The contributions of the actin machinery to endocytic membrane bending and vesicle formation

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Abstract

Branched and crosslinked actin networks mediate cellular processes that move and shape membranes. To understand the mechanisms of force-generation by actin, we used a hybrid imaging approach that combines live imaging with correlative microscopy to analyse deletion mutants of yeast actin network components. Our data show that the actin network needs to be physically linked to the plasma membrane to overcome a force threshold for initiating membrane bending. Two force-generating mechanisms reshape the membrane in successive phases. First, nucleation and polymerization of new actin filaments pull the membrane invagination. Then, rearrangement and expansion of the actin network provide the driving force for vesicle formation and propulsion. The transition between the phases occurs 2 seconds before scission when the filament nucleation rate drops. Our findings suggest that actin-based motility can be uncoupled from actin network growth and can be driven by the release of stress accumulated by the crosslinked actin network.

Introduction

Arp2/3-mediated actin filament networks play key roles in generating and controlling force for movement and reshaping of cellular membranes (Rotty et al., 2013). These cytoskeletal assemblies drive diverse processes such as cell motility at the leading edge, formation of lamellipodia, generation of actin comet-tails for the intracellular movement of endosomes and pathogens (Taunton et al., 2000, Pollard and Borisy, 2003, Welch and Way, 2013), as well as several endocytic uptake pathways including clathrin-mediated endocytosis (Pelkmans et al., 2002, Galletta et al., 2010, Collins et al., 2011, Boulant et al., 2011). These fundamental cellular functions rely on force generation by treadmilling of actin monomers through a dendritic actin network built from a set of conserved protein components. The network architecture and dynamics are modulated through its composition (Svitkina, 2013). Thus, actin network properties can adjust to meet the requirements of different processes. However, it remains poorly understood how variations in network properties control the mechanisms of force generation in cells, and how the force requirements vary during actin-driven processes.

Endocytosis in budding and fission yeasts is strictly dependent on Arp2/3-based actin polymerization (Kubler and Riezman, 1993, Kaksonen et al., 2003, Galletta and Cooper, 2009), which provides force that drives membrane invagination against membrane tension and against the turgor pressure inside yeast cells (Aghamohammadzadeh and Ayscough, 2009, Basu et al., 2014). The process generally conforms to the dendritic nucleation model (Berro et al., 2010, Sirotkin et al., 2010). The membrane is coupled to actin through endocytic adaptors at the tip of the invagination, while nucleation and filament elongation occur at the plasma membrane (Kaksonen et al., 2003, Idrissi et al., 2012, Skruzny et al., 2012, Chen and Pollard, 2013, Berro and Pollard, 2014, Picco et al., 2015). Most molecular constituents of the

yeast endocytic actin network have homologs in mammalian Arp2/3-mediated actin networks (Boettner et al., 2011a).

In this study, we aimed at mechanistically understanding how the endocytic actin network contributes throughout the process of membrane deformation and vesicle formation in the budding yeast *Saccharomyces cerevisiae*. We therefore deleted genes that encode proteins involved in coupling actin to the membrane, actin filament crosslinking, or regulation of actin nucleation. We studied these mutants using a hybrid imaging approach, which makes use of correlative microscopy data and of live cell imaging and particle tracking data (Kukulski et al., 2016, Picco et al., 2015, Picco and Kaksonen, 2017, Kukulski et al., 2011, Kukulski et al., 2012b, Kukulski et al., 2012a). This approach provides measurements of membrane morphology and actin network volume as well as high-resolution information on protein localisation and assembly dynamics. We could thus observe and quantify very subtle phenotypes caused by specific actin network perturbations. Our previously published wild type data sets served us as reference data for interpreting the results presented here (Kukulski et al., 2012a, Picco et al., 2015). Our findings show that during its initial assembly, the actin network overcomes a force threshold required for initiation of membrane bending. The further reshaping of the endocytic membrane is then driven by two subsequent phases of actin-based motility. The first phase occurs concurrently with actin filament nucleation and polymerization and is robust against perturbation of filament crosslinking. The second phase is uncoupled from actin network growth and is sensitive to perturbation of filament crosslinking.

Results:

Initiation of membrane bending requires actin-membrane coupling via clathrin adaptor proteins

Whether initial bending of the endocytic membrane requires actin polymerization is an open question. While our previous data indicated that initiation of membrane invagination coincides with the assembly of actin, and inhibiting actin polymerization with Latrunculin A prevents membrane bending at endocytic sites (Kukulski et al., 2012a, Picco et al., 2015), immunoelectron microscopy data suggested that curvature of the endocytic membrane is induced before actin network assembly (Idrissi et al., 2012). Indeed, endocytic adaptor proteins could modulate membrane curvature before actin assembly through their membrane binding domains or through crowding, as demonstrated *in vitro* (Boucrot et al., 2012, Skruzny et al., 2015, Busch et al., 2015, Stachowiak et al., 2012). To shed light on the requirements for initiation of membrane bending we addressed the role of adaptor proteins under conditions of unhindered actin assembly. Epsins and Sla2 (the yeast homolog of Hip1R), act cooperatively to couple the membrane to the actin network via their membrane- and actin-binding domains, and are essential for endocytic vesicle formation (Kaksonen et al., 2003, Sun et al., 2005, Boettner et al., 2011b, Skruzny et al., 2012). We deleted the actin-binding domains of both

Sla2 and Ent1 (*sla2ΔTHATCH ent1ΔACB-GFP*) to test if the presence of the membrane binding-domains of these endocytic adaptor proteins is sufficient for initiating membrane bending when the transmission of the force from the actin cytoskeleton is uncoupled from the plasma membrane (Skruzny et al., 2012). By applying correlative light microscopy and electron tomography to these cells, we targeted 12 sites marked by the colocalisation of GFP-labeled epsin and Abp1-mCherry as a marker for the presence of an actin network. In all the target localisations, we found no invaginations but only flat plasma membranes associated with large cytoplasmic zones excluding ribosomes (Figure 1A). In a similar experiment on cells in which the whole *SLA2* gene was deleted, all 11 target localisations of Sla1-GFP and Abp1-mCherry showed also flat plasma membranes associated with large exclusion zones (Figure 1B). The ribosome exclusion zones represent the extent of the endocytic machinery including the actin network (Kukulski et al., 2012a). In wild type cells, the presence of the actin network always coincides with membrane invaginations or vesicles (Kukulski et al., 2012a). Therefore, these results indicate that actin polymerization and the presence of all membrane-binding domains are not sufficient to induce membrane invagination, but the two need to be physically linked. Coupling the membrane to a polymerizing actin network via the actin-binding domains of Sla2 and Ent1 is thus essential for initiating endocytic membrane invagination.

Actin filament crosslinking is critical for the initiation of membrane bending and for reaching scission stage

The composition of actin networks modulates their mechanical properties. For instance, the stiffness of actin gels decreases with a lower density of actin filament crosslinkers (Blanchoin et al., 2014). In yeasts, the actin crosslinking activity of fimbrin has been shown to be essential for endocytic internalization (Kubler and Riezman, 1993, Kaksonen et al., 2005, Skau et al., 2011). Therefore, we deleted the yeast fimbrin Sac6 and studied *sac6Δ* cells by live imaging and correlative microscopy to investigate how the stiffness of the actin cytoskeleton regulates membrane invagination and vesicle formation.

