CHAPTER 9

Semaphorin Signaling during Cardiac Development

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A number of semaphorins have been shown to play crucial roles as axon guidance cues in the wiring of the nervous system, including axon fasciculation, branching, and target selection. However, increasing evidence has also attested to the significance of semaphorins in the development of other organ systems, including the cardiovascular system. Targeted disruption of certain semaphorins or their receptors has been shown to result in various defects in the vascular system. Furthermore, several studies have suggested that some semaphorins may contribute to the development of the cardiovascular system by controlling the migration of endothelial cells, cardiac myocytes, or their precursors. In this review, we will discuss how semaphorin signals are involved in regulation of cardiac cells and cardiac morphogenesis.

Cardiac Morphogenesis: An Overview

The heart is one of the first mesodermal tissues to differentiate just after gastrulation in the vertebrate embryo.[1,2] Cells that migrate anterior and lateral to the primitive streak in early gastrulation contribute to heart tissues (Fig. 1A). Soon after their specification, precursors of cardiac cells converge along the ventral midline of the embryo to form a linear heart tube composed of myocardial and endocardial layers separated by an extracellular matrix (Fig. 1B). In all vertebrates, the linear heart tube undergoes rightward looping, which is essential for proper orientation of the right and left ventricles, and for alignment of the heart chambers with the vasculature (Fig. 1C). The direction of cardiac looping is determined by an asymmetric axial signaling system involving Nodal, Lefty, and Pitx2, which also affects the position of the lungs, liver, spleen, and gut.[3] The linear heart tube becomes segmentally patterned along the cranial-caudal axis into precursors of the aortic sac, conotruncus, pulmonary and systemic ventricles, and atria. Upon rightward looping, the cranial (conotruncus) and the caudal portions of the cardiac tube juxtapose dorsally and fuse to form a single outflow tract (truncus arteriosus), and the middle portion of cardiac tube expands to form a single ventricle (Fig. 1D). In the developing ventricle, interventricular septation separates the right and left ventricles, each of which differs in its morphological and contractile properties. The left ventricle is composed of distinct outer (compact) and inner (trabecular) layers. Trabeculae, finger-like projections comprised of myocardial cells, are necessary to support the increasing hemodynamic load and to supply nutrients from inside without blood vessels during early embryonic heart development.[4]

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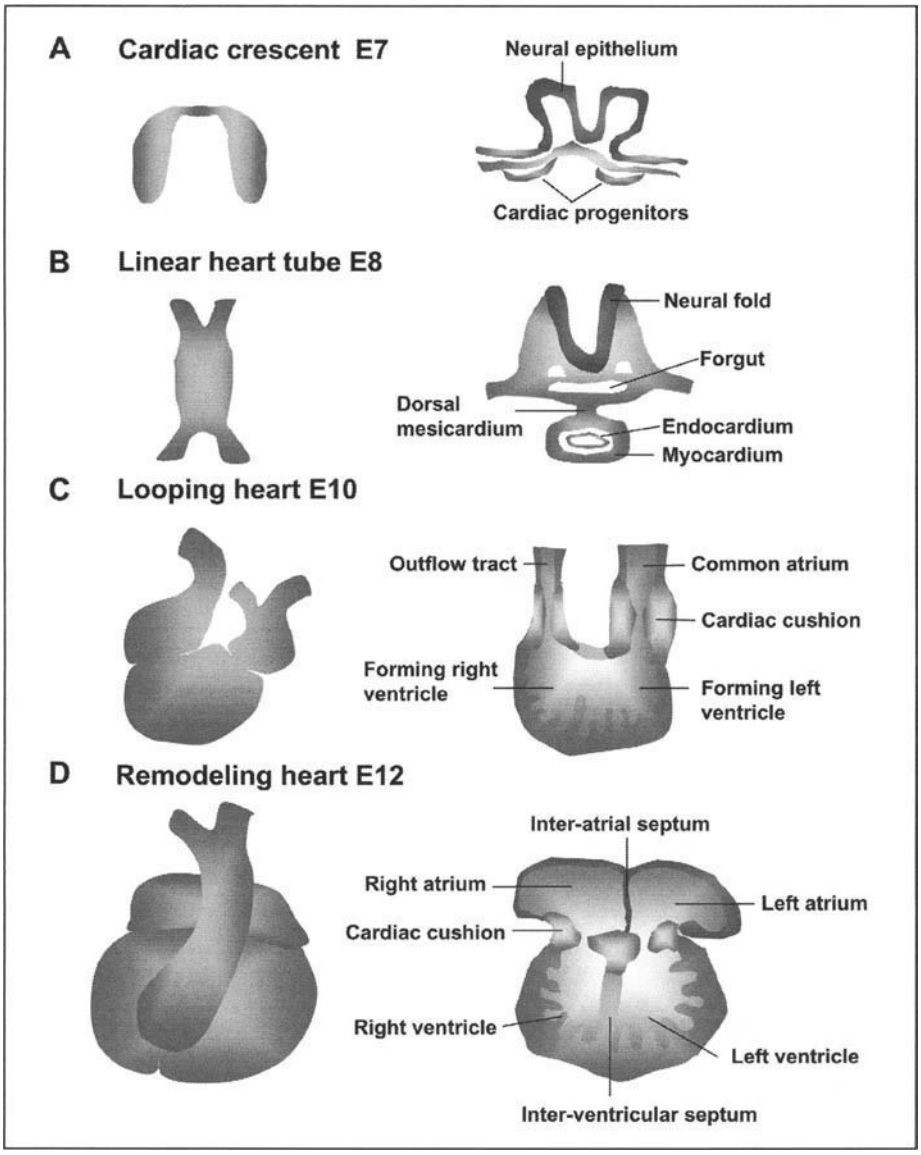

