Capping Protein Insulates Arp2/3-Assembled Actin Patches from Formins

Highlights

- Capping proteins prevent access of formins to Arp2/3-nucleated actin patches
- Formins at uncapped Arp2/3 patches form actin structures of mixed identity
- Formin activity at these ectopic sites leads to diversion of myosins and cargoes
- Capping protein β subunit shows specific competition with the formin knob region

In Brief

How do cells maintain functionally distinct actin structures in a common cytosol? Billault-Chaumartin and Martin show that competition between capping proteins and formins keeps formins off Arp2/3-assembled structures. Formin activity at uncapped Arp2/3 patches forms mixed-identity actin structures, which divert myosins from their site of action.
Capping Protein Insulates Arp2/3-Assembled Actin Patches from Formins

Ingrid Billault-Chaumartin and Sophie G. Martin
1Department of Fundamental Microbiology, Faculty of Biology and Medicine, University of Lausanne, 1015 Lausanne, Switzerland
2Lead Contact
*Correspondence: sophie.martin@unil.ch
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SUMMARY

How actin structures of distinct identities and functions coexist within the same environment is a critical self-organization question. Fission yeast cells have a simple actin cytoskeleton made of four structures: Arp2/3 assembles actin patches around endocytic pits, and the formins For3, Cdc12, and Fus1 assemble actin cables, the cytokinetic ring during division, and the fusion focus during sexual reproduction, respectively. The focus concentrates the delivery of hydrolases by myosin V to digest the cell wall for cell fusion. We discovered that cells lacking capping protein (CP), a heterodimer that blocks barbed-end dynamics and associates with actin patches, exhibit a delay in fusion. Consistent with CP-formin competition for barbed-end binding, Fus1, F-actin, and the linear filament marker tropomyosin hyper-accumulate at the fusion focus in cells lacking CP. CP deletion also rescues the fusion defect of a mutation in the Fus1 knob region. However, tropomyosin and exocytic cargoes are reduced at the fusion focus and diverted to ectopic foci, which underlies the fusion defect. Remarkably, the ectopic foci coincide with Arp2/3-assembled actin patches, which now contain low levels of Fus1. We further show that CP localization to actin patches is required to prevent the formation of ectopic foci and promote efficient cell fusion. During mitotic growth, actin patches lacking CP similarly display a dual identity, as they accumulate the formins For3 and Cdc12, normally absent from patches, and are co-decorated by the linear filament-binding protein tropomyosin and the patch marker fimbrin. Thus, CP serves to protect Arp2/3-nucleated structures from formin activity.

INTRODUCTION

Cells simultaneously contain several actin-based structures that need to be tailored to their specific function, with a specific architecture, size, lifetime, and set of actin-binding proteins. The architecture is defined in part by the nucleator [1, 2]: Arp2/3 promotes the assembly of branched structures, whereas other nucleators, in particular formins, assemble linear ones. Arp2/3-assembled dendritic networks generate pushing forces against membranes, for instance in the lamellipodium of migrating cells, to drive the movement of intracellular bacteria, or to promote internalization of endocytic vesicles in yeast actin patches. Formin-nucleated actin structures consist of linear filaments, which can be bundled for protrusive or contractile force generation, for instance in filopodia or cytokinetic contractile rings, or which underlie long-range myosin-based transport.

The principles underlying the assembly of distinct actin structures at the same time are beginning to be understood. First, competition is an important factor. For instance, diverse filamentous actin (F-actin) structures are in competition for a limited pool of actin monomers [3]. This competition is modulated by profilin, which favors F-actin assembly by formins and other nucleators over Arp2/3 [4, 5]. Second, specific actin nucleators confer part of the structure’s identity: formins promote the formation of more flexible filaments [6], favoring tropomyosin association [7]; different formins may even promote association of distinct tropomyosin isoforms [8]. Third, self-assembly principles govern the segregation of specific actin-binding proteins to diverse structures. For instance, cooperative loading and competition between fimbrin and tropomyosin drive their association to distinct actin structures [9]. Therefore, in vivo, tropomyosin preferentially associates with formin-assembled structures and fimbrin with Arp2/3-nucleated actin patches [10, 11].

Capping protein (CP) is present in cells in micromolar concentration, similar to the concentration of actin filament barbed ends, and binds the barbed end to arrest dynamics [12]. CP forms a heterodimer of structurally similar α and β subunits, both of which harbor a mobile C-terminal extension, called the tentacle, which contributes to barbed-end binding [13–15]. CP lacking both tentacles forms a stable complex but does not bind actin, with the α tentacle playing a more critical role than the β tentacle [14, 16]. CP activity is further regulated by binding partners bearing a CP interaction (CPI) motif [17]: CP-carrying mutations blocking CPI binding retain capping activity in vitro but lose localization and function in vivo, indicating that binding partners are required for activity in vivo [18]. The Aim21-Tda2 complex, which binds CP through the same residues, modulates CP recruitment and activity at actin patches in S. cerevisiae [19, 20]. By keeping filaments short, CP plays a major role in the force production of dendritic networks [21]. Indeed, absence of CP leads to loss of the lamellipodium in migrating cells [22, 23] and excess actin filaments in yeast actin patches, which exhibit a longer lifetime [16, 24–26].

As formins and CP both interact with actin filament barbed ends but promote opposite activities—i.e., extension versus
capping—they compete with each other in vitro [27–30]. Recent single-molecule work showed formin and CP simultaneously binding the filament barbed end, forming a ternary “decision complex” intermediate [31, 32]. Evidence for competition in vivo was shown in the fission yeast Schizosaccharomyces pombe, where CP deletion ameliorates the cell-division phenotype of a hypomorphic formin cdc12 allele [33]. CP may also compete with formins in filopodia [34]. In addition, CP is well established to compete with ENA/VASP both in vitro and in vivo, where this competition controls filopodium formation [12, 35]. Whether such competition contributes to actin structure identity has not been explored.

The fission yeast represents a simple system to dissect the mechanisms governing actin structure identities. This cell contains only four actin structures, actin patches, cables, ring, and focus, each of which fulfills a specific function [1, 36]. Arp2/3 nucleates actin patches, marked by fimbrin Fin1, around invaginating endocytic vesicles, which assemble in a stereotypical manner [37, 38] and provide force for vesicle internalization [39]. CP localizes to actin patches [33, 40, 41], where it limits actin incorporation and helps force production [16, 24–26]. Interestingly, although active CP is a heterodimer, deletion of the α and β subunits (Acp1 and Acp2, respectively) does not produce exactly the same phenotype [24]. Three formins nucleate distinct linear F-actin structures: Cdc12 assembles the contractile actin ring; For3 assembles linear actin cables that underlie long-range myosin-based transport for polarized cell growth; and Fus1 is expressed specifically during mating and nucleates the fusion focus, an F-actin aster that concentrates secretory vesicles transported by myosin V Myo52 [42, 43]. These vesicles carry cell-wall hydrolases, which digest the cell wall for cell fusion. The coalescence of the fusion focus also requires tropomyosin Cdc8 [44], and a visual screen revealed Cdc8 functions together with the type V myosin Myo51 [45]. This same screen identified fusion defects in cells lacking acp2.