We first tracked the dynamic behaviour of the coat protein Sla1-GFP in *sac6Δ* cells with high spatiotemporal resolution (Picco and Kaksonen, 2017). Sla1 is positioned close to the invagination tip and can be used to track the movement of the membrane invagination and the vesicle into the cell (Kukulski et al., 2016, Picco et al., 2015, Idrissi et al., 2008). In *sac6Δ* cells, we found three distinct types of Sla1-GFP behaviour (Figure 2A). The majority of Sla1 spots (78.4% ± 5.5%, mean ± SE, n = 9 cells; Figure 2A; see 'Materials and methods') remained immobile at the plasma membrane until they disassembled, consistent with earlier studies (Kaksonen et al., 2005, Gheorghe et al., 2008). However, 15.1% ± 4.4% (mean ± SE, n = 9 cells) of events revealed a distinct behaviour: The initial phase of centroid movement was very similar to that in wild type cells, but at a position approximately 90 nm away from the plasma membrane, the centroid started to retract towards the plasma membrane (Figure 2A

and 2B). The remaining 6.5% ± 2.2% (mean ± SE, n = 9 cells) of Sla1-GFP spots were motile and moved inward similarly to the Sla1-GFP wild type cells (Figure 2A and 2C).

To visualize the membrane morphology directly, we applied correlative microscopy to *sac6Δ* cells expressing Sla1-EGFP and Abp1-mCherry. In wild type cells, the presence of these proteins marks events that span from initiation of membrane bending until disassembly of the actin network from the newly formed endocytic vesicle (Kukulski et al., 2012a). In *sac6Δ*, we found that, in the presence of both Abp1 and Sla1, as well as in the presence of Abp1 only, the majority of endocytic sites were flat membranes (72% and 62%, respectively) and the remaining ones were invaginations (28% and 38%, respectively) (Figure 2D). We did not find endocytic vesicles at any of the sites, confirming that successful scission events are very rare in *sac6Δ* cells. These data also support the live cell imaging observation that the majority of the Sla1-GFP spots remained immobile throughout their lifetime. The immobile spots are thus likely events in which membrane bending is not initiated, despite assembly of the actin network.

We then measured the lengths of the invaginations marked by presence of Abp1-mCherry and Sla1-GFP, or by presence of Abp1-mCherry and absence of Sla1-GFP, in *sac6Δ* cells. We found that all the invaginations were less than 100 nm long, while in wild type cells invaginations are up to 140 nm long (Kukulski et al., 2012a) (Figure 2E). These measurements are in agreement with the live imaging finding that most invaginations retract before reaching 100 nm in length (Figure 2B).

We also measured the curvature of invagination tips in *sac6Δ* cells. Like in wild type cells, the tips reached a minimum radius for invaginations of about 40 nm in length. However, the average tip radius for invaginations longer than 40 nm was significantly larger in *sac6Δ* cells than in wild type cells (*sac6*Δ: 15.5 nm, SD 3.7 nm, n=12, wt: 11.6 nm, SD 2.6 nm, n=47; p=0.0003) (Figure 2—figure supplement 1). Thus, wider tips are either a property of all invaginations in *sac6Δ* cells, or widening of invagination tips occurs during retraction, possibly due to disassembly of coat proteins.

Taken together, our results indicate that in absence of the actin crosslinker Sac6, endocytosis is blocked either at the stage of initial membrane bending, or at an invagination length of approximately 100 nm, followed by a retraction of the invagination. Remarkably, in those events that initiate membrane bending, elongation of the invagination appears to be unaffected until retraction occurs. In rare cases, endocytosis progresses further and likely leads to successful vesicle formation.

Perturbed actin nucleation in *bbc1Δ* cells enhances propulsion of vesicles

Arp2/3-dependent nucleation of actin filaments is driven by nucleation promoting factors (NPFs) at cellular membranes (Pollard, 2007, Campellone and Welch, 2010). The NPF Las17, a yeast N-WASP ortholog, is required for actin assembly at endocytic sites and its activity is negatively regulated by Bbc1 *in vitro* (Rodal et al., 2003, Sun et al., 2006). We perturbed the rate of actin filament nucleation by deleting *BBC1*. In *bbc1*Δ cells, endocytic actin spots are brighter and the endocytic coat moves inward faster and over a longer distance than in wild type cells (Kaksonen et al., 2005).

To understand how actin nucleation in the absence of Bbc1 affects invagination dynamics, we first tracked Sla1-GFP dynamics in *bbc1Δ* cells. The average centroid trajectory of the Sla1-GFP in *bbc1Δ* cells shows an initial movement at the same speed as in wild type cells. About 7 seconds later, approximately when scission occurs in wild type cells, Sla1 centroid movement accelerated and it moved deeper into the cell than in the wild type (Figure 3A). We next investigated the dynamics of the amphiphysin Rvs167 in *bbc1Δ* cells. In wild type cells, the peak of Rvs167-GFP fluorescence intensity marks the scission event and coincides with a fast inward movement of the Rvs167-GFP centroid (Picco et al., 2015). In *bbc1*Δ cells, Rvs167-GFP showed a pronounced movement into the cytoplasm, which was concomitant with its peak in number of molecules (Figure 3B and Figure 3—figure supplement 1A), in 85% ± 6% (median ± SE , n = 14 cells) of the endocytic events. Like Sla1-GFP, motile Rvs167-GFP spots also moved deeper into the cell cytoplasm (Figure 3B). In the remaining 15% ± 6% (median ± SE , n = 14 cells) of events, Rvs167-GFP spots appeared immotile. The trajectories of Sla1-GFP and Rvs167-GFP indicate that *bbc1Δ* does not significantly affect the rate of invagination growth. However, they suggest that, after scission, vesicles are moved deeper into *bbc1Δ* cells compared to wild type.

We then imaged Abp1-GFP, which we used as a marker for the dynamics of the endocytic actin network (Kaksonen et al., 2003). In wild type cells, the recruitment of Abp1 coincides with the start of the Sla1 inward movement, which marks membrane invagination (Picco et al., 2015). In *bbc1Δ* cells, the recruitment of Abp1 preceded the start of Sla1-GFP inward movement by about 4 seconds, indicating that actin assembly does not immediately lead to membrane bending. The centroid of Abp1-GFP initially localised closer to the plasma membrane than in wild type cells (Figure 3C), suggesting a different distribution of the actin filaments on the membrane. Interestingly, the Sla1 inward movement began when the actin network reached the same height as in wild type cells.

The endocytic actin spots are brighter in *bbc1Δ* cells (Kaksonen et al., 2005). We therefore measured the number of Abp1-GFP and Arc18-GFP molecules present at endocytic sites over time (Joglekar et al., 2006, Lawrimore et al., 2011, Picco et al., 2015) (Figure 3D and Figure 3—figure supplement 1B, see Materials and Methods). Abp1 and Arc18 were recruited

to the endocytic sites at the same rate as in wild type cells (Picco et al., 2015). However, the actin network in *bbc1Δ* cells contained more Abp1 and Arc18 molecules because its assembly lasted for about 5 seconds longer. At the time when scission occurred, the number of Abp1 molecules recruited to the endocytic site was about doubled (Figure 3D), while the number of Arc18 was about 25% larger compared to wild type (Figure 3—figure supplement 1B).

The Abp1-GFP centroid in *bbc1Δ* cells began moving inward at the same speed as in wild type cells. However, the speed of the Abp1 centroid drastically increased when the invagination length reached 100 nm, about 2 seconds before scission (Figure 3C). Interestingly, the speed-up of the Abp1 centroid was not linked to an increase in assembly rate of Abp1 molecules (Figure 3D). This observation suggests that 2 seconds before scission, the actin network is undergoing a rearrangement, which leads to increased motility without extra addition of protein components.

