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The PCH family protein, Cdc15p, recruits two F-actin nucleation pathways to coordinate cytokinetic actin ring formation in *Schizosaccharomyces pombe*

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Cytokinetic actin ring (CAR) formation in *Schizosaccharomyces pombe* requires two independent actin nucleation pathways, one dependent on the Arp2/3 complex and another involving the formin Cdc12p. Here we investigate the role of the *S. pombe* Cdc15 homology family protein, Cdc15p, in CAR assembly and find that it interacts with proteins from both of these nucleation pathways. Cdc15p binds directly to the Arp2/3 complex activator Myo1p, which likely explains why actin patches and the Arp2/3 complex fail to be medially recruited during mitosis in *cdc15* mutants. Cdc15p also binds directly to Cdc12p. Cdc15p and Cdc12p not only display mutual dependence for CAR localization, but also exist together in a ring-nucleating structure before CAR formation. The disruption of these interactions in *cdc15* null cells is likely to be the reason for their complete lack of CARs. We propose a model in which Cdc15p plays a critical role in recruiting and coordinating the pathways essential for the assembly of medially located F-actin filaments and construction of the CAR.

Introduction

Cytokinesis is the final step of the cell cycle that results in the formation of two daughter cells from one (for reviews see Feierbach and Chang, 2001; Guertin et al., 2002). In *Schizosaccharomyces pombe*, as in many other eukaryotic organisms, cytokinesis requires major reorganization of the actin cytoskeleton to form an equatorial actomyosin-based cleavage apparatus (for reviews see Marks et al., 1986; Balasubramanian et al., 2000). In *S. pombe*, the cleavage apparatus, or cytokinetic actin ring (CAR), is assembled as cells enter mitosis and contains actin, type II myosin, and a number of other actin- and myosin-binding proteins (for reviews see Le Goff et al., 1999; Guertin et al., 2002). The CAR has been shown to be a highly dynamic structure, exhibiting continuous cycles of assembly and disassembly of actin and other ring components (Pelham and Chang, 2002; Wong et al., 2002).

In addition to the CAR, two other F-actin structures are detected in *S. pombe*, cables and patches, and both are involved in the cell division process. Cables accumulate in the medial region during the initial stages of ring assembly (Arai and Mabuchi, 2002). These preexisting cables are linked to the newly forming CAR and appear to be packed into this structure as it transitions from a thin primary ring to a thicker mature structure (Arai and Mabuchi, 2002). F-actin patches are highly dynamic, undergoing rapid movements and cycles of assembly and disassembly. Formation and motility of actin patches depends on Arp2/3 complex–driven F-actin polymerization (Li et al., 1995; Winter et al., 1997; Pelham and Chang, 2001). Patch formation during interphase is favored at growing ends of cells (Marks et al., 1986). At the onset of mitosis, patches cease to assemble at cell tips and, instead, form in the medial region of the cell (Marks and Hyams, 1985; Pelham and Chang, 2001), where the components of these patches presumably contribute to CAR formation (for review see Balasubramanian et al., 2000).

Actin filament formation occurs in two steps: nucleation, which is the rate-limiting step, followed by elongation through actin monomer addition (for review see Higgs and Pollard, 2001). In *S. pombe*, actin nucleation by the Arp2/3 complex is required for CAR formation (Pelham and

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Abbreviations used in this paper: CAR, cytokinetic actin ring; FH3, formin homology 3; MBP, maltose binding protein; PCH, pombe Cdc15 homology; SH3, Src homology 3.

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Chang, 2002). The Arp2/3 complex requires interaction with one of several activator proteins to achieve a high level of nucleation activity. Arp2/3 complex activators, such as the type I myosins and WASp/Scar protein family, bind directly to Arp2/3 complex and stimulate its ability to promote actin filament assembly (for reviews see Bear et al., 2001; Cooper et al., 2001; Higgs and Pollard, 2001). These activators possess COOH-terminal acidic domains by which they interact with the Arp2/3 complex (Lee et al., 2000). Arp2/3 complex activators in *S. pombe*, Wsp1p and Myo1p, are functionally redundant (Lee et al., 2000; Toya et al., 2001). Similar redundancy is seen in *Saccharomyces cerevisiae*, where the WASp homologue (Las17p/Bee1p) and type I myosins exist in a regulatory complex that includes verprolin (Vrp1p) (Evangelista et al., 2000; Lechler et al., 2000).

Recently, formins have been shown to represent a second F-actin nucleator family. The *S. cerevisiae* formin Bni1p nucleates linear, unbranched actin filaments while remaining bound to their barbed ends (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002). This mechanism is distinct from that of Arp2/3 complex, which nucleates de novo filaments by binding to pointed ends and creates branched actin meshworks by binding to the sides of existing filaments (for review see Higgs and Pollard, 2001). Bni1p and a second *S. cerevisiae* formin, Bnr1p, are important for the formation of actin cables and the CAR (Vallen et al., 2000; Tolliday et al., 2002). Additionally, in *S. pombe*, the formin Cdc12p is a component of the CAR and is required for the earliest steps of ring assembly (Arai and Mabuchi, 2002; Pelham and Chang, 2002). In interphase cells, Cdc12p is present in a single motile cytoplasmic spot (Chang et al., 1997). This motile particle is targeted to the medial region before CAR formation and serves as a nucleating structure for the CAR into which it is subsequently incorporated (for review see Chang, 2001).

Though much progress has been made toward elucidating formin function, the roles of many other proteins important for CAR formation are less well established. Furthermore, an important question still remains as to how these molecules are organized and coordinated to build the CAR. One protein linked to ring assembly, but whose function is poorly understood, is *S. pombe* Cdc15p. Cdc15p is the founding member (Fankhauser et al., 1995) of the pombe Cdc15 homology (PCH) family of proteins that localize to actin-rich regions, such as the CAR, and are important for actin cytoskeletal functions (for review see Lippincott and Li, 2000). These proteins characteristically have an NH_2-terminal FER-CIP4 homology (FCH) domain, a central coiled-coil region, and one or more COOH-terminal Src homology 3 (SH3) domains (for review see Lippincott and Li, 2000). Cdc15p is required for Cdc12p localization to both the cytoplasmic spot and the CAR (Chang et al., 1997). Furthermore, *cdc15* overexpression in interphase cells promotes medially directed actin rearrangements (Fankhauser et al., 1995). These observations have suggested that Cdc15p plays a key role early in the establishment of the CAR.

Here we investigate the role of Cdc15p in CAR formation. We find that Cdc15p interacts directly with both the Arp2/3 complex–dependent and formin-dependent actin nucleation pathways and is required for their medial recruitment during mitosis. As such, Cdc15p plays an integral role in, and is strictly required for, CAR formation during cytokinesis.

Results

Cdc15p is required for medial localization of Arp2/3 complex activators during mitosis

We showed previously that neither actin patches nor the Arp2/3 complex are recruited to the medial region of *cdc15-140* cells grown at its restrictive temperature (Balasubramanian et al., 1998). We therefore asked whether activators of the Arp2/3 complex are also not properly localized in this mutant. We examined in live cells the localization of endogenously GFP-tagged Myo1p and Wsp1p, as well as, verprolin (Vrp1p), a potential regulator of these activators. Consistent with known roles in actin patch regulation (Lee et al., 2000; Naqvi et al., 2001; Toya et al., 2001), Myo1p–GFP, Wsp1p–GFP, and Vrp1p–GFP were all localized as patches at growing cell ends during interphase, whereas in mitosis, they were detected in the medial region of cells (Fig. 1, A–C). In *cdc15-140* cells at 36°C, however, all three proteins were observed at the cell cortex and failed to localize to the medial region of cells (Fig. 1, A–C). Examination of *cdc15-140 myo1-GFP* cells, synchronized in G2 and released to either permissive or restrictive temperature, revealed that Myo1p was never detected in the medial region in the absence of Cdc15p function (Fig. 1 D). Similar results were obtained for Wsp1p–GFP and Vrp1p–GFP (not depicted). We conclude that Cdc15p function is required for medially directed mitotic recruitment of not only the Arp2/3 complex, but also of its known regulators.

