Fimbrins/plastins have been implicated in the generation of distinct actin structures, which are linked to different cellular processes. Historically, fimbrins/plastins were mainly considered as generating tight actin bundles. Here, we demonstrate that different members of the fimbrin/plastin family have diverged biochemically during evolution to generate either tight actin bundles or loose networks with distinct biochemical and biophysical properties. Using the phylogenetically and functionally distinct Arabidopsis fimbrins FIM4 and FIM5 we found that FIM4 generates both actin bundles and cross-linked actin filaments, whereas FIM5 only generates actin bundles. The distinct functions of FIM4 and FIM5 are clearly observed at single-filament resolution. Domain swapping experiments showed that cooperation between the conformationally plastic calponin-homology domain 2 (CH2) and the N-terminal headpiece determines the function of the full-length protein. Our study suggests that the structural plasticity of fimbrins/plastins has biologically meaningful consequences, and provides novel insights into the structure-function relationship of fimbrins/plastins as well as shedding light on how cells generate distinct actin structures.

The actin cytoskeleton has been implicated in numerous fundamental physiological cellular processes, including cell migration, cell division, cytokinesis, intracellular trafficking, and so on (1, 2). The multiplicity of actin-related functions correlates to some extent with the formation of distinct actin structures in cells. In fission yeast, actin filaments are packed into three well known actin structures, actin patches, actin cables, and actin rings, which carry out distinct functions (3). In metazoan cells, actin filaments are assembled into at least 15 distinct structures (4); well known examples are the branched actin networks and unbranched actin bundles in the lamellipodia and filopodia, respectively, of crawling cells (5, 6). In plant cells, formation of distinct actin structures with a range of potential functions has been reported (7). The level of actin bundling within plant cells appears to be temporally and spatially regulated during plant growth and defense (8–12). Although the importance of various actin structures is well appreciated, how cells generate and dynamically maintain them remains an open question.

The building block of different actin structures is the actin filament, but the assembly of various actin structures is tightly controlled by numerous actin-interacting proteins, such as actin nucleation factors, actin-bundling/cross-linking proteins, and actin capping proteins as well as filament severing proteins (13–16). Among these, actin-bundling/cross-linking proteins are of particular importance as they dictate the formation of various actin structures and integrate these structures into different physiological cellular processes through their responsiveness to diverse signals such as phosphorylation events and calcium fluxes (17, 18). Previous studies indicate that animal cells use different combinations and sequences of actin cross-linking proteins to assemble bundles with unique properties specific to their cellular functions (19). Different sets of actin-bundling/cross-linking proteins are employed to assemble distinct actin structures within different organisms (7, 19). In partial support of this, several well characterized mammalian actin-bundling proteins, including forked, fascin, espin, and quail, are absent in plants (20). Careful documentation of the activities and regulation of different actin-bundling/cross-linking proteins promises to provide important insights into the generation and maintenance of distinct actin structures in multicellular organisms. In metazoans, filament-bundling/cross-linking proteins are often represented by multiple family members (isovariants), each encoded by a separate gene. An intriguing possibility is that these isovariants may have diverged during evolution to generate the distinct actin structures that are necessary to meet the requirements of different cellular processes. However, it remains virtually unknown whether and how isovariants from the same actin-bundling/cross-linking factor families generate actin structures with distinct morphologies.

One large family of actin cross-linking proteins, including fimbrin, α-actinin, and β-spectrin, is characterized by the presence of a conserved filamentous actin (F-actin) binding domain (ABD) containing about 250 amino acids (21). These ABDs are

The abbreviations used are: ABD, F-actin-binding domain; F-actin, filamentous actin; CH, calponin-homology; N-ter, N-terminal headpiece; C-ter, C-terminal tail; ADF, actin depolymerizing factor; TIRF, total internal reflection fluorescence; SNAP, a mutant of O6-alkylguanine-DNA-alkyltransferase.
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composed of two tandemly arranged calponin-homology (CH) domains. Most of these ABD-containing proteins form noncovalent homodimers that allow the cross-linking of adjacent actin filaments to generate orthogonal actin networks. Spectrin and fimbrin are quite unique in terms of their active forms. Spectrin assembles into tetramers consisting of two heterodimers of the α- and β-subunits. Fimbrin, also known as plastin, was originally discovered in microvilli of the chicken intestinal brush border (22) and functions as a monomer because it possesses two tandem repeats of the AB within a single polypeptide chain. Generation of either orthogonal actin networks or tight actin bundles mediated by these ABD-containing proteins is believed to be determined by the spatial separation and relative orientation of the ABDs (21). Due to the close proximity of the two ABDs, fimbrin is believed to direct the formation of tightly bundled F-actin structures (18, 21, 23, 24). The crystal structure of the actin cross-linking core of fimbrin, consisting of ABD1 (CH1 and CH2) and ABD2 (CH3 and CH4), has been resolved (18). The cross-linking core (referred to as Core hereafter) forms a compact structure, determined by the interaction between CH1 and CH4, in which the two ABDs pack in an approximately antiparallel fashion. Interestingly, two structures of Core were captured in which CH2 adopted different orientations (18); however, it is unknown whether these structures are functionally relevant. Consistent with this evidence of plasticity, a later cryo-EM study of F-actin decorated with the ABDs of fimbrin showed that the F-actin/ABD1 interaction is polymorphic (25). To date, it remains unclear whether and how the conformational plasticity of fimbrin/plastin relates to its functions.

