The structural mechanism underlying the antithetic effect of homologous RND1 and RhoD GTPases in plexin regulation

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Abstract

Plexins are semaphorin receptors that play essential roles in neuronal axon guidance and in many other important biological processes. Plexin signaling depends on a semaphorin-induced dimerization mechanism, and is modulated by small signaling GTPases of the Rho family, of which RND1 serves as a plexin activator yet its close homolog RhoD an inhibitor. Using molecular dynamics (MD) simulations we showed that RND1 reinforces plexin dimerization interface whereas RhoD destabilizes it due to their differential interaction with cell membrane. Upon binding plexin dimers at the Rho-GTPase binding (RBD) domains, RND1 and RhoD interact differently with the inner leaflet of cell membrane, and exert opposite effects on the dimerization interface via an allosteric network involving the RBD domain, RBD linkers, and a buttress segment adjacent to the dimerization interface. The differential membrane interaction is attributed to the fact that, unlike RND1, RhoD features a short C-terminal tail and a positively-charged membrane interface.

Introduction

Plexins are a family of nine single-pass transmembrane receptor proteins including plexin A1–4, B1–3, C1 and D1. Plexins are best known as the receptors of extracellular semaphorin ligands (Nishide and Kumanogoh, 2018) that are guidance cue for neuronal axons. Plexins also help regulate other essential biological processes such as cell migration, angiogenesis and immune responses (Sakurai et al., 2012; Takamatsu and Kumanogoh, 2012). Aberrant plexin activity is associated with a plethora of diseases including neurological disorders and cancer metastasis (Gu and Giraudo, 2013; Tamagnone, 2012; Yaron and Zheng, 2007).

Plexin architecture is conserved across the family. Plexin consists of a large multi-domain extracellular module including the ligand-binding Sema domain, a single-pass transmembrane helix, and an intracellular module that includes a GTPase activating protein (GAP) domain and a Rho GTPase binding domain (RBD) (Figure 1A). In plexin signaling, semaphorin binds at the extracellular module, which leads to activation of the GAP domain. Structures (Janssen et al., 2010; Kuo et al., 2020; Liu et al., 2010; Nogi et al., 2010) showed that a semaphorin mediates plexin dimerization: a semaphorin dimer interacts with two plexins at the extracellular module, and this extracellular dimerization leads to dimerization at the intracellular module (Figure 1B), and in turn GAP activation for specific small GTPases Rap (Wang et al., 2012). The dimerization stabilizes the active conformation of the so-called activation segments of the GAP domains, which otherwise adopts an inactive conformation that precludes GTPase binding of the GAP domain (Wang et al., 2013). In plexin signaling, the GAP activity switches off the signaling of plexin substrate Rap by catalyzing its GTP hydrolysis and converting it from the GTP-bound state to the GDP-bound state. Besides a GAP domain, the intracellular module includes an RBD domain that binds Rho-family small signaling GTPases. The RBD of PlexinB1 has been shown to bind Rac1, Rac2, Rac3, Rnd1, Rnd2, Rnd3 and RhoD, but not RhoA, Cdc42, RhoG or Rif (Fansa et al., 2013).

The RBD-GTPase binding is integral to plexin regulation from the intracellular environment. Overexpression of Rac1 leads to higher cell surface expression of plexin and enhances plexin interaction with semaphorin, suggesting that Rac1 acts as an upstream activator of plexin (Vikis and H., 2002). Moreover, binding of over-expressed RND1 to plexin triggers cell collapse in the absence of semaphorin, suggesting that RND1 is a more potent activator than Rac1 for plexin (Zanata et al., 2002). Simultaneous extracellular binding of semaphorin and RBD-GTPase appears to be a prerequisite for full activation of at least some plexins (Bell et al., 2011), but RBD binding with some other Rho GTPases appears to attenuate plexin activity. RhoD binds plexin with similar affinity as RND1 (Fansa et al., 2013), presumably in the same mode as Rac1 and RND1, in sharp contrast it strongly inhibits plexin signaling (Zanata et al., 2002).

The structural mechanism of the antithetic effects Rho GTPases on plexin signaling, however, remains elusive. Activity assays in solution showed that the RhoGTPases do not alter the GAP activity of plexin either in the monomeric or the active dimer state (Wang et al., 2012). Resolved complex structures of plexin RBD with different RhoGTPase such as RND1 (PDB 2REX and 3Q3J) and Rac1 (Wang et al., 2012) showed that the RBD binding with these GTPases in a similar mode. The structure of plexin RBD in complex with RhoD is not available, but biophysical analyses have suggested that the binding mode is similar (Tong et al., 2007). The crystal structures and other biophysical data all suggest that the RBD domain does not undergo substantial and global conformational changes upon GTPase binding (Bell et al., 2011; Tong et al., 2007; Wang et al., 2012). Modulations of plexin activity from the RBD-GTPase binding thus are unlikely derived from major conformational changes within the RBD domain.

To understand the apparent paradox regarding the antithetic effects of RhoD and other RhoGTPase on plexin activation, we determined the crystal structure of the RhoD/plexin B2-RBD complex, which confirmed that the binding mode of RhoD to RBD is similar to that of other RhoGTPases. Furthermore,

we here modeled and simulated RND1 and RhoD complexes with plexin A4 (Figure 1C), to investigate the structural mechanisms underlying RND1 as an activator and RhoD as an attenuator in plexin regulation. The simulations showed that RND1 binding is compatible with the dimerization of plexin A4 while RhoD binding is likely disruptive to the dimerization. The simulations revealed two distinct modes of interactions of RND1 and RhoD with the membrane: RND1 interacts with the membrane loosely and its long C-terminal tail serves as a flexible tether from the membrane (Figure 1D), whereas RhoD interacts with the membrane in a specific manner using an electrostatically-positive membrane-contacting interface (Figure 1E) that is absent in RND1. As a result, RND1 binding strengthens plexin dimerization by stabilizing the RBD, RhoD interacts with the membrane closely and presents a hindrance to plexin dimerization.

Results

Crystal structure of the complex between RhoD and the plexin B2-RBD domain

Complex structures of the RBD domain with plexin activators such as RND1 or Rac1 have been previously resolved. To experimentally determine the binding mode between plexin and RhoD, a negative plexin regulator, we screened various combinations of RhoD and the intracellular region of plexin family members from different species for crystallization, which resulted in crystals of the complex of mouse plexin B2 and human RhoD bound to the GTP analogue GMP-PNP. Analyses of the diffraction data suggested that plexin B2 degraded during the incubation in crystallization drops, and the crystals only contained the complex between RhoD and the RBD of plexin B2. We solved the structure to 3.1 Å resolution by molecular replacement (Supplemental Table 1). See methods for details). The asymmetric unit of the crystal contains two RhoD molecules, each of which binds to one plexin B2 RBD molecule. Surprisingly, the two RBD domains form a domain-swapped dimer in the structure, with the N-terminal portion of one molecule fold together with the C-terminal portion of the other (Supplemental Figure 1). This domain-swapped dimer is likely a crystallization artifact because it cannot form in intact plexin. We therefore consider each RBD domain formed by the two halves of the two molecules as a representative of one intact, unswapped RBD, as its conformation is very similar to the structures of other RBDs (Figure 2).