Interactions of Arp2/3 complex regulators with one another

To understand possible interactions between Cdc15p and regulators of the Arp2/3 complex, we first needed to determine how these factors interacted with one another. The *S. pombe* verprolin homologue has not previously been characterized. Examination of the *S. pombe* genome sequence data in the Sanger Centre database revealed a protein (SPBC13E7.09) related to *S. cerevisiae* Vrp1p and human WASp-interacting protein (WIP), which we have called *S. pombe* Vrp1p (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200305012/DC1). We determined that *vrp1* is not an essential gene, but similar to the case in *S. cerevisiae* (Naqvi et al., 2001), *S. pombe vrp1Δ* cells display morphological defects at high temperatures and are cold sensitive (Fig. S1, B and C). At 18°C, cells became rounded, characteristic of a polarity defect.

In *S. cerevisiae*, Vrp1p, Las17p/Bee1p, and type I myosins form an Arp2/3 regulatory complex, with direct protein–protein interactions observed between Vrp1p and Las17p/Bee1p and between both of these proteins and the type I myosins (Evangelista et al., 2000; Lechler et al., 2000). Therefore, we asked whether the *S. pombe* homologues of these proteins also interact with one another. Similar to observations in *S. cerevisiae*, Wsp1p interacted strongly with the SH3 domain of Myo1p (residues 1077–1218) as well as with the COOH terminus of Vrp1p, by two hybrid analysis

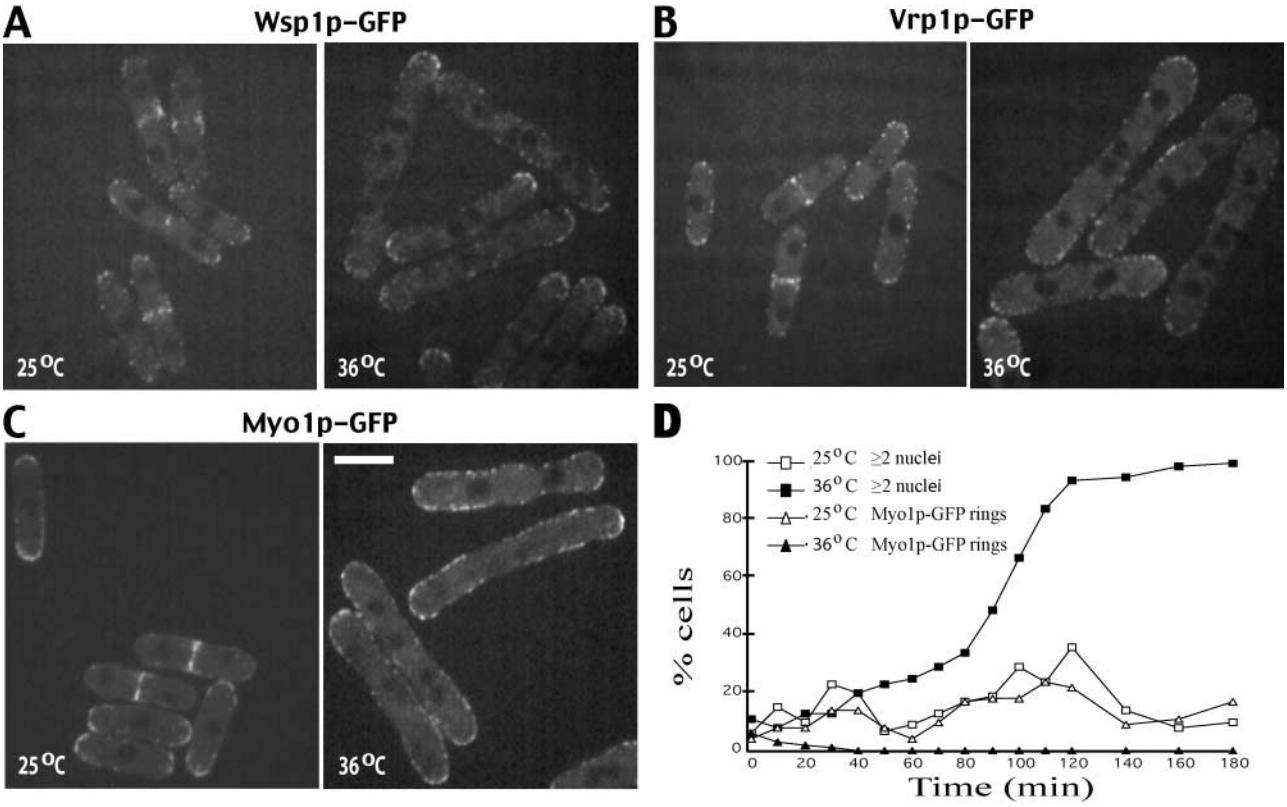

Figure 1. **GFP-tagged Arp2/3 activators fail to localize to the medial region in a *cdc15* mutant at the restrictive temperature.** (A–C) *cdc15-140* cells with either *wsp1-GFP* (KGY358), *vrp1-GFP* (KGY108), *or myo1-GFP* (KGY3963) alleles were grown to log phase at 25°C and shifted to 36°C for 4 h before live cells were imaged. Bar, 5 μm. (D) *myo1-GFP cdc15-140* cells (KGY3963) were synchronized by lactose gradients and released to 25°C or 36°C. Live cells were monitored for Myo1p–GFP, and parallel samples were fixed and stained with DAPI. Localization of Myo1p to the medial region is indicated by triangles, and cells with two or more nuclei are indicated by squares.

(Fig. 2 A). Further, GST–Myo1p(1077–1218) and maltose binding protein (MBP)–Vrp1p(206–309) fusion proteins bound directly to Wsp1p fragments produced in a coupled transcription/translation in vitro system (Fig. 2, B and C). These binding regions corresponded to those found to interact in the *S. cerevisiae* homologues (Evangelista et al., 2000; Lechler et al., 2000). Contrary to expectations, however, we found no evidence for an interaction between any regions of Myo1p and Vrp1p (unpublished data). Additionally, whereas *vrp1Δ wsp1Δ* cells were viable (unpublished data), deletion of both *vrp1* and *myo1* was synthetically lethal. Previous work has indicated that Wsp1p and Myo1p represent redundant pathways of Arp2/3 complex activation in *S. pombe* (Lee et al., 2000). Our data support this model and suggest that Vrp1p is most important for the Wsp1p pathway.

Cdc15p and Myo1p interact directly. Having observed that Arp2/3 complex regulators are not medially recruited in a *cdc15* mutant, we then asked whether any of these proteins physically interacted with Cdc15p. Extensive two-hybrid analysis revealed no detectable interactions between any regions of Cdc15p and either Wsp1p or Vrp1p (unpublished data). However, a strong interaction was observed between the NH_2 terminus of Cdc15p and the COOH terminus of Myo1p (Fig. 2 E). The Myo1p-interacting region of Cdc15p contained the conserved FER-CIP4 homology (FCH) domain and the two predicted coiled coils of the protein (Fig. 2, D and E). In Myo1p, residues 727–1041, which contains the IQ repeats as well as the tail homology 1 (TH1) and 60% of the tail homology 2 (TH2) motif, interacted strongly with Cdc15p (Fig. 2 E). Furthermore, the partial TH2 region from this construct also supported a significant interaction with Cdc15p.

We next tested whether Cdc15p and Myo1p could interact in *S. pombe* cell lysates. GST–Cdc15p(1–405) associated with Myo1–HA from lysates (Fig. 2 F, lane 3), whereas no interaction was seen with GST alone (lane 2). Furthermore, GST–Cdc15p(1–405) also interacted directly with both MBP–Myo1p(727–1041) (Fig. 2 G) and [^{35}S]Myo1p(727–1041), produced in a coupled transcription/translation in vitro system (Fig. 2 H), whereas GST alone was unable to bind Myo1p in either case. Therefore, Cdc15p and Myo1p are able to interact directly.

