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An Evolutionarily Conserved Autoinhibitory Molecular Switch in ELMO Proteins Regulates Rac Signaling

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Summary

Dedicator of cytokinesis (DOCK) proteins are guanine nucleotide exchange factors (GEFs) controlling the activity of Rac1/Cdc42 during migration, phagocytosis, and myoblast fusion [1–4]. Engulfment and cell motility (ELMO) proteins bind a subset of DOCK members and are emerging as critical regulators of Rac signaling [5–10]. Although formation of a DOCK180/ELMO complex is not essential for Rac1 activation, ELMO mutants deficient in binding to DOCK180 are unable to promote cytoskeleton remodeling [11]. How ELMO regulates signaling through DOCK GEFs is poorly understood. Here, we identify an autoinhibitory switch in ELMO presenting homology to a regulatory unit described for Dia formins. One part of the switch, composed of a Ras-binding domain (RBD) and Armadillo repeats, is positioned N-terminally while the other is housed in the C terminus. We demonstrate interaction between these fragments, suggesting autoinhibition of ELMO. Using a bioluminescence resonance energy transfer biosensor, we establish that ELMO undergoes conformational changes upon disruption of autoinhibition. We found that engagement of ELMO to RhoG, or with DOCK180, promotes the relief of autoinhibition in ELMO. Functionally, we found that ELMO mutants with impaired autoregulatory activity promote cell elongation. These results demonstrate an unsuspected level of regulation for Rac1 signaling via autoinhibition of ELMO.

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Supplemental Information
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Results and Discussion

The guanine nucleotide exchange factor (GEF) activity of dedicator of cytokinesis (DOCK) proteins is mediated by the DOCK homology region 2, a module exclusive to this family of GEFs [5, 12–14]. The identification of upstream regulators of the DOCK180-Rac pathway revealed a role for this GEF in developmental and pathological processes [2, 15–18]. Previous studies demonstrated a total requirement for engulfment and cell motility (ELMO) proteins in biological processes controlled by DOCK180 [1, 11]. Nevertheless, the molecular mechanisms by which ELMO orchestrates Rac signaling in concert with DOCK180 remain to be established. We used bioinformatics to search for novel structural elements in ELMO that could regulate Rac signaling. Threading analysis performed with the Phyre algorithm identified Armadillo repeats (ARR) in ELMO1–3 and *Drosophila* ELMO bearing structural homology to ARR found in the formin Dia1 [19] (see Figure S1A available online). Structural homology between ELMO1 and the formins Dia1 [20, 21] and FHOD1 [22] was also detected with the 3D-Jury structure prediction algorithm (Figure S1B). Finally, BLAST searches uncovered primary amino acid sequence similarity between ELMO1 and FHOD1 (Figure S1C). The region in Dia1 and FHOD1 sharing homology to ELMO is the diaphanous inhibitory domain (DID) and is characterized to engage in intramolecular interactions with a diaphanous autoregulatory domain (DAD) to maintain these proteins in a repressed state [23]. A hallmark of this regulatory switch is the presence of a GTPase-binding site N-terminal to the DID. Mechanistically, engagement of GTPases to autoinhibited formins disrupts the inhibitory DID-DAD interactions, thereby exposing their actin polymerization activity [20, 22]. Because the region in ELMO preceding the ARR interacts with RhoG [8], this led us to hypothesize that the N terminus of ELMO may constitute part of a similar autoinhibitory module. We therefore termed the ARR in ELMO as the ELMO inhibitory domain (EID) (Figure 1A). Based on sequence alignment with FHOD1, the EID is defined by one HEAT domain followed by four ARR (Figure 1B). Searching for the equivalent of the formins' DAD in ELMO is not straightforward because this functional region is not a domain but rather a short amphipathic helix. We nevertheless identified a C-terminal region in ELMO that resembles the formins' DAD [19, 24], and we named it the ELMO autoregulatory domain (EAD) (Figures 1A and 1C).