The structures confirm that RhoD binds the plexin B2 RBD in a mode similar to those of other complexes between RhoGTPases and plexin (Bell et al., 2011; Wang et al., 2011; Wang et al., 2012). The GTP analogue GMP-PNP and Mg^{2+} together stabilize the ligand-binding switch I and switch II regions in the active conformation, which make an extensive interface with one side of the beta-sheet of the RBD (Figure 2). All the residues in RhoD involved in interacting with the RBD are identical between human and mouse RhoD, suggesting that the cross-species complex that we crystallized is a

valid representative of the RhoD/plexin complex. Interestingly, a superimposition of the RhoD/Plexin B2-RBD complex with the RND1/Plexin B1-RBD complex based on the RBD domains shows that the orientation of RhoD and RND1 relative to the RBD domains are slightly different (Figure 2). Compared with that in RND1, the switch II helix in RhoD is placed further away from the RBD, which appears to be required to accommodate Phe85, which is larger than Cys81, the corresponding residue in RND1. This difference leads to different pivots of the two GTPases relative to the RBD, which propagates to a larger difference in the opposite side of the molecule where the insert helices (α_i), a unique helical segment present in the Rho subfamily but not other GTPases, is located (Figure 2). In the context of the active dimer of full-length plexin on the plasma membrane, the area near α_i of the RhoGTPases is placed to face the membrane surface. This orientational difference between RhoD and RND1 relative to plexin therefore may influence their interactions with the membrane, as analyzed in detail below. It is unclear how the subtle difference in orientation confers the opposite regulatory effects between RND1 and RhoD. To shed further light on this question, we performed further investigation using MD simulations of Rho GTPase-bound plexin systems.

RhoD and RND1 interacts differently with cell membrane

The membrane may play an important role in plexin regulation by the small GTPases, which are located adjacent to the membrane. Previous studies showed that RhoD does not alter the GAP activity of plexin A1 in a solvent environment (Pascoe et al., 2015; Wang et al., 2012). To investigate the underlying mechanism of this role, we simulated plexin A4 dimer in the membrane environment, respectively bound with RND1 and RhoD (Figure 1C). We first constructed a structural model of the transmembrane and the intracellular modules of the plexin A4 dimer with a membrane, primarily using homology modeling based on the resolved structure of the intracellular module of plexin C1 dimer (PDB 4M8N) (Wang et al., 2013). We then added two RND1 molecules to the plexin dimer. The GTP-bound catalytic domain of each RND1 is bound with a plexin RBD; the C-terminal tail of RND1

likely forms an amphipathic tail (Thiyagarajan et al., 2004) and serves as a membrane anchor. We additionally constructed a plexin dimer model in which each plexin RBD is bound with the catalytic domain of a GTP-bound RhoD, where the C-terminal tail is lipidated (Roberts et al., 2008) and anchored to the membrane.

We simulated the RND1-bound (Figure 3A) and the RhoD-bound (Figure 3B) plexin dimers, each for 1 μ s three times. In the simulations of the RND1-bound dimer, the amphipathic helices at the C-termini of the RND1 molecules remained anchored to the membrane, and the RND1 linkers between the catalytic domains and the amphipathic helices (residue 189-200) are sufficiently long to not affect the position of the catalytic domains (Figure 3A). The contact area between the membrane and the catalytic domains remains relatively small, with a mean at approximately 200 $\text{\AA}^2$ (Figure 3D). The two RND1 catalytic domains largely remained in their initial positions, with the root mean square deviation (RMSD) of the C α atoms with respect to their initial positions fluctuating around 6 $\text{\AA}$ (Figure 3E).

The C-terminal tail of the RhoD is shorter and more arginine-rich than the RND1, which is likely membrane-bound and hence restrains the RhoD catalytic domain to the membrane. In contrast to the RND1-bound plexin dimer, in the simulations of the RhoD-bound plexin dimer, the membrane interactions of the RhoD catalytic domains developed extensive and stable interactions with the membrane in the courses of the simulations (Figure 3D). The contact area of the two RhoD domains with the membrane fluctuated but generally trended upwards. It is apparent that the extent of the membrane interaction is closely correlated with the positioning of the catalytic domains in both the RND1- and the RhoD-bound plexin dimers. With the increase of the membrane interactions, the two RhoD domains deviated substantially from their initial positions, as shown by the RMSD of C α atoms with respect to their initial positions (Figure 3E). The RMSD fluctuation of the RhoD domains was larger than the RND1 domains (Figure 3E), indicating that the differential membrane interactions of RhoD and RND1 lead to differential positioning and dynamics of the two GTPases.

Further analysis showed that RhoD interacts with the membrane with a specific interface involving the insert helix (α_i) mentioned above and the α_4 helix (Figure 3C); Arg144, Arg145, His154, and Arg155 in this part of RhoD enjoyed stable interactions with the membrane (Figure 3F). The RhoD membrane-anchoring interface features pronounced positive electrostatic potential that is favorable for membrane interaction (Figure 3F). In contrast, RND1 interaction with the membrane is much less stable, without a specific membrane interface (Figure 3F). The positively-charged residues in the RhoD membrane interface are almost all replaced in RND1 (Figure 3G), and hence the strong electrostatic feature of RhoD in that region is absent in RND1 (Figure 3F). In conclusion, the tight membrane interaction of RhoD can likely be attributed to the short C-terminal tail and to the positively-charged surface patch, which distinguishes RhoD from RND1.

The differential membrane interactions lead to different RBD position and dynamics

In the plexin dimer, a RhoD or RND1 molecule is located in a space confined by the membrane and the RBD domain and interacts with both simultaneously (Figure 4A and 4B). In the simulations, the RBD interacts with both RhoD and RND1 stably, although RhoD interacts with RBD with a slightly larger interface than RND1 (Figure 4C). In addition to RND1- and RhoD-bound plexin dimers, we simulated plexin monomer and dimer with the RBD domains unoccupied, each for 500 ns. We analyzed the positions of the RBD domain with respect to the GAP domain in all our simulations. The RBD domain appeared to be inherently flexible with respect to the GAP domain (Figure 4E). This is suggested by existing crystal structures of plexins, in which the RBD domain exhibited substantial flexibility with respect to the GAP domain (Figure 4F). The interface between RBD and GAP appeared to be reduced by the presence of RhoD but not by the presence of RND1 (Figure 4D). More importantly, the RMSD of the RBD domains with respect to their initial positions were increased by RhoD and decreased by RND1 (Figure 4E), indicating that RhoD likely displaces the RBD from its native position while RND1 tends to stabilize RBD at that position. Since RBD binds stably with both RND1 and

RhoD, the differential RBD positioning and dynamics may likely be attributable to the differential membrane interactions of RND1 and RhoD.