Localization of Cdc15p

Given that medial mitotic recruitment of Arp2/3 regulators is dependent on Cdc15p function, we wished to determine whether loss of these proteins affected Cdc15p localization. Consistent with the nonessential nature of *wsp1*, *vrp1*, or *myo1* for cytokinesis, Cdc15p–GFP localization to the CAR appeared normal in the individual deletion strains (unpublished data). The live cell imaging analysis, however, revealed a previously unappreciated interphase localization of Cdc15p to numerous spots at cell tips in wild-type and

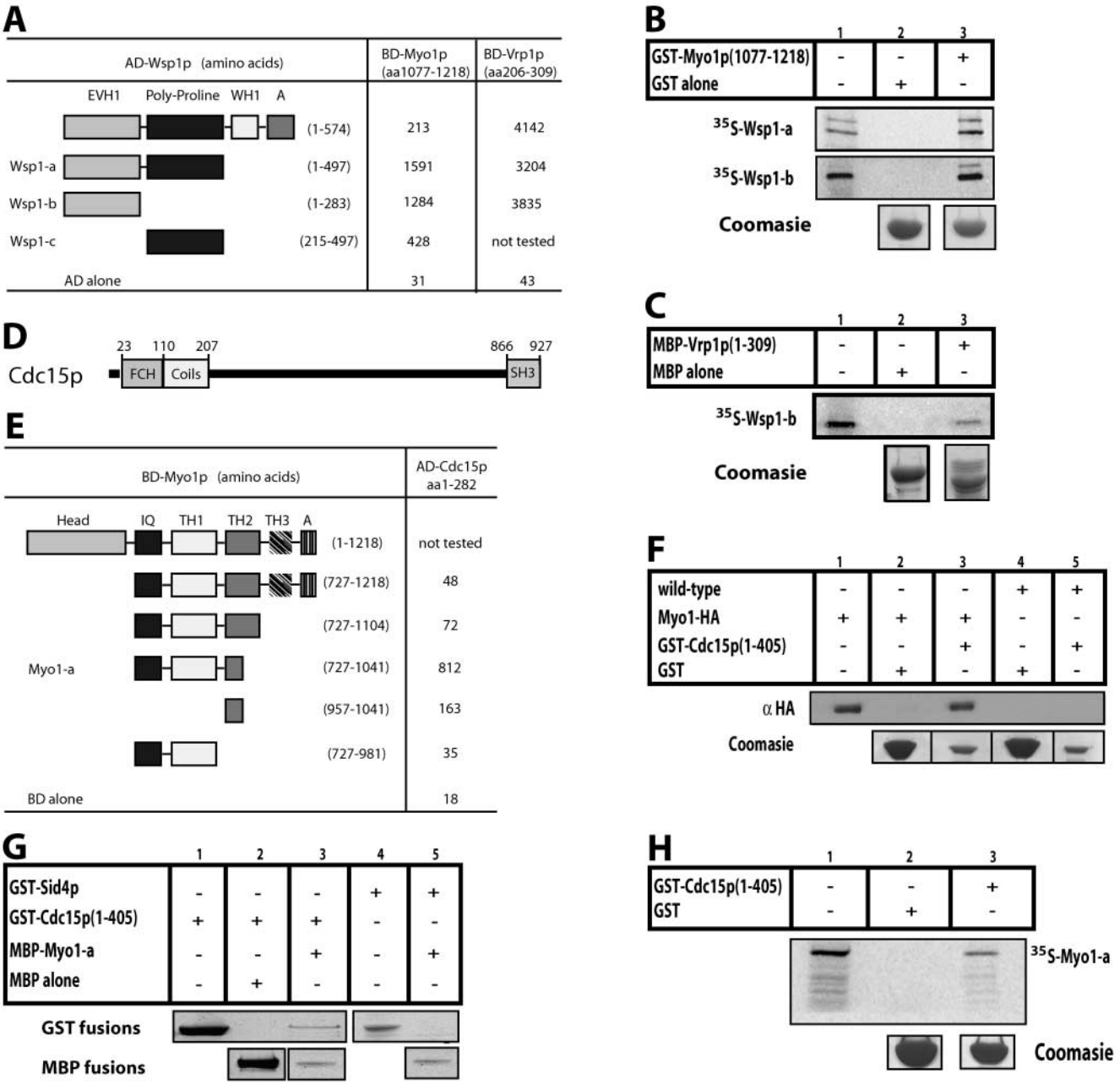

Figure 2. **Protein–protein interactions of Arp2/3 complex regulators.** (A) The indicated regions of Wsp1p were tested for interaction with Myo1p and Vrp1p by two-hybrid analysis. LEU$^+$ TRP$^+$ transformants were tested for growth on selective media (not depicted) and assayed for β-galactosidase activity measured in relative light units. (B) Approximately equal amounts of GST (lane 2, bottom) and GST–Myo1p(1077–1218) (lane 3, bottom) bound to glutathione beads were mixed with in vitro–translated Wsp1p-a (amino acids 1–497) (top) or Wsp1p-b (amino acids 1–283) (middle). Beads were collected, washed, and eluted as described in the Materials and methods. Proteins were resolved by SDS-PAGE and detected by fluorography (top two panels) or Coomassie staining (bottom). Only relevant portions of the Coomassie-stained gel are shown to indicate equal loading; however, all proteins ran at the predicted sizes. Lane 1 contains 10% of the input into the reactions. (C) Approximately equal amounts of MBP (lane 2, bottom) and MBP–Vrp1p(1–309) (lane 3, bottom) bound to amylose beads were mixed with in vitro–translated Ws1p-b (top). Beads were collected, washed, and eluted as described in the Materials and methods. Proteins were further analyzed as in B. (D) A graphic representation of Cdc15p. Amino acid residues at the borders of known domains are shown. (E) The indicated regions of Myo1p were tested for interaction with Cdc15p (amino acids 1–282) by two-hybrid analysis. LEU$^+$ TRP$^+$ transformants were tested for growth on selective media (not depicted) and assayed for β-galactosidase activity. (F) GST (lanes 2 and 4) or GST–Cdc15p(1–405) (lanes 3 and 5) bound to glutathione beads were incubated with protein lysates from a *myo1-HA* strain (KGY3960) and subsequently extensively washed in binding buffer. Bound proteins were then divided and analyzed by immunoblotting (top) and Coomassie staining (bottom). Lane 1 contained lysate from *myo1-HA* strain and is an input control. (G) Approximately equal amounts of MBP (lane 2, bottom) and MBP–Myo1-a (amino acids 727–1041) (lanes 3 and 5, bottom) bound to amylose beads were mixed with either soluble GST–Cdc15p(1–405) (lanes 2 and 3) or GST–Sid4p (lane 5). Beads were collected, washed, and eluted as described in the Materials and methods. Proteins were resolved by SDS-PAGE and detected by Coomassie staining. Only relevant portions of the Coomassie gel are shown to indicate equal loading; however, all proteins ran at the predicted sizes. Lanes 1 and 4 contain samples of the GST fusion proteins before the binding reactions. (H) Approximately equal amounts of GST (lane 2, bottom) and GST–Cdc15p(1–405) (lane 3, bottom) bound to glutathione beads were mixed with in vitro–translated Myo1-a (top). Beads were collected, washed, and eluted as described in the Materials and methods. Proteins were further analyzed as in B.