We next performed correlative microscopy of *bbc1Δ* cells expressing Sla1-GFP and Abp1-mCherry, as well as *bbc1Δ* cells expressing Rvs167-GFP and Abp1-mCherry. Similarly to wild type cells, we found endocytic invaginations and vesicles in the presence of Abp1 and Sla1 or Rvs167, as well as in the presence of Abp1 but the absence of Sla1 or Rvs167 (Figure 3E). We measured that endocytic vesicles were positioned up to 600 nm from the plasma membrane, which is significantly further away from the plasma membrane than in wild type cells (*bbc1Δ*: 288.7 nm, SD: 158.7 nm, n=47; wt: 129.5 nm, SD: 39.7 nm, n=62; P<0.0001) (Figure 3F). This result is in striking agreement with the enhanced movement of Sla1-GFP and Rvs167-GFP starting around scission. Taken together, these results suggest that vesicles are being propelled faster and deeper into the cytoplasm upon deletion of *bbc1Δ*.

To better resolve the effects of *bbc1Δ* on vesicle formation, we measured the surface areas of endocytic vesicles and compared them to wild type. We found that vesicles in *bbc1Δ* cells were on average 25% larger (*bbc1Δ*: 7'998 nm^2, SD: 2'838 nm^2, n=47, wt: 6'380 nm^2, SD: 1'929 nm^2, n=62; P=0.0004) (Figure 3G). The larger vesicle sizes indicate that scission either occurred when invaginations were longer, or that scission sites on the invaginations were positioned closer to the plasma membrane than in wild type cells. The putative sites of scission are the necks of invaginations, which we found to be positioned similarly to those in wild type cells (Figure 3—figure supplement 1C). However, we found that there were longer invaginations in *bbc1Δ* cells compared to the wild type (Figure 3—figure supplement 1D), suggesting that the membrane inward movement, observed through centroid tracking of Sla1-GFP (Figure 3A), might accelerate already shortly before scission, leading to longer invaginations that result in larger vesicles.

Actin assembly occurs on a larger membrane area in *bbc1Δ* cells

To detail whether the increase in Abp1 molecules is accompanied by an increase in actin network size in *bbc1Δ* cells, we measured the dimensions of the ribosome exclusion zones surrounding invaginations. We found that the volumes of exclusion zones in *bbc1Δ* cells were significantly larger compared to those in wild type cells (*bbc1Δ*: 0.0052 fl, SD=0.0029 fl, n=21. wt: 0.0028 fl, SD=0.0017 fl, n=62; P=0.0001), which was in line with the increased amounts of Abp1-GFP molecules that we measured by fluorescence microscopy. Interestingly, while the heights of the exclusion zones in *bbc1Δ* correlated with the invagination lengths as observed in wild type cells (Figure 3H) (Kukulski et al., 2012a), the exclusion zone diameters were independent of the stage of the invagination process and significantly larger than in wild type cells (Figure 3I, *bbc1Δ*: 235.1 nm, SD=48.2 nm, n=21, wt: 181.2 nm, SD=40.9 nm, n=62; P<0.0001). Note that to compute the above values we only considered exclusion zones of *bbc1Δ* invaginations below 135 nm in length so that the invagination lengths compare with the wild type population. The enlarged diameters of the exclusion zones indicate that the actin cytoskeleton is distributed over a larger surface area of the plasma membrane when nucleation of actin is initiated. These results are consistent with our observation that the initial position of the Abp1-GFP centroid in *bbc1Δ* cells is closer to the plasma membrane than in wild type cells (Figure 3C). Therefore, despite the height of the actin network being comparable to wild type during the invagination process, the volume of the actin network is larger.

Like in wild type cells, vesicles in *bbc1Δ* cells were surrounded by ribosome exclusion zones that were either disconnected or connected to the plasma membrane (Figure 3E). We previously suggested that the latter represent the earliest phase of the vesicle lifetime (Kukulski et al., 2012a). In *bbc1Δ* cells, only 20% of vesicles had exclusion zones connected to the plasma membrane, as compared to 70% in wild type cells. Therefore, we conclude that the density of the actin network near the plasma membrane decreases earlier in *bbc1Δ* cells than in wild type cells. This asymmetric change in density could shift the centroid of the Abp1-GFP towards the cell centre, contributing to enhance the movement of the Abp1-GFP centroid in *bbc1Δ* cells.

Actin nucleation ceases before scission, while network expansion and movement continue in *bbc1Δ* cells

We next investigated the number of molecules of Las17 at the endocytic site. Las17 is a NPF and putative target of Bbc1 (Kaksonen et al., 2005, Rodal et al., 2003). Las17 accumulates on the plasma membrane, where it activates the Arp2/3 complex to nucleate actin filament formation (Sun et al., 2006, Winter et al., 1999). We found that in *bbc1Δ* cells, Las17 accumulated over a longer time than in wild type cells (Figure 3J). In wild type cells, the number of Las17 molecules plateaus for about 5 seconds at about 40 molecules and then the number of molecules starts decreasing about 2 seconds before scission (Picco et al., 2015). In *bbc1Δ*, however, the number of Las17 molecules did not plateau but continued accumulating until it

reached about 80 molecules, and then started to disassemble about 2 seconds before scission like in wild type cells (Figure 3J). The assembly of the actin cytoskeleton in *bbc1Δ* cells began when approximately 40 Las17 molecules had accumulated, like in wild type cells. Taken together, these data show that in *bbc1Δ* cells, Las17 accumulation is unhindered and the number of accumulating Las17 molecules is larger than in wild type, supporting a role for Bbc1 in regulating the assembly of Las17. However, Bbc1 is not required for triggering disassembly of Las17 and thereby for ending the accumulation of actin network components.

In summary, in *bbc1*Δ cells, the actin network starts accumulating on a larger membrane area than in wild type cells, resulting in larger network volumes. Nevertheless, the rate at which the actin network expands towards the cytoplasm is similar to wild type cells up to about 2 seconds before scission: The velocity of the actin centroid movement as well as the height of the exclusion zones are similar to wild type cells. In the last 2 seconds before scission occurs, the rate of actin nucleation drops and a rearrangement of the actin network results in a significant speed-up of the actin centroid movement, as compared to its initial movement and to its movement in wild type cells. Thus, the rate and progression of the invagination process are unaffected until shortly before scission. The changes in actin network behaviour seem to interfere only with the shape of late invaginations, which can get longer than in wild type cells and therefore result in larger vesicles, and with the movement of vesicles, which are pushed further into the cytoplasm.

Discussion

The endocytic actin network is critical for invaginating the plasma membrane during endocytosis (Merrifield et al., 2002, Kaksonen et al., 2003, Boulant et al., 2011, Kukulski et al., 2012a). Here, we explored the requirements on the actin network for initiating membrane bending: When we deleted the THATCH and ACB actin-binding domains of the membrane binding proteins Sla2 and Ent1 respectively (Skruzny et al., 2012), the plasma membrane remained flat despite actin network assembly (Figure 4). Therefore, the start of membrane bending and the subsequent growth of the plasma membrane invagination require the plasma membrane to be physically linked to the actin network.

We identified two phases in the actin network lifetime that apply two different mechanisms to drive the membrane reshaping process (Figure 4). During the first phase, the nucleation and polymerization of actin filaments drive plasma membrane invagination. During the second phase, when the rate of filament nucleation and assembly drops, the actin network expands while the plasma membrane invagination undergoes scission into a vesicle. The transition between these two mechanisms occurs about two seconds before scission when the number of NPFs drops (Picco et al., 2015).