If the EID and EAD of ELMO behave like the analogous domains in formins, they should interact directly. We tested whether ELMO1$^{1–315}$ can interact with ELMO1$^{315–727}$ and found that these two ELMO1 fragments specifically coprecipitated with DOCK180 (Figure 1D). The critical residues of Dia1 and FHOD1 DIDs involved in binding the DAD, alanine 256 and valine 228, respectively, are located in a hydrophobic region of the last helix of the third ARR [19, 22]. Structure-based alignment of the ELMO EID with the DIDs of Dia1 and FHOD1 suggested L202, I204, and L205 as candidate residues potentially important for the function of the ELMO EID. By analyzing the Phyregenerated 3D model of the ELMO1 EID and comparing it to the structures of Dia1 and FHOD1, we found I204 to be surface exposed and thus likely to contribute to EAD binding (Figure S1D). We found that two mutants in this hydrophobic patch, ELMO1$^{1–315(I204D)}$ and ELMO1$^{1–315(L202E/I204D/L205E)}$, lost the ability to interact with ELMO1$^{315–727}$ in both coimmunoprecipitation and yeast two-hybrid assays (Figures 1D and 1E). Mutation of another nearby residue in the ELMO1 EID, Y216F,

did not affect the EID/EAD interaction (Figures 1D and 1E). Next, we investigated which residues in the EAD are critical in EID binding. To provide evidence that the EAD is included in the predicted α helix located between amino acids 681 and 701 of ELMO1 (Figure 1C), we used the yeast two-hybrid system. We found that nested C-terminal truncations (ELMO1$^{532–727}$ and ELMO1$^{532–707}$) maintained interaction with ELMO1$^{1–315}$, whereas further deletion of the region containing the predicted EAD (ELMO1$^{532–675}$) diminished the binding (Figure 1F). In both FHOD1 and Dia1, the conserved methionine of the DAD is responsible for extensive contacts with the DID [19, 24] (Figure 1C). Therefore, the equivalent methionine 692 and the highly conserved glutamate 693 of ELMO1 were both mutated to alanine. We found that ELMO1$^{1–315}$ was incapable of binding ELMO1$^{315–727(M692A/E693A)}$ in a yeast two-hybrid interaction assay, yet this mutant retained the ability to bind DOCK180 (Figure 1E; Figure S1E). Importantly, mutation of other residues in this region, namely R697A/L698A/L699A, had no effect on the EID/EAD interaction (Figures 1D and 1E).

The presence of GTPase-binding activity at the N terminus of ELMO proteins [8] suggests that the EID/EAD interactions could be regulated by engagement of active RhoG in a model suggestive of Dia-family formin activation [23]. Despite similarity in their DIDs, the GTPase-binding domains of Dia1 and FHOD1 are structurally unrelated [25]. In Dia1, this domain is solely α-helical and Rho selective, whereas in FHOD1, it is composed of a ubiquitin fold found in Ras-binding domains (RBDs) and is Rac specific [20, 22]. Our bioinformatic analyses uncovered that the GTPase-binding boundary of ELMO proteins belongs to the family of RBDs [26]. We found homology between ELMO, FHOD1, and c-Raf RBDs. Superimposition of FHOD1 and c-Raf RBD structures results in the alignment with ELMO shown in Figure 2A. This data allowed us to narrow in on leucine 43 as a likely candidate in the ELMO1 RBD to mediate contact to active RhoG on the basis that the analogous residue in c-Raf is in contact with Ras [26]. In GST pull-down assays, both ELMO1^{L43A} and ELMO1$^{1–315(L43A)}$ were incapable of binding RhoG^{V12} (Figure 2B). Similarly, ELMO1$^{1–315(L43A)}$ was impaired in RhoG^{V12} binding in coimmunoprecipitation assays (Figure 2C). Functionally, we found that ELMO1 mutants lacking RBD activity failed to synergize with DOCK180 and CrkII in promoting cell elongation (Figure 2D), suggesting that this domain is essential for the biological activity of the complex.

