The scaffold-protein IQGAP1 enhances and spatially restricts the actin-nucleating activity of Diaphanous-related formin 1 (DIAPH1)

The actin cytoskeleton is a dynamic array of filaments that undergoes rapid remodeling to drive many cellular processes. An essential feature of filament remodeling is the spatio-temporal regulation of actin filament nucleation. One family of actin filament nucleators, the Diaphanous-related formins, is activated by the binding of small G-proteins such as RhoA. However, RhoA only partially activates formins, suggesting that additional factors are required to fully activate the formin. Here we identify one such factor, IQ motif containing GTPase-activating protein-1 (IQGAP1), which enhances RhoA-mediated activation of the Diaphanous-related formin (DIAPH1) and targets DIAPH1 to the plasma membrane. We find that the inhibitory intramolecular interaction within DIAPH1 is disrupted by the sequential binding of RhoA and IQGAP1. Binding of RhoA and IQGAP1 robustly stimulates DIAPH1-mediated actin filament nucleation in vitro. In contrast, the actin capping protein Flightless-I, in conjunction with RhoA, only weakly stimulates DIAPH1 activity. IQGAP1, but not Flightless-I, is required to recruit DIAPH1 to the plasma membrane where actin filaments are generated. These results indicate that IQGAP1 enhances RhoA-mediated activation of DIAPH1 in vivo. Collectively these data support a model where the combined action of RhoA and an enhancer ensures the spatio-temporal regulation of actin nucleation to stimulate robust and localized actin filament production in vivo.

The actin cytoskeleton is a dynamic filamentous network that is crucially involved in directing cell migration, regulating cell shape, and enabling cell division (1, 2). Actin filaments are generated by the polymerization of actin monomers in vitro, polymerization of actin into filaments occurs at high concentrations of actin monomers; however, the lower concentration of free cytoplasmic actin monomers in vivo requires nucleators and polymerases for filament assembly within the cell (1, 2). Tight regulation of these actin effectors enables cells to spatio-temporally control actin filament networks and consequently to sequester a particular cellular process at a specific intracellular site (1–3).

The formin family of proteins comprises one critically important class of actin filament nucleators and elongators (3). In mammals, there are 15 formins that can be subdivided further into families, of which the Diaphanous-related formins (DRFs)2 is the largest (3, 4). The DRFs are autoinhibited through an intramolecular interaction between a C terminally located Diaphanous autoregulatory domain (DAD) and an N terminally located DAD interacting domain (DID). In the auto-inhibited state, the N terminus sterically hinders actin binding to the formin homology domain 2 (FH2), thereby preventing actin filament formation (5–7). Binding of a small G-protein of the Rac/Rho/Cdc42 family to the formin relieves the autoinhibited state, enabling the formin to nucleate actin filaments (5, 8). Structural studies reveal that Rho-GTP binding to DIAPH1 (also called mDia1, DRF1) elicits a conformational change that displaces the DAD from the DID (9, 10); however, this binding causes only partial activation of the formin (8, 11, 12). Therefore, other regulatory steps must be involved.

We previously showed that anillin, a protein essential for cytokinesis, enhances the RhoA-mediated activation of the formin DIAPH3 (also called mDia2, DRF3) by binding to the DID domain of DIAPH3 (13). Furthermore, anillin is required to target DIAPH3 to the cytokinetic furrow (13, 14) where DIAPH3 generates actin filaments required for successful cyto-

---

1 To whom correspondence should be addressed: Dept. Molecular Genetics, University of Toronto, 661 University Ave., Toronto, Ontario MSG 1M1, Canada. Tel.: 416-946-7714; Fax: 416-978-6885; E-mail: andrew.wilde@utoronto.ca.

2 The abbreviations used are: DRF, Diaphanous related formin; DIAPH1, Diaphanous related formin 1; DIAPH3, Diaphanous related formin 3; FH, formin homology domain; DAD, Diaphanous autoregulatory domain; DID, Diaphanous binding region; BLI, Bio-Layer interferometry; GLD, gelsolin-like domains; MOC, Manders overlapping coefficient; PCC, Pearson correlation coefficient; HGF, human gingival fibroblast; NT, N-terminal; CT, C-terminal; CB, column buffer; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione S-transferase; HIB, Hisincubation buffer; KB, kinetics buffer; DAPI, 4’,6-diamidino-2-phenylindole; IQGAP1, IQ motif containing GTPase-activating protein-1; tM1, autothreshold Manders’s overlapping coefficient.
kinesis (13). These data suggest that the targeting and full activation of formins may be linked to a common factor.

The mechanisms involved in the activation of other formins are less clear. The actin-binding protein Flightless-I (Fli-I) has been implicated in the activation of some formins based on in vitro experiments (12). Fli-I is a member of the gelsolin family of actin-binding proteins that has the capacity to remodel the actin cytoskeleton by capping and severing actin filaments (15, 16) and binds to a conserved region within the DAD of DRFs (12). In the case of the formins DAAM1 and DIAPH1, Fli-I binding enhances RhoA-mediated activation of the actin filament nucleation activity in vitro (12). Although Fli-I can bind and activate DIAPH1 in vitro, a role in targeting DIAPH1 to subcellular locations remains to be determined. Previous studies discovered that the N terminus of DIAPH1 is sufficient to target DIAPH1 to the plasma membrane (17, 18). Furthermore, the scaffold protein IQGAP1, a DID interacting partner, was required to target DIAPH1 to the plasma membrane (18). Taken together, these data suggest that DID and DAD interactors have the potential to regulate small G-protein–mediated activation and targeting of formins. Currently, it is not known whether both mechanisms further enhance the activation of formins in vitro and in vivo and whether these activation mechanisms are widespread and generally applicable.

We have previously shown that the DIAPH3 DID-binding factor anillin enhanced the G-protein–mediated activation of DIAPH3 (13). In this study, we sought to determine whether, in principle, this activation pathway was applicable to other formins. Furthermore, we assessed whether both DID- and DAD-dependent formin activation mechanisms are applicable to a single formin. Using DIAPH1 as a model formin, we examined the role of Fli-I, which interacts with the DAD, and IQGAP1, which interacts with the DID and targets DIAPH1 to the leading edge of migrating cells (18), on formin-dependent actin polymerization activity.

**Results**

**RhoA stimulates IQGAP1 binding to DIAPH1**

In the absence of an activating interaction, DRFs exist in an autoinhibited state due to the intramolecular interaction between its DAD and DID (Fig. 1A). Activation of the actin filament nucleation activity of DIAPH1 and other formins requires the binding of a small G-protein, often Rho, to partially release the formin from this autoinhibited state (8). Previously, we showed that the related formin DIAPH3 (also called mDia2, DRF3) requires the additional binding of anillin to the DID for the full activation of DIAPH3 and robust actin filament production in vitro and in vivo (13). Due to the degree of conservation of the amino acid sequences between DIAPH1 and -3, we sought to determine whether DIAPH1 was activated through an analogous mechanism.

