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A Steric-Inhibition Model for Regulation of Nucleotide Exchange via the Dock180 Family of GEFs

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Summary

CDM (CED-5, Dock180, Myoblast city) family members have been recently identified as novel, evolutionarily conserved guanine nucleotide exchange factors (GEFs) for Rho-family GTPases [1–7]. They regulate multiple processes, including embryonic development, cell migration, apoptotic-cell engulfment, tumor invasion, and HIV-1 infection, in diverse model systems [4, 6, 8–16]. However, the mechanism(s) of regulation of CDM proteins has not been well understood. Here, our studies on the prototype member Dock180 reveal a steric-inhibition model for regulating the Dock180 family of GEFs. At basal state, the N-terminal SH3 domain of Dock180 binds to the distant catalytic Docker domain and negatively regulates the function of Dock180. Further studies revealed that the SH3:Docker interaction sterically blocks Rac access to the Docker domain. Interestingly, ELMO binding to the SH3 domain of Dock180 disrupted the SH3:Docker interaction, facilitated Rac access to the Docker domain, and contributed to the GEF activity of the Dock180/ELMO complex. Additional genetic rescue studies in *C. elegans* suggested that the regulation of the Docker-domain-mediated GEF activity by the SH3 domain and its adjoining region is evolutionarily conserved. This steric-inhibition model may be a general mechanism for regulating multiple SH3-domain-containing Dock180 family members and may have implications for a variety of biological processes.

Results and Discussion

An Inhibitory Interaction between the SH3 and Docker Domains of Dock180

Previous studies showed a functional requirement for at least two regions of Dock180 during phagocytosis and cell migration [1, 11]. These include both the catalytic Docker domain that is located within the C-terminal half of Dock180 and mediates nucleotide exchange on Rac and an approximately 350 amino acid N-terminal region that contains an SH3 domain. To better define the molecular features of this N-terminal region of Dock180, we generated various Dock180 mutants (Figure S1 in the Supplemental Data available with this article online). Biochemical characterization of these mutants revealed two ELMO-interacting sites within this region. One interaction is mediated by the binding of the Dock180 SH3 domain to the PxxP motif of ELMO (Figure 1A). In addition, a second region adjacent to the SH3 domain also binds ELMO and was disrupted by a G171E mutation in Dock180 (Figure 1B; see below for description of G171E). It is noteworthy that these two types of interaction are independent of each other.

While characterizing these Dock180 mutants, we unexpectedly observed that the SH3-domain-deleted Dock180 was more active in a phagocytosis assay than wild-type Dock180 (Figure 1C, lanes 6 and 8), suggesting that the SH3 domain of Dock180 might play a basal inhibitory role. One possible mechanism for such SH3-mediated negative regulation is through binding to another region (or other regions) of Dock180. Consistent with this notion, the isolated SH3 domain (Dock1-83) or a slightly larger N-terminal fragment of Dock180 (Dock1-161), coprecipitated with Dock (ΔSH3) (Figure 1D and data not shown). No interaction was detectable between the SH3 domain and the C-terminal proline-rich tail of Dock180 (data not shown). When other regions of Dock180 were examined, the Dock1–161 fragment or the isolated SH3 domain readily bound the catalytic Docker domain (Figure 1E, lane 4; Figure S2) Intriguingly, the SH3:Docker interaction does not seem to be a typical SH3:PxxP binding; first, the W45A mutation within the Dock180 SH3 domain, which abolished the domain's binding to the PxxP motif of ELMO (Figure 1A), had no effect on binding to the Docker domain (Figure 1E, lane 5); second, disrupting the single PxxP motif within the Docker domain did not affect the Docker:SH3 interaction (Figure 1E, lane 6).

We then aligned the amino acid sequences from the SH3 domains of CDM family members and compared the consensus sequence with those from other SH3 domains. We identified a conserved isoleucine residue (I32 of Dock180) within CDM family members; in most other SH3 domains, this residue is a lysine or glutamate (Figure S3). Of note, this I32 residue resides in the ligand binding RT loop of the SH3 domains. Mutating this isoleucine residue to lysine (I32K) completely abrogated the SH3:Docker interaction (Figure 1F, lane 3).