Starting from the hypothesis that CP and formin Fus1 compete during fusion focus assembly, we discovered that CP protects actin patches against formin activity. In the absence of CP, Fus1 binds barbed ends in actin patches, forming ectopic foci that divert secretory vesicles away from the site of cell-cell contact and compromise fusion. Similarly, in proliferating cells lacking CP, the formins For3 and Cdc12 are ectopically recruited to actin patches, which exhibit a dual identity manifested by codecoration with fimbrin and tropomyosin. Thus, CP ensures actin structure identity by insulating Arp2/3-assembled structures against formins.

RESULTS

Capping Protein Is Required for Efficient Cell-Cell Fusion

To investigate the role of CP in cell fusion, we first assessed the fusion efficiency of strains lacking one or both CP subunits (acp1Δ, acp2Δ, acp1Δ acp2Δ). After 12 h of starvation, these strains exhibited a reduced fraction of fused zygotep (Figure 1A), indicating that CP deletion causes a fusion delay. The duration of the fusion process, from initial formation of the Myo52-labeled fusion focus in both partner cells [42] to cytoplasmic mixing, defined by entry in the M cell of cytosolic GFP expressed in P cells, was significantly longer in CP-lacking cells (Figures 1B and 1C). The fusion focus also persisted significantly longer post fusion in CP-lacking cells (Figures 1D and 1E). Both phenotypes were clear in all mutant combinations but strongest in acp2Δ single mutant, on which we focused most of our attention. Thus, CP promotes fusion, because its absence causes fusion delay and persistence of the fusion focus after fusion.

Formin Fus1 and F-Actin Excessively Accumulate at the Fusion Site in the Absence of Capping Protein

Consistent with CP preventing filament barbed-end extension, previous work reported that Arp2/3-assembled actin patches lacking CP accumulate more actin [24, 41]. To investigate the organization of F-actin during fusion, we first used GFP-CHD as F-actin marker [46, 47]. Like in interphase cells, actin patches appeared brighter in acp2Δ than WT cells during mating (Figure 2A) [24]. acp2Δ, acp1Δ, and acp1Δ acp2Δ cells also displayed slightly more F-actin at the position of the fusion focus (Figures 2A, 2D, and S1A). Because GFP-CHD measurements at the fusion site cannot distinguish between F-actin in the fusion focus or in surrounding patches, we probed the localization of specific fusion focus components. Fus1 accumulated approximately 4-fold more in acp2Δ, acp1Δ, and acp1Δ acp2Δ cells than in WT cells at the fusion focus (Figures 2B, 2D, and S1B). Global Fus1-sfGFP fluorescence levels also increased 1.6-fold (Figure S1D). Tropomyosin Cdc8, which preferentially binds formin-assembled filaments [7, 44, 48–50], increased about 2-fold at the fusion focus of acp2Δ compared to WT cells (Figures 2C, 2D, and S1C). Thus, the absence of CP leads to increased F-actin and associated proteins at the fusion focus.

The dramatic Fus1 increase in acp2Δ fusion foci is consistent with the proposed competition between formins and CP for barbed-end binding [31–33]. We note that CP levels were mildly increased in fus1Δ (1.1-fold; Figures S2A and S2B). To probe the hypothesis that CP promotes cell fusion by limiting Fus1-driven actin polymerization at the fusion focus, we tested whether (1) Fus1 overexpression mimics loss of CP, (2) reducing Fus1 activity ameliorates fusion efficiency in the absence of CP, and (3) CP localizes to the fusion focus. First, Fus1 overexpression had no effect on fusion duration, fusion focus persistence, or ectopic Myo52 foci (Figures S2C–S2E; see below regarding ectopic foci). However, the overexpression increased total Fus1 but not Fus1 at the fusion focus (Figure S2D). Thus, Fus1 overexpression does not directly mimic loss of CP. Second, we constructed four Fus1 alleles mutated in the FH2 domain. Although all mutants abolished actin assembly in vitro [51], they showed different phenotypes when introduced as sole copy at the native genomic locus of otherwise WT cells. Fus1K1087A and Fus1K1112A, which carry mutations in the FH2 lasso, were partly fusion competent, whereas Fus1T1087R and Fus1N1088P, which carry mutations in the FH2 knob and post, respectively, almost completely blocked cell fusion. Combining these fus1 alleles with the acp2Δ phenotype did not systematically ameliorate the fusion phenotype (Figures 2E and 2F). In particular, the two hypomorphic lasso mutants compromised fusion further in acp2Δ. By contrast, the fusion-incompetent fus1T1087R allele permitted high levels of fusion in acp2Δ. This reveals an allele-specific suppression, where the Fus1 knob but not lasso.
mutants compromise competition with CP. This finding is consistent with the recently proposed steric clash between the FH2 knob and the CP β tentacle [31]. Third, Acp1 and Acp2 tagged with sfGFP localized prominently to actin patches, but neither was detected at the fusion focus in either WT cells or cells lacking the other CP subunit (Figures 5A, 5C, 5D, and 5F). Thus, CPs are largely absent from the fusion focus, suggesting that competition with Fus1 principally takes place elsewhere.

**Absence of Capping Protein Leads to Reduced Levels of Myosin V and Cargoes at the Fusion Focus and Formation of Ectopic Foci**

Despite higher Fus1 levels, myosin Myo52 was reduced about 2-fold at the fusion focus in \( \text{acp2}D \) compared to WT cells (Figures 1C and 3F). A similar reduction was observed in \( \text{acp1}D \) and \( \text{acp1}D \ \text{acp2}A \) cells (Figure S3A). Similarly, exocytic vesicles marked by the exocyst subunits Exo84 and Exo70, the Rab11 homolog Ypt3, and the glucanase cargoes Agn2 and Eng2 were all reduced at the focus at fusion time in CP-lacking strains (Figures 3A–3F and S3B–S3F). Because these glucanases are responsible for degrading the cell wall for fusion [42], their reduced accumulation at the focus is the likely cause of the \( \text{acp2}D \) fusion delay.