We first confirmed that the Diaphanous-binding region (DBR) domain of IQGAP1 binds to the DID of DIAPH1 (18) using binding assays and Bio-Layer interferometry (BLI) (Fig. 1 and Fig. S1). A recombinant IQGAP1-DBR domain fused to glutathione S-transferase (GST-IQGAP1-DBR) was added to purified DIAPH1 N- or C-terminal fragments fused to His6 or maltose-binding protein (MBP), respectively, and interactions were determined by pulldown assays and Western blotting (Fig. 1). The N-terminal (NT) half of DIAPH1 (amino acids 1–575) contains the Rho-binding domain and DID, whereas the C-terminal (CT) half (amino acids 580–1272) includes the formin homology domains (FH1 and FH2) and the DAD region (refer to Fig. 1A). As expected, the GST-IQGAP1-DBR bound to the His6-DIAPH1-NT (Fig. 1B), with a Kd of 81.3 ± 9.2 nM, roughly 10-fold less than that observed for the binding between GST-RhoA and the His6-DIAPH1-NT (Kd = 8.3 ± 3.3 nM; Fig. S1). In contrast, no detectable interaction was observed between IQGAP1 and the reconstituted autoinhibited formin generated by preincubating the His6-DIAPH1-NT and MBP-DIAPH1-CT (Fig. 1C), indicating that IQGAP1 is insufficient to displace the DIAPH1-DID from the DIAPH1-DAD under these conditions. Consistent with these results, only a weak interaction between IQGAP1 and the reconstituted DIAPH1–NT–CT complex could be observed using BLI (Kd = 2.1 ± 0.145 μM; Fig. S1), suggesting that IQGAP1 has a strongly reduced (38-fold) affinity for the autoinhibited formin versus the isolated His6-DIAPH1-NT fragment. In contrast, the formin activator RhoA binds to the DIAPH1–NT–CT complex both by pulldown assay and by BLI (Kd = 113 ± 22.1 nM, Fig. S1), suggesting that activation of DIAPH1 is likely to be initiated through the binding by RhoA.

To determine whether RhoA facilitates the binding of IQGAP1 to DIAPH1, we again re-constituted autoinhibited DIAPH1 and assessed the ability of the GST-IQGAP1-DBR to bind to His6-DIAPH1-NT in the presence and absence of GST-RhoA. In the presence of GST-RhoA, the GST-IQGAP1-DBR now co-purified with His6-DIAPH1-NT in the pulldown assay with a Kd of 93.6 ± 27.8 nM, a 22-fold increase in affinity versus in the absence of GST-RhoA. Interestingly, in the presence of GST-IQGAP1-DBR, there was also a 2-fold increase in the amount of GST-RhoA co-purifying with His6-DIAPH1-NT (Fig. 1D), suggesting that the interaction of IQGAP1 to DIAPH1 may stabilize RhoA binding to DIAPH1. As GST-IQGAP1-DBR only bound to the His6-DIAPH1-NT in the presence of GST-RhoA (Fig. 1C), these data suggest that RhoA must first bind to the autoinhibited DIAPH1 to facilitate subsequent IQGAP1 binding.

**IQGAP1 enhances RhoA mediated release of DIAPH1 from the autoinhibited state**

The dependence of IQGAP1 on RhoA for binding to DIAPH1 is reminiscent of our previous work where we showed that anillin binding to DIAPH3 depended on RhoA (13). Our study also demonstrated that anillin enhanced the RhoA-dependent release of DIAPH3 from its autoinhibitory state. Based on this, we proposed that RhoA requires an enhancer to fully activate a formin. We therefore assessed if IQGAP1 similarly enhances the RhoA-mediated release of DIAPH1 from its autoinhibited state. The autoinhibited DIAPH1–NT–CT complex was formed by pre-binding His6-DIAPH1-NT to MBP–DIAPH1-CT. We then assessed the degree of MBP-DIAPH1-CT released from the His6-DIAPH1-NT in the presence of up to equimolar concentrations of GST–RhoA, GST-IQGAP1-DBR, or both. Although the incubation of GST or GST-IQGAP1-
DBR alone had little effect on the interaction of His<sub>n</sub>-DIAPH1-NT with MBP-DIAPH1-CT, the presence of GST-RhoA caused a partial release of the MBP-DIAPH1-CT fragment from His<sub>n</sub>-DIAPH1-NT (Fig. 1, E and F). Consistent with our model, we observed an increased release of MBP-DIAPH1-CT from His<sub>n</sub>-DIAPH1-NT in the presence of equimolar concentrations of GST-RhoA and GST-IQGAP1-DBR (Fig. 1, E and F), indicating that IQGAP1 enhances the RhoA-mediated release of DIAPH1 from its autoinhibited state at the equimolar conditions used in these experiments.

We next determined if individual formins with conserved DID regions require specific enhancers for their activation by asking if IQGAP1 and anillin can indiscriminately promote the activation of different Diaphanous family formins. We did observe specificity: IQGAP1 only bound to DIAPH1, whereas conversely, anillin only bound to DIAPH3 (Fig. S2). These data suggest that whereas a two-step formin-activation mechanism is likely to be generalizable to other formins, the specificity of formin activation relies on the enhancer rather than on the Rho-GTPase.

**IQGAP1 enhances DIAPH1 actin nucleation activity**

Our data shows that IQGAP1 enhances RhoA-mediated release of DIAPH1 from the autoinhibited state, suggesting that RhoA and IQGAP1 act together to increase the actin filament nucleation activity of DIAPH1. To test this directly, we performed in vitro pyrene-actin nucleation assays. We confirmed that the constitutively active MBP-DIAPH1-CT fragment, which contains the FH2 domain, nucleates actin filaments in vitro (Fig. 2A) (8, 12). When MBP-DIAPH1-CT was pre-bound to His<sub>n</sub>-DIAPH1-NT, the resulting complex exhibited no detectable actin nucleation activity (Fig. 2B). Consistent with previous studies (8, 12), GST-RhoA only weakly stimulated the actin nucleating activity of the DIAPH1–NT-CT complex (Fig. 2C), whereas GST-IQGAP1-DBR alone did not stimulate the actin filament nucleation activity of the DIAPH1–NT-CT complex (Fig. 2D). In contrast, in the presence of both GST-RhoA and GST-IQGAP1-DBR, actin nucleation activity was dramatically increased, approaching the levels observed with the constitutively active MBP-DIAPH1-CT alone (Fig. 2E).

The partial activation of DIAPH1 by RhoA could reflect that RhoA only weakly or transiently promotes the dissociation of the formin DID and DAD regions, or alternatively, could reflect that only a subset of DIAPH1 molecules are released from the autoinhibitory state, as our previous binding data suggested (Fig. 1). To address this, we determined the stoichiometry of complexes formed in the different reactions. Equimolar amounts of RhoA were added to preformed DIAPH1–NT-CT complexes, then the His<sub>n</sub>-DIAPH1-NT was re-isolated and the amounts of co-purifying MBP-DIAPH1-CT and GST-RhoA were determined by Western blotting. When all the reaction components were present in equimolar amounts, the molar ratio of the RhoA:DIAPH1-NT:DIAPH1-CT components in the reisolated complex was 1:2.3:1.2 (Fig. S3), suggesting that a single RhoA releases a single DIAPH1-CT from a formin dimer. We interpret this to mean that only a subset of the DIAPH1 molecules are activated in the presence of RhoA and thus only partial actin nucleation activity is observed. In contrast, when IQGAP1 was added to the reaction in equimolar amounts, the reisolated complexes contained no DIAPH1-CT, with a RhoA: IQGAP1:DIAPH1-NT:DIAPH1-CT stoichiometric ratio of near 1:1:1:0 (1:0.95:1.15:0.08; Fig. S3), suggesting that nearly every DIAPH1 molecule is released from the autoinhibited state, leading to the maximal expected increase in actin polymerization in the pyrene actin-assays (Fig. 2). Thus, IQGAP1 enhances the RhoA-mediated activation of DIAPH1 by enhancing the release of DIAPH1 from its autoinhibited state and leading to robust actin filament nucleation activity.

