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The Formin Daam1 and Fascin Directly Collaborate to Promote Filopodia Formation

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SUMMARY

Filopodia are slender cellular protrusions that dynamically extend and retract to facilitate directional cell migration, pathogen sensing, and cell-cell adhesion [1–4]. Each filopodium contains a rigid and organized bundle of parallel actin filaments, which are elongated at filopodial tips by formins and Ena/VASP proteins [5–10]. However, relatively little is known about how the actin filaments in the filopodial shaft are spatially organized to form a bundle with appropriate dimensions and mechanical properties. Here, we report that the mammalian formin Daam1 (Disheveled associated activator of morphogenesis 1) is a potent actin bundling protein and localizes all along the filopodial shaft, which differs from other formins that localize specifically to the tips. Silencing of Daam1 led to severe defects in filopodial number, integrity and architecture, similar to silencing of the bundling protein fascin. This led us to investigate the potential relationship between Daam1 and fascin. Fascin and Daam1 co-immunoprecipitated from cell extracts, and silencing of fascin led to a striking loss of Daam1 localization to filopodial shafts but not tips. Further, purified fascin bound directly to Daam1, and multi-color single molecule TIRF imaging revealed that fascin recruited Daam1 to and stabilized Daam1 on actin bundles *in vitro*. Our results reveal an unanticipated and direct collaboration between Daam1 and fascin in bundling actin, which is required for proper filopodial formation.

RESULTS AND DISCUSSION

Endogenous Daam1 localizes all along the filopodial shafts

Filopodia contain unbranched actin filaments that are organized into parallel arrays and cross-linked along their lengths in the filopodial shaft [11, 12]. There is little understanding of how the filaments in filopodia are organized, but the actin bundling protein fascin plays a critical role in keeping filopodia rigid and straight [11–13]. Silencing of fascin leads to filopodia with a “wavy” appearance, in which the actin bundle is bent, buckled, and loosely organized [12]. Importantly, fascin depletion does not abolish bundling of filaments in

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SUPPLEMENTAL INFORMATION

Supplemental Information, which includes three figures and corresponding figure legends, Supplemental Experimental Procedures, and five movies can be found with this article online.

filopodia, suggesting that other bundling proteins may contribute to this process [12]. Here, we report a role for the formin protein Daam1 in bundling actin to drive filopodia formation.

In a variety of cell types, formins localize to the tips of filopodial and lamellipodial protrusions, and have been demonstrated to be critical for actin nucleation and elongation at these sites [5, 7, 8, 14, 15]. However, many formins have the additional biochemical activity of bundling actin filaments, even though the potential relevance of this activity *in vivo* has remained largely unexplored. We performed immunostaining of endogenous Daam1 in B16F1 mouse melanoma cells and found that, unlike other reported formins, Daam1 localizes along the entire filopodial shaft and throughout the lamellipodium (Figure 1A and 1B). Line scan analysis revealed a strong co-localization of Daam1 with F-actin in these structures (Figure 1C). This localization to filopodia was verified with a second anti-Daam1 antibody (Figure S1A). Similar colocalization of Daam1 and F-actin in filopodia was observed in other cell lines, including NIH3T3 filbroblasts, N2A neuroblastoma cells, and Raw 264.7 macrophages (Figure S1B). Further, EGFP-tagged full-length Daam1 (EGFP-FL-Daam1) and constitutively active Daam1 (EGFP-Daam1ΔDAD) both localized to filopodia in B16F1 cells (Figure S1C).

Daam1 silencing causes severe defects in filopodia formation

To analyze the potential role of Daam1 in filopodia formation, we silenced Daam1 in B16F1 cells using si-oligonucleotides (siDaam1) directed against its N-terminus. Silencing was confirmed by Western blot analysis (Figure S1D) and by loss of Daam1 immunostaining in siDaam1-treated cells (Figure S1E). Daam1 silencing led to pronounced defects, including filpodia with a wavy appearance (Figure 1D), reminiscent of the defects caused by fascin silencing in B16F1 cells [12, 16]. This observation suggested that Daam1, like fascin, is required for proper structural integrity of filopodia. In addition, there was a ~2.5 fold decrease in the density of filopodia in siDaam1-treated cells compared to cells treated with control scrambled oligos (Figure 1E), again similar to defects caused by fascin silencing and consistent with a destabilization of filopodia structure. These phenotypes were also rescued by expression of an RNAi-resistant EGFP-CDaam1 (encompassing FH1-FH2-COOH) (Figure 1E), demonstrating that the filopodial phenotypes arise from loss of Daam1, and that the C-terminal half of Daam1 (used in all of the biochemical experiments below) is sufficient to perform Daam1's critical role(s) in filopodia formation. A similar striking loss of filopodia was observed upon Daam1 silencing in three additional cell lines (NIH3T3, N2A and Raw264.7; Figure S1B), indicating that Daam1 is critical for filopodia formation in diverse cells types.