Why do the actin-rich \( \text{acp2}D \) fusion foci accumulate fewer exocytic vesicles? We noticed that \( \text{acp2}D \) cells frequently form Myo52 foci away from the fusion focus (Figure 3G). Such ectopic foci formed in both P and M cells repeatedly during the fusion process. In time-lapse imaging at 5-min intervals, \( \text{acp2}D \) cells...
Figure 2. CP Deletion Leads to Increased Actin, Tropomyosin, and Fus1 at the Fusion Focus

(A–C) Myo52-tdTomato and (A) GFP-CHD labeling F-actin, (B) Fus1-sfGFP, and (C) GFP-Cdc8 in WT and acp2Δ at fusion time.

(D) Fusion focus fluorescence intensities normalized to WT at fusion time in strains as in (A)–(C). See Figures S1A–S1C for full fluorescence profile measurements.

(E) Spinning-disk confocal images of Myo52-tdTomato and WT or mutant Fus1-sfGFP, as indicated, in WT and acp2Δ. Yellow arrowheads point to unfused cell pairs, with a broad Fus1 and Myo52 distribution at the contact zone. Blue arrowheads point to fusion foci.

(F) Fusion efficiencies at 9 h after nitrogen removal in WT or acp2Δ strains carrying WT or mutant fus1, as indicated. All p values are relative to WT, except where indicated. Scale bars represent 5 μm. See also Figures S1 and S2.
displayed on average 7 time points with ectopic foci, acp1Δ and acp2Δ cells displayed 1, and we barely found any for WT cells with this set-up (a single ectopic focus in 32 mating pairs; Figure 3H). Camera upgrade (which happened in the course of the project) and spinning-disk imaging revealed more ectopic foci in all backgrounds, including WT (see Figures 5J and 4A, respectively). The rare, transient ectopic foci detected in WT mating pairs (see Figure 1C, 10-min time frames, for example) suggest that the acp2Δ behavior exists but is normally repressed in WT cells. These ectopic foci extensively colocalized with Exo84, Exo70, and Ypt3 (Figures 3A–3C and S3G–S3I). We could not detect glucanases at ectopic foci, likely because of low

Figure 3. CP Deletion Leads to Reduced Vesicular Markers at the Fusion Focus and Formation of Ectopic Foci

(A–E) Exo84-GFP, Myo52-tdTomato and (A) Myo52-GFP, (B) Exo70-GFP, (C) GFP-Ypt3, (D) Agn2 sfGFP, and (E) Eng2 sfGFP in WT and acp2Δ before fusion time. White arrows highlight ectopic foci. Note that these images, selected to show ectopic foci, stem from various time points during time-lapse imaging, for which specific timing in the fusion process and photobleaching may mask the difference in fusion focus intensity.

(F) Fusion focus fluorescence intensities at fusion time normalized to WT in the strains mentioned in (A)–(E).

(G) Time-lapse images of Myo52-tdTomato in WT and acp2Δ during the fusion process. White arrows show ectopic Myo52 foci. Time is in h:min.

(H) Number of time frames at which a Myo52 ectopic focus was observed during the fusion process in time-lapse imaging as in (G).

All p values are relative to WT. Scale bars represent 5 μm. See also Figure S3 and Video S1.
expression levels and reduction of function by sfGFP tagging (Figures 3D and 3E). These results suggest that the formation of ectopic foci is the cause of the reduced Myo52 and cargoes at the fusion focus.

**Myo52 Ectopic Foci Form at Actin Patches**

Higher-speed time-lapse spinning-disk imaging at 1-s intervals showed Myo52 ectopic foci did not appear randomly; they did not break off from the fusion focus; instead, they formed and stayed at remote locations at the cell periphery, occasionally moving back and fusing with the fusion focus (Figure 4A; Video S1). The colocalization with exocytic markers stood true at this higher temporal resolution (Figure 4B). Thus, ectopic foci are nucleated at a remote location in the absence of CP.

Interestingly, GFP-CHD as F-actin marker revealed that >90% of ectopic foci colocalized, at least transiently, with what looked like actin patches (Figure 4C). Ectopic foci also colocalized with Myo51, a second type V myosin that normally associates with tropomyosin and decorates linear actin structures [45, 52, 53] (Figure 4D). Whereas Myo51 principally decorates the fusion focus in WT cells, in acp2Δ it additionally localized to actin patches marked by the actin bundler Fim1 [54, 55] (Figure 4F). Importantly, the more sensitive spinning-disk microscopy also revealed Fus1 in Myo52 ectopic foci (Figure 4E; Video S2), which colocalized with Fim1 (Figure 4G; Video S3). Fus1 also colocalized with Myo52 ectopic foci in acp1Δ acp2Δ double mutants (Figure S4; Video S4). Thus, the absence of CP leads to recruitment of formin Fus1 and type V myosin to Arp2/3-nucleated actin patches.

Fus1 likely binds exposed filament barbed ends at actin patches in the absence of CP, and nucleates ectopic foci. To investigate whether CP’s capping function is required to protect patches marked by Fus1, we deleted Acp1 and Acp2 tentacles (acp1Δ and acp2Δt) to reduce CP affinity for actin barbed ends. These truncations compromised actin patch localization, with Acp2Δt retaining better localization than Acp1Δ (Figures 5B, 5E, and 5H), in agreement with previous in vitro work [14]. They also showed more frequent ectopic Myo52 foci than WT cells, in an
order consistent with their retained actin-binding capacity (Figures 5B, 5E, and 5I). We also generated acp2R12A,Y77A, predicted not to bind the CPI motif [18]. Acp2R12A,Y77A localized inefficiently to patches (Figures 5G and 5H), and exhibited ectopic foci and increased fusion times, to levels intermediate between WT and acp2Δ cells (Figure 5J). The reduced localization of Acp1Δt, Acp2Δt, and Acp2R12A,Y77A to actin patches was not due to reduced F-actin content in the patches, which instead exhibited increased LifeAct-mCherry fluorescence (Figures S5A and S5B), like acp2Δ cells [24]. These results indicate that localization of CP to patches is necessary to prevent the formation of ectopic foci.

Finally, to test whether ectopic foci cause the fusion delay by diverting vesicles away from the cell-cell contact zone, we artificially recruited Myo52-GFP to patches labeled with Fim1-GBP-mCherry by using the high affinity between GFP-binding protein (GBP) and GFP (Figures S5C–S5E). This led to fusion delay and focus persistence after fusion, replicating the acp2Δ phenotypes (Figures 1B, 1E, and S5C–S5E).

