The Chloroplast Division Protein ARC6 Acts to Inhibit Disassembly of GDP-bound FtsZ2

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ABSTRACT

Chloroplasts host photosynthesis and fulfill other metabolic functions that are essential to plant life. They have to divide by binary fission to maintain their numbers throughout cycles of cell division. Chloroplast division is achieved by a complex ring-shaped division machinery located on both the inner (stromal) and the outer (cytosolic) side of the chloroplast envelope. The inner division ring (termed the Z ring) is formed by the assembly of tubulin-like FtsZ1 and FtsZ2 proteins. ARC6 is a key chloroplast division protein that interacts with the Z ring. ARC6 spans the inner envelope membrane, is known to stabilize or maintain the Z ring, and anchors the Z ring to the inner membrane through interaction with FtsZ2. The underlying mechanism of Z-ring stabilization is not well understood. Here, biochemical and structural characterization of ARC6 was conducted using light scattering, sedimentation, and light and transmission electron microscopy. The recombinant protein was purified as a dimer. The results indicated that a truncated form of ARC6 (tARC6), representing the stromal portion of ARC6, affects FtsZ2 assembly without forming higher-order structures, and exerts its effect via FtsZ2 dynamics. tARC6 prevented GDP-induced FtsZ2 disassembly and caused a significant net increase in FtsZ2 assembly when GDP was present. Single particle analysis and 3D reconstruction were performed to elucidate the structural basis of ARC6 activity. Together, the data reveal that a dimeric form of tARC6 binds to FtsZ2 filaments and does not increase FtsZ polymerization rates but rather inhibits GDP-associated FtsZ2 disassembly.

Chloroplasts are essential plant organelles that host photosynthesis and fulfill many other important metabolic functions. They have to divide to maintain their numbers throughout cycles of cell division. Chloroplasts evolved from endosymbiotic cyanobacterial ancestors (1) and their division machineries share similarities with the bacterial division apparatus (2). Plastid division is accomplished by concentric contractile rings at the midpoint of the plastid, composed of FtsZ proteins on the stromal side of inner envelope membrane (IEM) (3-6) and Accumulation and Replication of Chloroplasts 5 (ARC5/Dynamin-Related Protein 5B/DRP5B) ring on cytosolic side of the outer envelope membrane (OEM) (7,8). Algae and plants encode two conserved FtsZ families, FtsZ1 and FtsZ2, that arose by gene duplication and divergent evolution from the
presumably single FtsZ gene in the cyanobacterial endosymbiont (9-11). Bacterial as well as plant FtsZ proteins are tubulin-like GTPases (12-14) that form filaments that ultimately assemble the FtsZ ring (Z ring) (6,15). The Z ring is a dynamic structure undergoing rapid exchange of FtsZ proteins between the assembled filamentous form and the unassembled soluble pool (16-19). Binding of GTP by the unassembled FtsZ promotes assembly of protofilaments where two adjacent FtsZ molecules form the catalytic site and the GTP is hydrolyzed soon thereafter. After GTP hydrolysis, the GDP-bound FtsZ dissociates from the filament and is replaced by a new GTP-bound FtsZ molecule. Localization of the division site to the mid-cell in bacteria or mid-chloroplast is accomplished by proteins that inhibit Z-ring assembly and/or promote its disassembly at “improper” sites. In plastids, this so-called Min system is composed of ARC3, a key negative regulator of FtsZ assembly (20-22), and additional proteins that are believed to modulate its activity, such as MinD (23), MinE (24), MCD1 (25) and PARC6 (26,27).