We then tested whether the I32K mutation introduced into the full-length Dock180 could functionally mimic the effects of deleting the SH3 domain. Remarkably, the I32K mutation enhanced the activity of Dock180 in phagocytosis in a manner similar to that of the SH3-domain deletion (Figure 1C, lanes 7 and 8). When one considers that the I32K mutation disrupted the SH3

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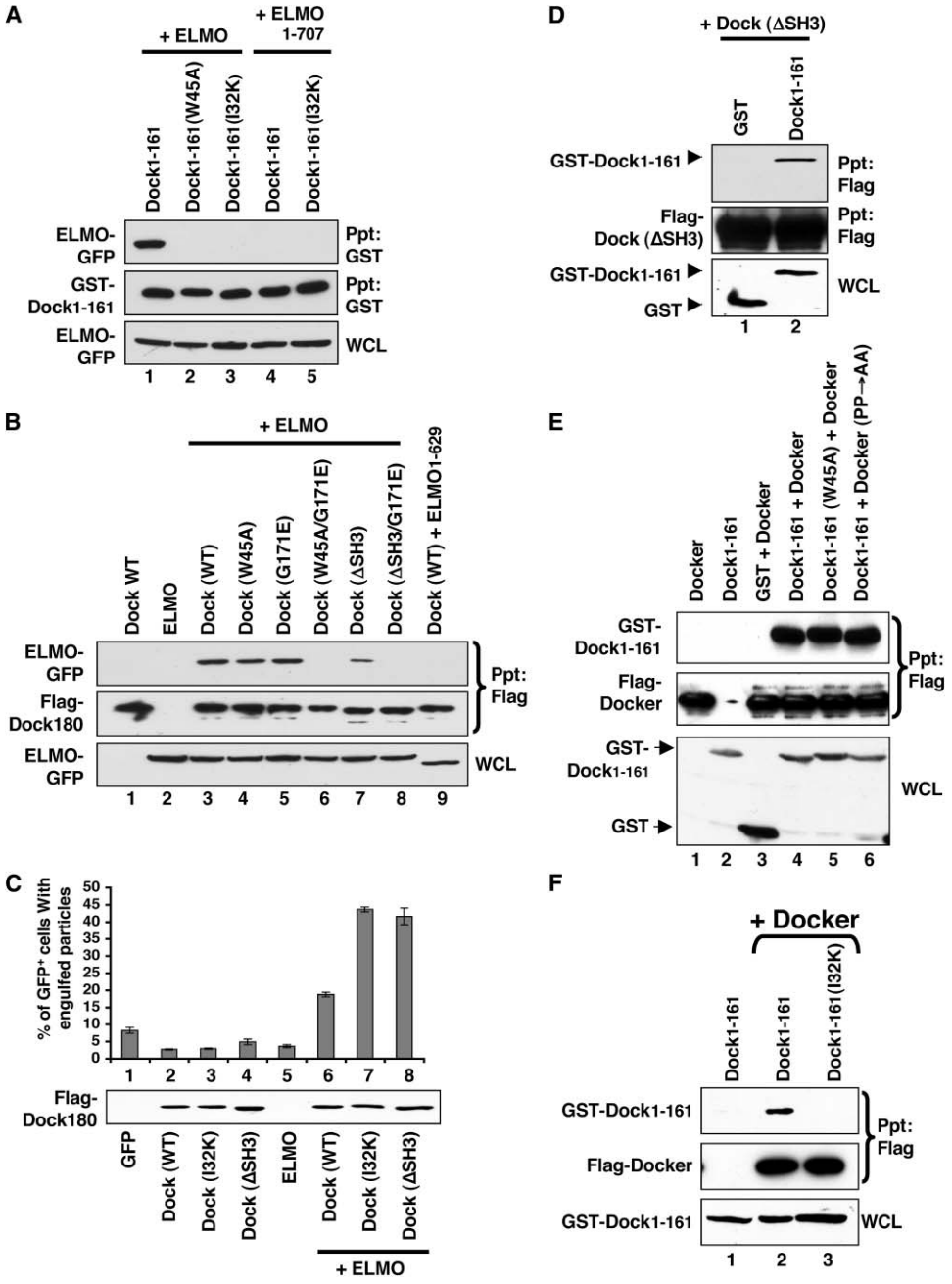


Figure 1. An Inhibitory Interaction between SH3 and Docker Domains of Dock180

(A) The PxxP motif of ELMO binds to the SH3 domain of Dock180. GST-Dock1-161 or its W45A or I32K mutant version was transfected into 293T cells with either full-length ELMO-GFP or its PxxP-deletion mutant. After GST precipitation, the bound proteins were analyzed by immunoblotting.