A number of formins, including *Drosophila* DAAM, are capable of bundling actin filaments *in vitro* [16–23], but there have been few studies addressing their *in vivo* roles as bundlers. We considered whether mouse Daam1 might function in this capacity within the filopodia shaft. Therefore, we next silenced fascin to directly compare the effects to Daam1 silencing. Silencing of fascin alone caused defects similar to those previously reported [12], which included a drastic reduction in filopodia density and the appearance of aberrant wavy filopodia (Figure 1F and 1H). Co-silencing of Daam1 and fascin led to even stronger defects in filopodia density, although some remaining short F-actin-rich foci or nubs were observed (Figure 1G and 1H). These results indicate that fascin and Daam1 are each critical for maintaining the normal density and appearance of filopodia. In addition, we observed in fascin-silenced cells a drastic reduction in Daam1 localization along filopodia shafts, although Daam1 still localized to filopodia tips (Figure 1I, S1F and 1J). These observations show that fascin is required for proper localization of Daam1 to filopodial actin bundles, and suggest that Daam1 may perform separate roles in the filopodia shafts versus tips.

Daam1 is a potent actin filament bundler

To better understand the activities of Daam1 that underlie its *in vivo* role(s) in filopodia formation, we purified the C-terminal half of human Daam1, CDaam1 (FH1-FH2-COOH), and analyzed its effects on actin filament dynamics and organization *in vitro*. Similar to many other formins, in pyrene-actin assembly assays CDaam1 directly stimulated actin polymerization and protected growing barbed ends of filaments from inhibition by capping protein (Figure 2A). Further, actin nucleation by CDaam1 was suppressed by profilin (Figure 2A) and an I698A mutation in CDaam1 abolished actin nucleation activity (Figure 2B) [24–26].

In addition to its polymerization effects, CDaam1 bundled actin filaments in a concentration dependent manner in low-speed sedimentation assays (Figure 2C and S2A). To examine actin filament bundling by CDaam1 in real time, we used total internal reflection fluorescence (TIRF) microscopy to visualize OG (Oregon Green)-labeled actin filaments. Nanomolar concentrations of CDaam1 induced bundle formation, and the abundance of bundles scaled with increasing CDaam1 concentrations (Figure 2D). Further, we observed zippering of polymerizing filaments into bundles (Figure S2B and Movie S1), reminiscent of previously reported fascin- or fimbrin-induced filament zippering [27, 28]. Analysis of bundle polarity by identification of the fast-growing barbed ends of filaments in time lapse imaging revealed that approximately 80% of CDaam1-induced bundles consisted of filaments with their barbed ends pointing in the same direction (Figure 2E, S2C, and Movie S1). This parallel arrangement is similar to the filament organization observed in filopodia [27, 29, 30]. We then tested whether, in addition to being able to bundle filaments that are actively growing, Daam1 could bundle preformed filaments. After polymerizing filaments in TIRF chambers, we flowed in 300 nM CDaam1 in the absence of actin monomers and observed rapid bundling, which reached completion by ~50 seconds after flow-in (Figure 2F and Movie S2).

Our *in vivo* observations showing that two different bundling proteins, fascin and Daam1, are required to organize actin into a structure that can support normal filopodial protrusion prompted us to compare the ultrastructural effects of fascin and Daam1 on bundle formation *in vitro* using transmission electron microscopy (TEM) (Figure 2G and S2D). Fascin produced very straight bundles consisting of flattened arrays of regularly spaced filaments, enabling measurement of average number of filaments per bundle (17.7 ± 1.6) and average inter-filament distance (8.2 ± 0.5 nm) [16, 31] (Figure S2D). In contrast, CDaam1 produced wavy, densely packed, rounded bundles, precluding any analysis of the number of filaments per bundle or inter-filament distances. Interestingly, bundles formed in the combined presence of CDaam1 and fascin showed intermediate levels of bundle straightness, roundedness, and width, and intermediate levels of inter-filament spacing and organization. Thus, the composite bundles appear to acquire distinct properties from each bundler, which may help explain their co-requirement for filopodia formation *in vivo*.