Under normal conditions, the action of the negative factors is balanced by the effect of the positive, FtsZ-assembly-promoting or stabilizing protein ARC6. Early studies showed that in mutants lacking ARC6, chloroplast division was almost completely blocked and instead of a single Z ring at mid-plastid, FtsZ formed numerous very short filamentous and dot-like assemblies. When ARC6 was overexpressed, FtsZ formed excessively long filaments (27,28). ARC6 is a bitopic protein localized to the IEM of the chloroplast, with its N-terminal portion exposed in the stroma and smaller C-terminal region located in the intermembrane space (IMS) (27,29,30). Consistent with this topology, the stromal portion of ARC6 can interact with FtsZ2 (31-34), while the C-terminal portion interacts directly with the C-terminal peptide of the OEM protein PDV2 and facilitates recruitment of the outer, cytosolic-side division ring components (7,32,35,36). TerBush et al., (37) showed that the stabilizing effect of ARC6 on FtsZ2 filaments is independent of membrane anchoring. Here we present new results that combine in vitro studies and live imaging in the Schizosaccharomyces pombe expression system to gain insight into the mechanism of ARC6 action on FtsZ2 assemblies.

RESULTS

Design of ARC6 and FtsZ2 Constructs and Purification of Recombinant Proteins—ARC6 contains several structural and functional domains depicted in Fig. 1A: chloroplast transit peptide (aa 1-67), conserved stroma-localized N-terminal region (aa 89-519) that includes a region identified in some ARC6 homologues as the J-like domain (89-159), the transmembrane domain (615-635) that is predicted to form a single helix (27) and the conserved C-terminal domain (679-774) located in the IMS where it interacts with chloroplast division protein PDV2 (27,32,36).

In all ARC6 constructs used in vitro in this study (Fig. 1A) the predicted chloroplast transit peptide that is cleaved upon import into the chloroplast (aa 1-67), as well as the non-conserved residues 68-88 in ARC6 have been omitted. Besides the longer form of ARC6 (aa 89-801), several deletion constructs were used to dissect the functional roles of the individual domains. The truncated, stromal portion of ARC6 (tARC6; aa 89-519, calculated molecular mass 48 kDa) representing the entire conserved N-terminal region was used to investigate how ARC6 affects FtsZ2 filament assembly. A similar construct from previous work, ARC6stromal (aa 68-614), which was tagged with mVenus (38) was used for FRAP assays in S. pombe also presented here. To test the role of the J-like domain of ARC6, the conserved N-terminal domain without the J-like domain was also constructed (tARC6AJ, aa 157-519, 40 kDa). The chloroplast J-like Domain 1 protein (CJD1) is known to interact with the N-terminal portion of ARC6 (aa 84-209) (39). We hypothesized that interaction with CJD1 is mediated by a different region of ARC6 than interaction with FtsZ2. Therefore, two constructs spanning residues 89-310 (tARC6AC) and 310-519 (tARC6AN) were also introduced to E. coli for expression (Fig. 1A).

The full length mature form of FtsZ2-1 (FtsZ2, aa 49-478, 45 kDa) without the predicted transit peptide (aa 1-48) (14) included the conserved C-terminal peptide (CTP, aa 463-478) (3,40,41) which is known to interact with ARC6(31). A truncated form of FtsZ2 (FtsZ2ANC, aa 118-424, 32 kDa) without the flexible N-terminal and conserved C-terminal regions was constructed in order to test whether
ARC6 also interacts with the central, core portion of FtsZ2 (Fig. 1B). *E. coli* Rosetta (DE3) pLysS was used to express all of constructs and achieved suitable expression level and solubility leading to successful purifications of all of proteins except tARC6ΔC and tARC6ΔN, which were not expressed sufficiently. Microscopic examination of bacterial cultures before and after induction indicated that with the exception of tARC6ΔC (data not shown) and tARC6ΔN, expression of the recombinant protein blocked bacterial cell division, causing filamentation of bacterial cells (Fig. 1C), possibly by interaction with the bacterial FtsZ that is evolutionarily and structurally related to chloroplast FtsZ2 (9,10).