Figure 1. The main transitions in early heart development. The whole isolated heart is shown on the left, whereas a representative section is presented on the right. Staging in days of embryonic development is based on mouse development. A) Cardiac progenitors are first recognizable as a crescent-shaped epithelium (the cardiac crescent). B) Heart progenitors move ventrally to form the linear heart tube, which is composed of an endothelial lining that is surrounded by a myocardial epithelium. C) The linear heart tube undergoes a complex progression termed cardiac looping, including endocardial cushion formation in the atrioventricular canal and outflow tract, and trabecular formation in the ventricle. D) During the remodeling phase of heart development, division of chambers by septation is completed. The chambers and vessels are now aligned as in the adult heart and become fully integrated.

Appropriate placement and function of cardiac valves is essential for division of the chambers and for unidirectional flow of blood through the heart. Septation of the cardiac tube into distinct chambers is achieved through regional swellings of extracellular matix with proliferating mesenchymal cells, known as cardiac cushions, that will form the atrioventricular and ventriculoarterial valves. The formation of cardiac cushions is a complex event characterized by the endothelial-to-mesenchymal transformation of a subset of endothelial cells in the cushion-forming region, where they subsequently proliferate and complete their differentiation into mesenchymal cells.[5]

The separation of the pulmonary and systemic circulations into two parallel circuits is established by the separation of the truncus arteriosus into the aorta and pulmonary artery, and in the formation of the conotruncal portion of the ventricular septum. This is accomplished in part by cardiac neural crest cells.[6,7] Neural crest cells, which originate from neuroepithelial cells at the dorsal edge of neural tube, undergo epithelial-to-mesenchymal transformation so that they migrate to form part of the mesenchyme of the outflow tract. After septation, the vessels rotate in a twisting fashion to achieve their connections with the right and left ventricles. Thus, the mechanism that regulates the positioning of myocardial cells in the dynamic remodeling of the cardiac tube may be necessary for the proper alignment and structure of each chamber in that structure. It has been recently shown that some semaphorins play roles in the positioning of cardiac cells during cardiac development by regulating their migration.

Sema6D-Plexin-A1 Axis in Cardiac Morphogenesis

One of the best characterized semaphorins in cardiac development is Sema6D, a member of the class VI transmembrane-type semaphorin subfamily.[8] The expression of Sema6D is first detected in the cardiac crescent and neural fold of E9 mouse embryo. Sema6D mRNA was observed throughout the entire heart, including the conotruncal (CT) segment, the atrioventricular segment, and the ventricular myocardium at E10.5. The expression of Sema6D is higher in myocardial cells than in endocardial cells. In chick embryonic heart, Sema6D exhibits a similar expression pattern.