In *sac6*Δ cells, most of the endocytic events fail to initiate the invagination process. Sac6-mediated crosslinking of actin filaments is thus important for initial bending of the plasma membrane. However, in about a fifth of the events, membrane bending is initiated and the first mechanism of invagination growth is sufficient to grow all invaginations to about 100 nm in length, which is the length that invaginations reach in wild type cells when the rate of filament nucleation drops, about 2 seconds before scission (Picco et al., 2015). Then, the majority of invaginations retract and only very few events proceed through apparently normal scission of the vesicle. Therefore, crosslinking is also necessary for the expansion of the actin network, which characterizes the second mechanism that drives the invagination growth through scission. In the rare successful events in *sac6Δ* cells, stochastic variations in the amount of other crosslinkers such as Scp1 (Aghamohammadzadeh and Ayscough, 2009), or in the timing of the nucleation drop, may allow the invagination to proceed to scission despite the absence of Sac6.

In *bbc1*Δ cells, the plasma membrane area on which actin filaments are assembled is larger than in wild type cells and this results in a larger actin network. The start of membrane bending in *bbc1*Δ cells is delayed with respect to the initiation of actin assembly. This might be because the actin assembly area is enlarged but the actin assembly rate is unaltered. Therefore, it would take longer for the actin network to grow to the height that may be required for the initiation of membrane bending. As soon as membrane bending is initiated, the first mechanism driving invagination growth proceeds normally and the plasma membrane invagination grows at the same rate as in wild type cells. When the rates of nucleation and assembly of actin filaments drop, 2 seconds before scission, the network expansion, comprising the second mechanism of invagination growth, is amplified, possibly by the larger number of actin filaments in *bbc1*Δ cells.

The force that is needed to pull a membrane tube depends on membrane shape and degree of protein coating (Powers et al., 2002, Koster et al., 2005, Derenyi et al., 2002, Dmitrieff and Nedelec, 2015). For endocytosis, it has been suggested that the force required for the initiation of membrane bending is higher than the force needed to subsequently shape the membrane into a tubular invagination (Dmitrieff and Nedelec, 2015). The two distinct mechanisms driving invagination growth, which we identified, suggest that the formation of the actin network might have evolved to adjust the force generation to the changing force requirements while pulling a plasma membrane tube. The crosslinking of actin filaments controls the mechanical properties of the actin network: A network of crosslinked actin filaments sustains larger stresses without deformation and has a higher elastic modulus than actin filaments alone (Sato et al., 1987, Wachsstock et al., 1994, Berro and Pollard, 2014, Bieling et al., 2016, Gardel et al., 2004). Crosslinking of the actin filaments is thus critical to initiate membrane bending when the force required is the highest. Once membrane bending is initiated, the force required to pull an invagination likely lowers and therefore, filament nucleation and

actin polymerization are sufficient to drive invagination growth in the absence of the cross-linker Sac6. However, when the nucleation of new filaments drops 2 seconds before scission, the mechanical properties of the actin network become important again to provide the force necessary to proceed to the scission of the vesicle: A crosslinked actin network might be able to store and then release the stress accumulated during the first part of the invagination growth, and thus expand and pull the invagination until scission occurs. The release of stress could occur either due to the elasticity of the whole actin network or as a consequence of crosslinker turnover, which could release filament contacts that were induced under higher loads. The enlarged actin network in *bbc1*Δ results in a deformation that is larger than in wild type cells and that drives the final stages of the invaginations and the vesicles deeper into the cytoplasm.

Materials and Methods

Strain generation

Yeast strains were generated by homologous recombination of the target genes with PCR cassettes encoding for the fluorescent tags (Janke et al., 2004). The C-terminal fluorescent tags were inserted from plasmids pFA6a-EGFP-His3MX6, pFA6a-mCherry-KanMX4 and pYM12-PKS134, which was used for monomeric GFP (myEGFP).

Strains used for correlative light and electron microscopy:

sla2Δ, Sla1-EGFP, Abp1-mCherry (Skruzny et al., 2012)(MKY1195):
MATa, his3Δ200, leu2-3,112, ura3-52, lys2-801, sla2:natNT2, SLA1-EGFP::HIS3MX6, ABP1-mCherry::kanMX4

ent1ΔACB-GFP, *sla2ΔTHATCH*, Abp1-mCherry (Skruzny et al., 2012)(MKY1846):
MATa/α, his3Δ200, ura3-52, lys2-801, ent1ΔACB(Δ amino acids 294–450)-EGFP::HIS3MX6, sla2ΔTHATCH::natNT2, ABP1-mCherry::kanMX4

sac6Δ, Sla1-EGFP, Abp1-mCherry (Kaksonen et al., 2005)(MKY2869):
MATa, his3Δ200, leu2-3,112, ura3-52, lys2-801, sac6:natNT2, SLA1-EGFP::HIS3MX6, ABP1-mCherry::kanMX4

bbc1Δ, Sla1-EGFP, Abp1-mCherry (Kaksonen et al., 2005)(MKY1805):
MATa, his3Δ200, leu2-3,112, ura3-52, lys2-801, bbc1::cgLEU2, SLA1-EGFP::HIS3MX6, ABP1-mCherry::kanMX4

bbc1Δ, Rvs167-EGFP, Abp1-mCherry (Kaksonen et al., 2005)(MKY1803):
MATa, his3Δ200, leu2-3,112, ura3-52, lys2-801, bbc1::cgLEU2, RVS167-EGFP::HIS3MX6, ABP1-mCherry::kanMX4

Strains used for fluorescence live microscopy:

*bbc1*Δ, Sla1-EGFP (Kaksonen et al., 2005)(MKY3412):
MATα , his3200, leu2-3,112, ura3-52, lys2-801, Sla1-EGFP::HIS3MX6, bbc1delta::natNT2

*bbc1*Δ, Abp1-EGFP (MKY3247):
MATa, his3200, leu2-3,112, ura3-52, lys2-801, Abp1-EGFP::HIS3MX6, bbc1delta::natNT2

*bbc1*Δ, Rvs167-EGFP (Kaksonen et al., 2005)(MKY3416):
MATα , his3200, leu2-3,112, ura3-52, lys2-801, Rvs167-EGFP::HIS3MX6, bbc1delta::natNT2

*bbc1*Δ, Arc18-myEGFP (MKY3016):
MATa, his3200, leu2-3,112, ura3-52, lys2-801, Arc18-myEGFP::NAT, bbc1Δ::natNT2

*bbc1*Δ, Las17-EGFP (MKY3418):
MATa, his3200, leu2-3,112, ura3-52, lys2-801, Las17-EGFP::HISMX6, bbc1Δ::natNT2

*bbc1*Δ, Sla1-EGFP, Abp1-mCherry (Kaksonen et al., 2005)(MKY2059):
MATa, his3200, leu2-3,112, ura3-52, lys2-801, Sla1-EGFP::HIS3MX6, Abp1-mCherry::KANMX4, bbc1delta::cgLEU2

*bbc1*Δ, Abp1-mCherry, Rvs167-EGFP (MKY2061):
MATa, his3200, leu2-3,112, ura3-52, lys2-801, Rvs167-EGFP::HIS3MX6, Abp1-mCherry, bbc1delta::cgLEU2

*bbc1*Δ, Arc18-myEGFP, Abp1-mCherry (MKY3015):
MATα, his3200, leu2-3,112, ura3-52, lys2-801, Arc18-myEGFP::NAT, Abp1-mCherry::KANMX bbc1Δ::natNT2

*bbc1*Δ, Las17-EGFP, Abp1-mCherry (Kaksonen et al., 2005)(MKY2103):
MATα, his3200, leu2-3,112, ura3-52, lys2-801, Las17-EGFP::HIS3MX6, Abp1-mCherry::KANMX, bbc1delta::cgLEU2