The ARC6 as well as the truncated tARC6 and tARC6ΔJ proteins were each purified as a single peak of approximate molecular mass 160, 100 and 80 kDa, respectively (Fig. 1D), which corresponds to dimeric forms of the proteins. The dimeric forms of tARC6 and ARC6 were confirmed by single particle analysis and 3D reconstruction described in this report. FtsZ2 and FtsZ2ΔNC proteins were also successfully purified as dimers (Fig. 1D), consistent with published results (42).

**Effects of ARC6 on the Assembly of FtsZ2 In Vitro**—The effects of ARC6 on FtsZ2 polymerization were assessed by three complementary techniques, namely 90° light scattering, sedimentation, and transmission electron microscopy. Assembly was performed at pH 7.5 and temperature of 25°C.

FtsZ2 assembly was initiated by addition of GTP to 1 mM final concentration and monitored for 5 minutes by light scattering. At FtsZ2:tARC6 equimolar ratio (2 µM each), FtsZ2 assembled at an approximately 2-fold higher rate than FtsZ2 alone. A 2:3 FtsZ2:tARC6 ratio yielded a 3-fold increase, confirming that tARC6 acts in a concentration-dependent manner to promote FtsZ2 assembly (Fig. 2A). A control reaction with tARC6 alone did not yield any increase in light scattering (Fig. 2A). When the same assay was conducted with the truncated form of FtsZ2 (FtsZ2ΔNC), FtsZ2ΔNC assembly was not affected by tARC6 (Fig. 2B). This is consistent with data showing that the C-terminus of FtsZ2 is required for interaction with ARC6 (30-33) and suggests that tARC6 does not interact with the conserved central region of FtsZ2 (aa 118-424). These control reactions also indicated that the light scattering assays in the presence of tARC6 were not influenced by overall elevated protein concentration, i.e., molecular crowding. The effect of ARC6ΔJ on FtsZ2 assembly was also tested and showed increased light scattering in a concentration-dependent manner similar to the effect of tARC6 (Fig. 2C). The initial rates of FtsZ2 assembly in reactions with tARC6 and ARC6ΔJ were significantly higher (P=0.01) than in FtsZ2-alone reactions, and not significantly different (P=0.05) from each other (Fig. 2D), indicating that the J-like domain does not play a role in facilitating FtsZ2 assembly *in vitro*.

To further analyze the interaction of FtsZ2 with tARC6, co-sedimentation assays were performed at a centrifugation speed where only large filaments may be pelleted with its interacting proteins (43). Assembly reactions with 2 µM FtsZ2 or FtsZ2ΔNC and increasing stoichiometric ratios of tARC6 were centrifuged and separated by SDS-PAGE. tARC6 co-pelleted with FtsZ2 filaments, confirming the interaction between FtsZ2 filaments and tARC6. Consistent with the light scattering assays, the presence of tARC6 resulted in a somewhat increased amount of FtsZ2 in the pellet, i.e., more FtsZ2 polymerization (Fig. 2E). The amount of tARC6 was less than that of FtsZ2 in the pellet when equimolar FtsZ2 and tARC6 were used, suggesting that tARC6 is not bound to FtsZ2 in the filament at 1:1 molar ratio. Nevertheless, the amount of co-pelleted tARC6 was increased in reactions with a higher tARC6 molar ratio (Fig. 2E), confirming its concentration-dependent interaction with FtsZ2. In control experiments with FtsZ2ΔNC, tARC6 did not promote FtsZ2ΔNC sedimentation. A weak tARC6 band was observed in all pellets from FtsZ2ΔNC mixtures (Fig. 2F), but this was not a result of FtsZ2ΔNC-tARC6 interaction, since control reactions with tARC6 alone showed a similar band (Fig. 2F, penultimate lane). Together with light scattering assays of FtsZ2ΔNC assembly in the presence of tARC6 (Fig. 2B), these results demonstrated that FtsZ2ΔNC assembly was independent of tARC6 and thus there was no functional interaction between FtsZ2ΔNC filaments and tARC6.
Analysis of FtsZ2 Filament Bundles Using Transmission Electron Microscopy—Light scattering and sedimentation assays demonstrated that tARC6 increased the abundance and/or size of FtsZ assemblies. This effect was not as dramatic as was reported in studies of positive regulators of bacterial FtsZ assembly that promote bundling of FtsZ filaments (44-46). To assess FtsZ2 filament morphology and size and to gain insight into how tARC6 promotes FtsZ2 filament assembly, transmission electron microscopy (TEM) was employed. The same assembly reactions used in the light scattering assays were prepared, negatively stained and examined by TEM (Fig 3, A, B).