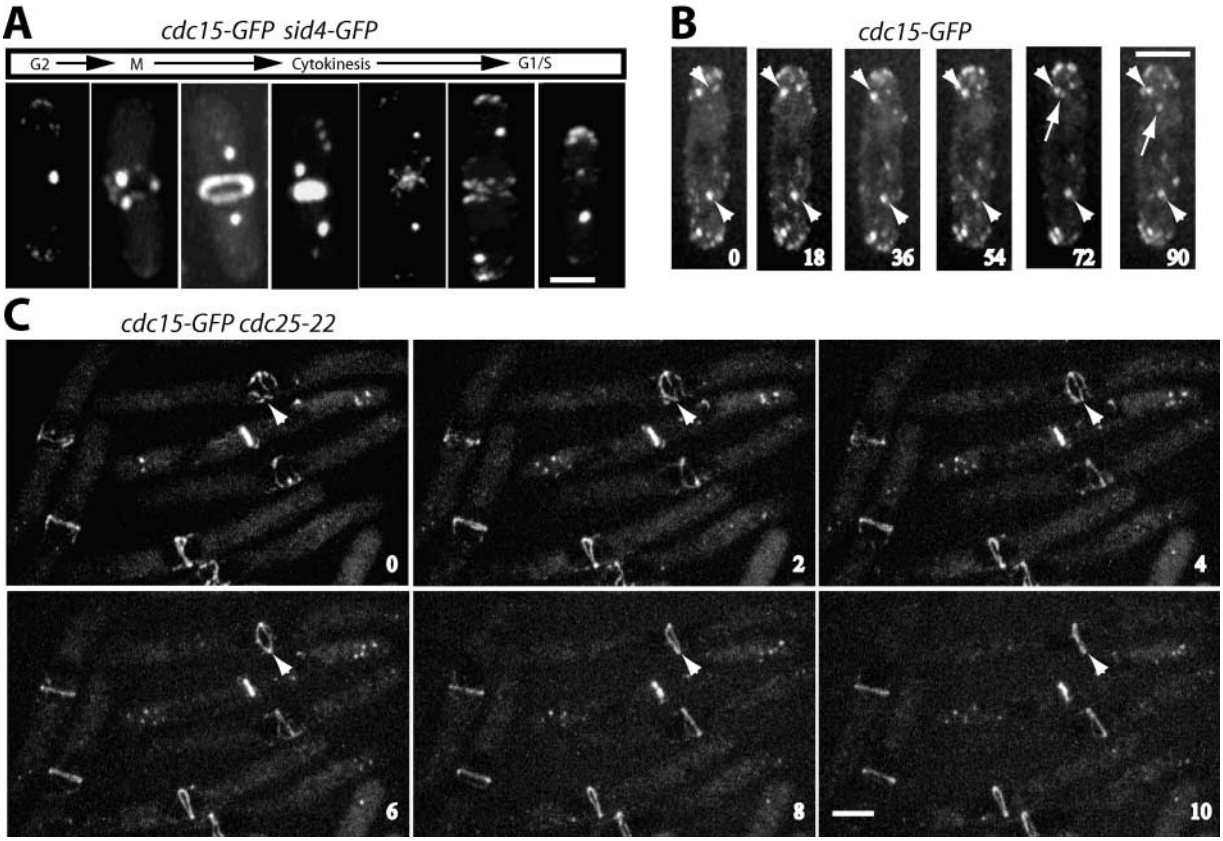

Figure 3. **Localization of Cdc15p–GFP.** (A) Representative live cell images of *cdc15-GFP sid4-GFP* cells (KGY3362) from various cell cycle stages. Sid4p–GFP localization was used to monitor spindle pole bodies and to indicate cell cycle stage of cells. (B) Time-lapse images of an interphase *cdc15-GFP* cell (KGY3019) with two nonmotile spots indicated by arrowheads. The arrow indicates the formation of a smaller patch from a larger one. Time is indicated in seconds. (C) Time-lapse images of synchronized *cdc15-GFP cdc25-22* cells (KGY3042) that had been arrested for 4 h at 36°C and released to 25°C for 10 min before the beginning of the time course at the early stages of ring formation. The arrowhead indicates a branched network of fibers encircling the medial region that is the initial Cdc15p ring structure. This network then coalesced to form a thick bright ring structure. Time is indicated in minutes. Bars, 5 μm.

wsp1, *vrp1*, or *myo1* deletion cells (Fig. 3 A; see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200305012/DC1). We typically observed a small number (two to five per cell) of large bright nonmotile spots as well as numerous smaller fast-moving spots (Fig. 3 B; see Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200305012/DC1). Small motile spots could occasionally be observed emerging from the larger static spots (Fig. 3 B). Upon entry into mitosis, Cdc15p–GFP spots moved to the medial region of the cell where they were seemingly incorporated into the forming Cdc15p ring structure (Fig. 4 A; see Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200305012/DC1). The Cdc15p ring initially appeared as a network of interconnected fibers encircling the medial region (Fig. 3 C; see Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200305012/DC1) that quickly coalesced into a thicker ring structure. The Cdc15p–GFP ring then constricted as cells divided, and subsequently, Cdc15p–GFP reformed as spots and relocalized to the old cell ends. Cdc15p–GFP spots were then detected at both cell ends when cells resumed bipolar growth. Because the localization and motility of Cdc15p–GFP spots were reminiscent of actin patches, we examined whether these structures were identical. We did not find a high degree of spatial overlap between Cdc15p and Myo1p, Wsp1p, Vrp1p, or the known actin patch protein Arc15p during interphase (unpublished data), suggesting that Cdc15p spots are distinct from actin patches.

As cells entered mitosis, a prominent Cdc15p–GFP spot could be detected associated with the nucleus before ring assembly (Fig. 4 A; Video 4). The nuclear-associated spot, and all other localizations of Cdc15p, was also detectable when expressing Cdc15p from a plasmid under the control of the *nmt81* promoter (Fig. 4 B, left). Furthermore, it appears that the localization domain may reside at the NH_2 terminus of the protein, as this region was sufficient for localization (Fig. 4 B, right; Ding et al., 2000). This medial spot of Cdc15p–GFP was reminiscent of a spot structure containing the formin, Cdc12p, that associates with the nuclear periphery just before ring assembly (Chang et al., 1997). The motile Cdc12p spot localizes to the medial cortex region as cells enter mitosis, presumably serving as a nucleating structure for the formation of the CAR (Chang, 1999). To ask whether the nuclear-associated spots of Cdc12p and Cdc15p were in fact the same structure, we expressed GFP–Cdc12p under control of the low strength *nmt81* promoter in a strain where the chromosomal locus of *cdc15* had been modified to produce a COOH-terminal fusion to CFP. In these

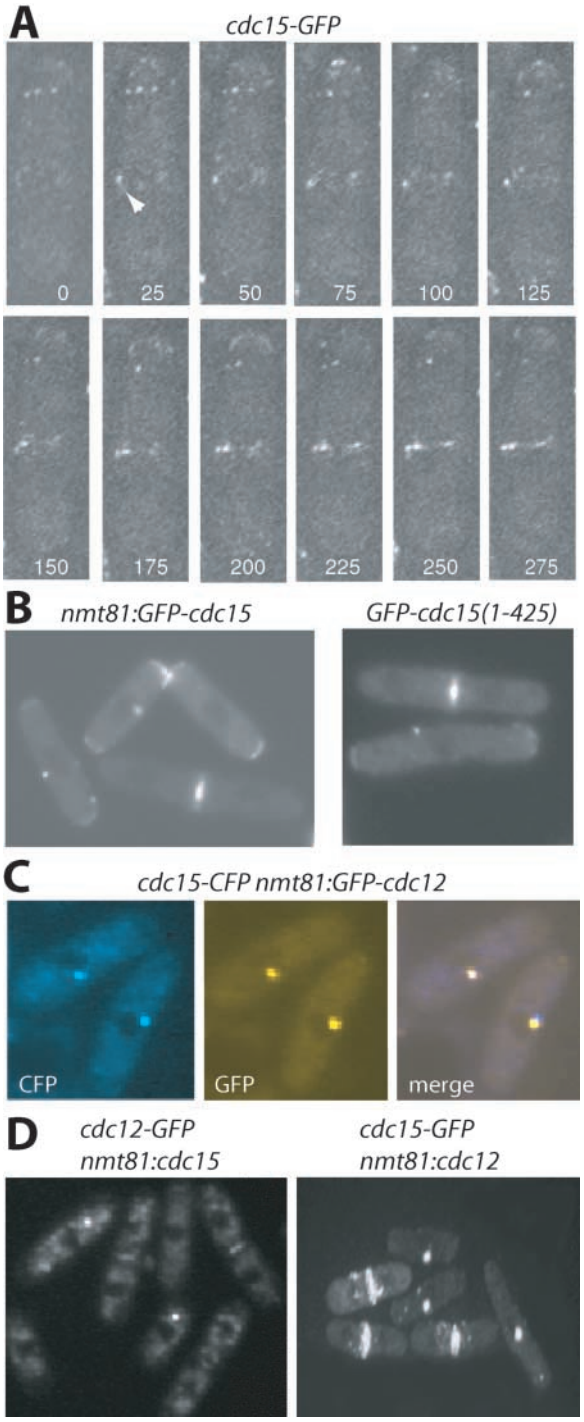

Figure 4. **Cdc15p colocalizes with the formin Cdc12p in a medial spot**. (A) Time-lapse images of a *cdc15-GFP* cell (KGY3019). The arrowhead indicates the initial localization of a Cdc15p–GFP spot to the medial region with subsequent incorporation of other Cdc15p patches into this structure. (B) GFP–Cdc15p, expressed under control of *nmt81* promoter, and GFP–Cdc15p(1–425), under control of the $cdc15^+$ promoter, were visualized in live wild-type cells. (C) GFP–Cdc12p was expressed under control of the *nmt81* promoter and visualized in live *cdc15-CFP* cells (KGY3352). (D) Cdc15p was produced under control of the *nmt81* promoter in *cdc12p-GFP* cells (KGY3066), and live cells were imaged (left). Cdc12p was produced under control of the *nmt81* promoter in *cdc15-GFP* cells (KGY3019), and live cells were imaged (right).

