation of the ectodomain of Robo2 might mask the binding site for NELL1/2, which is supported by a recent study showing that the Robo2 ectodomain has a hairpin-like architecture (Barak et al. 2019). Importantly, we observed that mild acidic conditions (pH 5.5–6.5) facilitate the interaction between NELL1/2 and intact Robo2, probably because of the conformational change to the Robo2 ectodomain (Fig. 4). Local extracellular acidification has been observed under many pathological conditions, including ischemia, inflammation, and tumor progression (Okajima 2013). Tissue acidosis causes a decrease in the bone mineral density by inducing cell death of osteoblasts and activation of osteoclasts to resorb bone (Arnett 2008; Arnett 2010). However, a recent study indicated that short-term acidic stimulation enhances the stem cell phenotype of mesenchymal stem cells, which facilitates differentiation to osteoblasts during bone tissue healing (Hazehara-Kunitomo et al. 2019). Although we have no direct evidence that NELL1 interacts with intact Robo2 *in vivo*, the

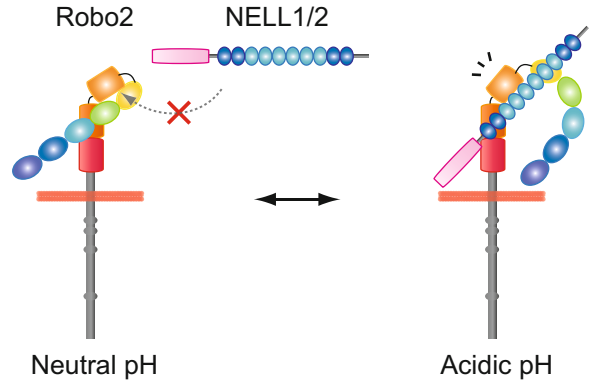

Fig. 4 Model showing the possible interaction between NELL proteins and Robo2 under acidic conditions. NELL1/2 only bind to Robo2 under acidic pH conditions. Conformational changes to the Robo2 ectodomain may unmask the binding site for NELL proteins under acidic conditions

temporal interaction between NELL1 and Robo2 in osteoblast precursors under acidic conditions may enhance bone formation to recover bone mass (Fig. 5).

The identification of the NELL proteins as novel ligands for the Robo receptors has raised questions concerning how NELL proteins activate Robo receptors and which signals are downstream of NELL-Robo interactions. Although NELL2 acts as repulsive axon guidance cue by binding to Robo3 and antagonizes Slits-Robo1/2-mediated repulsion, the molecular mechanisms of NELL2-Robo3 signaling remain unclear (Jaworski et al. 2015; Pak et al. 2020). In the case of the NELL1/2-Robo2 interaction, it is possible that Slit proteins and NELL1/2 act on Robo2 in cooperation because both ligands bind to Robo2 simultaneously. Thus, the signaling pathways downstream of the two classes of interaction, NELL-Robo2 and NELL-Robo3, may have different components. The identification of the NELL-Robo interaction has also stimulated further research. As described above, both NELL1 and NELL2 bind to Robo3, but NELL1 is not an effective ligand for Robo3 in commissural axons (Pak et al. 2020). It remains possible that NELL1 functions as a ligand for Robo3 at another spatiotemporal location. Because Robo3 is expressed in osteoclastic cell lineages (Kim et al. 2018a), NELL1 may have some effect on osteoclasts through binding to Robo3 (Fig. 5). In addition, conformational changes in the ectodomain of Robo2 allow both NELL1 and NELL2 to interact with Robo2, suggesting that NELL1/2-Robo2 interaction under acidic conditions functions in neuronal development.

6 Concluding Remarks

Because Slit ligands and their Robo receptors were originally identified in *Drosophila* mutants, many studies of *Drosophila* have been performed and the findings have contributed to the research progress on mammalian Slit-Robo signaling. However, *Drosophila* is not a suitable model to study bone metabolism because insects have an exoskeleton rather than a bony endoskeleton, which may be one of the reasons why studies of Slit-Robo signaling in bone remodeling have been recent. The identification of Slit3 as a coupling factor has revealed that Slit-Robo signaling acts with other axon guidance molecules in bone remodeling and the nervous system. As described above, Sema3A is a dual-acting factor that inhibits osteoclast differentiation and promotes osteoblast differentiation synchronously (Hayashi et al. 2012). Interestingly, neuron-specific, but not osteoblast-specific, *Sema3A*-deficient mice show a markedly low bone mass, suggesting that Sema3A affects bone remodeling through the nervous system. In comparison, osteoblast-specific, but not neuron-specific, *Slit3*-deficient mice show a significantly lower bone mass (Kim et al. 2018a; Xu et al. 2018). Thus, these two factors may act in a non-redundant and cooperative manner to fine-tune bone homeostasis. Recently, Ikebuchi et al. identified a unique

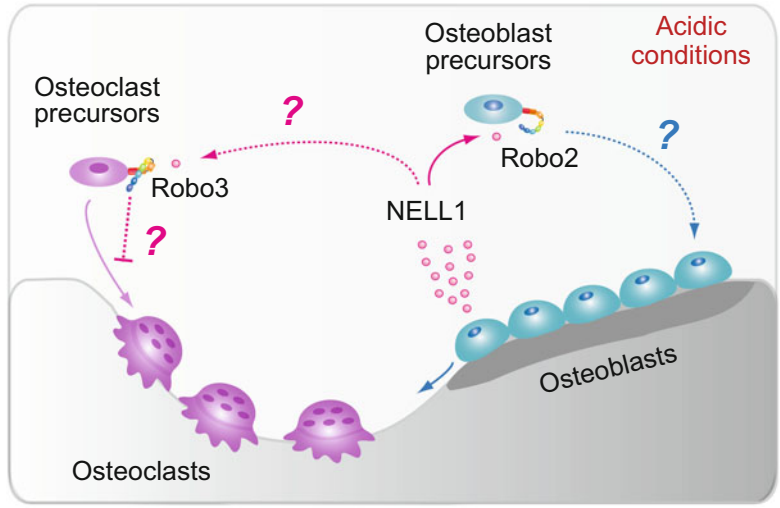

Fig. 5 Proposed model of the roles of NELL1-Robo signaling in bone remodeling. Robo2 is expressed in osteoblast precursors, whereas Robo3 is expressed in osteoclast precursors. NELL1 is possibly secreted by osteoblasts and may regulate osteoblast differentiation through Robo2 binding under acidic conditions. NELL1 may have some effects on osteoclasts through Robo3 binding

coupling factor, namely small extracellular vesicles (SEVs) containing receptor activator of nuclear factor-κB (RANK) secreted by mature osteoclasts (Ikebuchi et al. 2018). The RANK ligand (RANKL) produced by osteoblastic cell lineages acts on RANK expressed on the cell membrane of osteoclast precursor cells to differentiate osteoclasts. Ikebuchi et al. demonstrated that RANK-loaded SEVs secreted from mature osteoclasts act on RANKL on osteoblasts to promote osteoblast differentiation by activating the mTOR (mammalian target of rapamycin) pathway (RANKL reverse signaling). The interplay of these signaling systems, including RANKL-RANK, Sema3A-Plexin A1, Slit3-Robo, and other coupling factors, between osteoclasts and osteoblasts may contribute to the tight regulation of bone homeostasis. Future studies are required to elucidate how these factors coordinate bone-coupling events.

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Conflict of Interest The author declares that they have no conflicts of interest with the contents of this article.

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