Fascin directly binds and recruits Daam1 to actin filament bundles

Our *in vivo* observation that fascin is required for Daam1 localization to filopodial shafts prompted us to test whether CDaam1 and fascin physically associate. From B16F1 cells expressing GFP alone, GFP-CDaam1, or GFP-FL-Daam1 from plasmids, we found that endogenous fascin coimmmunoprecipitated with both GFP-Daam1 constructs but not with GFP alone, suggesting that fascin interacts with the C-terminal half of Daam1 (Figure 3A). Further, purified CDaam1 bound to immobilized GST-fascin but not GST (Figure 3B), demonstrating that fascin-Daam1 interactions are direct.

To better understand the functional relationship between fascin and Daam1, we employed two-color TIRF microscopy to directly visualize labeled Daam1 molecules interacting with actin bundles. A purified SNAP-CDaam1 fusion protein was labeled with Benzylguanine-Dy649. SNAP-649-CDaam1 nucleated actin assembly in bulk assays indistinguishably from unlabeled CDaam1 (Figure S3A). Further, it enhanced filament elongation rates in TIRF assays in a profilin-dependent manner (Figure S3B). Single SNAP-649-CDaam1 molecules processively moved on growing barbed ends of actin filaments in the presence and absence of profilin (Figure 3C, S3C and Movies S3) as observed for SNAP-mDia1 [32]. At concentrations above 100 nM, SNAP-649-CDaam1 induced actin bundle formation, similar to untagged CDaam1, and decorated the bundles (Figure 3D). Thus, SNAP-649-CDaam1 was functionally equivalent to CDaam1 in all of its effects on actin dynamics and organization.

To analyze dynamics of SNAP-649-CDaam1 molecules on actin bundles, we used low nanomolar concentrations of SNAP-649-CDaam1, which reduced the background and enabled detection of single-molecule binding events. Since these concentrations of SNAP-649-CDaam1 do not bundle filaments, we pre-bundled filaments with 300 nM of unlabeled CDaam1, fascin, or fimbrin (as a control), then flowed-in lower concentrations (1–10 nM) of SNAP-649-CDaam1. SNAP-649-CDaam1 molecules rarely interacted with the sides of single (non-bundled) filaments but readily associated with the barbed ends (Figure 3C and R.J. unpublished observations). When filaments were bundled by fascin or unlabeled CDaam1, SNAP-649-CDaam1 molecules bound to the sides of bundles (Figure 3E). In contrast, when filaments were bundled by fimbrin, SNAP-649-CDaam1 showed no interactions with the sides of bundles (Figure 3E). Thus, SNAP-649-CDaam1 recruitment to bundles is highly specific, consistent with the direct interaction between fascin and CDaam1.

As an additional control, we asked whether a different formin, mDia1, which lacks bundling activity and is absent from filopodial shafts [33–35], was recruited to the sides of bundles. SNAP-tagged mDia1 (SNAP-649-mDia1-C, FH1-FH2-COOH) readily associated with barbed ends of filaments and remained processively attached during elongation as expected [32]. However, no specific interactions of SNAP-649-mDia1-C with the sides of fascin-generated bundles were detected (Figure 3E). Thus, interactions of SNAP-649-CDaam1 with the sides of bundles are highly specific. Quantification of the number of SNAP-649-CDaam1 and SNAP-649-mDia1-C spots per unit length of bundle further revealed that SNAP-649-CDaam1 interactions are more pronounced for fascin-induced bundles compared to CDaam1-induced bundles (Figure 3F). Taken together with our other observations, fascin appears to directly recruit Daam1 to bundles.

Finally, we analyzed the dynamics of single SNAP-649-CDaam1 molecules in CDaam1- and fascin-generated actin bundles to gain insights into the mechanism of Daam1 interactions with these structures. SNAP-649-CDaam1 molecules were observed to transiently bind CDaam1-bundled filaments, with the majority of dwell times being shorter than 50 seconds (Figure 4A, 4C, S3D and Movie S4). However, in fascin-induced bundles SNAP-649-CDaam1 dwell times greatly increased (Figure 4B, 4D, S3D and Movie S4), suggesting that fascin stabilizes CDaam1 associations with the bundles. SNAP-649-CDaam1 molecules displayed 2-dimensional diffusion along CDaam1-induced bundles, as determined by mean-square displacement analysis (Figure 4A, 4E and Movie S5). In contrast, diffusion was abrogated in fascin-induced bundles as indicated by the spatially static spots (Figure 4B, 4D and Movie S4). Thus, fascin confines SNAP-CDaam1 in the bundle, which is remarkably consistent with our *in vivo* observations showing that Daam1 localization to filopodial shafts is diminished after fascin silencing.