RBD affects plexin dimerization via the buttress segment

The dimerization of plexins is mediated by their dimerization helices that are immediately C-terminal to the juxtamembrane helices (Figure 1B). The interaction between two dimerization helices in the dimer, which resembles coiled-coil interactions, is reinforced by Helix 11 of the GAP domain (Figure 1C) (Wang et al., 2013). In crystal structures, Helix 11 is a stable helix, but the segment to its N-terminal is more variable structurally—it takes the form of a 3-10 or an α helix in some crystal structures but in many other structures it is disordered. When it is a 3-10 or an α helix, it becomes an extension of Helix 11 and runs adjacent and in parallel to the dimerization helix, structurally it reinforces the interaction of the two dimerization helices in resemblance to a buttress. Based on this observation we refer to it the buttress segment (Figure 1A and 1B).

The RBD is connected to the plexin GAP domain by two linkers, a C-terminal and an N-terminal linker. The C-terminal linker (Residue 1597-1662) is followed immediately by sequentially connected with the buttressing segment. This linker is long and partially disordered in crystal structures, especially in the part closer to the buttressing segment. This suggests that this linker is conformationally highly flexible. The shorter N-terminal linker (residue 1482-1495) connects RBD to the bulk of the GAP domain and is packed against the buttress segment (Figure 5A and 5B). It is likely that the N and C linkers mediate the regulation of the buttress segment by the RBD since their conformations are expected to be closely coupled with the position of the RBD on one side and with the conformation of the buttress segment on the other.

Our simulations showed that the buttress interaction with the dimerization helix is minimal in a monomeric plexin, and this interaction increases substantially in plexin dimers (Figure 5D). Importantly, with RhoD binding at the RBD, the buttress interaction with the dimerization helix in the plexin dimer is much reduced compared to that in the RND1-bound dimer or in the dimer where the RBD domains are unoccupied (Figure 5D), suggesting that RhoD weakens the buttress interaction with the dimerization helix and potentially destabilizes the plexin dimer. In simulations of the RhoD-bound plexin dimer, the buttress segments lost its helical structure and gradually disengaged the dimerization helices (Figure 5C). In contrast, in simulations of the RND1-bound dimer both the helical structure and the interaction with the dimerization helices are much more stable (Figure 5B). The difference is reflected by the smaller contact area of the buttress segments and the dimerization helices in the RhoD-bound system than in the RND1-bound system (Figure 5D). Moreover, the simulations showed that in the RhoD-bound dimer the pair of the dimerization helices was conformationally more variable than that in an RBD-unoccupied plexin dimer, and the dimerization helices in an RND1-bound dimer was less variable than the unoccupied dimer (Figure 5E and 5F). This is consistent with the notion that RhoD binding destabilizes the plexin dimerization interface while RND1 binding may stabilize the dimer.

Based on these simulation results we suggest that the differential membrane interaction of RND1 and RhoD propagates to the plexin dimerization interface and confers antithetic impact to plexin dimerization through the RBD domain and its N and C linkers (Figure 5B). RhoD binding destabilizes the RBD with respect to the GAP domain, destabilizing the buttress segment with respect to the dimerization interface, and ultimately leads to destabilization of the dimer interface. In contrast, by the same RBD-centered route, RND1 binding helps stabilize the plexin dimer.

Discussion

Plexins function in ways similar to a transistor in that they take two inputs and their responses to the primary input of semaphorin are regulated by the secondary input in form of GTPase binding at the RBD domain. RND1 serves as a promoter of plexin signaling, while RhoD serves as an inhibitor. Our structural and molecular dynamics simulations and analyses suggest that the differential effects of RND1 and RhoD may arise from their differential interactions with the membrane. RND1 interacts with the membrane loosely and non-specifically, while RhoD interacts with the membrane tightly with a specific interface. This difference gives rise to different positioning and dynamics of the RBD domain, which dictates the conformation of the buttress segment adjacent to the dimerization interface of plexin. We further showed that RhoD binding destabilizes the dimerization interface while RND1 binding helps stabilize the interface. In short, we reveal an allosteric mechanism that regulates plexin dimerization involving cell membranes, the regulatory GTPases, the RBD domain, and the buttress segment (Figure 5F).

Our results on RND1 and RhoD offer a framework for the analysis of plexin regulation by Rho GTPases. We show that the antithetic roles of RND1 and RhoD result from two seemingly minor differences. First, RhoD furnishes a much shorter C-terminal tail than RND1, and consequently, RhoD is spatially more restrained to the membrane than RND1. Secondly, RhoD features a surface region that is rich of positively-charged residue, which serves as the interface with membranes; these positively charged residues are mutated in RND1. These two differences determine that RND1 and RhoD interact with the membrane differently. We analyzed the sequences of the members of the Rho GTPase family and, to our surprise, we found that these two features are indeed correlated. The Rho GTPases with longer C-terminal tails indeed tend to feature more positively-charged residues at the putative membrane interface (Figure 6). This suggests that, besides RND1 and RhoD, other Rho GTPases may also be involved in regulations of plexin signaling, and that the GTPases of the Rho family with short C-

terminal tails may likely be down-regulators and the other with long C-terminal tails likely up-regulators. In cell biology, similar to plexin regulation by Rho GTPases, there are many other cases in which similar protein in the same family interact with their target proteins almost identically yet achieve opposite regulatory effects. Simulations are an expedient platform to gain insight into such mechanisms.

In this study, we chose to focus on plexin A4 as a representative system, despite that crystal structures of the intracellular domains are not available for A4. Unlike A4, for those plexins for which better structural data are available, direct functional data of regulation by RBD-binding of small GTPases are lacking. C1, which is arguably the best structurally characterized plexin in terms of the intracellular domains, is such an example. Even for C1, the structural information is incomplete as the C1/RhoGTPase complex structure is not available. It is thus necessary to construct models from other plexins structures regardless of our choice of plexin system. We resorted to homology modeling (see Methods) to construct A4 structures for simulations, considering the high level of sequence (35% or above overall) and structure similarly among the plexin family members, in particular in the dimer interface. The binding mode between class A plexins and RhoGTPases is particularly highly conserved, as shown by the numerous crystal structures, including that of Plexin B2/RhoD presented in this paper. We therefore believe the models of the Plexin A4/RND1 and PlexinA4/RhoD complexes are highly reliable. Moreover, our conclusion is not sensitive to the fine structures, as the main driving force is the electrostatic interactions from the interface between the GTPases and membrane (Figure 3F), rather than any specific residue-residue interactions arising from a specific conformation. Reassuringly, our findings are supported by a recent study on plexin B1, which (Li et al., 2020) identified the functional importance of the buttress segment (or “activation switch loop” as is referred therein) based on analysis of plexin enzymatic turnover, and showed that the segment helps stabilizing the dimerization helix when the plexin active site is occupied by Rap.