The images revealed a mixture of FtsZ2 filaments and bundles as well as short protofilaments and non-assembled FtsZ2 subunits. FtsZ2 alone and FtsZ2+tARC6 assemblies were of similar appearance (Fig. 3, A, B). FtsZ2 did not form higher-order or different types of assemblies in the presence of tARC6. This was in contrast with the previously reported effect of prokaryotic positive regulators of FtsZ assembly, SepF (47) and FzLA (48,49), which induce tubules and curved bacterial FtsZ polymers, respectively (Fig. 3C).

The length and thickness of filament bundles were measured (Fig. 3, D, E). Filament bundles of FtsZ2 assembled alone (width 54.9 nm ± 21.2 nm S.D., n=1361) were approximately 7 nm thicker than bundles of FtsZ2 filaments assembled in the presence of tARC6 (47.2 nm ± 18.1 nm, n=1501), a statistically significant, but modest difference (p < 0.05, Student’s t-test). Filament bundle lengths (267.9 nm ± 243.0 nm, n=1361, and 240.2 nm ± 156.2 nm, n=1501) for FtsZ2 alone and FtsZ2 + tARC6, respectively, did not differ significantly (p > 0.05).

Effects of ARC6 on FtsZ2 and FtsZ2D322A in S. pombe—A previous FRAP study in the heterologous fission yeast system Schizosaccharomyces pombe suggested that turnover of subunits from FtsZ2 filaments may be slightly inhibited in the presence of the stromal portion of ARC6 (ARC6STROMAL) (37). Also, filaments composed of the GTPase-deficient FtsZ2D322A had significantly reduced turnover, confirming that GTP hydrolysis is an important factor in filament dynamics (19). To expand this analysis, we expressed FtsZ2 or FtsZ2D322A fused to eCFP either alone or coexpressed with an ARC6STROMAL–mVenus fusion construct (Fig. 1A) in S. pombe to test how the turnover of ARC6 is affected by the nucleotide form bound to FtsZ: FtsZ2D322A filaments contain GTP only, while the wild-type FtsZ2 filaments are capable of GTP hydrolysis and contain both GTP and GDP.

Consistent with previous results (37) ARC6STROMAL expressed well and adopted a diffuse localization pattern (Fig. 4A), while FtsZ2 and FtsZ2D322A assembled a network of interconnected filaments and aster-shaped structures, respectively (Fig. 4, B, C). Coexpression of ARC6STROMAL and FtsZ2 resulted in the proteins colocalizing to an interconnected network of filaments (Fig 4D), which were highly similar to those of FtsZ2 assembled alone. The Pearson’s Correlation Coefficient (PCC) was used to estimate the extent of fluorescence signal overlap and the degree to which the fluorescent signal intensities were correlated. FtsZ2 and ARC6STROMAL had a PCC of 0.92 ± 0.01 (± SEM), indicating that these proteins were highly colocalized in the filament network. Similarly, FtsZ2D322A and ARC6STROMAL colocalized to FtsZ2D322A-like structures when coexpressed (Fig. 4E). However, ARC6STROMAL had a more diffuse signal in this coexpression strain, with a PCC of 0.81 ± 0.03, a statistically significant reduction compared to ARC6STROMAL coexpressed with FtsZ2 (P-value = 0.0004), suggesting that ARC6STROMAL’s interaction with FtsZ2D322A is weaker than its interaction with FtsZ2.