We conclude that during fusion, CP insulates actin patches from Fus1. This ensures Fus1 activity is restricted, and myosin V-driven cargoes are directed to the site of cell-cell contact.

**Uncapped Actin Patches Recruit Formins and Acquire a Dual Identity in Interphase Cells**

To test whether CP more generally protects actin patch identity, we investigated the influence of CP deletion in interphase cells. In the absence of CP, actin patches are dispersed both in WT and acp2Δ cells (Figure 6J). This ensures Fus1 activity is restricted, and myosin V-driven cargoes are directed to the site of cell-cell contact.

![Figure 5. CP Recruitment to Actin Patches and Barbed-End Binding Is Required to Protect against Ectopic Foci](image-url)
Figure 6. CPs Insulate Actin Patches from Myo52 and Actin Cable Markers in Interphase Cells

(A–F) Spinning-disk confocal images of (A) Fim1-mCherry and Myo51-sfGFP, (B) Myo52-tdTomato and Myo51-sfGFP, (C) Myo52-tdTomato and Fim1-sfGFP, (D) Fim1-mCherry and GFP-Cdc8, (E) Fim1-mCherry and For3-3GFP, and (F) Fim1-mCherry and Cdc12-3GFP in WT and acp2Δ interphase cells. In (E), bottom panels show acp2Δ treated with 500 μM CK-666 for 5 min or 200 μM latrunculin A for 5 min. White arrows highlight colocalization events in acp2Δ, which do not occur in WT cells. Yellow arrowheads point to Cdc12 spots. The proportion of colocalization at ectopic sites along the cell sides is shown with the bar plot on the right.

(G) Spinning-disk confocal time-lapse images of Fim1-mCherry and For3-3GFP (green and gray) showing retrograde flow in WT (top) and acp2Δ cells (bottom two panels). Bottom: an example of retrograde flow starting at a lateral actin patch. Kymographs of the yellow dashed boxed regions are shown on the right. Green arrows point to For3 retrograde flow. Purple arrowheads in kymographs show lateral actin patches on which For3 localizes in acp2Δ but not WT cells.

(H) For3 retrograde flow rate.

Time is in s. Scale bars represent 5 μm. See also Figure S6 and Videos S5 and S6.

colocalized with Fim1 in acp2Δ (Figure 6D; Video S5). The coincidence of fimbrin and tropomyosin is particularly remarkable given recent data showing that competition between these two proteins drives their sorting to distinct Arp2/3- and formin-nucleated networks, respectively [9]. Thus, actin patches assume a dual identity in the absence of CP both during mating and during vegetative growth.

Because Fus1 is not expressed during mitotic growth [43], we monitored the localization of For3 and Cdc12 formins. For3, which only occasionally overlapped with actin patches in WT cells, was prominently present at actin patches in acp2Δ mutants (Figure 6E; Video S6). By contrast, in fim1Δ cells, in which tropomyosin also decorates patches [11], For3 was largely absent from patches like in WT cells (Figure S6A). In acp2Δ cells, disruption of patches with the Arp2/3 inhibitor CK-666 or F-actin depolymerization with latrunculin A restored For3 localization to cell poles (Figure 6E). Arp2/3 inhibition also promoted Fim1 re-localization to cell poles, which is likely due to excess formin-assembled cables [3], as complete actin depolymerization rendered Fim1 cytosolic (Figure 6F). Thus, uncapped actin patches ectopically recruit For3. Cdc12 also formed ectopic foci at actin patches in acp2Δ cells (Figure 6F). These ectopic...
foci were distinct from the previously reported spot of Cdc12
[57], which also occurs in WT cells, does not coincide with
patches, has a longer lifetime, and is more intense (Figure 6F).
We conclude that formins are recruited to uncapped actin
patches.

To address whether formins are active at patches, we first
tested whether their inactivation would alleviate the localization
of tropomyosin at acp2Δ actin patches. However, deletion of
for3 by itself led to significant Cdc8 enrichment on actin patches,
likely because actin cytoskeleton homeostasis is perturbed in
the absence of actin cables (Figure S6B). Therefore, not surpris-
ingly, Cdc8 also localized to actin patches in for3Δ acp2Δ and
cdc12-112 for3Δ acp2Δ mutants (Figures S6B and S6C). We
then directly probed for For3 activity by observing its retrograde
flow, which depends on actin assembly in cables [47]. For3 retro-
grade flow occurred at similar rates in WT and acp2Δ cells,
although fewer movements were observed in acp2Δ mutants [33, 41].
Interestingly, For3 linear movements could be observed to initiate from
actin patches, suggesting cable assembly from the patches (Fig-
ure 6G). We conclude that, independent of the specific formin,
CP insulates actin patches from formins, restricting their activity
to the proper location.

DISCUSSION

How cells simultaneously assemble functionally diverse actin
structures of distinct identity within a common cytosol is a
debated question. Arp2/3 and formins, respectively, assemble
branched and linear actin structures decorated by largely
distinct actin-binding proteins. A hallmark of formins is their abil-
ity to promote barbed-end growth against the growth-arrest
function of CP, a feature demonstrated in numerous in vitro
studies [27–32]. However, how CP may prevail against formins
in vivo was largely unexplored. Here, we have shown that CP
protects Arp2/3-assembled actin patches against formins, thus
preserving their identity and restricting formins to their proper
location.

Our interest in CP arose from the cell-fusion delay of acp2Δ
cells. For fusion, cells locally digest their cell wall by concent-
trating the exocytosis of secretory vesicles containing cell-wall
hydrolase at the site of cell-cell contact [42]. In WT cells, this is
achieved by the Fus1-assembled actin fusion focus. By contrast,
in cells lacking CP, despite strong Fus1 accumulation at the
fusion site, secretory vesicles are frequently diverted away
from the fusion site to ectopic Fus1 foci at actin patches. This
correlates with a reduction in secretory vesicles at the fusion
focus, which likely explains the fusion delay. Indeed, the forced
diversion of Myo52 to actin patches also yields extended fusion
times. Thus, the fusion defect of CP mutants likely results from
diversion of secretory vesicles to ectopic sites, rather than from
excessive actin assembly at the fusion site. Because CP
mutants exhibit longer actin patch lifetimes [24], which may
lead to slower endocytosis, the fusion delay may also partly
result from reduced recycling of the pheromone receptor from
the plasma membrane [58].

This raises the question of where CP acts—in formin-assem-
bled structures or at Arp2/3-assembled patches. We argue that
the formin-CP competition happens throughout the cell,
with distinct outcomes: in formin-assembled structures, the for-
min wins and CP is largely dispensable; at actin patches, CP
dominates and insulates this structure against formins. These
distinct outcomes are reflected in the exclusive localization of
CP at patches and restriction of formins to other cellular loca-
tions. The occasional ectopic foci detected in WT cells illustrate
that CP patch protection against formins is an active process
that can transiently fail even in WT cells.