Fimbrins/plastins have been implicated in numerous physiological cellular processes such as endocytosis, cytokinesis, cell migration, polarized cell growth (26–33), and host cell invasion by enteropathic bacteria (34–36) as well as assembly of specialized structures such as intestinal microvilli (37, 38) and auditory stereocilia (39). Fimbrin is so far the only CH domain-containing actin-bundling protein identified in plants, and the Arabidopsis genome has five fimbrin genes, FIM1–5, with distinct tissue expression patterns. Initial observations showed that two Arabidopsis fimbrins, FIM1 and FIM5, are able to generate higher-order actin structures mostly correlates with their phylogenetic grouping, as judged by the results of both low speed F-actin cosedimentation assays (Fig. 1B and supplemental Fig. S1B) and fluorescence light microscopy (Fig. 1C). The closely related FIM1 and FIM4 generate actin networks, whereas FIM3 and FIM5 generate actin bundles (Fig. 1C). Interestingly, the outlier, FIM2, also generates actin bundles (Fig. 1C). We next determined the potential biological significance of these distinct biochemical activities by examining the ability of different FIM genes to rescue fim5 under the control of the FIM5 promoter (27). Transgenic plants with FIM transcript levels that were similar to FIM5 in WT were selected for further analyses (supplemental Fig. S1C). FIM5 fully rescues the pollen germination and tube growth phenotypes in fim5 (supplemental Fig. S1D and Fig. 1, D and E (27)). The other four FIM genes differentially rescue the fim5 phenotypes. Specifically, FIM3, the closest homolog of FIM5, restores fim5 pollen germination and tube growth to a larger extent than the other FIM genes. We next determined the capability of these FIMs to rescue the actin defects in fim5 pollen by measuring the average number and width of the fluorescence peaks of actin filaments in fim5 pollen as described previously (27). Again, FIM5 fully rescues the actin defects in fim5 pollen grains and tubes (Fig. 1, F–H, and supplemental Fig. S1, E and F (27)). FIM3 rescues the fim5 actin defects more strongly than FIM1 and FIM4. Surprisingly, FIM2 restores fim5 actin structures to a similar degree to FIM3; this might be because FIM2 can generate actin bundles in vitro (Fig. 1C). These data suggest that FIM isoforms that generate tight actin bundles can partially replace the in vivo function of FIM5, implying that the biochemical distinction between FIMs is biologically significant.

FIM4 Generates Actin Networks in Vitro, Whereas FIM5 Generates Thick Bundles—To analyze the underlying difference between distinct actin structures generated by FIMs, we carefully compared the actin structures generated by two distinctly
FIGURE 1. *Arabidopsis* FIMs generate distinct actin structures in vitro and differentially rescue fim5. A, SDS-PAGE analysis of purified recombinant FIMs. 2 μg of protein was loaded per lane. B, a low-speed F-actin (3 μM) cosedimentation assay was performed in the presence of 100 nM FIMs. The abilities of different FIMs to generate higher-order actin structures were compared by determining the amount of actin in the supernatant (S) and pellet (P). C, micrographs of actin filaments formed in the presence of FIMs. Actin, 4 μM; FIMs, 1 μM. Micrographs of actin filaments formed in the presence of FIM2, FIM3, and FIM5 were from two independent microscopic fields that were separated by the white lines. Bar = 10 μm. D–H, FIMs differentially rescue fim5. Expression constructs harboring different FIMs driven by the FIM5 promoter were transformed into fim5. D, relative pollen germination percentage. WT pollen germination percentage was normalized to 100%. Statistical comparisons were performed with the χ² test: **, p < 0.01; ND, no significant difference. At least 30 micrographs were analyzed. E, pollen tube growth rate. Statistical comparisons were performed by Student’s t test: **, p < 0.01; ND, no significant difference, n = 50. F and G, the average number (E) and width (F) of fluorescence peaks in pollen grains were plotted to assess the ability of FIMs to rescue actin phenotypes in fim5 pollen grains. Statistical differences were determined by Student’s t test: *, p < 0.05; **, p < 0.01; ND, no significant difference, n = 20. H, average angles between shank-oriented actin cables and the tube growth axis. The angles formed between actin cables and the growth axis of pollen tube in the shanks were measured as described previously (27) and plotted.
related FIMs, FIM4 and FIM5. FIM4 and FIM5 were selected for comparison because both are expressed in pollen, which makes the subsequent functional assessment of their domain-swapped variants in pollen more biologically relevant (see below). We used laser scanning confocal microscopy to compare in more detail the actin structures generated by FIM4 and FIM5. Individual actin filaments or thin actin bundles intertwined within FIM4-generated actin structures, whereas only thick actin bundles were detected within FIM5-generated actin structures (Fig. 2A). This was confirmed by measuring the average width of filamentous structures in the absence or presence of FIM4 or FIM5 (Fig. 2, B and C). Consistent with the in vitro biochemical activities, overexpression of FIM4 generated an increased number of fine actin structures in tobacco pollen tubes, whereas overexpression of FIM5 generated an increased number of thick actin bundles (Fig. 2D). Thus, the data suggest that FIM4 and FIM5 generate loose actin networks and thick actin bundles, respectively.

**Actin Structures Generated by FIM4 and FIM5 Have Distinct Biochemical and Biophysical Properties in Vitro**—We next determined whether the actin structures generated by FIM4 and FIM5 have distinct biophysical and biochemical properties. We found that FIM4-generated actin structures are more viscous and have greater light-scattering ability than FIM5-generated actin structures (Fig. 3, A and B). The actin structures also differed in their stability. FIM5-generated actin structures are more resistant than FIM4-generated structures to depolymerization mediated by both dilution (Fig. 3, C and D) and ADF7 (Fig. 3, E and F). ADF7 (actin depolymerizing factor 7) was selected as the representative ADF because it is pollen-specific (41) and is thus likely to coordinate with FIM4 and FIM5 in pollen. The different stability of FIM4- and FIM5-generated actin structures may result from their differential capability to inhibit ADF7-induced filament fragmentation (Fig. 3, G and H).

Given that some actin filament side-binding and bundling proteins have been shown to inhibit Arp2/3-mediated actin nucleation activities (42, 43), we also asked whether FIM4 and FIM5 inhibit Arp2/3-mediated actin nucleation and if they have differential inhibitory effects. We found that both FIM4 and FIM5 inhibited Arp2/3-mediated actin nucleation in a dose-dependent manner, but interestingly, FIM4 has a more potent inhibitory effect than FIM5 (supplemental Fig. S2, A–C). Direct visu-
alization further confirmed that FIM4 is more potent than FIM5 at inhibiting Arp2/3-mediated formation of actin branches (supplemental Fig. S2, D–G). Taken together, these data suggest that actin structures generated by FIM4 and FIM5 have differential biophysical and biochemical properties.

**FIM4 Generates Actin Bundles and Networks in Vitro, Whereas FIM5 Only Generates Actin Bundles**—We next performed total internal reflection fluorescence (TIRF) microscopy to directly visualize the growth of individual actin filaments during the formation of distinct actin structures. This technology has been used successfully to determine the orientation of single actin filaments within actin bundles generated by yeast FIM1 and WLIM1 (28, 44). We initially determined the average peak width of actin structures formed in the absence or presence of FIM4 or FIM5, and found that the average peak width of actin structures increased in the presence of 1 mM FIM4 and FIM5, with FIM5 having a stronger effect (Fig. 4A).