The steady-state turnover properties of these filamentous structures were analyzed using fluorescence recovery after photobleaching (FRAP) (Fig. 4F). The time-course fluorescence recovery curves fit a two-binding state equation that yielded the fractions of bound molecules (c_{eq1} and c_{eq2}) and their respective unbinding rate constants (k_{off1} and k_{off2}, s^{-1}). The two-binding-state equation fit our FRAP data better than the simpler single-exponential equation we used previously (17,19,37). All results are summarized in Table 1, where we also include FRAP data from previous work, reprocessed with the new method. It should be noted that when recovery does not follow the single-binding state model, the half-time of recovery is not an ideal indicator of turnover dynamics, and is therefore not reported here. Most
of the FtsZ in filaments was included in the c_eq2 fraction, indicating that the k_{off} rate constant contributed the most to the recovery kinetics. Coexpression with ARC6_STROMAL had only a mild effect on FtsZ dynamics, with a similar amount of fluorescence recovery at 250 s (Fig. 4F, Table 1). As expected, the GTase-deficient FtsZD322A formed stable filaments that recovered to only 8% of the prebleach fluorescence intensity after 250 s, with greatly reduced unbinding rates compared to those of the wild-type FtsZ2. When coexpressed with ARC6_STROMAL, FtsZD322A also showed little fluorescence recovery.

ARC6_STROMAL was found to be much more dynamic than FtsZ2 in the coexpression strain, and at 250 s post-bleaching recovered to a higher extent than FtsZ2. Significantly, when coexpressed with FtsZD322A, ARC6_STROMAL showed a substantial increase of the fraction exhibiting fast unbinding, from c_eq1 = 4.1 e-2 to c_eq1 = 0.58, and in the extent of recovery at 250 s (Table 1), indicating that ARC6_STROMAL binds to FtsZD322A less tightly than it does to FtsZ2 and suggesting that ARC6 may preferentially bind to GDP-bound FtsZ2.

tARC6 Stabilizes FtsZ2 Filaments and Inhibits GDP-Induced Disassembly in Vitro—To further explore the effect of tARC6 on FtsZ2 assembly, FtsZ2 was polymerized in the presence of GTP for 5 minutes and then excess GDP was added to induce disassembly. Without tARC6, the addition of GDP led to FtsZ2 depolymerization (Fig. 5A). This decrease was substantially slower than previously reported for bacterial FtsZ GDP-induced disassembly (50,51), pointing to overall slower dynamics of plant FtsZ2 in comparison to its prokaryotic homologues. In the presence of tARC6, FtsZ2 disassembly was approximately two-fold slower (Fig 5, A, B). When the samples were examined by TEM, the large FtsZ filament bundles typically observed in FtsZ assembly reactions (insets in Fig. 5, C, D) were absent after GDP-induced disassembly reactions, regardless of whether tARC6 was present or not (Fig. 5, C, D). However, in the presence of tARC6, FtsZ2 was able to maintain some smaller and thinner filament bundles (Fig 5D) while only short protofilaments remained in reactions with FtsZ2 alone (Fig. 5C).

The dominant morphology of FtsZ2 filaments remaining after disassembly in the presence of tARC6 (Fig. 5D) was characterized as short and curved filament bundles. Curved filaments were previously reported with GDP-bound subunits of prokaryotic FtsZ (52,53). It is conceivable that the curved assemblies shown in Fig 5D represent GDP-bound FtsZ2, and that the role of tARC6 is to stabilize FtsZ2 filaments when GDP-bound FtsZ2 is present.