Although ELMO associates with RhoG [8], the minimal protein surface responsible for the interaction is poorly characterized. We investigated whether the RBD of ELMO is sufficient for membrane targeting by RhoG. We found that both the RBD (Myc-ELMO1$^{1–113}$) and the RBD-EID unit (Myc-ELMO1$^{1–315}$), but not the L43A mutant counterparts, relocalized to the membrane when coexpressed with RhoG^{V12} (Figure 3A; Figure S2A). ELMO1 lacking the RBD, ELMO1$^{113–727}$, also failed to relocalize to the membrane when coexpressed with RhoG^{V12} (Figure 3A). These results support the hypothesis that engagement of the RBD of ELMO proteins to GTPases may be a key event to localize and anchor the ELMO/DOCK complex at the membrane. To test whether the engagement of active RhoG to the RBD competes with the EID/EAD interaction, we performed a biochemical cell fractionation assay. We observed that, as expected, the RBD-EID unit of ELMO (Myc-ELMO1$^{1–315}$) was enriched in the membrane fraction when expressed with RhoG^{V12} (Figures 3B and 3C). Coexpression of an ELMO fragment containing the EAD (Myc-ELMO1$^{315–727}$) in this

system coerced the RBD-EID fragment away from $RhoG^{V12}$ at the membrane, increasing the proportion of Myc-$ELMO1^{1–315}$ in the cytosol (Figures 3B and 3C).

To address whether intramolecular interactions would take place in full-length ELMO, we developed a bioluminescence resonance energy transfer ($BRET^2$) ELMO conformation biosensor. We tagged ELMO2 at its extremities with GFP^{10} and *Renilla* luciferase (RlucII) tags (Figure 3D). ELMO2 was chosen because it is compatible for cloning in the $BRET^2$ vector and shares 88% similarity with ELMO1. Because our model predicts spatial proximity between the N- and C-terminal ends of ELMO proteins, the $BRET^2$ signal should occur in the autoinhibited state and decrease in the active conformation. Indeed, $BRET^2$ signal was detected when GFP^{10}-ELMO2-RlucII was expressed alone (Figure 3D). Importantly, the $BRET^2$ signal observed was independent of the concentration of ELMO2, indicating that intramolecular interactions instead of oligomerization events were being observed (Figure S2B). To test whether disturbing the EID/EAD interaction in ELMO2 leads to conformational changes, we expressed GFP^{10}-ELMO2-RlucII with function-inactivating mutations in the EID (I196D or L194E/I196D/L197E); we detected a decrease in $BRET^2$ signal, suggesting that these mutants are in an open conformation (Figure 3D). Mutation of residue L43A in the RBD did not affect $BRET^2$ signal (Figure 3D). We used this probe to test whether interaction of ELMO2 with its binding partners $RhoG^{V12}$ and DOCK180 could affect the conformation state of ELMO2. Unfortunately, the bulky tags on the GFP^{10}-ELMO2-RlucII almost totally abolished the interaction with $RhoG^{V12}$ (Figure S2C), preventing us from conclusively determining whether this GTPase can alter ELMO2 conformation in this assay. Interestingly, the binding of DOCK180 to GFP^{10}-ELMO2-RlucII, which still occurred, promoted conformational changes in ELMO2, suggesting that DOCK180 can participate in promoting the open conformation of ELMO (Figures S2D and S2E).

A previous report highlighted that ELMO can induce stress fibers [10], whereas other studies noted that ELMO has no effect on the cytoskeleton [6, 7, 11, 27]. We reasoned that if ELMO autoinhibition is important for regulating Rac signaling, activated mutants of ELMO1 should promote cytoskeletal changes. We studied the impact of ELMO1 in the presence and absence of $RhoG^{V12}$ on the morphology of HeLa cells grown on poly-L-lysine. Expression of $ELMO1^{WT}$, $ELMO1^{I204D}$, $ELMO1^{M692A/E693A}$, or $ELMO1^{L43A}$ did not induce morphological alteration in comparison to control cells (Figure S3A). In contrast, expression of $RhoG^{V12}$ amplified cell spreading as judged by morphology and quantification of the Feret's diameter (Figures 4A and 4B). When $RhoG^{V12}$ was coexpressed with $ELMO1^{WT}$ and $ELMO1^{L43A}$, membrane ruffles additionally characterized the cells, but notably, their Feret's diameters were unchanged with respect to cells expressing $RhoG^{V12}$ (Figures 4A and 4B). Strikingly, the active ELMO mutants $ELMO1^{I204D}$ and $ELMO1^{M692A/E693A}$ distinctly promoted cell elongation when expressed with $RhoG^{V12}$ (Figures 4A and 4B). We next analyzed the morphology of integrin-activated HeLa cells expressing ELMO1 and found that $ELMO1^{WT}$ and $ELMO1^{L43A}$ failed to induce cytoskeletal changes (Figures 4C and 4D). In agreement with our data suggesting a central role for the RBD in localizing ELMO at the membrane during integrin signaling, we noted that $ELMO1^{L43A}$ remained cytosolic (Figure 4C). Notably, ELMO1 mutants lacking autoregulatory properties ($ELMO1^{I204D}$ and $ELMO1^{M692A/E693A}$) efficiently accumulated