cells, a spot containing both GFP–Cdc12p and Cdc15p–CFP was observed in interphase cells (Fig. 4 C). Strains expressing only the GFP- or CFP-tagged protein were examined to ensure an absence of a fluorescent signal in the reciprocal channel (unpublished data). Furthermore, we observed that, although these spots were somewhat difficult to detect with endogenous levels of these proteins, mild overexpression from the *nmt81* promoter of either untagged Cdc12p or Cdc15p promoted higher levels of either endogenously tagged Cdc15p–GFP or endogenously tagged Cdc12p–GFP, respectively, into a spot structure (Fig. 4 D). We conclude that Cdc12p and Cdc15p exist together in a potential ring-nucleating structure at the onset of mitosis.

Cdc15p interacts directly with the formin Cdc12p

Given the colocalization of Cdc15p and Cdc12p to both the CAR and the interphase spot, we asked if these proteins could interact. By two-hybrid analysis, a strong interaction was detected between the NH_2 terminus of Cdc15p(1–282) and NH_2-terminal constructs of Cdc12p that included its formin homology 3 (FH3) domain (Fig. 5 A). Previous work has suggested that FH3 domains of formins are involved in targeting them to discrete locations within *S. pombe* cells (Petersen et al., 1998). The NH_2 terminus and FH3 domain of Cdc12p suffice not only for its localizations to the CAR and the motile spot, but also for its interaction with Cdc15p (Fig. 5 A). Notably, the COOH-terminal SH3 domain of Cdc15p is not involved in its interaction with Cdc12p. Indeed, a construct of Cdc15p lacking its SH3 domain is able to rescue *cdc15* null cells, indicating that it does not play an essential role in Cdc15p function during cytokinesis (unpublished data).

We next tested whether Cdc15p and Cdc12p could interact in *S. pombe* cell lysates. We found that MBP–Cdc12p (1–765), but not MBP, incubated with protein lysates from a *cdc15-HA* strain, interacted with Cdc15p–HA (Fig. 5 B, lane 3), whereas it did not interact with the unrelated protein Cut9p–HA (lanes 2 and 5). Furthermore, we observed that GST–Cdc12p NH_2 terminus, bound to glutathione beads, was able to bind the soluble recombinant Cdc15p NH_2-terminal region, whereas no binding was seen with an unrelated control protein (Fig. 5 C). Thus, Cdc15p and Cdc12p are able to bind directly to one another.

The evidence supporting the biological relevance of this interaction is twofold. First, although Cdc15p–GFP could be observed both in spots and in the medial cortex region, no Cdc15–GFP ring was detected in *cdc12-112* cells at the restrictive temperature (Fig. 5 D). Further, there are strong negative genetic interactions between *cdc15* and *cdc12* mutant alleles. Even at 25°C, cells were barely able to form colonies (Table I) with many cells in the population failing at cytokinesis (Fig. 5 E). We conclude that the interaction between Cdc15p and Cdc12p is important for their proper localization and in vivo function.

Cdc15p is required for medial actin ring formation. Recent work has established that formins represent an Arp2/3 complex–independent pathway for the nucleation of actin filaments (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002). Furthermore, formation of the CAR in

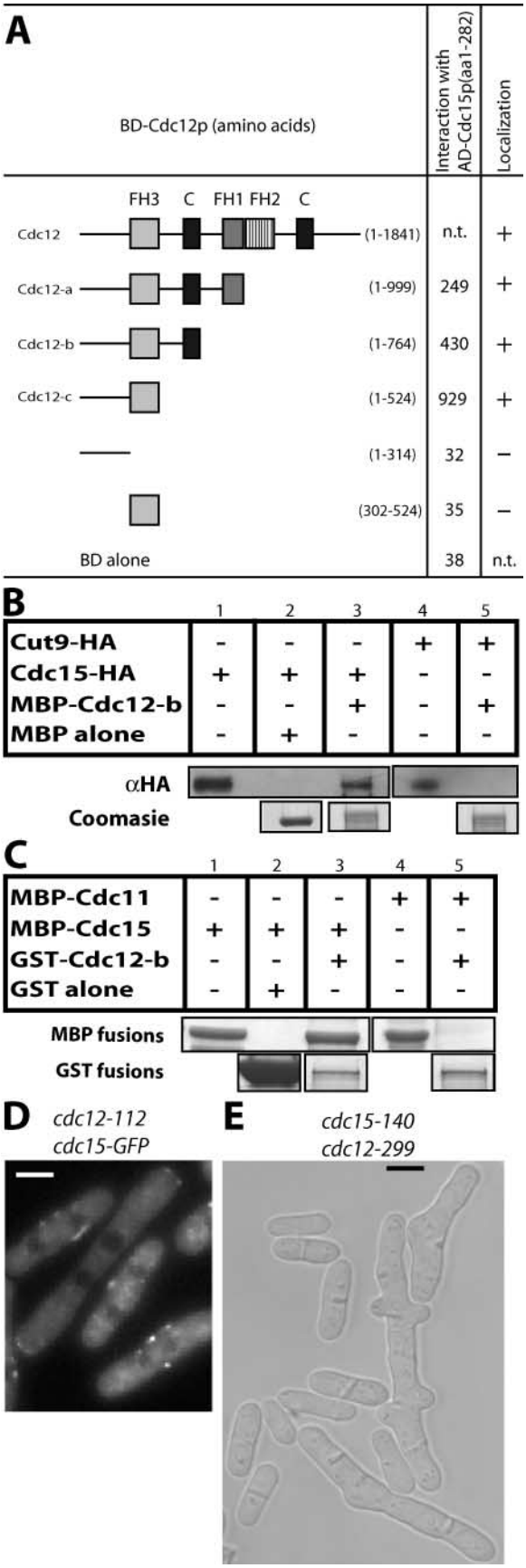

Figure 5. **Cdc15p interacts directly with Cdc12p.** (A) The indicated regions of Cdc12p were tested for interaction with Cdc15p (amino acids 1–282) by two-hybrid analysis. LEU^+ TRP^+ transformants were tested for growth on selective media (not depicted) and assayed for β-galactosidase activity measured in relative light units. GFP fusions of the *cdc12* constructs were also expressed under control of the *nmt81* promoter and visually examined for their ability (+) or lack of ability (−) to localize to an interphase spot structure and also to the CAR (column 3). (B) MBP (lane 2) or MBP–Cdc12p-b (amino acids 1–764) (lanes 3 and 5) both bound to amylose beads were incubated with protein lysates from either a *cdc15-HA* strain (KGY3020) or a *cut9-HA* strain (KGY1463) and subsequently extensively washed in binding buffer. Bound proteins were then divided and analyzed by immunoblotting (top) and Coomassie staining (bottom). Only relevant portions of the gels are shown; however, all proteins ran at the predicted sizes. (C) GST (lane 2, bottom) or GST–Cdc12-b (lanes 3 and 5, bottom) both bound to glutathione beads were mixed with either soluble MBP–Cdc15p(1–282) (lanes 1–3, top) or soluble MBP–Cdc11p(1–660) (lanes 4 and 5, top). Proteins were resolved by SDS-PAGE and detected by Coomassie staining. Only relevant portions of the Coomassie gel are shown to indicate loading; however, all proteins ran at the predicted sizes. Lanes 1 and 4 contain samples of the MBP fusion proteins before the binding reactions. (D) Localization of Cdc15p–GFP in *cdc15-GFP cdc12-112* cells after 4 h at 36°C. (E) Phase contrast image of *cdc15-140 cdc12-299* cells grown at 25°C. Bars, 5 μm.