Mechanistic Role of tARC6 in FtsZ2 Filament Stabilization—Two possible modes by which tARC6 could modulate FtsZ2 dynamics to stabilize FtsZ2 filaments were tested. In the first mode, tARC6 inhibits GTase activity of FtsZ2, rendering the FtsZ2 polymers less prone to disassembly and less dynamic (54,55). However, the results showed that the GTase activity was either not significantly different or slightly elevated when tARC6 was present (Fig. 6A).

For the second mode, tARC6 stabilizes FtsZ2 filaments by preventing dissociation of FtsZ2 subunits after GTP hydrolysis. The ratio of GTP to GDP is known as a critical factor for FtsZ filaments (56), because GDP can trigger disassembly (Fig. 5A) and allow filament remodeling and turnover (54). To test this hypothesis, the non-hydrolyzable GTP analogue GpCpp was used for FtsZ2 assembly in the presence or absence of tARC6. Because FtsZ2 subunit turnover and filament disassembly require the GDP-bound form of FtsZ2, the non-hydrolyzable analogue yields stable FtsZ2 filaments that are not prone to disassembly. When GpCpp was used, FtsZ2 assembly rate was not affected by tARC6 (initial assembly rate normalized to FtsZ2-alone reactions was 0.999 ± 0.102 S.D, n=6), as demonstrated in Fig. 6B, reactions with GpCpp only. This indicated that the dynamic assembly and disassembly of FtsZ2, driven by GTP binding and hydrolysis, are required for tARC6 to exert an effect on FtsZ2. In other words, tARC6 does not promote FtsZ polymerization but rather inhibits disassembly of existing FtsZ2 filaments. Since tARC6 did not have any effect when the stable GTP analogue GpCpp was used alone in FtsZ2 assembly reactions, a mixture of 0.1 mM GpCpp and 0.2 mM GDP was used to produce assemblies containing predominantly GDP-bound FtsZ2 that are more prone to FtsZ2 subunit dissociation and filament disassembly. In these reactions GTP-to-GDP hydrolysis was neither required nor involved.
in FtsZ disassembly/turnover since GpCpp was not readily hydrolyzed and GDP was readily supplied. In reactions with both GpCpp and GDP, light scattering in FtsZ2-alone reactions increased at a much lower rate than in GpCpp-only reactions, indicating that the balance has been tipped towards accelerated disassembly of filaments (Fig. 6B). The presence of tARC6 largely compensated for this effect and produced an approximately 3-fold increase in net assembly rates compared to FtsZ2-alone reactions (Fig. 6, B, C).

Taken together, these results strongly suggest that tARC6 exerts its effect through GDP-induced FtsZ2 dynamics within filament bundles by preventing immediate dissociation of GDP-bound FtsZ2 molecules.

3D Reconstruction of tARC6, ARC6 and the Binding Model—Gel filtration chromatography of the recombinant tARC6 showed that the protein purified as a dimer. The full-length mature form of the protein, ARC6, purified mainly as a dimer with a minor monomeric fraction (Fig. 1E). The fraction corresponding to the dimeric form (~160 kDa) was used for TEM. Images of negatively stained monodispersed tARC6 and ARC6 proteins (Fig. S1, A, A') were used for single particle analysis and 3D reconstruction (Fig. 7). Reconstruction of tARC6 dimer at around 2 nm resolution (Fig. S1E) revealed a horseshoe-like-shaped molecule with an enclosed volume corresponding to approximately 100 kDa (Fig. 7B). In contrast, 3D reconstruction of the ARC6 dimer revealed an oval shape (Fig. S1G') at approximately 2.5 nm resolution (Fig. S1E') with an enclosed volume corresponding to approximately 160 kDa. The difference in molecular mass of approximately 30 kDa per monomer is in agreement with the difference in molecular mass between the tARC6 and ARC6 monomers.