Concluding Remarks

In summary, our results reveal a critical role for the formin Daam1 in organizing filaments in the filopodial shaft, and in maintaining the structural integrity of filopodia. Until now, fascin has been the only bundler shown to both localize all along the filopodia shaft and to be required for filopodia integrity. We found that Daam1 similarly localizes along the shafts, and demonstrated that both Daam1 and fascin are required for proper filopodia formation. Further, we found that fascin and Daam1 directly associate, and that fascin recruits Daam1 to filopodial shafts *in vivo. In vitro*, fascin also is sufficient to recruit Daam1 to actin bundles and to restrict Daam1 diffusion along bundles. Together, these data reveal an unanticipated and direct collaboration between these two actin bundlers, where each is critical *in vivo* for the proper formation of filopodia.

Finally, our results expand the *in vivo* roles of formins to include actin bundling. Until now, formin cellular functions in regulating the actin cytoskeleton have focused primarily on actin filament nucleation and elongation, and there have been few *in vivo* investigations into their potential roles as bundlers. Even though bundling activities have been described *in vitro* for many formins [17, 33], the *in vivo* relevance of these activities has remained in question. Our findings demonstrate a critical role for the formin Daam1 in bundling filaments and collaborating with fascin to give the filopodia shaft structural integrity. Formins also decorate a number of other actin arrays *in vivo* that contain bundled filaments (e.g. stress fibers, cytokinetic rings, stereocilia, invadopodia, bristles, and sarcomeres) [36–41], where their bundling activities may be relevant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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RESEARCH HIGHLIGHTS

- Daam1 is a potent actin bundling protein and localizes to filopodial shafts
- Silencing of Daam1 disrupts filopodia formation and architecture
- Fascin binds to and recruits Daam1 to filopodial shafts *in vivo*
- Fascin stabilizes Daam1 association with actin bundles *in vitro*