**Fli-I enhances RhoA-mediated release of DIAPH1 from the autoinhibited state**

Our data suggest that DIAPH1 function is regulated through the interaction of its DID region with IQGAP1. In a previous study, the regulation of DIAPH1 and DAAM1 was proposed to occur through an interaction between the formin DAD regions and the gelsolin-like domains (GLD) of Fli-I. Fli-I binding to the DAD of either DAAM1 or DIAPH1 enhanced RhoA-mediated activation of the formins’ actin nucleation activity (12). We confirmed this using a fragment of Fli-I (amino acids 398–1271) containing the GLDs. GST-Fli-I-GLD bound to MBP-DIAPH1-CT in the pulldown assay (Fig. 3A) with a K<sub>d</sub> of 139.3 ± 7.6 nM (Fig. S1). However, there was no detectable interaction with His<sub>n</sub>-DIAPH1-NT (Fig. 3A). Likewise, there was no detectable interaction between GST-Fli-I-GLD and preformed DIAPH1–NT-CT complexes in pulldown assays (Fig. 3B), consistent with the weak interaction detected by BLI (K<sub>d</sub> = 3.93 ± 0.18 µM) (Fig. S1). However, in the presence of GST-RhoA, GST-Fli-I-GLD bound to MBP-DIAPH1-CT in the pull down assay (Fig. 3B) with a K<sub>d</sub> of 166.5 ± 47.5 nM. These data suggest that RhoA binds to the DIAPH1–NT-CT complex to facilitate the subsequent binding to Fli-I to the DAD region of the DIAPH1-CT.

To determine whether Fli-I binding could release DIAPH1 from its autoinhibited state, we next preformed the DIAPH1–NT-CT complex and incubated the complex with GST-RhoA, GST-IQGAP1-DBR, GST-Fli-I-GLD, or a combination of these reagents (Fig. 3, C and D). Fli-I alone did not release the MBP-DIAPH1-CT from the His<sub>n</sub>-DIAPH1-NT. However, GST-Fli-I-GLD did enhance the GST-RhoA–mediated release of MBP-DIAPH1-CT from the His<sub>n</sub>-DIAPH1-NT to a level...
**IQGAP1 enhances DIAPH1 activity**

---

**Figure 2. IQGAP1 enhances RhoA-dependent activation of DIAPH1 actin nucleation activity.** A–E, pyrene-actin nucleation assays performed in the presence of recombinant proteins and 2 μM pyrene-labeled actin. Fluorescence intensity measure is in arbitrary units (A.U.). The fluorescence of pyrene-labeled actin is much higher after actin filament polymerization. CT, MBP-DIAPH1-CT; NT, His<sub>6</sub>-DIAPH1-NT; RhoA, GST-RhoA; IQGAP1, GST-IQGAP1-DBR. F, normalized actin polymerization rates at t<sub>1/2</sub> in the different conditions shown in A–E. Abbreviations as in A–E, except IQ, GST-IQGAP1-DBR (n = 3, error bars indicate ± S.D.).

---

Comparable with IQGAP1. Consistent with this, *in vitro* pyrene-actin filament nucleation assays showed that Fli-1 enhances RhoA-mediated activation of the actin filament nucleation activity of the DIAPH1–NT–CT complex (Fig. 4). Fli-1 was, however, a weaker enhancer of DIAPH1 activity relative to IQGAP1. In the presence of GST-RhoA and GST-Fli-I-GLD, MBP-DIAPH1-CT exhibited a 35% reduction in the actin polymerization rate (t<sub>1/2</sub>) relative to that observed in the presence of GST-RhoA and GST-IQGAP1-DBR (Fig. 4, C and D). In the presence of both GST-IQGAP1-DBR and GST-Fli-I-GLD, we observed an increase in MBP-DIAPH1-CT release from the His<sub>6</sub>-DIAPH1-NT; however, this did not increase the DIAPH1 actin nucleation activity (Fig. 3, C and D), suggesting that either the DAD region plays additional roles in actin polymerization or that Fli-I recruitment is itself inhibitory to actin filament production.

**IQGAP1 but not Fli-I targets DIAPH1 within the cell**

We previously showed that anillin both enhances DIAPH3 actin nucleation activity, and targets DIAPH3 to the cytokinetic furrow (13). As Fli-I and IQGAP1 similarly enhance DIAPH1 activity, we sought to assess their ability to target DIAPH1
IQGAP1 enhances DIAPH1 activity

A

Fli-1

Fli-1-GLD

leucine rich repeats

G1 G2 G3 G4 G5 G6

597 745 858 1039 1056 1152

363 499 617 728 817 1167

50- 60- 100- 120-

His - DIAPH1 NT pull-down

MBP - DIAPH1-CT

GST-Fli-I-GLD

B

His - pull down

MBP pull down

His - DIAPH1-NT

MBP-DIAPH1-CT

GST-Fli-I-GLD

C

MBP-DIAPH1 CT pull down

+GST-Fli-I +GST-Fli-I-GLD

+GST-RhoA

+GST-RhoA

His - DIAPH1 NT pull down

+GST-RhoA +GST-Fli-I-GLD

+GST-RhoA

D

DIAPH1-CT-NT complex

GST

GST-IQGAP1-DBR

GST-RhoA

GST-Fli-I-GLD

Ratio of MBP-DIAPH1-CT compared to control

MBP-DIAPH1-CT

His - DIAPH1-NT

Loading controls

G1 G2 G3 G4 G5 G6

70- 60- 50- 60- 50- 60-

70- 60- 50- 60- 50- 60-

70- 60- 50- 60- 50- 60-

70- 60- 50- 60- 50- 60-

70- 60- 50- 60- 50- 60-

70- 60- 50- 60- 50- 60-
IQGAP1 enhances DIAPH1 activity

activity to the plasma membrane. In MDA-MD-231 cells, DIAPH1 predominantly localizes to the plasma membrane (Fig. 5). IQGAP1 colocalizes with DIAPH1 at the plasma membrane (Manders overlapping coefficient (MOC) of 0.819 ± 0.012 and a Pearson correlation coefficient (PCC) of 0.565 ± 0.018, Fig. 5, A and C). In contrast, Fli-I did not co-localize with DIAPH1 at the plasma membrane (MOC and PCC to 0.522 ± 0.020 and 0.320 ± 0.026, respectively, Fig. 5, B and D). Consistent with these results, depletion of IQGAP1 by siRNA reduced DIAPH1 targeting to the plasma membrane (Fig. 5A), whereas depletion of Fli-I did not (Fig. 5B). Similar observations were made in human gingival fibroblast (HGF) cells (Fig. S4).