CP-formin competition is best revealed by alteration of Fus1
and/or CP function. At the fusion focus, the competition is
exposed by Fus1 FH2 mutations. A first observation is that
FH2 mutations compromise fusion to various extents in vivo,
although they all fully abrogated actin assembly in vitro [51].
The poor predictive value of the in vitro activity may be due to
high local Fus1 concentration in the focus, which likely exceeds
the concentrations tested in vitro. A second observation is the
strong allele-specific suppression by acp2Δ of the fusion defect
of fus1ΔI951A, which carries a mutation in the knob. This allele-spe-
cific suppression is consistent with the proposed structural
arrangement of the formin-CP ternary complex at the barbed
end, which shows a steric clash between the formin knob and
CPβ [31]. The I951A mutation likely favors the binding of CPβ,
thus promoting capping function in the ternary complex, which
is relieved upon CPβ deletion. In the cell, the inability of Fus1ΔI951A
assembled a fusion focus must be due to CP competing with
the formin at the fusion site. Further evidence for competition
at this location comes from increased Fus1 intensity at the fusion
focus and the extended focus lifetime in CP mutants. This sug-
ests that small amounts (below detection levels) of CP may
compete with Fus1 in the fusion focus, although the increased
Fus1 intensity may also be due to Fus1 recruitment to adjacent
patches. We conclude that some Fus1-CP competition can
take place at the fusion site, where Fus1 normally dominates.

Our data indicate that the principal sites of formin-CP compe-
tition are actin patches, where CP efficiently outcompetes Fus1.
In WT cells, CP is strongly enriched at actin patches and pre-
vents formin binding. In cells lacking CP function, all three for-
mins localize to actin patches. The strength of CP protection
against formins largely scales with its barbed-end binding affinity
as measured in vitro [14], with acp2Δ showing fewer ectopic foci
than acp1Δ, and acp1Δ or acp2Δ. When CP is absent from
patches, these acquire characteristics of linear actin structures:
they are decorated by myosin V Myo51 and tropomyosin, which
normally preferentially associate with actin cables, ring, and
focus [7, 44, 45, 48–50, 52, 53]; they also accumulate the myosin
V Myo52, which erroneously transports its cargoes to these loca-
tions. The coincidence of tropomyosin and fimbrin at patches
devoid of CP is striking given recent findings that competition
between these actin-binding proteins drives their specific asso-
ciation with formin- and Arp2/3-assembled structure in vitro,
respectively [9–11]. Importantly, For3 is absent from tropomy-
osin-decorated fim1Δ patches, indicating that formins localize
to patches because of a specific defect in capping rather than a
global change in actin cytoskeleton homeostasis. We conclude
that, in the absence of CP, actin patches acquire a double
identity.

One important question is whether the double identity of CP-
devoid patches arises from ectopic formin activity or simply
from uncapping. This question is difficult to address because
any perturbation in actin structures will perturb homeostasis [3]. For instance, formin deletion frees G-actin and tropomyosin, which now incorporate in actin patches. Conversely, actin patch disruption enhances formin-assembled structures, now more permissive for fimbrin association. However, two observations argue for formin activity at CP-devoid patches. First, during mating, Myo52 was not present at all actin patches, but was always there when Fus1 was. This argues that Fus1 activity is the driving force for vesicular cargo recruitment. Second, the For3 flow from patches indicates assembly of cables from this location [47]. As a side note, although the mechanism and function of For3 retrograde flow remain unknown, it occurred, and at similar rates, in acp2Δ cells, indicating that flow is not due to arrest of For3-dependent filament elongation upon formation of a ternary formin-CP complex. Together, these observations indicate that patch-localized formins actively assemble linear actin filaments.

An interesting observation is that acp2Δ cells consistently displayed stronger phenotypes not only than acp1Δ but also than acp1Δ acp2Δ double mutants. Berro and Pollard also previously noted that the phenotypes of acp2Δ and acp1Δ are not identical [24]. We note that our data show a quantitative, but not qualitative, difference between these genotypes. It is formally possible that the stronger phenotype of acp2Δ cells is due to a CPβ function partly unrelated to capping activity. However, the weaker phenotype of acp1Δ acp2Δ instead indicates that Acp1 not bound by Acp2 enhances the phenotype. One interpretation is that, although formins gain access to actin barbed ends in the absence of Acp2, they may gain better access if Acp1 is still present. Because Acp1 still weakly binds the actin barbed end in the absence of Acp2 in vitro [16], its presence may somehow help recruit formins to the barbed end. This interpretation predicts an interaction between formins and CPα, a hypothesis consistent with human INF2 formin association with CPα [59].

Because CP-formin competition yields distinct outcomes at the sites of formin action and Arp2/3-assembled patches, one question is what defines the competition outcome. Part of the answer comes from the Acp2R12A,Y77A allele, carrying mutations in the CPI-binding residues, which compromises CP localization to actin patches. This finding agrees with previous data in human cells that CP localization relies on interaction with CPI-containing proteins [18]. The specific CP-binding partners are unknown in S. pombe, but may involve the homolog of S. cerevisiae Aim21, which binds CP through the CPI-binding residues and contributes to its localization to actin patches [19], although this finding was not reproduced in a second study [20]. Consistent with this hypothesis, deletion of S. pombe Aim21 was identified in our genome-wide screen to have fusion defects [45]. The phenotype of acp2R12A,Y77A cells indicates that CP recruitment to actin patches by pre-localized partners is required to protect them against formin activity. Thus, barbed-end-independent recruitment of CP may tip the CP-formin competition in favor of CP in Arp2/3-assembled structures.

The findings described in our study present the CP-formin competition in a new light, where CP protects Arp2/3 structures against ectopic localization of formins. In fission yeast acp2Δ cells, formin localization to actin patches has important consequences for cellular organization: during mating, Fus1 diverts cargoes away from the fusion site, slowing the fusion process; in interphase cells, For3 activity at patches may cause the previously noted actin cable disorganization and partial loss of cell polarity, leading to actin patch depolarization [24, 33]; in dividing cells, the reason for the cytokinetic defect of cells lacking CP [33] may also be the titration of Cdc12 to actin patches. Because CP is ubiquitous in eukaryotic cells, it likely protects the identity of Arp2/3 actin assemblies and prevents formin ectopic activity in a vast range of organisms.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.07.088.