Table I. **Genetic interactions of *cdc15* with *cdc12***

Genotype	25°C	29°C	32°C	36°C
cdc15-140	++	++	+/−	−
cdc12-299	++	++	−	−
cdc15-140 cdc12-299	+/−	−	−	−
cdc12-112	++	++	+/−	−
cdc12-112 cdc15-140	+	−	−	−

S. pombe depends upon both the Arp2/3 complex and Cdc12p (Pelham and Chang, 2002). As Cdc15p is involved in recruitment of both actin nucleation pathways required for CAR formation, we predicted that in the absence of Cdc15p function, actin rings should not be able to form. Contrary to this prediction, however, actin rings were reported to form in *cdc15-140* cells (Balasubramanian et al., 1998). Re-examination of this issue indicated that although rings could be detected after 4 h incubation at the restrictive temperature of 36°C in *cdc15-140* cells, this occurred in <2% of cells (vs. 12% in a similarly treated wild-type culture), and in accordance with another report on CAR formation in *S. pombe* (Arai and Mabuchi, 2002), these rings were poorly organized and incomplete (unpublished data). We also examined CAR formation in *cdc15::ura4* cells. Spores from a $cdc15^+/cdc15::ura4^+$ heterozygous diploid were inoculated into liquid medium either with or without selection for $Ura4^+$ growth. Under selective conditions, no CARs were detected as spores germinated and underwent their first mitosis (Fig. 6, A and B). Rather, actin patches remained primarily at cell tips. This is in contrast to spores released into nonselective medium, where CARs were observed in ~50% of binucleate cells and a significant portion of germinated mononucleate cells (Fig. 6 A), as would be predicted.

As Cdc15p overexpression is sufficient for directing medial actin rearrangement in G2-arrested cells (Fankhauser et al., 1995), we tested whether it was also sufficient for accumulation of F-actin nucleators. Myo1p–GFP localized pre-

A

Ura Selection	nuclei	% rings	total cells
+	mononucleates	0	120
+	binucleates	0	111
-	mononucleates	19	108
-	binucleates	47	125

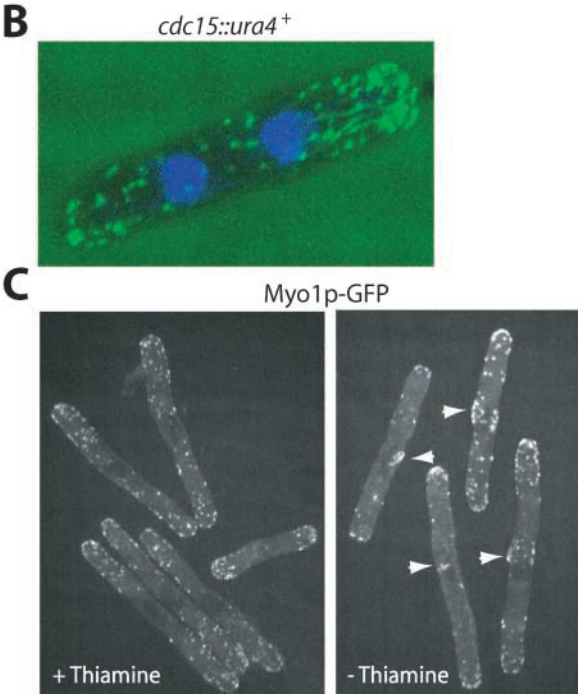

Figure 6. **Cdc15p is required for formation of the medial actin ring.** (A) Spores from the heterozygous *cdc15*$^+$/*cdc15::ura4*$^+$ diploid were released into selective media (−uracil), indicated by +, or nonselective media (YE), indicated by −, and after 16 h were fixed and stained with AlexaFluor 488–phalloidin. The presence of a CAR in germinated cells was determined in binucleates and mononucleates. (B) A representative image of a binucleate *cdc15::ura4* cell, fixed and stained with AlexaFluor 488–phalloidin to visualize actin (green) and DAPI to visualize DNA (blue). (C) Myo1p–GFP localization in G2-arrested *cdc25-22* cells either expressing (induced) or not expressing (uninduced) *nmt1cdc15*.

dominantly at cell ends in *cdc25-22* G2-arrested cells (Fig. 6 C). In contrast, induction of *cdc15* expression led to medial accumulation of Myo1p–GFP in *cdc25-22* arrested cells (Fig. 6 C). We conclude that Cdc15p function is strictly required for assembly of the CAR and is able to direct medial recruitment of key factors involved in this process.

Discussion

cdc15 encodes a member of the PCH protein family, conserved from yeast to mammals, that has been linked to actin cytoskeletal functions (Lippincott and Li, 2000). Though Cdc15p is the founding member of this protein family, little progress has been made toward understanding its precise function in cytokinesis. Here we have provided the first clues as to its critical role in this process. Cdc15p interacts directly with both the Arp2/3 complex activation machinery and the formin Cdc12p to orchestrate early events in CAR formation. As predicted by these interactions, the CAR does not form in the absence of Cdc15p function.

The critical role of Cdc15p in CAR formation was suggested by the observation that overexpression of Cdc15p was sufficient to drive medial recruitment of actin during interphase (Fankhauser et al., 1995). We have extended this observation to show that Cdc15p recruits Myo1p and other F-actin nucleators (this study; unpublished data) that presumably allow this to occur. Plo1p overproduction has similarly been shown to lead to actin ring assembly in G2-arrested cells (Ohkura et al., 1995). However, other ring proteins, such as Cdc12p, do not appear to drive CAR formation in interphase (Chang, 1999; Balasubramanian et al., 2000). Therefore, our results solidify the role of Cdc15p as a critical factor in ring nucleation.

Formins have recently emerged as key factors in a novel actin nucleation pathway. The conserved NH_2-terminal region of Cdc15p interacts directly with the *S. pombe* formin, Cdc12p, which is required for CAR assembly (Pelham and Chang, 2002). Further, these two proteins exist together in a medially located structure in cells before CAR formation, and they show a mutual dependence for localization to both this structure and the CAR (Fig. 5 D; Chang et al., 1997). This Cdc12p-containing structure has previously been reported to be the site of nucleation for the CAR (Chang, 1999). Furthermore, the initial actin filaments of the ring have also been shown to originate from a similar structure, termed a medial aster (Arai and Mabuchi, 2002). It seems likely that the Cdc15p–Cdc12p spot and the actin aster are identical structures. The type II myosin Myo2 also localizes to a medial spot structure before ring formation (Naqvi et al., 1999; Motegi et al., 2000). Although actin and myosin localization to the cleavage furrow occurs partially independently (Naqvi et al., 1999; Motegi et al., 2000), there must be a mechanism for coordinating final CAR assembly, and it will be interesting to determine whether the Cdc15p–Cdc12p spot structure also contains Myo2p.