at cell extremities and induced cell elongation (Figures 4C and 4D). We found that control mutants in which the EID/EAD interaction was not abrogated, ELMO1^{Y216F} and ELMO1$^{R697A/L698A/L699A}$, behaved like ELMO1^{WT} (Figures 4C and 4D). Furthermore, we found that constitutively activated ELMO1 variants (ELMO1^{I204D} and ELMO1$^{M692A/E693A}$) were sufficient to induce a more than 2-fold increase in cell motility (Figure S3B). Significantly, uncoupling DOCK180 binding from these constitutively activated ELMO1 mutants abrogated the cell elongation phenotype, indicating that they are dependent on DOCK180-mediated activation of Rac (Figure S3C). Finally, we observed little impact on global Rac activation in either 293T or LR73 cells expressing active ELMO1 mutants (Figure S3D). Instead, we found that these mutants promote cell elongation by localizing DOCK180 at the membrane (Figure 4C).

In this study, we identified three novel domains in ELMO proteins: the RBD, EID, and EAD. We propose that the activation state of ELMO proteins is regulated, much like in Dia-family formins, via interaction with other proteins. We provided biochemical evidence that active RhoG and the ELMO EAD compete for binding to the ELMO RBD-EID unit, suggesting that RhoG could actively participate in unleashing the EID/EAD negative regulation. However, we cannot rule out the alternative hypothesis that RhoG recruits “inactive” ELMO to the membrane, where an additional interaction partner comes into play to stabilize ELMO in an active conformation. We also found that DOCK180 binding to ELMO promotes conformational changes in ELMO. This result is in agreement with several reports suggesting that coexpression of ELMO and DOCK180 is essential for optimal activity of the complex, and we now propose that this may be a consequence of favoring the open conformation of ELMO.