Live cell imaging

Yeast strains were imaged as described before in (Picco et al., 2015) and (Picco and Kaksonen, 2017). In short, yeast cells were grown overnight at 25ºC in SC-Trp. They were diluted in the morning and grown to log phase at 25ºC. Cells were adhered to 25 mm coverslips (Menzel-Gläser #1), which were coated with Concanavalin A (100 µg/ml). Adhered cells were imaged in SC-Trp medium with an Olympus IX81 microscope and an Olympus 100 x/1.45 NA TIRF objective. Cells were excited for 80-250 ms per exposure with a 488 nm laser or simultaneously with 488 nm and 561 nm lasers. For GFP only images the emission light was filtered using the GFP-3035C-OMF single-band filter set (Semrock, Rochester, NY). For GFP and mCherry two-color simultaneous imaging, the excitation light was reflected with the OBS-U-M2TIR 488/561 (Semrock, Rochester, NY) dichroic mirror and the emission light was split and filtered with the DUAL-view beam splitter (Optical Insights, LLC, Tucson, AZ).

Image analysis

Images were background subtracted and corrected for photobleaching. Before tracking the endocytic events, the cytoplasmic fluorescent signal was removed as described in (Picco et al., 2015) and (Picco and Kaksonen, 2017). Briefly, a median filter of radius 6 pixels was used to estimate the cytoplasmic fluorescent signal, which was then subtracted from the image it-

self. The endocytic events were tracked using the Particle Tracker plugin in Fiji/ImageJ (Sbalzarini and Koumoutsakos, 2005). All trajectory positions were corrected for chromatic aberrations by applying a warping transformation that was computed from TetraSpeck bead images acquired with the same imaging conditions (Picco et al., 2015). After that, the trajectories of each protein, with the exception of Las17-GFP, were aligned in space and in time and averaged using the trajalign module distribution available in http://apicco.github.io/trajectory_alignment/ (Picco and Kaksonen, 2017). Sla1-GFP average trajectories in Figure 2B and C were aligned in time manually to compare their invagination dynamics. All other average trajectories were aligned in space and time using their position and appearance relative to Abp1 (Picco et al., 2015, Picco and Kaksonen, 2017). Las17-GFP trajectories were tracked simultaneously with Abp1-mCherry trajectories. They were then aligned and averaged using the software in (Picco et al., 2015), which used the spatial and temporal alignment that aligned the Abp1-mCherry trajectories together to compute the transformations that aligned Las17-GFP trajectories.

Quantification of the number of proteins

The average protein amounts were estimated by comparing the fluorescence intensities of the endocytic spots of the proteins of interest with the fluorescence intensity of Nuf2-GFP spots in cells imaged during their anaphase-telophase (Picco et al., 2015, Joglekar et al., 2006). The average amount of Arc18-myGFP was estimated by comparing to Nuf2-GFP spot intensities and by applying a correction constant of 0.68 ± 0.14 (Picco et al., 2015). The average protein amounts were then used to calibrate the fluorescence intensity curves by scaling the average fluorescence intensity value (Picco et al., 2015).

Correlative light and electron microscopy

Correlative light and electron microscopy was carried out as described before (Kukulski et al., 2011, Kukulski et al., 2012b). In short, yeast cultures were grown at 25°C in SC-Trp medium to exponential phase. Cells were pelleted using vacuum filtration and high pressure frozen using a Bal-tec HPM010 (McDonald, 2007). Freeze substitution, Lowicryl HM20 embedding and sectioning were done according to the protocol in (Kukulski et al., 2012a). As fiducial markers for the correlation procedure, we used either blue 20 nm FluoSpheres (Molecular Probes) (Kukulski et al., 2012a) or 50 nm TetraSpeck beads (Life Technologies) (Suresh et al., 2015). Fluorescence microscopy of the resin sections on electron microscopy grids was performed immediately after sectioning, as detailed in (Suresh et al., 2015, Kukulski et al., 2012b). Low magnification (2.53 nm pixel size) and high magnification (1.18 nm pixel size) electron tomographic tilt series were acquired on a FEI Tecnai F30 microscope, set up as described in (Kukulski et al., 2012a), using Serial EM (Mastronarde, 2005) and reconstructed with IMOD (Kremer et al., 1996). Centroids of GFP and mCherry signals were correlated to virtual slices of the low magnification electron tomograms based on the fluorescent fiducial makers and in a second step to virtual slices of the high magnification tomograms based on

gold fiducial markers using MATLAB-based correlation procedures described in (Kukulski et al., 2011).

Quantification of membrane shape parameters and ribosome exclusion zones

To quantify shapes of invaginations and vesicles as well as ribosome exclusion zones, we used precisely the same procedures as for the wild type data set (Kukulski et al., 2012a). This allowed us to directly compare the parameters measured in the mutant cells with the wild type data. In short, the Amira EM package (Pruggnaller et al., 2008) was used to click points along the cytoplasmic leaflet of plasma membrane invaginations, in a selected virtual slice of the electron tomogram which was positioned to contain the long axis of the invagination. The groups of points were transferred into a coordinate system such that the x-axis corresponded to the plasma membrane. A local second-degree polynomial was fitted through the points using MATLAB. This fit was used to measure invagination length and radius of the invagination tip, as well as appearance and position of a neck. The Amira EM package was also used to click points along the cytoplasmic leaflet of the vesicle membrane. This was done in the set of virtual slices that comprised the whole vesicle, allowing us to fit an ellipsoid through the set of points. From the major axes of the ellipsoid, we calculated the vesicle surface area. In tomograms that contained vesicles, we also clicked points on the plasma membrane throughout the tomographic volume. We then fitted a sphere through these points and determined the shortest distance of the vesicle centre to the plasma membrane. To quantify the sizes of ribosome exclusion zones, we first generated an average of 10 consecutive virtual slices of which the central slice contained the invagination or the vesicle center. We overlaid this average with a hexagonal mesh of 50 nm spacing and determined which hexagons did not contain ribosomes, as well as which hexagons contained the plasma membrane. These data provided us with estimates for the diameter of the exclusion zone (parallel to the plasma membrane) as well as the height of the exclusion zone. To calculate the volume of the exclusion zones of invaginations, we assumed the shape of a half-ellipsoid with two equal short axes.

Statistical tests

For statistical analysis of the membrane shapes and exclusion zones, we used GraphPad Prism or R (https://cran.r-project.org/). All mutant data were compared to wild type data (Kukulski et al., 2012a). For the data that are normally distributed, we used an unpaired two-tailed Welch's test. To compare the exclusion zone volumes, the vesicle surfaces and their distance from the plasma membrane, we used a Mann-Whitney unpaired nonparametric test.

Endocytic sites with multiple invaginations or vesicles

As described for wild type cells, in the correlative microscopy data set of mutant strains described here we found endocytic sites that contained multiple endocytic events within the same exclusion zone (Kukulski et al., 2012a). In the *sac6*Δ data set, one Sla1-GFP Abp1-mCherry spots contained two invaginations. These were included in all analysis.

In the *bbc1*Δ data set, of the 26 Rvs167-GFP/Abp1-mCherry spots, 6 contained multiple endocytic events. One site contained an invagination and a vesicle, one contained two invaginations, two contained two vesicles and two contained three vesicles each. Of 25 Sla1-GFP/Abp1-mCherry spots containing either invaginations or vesicles, four contained multiple events. One contained a vesicle and an invagination, two contained two invaginations and one contained two vesicles. Of the 14 Abp1-mCherry spots in absence of Sla1-GFP, two contained multiple events. One contained an invagination and a vesicle, and one contained three vesicles. We included these events into all analysis of membrane shape parameters. However, we did not use ribosome exclusion zones that contained multiple events for any quantification of exclusion zone sizes.