The role of Sema6D in the developing heart has been revealed by a series of studies using the chick embryo system. Inoculation of transfected cells that release a large amount of soluble Sema6D into cultured chick embryos at Hamberger and Hamilton (HH) stage 9 results in enhanced looping of the cardiac tube and enlargement of the ventricular region. In ovo inoculation of Sema6D producing cells or recombinant soluble Sema6D into HH stage 29 embryos also results in expansion of the ventricular cavity with a thin myocardial layer and an enlarged endocardial cushion. In contrast, RNAi-mediated knockdown of Sema6D inhibits looping of the cardiac tube. In the developing heart, Sema6D signals are largely mediated by Plexin-A1, which is also expressed in the embryonic heart. Indeed, RNAi-mediated knockdown of Plexin-A1 or expression of truncated Plexin-A1 results in decreased ventricular size. Therefore, the Sema6D-Plexin-A1 axis is critically involved in the dynamic remodeling of the cardiac tube and formation of the ventricle and endocardial cushion.

Sema6D Differentially Regulates Migration of Endothelial Cells in Distinct Regions of Cardiac Tube

Sema6D exerts distinct biological activities on endothelial cells in different regions of the cardiac tube. For instance, Sema6D inhibits migration of outgrowing cells from the ventricular segment. On the other hand, Sema6D promotes migration of outgrowing cells from the conotruncal and atrioventricular valve segments, both of which fuse to form the endocardial cushion later. These biological activities of Sema6D appear to be mediated by Plexin-A1, because they are abrogated by RNAi-mediated knockdown of Plexin-A1 or

expression of truncated Plexin-A1 in endothelial cells from the ventricle as well as the conotruncal segments. How are two distinct biological activities mediated through the same ligand binding receptor? In the nervous system of *Drosophila*, Plexin-A, a homologue of mammalian Plexin-A family members, forms a complex with off-track (OTK), to transduce the repulsive signaling of Sema-1a, a homologue of mammalian class VI semaphorins.[9] Like *Drosophila* Plexin-A, Plexin-A1 forms a complex with a vertebrate homologue of OTK in the endothelial cells of the ventricular region of the cardiac tube. The migration-inhibitory activity of Sema6D is suppressed by RNAi-mediated knockdown of OTK in ventricular endothelial cells. OTK is a member of the membrane-type tyrosine kinase family, although its kinase activity is lost because of the absence of critical residues in the kinase domain (so-called kinase dead). Notably, mice lacking the mammalian homologue to OTK exhibit several defects, including failure of tube closure, which are known to be associated with defective planar cell polarity.[10] Thus, Sema6D functions as a positional cue for migrating cells through the Plexin-A1/OTK receptor complex so as to regulate the shape and rotation of the cardiac ventricle (Fig. 2).

On the other hand, Plexin-A1 forms a complex with VEGF receptor type 2 (VEGFR2) in endothelial cells of conotruncal segments. The Sema6D-induced migration of endothelial cells is suppressed not only by RNAi against Plexin-A1, but also by RNAi against VEGFR2. A narrow range of VEGF levels is critical for cardiac cushion formation, because either induction or suppression of VEGF induces similar defects in cardiac cushion formation.[11,12] The VEGF-mediated tyrosine phosphorylation of VEGFR2, an initial step in the VEGF signaling pathway, is enhanced by Sema6D. Thus, Sema6D functions to regulate cardiac-cushion formation by modifying the signaling of VEGFR2 through Plexin-A1 on endothelial cells in the endocardiac cushion-forming region (Fig. 2). Indeed, overexpression of Sema6D results in enlargement of endocardiac cushion. Thus, the differential association of Plexin-A1 with additional receptor components enables Sema6D to exert distinct biological activities in adjacent regions, which is critical for complex cardiac morphogenesis.