This is consistent with the formation of actin bundles. In support of this, the skewness of actin filaments significantly increased in the presence of both FIM4 and FIM5, again with...
FIM5 having the stronger effect (Fig. 4B). Using TIRF microscopy, we next performed real-time visualization of growing actin filaments to trace the process of bundle formation. Compared with actin alone (Fig. 4C), “capturing and zipping” of actin filaments were frequently detected in the presence of 1 μM FIM4 (Fig. 4D) and 1 μM FIM5 (Fig. 4H). Considering that some actin-bundling proteins have preference for one filament orientation over the other (45–47), we sought to determine whether FIM4 and FIM5 have selectivity for filament orientation during the formation of higher-order actin structures. We found that both FIM4 and FIM5 are able to generate parallel and anti-parallel actin bundles (Fig. 4, D–K), and the parallel and anti-parallel actin bundles occupy roughly about 56 and 44%, and 63 and 37%, of the total bundles in the presence of 1 μM FIM4 and FIM5, respectively (Fig. 4L). Surprisingly, in the presence of FIM4, actin filament fragments are recruited to an existing actin filament and then stably grow out from the existing filament at relatively large angles to generate branches (Fig. 4, M and N), which presumably results from the filament cross-linking activity of FIM4. We showed that
FIM4 increased the number of actin branches in a dose-dependent manner (Fig. 4O) and the newly recruited actin filaments form different angles with the existing ones (Fig. 4P). This presumably explains why FIM4 generates loosely constructed actin structures.

To directly visualize how exactly FIM4 and FIM5 function differentially in generating distinct actin structures, we generated functional F4-SNAP and F5-SNAP recombinant fusion proteins (supplemental Fig. S3, A–D). We found that F4-SNAP is able to generate cross-linked actin filaments and the cross-linking point occurs on the filament where F4-SNAP is located (supplemental Fig. S3E), suggesting that two actin filaments are directly conjugated by FIM4 at the cross-linking point. In addition, we found that F4-SNAP (supplemental Fig. S3, F and G) and F5-SNAP (supplemental Fig. S3, H and I) were able to generate both parallel and anti-parallel actin bundles. The capturing and zipping of actin filaments occurs on the existing actin filament where F4-SNAP or F5-SNAP is located (supplemental Fig. S3, F–I). Based on the single filament growth data, a simplified schematic diagram describing the generation of tight actin bundles and cross-linked actin filaments mediated by two distinct fimbrin isovariants is presented in Fig. 4Q. Taken together, our data show that FIM4 generates cross-linked actin filaments and actin bundles, whereas FIM5 only generates tight actin bundles.

The CH2 and N-ter Domains Contribute Together to the Distinct Functions of Fimbrins/Plastins—We next decided to determine which domains contribute to the differential activity of FIM4 and FIM5. Besides the Core, which is implicated in direct binding to F-actin, Arabidopsis fimbrins contain an N-ter and a C-terminal tail (called C-ter hereafter) (Fig. 5A) (48). We performed domain swapping experiments to determine the relative contribution of each domain to the distinct functions of FIM4 and FIM5 (Fig. 5A). Recombinant FIM4 and FIM5 variant proteins were generated (Fig. 5B) and functionally assessed. The morphology of the generated actin structures, judged by epifluorescence light microscopy, was greatly affected by substitution of the Core and N-ter, but not by substitution of C-ter (Fig. 5C). We quantitatively evaluated the morphology of the structures by determining the coefficient of variation of the fluorescence of the actin structures (supplemental Fig. S4). This showed that both the Core and N-ter make important contributions to the generation of distinct actin structures (Fig. 5, D and E).

We next analyzed the domains within the Core in the presence of the corresponding N-ter. Given that the Core contains two ABDs, each contributing two F-actin binding sites, we asked which ABD is mainly responsible for the distinct activity of FIM4 and FIM5. We therefore generated four recombinant FIM4 and FIM5 variants by exchanging either ABD1 or ABD2 along with the corresponding N-ter: F4–5(N-ABD1), F4–5(N-ABD2), F5–4(N-ABD1), and F5–4(N-ABD2) (Fig. 5, A and F). Our results showed that the morphology of the actin structures generated by F4–5(N-ABD2) and F5–4(N-ABD1) is similar to those generated by FIM5 and FIM4, respectively (Fig. 5, G–I). Thus, the data suggest that the N-ter along with ABD1 is vital for the distinct activity of FIM4 and FIM5. Given that ABD1 contains two tandemly arranged CH domains, CH1 and CH2, we next asked whether CH1, CH2, or both, are important for the distinct activity of FIM4 and FIM5 in the presence of N-ter. We therefore generated four recombinant proteins, F4–5(N-CH1), F4–5(N-CH2), F5–4(N-CH1), and F5–4(N-CH2) (Fig. 5, A and F). Our results showed that the morphology of actin structures generated by F4–5(N-CH2) and F5–4(N-CH2) appears more similar to those generated by FIM5 and FIM4, respectively (Fig. 5, K–M). This suggests that CH2 determines the distinct function of FIM4 and FIM5 along with the N-ter. In support of this, we found that F4–5(N-CH2) functions significantly better than FIM4 in restoring the pollen germination and tube growth phenotypes associated with fim5 (supplemental Fig. S5). Thus, these data suggest that the CH2 and N-ter domains together determine the distinct functions of fimbrin.