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Figure 1

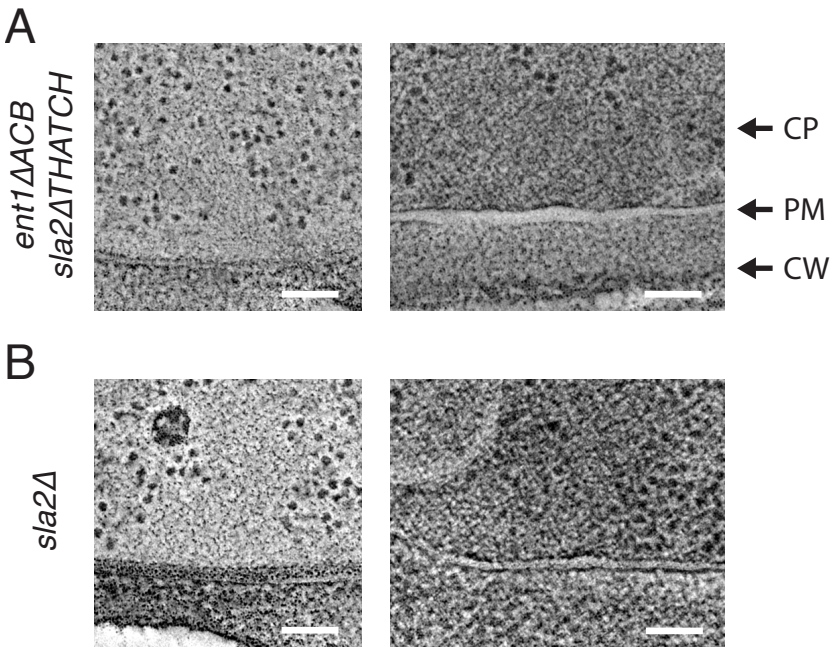

Figure 1: Endocytosis in cells with the actin cytoskeleton uncoupled from the plasma membrane. (**A**) Endocytic sites in cells expressing *ent1⊿ACB-GFP*, *sla2⊿THATCH* and Abp1-mCherry (**B**) Endocytic sites in *sla2⊿* cells expressing Sla1-GFP and Abp1-mCherry. All panels show virtual slices through electron tomograms of endocytic sites that correspond to the localisation of GFP and mCherry signals, identified by correlative microscopy. The panels to the left show examples of the flattest membranes in each dataset. The panels on the right show examples of the most bent membranes in each dataset. All panels are oriented so that the cytoplasm (CP) is above the plasma membrane (PM), and the cell wall (CW) is below. Scale bars are 100 nm.

Figure 2

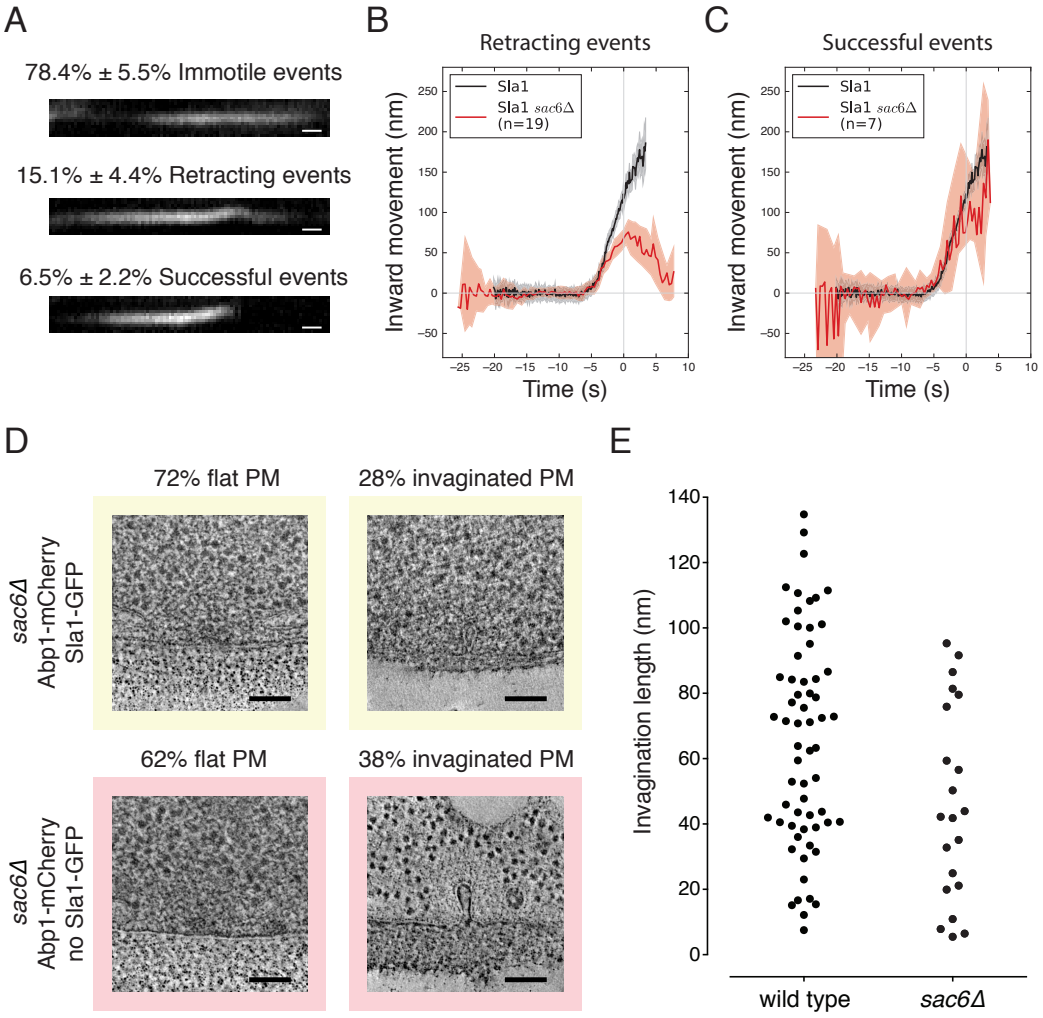

Figure 2: Endocytosis in cells with impaired actin crosslinking. (**A**) Representative kymographs of the three distinct behaviours of Sla1-GFP spots in *sac6Δ* cells and the percentage of occurrence for each type of event (mean ± SE, n = 9 cells). Scale bars correspond to 10 seconds. (**B**) The inward movement of Sla1-GFP average trajectories of retracting events in *sac6Δ* cells (red) and of wild type cells (black, (Picco et al., 2015)). (**C**) The inward movement of Sla1-GFP average trajectories of successful events in *sac6Δ* cells (red) and of wild type cells (black, (Picco et al., 2015)). The shadows in B and C correspond to the 95% confidence interval. Time 0 in B and C marks the scission event (Kukulski et al., 2012a, Picco et al., 2015). (**D**) Virtual slices through electron tomograms showing endocytic sites in *sac6Δ* cells expressing Sla1-GFP and Abp1-mCherry imaged by correlative microscopy. The upper panels (yellow) show endocytic sites marked by the presence of both Sla1-GFP and Abp1-mCherry signals (n = 25). The lower panels (pink) show endocytic sites marked only by Abp1-mCherry (n = 34). Examples of a flat plasma membrane and of an invagination are shown for each case, together with the percentages of sites corresponding to each ultrastructure. Scale bars are 100 nm. (**E**) The distribution of invagination lengths measured in the electron tomograms of *sac6Δ* cells. The wild type data (Kukulski et al., 2012a) is shown for comparison.