The physiological relevance of the RhoG/ELMO/DOCK180 interaction is not clear. In fact, new lines of evidence suggest that RhoG may contribute modestly to the regulation of this pathway. First, whereas DOCK180 mutant mice suffer from defects in myoblast fusion [4], mice lacking RhoG undergo normal development [28], suggesting that this GTPase cannot be a master regulator of DOCK180 signaling. Second, although RhoG is a bona fide ELMO binder, it is not activated by integrin engagement and is not an essential upstream component of DOCK180 in cell spreading [29]. Here, we demonstrated that ELMO recruitment at the membrane is dependent on the activity of the RBD during integrin signaling, suggesting that one or more additional GTPases, activated by integrins, must bind ELMO. The exact mechanism whereby open ELMO mutants are able to promote polarity is not understood. Our model is that ELMO may enter a repressed state to mask an intrinsic enzymatic activity much like formins do to control their actin nucleation potential. The central region of ELMO contains an uncharacterized ELM domain suspected to house GAP activity toward Arf GTPases [30]. Our structure/function analysis suggests that the ELM is essential for the polarization activity of the ELMO/DOCK180 complex (data not shown). We are currently testing whether the ELM carries GAP enzymatic activity and, more importantly, whether the autoinhibitory switch regulates it.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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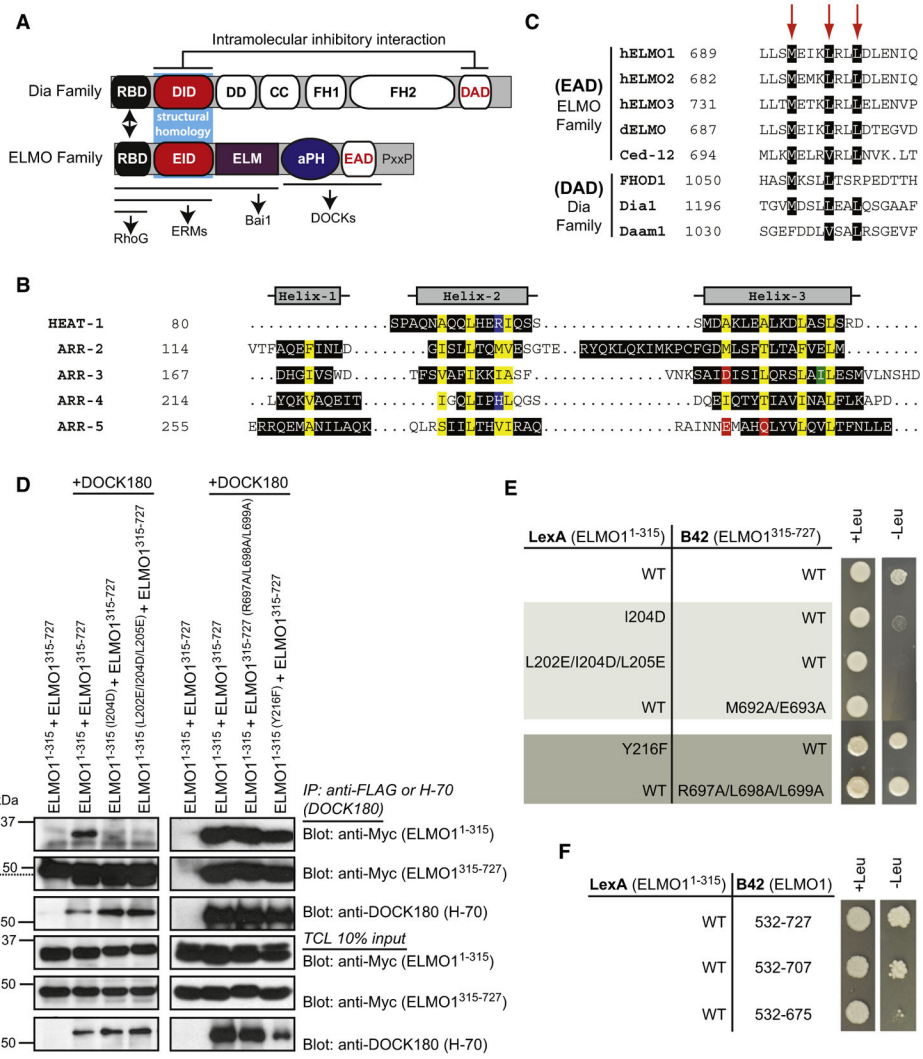


Figure 1. Intramolecular Interactions in ELMO1 through Novel Domains

(A) Schematic representation of the structural homology between ELMO and Dia-family formins.

(B) The ELMO1 EID domain is composed of HEAT and Armadillo repeats (ARR). Predicted α helices are shown in gray, hydrophobic residues of the ARR consensus sequence in yellow, and polar residues in blue and red. I204 in ARR-3 (green) is a conserved residue of ELMO proteins.

(C) Sequence alignment of the autoregulatory domains of ELMO (EAD) and Dia-related formins (DAD). Red arrows indicate highly conserved residues forming the core motif.