(B) The second interaction between Dock180 and ELMO and its disruption by G171E mutation. Flag-Dock180 or one of its mutants was coexpressed with ELMO-GFP in 293T cells. Pre-cleared cell lysates were subject to anti-Flag precipitation. The bound proteins were analyzed by immunoblotting. The ELMO1-629, which does not bind Dock180 [1], was used as a negative control.

(C) Phagocytosis assay. LR73 cells were transfected with wild-type Flag-Dock180 or one of its mutants together with either GFP (lanes 2–4) or ELMO-GFP (lanes 6–8). The percentage of GFP-positive cells with engulfed particles was determined in a flow-cytometry-based engulfment assay. The error bar represents the standard deviation.

(D) The SH3 domain of Dock180 binds to the SH3 domain-deleted Dock180 mutant. Flag-Dock (ΔSH3) was coexpressed in 293T cells with either GST or GST-Dock1-161. Cleared cell lysates were precipitated with anti-Flag and immunoblotted with the indicated antibodies.

(E) Interaction between the SH3 domain and the Docker domain of Dock180. Wild-type or mutant Flag-Docker domain and the indicated GST-Dock1-161 or its W45A mutant were expressed in 293T cells, precipitated with anti-Flag antibody, and immunoblotted with the indicated antibodies. The PP→AA mutation disrupted the single PxxP motif within the Docker domain.

(F) I32K mutation within the SH3 domain disrupts SH3 interaction with the Docker domain. Flag-Docker domain was cotransfected with GST-Dock1-161 or its I32K mutant into 293T cells, precipitated with anti-Flag antibody, and immunoblotted with the indicated antibodies.

binding to Docker, these data support the hypothesis that the SH3:Docker interaction inhibits the functional activity of Dock180.

SH3 Domain Blocks Rac Access to the Docker Domain

Previous studies have shown that the binding of nucleotide-free Rac to the Docker domain is critical for Rac activation by the Dock180/ELMO complex [1]. We hypothesized that the SH3 domain might exert its inhibitory effect by blocking the access of Rac to the Docker domain. Interestingly, the Dock (ΔSH3) mutant associated with nucleotide-free Rac significantly better than wild-type Dock180 (Figure 2A). A similar result was seen with another CDM family member, Dock2 [17], wherein deletion of the N-terminal region resulted in better Rac binding (Figure 2B). Moreover, addition of nucleotide-free Rac inhibited the SH3 domain binding to the Docker domain (Figure 2C, lanes 2 and 3), suggesting that the SH3 domain and nucleotide-free Rac competitively bind the Docker domain. Under the same conditions, the GDP bound Rac, which does not bind the Docker domain with high affinity, did not reduce the SH3:Docker interaction (Figure 2C, lanes 5 and 6).

Compared with wild-type Dock180, the Dock (ΔSH3) mutant also showed increased basal Rac activation in cells (increase of 1.6-fold $\pm$ 0.2-fold, $p < 0.05$, $n = 3$) (Figure 2D). Deleting the SH3 domain from Dock180 also rendered it more active in the in vitro GEF assay (Figure S4). Recent studies suggest that wild-type Dock4 can promote Rac-GTP loading within cells when it is overexpressed (M.H. and V.Y., unpublished data). Also, compared to wild-type Dock4, Dock4 in which the SH3 domain was deleted led to increased Rac-GTP loading in cells (increase of 1.7-fold $\pm$ 0.3-fold, Figure 2D). These results suggested that the SH3 domain of CDM proteins has a negative regulatory role with regard to the Docker-domain-mediated Rac activation and that the removal of such inhibition may be required for the optimal activation of this family of proteins.

