Published in final edited form as:
*Curr Opin Hematol*. 2011 May ; 18(3): 186–190. doi:10.1097/MOH.0b013e328345a4b9.

# Robo4-dependent Slit signaling stabilizes the vasculature during pathologic angiogenesis and cytokine storm

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## Abstract

**Purpose of review—**The endothelium is bombarded with and must respond to multiple destabilizing proangiogenic and inflammatory cytokines.

**Recent findings—**Endogenous cell signaling systems such as Roundabout (Robo)4-dependent Slit signaling are in place to help maintain homeostatic balance and prevent excessive destabilization. Upon Robo4 activation by Slit, paxillin is recruited to the cytoplasmic domain along with an ArfGAP known as GIT1. GIT1 recruitment results in inactivation of Arf6, a protein shown to regulate cadherin cell surface localization. Slit increases vascular endothelial-cadherin presentation at the cell surface and enhances vascular barrier function in the presence of inflammatory cytokines.

**Summary—**Through harnessing Robo4-dependent Slit signaling, survival can be enhanced in mouse models of sepsis and avian flu infection. This effect is achieved by blunting the host vascular response to cytokines. Thus, vascular stabilizing programs should be investigated as potential therapeutics for infectious disease characterized by cytokine storm.

### Keywords

Arf6; cytokine storm; GIT1; roundabout 4; Slit

## Introduction

Roundabout (Robo) was originally defined as a mediator of repulsive axon guidance signaling [1,2]. Slit proteins act as ligands for Robo receptors and are known to initiate an inhibitory effect on neural guidance [3–5]. Wu *et al.* [6] first described an inhibitory role for Slit proteins outside the nervous system. Robo expression was observed in the thymus, spleen, and lymph nodes, leading them to speculate an additional role for Slit–Robo signaling in leukocytes. Indeed, HL-60-derived neutrophils and rat lymphocytes were repelled by Slit2 [6]. Since then, an endothelial-specific Robo family member was discovered termed Robo4 [7]. Park *et al.* [8] hypothesized that Slit may also inhibit endothelial cell migration. Indeed, Slit2 inhibited microvascular endothelial cell migration and this repulsive effect was lost when Slit2 was depleted from the sample.



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As Robo was originally defined in neural guidance, it could be hypothesized that an endothelial-specific Robo may be important for vascular guidance *in vivo*. To answer this question, *Robo4*-null (*Robo4*$^{AP/AP}$) mice were generated [9•]. Surprisingly, no vascular guidance defects were discovered in multiple vascular beds. Furthermore, *Robo4*$^{AP/AP}$ mice were viable and fertile. As a potential explanation for this result, Robo4 expression was found to be predominantly localized to the endothelial stalk cells rather than the tip cells, perhaps suggesting rather a role for Robo4-dependent Slit signaling in vascular stability. This is supported by evidence demonstrating that recombinant Slit protein can inhibit vascular endothelial growth factor (VEGF)-induced vascular destabilization in a Robo4-dependent manner [9•]. A recent study using a genetic approach instead of recombinant protein to understand the role of Slit in an endogenous context was consistent with these findings [10••].

Marlow *et al.* [10••] found that Slit from a stromal source restrains mammary gland endothelial cell growth. This was demonstrated by a significant increase in vascular network density and complexity in the mammary glands of *Slit2*$^{+/-}$; *Slit3*$^{-/-}$ mice. Next, they evaluated whether *Robo1*-knockout or *Robo4*-knockout mice phenocopied this result. However, neither loss of Robo1 or Robo4 individually caused a significant difference in blood vessel growth. Interestingly, *Robo1*$^{-/-}$; *Robo4*$^{AP/AP}$ mice demonstrated an approximately two-fold increase in blood vessel density. To determine the tissue specificity of this effect, *Robo1*$^{-/-}$ epithelium was transplanted into *Robo4*$^{AP/AP}$ fat pads and a significant increase in neovascularization was observed. Loss of Robo1 in the epithelium caused an increase in VEGF and stromal derived factor-1. This increased expression was insufficient itself to stimulate increased neovascularization but required the loss of Robo4. This observation is supported in a second setting of sprouting angiogenesis driven by VEGF. During pregnancy, there is a significant increase in blood vessel density in mammary glands. As such, mammary glands from mid-pregnant *Robo4*$^{AP/AP}$ mice displayed a hypervascular response. Taken together, these data demonstrate an inhibitory role for Slit–Robo4 signaling in angiogenesis as elucidated by a genetic system rather than recombinant protein. Furthermore, these data suggest a genetic interaction between Slit and Robo4 [10••].