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**AUTHOR CONTRIBUTIONS**

I.B.-C. performed all experiments; I.B.-C. made the figures, with contributions from S.G.M.; I.B.-C. and S.G.M. wrote the manuscript; S.G.M. provided vision; and S.G.M. acquired funding.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, Peptides, and Recombinant Proteins |  |  |
| LatrunculinA | Enzo Life Science | Cat# T-119-0500 |
| CK666 | Sigma | Cat# SML0006 |
| AgaPure Agarose LE | Canvax | Cat# AG006 |
| Vaseline | Reactolab | Cat# 99813 |
| Lanolin | Sigma | Cat# L7387 |
| Paraffin | Reactolab | Cat# 99756 |
| Dimethyl sulfoxide (DMSO) | Applichem | Cat# 1584 |
| Poly(ethylene glycol) BioUltra, 4,000 | Sigma | Cat# 95904 |
| Lithium Acetat Dihydrate | Applichem | Cat# A3478 |
| EDTA Disodium Salt 2-hydrate | Applichem | Cat# A2937 |
| Tri(hydroxy)methylaminoethane | Biosolve | Cat# 200923 |

## Experimental Models: Organisms/Strains

| h90 myo52-tdTomato:natMX ura4-294:p^{pmap3}.GFP:ura4+ | Lab Stock | YSM2535 |
| h90 myo52-tdTomato:natMX ura4-294:p^{pmap3}.GFP:ura4+ acp1::kanMX | Lab Stock | YSM307 |
| h90 myo52-tdTomato:natMX ura4-294:p^{pmap3}.GFP:ura4+ acp2::kanMX | Lab Stock | YSM2955 |
| h90 myo52-tdTomato:natMX ura4-294::p^{pmap3}.GFP-ura4+ acp1::kanMX acp2::kanMX | This work | YSM3008 |
| h90 myo52-tdTomato:natMX leu1-32:p^{pmap41}.GFP-CHD:leu1+ | Lab Stock | YSM2515 |
| h90 myo52-tdTomato:natMX leu1-32:p^{pmap41}.GFP-CHD:leu1+ acp1::hphMX | This work | YSM3009 |
| h90 myo52-tdTomato:natMX leu1-32:p^{pmap41}.GFP-CHD:leu1+ acp2::bleMX | This work | YSM3010 |
| h90 myo52-tdTomato:natMX leu1-32:p^{pmap41}.GFP-CHD:leu1+ acp1::hphMX acp2::bleMX | This work | YSM3011 |
| h90 myo52-tdTomato:natMX fus1-sfGFP:kanMX | This work | YSM3012 |
| h90 myo52-tdTomato:natMX fus1-sfGFP:kanMX acp1::hphMX | This work | YSM3013 |
| h90 myo52-tdTomato:natMX fus1-sfGFP:kanMX acp2::bleMX | This work | YSM3014 |
| h90 myo52-tdTomato:natMX fus1-sfGFP:kanMX acp1::hphMX acp2::bleMX | This work | YSM3015 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ | This work | YSM3016 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp2::bleMX | This work | YSM3017 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3018 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3019 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3020 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3021 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3022 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3023 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3024 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3025 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3026 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3027 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3028 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3029 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3030 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3031 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3032 |
| h90 myo52-tdTomato:natMX fus1::p^{pmap41}.GFP-cdc8::ura4+ acp1::hphMX acp2::bleMX | This work | YSM3033 |

(Continued on next page)
### Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| h90 myo52-tdTomato:natMX exo70-GFP:kanMX acp1::hphMX | This work | YSM3334 |
| h90 myo52-tdTomato:natMX exo70-GFP:kanMX acp2::bleMX | This work | YSM3335 |
| h90 myo52-tdTomato:natMX ura4-294:pnmt41-GFP-ypt3:ura4+ | This work | YSM3337 |
| h90 fim1-mCherry:natMX leu1::pinst1::LifeAct::mCherry::leu1+ | Lab Stock | YSM3434 |
| h90 fim1-mCherry:natMX for3-3GFP:ura4+ | This work | YSM3365 |
| h+ fim1-mCherry:natMX cdc12-3GFP:ura4+ | This work | YSM3366 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sophie Martin (sophie.martin@unil.ch). Yeast strains generated in this study have not been deposited on an external repository but are available for distribution on request from the Lead Contact.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*S. pombe* strains used in this study are listed in the Key Resources Table. Homothallic (h90) strains able to switch mating types were used for all mating experiments, where cells were grown in liquid or agar Minimum Sporulation Media (MSL), with or without nitrogen (+/− N) [61, 62]. For interphase experiments, cells were grown in liquid or agar Edinburgh minimal medium (EMM) supplemented with amino acids as required.

METHOD DETAILS

Strain Construction

Strains were constructed using standard genetic manipulation of *S. pombe* either by tetrad dissection or transformation.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| h90 for3-3GFP:ura4+ acp2-mCherry:natMX | This work | YSM3438 |
| h90 for3-3GFP:ura4+ acp2-mCherry:natMX fim1::bleMX | This work | YSM3439 |

Oligonucleotides

Primers for PCR, see Table S1

Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pFA6a vector | [60] | pSM644 |
| pFA6a-sfGFP-kanMX (to generate Myo51-sfGFP, Fim1-sfGFP, acp1-sfGFP, acp1^{ΔsfGFP}, acp2-sfGFP and acp2^{ΔsfGFP}) | Lab Stock | pSM1538 |
| pFA6a-mCherry-natMX (to generate Fim1-mCherry and Acp2-mCherry) | Lab Stock | pSM684 |
| pFA6a-GFP-mCherry-natMX (to generate Fim1-GFP-mCherry) | Lab Stock | pSM1768 |
| pFA6a-hphMX (to generate acp1Δ) | Lab Stock | pSM693 |
| pFA6a-bleMX (to generate acp2Δ) | Lab Stock | pSM694 |
| pSP72 vector | Promega | pSM1232 |
| pSP72-5′UTR-acp2-sfGFP-kanMX-3′UTR | This work | pSM2194 |
| pSP72-5′UTR-acp2^{R12A}-sfGFP-kanMX-3′UTR | This work | pSM2201 |
| pSP72-5′UTR-acp2^{R12A,Y77A}-sfGFP-kanMX-3′UTR | This work | pSM2203 |
| pSP72-5′UTR-fus1-sfGFP-kanMX-3′UTR | This work | pSM2218 |
| pSP72-5′UTR-fus1^{D979A}-sfGFP-kanMX-3′UTR | This work | pSM2251 |
| pSP72-5′UTR-fus1^{D951A}-sfGFP-kanMX-3′UTR | This work | pSM2252 |
| pSP72-5′UTR-fus1^{Y1087,1088R}-sfGFP-kanMX-3′UTR | This work | pSM2253 |
| pSP72-5′UTR-fus1^{K1112A}-sfGFP-kanMX-3′UTR | This work | pSM2254 |
| pREP1 vector | Lab Stock | pSM1758 |
| pUra4^{Δneo} vector | Lab Stock | pAV133 |
| pUra4^{Δneo} p^{Δml}-fus1-sfGFP | This work | pSM2282 |
| pREP41-GFP-ypt3 | Lab Stock | pSM893 |
| pUra4^{Δneo} pmnt41-GFP-ypt3 | This work | pSM2250 |