To determine the role of DIAPH1, IQGAP1, and Fli-I in actin filament production, we analyzed actin accumulation at the leading edge of MDA-MB-231 cells using rhodamine-phalloidin staining in the presence and absence of DIAPH1, IQGAP1, and Fli-I (Fig. 6). In control siRNA-treated cells, we observed a strong actin signal at the leading edge that co-localized with IQGAP1 (MOC = 0.809 ± 0.019, PCC = 0.533 ± 0.018, Fig. 6, B, D, and E) and also in part with DIAPH1, reflecting that a pool of DIAPH1 also localized to actin-poor perinuclear locations (MOC = 0.481 ± 0.031, PCC = 0.45 ± 0.030, Fig. 6, A, D, and E). Fli-I localized primarily to actin-poor perinuclear regions and thus did not co-localize with actin at the leading edge of cells (MOC = 0.256 ± 0.023, PCC = 0.059 ± 0.013, Fig. 6, C–E), suggesting that it is unlikely to play a role in regulating actin dynamics at the plasma membrane. Consistent with these observations, depletion of either IQGAP1 or DIAPH1 reduced the actin staining at the leading edge (Fig. 6, A and B), whereas depletion of Fli-I did not (Fig. 6C). Taken together our biochemical and microscopy data suggest that in the cell lines examined, IQGAP1 enhances RhoA-mediated activation of DIAPH1 actin nucleation activity at the plasma membrane whereas Fli-I exerts little effect in the cell lines and conditions examined.

Discussion

The precise control of when and where actin filaments are generated in response to different stimuli is a fundamental aspect of cytoskeletal regulation. Small G-proteins are instrumental in activating formins, but in vitro, small G-proteins only partially overcome formin autoinhibition, and do so indiscriminately (5, 8, 11–13). We previously demonstrated that the cyto-kinetic factor anillin enhances RhoA-mediated activation of the formin DIAPH3 by binding to the DID and directs actin filament production to the cytokinetic furrow (13). Anillin is therefore a bifunctional regulator of DIAPH3, ensuring that actin nucleation is both enhanced and targeted to a specific subcellular location, the cytokinetic furrow. In this study we extend this principle of coupling formin activation with subcellular localization to a second formin, DIAPH1. In this work, we show that RhoA-mediated activation is enhanced by the binding of IQGAP1 to the DID of DIAPH1, which both targets the formin to its subcellular localization and promotes its full activation. A previous study demonstrated that IQGAP1 recruits DIAPH1 to the leading edge of migrating cells and the phagocytic cup, but found no role for IQGAP1 in DIAPH1 activation (18), possibly because the authors reported maximal activation of DIAPH1 in the presence of RhoA. In contrast, other studies, including our own, report only the partial activation of DIAPH1 by RhoA (including but not limited to Refs. 5, 8, 11, and 12), suggesting that in vivo actin nucleation is modulated by additional factors that control both formin localization and function. By exploiting a two-factor activation mechanism in the control of formin activity, more precise spatio-temporal regulation can be exerted as both factors must be present in one location for maximal activation.

There are clear parallels between the activation mechanisms of DIAPH1 and DIAPH3: both formins exist in an autoinhibited state that is mediated by an intramolecular interaction between the DID and DAD regions, and both formins exploit a DID-binding factor to augment RhoA-mediated activation. In both cases the enhancer has a dual function, to both fully activate and target actin nucleating activity to a specific subcellular region. We propose that this general activation mechanism is likely to be a common feature of other Diaphanous-related formins (Fig. 7).

Additional regulatory pathways are likely to contribute to the correct spatio-temporal regulation of formin activity. Fli-I, a protein with actin capping and filament severing activities (19), binds to the DAD of DIAPH1 and DAAM1 at a site conserved across DRFs. Our work, and that of others (12), demonstrated that Fli-I binding to DIAPH1 released DIAPH1 from its autoinhibited state and enhanced RhoA-dependent activation of DIAPH1 in vitro. We extend these results to show that in our assays Fli-I is a less potent enhancer of DIAPH1 activation than IQGAP1.

The mechanism underlying the different formin enhancing activities of IQGAP1 and Fli-I is unclear. Full activation of formins must require structural changes to the formin that reorient the N- and C-terminal regions such that the N terminus of the formin no longer sterically blocks the interaction of actin monomers with the actin nucleating FH2 domain located in the formin C terminus (5). If DID-binding factors such as IQGAP1 and anillin more efficiently facilitate or stabilize this “open” form of the formin than DAD interacting factors such as Fli-I, then IQGAP1 and anillin would be expected to have a greater stimulatory effect on formin actin nucleation activity.

Figure 3. In vitro Fli-I enhances RhoA-dependent release of DIAPH1-NT from DIAPH1-CT. A, domain organization of Flightless-I and the recombinant fragment used in this study. The domain boundaries are denoted as the amino acid number in the sequence. B, in vitro binding assay between either bacterially expressed His6-DIAPH1-0 NT and GST-Fli-I-GLD or MBP-DIAPH1-CT and GST-Fli-I-GLD. C, preformed His6-DIAPH1-NT plus MBP-DIAPH1-CT complexes (0.05 nmol) were immobilized on amyllose beads and incubated with increasing concentrations of GST-Fli-I-GLD ± GST-RhoA (upper panel) or immobilized on nickel-Sepharose and incubated with increasing amounts of GST-RhoA ± GST-Fli-I-GLD (lower panel). The DIAPH-loaded beads were then isolated and analyzed by immunoblotting to detect co-purifying GST-Fli-I-GLD or GST-RhoA as indicated. D, preformed DIAPH1–NT–CT complexes as described above were immobilized on Ni-Sepharose, which binds His6-DIAPH1-NT, and incubated with GST, GST-RhoA, GST-IQGAP-DBR, GST-Fli-I-GLD or combinations thereof in an equimolar ratio. The Ni-Sepharose beads were then washed and analyzed by immunoblotting for the co-purifying MBP-DIAPH1-CT. Quantitation of the immunoblots indicates the ratio of co-purifying DIAPH1-CT recovered under each condition relative to that found in the input lane with the DIAPH1–NT + CT complex (leftmost lane).
IQGAP1 enhances DIAPH1 activity

Fli-I is a modulator of the actin cytoskeleton in its own right, and it may be that these activities account for its weaker enhancement of DIAPH1 activation compared with IQGAP1. The actin capping and severing activities of Fli-I may counteract filament elongation by DIAPH1. At much higher concentrations of the Fli-I fragment that contains all six gelsolin-like domains, we observed such activities over extended periods (19). These data raise the interesting possibility that formin activity could be fine-tuned by recruiting capping/severing proteins depending on the needs of the cell. Alternatively, Fli-I could interfere with actin binding to the formin. The DAD is required for full DIAPH1 actin nucleating activity in vitro through an as yet undefined interaction with actin (20). Mutations in the DAD perturb the interaction of actin with DIAPH1. Interestingly the mutations are directly adjacent to mutations that disrupt the formin–Fli-I interaction (12), suggesting that both actin and Fli-I may interact with formins at the same site. Consequently, Fli-I binding could attenuate formin-mediated actin nucleation and elongation activities.

Although we propose that the activation of Diaphanous family formins is a two-step process, we do not envisage that indiscriminate protein binding to the DID necessarily confers a formin-activating function. For instance, the binding of Liprin-α3 to the DID of DIAPH1 has an inhibitory effect, as it prevents the correct targeting of DIAPH1 to the membrane (21, 22). Liprin-α3 has been proposed to displace IQGAP1 from DIAPH1 (21), suggesting that the control of formin activity can occur through both positive and negative effectors, and providing yet greater scope for the multi-layered regulation of the actin cytoskeleton.