## Robo4 downstream signaling

Previously, Stein and Tessier-Lavigne [11] utilized chimeric Robo receptors to ascertain whether Slit silenced netrin-1-mediated neural attraction through a receptor-mediated or another mechanism. By fusing the cytoplasmic domain of Robo1 to the ectodomain of either Met or trkA and utilizing either hepatocyte growth factor or nerve growth factor, respectively, they observed activation of the cytoplasmic receptor. This demonstrated precedence that chimeric Robo receptors could be activated by a heterologous ligand. A similar approach was taken to determine whether the Robo4 cytoplasmic domain possessed downstream signaling capabilities. A chimera was generated by fusing the cytoplasmic domain of Robo4 to αIIb integrin, thus allowing for cytoplasmic activation using fibrinogen, an activator of αIIb:β3 integrin [12••]. Using this system, a decrease in cell spreading was observed, consistent with a response seen with Slit-mediated activation of Robo4. This result demonstrated that the cytoplasmic domain of Robo4 possessed downstream signaling capabilities.

Wong *et al.* [13] have provided an excellent roadmap for dissecting components of Robo signaling through their detailed analysis of Robo1. First, a yeast two-hybrid screen was conducted to ascertain binding partners of the Robo1 cytoplasmic domain. This screen uncovered an interaction with rhoGAP proteins referred to as slit-robo GAPs (srGAPs). To determine the site of the interaction, mutant forms of the Robo1 cytoplasmic domain and srGAP1 were generated. These experiments identified the important interaction domains

both on the receptor's cytoplasmic domain and on the srGAP1 itself, thus providing strong evidence as to the veracity of the interaction.

Using this outline, a yeast two-hybrid screen using the N-terminal half of the cytoplasmic tail of Robo4 was conducted [12••]. The N-terminal fragment was used as it was determined to be the fragment necessary for Slit-mediated inhibition of cell migration. The yeast two-hybrid screen identified a member of the paxillin family of adaptor proteins as a potential Robo4-interacting protein. The Robo4–paxillin interaction was confirmed *in vitro* and was found to be enhanced by Slit2. Following the pattern previously described, the domain of Robo4 necessary for its interaction with paxillin was mapped. Using glutathione-S-transferase (GST)–Robo4 fusion proteins and purified paxillin protein, a 35 amino acid fragment of Robo4 was identified as necessary for the interaction with paxillin, termed the paxillin interaction motif (PIM). Furthermore, the PIM domain was determined to be functionally important as Slit2 could inhibit the migration of Robo4 but not Robo4ΔPIM expressing human embryonic kidney cells. Next, the domain of paxillin necessary for its interaction with Robo4 was determined. Using serial deletions of the Lim domains of paxillin, it was discovered that deletion of the Lim4 domain resulted in the loss of the interaction between paxillin and Robo4. The Lim4 domain was found to be functionally important in cells depleted of endogenous paxillin by siRNA and reconstituted with wild-type chicken paxillin or chicken paxillinΔLim4. Whereas Slit2 retained its activity in cells reconstituted with wild-type chicken paxillin, its effect was lost in cells reconstituted with chicken paxillinΔLim4. These data demonstrated that the PIM domain of Robo4 and the Lim4 domain of paxillin are necessary for the functional effect of Slit.

In addition to binding Robo4, paxillin also recruits an ArfGAP known as GIT1, resulting in inhibition of Arf6-GTP and downstream Rac activation. Ectopic expression of the GIT1–paxillin binding sequence (GIT1-PBS), which blocks the interaction of paxillin and GIT1, also abrogated the inhibitory effect of Slit2. Furthermore, overexpression of an ArfGEF, ARF nucleotide-binding site opener (ARNO), blocked the inhibitory effect of Slit2. This signaling system was then moved from a heterologous system and studied in endothelial cells. Slit 2 inhibited VEGF-induced Arf6 activation and the Robo4 PIM domain was found to be necessary for the effect of Slit2 in endothelial cell migration. SecinH3, a small molecule inhibitor of a set of ArfGEFs known as cytohesins, was then used to test the validity of this pathway *in vivo*. Recombinant Slit2 had been previously discovered to inhibit VEGF-induced retinal permeability as well as neovascularization in models of pathologic angiogenesis [9•]. SecinH3 was found to phenocopy the effect of Slit2 and inhibit VEGF-induced retinal permeability and pathologic neovascularization in mice [12••]. Thus, Slit-mediated activation of Robo4 recruits a paxillin–GIT1 complex that inactivates Arf6 to enhance vascular stability.