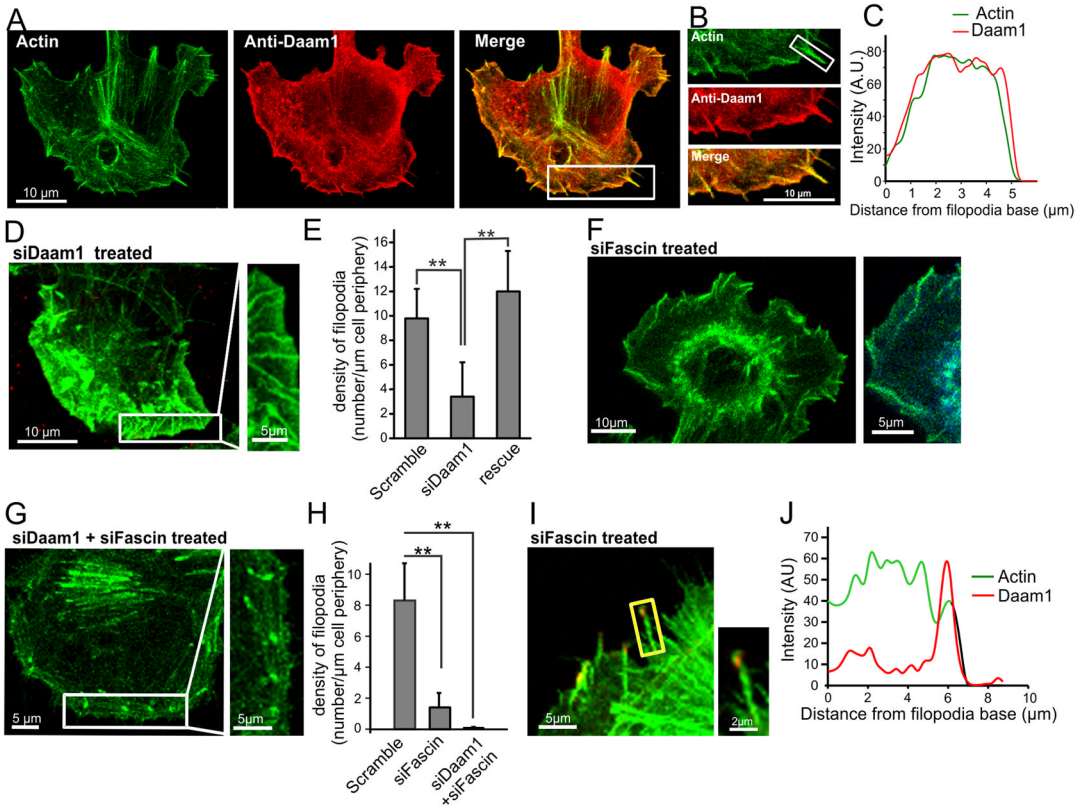

Figure 1. Daam1 localizes to filopodia shafts and is required for filopodia integrity

(**A**) Co-localization of Daam1 and actin. B16F1 cells were fixed and stained with rhodamine phalloidin and anti-Daam1 antibody. (**B**) Magnified view of boxed region in '**A**'. (**C**) Line scan analysis of Daam1 and actin fluorescence intensities along the filopodium boxed in '**B**'. (**D**) Daam1 silencing leads to a decrease in filopodia density and altered filopodial morphology. (**E**) Average filopodia density (number of filopodia per 20 μm of cell periphery) in siDaam1-treated cells, and cells rescued by expression of EGFP-CDaam1 (n=10 cells, $p<0.001$). Error bars represent SD. (**F**) Silencing of fascin leads to a decrease in filopodia density and altered filopodial morphology, reminiscent of Daam1 silencing. (**G**) F-actin staining in cells where fascin and Daam1 were co-silenced. (**H**) Quantification of average filopodia densities. Error bars represent SD (n = 10 cells; $p<0.001$). (**I**) Silencing of fascin reduces Daam1-localization to filopodia shafts but not tips. (**J**) Line scan analysis of fluorescence intensities of Daam1 and actin along the filopodium boxed in '**I**'.