Software and Algorithms

| SOFTWARE AND ALGORITHMS | SOURCE | RRID |
|-------------------------|--------|------|
| ImageJ (Fiji) | NIH | RRID: SCR_002285 |
| S. pombe database (released in August, 2013) | PomBase | RRID: SCR_006586 |
| Image Lab | Bio-Rad | RRID: SCR_014210 |
| Volocity | PerkinElmer | RRID: SCR_002668 |
| softWoRx v4.1.2 | Applied Precision, GE Healthcare | No direct download |
| MATLAB | MathWorks | RRID: SCR_001622 |
With two exceptions (cdc8 and ypt3), genes were tagged at their endogenous genomic locus at their 3’ end, yielding C-terminally tagged proteins, or deleted by replacing their ORF by a resistance cassette. This was achieved by PCR amplification of a fragment from a template plasmid with primers carrying 5’ extensions corresponding to the last 78 coding nucleotides of the ORF (for C-terminal tagging) or the last 78 nucleotides of the 5’UTR (for gene deletion) and the first 78 nucleotides of the 3’UTR, which was transformed and integrated into the genome by homologous recombination, as previously described [60]. For tagging of genes with sfGFP, a pFA6a-sfGFP-kanMX plasmid (pSM1538) was used as a template for PCR-based targeted tagging of myo51, fim1, acp1 and acp2. For tagging of genes with mCherry, a pFA6a-mCherry-natMX plasmid (pSM684) was used as a template for PCR-based targeted tagging of fim1 and acp2. For tagging of genes with GBP-mCherry, a pFA6a-GBP-mCherry-natMX plasmid (pSM7186) was used as a template for PCR-based targeted tagging of fim1. For PCR-based targeted deletion, a pFA6a-hphMX plasmid (pSM693) or a pFA6a-bleMX plasmid (pSM694) was used as a template for acp1 and acp2 respectively. For deletion of the tentacle of Acp1 and Acp2, PCR-based targeted tagging was done using the last 78 nucleotides upstream of the C-terminal tentacle (R233-T256 for Acp1 and R244-I268 for Acp2). The functionality of the tagged proteins was verified by comparing the phenotype of the tagged strain with that of the deletion strain. For tagged Acp1 and Acp2 alleles, the number of ectopic foci was quantified and found to be as in wild-type cells. Reduced functionality was only observed for tagged Agn2 and Eng2 alleles, which in combination (but not individually) showed reduced fusion efficiency.

For point mutations of acp2 (R12A-Y77A) and fus1 (K879A, I951A, N1087-1088RP, K1112A), a plasmid containing the full 5'UTR-ORF-sfGFP-kanMX-3'UTR sequence was first constructed by PCR amplification of the full fragment from strains YSM3312 or YSM3355 with primers carrying unique restriction sites, which were then cloned into a pSP72 plasmid (pSM1232). Site-directed mutagenesis was then conducted on these plasmids with primers containing the desired mutations. The resulting plasmids (pSM2203, pSM2251, pSM2252, pSM2253 and pSM2254, respectively) were sequenced, linearized and transformed into recipient strains YSM2440 for acp2 and IBC178 for fus1 mutants.

Construction of the strain overexpressing fus1 (p<nmt1>-fus1-sfGFP) was done by integration of fus1-sfGFP under the nmt1 promoter at the ura4+ locus. First, the nmt1 promoter was amplified from a pREP1 plasmid (pSM7186) with primers carrying KpnI and NotI extensions. Second, the fus1-sfGFP fragment was amplified from strain YSM3312 with primers carrying NotI and SacI extensions. These two fragments were cloned by 3-point ligation into the vector pAV133 (pJK211, a kind gift from Dr. Aleksandar Vjestica, UNIL) digested with KpnI and SacI. The resulting plasmid (pSM2282) was sequenced, digested with Afel and stably integrated as a single copy at the ura4+ locus into strain YSM2440.

Construction of the strain expressing ypt3 (p<nmt1>-GFP-ypt3) was done by integration of GFP-ypt3 under the nmt41 promoter at the ura4+ locus. The p<nmt1>-GFP-ypt3 fragment was digested from a pREP41-GFP-ypt3 plasmid (pSM693) with PstI and Xmal and cloned into the vector pAV133 digested with the same enzymes. The resulting plasmid (pSM2250) was linearized by Afel and transformed into strain YSM2440. This construct is in principle similar to that published by Cheng et al. [63], which was shown to rescue the ypt3-5 mutant phenotype. However, we note that we were unable to construct a GFP-ypt3 expressed as single copy from the native genomic locus, suggesting that GFP tagging impairs Ypt3 function.

The p<nmt1>-GFP-cdc8 construct is integrated at the leu1 locus and was shown to rescue the cdc8-110 mutant phenotypes [64] (gratefully received from Prof David Kovar (Chicago University)). It is expressed in addition to the untagged cdc8 gene copy at the endogenous genomic locus.

Mating Assays

Live imaging of S. pombe mating cells protocol was adapted from [62]. Briefly, cells were first pre-cultured overnight in MSL+N at 25°C, then diluted to OD<sub>600</sub> = 0.05 into MSL+N at 25°C for 20 hours. Exponentially growing cells were then pelleted, washed in MSL-N by 3 rounds of centrifugation, and resuspended in MSL-N to an OD<sub>600</sub> of 1.5. Cells were then grown 3 hours at 30°C overnight imaging, or for 3 h before high speed imaging.

For interphase imaging, cells were grown to exponential phase at 30°C in EMM+ALU media, pelleted and added to 2% agarose EMM+ALU pads. For CK-666 (Sigma) and LatA (Enzo Life Sciences) treatments, the drugs were added directly before imaging to the final resuspension, to a final concentration of 500μM and 200μM, respectively. In this case, cells were simply imaged without pad between slide and coverslip. The slide was allowed to rest for 5 minutes before imaging.