Figure 3

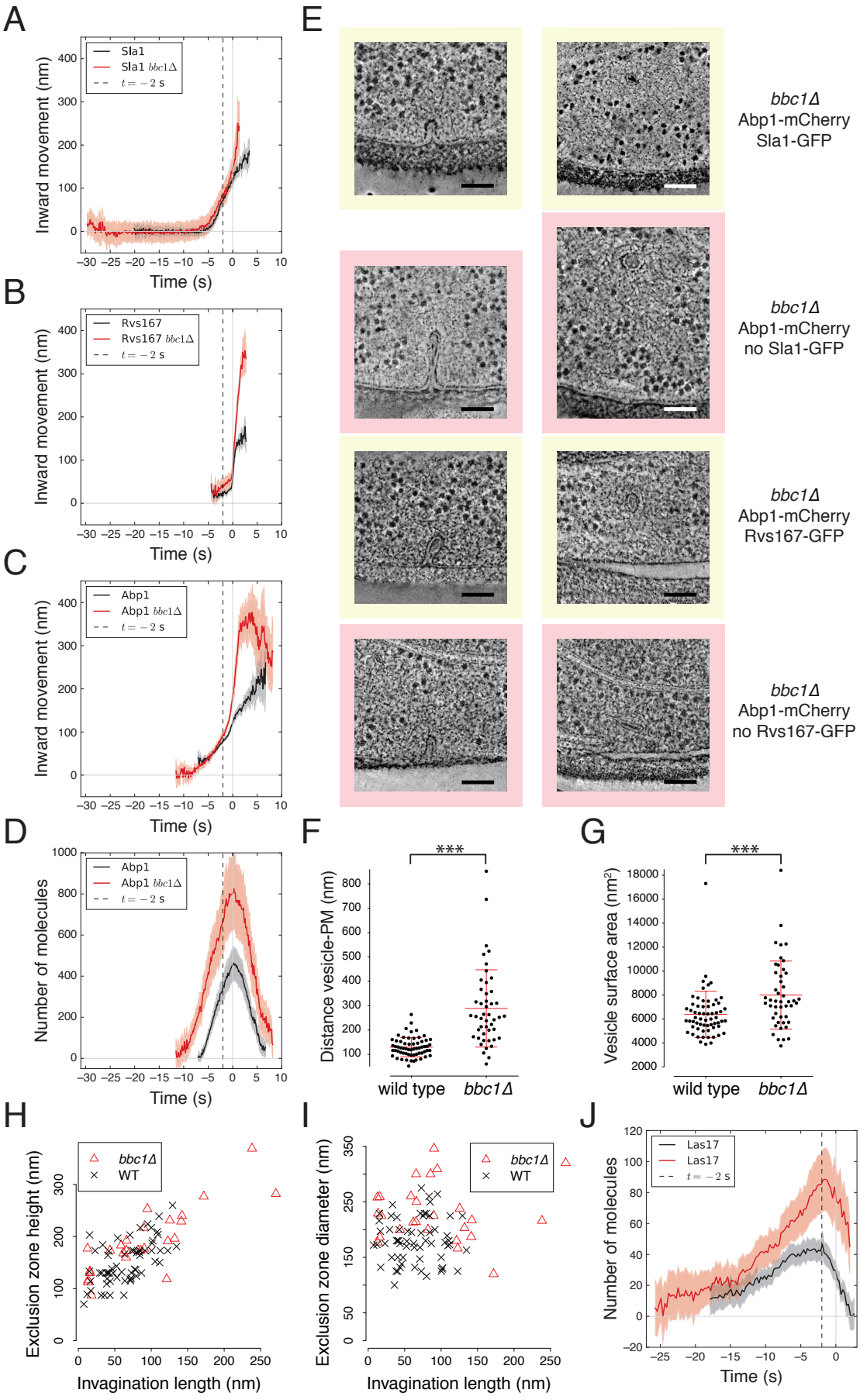

Figure 3: Endocytosis in cells with perturbed actin nucleation. (**A**) The inward movement of Sla1-GFP average trajectories in *bbc1*Δ (red) and in wild type (black) cells. (**B**) The inward movement of Rvs167-GFP average trajectories in *bbc1Δ* (red) and wild type (black) cells. (**C**) The inward movement of Abp1-GFP average trajectory in *bbc1Δ* (red) and in wild type (black) cells. (**D**) The number of Abp1-GFP molecules over time in *bbc1Δ* (red) and in wild type (black) cells. (**E**) Virtual slices through electron tomograms showing examples of endocytic sites identified by correlative microscopy of *bbc1Δ* cells expressing Abp1-mCherry and either Sla1-GFP or Rvs167-GFP. Yellow and pink frames indicate presence or absence of Sla1-GFP and Rvs167-GFP, as indicated. Scale bars are 100 nm. (**F**) The shortest distance between vesicle centres and the plasma membrane (PM) in wild type and *bbc1Δ* cells (**G**) The surface areas of vesicles in *bbc1Δ* cells. Red lines in F and G represent the mean and the standard deviation. (**H**) The heights of exclusion zones surrounding invaginations plotted against invagination lengths. (**I**) The diameters of exclusion zones surrounding invaginations plotted against invagination lengths. (**J**) The number of Las17-GFP molecules over time in *bbc1Δ* (red) and in wild type (black) cells. The shadows in A-D and J correspond to the 95% confidence interval. Time 0 in A-D and J marks the scission event (Kukulski et al., 2012a, Picco et al., 2015). The wild type trajectories in A-D and J are from (Picco et al., 2015). The correlative microscopy data on wild type cells in F-I are from (Kukulski et al., 2012a)