ELMO Binding to the SH3 Domain Relieves the Inhibitory SH3:Docker Interaction

Compared with Dock180 alone, the Dock180/ELMO complex binds to the nucleotide-free Rac more efficiently and shows higher Rac-GEF activity [1, 10, 18]. Based on previous studies [1, 2, 10, 11, 19, 20], ELMO was considered as a prime candidate for disrupting the SH3:Docker interaction via binding to the Dock180 SH3 domain (Figure 3A). Consistent with this model, coexpression of ELMO reduced the amount of Dock1–161 that coprecipitated with the Docker domain (Figure 3B, lanes 1 and 3). In contrast, the Dock1–161:Docker interaction was not reduced by an ELMO mutant that lacks the PxxP motif (lanes 1 and 5). Similarly, the binding of the Docker domain to the Dock1–161 (W45A) mutant was not reduced by coexpression of ELMO (lanes 2 and 4). The Dock180 SH3 domain did not appear to simultaneously bind the Docker domain and ELMO because no detectable ELMO coprecipitated with the SH3 domain bound to the Docker domain (data not shown). Furthermore, the I32K mutation within the SH3 domain disrupted its binding to both the Docker domain and ELMO (Figures 1A and 1F). Collectively, these results suggest that the binding of the PxxP motif of ELMO to the SH3 domain of Dock180 can disrupt the SH3:Docker interaction.

Consistent with the above observations, the binding of an ELMO fragment carrying the PxxP motif enhanced Rac binding to Dock180 (Figure 3C, lanes 2 and 5) to an extent roughly equal to that observed with the Dock (ΔSH3) mutant (lanes 1 and 5). In contrast, coexpression of ELMO (lanes 3 and 6) did not affect the binding of nucleotide-free Rac to the Dock (W45A) mutant. Of note, here we used the W665A mutant of ELMO to avoid the ELMO PH-domain-mediated contribution to Rac binding [18]. Similarly, ELMO interaction with Dock2 contributed to enhanced Rac binding (Figure 2B). Collectively, these data suggested that the PxxP motif of ELMO binding to the SH3 domain of CDM proteins could relieve the SH3-domain-mediated steric hindrance.

We next assessed the functional relevance of the Dock-SH3:ELMO-PxxP interaction. In a fluorescence-based GEF assay, in which the association between Dock180 and ELMO was primarily mediated via the SH3:PxxP interaction, the Dock180/ELMO complex showed 2-fold enhancement in GEF activity over Dock180 alone (Figure 3D, curves e and i). This enhancement was similar to that observed with wild-type Dock180 (curves d and g). In contrast, the SH3 mutant Dock (W45A) failed to synergize with ELMO (W665A) in the in vitro GEF assay (data not shown). When examined in the Dock180/ELMO-mediated, Rac-dependent Transwell migration of LR73 cells, the Dock (W45A) mutant failed to synergize with ELMO in promoting cell migration, whereas wild-type Dock180 promoted migration (Figure 3E, lanes 5 and 6).

At the level of the whole organism, we assessed the effect of disrupting the PxxP:SH3 interaction in *C. elegans* gonadal distal-tip cell (DTC) migration. During *C. elegans* development, the distal-tip cell migrates in a stereotypical U-shaped pattern to define the shape of the adult hermaphrodite gonad. In worms with null mutations in *ced-12* or *ced-5*, the DTC frequently mismigrates with extra or wrong turns. This phenotype can be rescued, respectively, by transgenic expression of wild-type *ced-12* or *ced-5*. However, a *ced-12* transgene with mutations of its PxxP motif was less efficient in rescuing the DTC-migration defects in CED-12 null animals (Table 1). Similarly, a *ced-5 (ΔSH3)* mutant was also less efficient in rescue of DTC mismigration in CED-5 null worms (Table 1). Taken together, these data suggested that the Dock-SH3:ELMO-PxxP interaction is functionally relevant in vitro and in vivo and that a requirement for this interaction is evolutionarily conserved.