Our previous work demonstrated that during cytokinesis, anillin interacts with DIAPH3, recruiting the formin to the cytokinetic furrow and enhancing its RhoA-dependent activation (13). Here we find that IQGAP1 performs an analogous role in regulating DIAPH1: IQGAP1 targets DIAPH1 to the plasma membrane and there enhances DIAPH1 actin nucleation activity. In contrast, Fli-I was not required for DIAPH1 targeting in the cell lines tested (MDA-MB-231, Figs. 5 and 6, and HGF, Fig. S4). It still remains possible that in specialized cell lines, Fli-I could be involved in regulating DIAPH1 activity. Alternatively, Fli-I activity may be preferentially directed toward other formins in vivo, as the Fli-I interaction site on DIAPH1 and DAAM1 is conserved in other Diaphanous-related formins.

Our studies define one regulatory mechanism for the spatio-temporal control of formin activity. However, in vivo formin regulation is likely to be more complex, as formins can undergo post-translational modifications that may modulate their activity (23–30). Taken together, these observations suggest that formins are subject to a rich array of regulatory mechanisms, allowing for the dynamic control of distinct actin superstructures within the cell.

Experimental procedures

cDNA cloning

pGEX-6P-2-DIAPH1-CT(580–1272) and pET30a-DIAPH1-NT(1–575) plasmids were previously generated (by J.C.). The IQGAP1 full-length cDNA was a synthetic gene corresponding to NP 003861, a gift from Drs. Y. Tong and C. Arrowsmith.
**IQGAP1 enhances DIAPH1 activity**

Figure 5. DIAPH1 recruitment requires IQGAP1 but not Fli-I. A, immunofluorescence micrographs of siRNA-treated MDA-MB-231 cells stained with DIAPH1 and IQGAP1 antibodies. Arrows indicate IQGAP1 and DIAPH1 at the plasma membrane. Scale bars = 10 μm. B, siRNA-treated MDA-MB-231 cells stained with DIAPH1 and Fli-I antibodies. Arrows point to DIAPH1 at the plasma membrane. Scale bars = 10 μm. C, The relative co-localization of DIAPH1 (DIA1) with IQGAP1 or Fli-I was measured using a tM1 over both the whole image or a region of interest (ROI) at the leading edge of the plasma membrane, where a tM1 value of 1 = 100% colocalization. A positive control of a DIAPH1 image compared with itself (DIA1 single channel 0°) and a negative control of a DIAPH1 image compared with the same image after a 90° rotation (DIA1 single channel 90° rotation) are included. D, the relative co-localization of DIAPH1 (DIA1) with IQGAP1 or Fli-I was measured using a Pearson correlation coefficient, R(coloc), over both the whole image or a region of interest the leading edge of the plasma membrane, where a R(coloc) of 1 = 100% colocalization. Positive and negative controls were as described in C (n = 30, red bar = average, whiskers indicate 25–75 percentile).

(Structural Genomics Consortium, Toronto, Ontario, Canada). pGEX-4T-2-Fli-I-GLD (1–6) plasmid was previously described (16). Complementary DNA (cDNAs) fragments of human DIAPH1-CT(583–1272) and human IQGAP1-DBR(1500–1657) were amplified by PCR using the i-Max II DNA polymerase (Froggalab) and the oligonucleotide primers (Integrated DNA Technologies) listed in Table S1. PCR fragments of IQGAP1-DBR were cloned into a pDEST15 destination vector (Life Technologies) using In-Fusion Cloning Kit (Clontech) to generate GST fusion proteins. PCR fragments of DIAPH1-CT were cloned using the TOPO Gateway system (Life Technologies) being first cloned into the entry plasmid vector pCR8/GW/TOPO, then moved into the destination vectors pKM596 (Addgene plasmid 8837) to generate MBP-fusion proteins.
Protein expression and purification

Recombinant proteins were purified from BL21 *Escherichia coli* cells transformed with plasmids containing His6 or GST-fusion proteins, or ER2523 *E. coli* (New England Biolabs) with plasmids containing MBP-fusion proteins. Cells were grown in LB media at 37 °C to an optical density of 0.6 at A600. Recombinant protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and further incubated at 16 °C overnight. Cells were harvested by centrifugation and stored in −80 °C.

To purify GST-fusion proteins, BL21 *E. coli* cells were re-suspended in 25 mM HEPES, pH 7.5, 250 mM NaCl, 100 mM KCl,
**IQGAP1 enhances DIAPH1 activity**

**Figure 7. Schematic outlining Diaphanous-related formin activation pathway.** Autoinhibited Diaphanous-related formins are activated by the sequential binding of a small GTPase (e.g., Rho) then an enhancer, IQGAP1 in the case of DIAPH1 or anillin in the case of DIAPH3. The enhancer is bifunctional as it also targets the formin to its site of action. GBD, G-protein binding domain.

0.5 mM β-mercaptoethanol, 1 mM PMSF, and lysed by sonication. The lysates were cleared by centrifugation at 10,000 × g for 30 min at 4 °C and supernatant applied to GSH beads (Invitrogen). The GSH beads were washed with 10 column volumes of column buffer (CB) containing 25 mM HEPES, pH 7.5, 250 mM NaCl, 100 mM KCl, 0.5 mM β-mercaptoethanol, 1 mM PMSF, 0.1% (v/v) Triton X-100. The GST-fusion proteins were eluted in CB containing 10 mM GSH.

To purify MBP-fusion proteins, ER2523 E. coli cells were harvested and lysed as above. The lysates were cleared by centrifugation as above, then the supernatant was applied to amylose resin (New England Biolabs). The resin was washed with 10 column volumes of CB and the MBP-fusion proteins were eluted in CB containing 10 mM maltose.

To purify His6-fusion proteins, BL21 E. coli cells were re-suspended in 25 mM HEPES, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol, 5 mM imidazole, 0.5 mM β-mercaptoethanol, 1 mM PMSF, and lysed by sonication. The lysates were cleared by centrifugation as above then the supernatant was applied to nickel-Sepharose beads (Amersham Biosciences). The beads were washed with 10 column volumes of His6 column buffer containing 25 mM HEPES, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol, 5 mM imidazole, 0.5 mM β-mercaptoethanol, 1 mM PMSF, 0.1% (v/v) Triton X-100. The His6-fusion proteins were eluted in His6 column buffer containing 500 mM imidazole.

Eluted proteins were dialyzed against 10 mM HIB (all subsequent His6 protein washes were done in this buffer, unless otherwise noted) and blocked with 3% (w/v) BSA for 20 min. The beads were then washed and mixed with 0.05 nmol of GST-IQGAP1-DBR and incubated for 2 h at 4 °C. Unbound protein was removed by washing the beads in HIB. The beads were re-isolated by centrifugation and boiled in SDS sample buffer, then analyzed by Western blotting using an anti-IQGAP1 polyclonal antibody (ab109292, Abcam, 1:250 dilution) to detect co-purifying IQGAP1. To determine whether IQGAP1 binds to DIAPH1-CT, 0.05 nmol of MBP-DIAPH1-CT was immobilized onto 25 μl of amylose resin in 100 μl of incubation buffer containing 25 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EGTA, 0.3% (v/v) Triton X-100, 1 mM β-mercaptoethanol and incubated for 1 h at 4 °C. The beads were washed, incubated with GST-IQGAP1-DBR, reisolated, and analyzed by Western blotting as described above to detect co-purifying IQGAP1.