Reverse Signaling of Sema6D in the Cardiac Ventricle

The developing ventricular wall is composed of the outer myocardial layer (compact layer) and trabeculae. Myocardial cells in the former express both Sema6D and Plexin-A1, and cells in the latter express Sema6D but not Plexin-A1.[13] Knockdown of either Sema6D or Plexin-A1 leads to the generation of a small, thin ventricular compact layer and to defective trabeculation. Ectopic expression of the Plexin-A1 extracellular domain alone can rescue the defective trabeculation induced by the suppression of Plexin-A1 but not that induced by suppression of Sema6D, indicating a role for Sema6D cytoplasmic signaling in trabeculation (Fig. 3A). The Sema6D cytoplasmic region can associate with two molecules; Abl kinase and Enabled (Ena), a member of the Ena/VASP family. Abl kinase and Ena are known to play opposing roles in the downstream regulation of *Drosophila* Robo, an axonal guidance receptor for Slit.[14] Ena has also been implicated in reverse signaling by *Drosophila* Sema1a. Upon binding to Plexin-A1, Abl kinase is recruited to the cytoplasmic tail of Sema6D and activated, which results in phosphorylation of Ena and its dissociation from Sema6D. In fate-mapping studies, myocardial cells carrying defects in reverse signaling by Sema6D arrest in the compact layer, whereas expression of constitutively active Abl kinase enhances the migration of cells from the compact layer to the trabeculae. Thus, Sema6D acts through its cytoplasmic domain as a reverse signal to regulate trabeculation.

Semaphorin Signaling in Vascular Connections to the Heart

Neuropilins, a receptor for class-III semaphorins, are widely expressed in the developing vasculature, and mice lacking neuropilin-1 or neuropilin-1 and -2 exhibit branching and