Our results suggest that, similar to many other signaling proteins, for plexin the membrane also plays an important role in its regulation. In a membrane environment of a high composition of negatively-charged lipids such as POPS, PIP2, and PIP3, plexin signaling is likely more susceptible to negative regulation by RhoD. There are reports that plexin signaling activates the PI3K/AKT pathway, upon which PIP2 lipids in the membrane are phosphorylated and converted to more negatively charged PIP3 lipids. Our findings raise the question as to whether down-regulation associated with RhoD binding may be part of a negative feedback mechanism for plexin signaling involving the PI3K/AKT pathway.

Supplemental Information

Supplemental information includes the diffraction data and refinement statistics of the crystal structure and orthogonal views of the asymmetric unit of the RhoD/plexin B2-RBD complex crystal.

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Data accessibility. The diffraction data and atomic coordinates of the crystal structure of the RhoD/plexin B2-RBD complex have been deposited into the PDB database under the access code 7KDC.

References

1. Bell, C.H., Aricescu, A.R., Jones, E.Y., and Siebold, C. (2011). A Dual Binding Mode for RhoGTPases in Plexin Signalling. *Plos Biology* 9.
2. Benjamin, Webb, Andrej, and Sali (2014). Comparative Protein Structure Modeling Using MODELLER. *Current Protocols in Bioinformatics*.
3. Berendsen, H.J.C., Postma, J.P.M., Vangunsteren, W.F., Dinola, A., and Haak, J.R. (1984). MOLECULAR-DYNAMICS WITH COUPLING TO AN EXTERNAL BATH. *Journal of Chemical Physics* 81, 3684-3690.
4. Bertoni, M., Kiefer, F., Biasini, M., Bordoli, L., and Schwede, T. (2017). Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology. *Scientific Reports* 7.
5. Darden, T., York, D., and Pedersen, L. (1993). PARTICLE MESH EWALD - AN N.LOG(N) METHOD FOR EWALD SUMS IN LARGE SYSTEMS. *Journal of Chemical Physics* 98, 10089-10092.
6. Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. *Acta Crystallographica Section D-Biological Crystallography* 66, 486-501.
7. Fansa, E.K., Dvorsky, R., Zhang, S.C., Fiegen, D., and Ahmadian, M.R. (2013). Interaction characteristics of Plexin-B1 with Rho family proteins. *Biochemical & Biophysical Research Communications* 434, 785-790.

8. Fiser, A., Do, R.K.G., and Sali, A. (2000). Fiser, A., Do, R. K. & Sali, A. Modeling of loops in protein structures. *Protein Sci.* 9, 1753-1773. *Protn ence* 9, 1753-1773.
9. Gu, C., and Giraudo, E. (2013). The role of semaphorins and their receptors in vascular development and cancer. *Experimental Cell Research* 319, 1306-1316.
10. Guex, N., Peitsch, M.C., and Schwede, T. (2009). Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. *Electrophoresis* 30, S162-S173.
11. Hess, B., Bekker, H., Berendsen, H.J.C., and Fraaije, J. (1997). LINCS: A linear constraint solver for molecular simulations. *Journal of Computational Chemistry* 18, 1463-1472.
12. Janssen, B.J.C., Robinson, R.A., Perez-Branguli, F., Bell, C.H., Mitchell, K.J., Siebold, C., and Jones, E.Y. (2010). Structural basis of semaphorin-plexin signalling. *Nature* 467, 1118-U1132.
13. Jo, S., Kim, T., and Im, W. (2007). Automated Builder and Database of Protein/Membrane Complexes for Molecular Dynamics Simulations. *Plos One* 2.
14. Jo, S., Kim, T., Iyer, V.G., and Im, W. (2008). Software news and updates - CHARMM-GUI: A web-based graphical user interface for CHARMM. 29, 1859-1865.
15. Jo, S., Lim, J.B., Klauda, J.B., and Im, W. (2009). CHARMM-GUI Membrane Builder for Mixed Bilayers and Its Application to Yeast Membranes. *Biophysical Journal* 97, 50-58.
16. Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W., and Klein, M.L. (1983). COMPARISON OF SIMPLE POTENTIAL FUNCTIONS FOR SIMULATING LIQUID WATER. *Journal of Chemical Physics* 79, 926-935.
17. Kuo, Y.C., Chen, H., Shang, G., Uchikawa, E., and Zhang, X. (2020). Cryo-EM structure of the PlexinC1/A39R complex reveals inter-domain interactions critical for ligand-induced activation. *Nature Communications* 11, 1953.
18. Lee, J., Cheng, X., Swails, J.M., Yeom, M.S., Eastman, P.K., Lemkul, J.A., Wei, S., Buckner, J., Jeong, J.C., Qi, Y.F., *et al.* (2016). CHARMM-GUI Input Generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM Simulations Using the CHARMM36 Additive Force Field. *Journal of Chemical Theory and Computation* 12, 405-413.
19. Li, Z.L., Müller-Greven, J., Kim, S.J., Tamagnone, L., and Buck, M. (2020). Plexin-Bs enhance their GAP activity with a novel activation switch loop generating a cooperative enzyme. *Cellular and Molecular Life sciences CMLS*.

20. Liebschner, D., Afonine, P.V., Baker, M.L., Bunkoczi, G., Chen, V.B., Croll, T.I., Hintze, B., Hung, L.-W., Jain, S., McCoy, A.J., *et al.* (2019). Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. *Acta Crystallographica Section D-Structural Biology* 75, 861-877.
21. Liu, H., Juo, Z.S., Shim, A.H., Focia, P.J., Chen, X., Garcia, K.C., and He, X. (2010). Structural basis of semaphorin-plexin recognition and viral mimicry from Sema7A and A39R complexes with PlexinC1. *Cell* 142, 749-761.
22. Lladós, J., Cores, F., and Guirado, F. (2018). Scalable Consistency in T-Coffee Through Apache Spark and Cassandra Database. *Journal of Computational Biology* 25, 894-906.
23. McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J. (2007). Phaser crystallographic software. *Journal of Applied Crystallography* 40, 658-674.
24. Nishide, M., and Kumanogoh, A. (2018). The role of semaphorins in immune responses and autoimmune rheumatic diseases. *Nature Reviews Rheumatology*.
25. Nogi, T., Yasui, N., Mihara, E., Matsunaga, Y., Noda, M., Yamashita, N., Toyofuku, T., Uchiyama, S., Goshima, Y., Kumanogoh, A., *et al.* (2010). Structural basis for semaphorin signalling through the plexin receptor. *Nature* 467, 1123-U1138.
26. Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Macromolecular Crystallography, Pt A* 276, 307-326.
27. Pascoe, H.G., Wang, Y., and Zhang, X. (2015). Structural mechanisms of plexin signaling. *Progress in Biophysics & Molecular Biology* 118, 161-168.
28. Roberts, P.J., Mitin, N., Keller, P.J., Chenette, E.J., Madigan, J.P., Currin, R.O., Cox, A.D., Wilson, O., Kirschmeier, P., and Der, C.J. (2008). Rho family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. *Journal of Biological Chemistry* 283, 25150-25163.
29. Sakurai, A., Doci, C.L., and Gutkind, J.S. (2012). Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer (vol 22, pg 23, 2012). *Cell Research* 22, 441-441.
30. Takamatsu, H., and Kumanogoh, A. (2012). Diverse roles for semaphorin-plexin signaling in the immune system. *Trends in Immunology* 33, 127-135.
31. Tamagnone, L. (2012). Emerging Role of Semaphorins as Major Regulatory Signals and Potential Therapeutic Targets in Cancer. *Cancer Cell* 22, 145-152.