To assess the roles of IQGAP1 and RhoA in regulating the interaction between DIAPH1-NT and DIAPH1-CT, 0.05 nmol of His6-DIAPH1-NT was immobilized onto 25 μl of nickel-Sepharose beads in 100 μl of HIB as described above and incubated for 1 h at 4 °C. The beads were washed to removed unbound protein, then blocked with 3% (w/v) BSA for 45 min and further washed. 0.05 nmol of MBP-DIAPH1-CT was then incubated with the beads for 2 h at 4 °C, followed by a washing step. GST-IQGAP1-DBR or GST-RhoA were then added to the beads, incubated for 2 h, then washed in HIB to remove unbound protein. The beads were re-isolated by centrifugation, boiled in SDS sample buffer, then analyzed by Western blotting using an anti-MBP mAb (E8032, New England Biolabs, 1:2500 dilution) to detect co-purifying MBP-DIAPH1-CT.

To assess the roles of Fli-I and RhoA in regulating the interaction between DIAPH1-NT and DIAPH1-CT, the DIAPH1 CT-NT complex was reconstituted as described above. GST-Fli-I-GLD or GST-RhoA were then added to the beads, incubated for 2 h, then washed in HIB and further washed. 0.05 nmol of MBP-DIAPH1-CT was then incubated with the beads for 2 h at 4 °C, followed by a washing step. GST-IQGAP1-DBR or GST-RhoA were then added to the beads, incubated for 2 h, then washed in HIB to remove unbound protein. The beads were re-isolated by centrifugation, boiled in SDS sample buffer, then analyzed by Western blotting using an anti-MBP mAb (E8032, New England Biolabs, 1:2500 dilution) to detect co-purifying MBP-DIAPH1-CT.
To determine whether IQGAP1 or RhoA bound to the DIAPH1–CT-NT complex first, the same protocol as above was used, except analysis was carried out with an anti-IQGAP1 polyclonal antibody (ab109292, Abcam, 1:250 dilution) to detect co-purifying IQGAP1 or an anti-RhoA mAb (ab86297, Abcam, 1:200 dilution) to detect co-purifying RhoA. To determine whether Fli-I or RhoA bound to the DIAPH1–CT-NT complex first, the same protocol as above was used, except analysis was carried out with the homemade anti-GST polyclonal antibody to detect co-purifying Fli-I or an anti-RhoA mAb (ab86297, Abcam, 1:200 dilution) to detect co-purifying RhoA.

**Biolyser interferometry assays**

Dissociation constants between different proteins were determined by the Octet RED96 system (FortéBio), which measures association onto and dissociation from a sensor surface using BLI as previously described (32, 33). Briefly, purified GST-tagged ligands (RhoA, IQGAP1, Fli-I, DIAPH1-CT or preformed DIAPH1–NT-CT complex) were equilibrated into kinetics buffer (KB), which contains phosphate-buffered saline (PBS), 0.002% Tween 20, and 0.1 mg/ml of BSA, to a concentration of 25 μg/ml. The GST biosensors (FortéBio) were first equilibrated in KB for 60 s and then followed by incubation with GST ligands in KB for 300 s. Sensors were then rinsed in KB for 250 s to obtain a baseline of the levels of GST ligands loading onto the biosensors. Binding assays were then performed in a series of increased concentration of analyte (DIAPH1–NT, GST tag-removed RhoA or IQGAP1 or DIAPH1-CT) from 0.5 nM to 5 μM, from the lowest concentration to the highest. Each binding sequence started with a baseline incubation in KB (150 s), followed by the association with the analyte (300 s), the dissociation in KB (250 s), and a regeneration step (200 s) where sensors were then rinsed in KB for 250 s to obtain a baseline of the levels of GST ligands loading onto the biosensors. Binding assays were then performed in a series of increased concentration of analyte (DIAPH1–NT, GST tag-removed RhoA or IQGAP1 or DIAPH1-CT) from 0.5 nM to 5 μM, from the lowest concentration to the highest. Each binding sequence started with a baseline incubation in KB (150 s), followed by the association with the analyte (300 s), the dissociation in KB (250 s), and a regeneration step (200 s) where sensors were then rinsed in regeneration buffer (100 mM sodium citrate, pH 4.5, 50 mM EDTA, and 150 mM NaCl), to remove analyte still bound to GST ligands. Reference sensors that were loaded with GST ligands but only assayed in a series of pure KB, or a series of increasing concentration of MBP were also measured as controls. All incubation steps were performed at 30 °C with a shaking speed of the plates at 1000 rpm.

Data analysis was performed using Octet Software (FortéBio) and GraphPad Prism (8.2.0). The control signals measured by the reference sensors were subtracted from the signals measured by analyte-bound sensors. Steady-state analysis was performed using FortéBio Data Analysis 9 software to obtain the dissociation constant (K_d) from the equilibrium response. Each binding sequence was repeated >4 times. The resulted K_d values and averages were plotted in GraphPad Prism, with error bars representing mean ± S.D.

**Quantification of Western blots**

The polyvinylidene difluoride membrane of Western blots was developed by chemiluminescent solution (Life Technologies) for 5 min at room temperature and visualized using a Bio-Rad MP Imager (Bio-Rad, Canada). The intensities of individual bands on the blots were measured using ImageLab software (Bio-Rad). To determine the relative binding in the in vitro competition assays (Fig. 3B), a control pulldown reaction of WT “bait” protein that bound to beads and prey protein was performed and run on each gel. The intensity of the of bait protein band that bound to beads and the band of prey protein that was pulled down were set as the control standard to 1. In the comparative reactions, where potential competitor proteins were added or changing concentrations of proteins were added, the band intensity of the bait protein in each reaction was first compared and the differences used to adjust the band intensity of the amount of co-purifying prey protein to allow comparison. The adjusted prey band intensities were then compared with the intensity of the prey in the control reaction. Each binding assay was repeated at least three times. Analogous strategies were used to compare direct in vitro binding assays in Figs. 1, E and F, and 3. The relative intensities of different bands were compared using a Student’s t test to calculate the p value. Error bars indicate mean ± S.D.