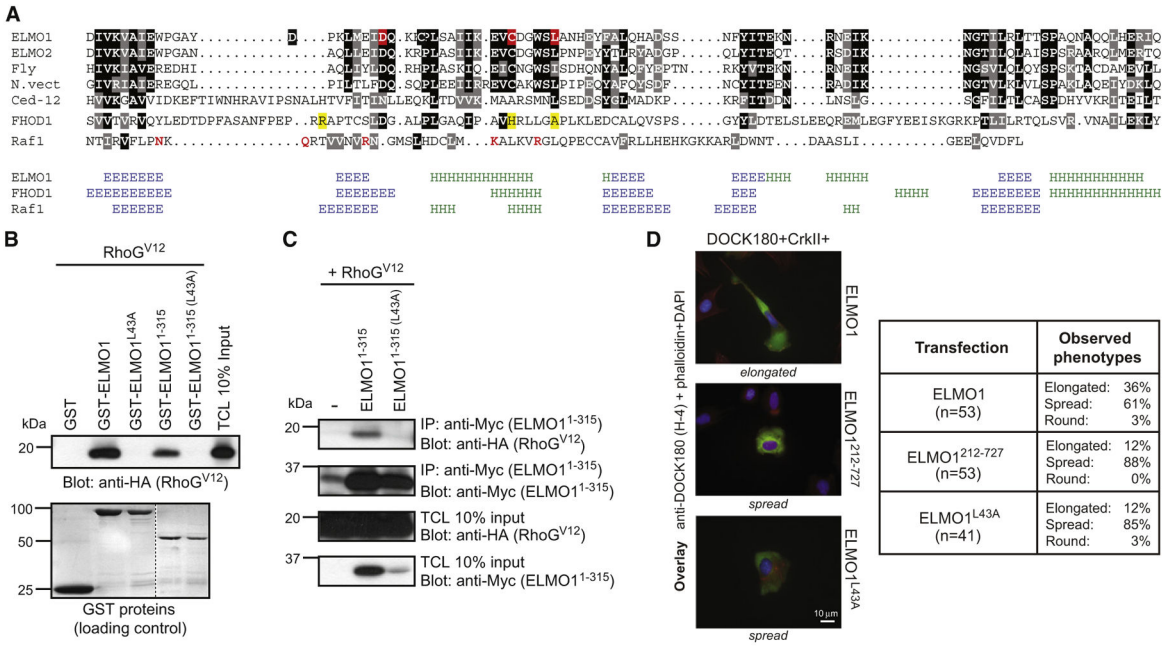
(D and E) Mutation of critical EID or EAD residues disrupts EID/EAD interaction in coimmunoprecipitation (D) and the yeast two-hybrid system (E).

(D) Lysates of HEK293T cells transfected with the indicated plasmids were subjected to immunoprecipitation with an anti-FLAG (lanes 1–4) or anti-DOCK180 H-70 (lanes 5–8) antibody. Immunoblots were analyzed with anti-Myc (ELMO1) and anti-DOCK180 (H-70) antibodies. “HC” indicates IgG heavy chain.

(E) Yeasts cotransformed with LexA fusion construct of ELMO1$^{1–315}$ and B42 fusion constructs of ELMO1$^{315–727}$ were grown on nonselective and selective (–Leu) media for a nutrient-selective growth assay.

(F) Mapping of critical EAD region boundaries. Yeasts cotransformed with the indicated plasmids were assayed as in (E).

See also Figure S1.



Transfection	Observed phenotypes	
ELMO1 (n=53)	Elongated:	36%
	Spread:	61%
	Round:	3%
ELMO1$^{212-727}$ (n=53)	Elongated:	12%
	Spread:	88%
	Round:	0%
ELMO1^{L43A} (n=41)	Elongated:	12%
	Spread:	85%
	Round:	3%

Figure 2. The N Terminus of ELMO1 Contains a Ras-Binding Domain

(A) Secondary structure and sequence comparison between ELMO-family proteins, FHOD1, and Raf1 indicates an evolutionarily conserved Ras-binding domain (RBD) characterized by the presence of a ubiquitin-like subdomain. ELMO secondary structure was predicted with Jpred3. FHOD1 (Protein Data Bank ID code 3DAD) and Raf1 (Protein Data Bank ID code 1GUA) structures were used for the manual alignment with the ELMO RBDs. Residues potentially involved in contacting RhoG are shown in red. E indicates β strand, H indicates α-helical.

(B and C) L43A mutation in the ELMO1 RBD abolishes the interaction with RhoG^{V12}.

(B) GST-tagged versions of the indicated ELMO1 proteins were used to pull down HA-tagged RhoG^{V12} from HEK293T lysates. Bound proteins were detected by immunoblotting with an anti-HA antibody.

(C) Transfected HEK293T cells were subjected to immunoprecipitation against Myc-tagged ELMO1. Bound proteins were analyzed by immunoblotting with anti-HA (RhoG^{V12}) and anti-Myc (ELMO1) antibodies.

(D) Mutational inactivation or deletion of the ELMO1 RBD results in defective cell elongation. Left: images show overlay of anti-H-4 (DOCK180), rhodamine phalloidin, and DAPI stains. Scale bar represents 10 μm. Right: several independent fields of the experiments were scored for the indicated phenotypes.