Substitution of Both CH2 and N-ter Is Sufficient to Convert the Activity of Fimbrins/Plastins—We next asked whether control of fimbrin function by the CH2 and N-ter domains is a universal mechanism. We initially selected another two phylogenetically and functionally distinct Arabidopsis FIMs, FIM1 and FIM3 (Fig. 1C), and swapped their N-ter and CH2 domains
(Fig. 6A) to generate the FIM1 and FIM3 protein variants F1–3(N-CH2) and F3–1(N-CH2) (Fig. 6B). The results showed that the morphology of actin structures generated by F1–3(N-CH2) and F3–1(N-CH2) appears more similar to those generated by FIM3 and FIM1, respectively (Fig. 6C and D). This suggests that mutual exchange of both N-ter and CH2 between FIM1 and FIM3 is sufficient to convert their functions. To determine whether this also applies to functionally distinct plastin isovariants in mammals, we analyzed the three plastin proteins from mouse, PLS1–3. We initially generated recombinant PLS1, PLS2, and PLS3 proteins (Fig. 6E) and found that they generated actin structures with distinct morphologies (Fig. 6F). Interestingly, the actin structures generated by PLS2 and PLS3 are similar to those generated by FIM5 and FIM4, respec-
FIGURE 6. Substitution of both the N-ter and CH2 is sufficient to convert one distinct fimbrin/plastin to another from the same multicellular organism. A, schematic map of domain organization of FIM1 and FIM3 and their variants F1–3(N-CH2) and F3–1(N-CH2). In F1–3(N-CH2), the N-ter and CH2 of FIM1 are replaced with those from FIM3; in F3–1(N-CH2), the N-ter and CH2 of FIM3 are replaced with those from FIM1. B, SDS-PAGE analysis of recombinant F1–3(N-CH2) and F3–1(N-CH2). C, fluorescence micrographs of actin structures stained with rhodamine/phalloidin in the absence or presence of FIM1 or FIM3 or their variants. [Actin], 4 μM; [FIM], 1 μM. Ctrl, actin alone. Bar = 10 μm. D, plot of the coefficient of variation of actin structures generated by FIM1, FIM3, and their variants. Values represent mean ± S.D., n = 30. The values were compared statistically by Student’s t test, **, p < 0.01; ND, no significant difference. E, SDS-PAGE analysis of recombinant mouse plastins. F, micrographs of actin structures generated by the mouse plastins PLS1–3. Actin structures were revealed by staining with rhodamine/phalloidin in the absence or presence of mouse plastins. Ctrl, actin alone. Bar = 10 μm. G, schematic map of domain organization of PLS2 and PLS3 and their variants P2–3(N-CH2) and P3–2(N-CH2). In P2–3(N-CH2), the N-ter and CH2 of PLS2 are replaced with those from PLS3; in P3–2(N-CH2), the N-ter and CH2 of PLS3 are replaced with those from PLS2. H, SDS-PAGE analysis of recombinant mouse plastins PLS1–3. Actin structures were revealed by staining with rhodamine/phalloidin in the absence or presence of mouse plastins. Ctrl, actin alone. Bar = 10 μm. J, plot of the coefficient of variation of actin structures generated by PLS2, PLS3, and their variants. Values represent mean ± S.D., n = 30. The values were compared statistically by Student’s t test, **, p < 0.01; ND, no significant difference.
PLS2 and PLS3 were therefore selected for the subsequent domain swapping experiment (Fig. 6G). Recombinant proteins, P3–2(N-CH2) and P2–3(N-CH2), were created (Fig. 6H), and shown to generate actin structures with similar morphology to those generated by PLS2 and PLS3, respectively (Fig. 6I and J). This suggests that mutual exchange of N-ter and CH2 is sufficient to convert the functions of two functionally distinct plastins from the same organism. Thus, these data suggest that the distinct activities of fimbrins/plastins are universally determined by CH2 and the corresponding N-ter.

**CH2 Is Sufficient to Determine the Distinct Functions of the Fimbrin/Plastin Core**—The above results showed that cooperation of CH2 with its corresponding N-ter is required for fimbrin/plastin isovariants to perform distinct functions. Considering that the interaction between fimbrins/plastins and F-actin is mediated by the Core, we asked whether CH2 is sufficient to determine the distinct function of the Core. To answer this question, we initially determined whether the Core could fully represent the full-length fimbrin in terms of generating distinct actin structures. Indeed, recombinant proteins containing only the Core of FIM4 or FIM5 (Fig. 7A and B) were able to generate actin structures that were morphologically similar to those formed by the full-length proteins (Fig. 7C and D). This suggests that the ability of full-length fimbrin to generate distinct actin structures resides in the Core. We next replaced CH2 of the FSCore with the corresponding CH2 from

![Figure 7](image-url)
Distinct Functions of Fimbrin/Plastin Isovariants

The Structurally Plastic CH2 Determines the Distinct Functions of Fimbrins/Plastins—It is generally believed that the interaction between fimbrin/plastin and F-actin is mainly mediated by its Core. In support of this, a previous report showed that the ability of the Core to generate higher-order structures is only slightly weaker than the full-length protein (18). However, there is no direct evidence that the Core fully represents the full-length protein in generating distinct actin structures. We demonstrate that actin structures generated by the Core are indistinguishable from those generated by the full-length protein in terms of their overall morphology (Fig. 7). Interestingly, we found that the functional distinction between two distinct fimbrin isovariants is mainly determined by CH2 (Fig. 7). A previous crystallographic study showed that CH2 of FIM1 adopts two orientations (18), but it is unclear whether this is a crystallographic artifact or a physiologically relevant result. Given that FIM1 and FIM4 are phylogenetic and functional homologs (Fig. 1 and supplemental Fig. S1, A and B), it is tempting to speculate that CH2 of FIM4 might also adopt two orientations, which will lead to different positioning of ABD1 and ABD2 relative to each other, and facilitate the formation of either tight actin bundles or cross-linked actin filaments (Fig. 4 and supplemental Fig. S3). However, in the case of FIM5, we hypothesize that CH2 adopts one orientation, which results in a single orientation of ABD1 relative to ABD2, and facilitates the generation of tight actin bundles only. The speculated mode of action of FIM4 and FIM5 is illustrated in Fig. 8. Direct visualization of the structure of actin filaments decorated with different fimbrin isovariants by cryo-EM promises to provide a conclusive answer to this question in the future. Nonetheless, direct visualization of single filament growth, which shows that FIM4 generates both tight actin bundles and cross-linked actin filaments, suggests to some extent that the conformation of the fimbrin/plastin Core is flexible and adjustable.