## GIT1 mediated Arf6 inactivation: a common stabilizing system

Robo4 was not the first transmembrane protein found to utilize the downstream signaling properties of a paxillin–GIT1–Arf6 module. Previously, $\alpha_4$ integrins have been shown to utilize this pathway and bind paxillin [14,15]. The interaction site was mapped to a nine amino acid sequence, which could then be further reduced to single critical amino acids [16]. In addition, the fragment of paxillin sufficient to bind $\alpha_4$ integrins has also been elucidated [17]. Interestingly, this interaction was shown to be sufficient to inhibit Rac activation, a key step in cell spreading, by utilizing an $\alpha_4$–paxillin fusion protein [14]. Furthermore, an integrin–paxillin fusion protein lacking the LD4 domain was unable to block Rac activation or cell spreading. However, a fusion protein possessing only the LD3 and LD4 regions of paxillin reduced both Rac activation and cell spreading. Next, GIT1 was found to form a ternary complex in combination with paxillin. A GIT1–PBS dissociated

GIT1 from paxillin and negated the effect of integrin–paxillin binding. The Arf–GAP domain was found to be necessary and sufficient for the inhibition of Rac activation and cell spreading. Lastly, adhesion-mediated Arf6 activation was inhibited by integrin association with either paxillin or the GAP domain of GIT1. Thus, $\alpha_4$ integrins utilize a paxillin–GIT1–Arf6 module to reduce Rac activation and inhibit cell spreading [14].

A similar signaling platform has been described for EphA2 [18•]. Upon ephrinA1 ligand activation of EphA2, an adaptor protein known as Nck1 is recruited to the receptor. Nck1 constitutively binds GIT1 and, thus, localizes GIT1 to the receptor and results in the inactivation of Arf6. The importance of these signaling events in ephrinA1-induced cell compaction was also demonstrated as a constitutively active Arf6 construct reversed the effect of ephrinA1. Interestingly, a separate group has found that ephrinA1 inhibits measurements of angiogenesis *in vitro* and *in vivo*. Ojima *et al.* [19] demonstrated that ephrinA1 inhibited VEGF-induced tube formation *in vitro* as well as VEGF-induced retinal permeability *in vivo*. Furthermore, in a mouse model of proliferative retinopathy, ephrinA1 reduced neovascularization. These data demonstrate that ephrinA1 can also act as a vascular stabilizing cue.

## Robo4-dependent Slit signaling stabilizes vasculature during cytokine storm

With the understanding that Slit stabilizes the vasculature and inhibits Arf6 activation, and that dominant-negative Arf6 blocks cadherin internalization in epithelial cells, one can logically hypothesize that Slit may enhance vascular endothelial-cadherin localization to the cell surface [20]. Indeed, Slit2N, an active fragment of Slit2, can enhance vascular endothelial-cadherin and p120-catenin localization to the endothelial cell surface [21••]. As Slit2N enhanced the surface localization of the cell machinery important for regulating vascular permeability, one could also hypothesize that Slit2N may inhibit permeability induced by multiple factors. Indeed, Slit2N was found to inhibit interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and lipopolysaccharide (LPS)-induced endothelial permeability. Aside from directly causing permeability *in vitro*, LPS administration to the lungs of animals ultimately stimulates the release of multiple permeability inducing factors, including IL-1β and TNF-α. This may also be known as cytokine storm. Interestingly, recombinant Slit2N was found to significantly inhibit LPS-induced Evans Blue dye leak, a measure of vascular permeability, in mice [21••]. Furthermore, Slit2N inhibited the accumulation of neutrophils and protein exudates in the alveolar space of LPS-treated mice. A second recent study has found that LPS-induced neutrophil accumulation is diminished in Slit2 transgenic mice [22•]. The effect of Slit2N was lost in *Robo4*$^{AP/AP}$ mice, demonstrating that Robo4 is necessary for the effect of Slit2N, and also suggesting that the effect of Slit2N is vascular specific as Robo4 is regarded as an endothelial-specific protein [21••]. Lastly, the effect of Slit2N was lost in the presence of a vascular endothelial-cadherin blocking antibody, confirming the importance of the action of Slit2N on vascular endothelial-cadherin *in vivo*.