**In vitro pyrene-actin polymerization assays**

To determine the actin polymerization activity of DIAPH1, 1 mg of lyophilized pyrene-labeled or unlabeled actin (Cytoskeleton Inc.) was re-suspended in 50 μl of H₂O at 4 °C, then 150 μl of G-buffer (monomer actin buffer: 2 mM Tris, pH 8.0, 0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM β-mercaptoethanol) was added to make an actin stock (58 μM) and incubated on ice for 2 h. For individual assays, the actin stock was further diluted in G-buffer and the pyrene-labeled actin was mixed with unlabeled actin in a 15:85% ratio to a final concentration of 2 μM total actin. Freshly purified proteins were added to actin at a concentration of 2.5 nM and incubated for 5 min at room temperature. The reaction was initiated by adding polymerization buffer (25 mM Tris, pH 7.0, 50 mM KCl, 2 mM MgCl₂, 0.1 mM ATP). The increase in fluorescence intensity was monitored in a PTI fluorimeter with excitation at 365 nm and emission at 386 nm (34). The initial time point was set to when the polymerization buffer was added and the reaction was monitored for 800 s. The t½ was defined as the time point when half-amount of total actin monomers polymerized to filaments (35). Actin polymerization rates at t½ were calculated using the same methods as previously described (35). Briefly, 10 data points at the minimum and maximum intensities were chosen and used to calculate I_min (the average minimum intensity) and I_max (the average maximum intensity). The data point with intensities between 0.48 × (I_max − I_min) + I_min and 0.52 × (I_max − I_min) + I_min was chosen, fitted to a linear curve, and where the slope = m½ and the intercept = b½. Subsequently the t½ (time point when half-amount of total actin monomers polymerize to filaments) was calculated as: t½ = (0.5 × (I_max − I_min) + I_min + b½)/m½, the APt½ (actin polymerization rate at t½) as APt½ = 1.88 × m½/(I_max − I_min).

**siRNA-knockdown assays**

MDA-MB-231 cells (a gift from Dr. L. Attisano, University of Toronto) and HGF cells were transfected with 40 nM double strand IQGAP1 or Fli-I siRNA using Lipofectamine 2000 Reagent (Invitrogen) and DIAPH1 localization was analyzed by immunofluorescence. Alternatively, MDA-MB-231 cells were transfected with 40 nM double strand DIAPH1 siRNA as described above and IQGAP1 or Fli-I localization was analyzed by immunofluorescence. siRNAs were obtained from Inte-
**IQGAP1 enhances DIAPH1 activity**

The quantification of the actin polymerization rate ($Ap_{t_1/2}$) (Figs. 2F and 4D) in the *in vitro* actin polymerization assays was performed using GraphPad Prism (v8.1.2). Each actin polymerization assay was independently repeated three times and the average actin polymerization rate of actin alone (2 μM) with polymerization buffer was set to 1.0. Dots presented in graphs indicate individual data points. Red bars indicate the average value of the data points in each experimental group. The quantification of co-localization analysis of DIAPH1, IQGAP1, Fli-I, or actin in MDA-MB-231 cells were performed using the Coloc 2 plugin of ImageJ (Fiji package, version 2.0.0-rc-69). A total of 30 sets of images in each co-staining group were analyzed to calculate both the autothreshold Mander’s overlapping coefficient (tM) or PCC (above threshold). For the analysis of Mander’s overlapping coefficient or Pearson’s correlation coefficient of the whole image, two channels of a co-staining set were first subjected to background subtraction with a rolling ball algorithm (radius = 50.0) in ImageJ. The two channels were then analyzed by Coloc 2 plugin using Costes method threshold regression (37). The point spread function was set to 10, with Costes randomization set to 100. The M1 channel was assigned as DIAPH1 (Fig. 5, *Cand D*) or phalloidin staining (Fig. 6, *D and E*), whereas the M2 channel was assigned as the co-staining protein in each group as indicated in the graph. The values of tM1 and PCC (above threshold) were exported for statistical analysis. The control groups were set up to compare a DIAPH1 (or phalloidin) single channel image with the exact same image (0° rotation, positive control) or rotating it 90° (negative control). For the analysis of tM or PCC at the leading edge, a region of interest was first drawn using the Freehand tools of ImageJ to generate the leading edge segment of both channels of a co-staining set. The images were then analyzed by Coloc 2 to calculate the tM1 or PCC using the same methods as described above. All graphs and statistical tests in co-localization analysis were generated by GraphPad Prism (version 8.1.2). The non-parametric unpaired Mann Whitney *t* test (*n* = 30) was used to calculate *p* values. No statistical method was used to predetermine sample size.

**Author contributions**—A. C., T. F. M., C. A. M., B. D. L., and A. W. conceptualization; A. C., P. D. A., C. C. L., and A. W. formal analysis; A. C., P. D. A., T. F. M., C. A. M., B. D. L., and A. W. supervision; A. C., C. A. M., B. D. L., and A. W. funding acquisition; A. C. and P. D. A. investigation; A. C. and P. D. A. writing-original draft; A. C., J. W. C., T. F. M., C. A. M., B. D. L., and A. W. writing-review and editing; P. D. A., J. W. C., C. A. M., and B. D. L. methodology.

**References**

1. Skau, C. T., and Waterman, C. M. (2015) Specification of architecture and function of actin structures by actin nucleation factors. *Annu. Rev. Biophys.* **44**, 285–310 CrossRef Medline
2. Blanchin, L., Boujemaa-Paterski, R., Sykes, C., and Plastino, J. (2014) Actin dynamics, architecture, and mechanics in cell motility. *Physiol. Rev.* **94**, 235–263 CrossRef Medline
3. Kühn, S., and Geyer, M. (2014) Formins as effector proteins of Rho GTPases. *Small GTPases* **5**, e29513 Medline
4. Schönichen, A., and Geyer, M. (2010) Fifteen formins for an actin filament: a molecular view on the regulation of human formins. *Biochim. Biophys. Acta* **1803**, 152–163 CrossRef Medline
5. Maiti, S., Michelot, A., Gould, C., Blanchon, L., Sokolova, O., and Goode, B. L. (2012) Structure and activity of full-length formin mDia1. Cytoskeleton (Hoboken) 69, 393–405 CrossRef Medline

6. Otomo, T., Tomchick, D. R., Otomo, C., Panchal, S. C., Machius, M., and Rosen, M. K. (2005) Structural basis of actin filament nucleation and pro- cessive capping by a formin homology 2 domain. Nature 433, 488–494 CrossRef Medline

7. Nezami, A., Poy, F., Toms, A., Zheng, W., and Eck, M. J. (2010) Crystal structure of a complex between amino and carboxy terminal fragments of mDia1: insights into autoinhibition of diaphanous-related formins. PLoS One 5, e12992 CrossRef Medline

8. Li, F., and Higgs, H. N. (2003) The mouse formin mDia1 is a potent actin nucleation factors regulated by autoinhibition. Curr. Biol. 13, 1335–1340 CrossRef Medline

9. Rose, R., Weyand, M., Lammers, M., Izhizaki, T., Ahmadian, M. R., and Wittinghofer, A. (2005) Structural and mechanistic insights into the interaction between Rho and mammalian Dia. Nature 435, 513–518 CrossRef Medline

10. Lammers, M., Rose, R., Scrima, A., and Wittinghofer, A. (2005) The regulation of mDia by autoinhibition and its release by Rho*GTP. EMBO J. 24, 4176–4187 CrossRef Medline

11. Li, F., and Higgs, H. N. (2005) Dissecting requirements for auto-inhibition of actin nucleation by the formin, mDia1. J. Biol. Chem. 280, 6986–6992 CrossRef Medline

12. Higashi, T., Ikeda, T., Murakami, T., Shirakawa, R., Kawato, M., Okawa, K., Furuse, M., Kimura, T., Kita, T., and Horiuchi, H. (2010) Flightless-I (Fli-I) regulates the actin assembly activity of diaphanous-related formins (DRFs) Daam1 and mDia1 in cooperation with active Rho GTPase. J. Biol. Chem. 285, 16231–16238 CrossRef Medline