Our study is consistent with previous studies showing that the structural plasticity of CH2 and ABD1 is a fundamental mechanistic feature of fimbrin and other CH domain-containing proteins during interaction with F-actin (25, 49–52). Meanwhile, our study suggests that modulation of conformational plasticity may be one of the main regulatory mechanisms used by fimbrins/plastins, with CH2 acting as the main regulatory region. In support of this notion, a previous report showed that the activity of α-actinin is regulated by phosphatidylinositol 4,5-bisphosphate through binding to CH2 (53). However, given that it was reported that CH2 can act as either a negative or positive regulator of CH/F-actin binding (54, 55), the exact role of CH2 in regulating the interaction of CH domain-containing proteins with F-actin needs to be considered on a case-by-case basis. Considering that FIM4 exhibits both actin-bundling and cross-linking activities, regulation of the balance of activities for a particular fimbrin/plastin isovariant via modulation of its conformational plasticity should be considered. We found that CH2 determines the distinct functions of fimbrin/plastin iso-variants (Figs. 5–7 and supplemental Fig. S5), which, to some extent, is consistent with previous studies showing that missense mutations of residues in ABD1 of CH domain-containing proteins cause human disease (52, 56–58). Our study provides the first in vitro functional data showing that the structural

FIM4 to create F5Core-4(CH2). Interestingly, F5Core-4(CH2) generates actin structures similar to those formed by FIM4 (Fig. 7, C and E), suggesting that CH2 is sufficient to confer distinct functions upon fimbrin. Surprisingly, we found that a similar substitution in full-length FIM5, F5-4(CH2), is not sufficient to convert it into a FIM4-like protein (Fig. 7, C and F). This suggests that the N-ter of FIM5 inhibits the flexibility of the FIM4 CH2 domain.

Discussion

Here, we demonstrate that fimbrins/plastins are able to generate loose actin networks besides their expected role in generating tight actin bundles. We provide for the first time data showing growth of individual filaments during the generation of complex actin networks. We also identify that the CH2 domain, previously shown to be conformationally plastic (18), is the major determinant of the distinct functions of fimbrins/plastins. Interestingly, in the context of the full-length protein, CH2 requires the presence of the corresponding N-ter to confer its functional properties. We assume that the N-ter modulates the orientation of CH2 so that ABD1 is properly positioned relative to ABD2. Our results suggest that structural plasticity of fimbrins/plastins is biologically relevant and also provide a biochemical explanation for why multiple isovariants of fimbrin/plastin are needed within a single cell in multicellular organisms. Furthermore, direct visualization of individual filament growth provides general insights into the construction of complex actin networks in cells.

The Basis of Individual Filament Growth Underlying Fimbrin-mediated Generation of Higher-order Actin Structures—We used TIRF microscopy to uncover the underlying basis of individual filament growth during the generation of distinct actin structures by the phylogenetically and functionally distinct FIM4 and FIM5 (Fig. 1). We found that both FIM4 and FIM5 are able to generate actin bundles with mixed polarity, either parallel or anti-parallel (Fig. 4 and supplemental Fig. S3). In this case, we speculate that two adjacent actin filaments, bridged by either FIM4 or FIM5, form small angles. Further bridging by other FIM4 or FIM5 molecules at other locations along those two actin filaments is allowed and therefore induces “zipping” and finally bundling (Fig. 4Q). The mechanism by which FIM4 and FIM5 generate actin bundles is consistent with the capturing and zipping model proposed previously (28). Interestingly, we found that FIM4 can also recruit actin filament fragments to existing actin filaments; these recruited actin filaments continue to grow out at a fixed large angle to the original filament. The recruited filaments will not be zipped and will therefore lead to the generation of cross-linked actin filaments (Fig. 4, M and N, and supplemental Fig. S3 E). We speculate that the cross-linking growth cascade of actin filaments accounts for the generation of loose actin networks mediated by FIM4 (Figs. 1 and 2). To the best of our knowledge, our study is the first to describe the growth of individual filaments during the generation of complex cross-linked actin networks. Given that two F-actin binding surfaces are contributed by ABD1 and ABD2, the TIRF microscopy data allow us to speculate that the ABDs of FIM4 adopt two orientations, whereas the ABDs of FIM5 adopt a single orientation.

The Structurally Plastic CH2 Determines the Distinct Functions of Fimbrins/Plastins—It is generally believed that the interaction between fimbrin/plastin and F-actin is mainly mediated by its Core. In support of this, a previous report showed that the ability of the Core to generate higher-order structures is only slightly weaker than the full-length protein (18). However, there is no direct evidence that the Core fully represents the full-length protein in generating distinct actin structures. We demonstrate that actin structures generated by the Core are indistinguishable from those generated by the full-length protein in terms of their overall morphology (Fig. 7). Interestingly, we found that the functional distinction between two distinct fimbrin isovariants is mainly determined by CH2 (Fig. 7). A previous crystallographic study showed that CH2 of FIM1 adopts two orientations (18), but it is unclear whether this is a crystallographic artifact or a physiologically relevant result. Given that FIM1 and FIM4 are phylogenetic and functional homologs (Fig. 1 and supplemental Fig. S1, A and B), it is tempting to speculate that CH2 of FIM4 might also adopt two orientations, which will lead to different positioning of ABD1 and ABD2 relative to each other, and facilitate the formation of either tight actin bundles or cross-linked actin filaments (Fig. 4 and supplemental Fig. S3). However, in the case of FIM5, we hypothesize that CH2 adopts one orientation, which results in a single orientation of ABD1 relative to ABD2, and facilitates the generation of tight actin bundles only. The speculated mode of action of FIM4 and FIM5 is illustrated in Fig. 8. Direct visualization of the structure of actin filaments decorated with different fimbrin isovariants by cryo-EM promises to provide a conclusive answer to this question in the future. Nonetheless, direct visualization of single filament growth, which shows that FIM4 generates both tight actin bundles and cross-linked actin filaments, suggests to some extent that the conformation of the fimbrin/plastin Core is flexible and adjustable.