The Region Adjacent to the SH3 Domain Also Affects the GEF Activity of Dock180

The regulation of the basal activity of Dock180 by its N-terminal region was also supported by another set of observations. In *Drosophila* Myoblast city, a single point mutation within the region adjacent to its SH3 domain (corresponding to G171E mutation in Dock180; Figure S5) resulted in dorsal-closure and myoblast-fusion defects comparable to those of *Mbc* null alleles [9]. Inter-

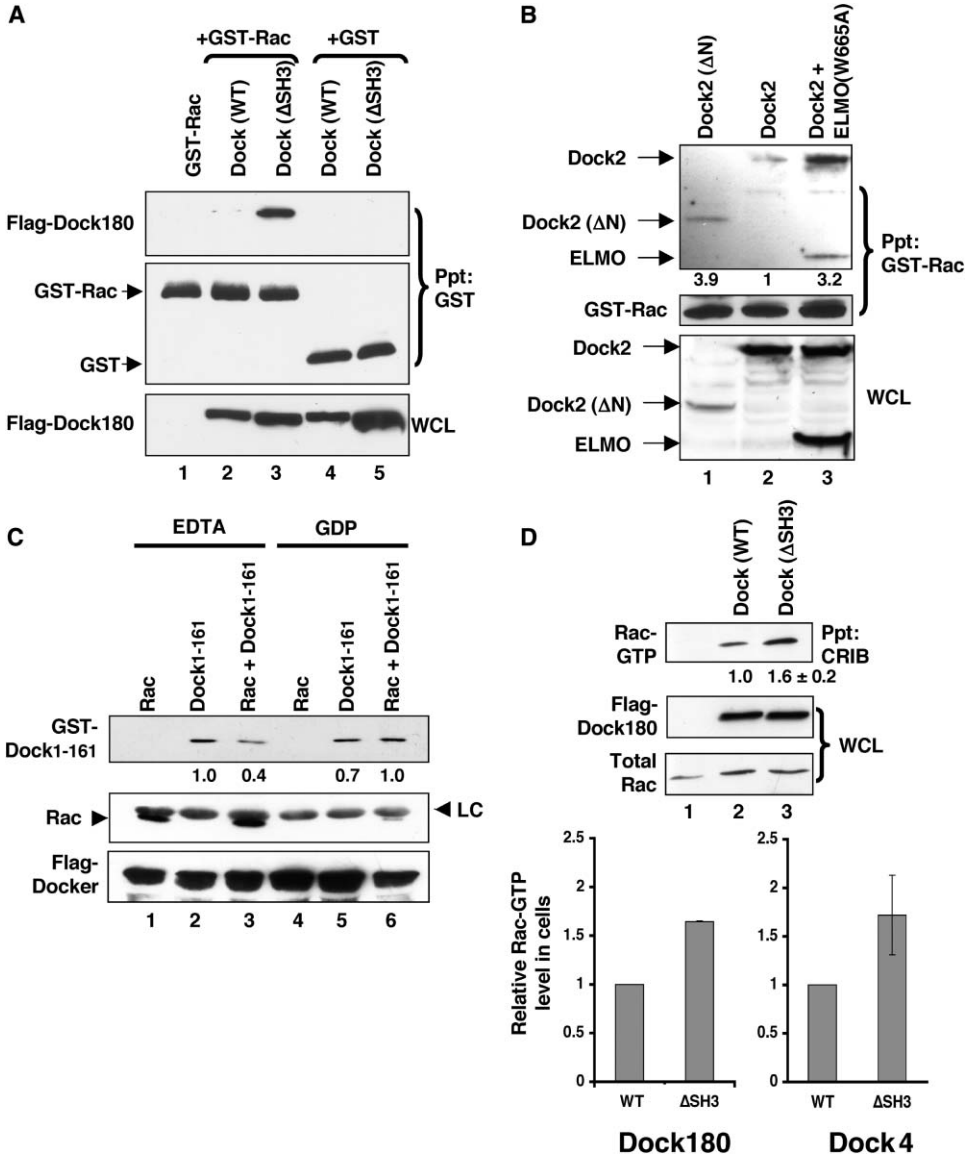


Figure 2. SH3 Domain Blocks the Access of Rac to the Docker Domain

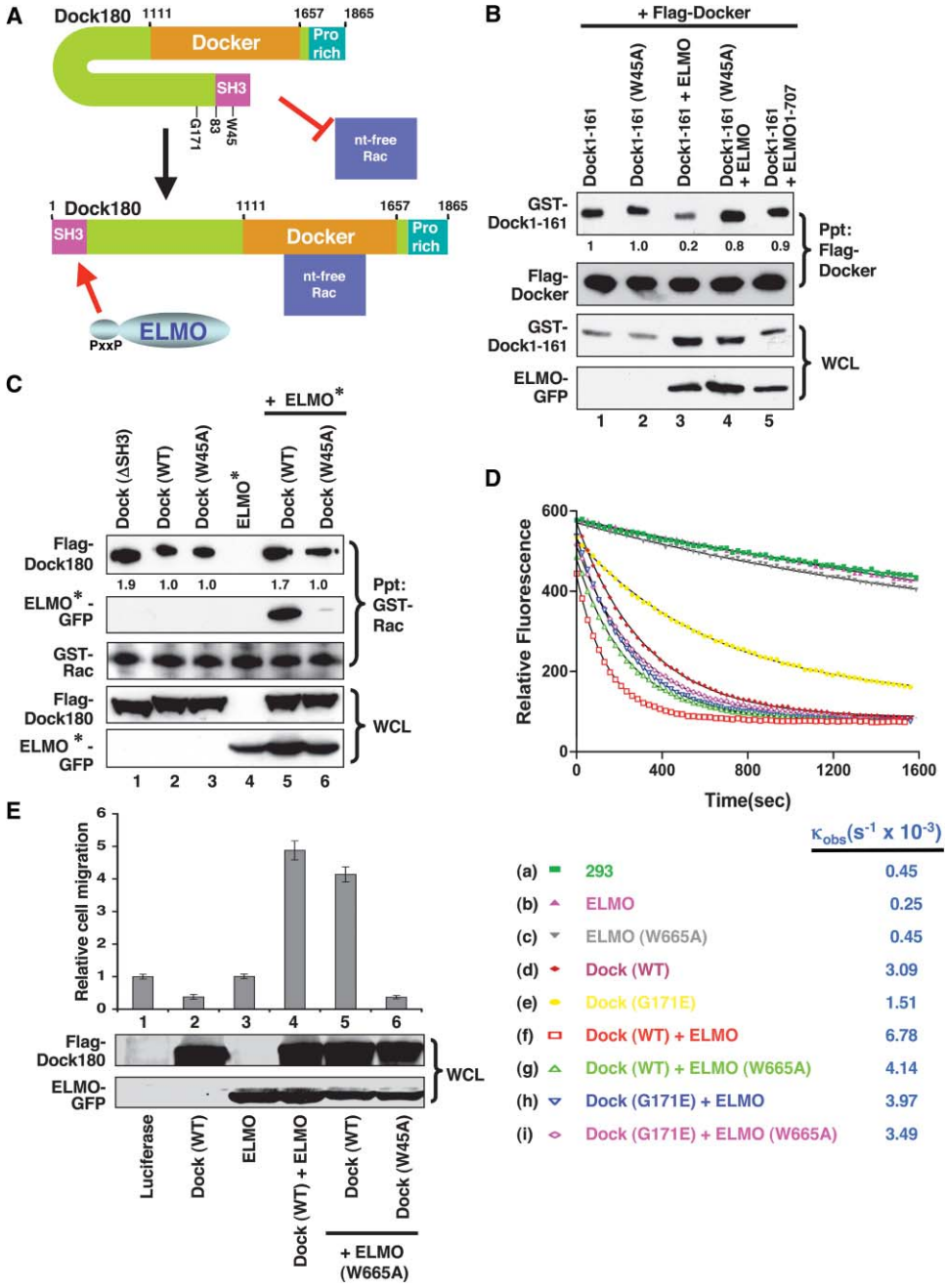
(A) Deletion of the SH3 domain promotes the binding of nucleotide-free Rac to Dock180. Flag-Dock180 or its SH3-deletion mutant was transfected into 293T cells with either GST or GST-Rac. The total cell lysates were subjected to GST precipitation in the presence of 10 mM EDTA and immunoblotted with the indicated antibodies.

(B) Deletion of the N-terminal region of Dock2 or association with ELMO enhances nucleotide-free Rac binding to Dock2. A Dock2 (ΔN) construct [17] or wild-type Dock2 with or without ELMO was expressed in 293 T cells, and lysates were precipitated with coexpressed GST-Rac (in the presence of EDTA) and assessed for coprecipitation of Dock2 or its ΔN mutant. It is notable that the Dock2 (ΔN) construct was poorly expressed in comparison to wild-type Dock2. After densitometry, the signals for Rac-associated Dock2 were normalized against protein expression in the total lysates, and the signal intensity for wild-type Dock2 alone was arbitrarily set to 1.