Microscopy

Images presented in Figures 1, 2, 3, 5, S2, S5C, and S5D, except Figure 2E were obtained using a DeltaVision platform (Applied Precision) composed of a customized inverted microscope (IX-71; Olympus), a UPlan Apochromat 100x /1.4 NA oil objective, a camera (CoolSNAP HQ2 or 4.2Mpx PrimeBSI sCMOS camera; Photometrics), and a color combined unit illuminator (Insight SSI 7; Social Science Insights).

Figures were acquired using softWoRx v4.1.2 software (Applied Precision). Images were acquired every 5 minutes during 9 to 15 hours. To limit photobleaching, overnight videos were captured by optical axis integration (OAI) imaging of a 4.6-μm z section, which is essentially a real-time z-sweep.

Images presented in Figures 4, 5, S4, S5A, S6, and 2E were obtained using a spinning-disk microscope composed of an inverted microscope (DMi4000B; Leica) equipped with an HCX Plan Apochromat 100x /1.46 NA oil objective and an UltraVIEW system.
(PerkinElmer; including a real-time confocal scanning head [CSU22; Yokagawa Electric Corporation], solid-state laser lines, and an electron-multiplying charge coupled device camera [C9100; Hamamatsu Photonics]). Time-lapse images were acquired at 1 s interval using the Volocity software (PerkinElmer).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Fusion efficiencies were calculated as in [42]. Briefly, at the specified time post-starvation, mating pairs and fused pairs were quantified using the ImageJ Plugin ObjectJ, and the subsequent fusion efficiency was calculated using the following equation:

\[
\text{Fusion Efficiency} = \frac{\text{Fused Pairs}}{\text{Mating Pairs}} \times 100
\]

Fusion Times were calculated using the 5-minutes time lapse overnight movies using the 2-dots Myo52-tdTomato stage [42] as a marker for the beginning of the fusion process and either the entry of GFP expressed under control of the P-cell-specific pmap3 promoter into the h-partner, or the maximum intensity of the Myo52-tdTomato dot, the two of which perfectly correlate [42], as a marker for the end of the process. Persistence times were calculated using the 5-minutes time lapse overnight movies using fusion time as beginning and last appearance of the Myo52-tdTomato dot as end of the post-fusion focus lifetime. Fusion times and persistence times vary between experiments because, for all experiments but Figures 1A and 1C, only early time points were considered to avoid Myo52 bleaching, which most certainly induces a bias toward quickly fusing cells. The WT control was imaged and quantified for each experiment to allow comparison within an experiment.

Fusion Focus intensities at fusion time were obtained using the 5-minutes time lapse overnight movies using either the entry of GFP into the h-partner, or the maximum intensity of the Myo52-tdTomato dot to determine the moment of fusion. On that time frame, a fluorescence profile across the fusion focus perpendicular to the long axis of the mating pair was recorded.

Profiles were background-subtracted and corrected for bleaching as follows: First, the cell fluorescence intensity was recorded over time in a square of 7x7 pixels in 12 control (non-mating) cell. These fluorescence profiles were averaged, and the mean was fitted to a double exponential:

\[
\text{Signal}_{\text{photobleaching correction}}(t) = Ae^{-at} + Ce^{-bt}
\]

We then used this fit to correct the fluorescence profiles across the fusion focus for photobleaching. After subtracting background signal, the value at each time point was divided by the photo-bleaching correction signal:

\[
\text{Corrected Profiles} = \frac{\text{Signal} - \text{Signal}_{\text{background}}}{\text{Signal}_{\text{photobleaching correction}}(t)}
\]

Corrected profiles were then either directly averaged and plotted, or further normalized to the mean of the WT maximum.

Patch to cytosol ratios were calculated from the ratio of the mean fluorescence intensity of 5 circular ROIs centered on patches (which gives the patch intensity) to the mean fluorescence signal of 5 circular ROIs centered on cytosolic signal per cell. The same operation was repeated on 36 cells and plotted.

Total fluorescence intensities in mating pairs were obtained using single snapshots on regular slides, 7h post-starvation, by outlining the mating pairs and recoding the mean fluorescence intensity for each of them. Background fluorescence was assessed by a small square ROI in an area devoid of cells on each image, averaged over all images, and subtracted from fluorescence intensity measurements.

The number of ectopic foci was assessed using the 5-minutes time lapse overnight movies during the fusion process between the 2-dot stage and the fusion time by simply counting the number of time-frames showing an ectopic Myo52-tdTomato or any other marker. The numbers vary between experiments because the DeltaVision camera was upgraded in the middle of the project, allowing us to detect more delocalization events. To assess colocalization at ectopic foci, single and double-color ectopic foci were identified as above and the colocalization was calculated with the following formula:

\[
\text{Percentage of Colocalization} = \frac{2 \times \text{Ectopic Foci Both Markers}}{\text{Ectopic Foci Marker1} + \text{Ectopic Foci Marker2}} \times 100
\]

The proportion of ectopic foci containing a given marker was derived from the ratio of ectopic foci containing the given marker to the total number, which gave a ratio for each cell. That ratio was then averaged over all the recorded cells. For all experiments shown in Figure 6, only ectopic foci present at the cell sides were considered.

For3-3GF retrograde flow was identified by visual inspection of spinning disk time-lapse imaging acquired at 1 s interval. Only linear For3 dot movements present over at least 5 consecutive time frames were considered. Dots were manually tracked using the ImageJ multi-point tool and instantaneous speeds calculated and averaged per track. Kymographs were constructed using the ImageJ reslice tool along a 5-pixel-wide line along the For3 dot track.

All plots, fittings, corrections and normalizations were made using MATLAB home-made scripts. For boxplots, the central line indicates the median, the circle, if present, indicates the mean, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points not considered outliers. For bar plots, error bars represent the standard deviation. Statistical p values were obtained using a two-sided t test, after normal distribution had been
visually checked using a simple histogram. No further verification was made to ascertain that the data met assumptions of the statistical approach. All values below 0.05 are mentioned in the Figures, including sample size. Comparison with values above 0.05 are not shown (Figure 1A, acp1Δ and acp1Δ acp2Δ versus WT at 36h; Figure 2F, fus1ΔG1087R,N1088P acp2Δ versus acp2+; Figure 3F, Eng2-sfGFP in acp2Δ versus WT; Figure 6H). For all the supplementary figures, we consider, * < 5.10^-2, ** < 5.10^-5, *** < 5.10^-8. All experiments were replicated at least 3 times. There was no pre-selection of sample size, nor randomization or blinding. No data was excluded from analysis.

**DATA AND CODE AVAILABILITY**

This study did not generate any substantial code or dataset.