In order to understand whether Slit2N was also effective in systemic rather than localized inflammation, the cecal ligation and puncture (CLP) mouse model of sepsis was utilized. Slit2N inhibited Evans Blue dye leak in the kidney and spleen of CLP-treated mice. Furthermore, Slit2N significantly enhanced survival. Slit2N did not significantly affect the level of a panel of cytokines and chemokines in the serum, suggesting that the effect of Slit2N was not simply by reducing inflammation. The effect of Slit2N was also investigated in a mouse model of avian flu (H5N1) infection. Slit2N significantly inhibited H5N1-induced permeability in the lung and also enhanced survival. Slit2N did not significantly alter cytokine levels in the lungs of these mice nor was there a significant change in viral

titer loads. Together, these data suggest a platform whereby specifically targeting the host vascular response to cytokines may enhance survival during multiple settings of infectious disease characterized by cytokine storm [21••,23].

## Questions in the field

Although a direct interaction between Slit and Robo4 is difficult to detect by BiaCore *in vitro* [24], another study supports a genetic interaction *in vivo* [10••]. Additional experiments have demonstrated the Robo4-dependency of Slit signaling *in vitro* and *in vivo* [9•,21••]. A possible explanation is the requirement of a coreceptor. Indeed, others have shown that syndecans or cell surface heparan sulfate are important for Robo signaling [5,25]. Alternatively, Suchting *et al.* [24] and Sheldon *et al.* [26•] have proposed a Robo1/Robo4 heterodimer. Consistent with this proposed model, either siRNA knockdown of Robo1 or Robo4 abrogates the inhibitory effect of Slit in human umbilical vein endothelial cells (HUVECs) [21••]. However, whereas HUVECs express a significant level of both Robo1 and Robo4, human microvascular endothelial cells (HMVECs) express a significant level of Robo4 and very little Robo1. Whereas Robo4 siRNA in these cells abrogates the effect of Slit, Robo1 siRNA does not, suggesting the Robo1/Robo4 heterodimer mechanism may be context-dependent [10••].

Although many have demonstrated the repulsive properties of Slit in relation to endothelial cell migration [9•,27•,28], others have also demonstrated a pro-migratory effect [26•,29•,30]. A recent study postulates an explanation as to these results [31••]. Although Slit2 and ephrinA1 were found to be pro-angiogenic as single agents, when combined together, an antiangiogenic function of Slit2 was concluded. This group hypothesized that perhaps ephrinA1 could be inhibiting mammalian target of rapamycin (mTOR) activity in the endothelium, which could impair Slit2-induced activation of Akt and/or Rac [31••]. This study offers a possible explanation defining contexts in which Slit is anti versus pro-migratory.

## Conclusion

Robo was originally discovered as a mediator of repulsive axon guidance signaling. Although a role for an endothelial-specific Robo, Robo4, in vascular guidance was anticipated, a role for Robo4 as a vascular stability program was unexpectedly uncovered. Robo4 activation results in the recruitment of a paxillin–GIT1–Arf6 module. A similar signaling module is utilized by multiple signaling systems, including $\alpha_4$ integrins and EphA2. Slit2N enhances vascular endothelial-cadherin cell surface presentation and reduces permeability induced by multiple inflammatory cytokines. Through harnessing Robo4-dependent Slit signaling, survival can be enhanced in mouse models of sepsis and avian flu. Novel ways to treat infectious diseases characterized by cytokine storm should be investigated using known vascular stabilizing pathways [23].

## Acknowledgments

The present work was supported by National Heart, Lung, and Blood Institute (NHLBI); National Institute of Allergy and Infectious Diseases (NIAID); Rocky Mountain Regional Center of Excellence in Biodefense and Emerging Infectious Disease; Juvenile Diabetes Research Foundation; HA and Edna Benning Foundation; American Asthma Foundation; National Center for Research Resources Public Health Services research grant UL1-RR025764; and Department of Defense. D.Y.L. is a Burroughs Wellcome Foundation Clinical Scientist in Translational Research and an established investigator of the American Heart Association. N.R.L. and D.Y.L. are employed by the University of Utah, which has filed intellectual property surrounding the therapeutic uses of targeting Robo4 with the intent of commercialization. The University of Utah has licensed Robo4 technology to Navigen, a biotechnology company owned in part by the University of Utah Research Foundation. N.R.L. is a paid consultant for Navigen and D.Y.L. is a founder of and on the Board of Directors of Navigen.

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 200–201).

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**Key points**

- Roundabout 4-dependent Slit signaling stabilizes the vasculature during pathologic angiogenesis and cytokine storm.
- A paxillin–GIT1–Arf6 module is a common stabilizing signaling mechanism.
- Blunting the host response to cytokine storm enhances survival in mouse models of sepsis and avian flu infection.