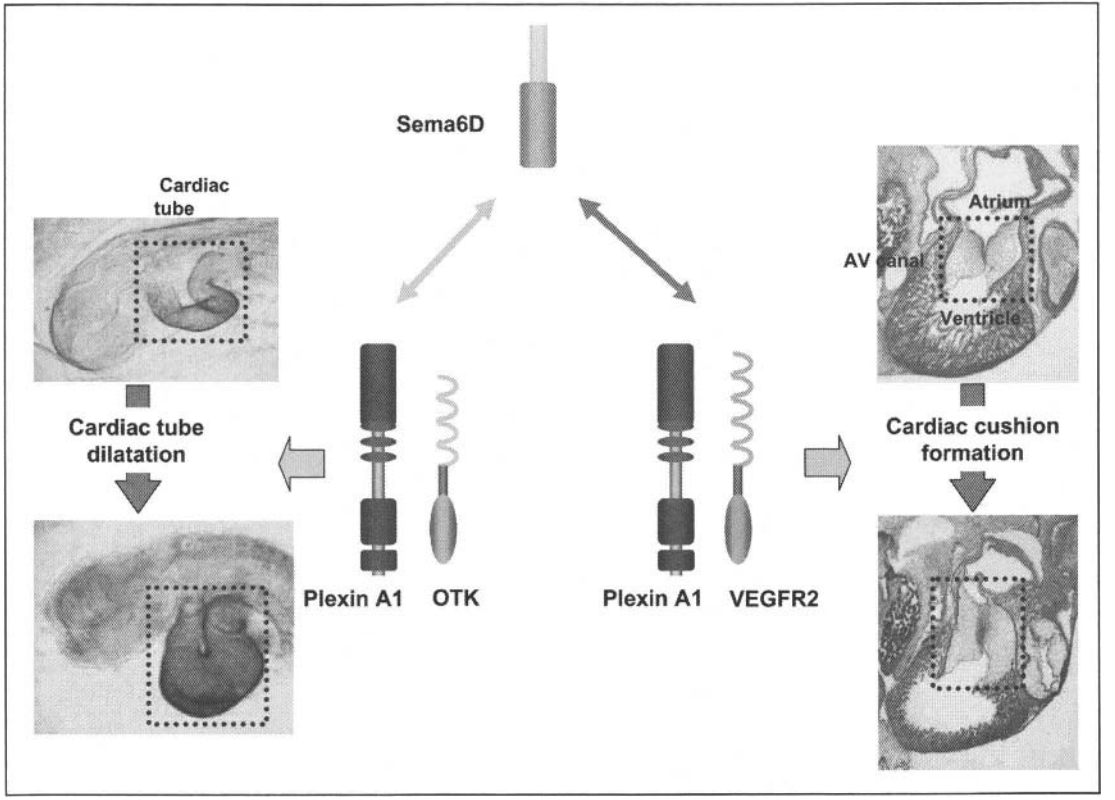

Figure 2. The role of Sema6D in cardiac expansion and cardiac cushion formation. In the ventricle-forming region of the looped cardiac tube, Plexin-A1 and OTK form a receptor complex and mediate Sema6D-induced suppression of endothelial cell motility, leading to cardiac expansion. In the cardiac cushion-forming region, Plexin-A1 and VEGFR2 form a receptor complex and mediate Sema6D-induced enhancement of endothelial cell motility, leading to cardiac-cushion formation.