32. Thiagarajan, M.M., Stracquatano, R.P., Pronin, A.N., Evanko, D.S., Benovic, J.L., and Wedegaertner, P.B. (2004). A Predicted Amphipathic Helix Mediates Plasma Membrane Localization of GRK5. *Journal of Biological Chemistry* 279, 17989-17995.
33. Tong, Y., Chugha, P., Hota, P.K., Alviani, R.S., Li, M., Tempel, W., Shen, L., Park, H.-W., and Buck, M. (2007). Binding of Rac1, Rnd1, and RhoD to a novel Rho GTPase interaction motif destabilizes dimerization of the plexin-B1 effector domain. *Journal of Biological Chemistry* 282, 37215-37224.
34. Vikis, and H., G. (2002). The Plexin-B1/Rac interaction inhibits PAK activation and enhances Sema4D ligand binding. *Genes Dev* 16, 836-845.
35. Wang, H., Hota, P.K., Tong, Y., Li, B., Shen, L., Nedyalkova, L., Borthakur, S., Kim, S., Tempel, W., Buck, M., *et al.* (2011). Structural Basis of Rnd1 Binding to Plexin Rho GTPase Binding Domains (RBDs). *Journal of Biological Chemistry* 286, 26093-26106.
36. Wang, Y., He, H., Srivastava, N., Vikarunnessa, S., Chen, Y.-b., Jiang, J., Cowan, C.W., and Zhang, X. (2012). Plexins Are GTPase-Activating Proteins for Rap and Are Activated by Induced Dimerization. *Science Signaling* 5.
37. Wang, Y., Pascoe, H.G., Brautigam, C.A., He, H., and Zhang, X. (2013). Structural basis for activation and non-canonical catalysis of the Rap GTPase activating protein domain of plexin. *Elife* 2.
38. Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., *et al.* (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Research* 46, W296-W303.
39. Williams, C.J., Headd, J.J., Moriarty, N.W., Prisant, M.G., Videau, L.L., Deis, L.N., Verma, V., Keedy, D.A., Hintze, B.J., Chen, V.B., *et al.* (2018). MolProbity: More and better reference data for improved all-atom structure validation. *Protein Science* 27, 293-315.
40. Wu, E.L., Cheng, X., Jo, S., Rui, H., Song, K.C., Davila-Contreras, E.M., Qi, Y., Lee, J., Monje-Galvan, V., Venable, R.M., *et al.* (2014). CHARMM-GUI Membrane Builder Toward Realistic Biological Membrane Simulations. *Journal of Computational Chemistry* 35, 1997-2004.
41. Yaron, A., and Zheng, B. (2007). Navigating their way to the clinic: Emerging roles for axon guidance molecules in neurological disorders and injury. *Developmental Neurobiology* 67, 1216-1231.
42. Zanata, S.M., Hovatta, I., Rohm, B., and Puschel, A.W. (2002). Antagonistic effects of Rnd1 and RhoD GTPases regulate receptor activity in semaphorin 3A-induced cytoskeletal collapse. *Journal of Neuroscience* 22, 471-477.

Figure 1

Figure 1. Architecture, dimerization, and RBD-binding of GTPase of plexins. (A) Components of a plexin molecule. Each RBD domain is connected with a GAP domain by the N and C linkers. (B) Architecture of the semaphorin-induced plexin dimer. A buttress segment is positioned between the RBD domain and the dimerization helix and Helix 11 is C-terminal to the buttress. The activation segments are held in the active conformations by the dimerization helices in trans. A Rap GTPase is bound to each GAP domain as a substrate at the active site. (C) The RND1- or RhoD-bound plexin dimer systems simulated in this study. The extracellular portions of the dimers were excluded. (D) and (E) Structural basis of RND1 stabilization and RhoD destabilization of the plexin dimer according to this study. The key difference is that the catalytic domain of RND1 is relatively detached from the membrane.

Figure 3

A Activator RND1

B Inhibitor RhoD

Figure 3. Plexin-bound RND1 and RhoD interact with the membrane differently. (A) and (B) Representative snapshots of the simulations of RND1- and RhoD-bound plexin dimer. (C) Close-up view of the membrane interaction of RhoD bound with the plexin dimer. Primarily the membrane interface consists of the positive-charged residues of RhoD at the $\alpha 4$ and the αi helices. (D) Distributions of the membrane contact area of RND1 and RhoD bound with the plexin dimer. Two RND1 or RhoD molecules are bound to the plexin dimer and the RND1- and RhoD-bound dimers are each simulated three times, hence the six sets of data for RND1 and RhoD, respectively. (E) The time series of the membrane contact area of RND1 or RhoD in juxtaposition with the time series of the RMSD of the RND1 or RhoD catalytic domains with respect to their initial positions in two representative simulations. These two quantities appear to be correlated. (F) Upper panels: the membrane contact residues of RND1 and RhoD indicated by color coding (the color coding indicates the number of lipid residues within 5 Å of the residue average in all simulations of the RND1- or RhoD-bound plexin dimer); lower panels: the surface electrostatic properties of RND1 and RhoD around their respective membrane-contacting regions. (G) Sequence alignment of RND1 against RhoD showing that 1) the positively-charged membrane-contacting residues of RhoD are mostly not conserved in

RND1, and 2) the C-terminal tail of RhoD is much shorter than that of RND1. The color-coding of the membrane-contact residues is inherited from (F).

Figure 4

Figure 4. RND1- and RhoD-bound RBD domains are positioned differently with respect to their respective GAP domains. (A) RhoD-bound plexin dimer. (B) A close-up of a part of the plexin dimer illustrating the relative positions of the membrane, the RhoD (or RND1) GTPase (purple), the RBD domain (blue), the GAP domain (Green), and the dimerization helices (yellow). (C) The RBD contact area of RND1 and RhoD, together with the relative flexibility of RND1 or RhoD relative to the respective RBD domains in terms of RMSD of the catalytic domains with the RBD domains aligned. The RBD complexes of both RND1 and RhoD appear stable. (D) The contact area of the RBD domains with their respective GAP domains. In addition to simulations of the RND1- and RhoD-bound plexin dimers, simulations of the plexin dimer and monomer with the RBD domains unoccupied are also included. RhoD-binding appears to moderately reduce the RBD-GAP contact area. (E) RBD flexibility relative to the GAP domain indicated by RMSD of the RBD domain measured with the GAP domain aligned. The data suggest that RND1-binding stabilizes the RBD conformation and RhoD-binding destabilizes it. The data from simulations of the plexin monomer and dimer with unoccupied RBD

suggests that the RBD domain is inherently flexible relative to the GAP domain. (F) Conformation of the RBD domain relative to the GAP domain in existing crystal structures of plexins. The GAP domain is aligned in generating this figure.