(C) Nucleotide-free Rac competes with Dock1-161 in binding to the Docker domain. Flag-Docker was expressed and precipitated from transfected 293T cells. Precipitates were then incubated with bacterially produced Dock1-161 or Rac (1 μg of each) as indicated. Normalized quantitation of the intensity of the GST-Dock1-161 bands is indicated below each band. The results are representative of two independent experiments. LC indicates the light chain of antibody.

(D) SH3-domain-deleted Dock180 and Dock4 are more active in Rac activation within cells. 293T cells were cotransfected with Rac and wild-type Dock180 or its ΔSH3 mutant. The intracellular Rac-GTP level was assessed, in triplicates, by precipitation with GST-CRIB beads and immunoblotting for Rac (upper panel). Rac-GTP levels were normalized against the expression level of Rac and Dock180 proteins (lower left panel). Results from similar experiments comparing wild-type and ΔSH3 versions of Dock4 were also presented (lower-right panel). The error bars denote one standard deviation.

estingly, G171E mutation within Dock180 resulted in an approximately 50% reduction in the basal in vitro GEF activity (Figure 3D, curves d and e). This G171E mutation also disrupted the second interaction of Dock180 with ELMO (Figure 1B). These combined effects might explain the severe phenotypes associated with this mutation in *Drosophila*. When tested in CED-5-deficient worms, the Dock (G171E) mutant was completely defec-



		$\kappa_{obs}(s^{-1} \times 10^{-3})$
(a)	293	0.45
(b)	ELMO	0.25
(c)	ELMO (W665A)	0.45
(d)	Dock (WT)	3.09
(e)	Dock (G171E)	1.51
(f)	Dock (WT) + ELMO	6.78
(g)	Dock (WT) + ELMO (W665A)	4.14
(h)	Dock (G171E) + ELMO	3.97
(i)	Dock (G171E) + ELMO (W665A)	3.49

Figure 3. ELMO Relieves the SH3-Mediated Inhibition of Dock180

(A) Model figure depicting the binding of ELMO to the SH3 domain of Dock180. This binding disrupts the SH3:Docker interaction and allows Rac better access to the Docker domain.

(B) Binding of the PxxP motif of ELMO to the SH3 domain of Dock180 disrupts the SH3:Docker interaction. Flag-Docker domain was coexpressed in 293T cells with wild-type or mutant GST-Dock1-161 in the presence or absence of ELMO-GFP. Cell lysates were subject to anti-Flag-antibody precipitation, and the coprecipitation of Dock1-161 in the presence or absence of ELMO was assessed by immunoblotting. After densitometry, the signal for GST-Dock1-161 in the precipitates was normalized against its expression level in the total cell lysates, and the GST-Dock1-161 value was set at 1 for the purpose of comparison. The results are representative of three independent experiments.

(C) ELMO enhances the binding of nucleotide-free Rac to wild-type Dock180 but not its W45A mutant. Indicated Dock180 or its mutant was expressed in 293T cells with ELMO532-727 (W665A)-GFP (denoted as ELMO*). Cell lysates were subject to precipitation with bacterially produced GST-Rac in the presence of EDTA.

(D) In vitro GEF assay. Flag-Dock180 or its G171E mutant was expressed in 293T cells either alone or together with ELMO-GFP or its W665A mutant as indicated. The proteins were precipitated with anti-Flag antibody and eluted with Flag peptide. The eluted proteins were quantitated after anti-Flag blotting, and equal amounts of Dock180 protein were used in a mant-GDP fluorescence-based in vitro GEF assay. The observed mant-GDP dissociation-rate constants (κ_{obs}) were indicated.

(E) Transwell migration assay. Wild-type Dock180 or its W45A mutant was transfected into LR73 cells with ELMO (W665A)-GFP. The relative cell migration of transfected cells through a Transwell was scored.