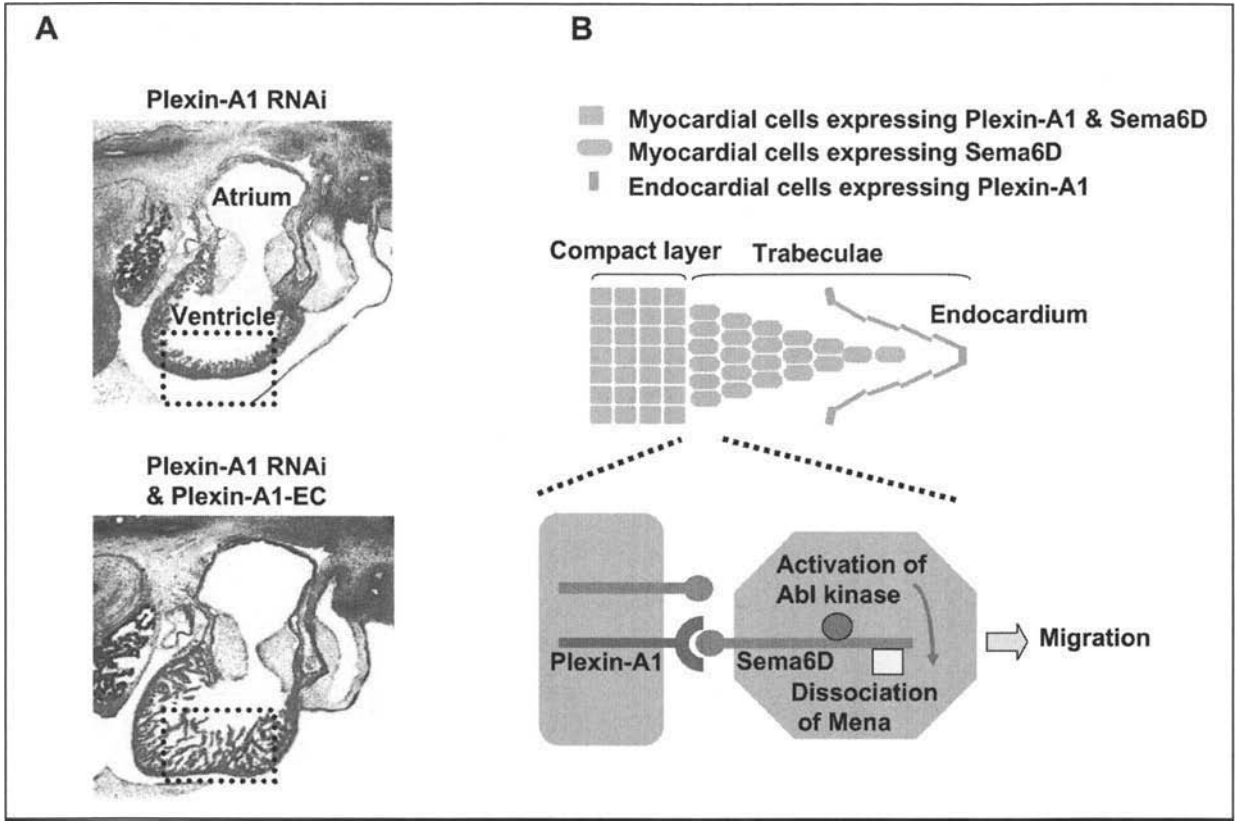

Figure 3. The role of Sema6D in cardiac trabeculation. A) Plexin-A1 RNAi suppresses trabecular formation, which is rescued by recombinant extracellular domain of plexin-A1 (B) The role of Sema6D in cardiac trabeculation through its cytoplasmic region. Plexin-A1 binding to Sema6D activates Abl kinase, and activated Abl kinase phosphorylates the associated Mena, resulting in its dissociation. This process allows myocardial cells to migrate into trabeculae.

remodeling defects, improper routing and connections, and ectopic termination of vessels.[15] Sema3A inhibits formation of endothelial lamellipodia and vessels.[16-18] However, neuropilins are also receptors for a specific VEGF isoform (VEGF165) and modulate the activity of VEGF receptors;[19] moreover, VEGF165 competes with Sema3A for binding to neuropilins.[16] Thus, the vascular effects of neuropilin-1 may reflect loss of VEGF rather than Sema3A signaling. Mice expressing a variant of neuropilin-1 that are only capable of binding to VEGF, but not to semaphorins, do not exhibit vascular defects,[20] indicating that neuropilin-1 plays a major role in vascular patterning as a VEGF coreceptor.

Mice lacking Sema3C exhibit persistent truncus arteriosus.[21] Plexin-A2, which is usually detected in cardiac neural crest, is patterned abnormally in several mutant mouse lines with congenital heart disease, including those lacking the secreted signaling molecule Sema3C.[22] It is noteworthy that Sema3C shows an attractive effect on cortical neurons through neuropilins-1.[23] Together with these findings, the complementary expression pattern of Sema3C and Pexin-A2 raises the possibility that cardiac neural crest cells navigate from the neural tube to the outflow tract of the cardiac tube by attractive signals involving the Sema3C-Plexin-A2 axis.

Signals of Sema3A in Endothelial Cell Migration

As described above, class III semaphorins such as Sema3C may be involved in cardiac morphogenesis. However, it remains largely unclear how class III semaphorins regulate migration of cardiac cells. A recent report showing the involvement of Sema3A in the regulation of vascular endothelial cells may provide a clue to this issue. Serini et al[18] showed that Sema3A suppresses adhesion of endothelial cells, which may contribute to the regulation of endothelial cell migration during vasculogenesis. In fact, Sema3A inhibits the function of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins in endothelial cells, although the molecular mechanism underlying this regulation is not known.

We have recently shown that the FERM domain-containing guanine nucleotide exchange factor (GEF) FARP2 functions as an immediate downstream signal transducer of the Plexin-A1-neuropilin-1 receptor complex in Sema3A-mediated repulsion of axons[24] (Fig. 4). Sema3A-induced dissociation of FARP2 from Plexin-A1 and activation of its Rac-GEF activity triggers a series of biochemical events including Rac activation and the binding of Rnd1, a member of the Rho GTPase family, to Plexin-A1.[25] This binding stimulates the GAP activity of Plexin-A1 for R-Ras, a member of the Ras GTPase family. Thus, the downregulation of R-Ras leads to cytoskeletal disassembly, which is critical for Plexin-A1-mediated growth-cone collapse.[26] In parallel with this event, dissolved FARP2 competes with an isoform of type-1 phosphatidylinositol phosphate kinase, PIPKIγ661, for the FERM domain of talin. PIP_2, catalyzed by PIPKIγ661 in association with talin, is important for the stability of integrin-mediated focal adhesion,[27,28] and the inhibition of PIPKIγ661 kinase activity by binding to FARP2 downregulates integrin function. Such a mechanism for class III semaphorin-mediated regulation of cell adhesion may also be involved in control of migration of precursors of cardiac cells during embryonic development.