Figure 5

Figure 5. Interaction between the buttress segment and the dimerization helices. (A) RhoD- or RND1 bound plexin dimer. (B) Close-up of the RND1-bound dimer centered at the dimerization helices (yellow). The buttress segments (red), Helix 11 (green), the RBD domains (blue), the N (orange) and C (cyan) linkers of the RBD domains, and the activation segments (purple) are shown. (C) A similar close-up of the RhoD-bound dimer. (D) Contact area of the buttress segment with the dimerization helix. As shown, RND1-binding moderately raises the contact area, and RhoD reduces the contact area. (E) The RMSD of the dimerization helices in plexin dimers as a measurement of the stability of the dimerization interface. RhoD-binding clearly destabilizes the dimerization interface. (F) Snapshots of the dimerization helices. As shown, the dimerization helices are more flexible with respect to one another in the RhoD-bound plexin dimer. (G) A schematic summary of the mechanism by which RhoD and RND1 binding regulate plexin dimerization.

Figure 6

Figure 6. Correlation of the length of the C-terminal tail and the charge distribution at the putative membrane interface of Rho GTPases. (A) Sequence alignment of Rho GTPases at the region of the putative membrane interface; red denotes negatively charged residues and blue denotes positively charged residues. All members of the Rho family are included in this analysis with exception of RHBT1, RHBT2, and RHBT3, which furnish another domain C-terminal to the catalytic domain. (B) The number of positively- and negatively-charged residues at the membrane interface. The protein name and the number of residues of its C-terminal tail are marked next to each data point herein.

Methods

1. Construction of the simulation systems

This research included four simulation systems: a plexin monomer, a plexin dimer with RBD unoccupied, an RND1-bound dimer, and a RhoD-bound dimer. In each of these systems, the extracellular domains were absent, but the membrane and the transmembrane helix were included.

Lacking of the crystal structure for plexinA4, we constructed one monomeric structure of the intracellular portion of plexin A4 using homology modelling. The sequence of mouse plexinA4 was taken from NCBI website (<http://www.ncbi.nlm.nih.gov/protein>). The templates were selected according to the SWISS-MODEL searching results (<http://swissmodel.expasy.org/>) (Bertoni et al., 2017; Guex et al., 2009; Waterhouse et al., 2018), which were mainly the intracellular domain including mouse PlexinA1 (PDB entry 3RYT), mouse PlexinA3 (PDB entry 3IG3), mouse PlexinB1 (PDB entry 3SU8), and human PlexinC1 (PDB entry 4M8N), respectively. All the homology sequence identities of human PlexinA4 with the mouse PlexinA1, A3, B1, *h*PlexinC1 were higher than 35%. The sequence alignment was done by T-coffee (Llados et al., 2018), and the output alignment file was used to do homology modelling with Modeller 9.17 (Benjamin et al., 2014; Fiser et al., 2000). Modeller generated 100 structural models for the query sequence, and the one with the lowest estimated energy was selected for the construction of our simulation systems.

The plexinA4 dimer structure was obtained from superimposing the monomeric model of plexin A4 onto each protomer of the crystal structure of plexin C1 dimer (PDB entry 4M8N).

We also constructed the complex structure of RND1 with the RBD domain using Modeller 9.17. All the template structures selected in this research were downloaded from the Protein Data Bank (PDB) database. The templates for constructing RND1 we selected were the resolved crystal structures PlexinA2 *h*RND1(PDB entry 3Q3J). The process of the Modeller generating structures and the selection standard were the same as used in constructing plexin structure. We separately

resolved the RhoD-RBD complex structure using crystallography. This structure was incorporated into the simulation systems.

The CharmmGUI website (<http://www.charmm-gui.org/>) (Jo et al., 2007; Jo et al., 2008; Wu et al., 2014) was used to construct the systems containing the membrane. The membrane in the simulation systems was comprised of heterogeneous lipids. There were 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) molecules in the upper leaflet and POPC and negatively-charged 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) molecules with a ratio of 7:3 in the lower leaflet, so there were negative charges in the inner membrane (Jo et al., 2009).

The monomer system was a cubic box of 120 x 120 x 140 Å³ that contained 198,176 atoms in total, including water molecules and Na⁺ and Cl⁻ ions. The plexin dimer system with the RBD domains unoccupied was a cubic box of 190 x 190 x 170 Å³ that contained 639,794 atoms in total. The RND1-bound plexin dimer system was a cubic box of 190 x 190 x 170 Å³ that contained 679,235 atoms in total. The dimensions of the simulation boxes were chosen so that the minimum distance of any protein in a system was greater than 10 Å to the edge. Na⁺ and Cl⁻ ions were added to maintain physiological salinity (150 mM) and to obtain a neutral charge for the system. The system was parameterized using the CHARMM36 force field (Lee et al., 2016) and TIP3P water model (Jorgensen et al., 1983).

The above dimer system with the RBD domains unoccupied system was also used to set up the G-protein-bound dimer system. When the system of two plexin monomer inserted into the membrane was generated, the initial placement of the RND1 molecule bound to RBD of plexin was determined by firstly superimposing one monomer structure in the dimer on the complex of RBD bound with RND1 (PDB entry 3Q3J) with the RBD domains aligned, followed by superimposing one RND1 structure on the RND1 in the complex structure encoded 3Q3J. The RND1 structure was placed at the targeting position. Meanwhile, both the GTP molecule and Magnesium (Mg²⁺) ion in the complex (PDB entry 3Q3J) were superimposed on the RND1 structure. For the other

monomer, the RND1 structure as well as the GTP molecule and Mg^{2+} were also placed at the corresponding positions in the same way. Finally, the whole system of the RND1-bound plexin dimer inserted into the membrane was set up. The RND1-bound dimer system was placed in a cubic box of $190 \times 190 \times 170 \text{ \AA}^3$ and 679,235 atoms in total in the system. The RhoD-bound dimer system was a cubic box of $190 \times 190 \times 170 \text{ \AA}^3$ that contained 679,086 atoms in total. Both RhoD and RND1 were GTP-bound in the systems with Mg^{2+} coordinating the GTP binding.

2. MD Simulations

Each initial simulation system was equilibrated under NPT ensemble at 1 bar and 300 K for 5 ns, after energy minimization (50,000 steps) and a preliminary NVT equilibration (500 ps) with the position restraint applied on the heavy atoms of the protein with a force constant of $10 \text{ kJ/mol/\AA}^2$. Periodic boundary condition (PBC) was imposed on the system to eliminate the boundary effect. A cutoff distance of $12 \text{ \AA}$ was set for *van der Waals* interactions and the long-range electrostatic interactions were treated by the Particle Mesh Ewald (PME) method (Darden et al., 1993). LINCS algorithm (Hess et al., 1997) was used to constrain the covalent bonds involving hydrogen atoms. The time step was set to 2.5 fs. The temperature was controlled by the Langevin thermostat with a collision frequency of 2.0 ps^{-1} and the Berendsen barostat (Berendsen et al., 1984) was used to control the pressure at 1.0 atm. All MD simulations were performed using Gromacs 5.1.3 on Tianhe Supercomputer. Each of the simulations of the monomer and the dimer system with unoccupied RBD domains was 0.5- μs long, and each of the simulations of the RND1- and RhoD-bound dimer was 1- μs long.