13. Chen, A., Arora, P. D., McCulloch, C. A., and Wilde, A. (2017) Cytokinesis requires localized β-actin filament production by an actin isoform specific nucleator. Nat. Commun. 8, 1530 CrossRef Medline

14. Watanabe, S., Okawa, K., Miki, T., Sakamoto, S., Morinaga, T., Segawa, K., Arakawa, T., Kinoshita, M., Izhizaki, T., and Narumiya, S. (2010) Rho and anillin-dependent control of mDia2 localization and function in cytokinesis. Mol. Biol. Cell 21, 3193–3204 CrossRef Medline

15. Arora, P. D., Di Gregorio, M., He, P., and McCulloch, C. A. (2017) TRPV4 mediates the Ca2⁺ influx required for the interaction between flightless-I and non-muscle myosin, and collagen remodeling. J. Cell Sci. 130, 2196–2208 CrossRef Medline

16. Arora, P. D., Wang, Y., Bresnick, A., Janney, P. A., and McCulloch, C. A. (2015) Flightless I interacts with NMMIIA to promote cell extension formation, which enables collagen remodeling. Mol. Biol. Cell 26, 2279–2297 CrossRef Medline

17. Copeland, S. J., Green, B. J., Burchat, S., Papalia, G. A., Banner, D., and Copeland, J. W. (2007) The diaphanous inhibitory domain/diaphanous autoregulatory domain interaction is able to mediate heterodimerization between mDia1 and mDia2. J. Biol. Chem. 282, 30120–30130 CrossRef Medline

18. Brandt, D. T., Marion, S., Griffiths, G., Watanabe, T., Kaibuchi, K., and Grosse, R. (2007) Dia1 and IQGAP1 interact in cell migration and phagocytic cup formation. J. Cell Biol. 178, 193–200 CrossRef Medline

19. Mohammad, I., Arora, P. D., Naghibzadeh, Y., Wang, Y., Li, J., Macarenhas, W., Janney, P. A., Dawson, J. F., and McCulloch, C. A. (2012) Flightless I is a focal adhesion-associated actin-capping protein that regulates cell migration. FASEB J. 26, 3260–3272 CrossRef Medline

20. Gould, C. J., Maiti, S., Michelot, A., Graziano, B. R., Blanchon, L., and Goode, B. L. (2011) The formin DAD domain plays dual roles in autoinhibition and actin nucleation. Curr. Biol. 21, 384–390 CrossRef Medline

21. Brenig, J., de Boor, S., Knypsyansen, P., Kuhlmann, N., Wrooblowski, S., Baldus, L., Scisłowski, L., Artz, O., Trauschies, P., Baumann, U., Neundorf, I., and Lammers, M. (2015) Structural and biochemical basis for the inhibitory effect of liprin-α3 on mouse diaphanous 1 (mDia1) function. J. Biol. Chem. 290, 14314–14327 CrossRef Medline

22. Sakamoto, S., Ishizaki, T., Okawa, K., Watanabe, S., Arakawa, T., Watanabe, N., and Narumiya, S. (2012) Liprin-α controls stress fiber formation by binding to mDia and regulating its membrane localization. J. Cell Sci. 125, 108–120 CrossRef Medline

23. Floyd, S., Whiffin, N., Gavilan, M. P., Kutscheidt, S., De Luca, M., Marcozzi, C., Min, M., Watkins, J., Chung, K., Fackler, O. T., and Lindon, C. (2013) Spatiotemporal organization of Aurora-B by APC/CCdh1 after mitosis coordinates cell spreading through FHOD1. J. Cell Sci. 126, 2845–2856 CrossRef Medline

24. Iskratsch, T., Reijntjes, S., Dwyer, J., Toselli, P., Dégano, I. R., Domínguez, L., and Ehler, E. (2013) Two distinct phosphorylation events govern the function of muscle FHOD3. Cell. Mol. Life Sci. 70, 893–908 CrossRef Medline

25. Staus, D. P., Taylor, J. M., and Mack, C. P. (2011) Enhancement of mDia2 activity by Rho-kinase-dependent phosphorylation of the diaphanous autoregulatory domain. Biochem. J. 439, 57–65 CrossRef Medline

26. Cheng, L., Zhang, J., Ahmad, S., Rozier, L., Yu, H., Deng, H., and Mao, Y. (2011) Aurora B regulates formin mDia3 in achieving metaphase chromosome alignment. Dev. Cell 20, 342–352 CrossRef Medline

27. Wang, Y., El-Zarur, M. R., Surks, R. K., and Mendelsohn, M. E. (2004) Formin homology domain protein (FHOD1) is a cyclic GMP-dependent kinase linking protein and substrate in vascular smooth muscle cells. J. Biol. Chem. 279, 24420–24426 CrossRef Medline

28. Vogt, T. F., Jackson-Grusby, L., Rush, J., and Leder, P. (1993) Formins: phosphoprotein isosforms encoded by the mouse limb deformity locus. Proc. Natl. Acad. Sci. U.S.A. 90, 5554–5558 CrossRef Medline

29. Zhou, Q., Wei, S. S., Wang, H., Wang, Q., Li, W., Li, G., Hou, J. W., Chen, X. M., Chen, J., Xu, W. P., Li, Y. G., and Wang, Y. P. (2017) Crucial role of ROCK2-mediated phosphorylation and upregulation of FHOD3 in the pathogenesis of angiotensin II-induced cardiac hypertrophy. Hypertension 69, 1070–1083 CrossRef Medline

30. Greseth, M. D., Carter, D. C., Terhune, S. S., and Traktman, P. (2017) Pro tematic screen for cellular targets of the vaccinia virus F10 protein kinase reveals that phosphorylation of mDia regulates stress fiber formation. Mol. Cell Proteomics 16, S124–S143 CrossRef Medline

31. Liu, J., Fairn, G. D., Ceccarelli, D. F., Sichieri, F., and Wilde, A. (2012) Cleaveage furrow organization requires PIP2-mediated recruitment of anillin. Curr. Biol. 22, 64–69 CrossRef Medline

32. Pogust, A. K., Lai, C. C., Ostman, N., Yu, R. H., Schryvers, A. B., and Moraes, T. F. (2016) A method for measuring binding constants using unpurified in vivo biotinylated ligands. Anal. Biochem. 501, 35–43 CrossRef Medline

33. Abdiche, Y. N., Malashock, D. S., Pinkerton, A., and Pons, J. (2009) Exploring blocking assays using Octet, ProteOn, and Biacore biosensors. Anal. Biochem. 386, 172–180 CrossRef Medline

34. Arora, P. D., and McCulloch, C. A. (1996) Dependence of fibroblast migration on actin severing activity of gelsolin. J. Biol. Chem. 271, 20516–20523 CrossRef Medline

35. Doolittle, L. K., Rosen, M. K., and Padrick, S. B. (2013) Measurement and analysis of in vitro actin polymerization. Methods Mol. Biol. 1046, 273–293 CrossRef Medline

36. Renshaw, M. K. (2005) Structural basis of actin filament nucleation and phagocytic cup formation by binding to mDia and regulating its membrane localization. J. Cell Sci. 125, 108–120 CrossRef Medline

37. Costes, S. V., Daelmans, D., Cho, E. H., Dobbin, Z., Pavlakis, G., and Lockett, S. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells. Biophys. J. 86, 3993–4003 CrossRef Medline

IQGAP1 enhances DIAPH1 activity