Table 1. The SH3 and Adjacent Regions of CED-5/Dock180 and the PxxP Motif of CED-12 Influence DTC Migration In Vivo

Genotype	Mismigration (Percent)	*n*
(A) Effect of SH3 Deletion of CED-5 or PxxP Mutation of CED-12 on DTC Migration		
DTC Mismigration in *ced-5*- and *ced-12* Null Worms Is Rescued by *ced-5* and *ced-12*		
Wild-type	0	204
ced-5(n1812)	35	314
ced-12(k149)	36	305
ced-5(n1812); opEx732 [P_{eft-3}::ced-5(WT)]	5	173
ced-12(k149); opEx872 [P_{eft-3}::ced-12(WT)::yfp]	9	302
Partial Rescue of Mismigration in *ced-12* Null Worms by the PxxP Mutant of *ced-12*		
ced-12(k149); opEx874 [P_{eft-3}::ced-12(AxxA)::yfp]	18	305
ced-12(k149); opEx873 [P_{eft-3}::ced-12(AxxA)::yfp]	21	302
Partial Rescue of Migration Defects in *ced-5* Null Worms by *ced-5(ΔSH3)*		
ced-5(n1812); opEx723 [P_{eft-3}::ced-5(ΔSH3)]	22	305
ced-5(n1812); opEx725 [P_{eft-3}::ced-5(ΔSH3)]	23	326
ced-5(n1812); opEx726 [P_{eft-3}::ced-5(ΔSH3)]	24	315
(B) Effect of G171E Mutation of Dock180 on Rescuing DTC-Migration Defects in *CED-5*-Deficient Worms		
Partial Rescue of DTC-Migration Defects in *ced-5* Null Worms by Wild-Type *Dock180*		
ced-5(n1812); opEx763 [P_{eft-3}::Dock180(WT)]	24	310
No Rescue of DTC-Migration Defects in *ced-5* Null Worms by *Dock(G171E)*		
ced-5(n1812); opEx379 [P_{eft-3}::Dock180(G171E)]	44	302
ced-5(n1812); opEx371 [P_{eft-3}::Dock180(G171E)]	53	405
ced-5(n1812); opEx382 [P_{eft-3}::Dock180(G171E)]	50	304

During *C. elegans* development, the distal-tip cell (DTC) migrates in a stereotypical U-shaped pattern to define the shape of the adult hermaphrodite gonad (wild-type). In worms with null mutations in *ced-5* or *ced-12*, the DTC frequently migrates incorrectly with extra or wrong turns (mismigrated). The *ced-5(n1812)* mutant or the *ced-12(k149)* was made transgenic for the indicated constructs. The percentage of gonadal arms with migration defects was scored in multiple independent transgenic lines for each construct. All lines were coinjected with the P_{lim_7}*::GFP* marker for visualizing the gonadal arms. All of the CED-5, Dock180 and CED-12 constructs were expressed under the P_{eft-3} promoter.

tive in rescuing the DTC-migration defect, and in fact it enhanced the DTC mismigration (Table 1). Taken together, these data suggest that the SH3 domain and its adjoining region critically regulate the GEF activity of Dock180 and that the ELMO binding to the N-terminal region of Dock180 may alleviate the inhibitory SH3:Docker interaction and fully activate Dock180.

Our data suggest a steric-inhibition model for regulation of the GEF activity of CDM family members. In this model, the SH3 domain binding to the catalytic Docker domain blocks the access of Rac to the Docker domain; this inhibition is relieved, at least in part, by the binding of the PxxP motif of ELMO to the SH3 domain and allows Rac to have better access to the Docker domain. Although the SH3 domain in the basal state negatively regulates the Rac-GEF activity of Dock180, it provides one of the two contact sites for ELMO and thereby positively affects Rac-GEF activity of the Dock180/ELMO complex. The latter effect was best revealed in the *C. elegans* in vivo rescue studies involving CED-5 mutants lacking the SH3 domain. Interestingly, a self-inhibitory mechanism is also utilized by certain conventional DH-PH-containing Rho-GEFs to regulate their basal catalytic activity [21]. The SH3 domain is present in the five mammalian CDM family members, with at least four of them shown to bind ELMO [1, 2, 11, 13]. The data presented here on Dock180, Dock2, and Dock4, along with the worm rescue studies using mutants of Dock180 and CED-5, support a novel steric-inhibition model for the regulation of GEF activity of several CDM family members, and this model has implications for multiple biological processes.

Supplemental Data

Supplemental Experimental Procedures, three figures, and a table are available with this article online at http://www.current-biology.com/cgi/content/full/15/4/371/DC1/.

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