Our study is consistent with previous studies showing that the structural plasticity of CH2 and ABD1 is a fundamental mechanistic feature of fimbrin and other CH domain-containing proteins during interaction with F-actin (25, 49–52). Meanwhile, our study suggests that modulation of conformational plasticity may be one of the main regulatory mechanisms used by fimbrins/plastins, with CH2 acting as the main regulatory region. In support of this notion, a previous report showed that the activity of α-actinin is regulated by phosphatidylinositol 4,5-bisphosphate through binding to CH2 (53). However, given that it was reported that CH2 can act as either a negative or positive regulator of CH/F-actin binding (54, 55), the exact role of CH2 in regulating the interaction of CH domain-containing proteins with F-actin needs to be considered on a case-by-case basis. Considering that FIM4 exhibits both actin-bundling and cross-linking activities, regulation of the balance of activities for a particular fimbrin/plastin isovariant via modulation of its conformational plasticity should be considered. We found that CH2 determines the distinct functions of fimbrin/plastin iso-variants (Figs. 5–7 and supplemental Fig. S5), which, to some extent, is consistent with previous studies showing that missense mutations of residues in ABD1 of CH domain-containing proteins cause human disease (52, 56–58). Our study provides the first in vitro functional data showing that the structural
plasticity of fimbrins/plastins is biologically significant. Our work therefore complements previous structural studies (18, 25) and provides new insights into the mechanism of action of fimbrins/plastins.

The N-ter Has Coevolved to Match CH2 in Conferring Distinct Functions upon Fimbrin/Plastin Isovariants—We found that one distinct fimbrin/plastin can be converted into another by replacing CH2 together with the corresponding N-ter (Figs. 5 and 6). Swapping other domains had no effect in our assays. These results suggest that the N-ter has evolved to match the Core. Given that the N-ter and CH2 are separated by CH1, the coordination is very likely through hydrophobic interaction. The N-ter most likely acts by modulating the spatial orientation of CH2 and consequently the spatial orientation of ABD1. In addition, because the N-ter is physically linked with CH1, it may contribute directly to the binding of CH1 with F-actin. In favor of this notion, it was reported that the affinity of Core binding to F-actin decreased in the absence of the N-ter (18), and the N-ter plus ABD1 binds to actin filaments with higher affinity than ABD1 alone (28). Given that the N-ter and Core are relatively conserved between different fimbrin/plastin isoforms, at least in Arabidopsis fimbrins (27), the functional distinction of CH2 between functionally distinct fimbrin isoforms suggests that subtle differences in CH2 have been biologically advantageous during evolution. More work is needed to fully understand how CH2 specifies function in different fimbrin/plastin isoforms, and how it coordinates with the N-ter. Previous reports showed that the calcium-mediated regulation of bundling/cross-linking activity of plastins from mammalian cells and Dictyostelium occurs through the N-terminal EF-hand motif (59–61). Although the exact mechanism of calcium-induced regulation of plastin-mediated bundling remains obscure, it is possible that the calcium-induced conformational change of the N-ter alters the spatial orientation of CH2 and consequently hides the F-actin binding surface of ABD1. Although the bundling activity of plant fimbrins is insensitive to calcium (27, 32, 40), our study suggests that regulation of plant fimbrins through their N-ter should be considered.

Biochemically Distinct Fimbrin/Plastin Isovariants Might Be Involved in the Generation of Distinct Actin Structures in Cells—Our observations suggest that generation of distinct actin structures by fimbrin isoforms is biologically significant, which is evidenced by the data showing that fimbrin isoforms with biochemical activities similar to FIM5 complement fim5 better (Fig. 1 and supplemental Fig. S1). This notion was further strengthened by the data showing that overexpression of biochemically distinct FIM4 and FIM5 generates differential actin structures in tobacco pollen tubes (Fig. 2D). This suggests that generation of distinct actin structures by fimbrin isoforms may represent one strategy by which cells can assemble distinct actin structures. In terms of FIM4 and FIM5, they likely cooperate in driving the construction and maintenance of distinct actin structures in the pollen tube (62), because both express in pollen. More work will be needed to uncover the underlying details.

Experimental Procedures

Protein Production—Total RNA was isolated from Arabidopsis seedlings and inflorescence tissues to amplify full-length cDNAs for FIM1, FIM2 and FIM3, and FIM4 and FIM5, respectively, and from mouse intestinal epithelium to amplify full-length cDNAs for the plastins PLS1, PLS2, and PLS3. Total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. To generate recombinant FIM1–5 proteins, cDNAs were amplified with primer pairs F1KGFor/ F1KGR, F2KGFor/F2KGR, F3KGFor/F3KGR, F4KGFor/ F4KGR, and F5KGFor/F5KGR (supplemental Table 1),
respectively. To generate recombinant proteins for PLS1–3, cDNAs were amplified with primer pairs P1KGF/or/P1KGR, P2KGF/or/P2KGR, and P3KGF/or/P3KGR (supplementary Table 1), respectively. To generate recombinant protein variants for FIM1, FIM3, FIM4, FIM5, PLS2, and PLS3, full-length cDNAs were used as templates to perform overlap PCRs (for primers and detailed construction schemes, see supplementary Table 1). After their sequences were verified, these fragments were moved into the pGEX-KG vector to generate various prokaryotic expression constructs, which were subsequently transformed into Escherichia coli BL21(DE3). Expression of recombinant proteins was induced after the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside at 16 °C overnight. Recombinant proteins were purified with glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions, and GST was cleaved by digestion with thrombin (Sigma) at 4 °C overnight. The purified proteins were dialyzed against 10 mM Tris-HCl, pH 8.0, and subsequently flash-frozen in liquid nitrogen and stored at −80 °C. To generate FIM4-SNAP (F4-SNAP) and FIM5-SNAP (F5-SNAP), the coding sequences for FIM4 and FIM5 were amplified from full-length cDNAs with primer pairs F4-SNAPF/or/F4-SNAPR and F5-SNAPF/or/F5-SNAPR (supplementary Table 1), respectively. The sequences of the products were verified, then the products were moved into pSNAP-tag (T7)-2 vector (New England Biolabs) after digestion with NheI and EcoRI. The resulting expression constructs were subsequently transformed into Escherichia coli BL21(DE3). Expression of recombinant proteins was induced after the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside at 16 °C overnight. Recombinant proteins were purified with nickel-nitrioltriacetic acid beads (Novagen) according to the manufacturer’s instructions, then dialyzed with 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4).

Fluorescent Labeling of SNAP-tagged Proteins—To fluorescently label F4-SNAP or F5-SNAP, purified recombinant proteins at 5 μM were incubated with 10 μM SNAP-Surface 549 (New England Biolabs) in 1× PBS containing 1 mM DTT at 4 °C overnight. The unreacted substrate (SNAP-Surface 549) was separated from the labeled SNAP-tag fusion protein by dialysis.