3. Trajectories Analysis

3.1 Protein-protein Contact Area Calculation

All the protein-protein contact areas were calculated using Gromacs tools.

3.2 RMSD Analysis

RMSD of an RND1 or RhoD as an indicator of its position relative to the plexin

The RMSD calculation was carried out by first aligning the system by the C α atoms of the GAP domain of the plexin protomer to which the GTPases is bound to, and then the RMSD was calculated using the C α atoms of the GTPase with respect to their initial positions in the aligned simulation system.

RMSD of RND1 or RhoD as an indicator of its position relative to the RBD domain

The RMSD calculation was carried out by first aligning the system by the C α atoms of the RBD domain of the plexin protomer to which the GTPases is bound to, and then the RMSD was calculated using the C α atoms of the GTPase with respect to their initial positions in the aligned simulation system.

RMSD of RBD as an indicator of its position relative to the corresponding GAP domain

The RMSD calculation was carried out by first aligning the system by the C α atoms of the GAP domain of the same plexin protomer, and then the RMSD was calculated using the C α atoms of the RBD with respect to their initial positions in the aligned simulation system.

RMSD of the dimerization helix as an indicator of the stability of the dimer interface

The dimerization helices were first aligned using their C α atoms, and then the RMSD was calculated using the C α atoms with respect to their initial position.

3.3 The metric for protein-membrane interaction

For each residue of the protein in each simulation snapshot, the number of any lipid molecules within 5 Å of any atom of the residue is calculated. This number was averaged over each simulation (with the first 0.3 μ s of the simulation ignored) for each protein residue as a metric for the residue's membrane interaction.

4. Sequence Alignment

The sequences of human RND1 and RhoD were downloaded from NCBI website. The sequence alignment of the GTPases of the Rho family was performed using the UniProt website (<https://www.uniprot.org/>).

5. Protein expression and purification

The coding regions of the intracellular region of mouse plexin B2 with the juxtamembrane region removed (residues 1274-1842) was cloned into a modified pET-28(a) vector (Novagen) that encodes an N-terminal His6-tag followed by a recognition site for human rhinovirus 3C protease. The plasmid was transformed into the E. coli strain ArcticExpress (DE3) (Stratagene). ArcticExpress (DE3) carrying the expression plasmid was cultured at 37 °C in 100~120 mL LB medium in the presence of Gentamycin overnight. Bacterial cells were scaled up at 30 °C to reach OD600 2.0 in TB medium. Protein expression was induced by 0.2 mM IPTG at 10°C overnight. Cells were harvested by centrifugation and resuspended in Buffer A containing 10 mM Tris (pH 8.0), 500 mM NaCl, 5 % glycerol (v/v), 20 mM Imidazole, and 3 mM β -mercaptoethanol. Cells were lysed with a Avestin C3 disruptor (Avestin) and subjected to centrifugation. The plexin protein in the supernatant was captured using a 1 mL HisTrap FF column (GE Healthcare) and eluted by Buffer B containing 10 mM Tris (pH 8.0), 500 mM NaCl, 5 % glycerol (v/v), 250 mM Imidazole, and 3 mM β -mercaptoethanol. The protein was treated with recombinant human rhinovirus 3C protease at 4 °C overnight to remove the N-terminal His6-tag. The tag-removed protein was loaded to a Resource Q anion-exchange column (GE Healthcare) and eluted with a linear NaCl gradient (10 mM to 300 mM). Fractions containing plexin B2 were pooled and subjected to size exclusion chromatography with a Superdex 200 GL 10/30 column (GE Healthcare) equilibrated with Buffer C containing 20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol (v/v), and 2 mM DTT. Purified proteins were concentrated and stored at -80 °C.

The coding regions of human RhoD (residues 8–194) with the Q75L mutation, which renders the protein catalitically dead and therefore does not hydrolysize GTP, was cloned into the above-mentioned modified pET-28(a) vector. The plasmid was transformed into the bacterial strain BL21 (DE3). Protein expression was induced by 0.2 mM IPTG at 16 °C overnight. The protein

purification procedure was similar to that for plexin B2, except that all the buffers contained 2 mM MgCl₂. The RhoD protein with the Hist6-tag removed was subjected to the final purification step with a Superdex 75 GL 10/30 column with Buffer D containing 20 mM Tris (pH 8.0), 250 mM NaCl, 10 % glycerol (v/v), 2 mM MgCl₂, and 2 mM DTT.

To load the protein with GMP-PNP (guanosine 5'-[β,γ -imido]triphosphate) for crystallization, the purified RhoD protein was incubated with GMP-PNP at 20-fold molar ratio to the protein in the exchange buffer containing 20 mM Tris (pH 8.0), 250 mM NaCl, 5 % glycerol (v/v), 7.5 mM EDTA, and 1 mM DTT at RT for 2 hours. After the incubation, 20 mM MgCl₂ was added to stop exchange reaction. The protein was then subjected to gel filtration chromatography on a Superdex 75 GL 10/30 column equilibrated with Buffer D to remove excess GMP-PNP.

6. Crystallization, X-ray data collection and structure determination

Plexin B2 and GMP-PNP-loaded RhoD were mixed at 1:1 molar ratio in a buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10 % glycerol (v/v), 2 mM MgCl₂, 2 mM TCEP, and 100 μ M GMP-PNP to form the complex. The total protein concentration of the complex for crystallization was 6 mg/mL. The complex was crystallized initially at 20 °C in 0.2 M MgCl₂ and 20 % PEG3350 (w/v) in sitting-drop 96-well plates. Crystals large enough for data collection were grown for over a month with sitting-drop or hanging-drop vapor diffusion at 20 °C in 0.2 M MgCl₂, 22 % PEG3350 (w/v), and 100 mM MIB (pH 6.8, sodium malonate, imidazole, and boric acid mixed at 2:3:3 molar ratio). Crystals were cryo-protected using the crystallization buffer supplemented with 25% glycerol and flash cooled in liquid nitrogen. Diffraction data were collected at 100 K at the beamline 19ID at the advance photon source (Argonne, IL). Data were indexed, reduced and scaled with the software HKL2000 (Otwinowski and Minor, 1997). Molecular replacement using RND1 (PDB ID: 2REX) as the search model with the program phaser (McCoy et al., 2007) found two copies of RhoD in the asymmetric unit. However, repeated search using various full-length intracellular region of plexin models failed to yield any solution. In the end, the RBD of Plexin B1 (PDB ID: 2REX) as the search model lead to the solution of two copies of Plexin B2-RBD in the asymmetric unit. It is likely that the full-length intracellular region of Plexin B2 was degraded during the