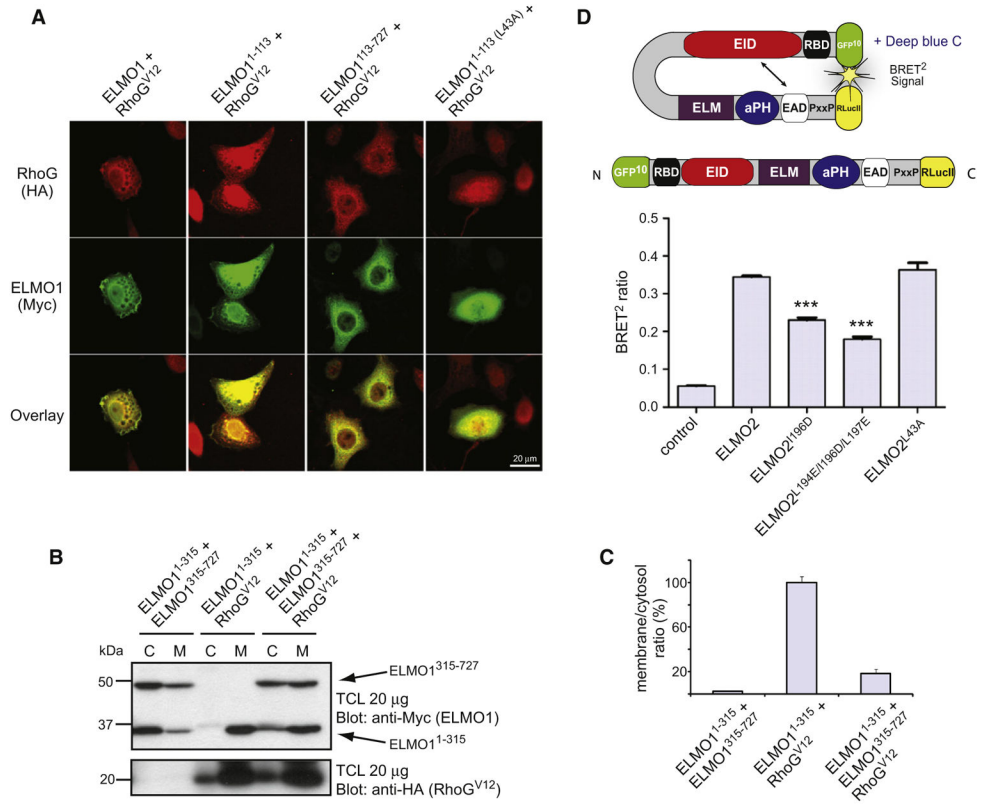


Figure 3. Full-Length ELMO2 Is Autoinhibited and Regulated by RhoG Binding to the RBD

(A) Membrane recruitment of the ELMO1 RBD by RhoG. HeLa cells were transfected with the indicated plasmids, and ELMO1 and RhoG^{V12} localization was analyzed with anti-Myc and anti-HA antibodies, respectively. Scale bar represents 20 µm.

(B and C) In the presence of RhoG^{V12}, the ELMO1 EAD-containing fragment coerces ELMO1 RBD-EID away from the membrane and into the cytosol.

(B) HEK293T cells were transfected with the indicated plasmids, and cytosolic and membrane fractions were biochemically purified and analyzed via immunoblotting with the indicated antibodies.

(C) Quantification of band intensity was used to calculate the ratio of protein found in the membrane versus the cytosol. Error bars represent standard deviation; n = 3.

(D) Disrupting the EID/EAD interaction leads to conformational changes in ELMO2. Top: schematic model of the GFP^{10}-ELMO2-RlucII conformation biosensor. Bottom: luminescence at 400 nm and 510 nm was measured upon addition of DeepBlueC in HEK293T cells expressing the indicated proteins. Analysis of variance and Bonferroni's multiple comparison were performed to compare each condition. ***$p < 0.001$; error bars represent standard error of the mean; n = 3.

See also Figure S2.