F-Actin Cosedimentation Assays—Low speed F-actin cosedimentation assays were performed to determine the bundling activity of fimbrin as described previously (40, 63). Preassembled F-actin at 3 μM was incubated with various concentrations of fimbrin proteins in 1× KMEI (50 mM KCl, 1 mM MgCl2, 1 mM EGTA, and 10 mM imidazole, pH 7.0) at room temperature for 30 min, and then spun at 13,600 × g for 30 min at 4 °C. High speed F-actin cosedimentation assays were performed roughly according to previous reports (27, 64) to assess the antagonizing effect of FIM4 or FIM5 on ADF-mediated actin depolymerization. Preassembled F-actin at 3 μM was incubated with or without 1 μM FIMBRINs in 1× KMEI at room temperature for 5 min. 5 μM ADF7 was then added and incubated further for 30 min. The samples were subsequently spun at 100,000 × g for 30 min at 4 °C. For both low speed and high speed F-actin cosedimentation assays, the supernatant and pellets resulting from centrifugation were resolved by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. The amount of actin in the supernatant and pellet was quantified by densitometry with ImageJ Software (rsbweb.nih.gov/ij; version 1.48).

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Direct Visualization of FIMBRIN-decorated Higher-order Actin Structures by Microscopy—Various concentrations of recombinant fimbrin/plastin or their variants were incubated with 4 μM preassembled actin in 1× KMEI buffer for 60 min at room temperature. Subsequently, the reactions were diluted 20-fold in fluorescence buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl2, 100 mM DTT, 10 μg/ml of glucose oxidase, 15 mg/ml of glucose, 20 μg/ml of catalase, and 0.5% methylcellulose) before imaging. To visualize FIMBRIN-decorated higher-order actin structures, actin filaments were labeled with equimolar amounts of rhodamine/phalloidin then observed under an Olympus IX71 microscope equipped with a ×60 objective (1.42 NA) as described previously (65). Images were captured with a Retiga Exi Fast 1394 CCD camera (QImaging) using Image-Pro Express 6.3 software. To visualize higher-order actin structures at a three-dimensional level, they were labeled with equimolar Alexa-488 phalloidin and observed under an Olympus FV1000MPE multiphoton laser scanning confocal microscope equipped with a ×100 objective (1.4 NA). Samples were excited by a laser with the excitation wavelength set at 488 nm, and the images were collected with the emission wavelength set at 505–605 nm.

Determination of Coefficient of Variation of the Fluorescence Images of Actin Structures in Vitro—To quantitatively assess the morphology of actin structures generated by fimbrin/plastin and its variants, the coefficient of variation of the fluorescence images of actin structures was measured. Coefficient of variation is defined as the ratio of the standard deviation to the mean values of the fluorescence pixel intensity of the fluorescence images, and allows the comparison of the degrees of variation between actin structures. Briefly, regions of interest were selected randomly from images of the actin structures using the polygon selection tool in ImageJ software. The values of standard deviation and the mean of each area were obtained and the coefficient of variation was calculated. A minimum of 30 images were analyzed to calculate the coefficient of variation in each experiment, and the experiments were repeated at least three times.

Actin Depolymerization Assays—Dilution-mediated actin depolymerization was performed to test the ability of fimbrin proteins to stabilize actin filaments as described previously (27). Various concentrations of FIM4 or FIM5 were incubated with 5 μM preassembled actin filaments (100% pyrene labeled) at room temperature for 5 min. The mixtures were subsequently diluted 25-fold in G buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM CaCl2, 0.5 mM DTT, 0.2 mM ATP, 0.01% NaN3), and the change of fluorescence accompanying depolymerization was monitored by a spectrofluorometer (Photon Technology International, Inc.). The stabilizing effect of FIM4 and FIM5 on actin filaments was also assayed by determining their ability to antagonize ADF-mediated actin depolymerization. Preassembled actin filaments at 5 μM (80% pyrene labeled) were incubated with or without 1 μM FIM4 or FIM5 in the presence of 2 μM ADF7 for 5 min at room temperature followed by 10-fold dilution in G buffer.
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Actin Nucleation Assays—Mg-ATP-monomeric actin (10% pyrene labeled) was incubated with GST-VCA (200 nM) and Arp2/3 (10 nM, from Cytoskeleton, Inc.) in the presence of varying concentrations of FIM4 or FIM5 for 10 min at room temperature, and actin polymerization was initiated by adding one-tenth volume of 10× KMEI. The change of fluorescence was monitored by a spectrofluorometer (Photon Technology International) with excitation and emission wavelengths set at 365 and 407 nm, respectively.

Light Scattering Assays—Light scattering assays were performed to test the formation of higher-order actin structures as described previously (66). Briefly, various concentrations of FIM4 or FIM5 were incubated with 3 μM actin in a microcapillary tube (100-μl calibrated pipettes, Sigma) in 1× KMEI for 2 h at room temperature. Microcapillary tubes were sealed with plasticine at one end and stood on a steady platform at a 45 degree angle. A small steel ball was placed in the tube and the time for the ball to run a distance of 5 and 10 cm from the starting line was recorded. At least three independent experiments were conducted.

Falling Ball Assays—To test the effect of FIM4 and FIM5 on the viscosity of actin filaments solution, the falling ball assay was performed as reported previously (67). Briefly, various concentrations of FIM4 or FIM5 were incubated with 3 μM actin in a microcapillary tube (100-μl calibrated pipettes, Sigma) in 1× KMEI for 2 h at room temperature. Microcapillary tubes were sealed with plasticine at one end and stood on a steady platform at a 45 degree angle. A small steel ball was placed in the tube and the time for the ball to run a distance of 5 and 10 cm from the starting line was recorded. At least three independent experiments were conducted.