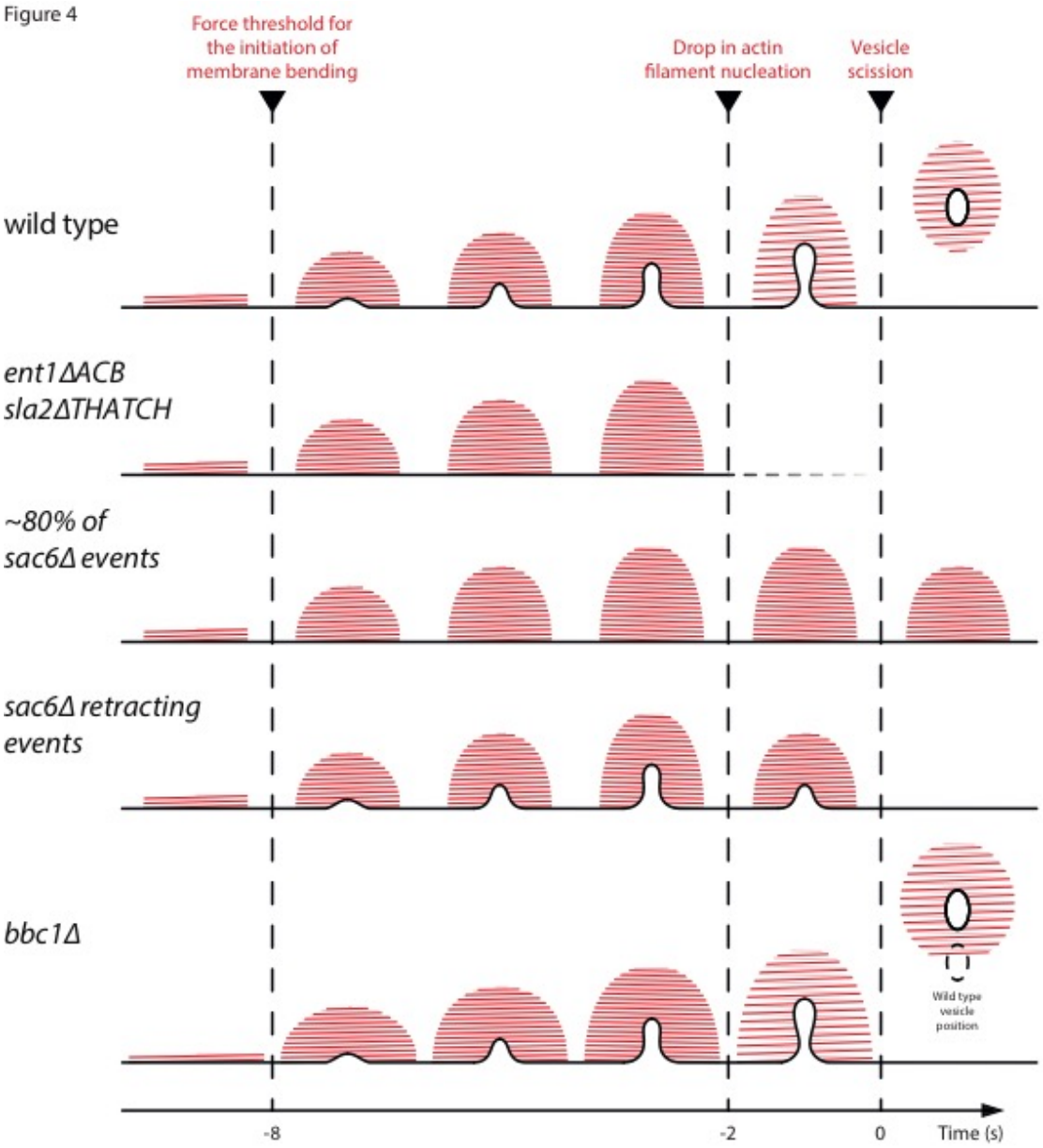

Figure 4: The actin network pulls the plasma membrane invagination in two distinct phases. The beginnings of the two phases are marked by the dashed lines and correspond to the initiation of the membrane bending, which requires a force threshold to be overcome, and the elastic recoil of the actin network when the nucleation of new actin filaments drops 2 seconds before scission. *ent1ΔACB*, *sla2ΔTHATCH* prevents membrane bending. Likewise, *sac6Δ* hinders initiation of membrane bending in the majority of cases. If membrane bending is initiated, the plasma membrane invagination proceeds until 2 seconds before scission and then retracts. In rare cases, invagination proceeds further through scission of the vesicle, like in wild type cells. *bbc1Δ* allows the plasma membrane to invaginate normally but the actin network is larger and expands about 2 seconds before scission. In *bbc1Δ* the vesicle is propelled deeper into the cytoplasm than in wild type cells.

Figure 2—figure supplement 1

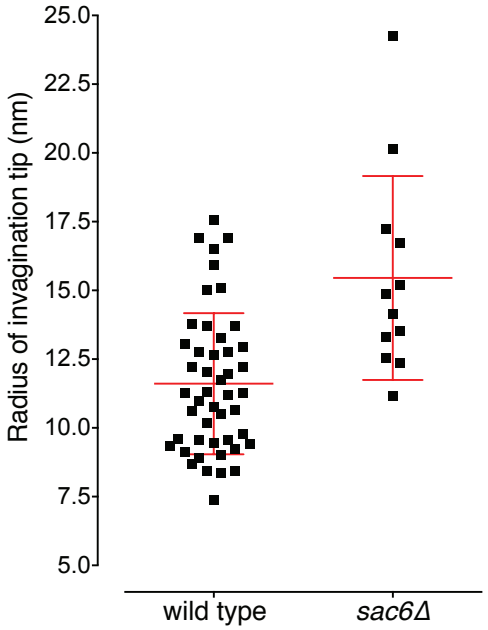

Figure 2—figure supplement 1: The radii of the tips of the invaginations that were longer than 40 nm, in wild type (Kukulski et al., 2012a) and *sac6Δ* cells.

Figure 3—figure supplement 1

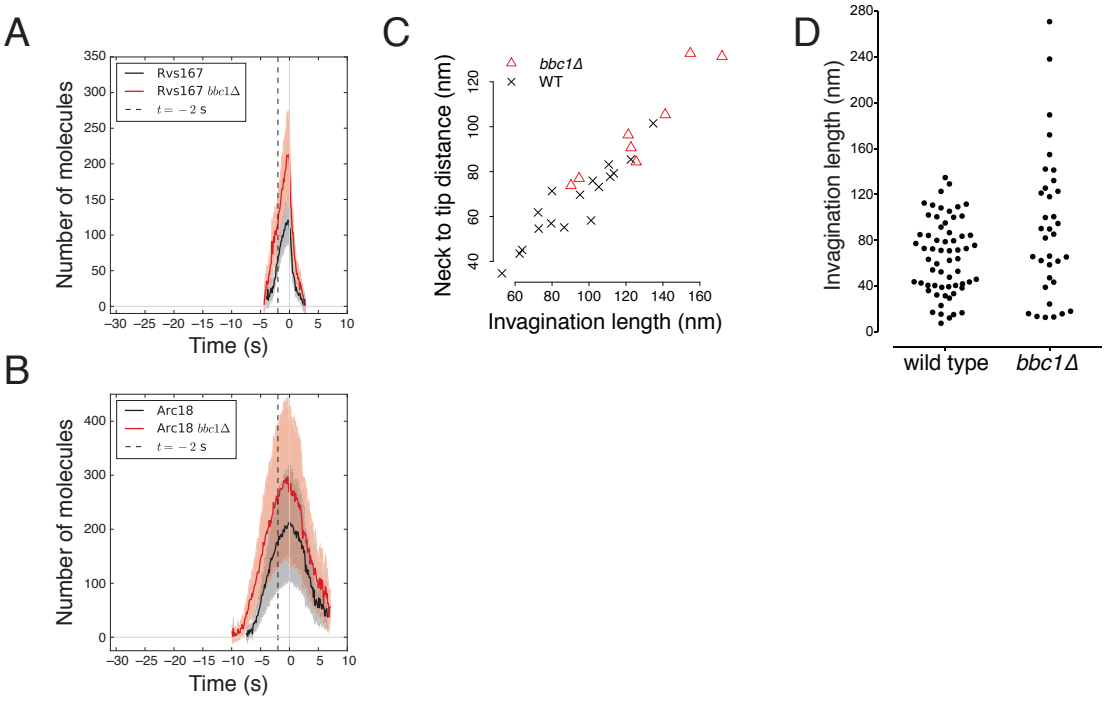

Figure 3—figure supplement 1: (**A**) The number of Rvs167-GFP molecules that accumulate over time in *bbc1Δ* (red) compared to wild type (black, (Picco et al., 2015)) cells. (**B**) The number of molecules of Arc18-GFP in *bbc1Δ* (red) and in the wild type (black, (Picco et al., 2015)) cells. In A and B the time point 0 marks the scission event (Kukulski et al., 2012a, Picco et al., 2015) (**C**) The position of invagination necks in *bbc1Δ* (red triangles) and in wild type (black crosses (Kukulski et al., 2012a)) cells. (**D**) The invagination lengths in *bbc1Δ* compared to the invagination lengths in wild type cells (Kukulski et al., 2012a).