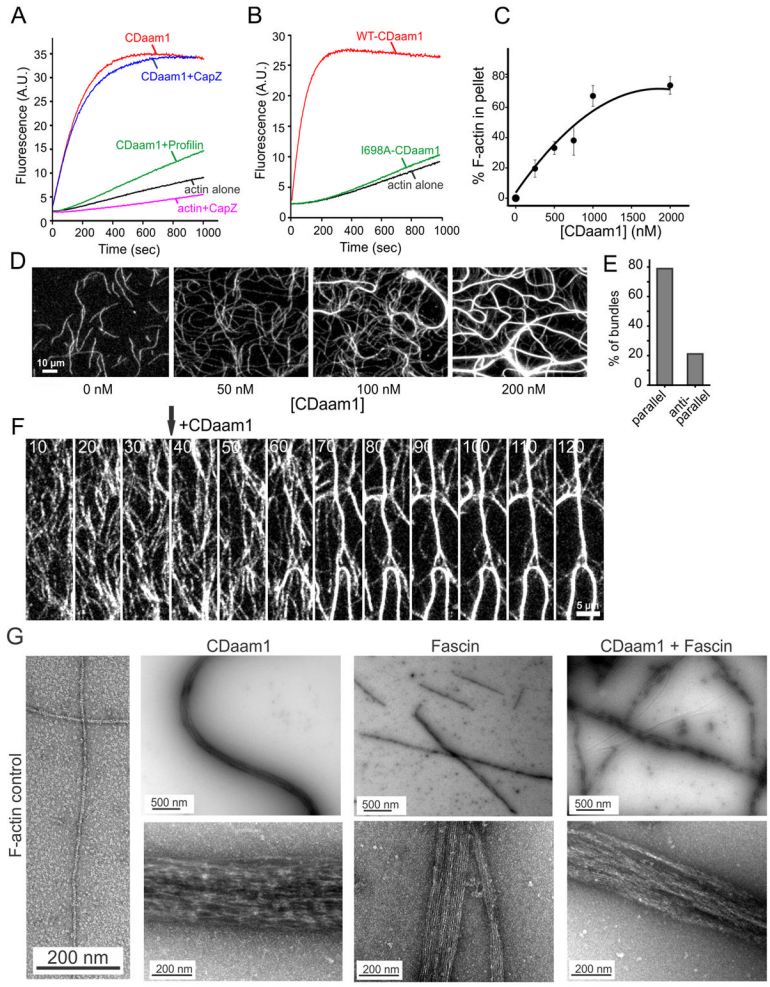

Figure 2. CDaam1 promotes actin filament assembly and bundling *in vitro*

(**A**) Assembly of 2 μM G-actin (5% pyrene-labeled) in the presence of 20 nM CDaam1 (red curve) or 20 nM CDaam1 and 2 nM CapZ (blue curve). CDaam1-induced actin assembly is suppressed by 3 μM profilin (green curve). (**B**) Comparison of actin assembly stimulated by WT-CDaam1 (50 nM, red curve) and I698A-CDaam1 (200 nM, green curve). (**C**) Low-speed pelleting of F-actin in the presence of different concentrations of CDaam1. Gels of pellets and supernatants from one experiment are shown in Figure S2A. Error bars represent SD; n=3 experiments. (**D**) TIRF microscopy images (taken 10 min into reactions) of F-actin bundle formation (1 μM actin, 10% Oregon-Green (OG)-labeled) in the presence of different concentrations of CDaam1. (**E**) Quantification of the polarity of actin filaments in CDaam1-induced bundles. (**F**) TIRF microscopy time-lapse imaging of CDaam1 bundling effects on pre-formed actin filaments. Arrow indicates when 300 nM CDaam1 was introduced into the reaction. (**G**) Electron micrographs of negatively stained actin filaments (far left) or bundles of filaments generated by CDaam1 (left), fascin (center) or CDaam1 and fascin together (right), imaged after 30 min (upper panels) or 18 hours (lower panels) of incubation.