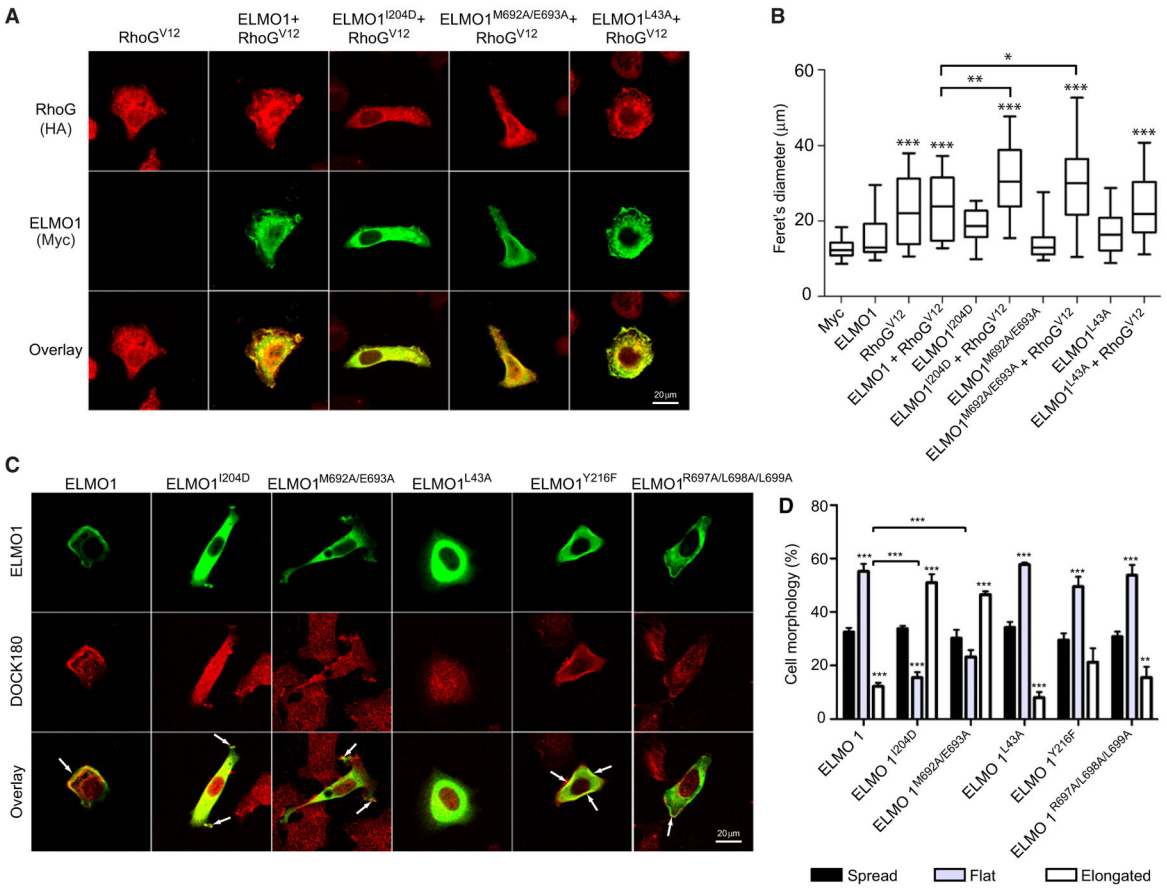


Figure 4. The EID/EAD Intramolecular Interaction Is a Regulatory Feature of ELMO in Cells

(A) Activated ELMO1 mutants synergize with RhoG to promote cell elongation. HeLa cells were transfected with the indicated plasmids, and ELMO1 and RhoG^{V12} localization was observed with anti-Myc and anti-HA antibodies, respectively. Scale bar represents 20 μm.

(B) Quantification of the effect of ELMO1 mutations on cell elongation. The morphology of cells in (A) was analyzed with anti-Myc antibodies. For each condition, the Feret's diameter of >40 cells was measured (bars represents lowest and highest values; see Supplemental Experimental Procedures).

(C) Activated ELMO1 mutants promote cell elongation on fibronectin. Serum-starved LR73 cells transfected with the indicated ELMO1 plasmids were detached and plated on fibronectin-coated chambers for 2 hr. Cells were stained for ELMO1 (anti-Myc) and DOCK180 (H-70). Scale bar represents 20 μm.

(D) Quantification of cell morphology (see Supplemental Experimental Procedures). For each condition, >100 cells were analyzed. In (B) and (D), analysis of variance and Bonferroni's multiple comparison were performed to compare each condition. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$; error bars represent standard error of the mean; n = 3.

See also Figure S3.