prolonged incubation at 20 °C in the crystallization drops, which separated the RBD from the rest of the protein. The RBD formed the complex with RhoD, which crystallized at the end. The initial model from molecular replacement was manually modified in Coot (Emsley et al., 2010) and refined using Phenix (Liebschner et al., 2019). The density clearly showed that the two RBD domains were formed a domain-swapped dimer, with the swap occurring between residues 1509 and 1510. As a result, the N-terminal segment (residues 1463-1509) from the first molecule and the C-terminal segment (residues 1510-1565) from the second molecule pack together to form one RBD, and vis versa. The conformation of the RBD formed in this manner is very similar to other RBD structures in the database, and its binding mode with RhoD is very similar to that in other RhoGTPase/RBD complexes. This domain-swapped dimer cannot form in the context of the intact plexin, and therefore is unlikely have any biological significance. The refined structure were validated by using Molprobit as implemented in Phenix (Williams et al., 2018). The data collection and structure refinement statistics are summarized in Supplemental Table 1.

The structural mechanism underlying the antithetic effect of homologous RND1 and RhoD GTPase in plexin regulation

(Supplemental Information)

Supplemental Table 1. Diffraction data and structure refinement statistics.

Data collection	
Space group	P3 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	82.8, 82.8, 136.8
α, β, γ (°)	90, 90, 120
Resolution (Å)	50.0-3.10(3.15-3.10)
R _{sym} (%)	7.5(51.4)

$R_{\text{pim}}(\%)$	5.9(46.5)
I/σ	23.2(1.3)
$CC_{1/2}^{\#}$	0.843
Completeness (%)	99.2(91.2)
Redundancy	5.4(3.5)
Refinement	
Resolution (Å)	39.63-3.10(3.26-3.10)
No. reflections	18763
$R_{\text{work}}/R_{\text{free}}$ (%)	21.5(35.9)/26.6(37.6)
No. atoms	
Protein	4382
Ligand/ion	68
Water	8
B-factors	

Protein	123.6
Ligand/ion	115.9
Water	85.1
R.m.s deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.49
Ramachandran plot	
Favored (%)	97.1
Allowed (%)	2.9
Disallowed (%)	0
Rotamer outlier(%)	0
All-atom clashscore	5.88

*Numbers in parenthesis are for the highest resolution shell. # CC_{1/2} values shown are for the highest resolution shell.

Supplemental Figure 1. Two orthogonal views of the asymmetric unit of the RhoD/plexin B2-RBD complex crystal. The two copies of the RBD, colored blue and cyan respectively, form a domain-swapped dimer, which binds two RhoD molecules (magenta).

Supplementary Figure 1

Figure 1

Figure 2

Figure 3

A Activator RND1

B Inhibitor RhoD

C

D

E

F

G

Figure 4

Figure 5

Figure 6

A the membrane interface regions of Rho GTPases

<i>RhoD</i>	K	T	D	L	R	K	D	K	S	L	V	N	K	L	R	R	N	-	G	L	E	P	V	T	Y	H	R	G	Q	E	M	A	R	S	V	G	
<i>RND1</i>	K	T	D	L	R	T	D	L	S	T	L	M	E	L	S	H	Q	-	K	Q	A	P	I	S	Y	E	Q	G	C	A	I	A	K	Q	L	G	
<i>RND2</i>	K	L	D	M	R	T	D	L	A	T	L	R	E	L	S	K	Q	-	R	L	I	P	V	T	H	E	Q	G	T	V	L	A	K	Q	V	G	
<i>RND3</i>	K	S	D	L	R	T	D	V	S	T	L	V	E	L	S	N	H	-	R	Q	T	P	V	S	Y	D	Q	G	A	N	M	A	K	Q	I	G	
<i>RAC1</i>	K	L	D	L	R	D	D	K	D	T	I	E	K	L	K	E	K	-	K	L	T	P	I	T	Y	P	Q	G	L	A	M	A	K	E	I	G	
<i>RAC2</i>	K	L	D	L	R	D	D	K	D	T	I	E	K	L	K	E	K	-	K	L	A	P	I	T	Y	P	Q	G	L	A	L	A	K	E	I	D	
<i>RAC3</i>	K	L	D	L	R	D	D	K	D	T	I	E	R	L	R	D	K	-	K	L	A	P	I	T	Y	P	Q	G	L	A	M	A	R	E	I	G	
<i>RhoA</i>	K	K	D	L	R	N	D	E	H	T	R	R	E	L	A	K	M	-	K	Q	E	P	V	K	P	E	E	G	R	D	M	A	N	R	I	G	
<i>RhoB</i>	K	K	D	L	R	S	D	E	H	V	R	T	E	L	A	R	M	-	K	Q	E	P	V	R	T	D	D	G	R	A	M	A	V	R	I	Q	
<i>RhoC</i>	K	K	D	L	R	Q	D	E	H	T	R	R	E	L	A	K	M	-	K	Q	E	P	V	R	S	E	E	G	R	D	M	A	N	R	I	S	
<i>RhoU</i>	Q	S	D	L	R	E	D	V	K	V	L	I	E	L	D	K	C	-	K	E	K	P	V	P	E	E	A	A	K	L	C	A	E	E	I	K	
<i>RhoG</i>	K	K	D	L	R	A	Q	P	D	T	L	R	R	L	K	E	Q	-	G	Q	A	P	I	T	P	Q	Q	G	Q	A	L	A	K	Q	I	H	
<i>RhoQ</i>	Q	I	D	L	R	D	D	P	K	T	L	A	R	L	N	D	M	-	K	E	K	P	I	C	V	E	Q	G	Q	K	L	A	K	E	I	G	
<i>RhoF</i>	K	T	D	L	R	K	D	K	E	Q	L	R	K	L	R	A	A	-	Q	L	E	P	I	T	Y	M	Q	G	L	S	A	C	E	Q	I	R	
<i>RhoH</i>	Q	T	D	Q	R	E	-	-	-	-	-	-	-	M	G	P	H	-	R	A	S	C	V	N	A	M	E	G	K	K	L	A	Q	D	V	R	
<i>RhoJ</i>	Q	I	D	L	R	D	D	P	K	T	L	A	R	L	L	Y	M	-	K	E	K	P	L	T	Y	E	H	G	V	K	L	A	K	A	I	G	
<i>RhoV</i>	Q	A	D	L	R	D	D	V	N	V	L	I	Q	L	D	Q	G	G	-	R	E	G	P	V	P	Q	P	Q	A	Q	G	L	A	E	K	I	R
<i>CDC42</i>	Q	I	D	L	R	D	D	P	S	T	I	E	K	L	A	K	N	-	K	Q	K	P	I	T	P	E	T	A	E	K	L	A	R	D	L	K	

B Charges at the membrane interface regions and the C tail