Direct Visualization of Actin Dynamics in Vitro by TIRF Microscopy—TIRF microscopy was used to observe dynamic actin events, such as bundling, ADF-mediated severing, and Arp2/3-mediated branch nucleation. Flow cells were prepared according to a previously published method (68). Flow cells were finally washed with 1× TIRF buffer (1× KMEI buffer, 100 mM DTT, 0.2 mM ATP, 15 mM glucose, 20 μg/ml of catalase, 100 μg/ml of glucose oxidase, 0.2% BSA, 0.5% methylcellulose). To trace the process of actin bundle formation mediated by FIM4 or FIM5, different concentrations of FIM4 or FIM5 were mixed with 1.5 μM Mg-ATP actin (33.3% rhodamine labeled) in 1× TIRF buffer. Images were acquired immediately after the mixtures were injected into flow cells. The polarity of bundles was determined by the relative direction of the two fast growing ends and the percentage of both parallel and anti-parallel bundles was then calculated. The angles of cross-linked actin filaments refers to the angles formed between two fast-growing actin filaments during the generation of cross-linked actin filaments in the presence of FIM4. The activity of FIM4 in generating cross-linked actin structures was quantified by measuring the number of branches formed per unit length of actin filament over time. The angles of cross-linked actin bundle formation in the presence of FIM4 were determined and quantified. To perform dual color visualization of fimbrin and actin filaments, the procedures were exactly the same except that FIM4 or FIM5 were replaced with F4-SNAP or F5-SNAP. To test the antagonizing effect of FIM4 or FIM5 on ADF-mediated actin depolymerization and severing, reassembled F-actin (33.3% rhodamine labeled) at 1.5 μM was incubated with or without 1 μM FIM4 or FIM5 for 5 min and the mixtures were subsequently perfused into the flow cells. The flow cell was allowed to settle, then placed on the stage of a microscope for imaging. After the focal plane was found, 1 μM ADF7 in 1× TIRF buffer was perfused into the flow cell and images were acquired immediately. To determine the effect of FIM4 or FIM5 on Arp2/3-mediated actin branch formation, 1 μM Mg-ATP actin (33.3% rhodamine labeled) was incubated with 100 nM GST-VCA, 5 nM Arp2/3, and 1 μM FIM4 or 1 μM FIM5 in 1× TIRF buffer, and images were acquired immediately after the mixtures were injected into the flow cell. The samples were illuminated by TIRF illumination under an Olympus IX81 microscope equipped with a ×100 oil objective (1.49 numerical aperture) and images were captured with MicroManager imaging software (Micro-Manager) at 2- or 3-s intervals.

Transient Expression in Tobacco Pollen Tubes—To determine the effect of gain-of-function of FIM4 or FIM5 on actin structures in pollen tubes, microparticle bombardment was employed according to a previously published method (69). Overexpression of FIM4 or FIM5 in pollen tubes was carried out with the PLIM2a promoter. To generate the overexpression constructs, the PLIM2a promoter was initially amplified with primer pair PLIM2aproF1/PLIM2aproRev, and cloned into pEASY-blunt vector (TransGen Biotech) to generate pEASY-blunt-PLIM2aapro. The coding regions of FIM4 and FIM5 were amplified with primer pairs F4For/F4Rev and F5For/F5Rev, respectively, and were moved into pEASY-blunt-PLIM2aapro after digestion with XbaI/EcoRI to generate pEASY-blunt-PLIM2aapro-FIM4/FIM5. After sequence verification, the PLIM2apro-FIM4 and PLIM2apro-FIM5 fragments were moved into pdGN vector (70) to generate pdGN-PLIM2aapro::FIM4/FIM5 after digestion with SmaI/XbaI. The fragment containing Lat52, Lifeact-EGFP, and Nos terminator was amplified with primer pairs Lat52For/NOSF4Rev and Lat52For/NOSF5Rev, respectively, using the plasmid pCambia 1301-Lat52::lifeact-EGFP-nos (71) as the template. The PCR products were moved into pdGN-PLIM2aapro::FIM4 and pdGN-PLIM2aapro::FIM5 after being digested with KpnI/SmaI and XhoI/SmaI, respectively, to yield the final constructs pdGN-FIM4::FIM5 after digestion with SmaI/XbaI. The fragment containing Lat52, Lifeact-EGFP, and Nos terminator was amplified with primer pairs Lat52For/NOSF4Rev and Lat52For/NOSF5Rev, respectively, using the plasmid pCambia 1301-Lat52::lifeact-EGFP-nos (71) as the template. The PCR products were moved into pdGN-PLIM2aapro::FIM4 and pdGN-PLIM2aapro::FIM5 after being digested with KpnI/SmaI and XhoI/SmaI, respectively, to yield the final constructs pdGN-PLIM2aapro::FIM4::Lat52::lifeact-EGFP-nos and pdGN-PLIM2aapro::FIM5::Lat52::lifeact-EGFP-nos. Tobacco pollen grains on nitrocellulose membranes were bombarded three times with a PSD-1000/He particle delivery system (Bio-Rad), and 5 μg of plasmid DNA was used each time. The bombarded pollen grains were germinated on germination medium and pollen tubes were observed under an Olympus FV1000MPE multiphoton laser scanning confocal microscope equipped with a ×100 oil objective (1.4 numerical aperture). The excitation wavelength was 488 nm and the emission wavelength was 505–605 nm.

Genetic Rescue Experiments—The ability of different FIM genes to rescue the phenotype of the loss-of-function mutant fim5 was tested according to previously published methods (27). FIM1, FIM2, FIM3, FIM4, FIM5 and recombinant variants of FIM4 and FIM5 were driven by the native FIM5 promoter. RT-PCR was employed to screen transgenic lines in which the gene expression level was close to that of FIM5 in wild type. Analysis of the pollen germination percentage and pollen tube growth rate were performed as described previously (72, 73).
Actin staining of pollen grains and pollen tubes and subsequent quantitative analysis, such as peak number and peak width for pollen grains as well as the angles formed between actin cables and the pollen tube growth axis, were performed exactly as described previously (27). Statistical Analysis—Besides the statistical analysis of pollen germination percentage data performed by the $\chi^2$ test with SPSS 13.0, the statistical analysis of other data were performed using Student’s t test.

Author Contributions—R. Z. and S. H. conceived and designed the research; R. Z., M. C., M. Z., X. Q., and Y. W., performed the research; R. Z., Y. W., M. Z., M. C., X. Q., and S. H. analyzed the data; R. Z. and S. H. wrote the paper.

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Note Added in Proof—In the version of this article that was published as a paper in press on June 3, 2016, Figs. 1C, 6H, and 7C did not clearly indicate the borders between different sections of different microscopy fields or the same gel. These errors have now been corrected and do not affect the results or conclusions of this work.

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