Cdc15p also interacts directly with the Arp2/3 complex regulatory factor Myo1p and is required for medial localization of all known Arp2/3 complex regulators in *S. pombe*. This is likely the explanation for the previously observed failure of the Arp2/3 complex itself to localize to the CAR in *cdc15-140* cells (Balasubramanian et al., 1998) and a major reason for CAR formation failure. Cdc15p appears to be the first reported factor required for proper localization of both Arp2/3 complex and its activator proteins. Other Cdc15-related proteins have previously been shown to interact with regulators of actin nucleation, although the functional consequences of these interactions are not clear. For example, the mammalian PCH proteins PSTPIP and CIP4 bind to WASP (Wu et al., 1998; Tian et al., 2000), WRP binds to WAVE-1 (Soderling et al., 2002), and in *S. cerevisiae*, Bzz1p and Hof1p have also been observed to interact with Las17/Bee1p, Vrp1p, and Myo1p (Kamei et al., 1998; Vallen et al., 2000; Naqvi et al., 2001; Soulard et al., 2002). However, all of these reported interactions occur via the SH3 domains of

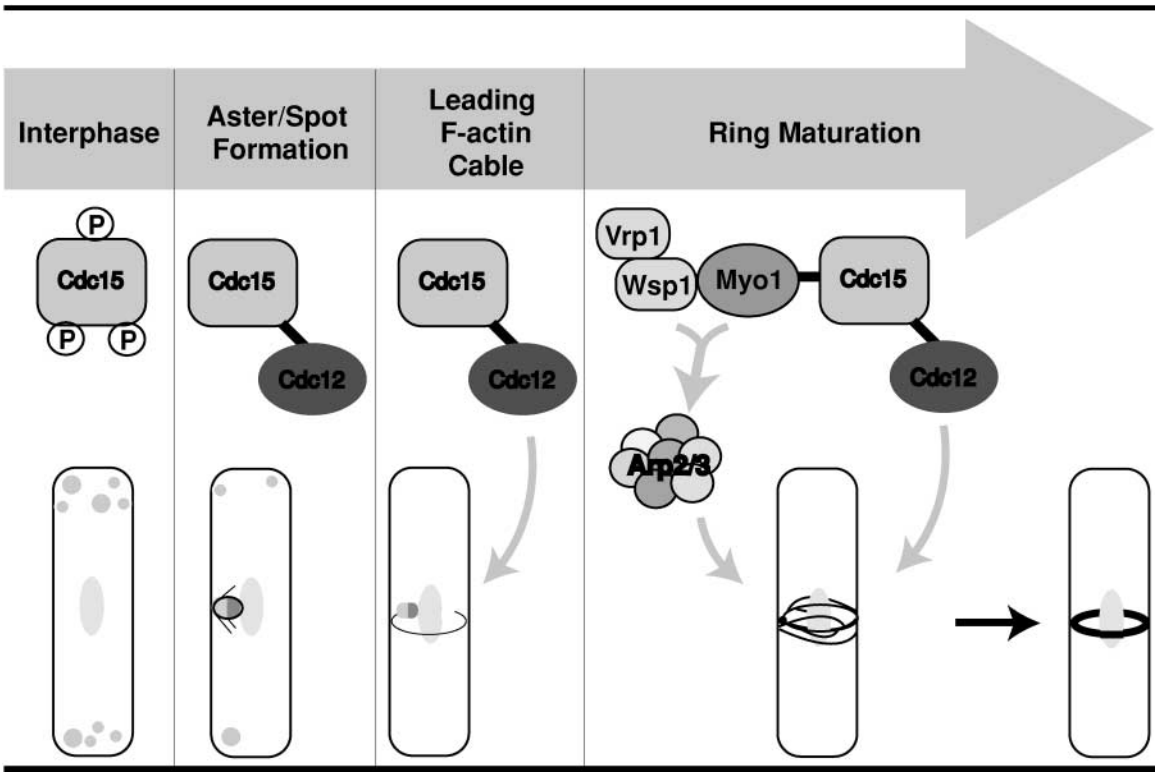

Figure 7. **Model of Cdc15p involvement in ring formation.** Hyperphosphorylated Cdc15p resides in patches at cell ends during interphase (indicated in gray spots). At the G2/M transition, Cdc15p becomes hypophosphorylated and appears in a medial spot structure (gray spot) with Cdc12p (darker gray spot) near the nucleus (oblong gray structure). This complex recruits actin (black spot and lines) and, through nucleation by Cdc12p, initiates the assembly of the primary F-actin ring. Cdc15p also recruits the Arp2/3-dependent actin nucleation machinery through its direct association with Myo1p. The Arp2/3 complex promotes the formation of the medial actin cable meshwork seen subsequent to primary ring formation. Finally, these actin fibers are bundled to create the mature CAR.

the PCH proteins, whereas, we find that the SH3 domain of Cdc15p is not essential for the observed interactions. Nevertheless, it is worth noting that a similar network of physical interactions, as we describe here, may well take place in *S. cerevisiae* because two-hybrid interactions link Bni1p/Bnr1p–Myo3/5p–Bzz1p–Las17p (Tong et al., 2002). It was surprising that we were unable to detect association between Cdc15p and Wsp1p or Vrp1. This is consistent, however, with our observation that the SH3 domain of Cdc15p is not essential for its function (unpublished data).

S. cerevisiae Hof1p has been previously suggested to be a functional homologue of Cdc15p (Lippincott and Li, 2000). However, unlike Cdc15p, Hof1p plays no known role in CAR formation but, rather, seems to be involved in coordinating ring contraction and septation (Lippincott and Li, 1998; Vallen et al., 2000). Indeed, Hof1p appears to be more functionally related to a second *S. pombe* PCH protein, Imp2p, which is involved in CAR dynamics during contraction (Demeter and Sazer, 1998).

Surprisingly, we found that Cdc15p also localizes to cell ends during interphase in a pattern very similar to that of actin patches. Though we found very little colocalization of these structures with actin patches, this localization pattern suggests that Cdc15p regulation of the actin cytoskeleton might not be limited to mitosis.

Cdc15p is heavily phosphorylated in interphase (Fankhauser et al., 1995; unpublished data). Immediately before CAR formation, Cdc15p becomes hypophosphorylated. Then, as cytokinesis begins, Cdc15p returns to its hyperphosphorylated state (Fankhauser et al., 1995). Integration of these observations with our results leads one to speculate that the changes in Cdc15p phosphorylation might affect its interactions with other proteins. Consistent with this possibility, bacterially produced and presumably unphosphorylated Cdc15p binds to both Cdc12p and Myo1p. It will be interesting to determine if these interactions can be modulated by the phosphorylation state of Cdc15p, and if Cdc15p dephosphorylation might serve as a trigger for CAR formation.

Placing our results in the context of previous studies, particularly the careful analysis of CAR formation in Arai and Mabuchi (2002), leads us to propose a model for ring formation that includes Cdc15p (Fig. 7). In interphase, hyperphosphorylated Cdc15p is prevented from associating with Cdc12p. Upon commitment to mitosis, Cdc15p is dephosphorylated and associates with Cdc12p and actin to form the pre-ring medial aster/spot. In metaphase, the primary F-actin ring is formed. This step has been suggested to be dependent on Cdc12p (Chang et al., 1997), a hypothesis consistent with the role of formins in nucleating linear unbranched filaments (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002). The presence of Cdc15p in this primary ring leads to medial recruitment of the Arp2/3 complex and the subsequent creation of an actin network encir-

Table II. **Strains used in this study**

Strain	Genotype	Source
KGY188	*cdc15-140 h⁻*	P. Nurse
KGY246	*ade6-M210 ura4-D18 leu1-32 h⁻*	Lab stock
KGY3958	*myo1-GFP ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY3963	*myo1-GFP cdc15-140 leu1-32 h⁻*	This study
KGY3966	*vrp1-GFP ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY108	*vrp1-GFP cdc15-140 leu1-32 h⁻*	This study
KGY358	*wsp1-GFP cdc15-140 ura4-D18 leu1-32 h⁺*	This study
KGY3019	*cdc15-GFP ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY3020	*cdc15-HA ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY3960	*myo1-HA ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY3359	*cdc15-GFP cdc12-112 ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY3066	*cdc12-GFP ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY3362	*cdc15-GFP sid4-GFP ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY746	*vrp1::ura4 ade6-M210 ura4-D18 leu1-32 h⁺*	This study
KGY586	*myo1::kanR ade6-M210 leu1-32 h⁻*	M. Yamamoto
KGY318	*cdc12-112 ura4-D18 h⁻*	P. Nurse
KGY748	*cdc12-299 h⁻*	P. Nurse
KGY1463	*cut9-HA ura4-D18 h⁻*	Lab stock
KGY3042	*cdc15-GFP cdc25-22 ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY4224	*cdc15/cdc15::ura4 ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h⁺/h⁻*	This study
KGY3352	*cdc15-CFP ade6-M210 ura4-D18 leu1-32 h⁻*	This study
KGY751	*cdc15-140 cdc12-112 ura4-D18 leu1-32 h⁺*	This study
KGY752	*cdc15-140 cdc12-299 h⁺*	This study
KGY753	*vrp1::ura4 wsp1::ura4*	This study

cling the equator of the cell. Finally, in late anaphase, these thinner filaments coalesce into what is recognized as the mature CAR structure. This places Cdc15p at a major convergence point for coordination of events required for initiation and formation of the CAR in *S. pombe*. In the future, it will be interesting to determine if Cdc15p's function is limited to that of recruitment, or whether it might also have a catalytic role in regulating the activity of the proteins it recruits. Domain architecture and localization to actin-rich regions are conserved across the PCH protein family. Given this structural conservation, it is likely that other family members are similarly involved in the organization of dynamic actin structures in higher eukaryotes.