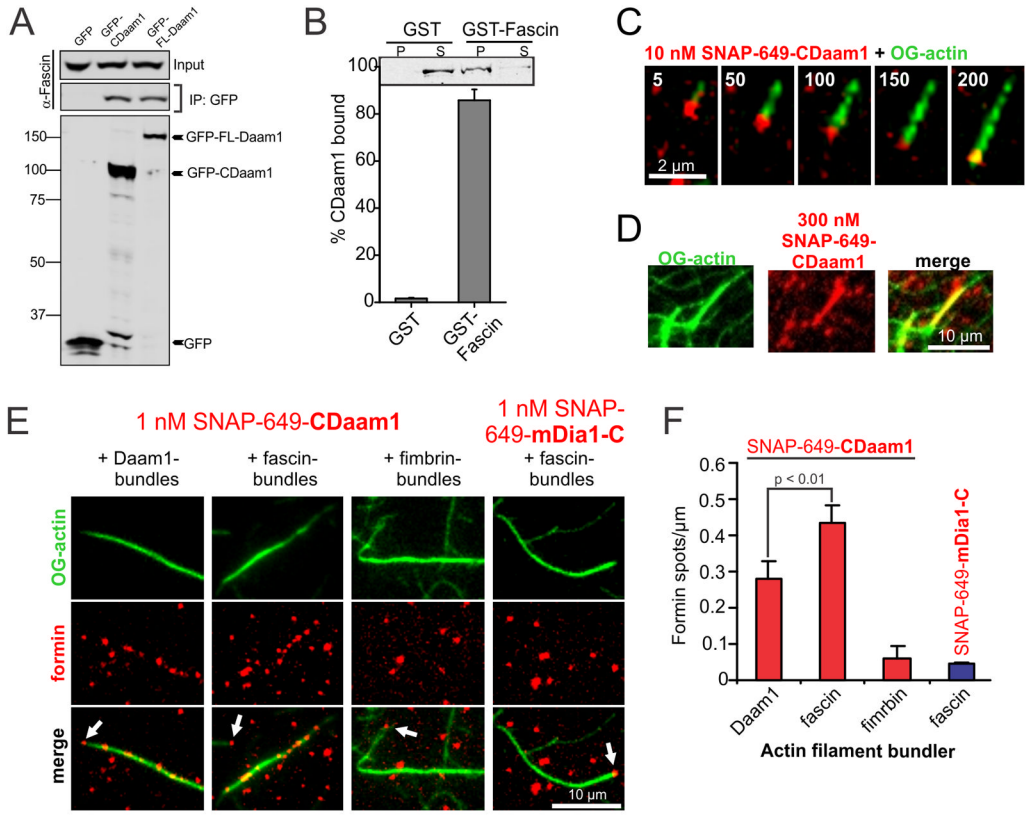

Figure 3. Fascin directly binds and recruits Daam1 to actin filament bundles

(**A**) Coimmunoprecipitation in B16F1 cells of endogenous fascin with plasmid-expressed GFP–CDaam1 and GFP-FL-Daam1 but not GFP. Blots were probed with antibodies to fascin (top) and GFP (bottom). (**B**) Direct binding between purified fascin and CDaam1. Immunoblot probed with anti-Daam1 antibodies and quantification of levels of soluble CDaam1 in pellet fractions after incubation with GST or GST–fascin beads. Error bars represent SD, n = 3. (**C**) Dual color TIRF microscopy of an elongating actin filament (green), conditions as above, processively capped by a SNAP-649-CDaam1 molecule (red). Time is given in sec. (**D**) F-actin bundling by SNAP-649-CDaam1. 1 μM actin (10% OG-labeled) was polymerized in the presence of 300 nM SNAP-649-CDaam1, producing bundles heavily decorated with SNAP-649-CDaam1. (**E**) Single-molecule binding of SNAP-649-CDaam1 to pre-formed F-actin bundles. 1 μM actin (10% OG-labeled) was polymerized in the presence of 300 nM CDaam1, fascin or fimbrin (Sac6) to generate actin filament bundles, then 1 nM SNAP-649-CDaam1 or SNAP-649-mDia1-C was introduced, and binding of formin molecules (red) to bundles (green) was monitored. Arrows mark formin molecules associated with filament/bundle barbed ends. (**F**) Quantification of the number of SNAP-649-CDaam1 or SNAP-649-mDia1-C spots per micron length of bundle.

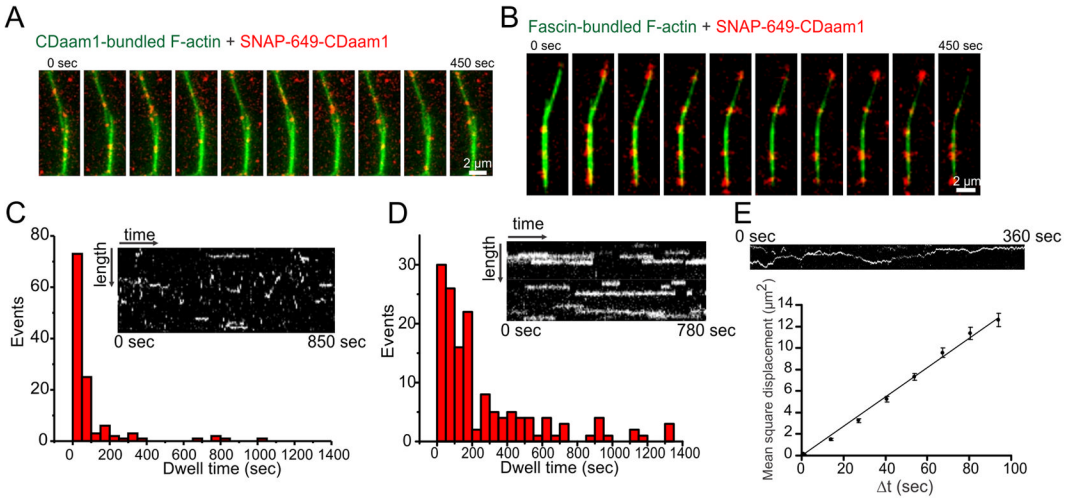

Figure 4. Single molecule analysis of SNAP-649-CDaam1 dynamics on actin bundles generated by unlabeled CDaam1 or fascin

(**A** and **B**) Time-lapse imaging of SNAP-649-CDaam1 molecules binding to actin filament bundles produced by CDaam1 (**A**) or fascin (**B**). (**C**) and (**D**) Kymographs of SNAP-649-CDaam1 binding to bundles from experiments as in '**A**' and '**B**'. Histograms show dwell-times of SNAP-649-CDaam1 molecules on CDaam1 (**C**) and fascin (**D**) bundles. (**E**) Mean-square displacement of the diffusion of a single SNAP-649-CDaam1 molecule (as shown in kymograph above) on a CDaam1-generated bundle plotted against the time interval. A linear curve fit yields a diffusion coefficient of $D = 0.06825\ \mu m^2 s^{-1}$.