Summary and Perspectives

As discussed in this review, the patterning and morphogenesis of the heart can be described in some detail, but the relevant molecular details are not completely understood. To further comprehend heart development, we must completely define its basic units, then seek the points of integration between various molecular systems affecting the developing heart tube. The recent discovery that guidance molecules such as semaphorins regulate the dynamic remodeling of the cardiac tube should provide insights into the molecular mechanisms underlying cardiac morphogenesis.

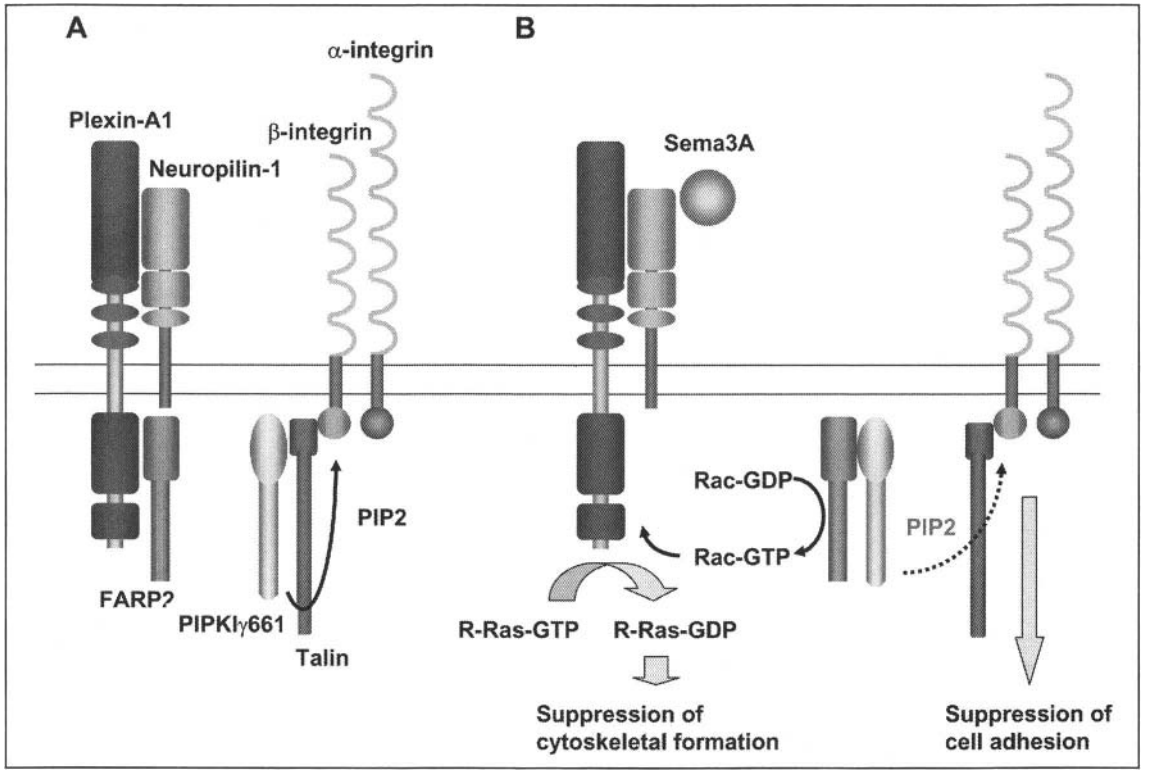

Figure 4. A schematic diagram depicting the roles of FARP2 in the initial steps of Sema3A-Plexin-A1 signaling. Sema3A binding to the receptor complex comprising Neuropilin-1 as the ligand-binding subunit and Plexin-A1 as the signal-transducing subunit triggers the dissociation of FARP2. Released FARP2 has two major roles in the downstream signaling of Plexin-A1. First, the RacGEF activity of FARP2 is turned on. This activity is essential for subsequent Rnd1 recruitment to Plexin-A1 and activation of Plexin-A1 downstream signaling events such as activation of R-Ras GAP activity of Plexin-A1 and downregulation of R-Ras. Second, released FARP2 binds to PIPKIγ661 and inhibits its PIPKIγ kinase activity, resulting in an inhibition of focal adhesion.

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