Materials and methods

Strains, media, and molecular biology methods

S. pombe strains used in this study (Fig. S1; Table II) were grown in YE medium or EMM minimal medium with the appropriate supplements (Moreno et al., 1991). Expression of constructs under control of the thiamine-repressible *nmt* promoter system was performed as previously described (Maundrell, 1993). Cells were synchronized using arrest release of a *cdc25-22* mutant, as previously described (Fankhauser et al., 1995), or lactose gradients (Barbet and Carr, 1993). Standard genetic and recombinant DNA methods were used except where noted. Gene fragments were obtained by PCR amplification from either *S. pombe* genomic DNA or a cDNA library, as appropriate. pK294, a plasmid expressing GFP fused to Cdc15p(1–425), under control of the native promoter, was obtained from Y. Hiraoka (Kansai Advanced Research Center, Kobe, Japan) (Ding et al., 2000). Sequencing was performed using Thermosequenase (USB) and Redivue ^{33}P terminator kit (Amersham Biosciences). Yeast transformations were performed using either a lithium acetate method (Keeney and Boeke, 1994) or electroporation (Prentice, 1992).

In vivo tagging and gene deletion

Strains expressing epitope-tagged versions of proteins were constructed using a PCR-based approach as previously described (Bahler et al., 1998). Each ORF was tagged at its endogenous locus at its 3′ end with a variety of tag-Kan^r cassettes. Appropriate tagging was confirmed by PCR and either microscopic or immunoblot inspection, as appropriate. All tagged strains were viable at temperatures ranging from 25°C to 36°C. Unless otherwise noted, proteins are detected using endogenous tags throughout this study. Coding sequences were replaced with the *ura4*$^+$ gene using a one-step PCR-based approach as previously described (Bahler et al., 1998). Deletions were confirmed by PCR amplification using primers inside the *ura4*$^+$ gene and primers outside the disruption cassette. For phenotypic analysis of *cdc15::ura4* cells, the heterozygous *cdc15*$^+$/*cdc15::ura4*$^+$ diploid was induced to sporulate on glutamate plates, and spores were isolated after glusulase-treatment and inoculated into YE media for 3 h to assist spore germination. Spores were washed extensively and then incubated in media without uracil to allow growth of *cdc15::ura4*$^+$ cells, but not of the Ura$^-$ *cdc15*$^+$ cells. Spores were grown for 10–12 h in selection media before being harvested and processed for actin staining.

Microscopy

All microscopy, except germination of *cdc15::ura4* cells, was performed on a Carl Zeiss MicroImaging, Inc. Aviovert II inverted microscope equipped with a Plan Apo 100/1.40 lens, a piezo-electric Z-axis stepper objective motor (Physik Instumente), an UltraView LCI real-time scanning head confocal (PerkinElmer), a 488-nm argon ion laser (for GFP and YFP excitation), and a 442-nm helium cadmium laser (for CFP excitation), and images were captured on an Orca-ER charge-coupled device camera (Hamamatsu). Images were captured using Ultra-View software (PerkinElmer) and subsequently processed using Volocity 2.0 software (Improvision). For time-lapse experiments, cells were placed on a YE agar pad, and a coverslip was then sealed over the sample using VALAP. For spore germination experiments, fluorescence microscopy was performed on a Carl Zeiss MicroImaging, Inc. Axioskop II equipped with a z-focus motor drive, and images were captured with an Orca II charge-coupled device camera (Hamamatsu). Images were obtained, processed, and analyzed with OpenLab 2.1.3 software (Improvision). In all microscopy experiments on temperature-sensitive strains, the temperature of the sample was maintained during examination using an objective heater (Bioptechs). For visualization of actin structures, cells were fixed and processed as previously described (Pelham and Chang, 2001).

Yeast two-hybrid analysis

The yeast two-hybrid system used in this study was described previously (James et al., 1996). Various portions of the *cdc15*$^+$, *myo1*$^+$, *wsp1*$^+$, *vrp1*$^+$,

and *cdc12*$^{+}$ cDNAs were cloned into the bait plasmid pGBT9 and/or the prey plasmid pGAD424 (CLONTECH Laboratories, Inc.) and sequenced to ensure the absence of PCR-induced mutations and that the correct reading frame had been retained. To test for protein interactions, both bait and prey plasmids were cotransformed into *S. cerevisiae* strain PJ69-4A. β-galactosidase reporter enzyme activity in the two-hybrid strains was measured using Galacto-Star™ chemiluminescent reporter assay system according to the manufacturer's instructions (Tropix Inc.), with the exception that cells were lysed by glass bead disruption. Each sample was measured in triplicate. Reporter assays were recorded on the Mediators PhL luminometer (Aureon Biosystems).

In vitro binding assays of recombinant proteins

GST or MBP fusion proteins were produced in *Escherichia coli* from pGEX-2T or pMAL-2C, respectively, and purified on either glutathione-Sepharose beads (for GST) or amylose-Sepharose beads (for MBP). To elute fusion protein from beads, they were incubated in the presence of excess amounts of either glutathione or maltose, as appropriate. The supernatant, containing the eluted proteins, was then separated from the beads. For binding reactions, bead-bound recombinant proteins were incubated for 1 h at 4°C with the eluted fusion protein indicated in binding buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40). The beads were washed extensively in binding buffer, and the proteins were resolved by SDS-PAGE followed by Coomassie blue staining to visualize the proteins.

In vitro binding assays in reticulocyte lysates

As attempts to express fragments of Wsp1p in *E. coli* were unsuccessful, the indicated fragments were cloned into pSK(+) and translated in vitro in the presence of ^{35}S-Trans label (ICN Biomedicals) with the use of the T_NT-coupled reticulocyte lysate system (Promega). Recombinant fusion proteins and binding reactions were performed as described above, except that SDS-PAGE gels were treated with Amplify (Amersham Biosciences), and bound [^{35}S]Wsp1p was visualized by a Storm 860 phosphoimager (Amersham Biosciences).

Protein lysates, lysate bindings, and immunoblotting

Protein lysates were prepared in NP-40 buffer (Gould et al., 1991). For lysate binding experiments, the indicated bead-bound recombinant proteins were added to lysates, incubated for 1 h at 4°C, and extensively washed with NP-40 buffer. Proteins were resolved by SDS-PAGE and transferred by electroblotting to a PVDF membrane (Immobilon P; Millipore). Anti-HA (12CA5) antibodies were detected using horseradish peroxidase–conjugated goat anti–mouse secondary antibodies (0.8 mg/ml; Jackson ImmunoResearch Laboratories) at a dilution of 1:50,000. Immunoblots were visualized using ECL reagents (Amersham Biosciences).

Online supplemental material

The supplemental material for this article is available at http://www.jcb.org/cgi/content/full/jcb.200305012/DC1. Fig. S1 A shows the sequence alignment of *S. pombe* Vrp1p with *S. cerevisiae* Vrp1p and human WASp-interacting protein (WIP). Fig. S1 (B and C) shows the growth of *vrp1* and *wsp1* single and double deletion strains on plates at 18°C and 36°C, respectively. The following four Quicktime movie files of live cells are available online: three-dimensional rotation of Cdc15p–GFP and Sid4p–GFP (Video 1), Cdc15p–GFP spots at cells ends (Video 2), Cdc15p–GFP spots arriving into the medial region (Video 3), and Cdc15p fibers coalescing into a ring (Video 4).

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