The dimers of tARC6, which represents most of the stromal portion of ARC6, showed a good fit with the bottom portion of the full-length ARC6 dimer (Fig. 7C). The protein densities extending past the stromal portion agreed with the crystal structures of C-terminal domains (CTDs) of ARC6 (36) (Fig. 7C). Together with the biochemical assays showing ARC6 membrane topology (27), these findings were consistent with the membrane topology of ARC6 dimers depicted in Fig. 7C. The model also hints at the possibility that the stromal portion of the ARC6 dimer binds two adjacent GDP-bound FtsZ2 molecules to stabilize the FtsZ protofilament.

DISCUSSION

Conservation of Interactions between Bacterial Cell and Chloroplast Division Proteins—Both full-length and truncated forms of ARC6 and FtsZ2 were introduced into E. coli Rosetta (DE3) pLysS for protein expression. Expression of FtsZ2 and FtsZ2ΔNC significantly inhibited cell division, pointing to functional and structural similarities between the prokaryotic cell and chloroplast division machineries (34,57). Similar effects were observed previously in our and others’ experiments with other chloroplast division proteins expressed in E. coli, such as ARC3, MinD, MinE (58,59), as well as with the ARC6, tARC6 and ARC6ΔJ constructs in this report, probably due to their interactions with the bacterial FtsZ protein, an ancestor that is structurally very close to the chloroplast division protein FtsZ2 (10,60). The CTP of FtsZ2 (aa 463-470) shows 62.5 % sequence identity with the corresponding region of E. coli FtsZ (374-381). The CTP in both bacterial FtsZ and in plant FtsZ2 plays an essential role in interactions with membrane-localized components of the division machinery, such as FtsA and ZapA in E. coli (61-63) and PARC6 and ARC6 in chloroplasts (26,34,64,65).

Analysis of FtsZ2 and tARC6 Interactions by Light Scattering and TEM—FtsZ2 assembled at a higher rate when tARC6 or tARC6ΔJ was present and this effect was concentration-dependent (Fig. 2, A, C). This was not surprising since it was known that the conserved stromal portion of ARC6 interacts with the C-terminus of FtsZ2 (30-34) and facilitates FtsZ2 assembly (27,31,33,38). Interestingly, in reactions containing equimolar tARC6 and FtsZ2, there was substantially less tARC6 than FtsZ2 in the pellet (Fig. 2E), suggesting that tARC6 and FtsZ2 interact in filaments at a ratio of approximately 1:2. Furthermore, FtsZ2 and tARC6 did not co-migrate as a complex in gel permeation chromatography (data not shown), suggesting a rather weak or
transient interaction between FtsZ2 and tARC6. Results from assembly of FtsZ2ANC in the presence of tARC6 confirmed that the central portion of FtsZ2 is not involved in interactions with tARC6 and together with the previous reports suggested that the C-terminus of FtsZ2 is as essential for in vitro ARC6-FtsZ2 interaction as it is in in-vivo experiments (31,34,37). The role of the J-like domain of ARC6 remains unclear. Maple et al., (31) demonstrated that it is not required for FtsZ binding and data herein confirm that it does not change how tARC6 affects FtsZ2 assembly (Fig. 2, C, D).

In contrast with the effect of prokaryotic positive regulators of FtsZ assembly that induce FtsZ to form rings (ZipA (44)), thick bundles (FtsA (45)) cross-linked or bundled filaments (ZapA (66)), bundled polymers (ZapD (46)), tubules (SepF (47)), and curved polymers (FzlA (48,49)), tARC6 did not induce formation of higher-order FtsZ2 structures. This indicated that tARC6 does not have a scaffolding function within FtsZ2 structures.

**ARC6, FtsZ2 Turnover and the Effect of Nucleotides**—FtsZ filament assembly is promoted in the presence of GTP. Once the GTP bound in the filament is converted to GDP, the filament becomes unstable, leading to depolymerization (54). GTPase activity of FtsZ is generally correlated with the rate of assembly and FtsZ subunit turnover in and out of the filament in prokaryotes (18,67) and the same has also been shown for FtsZ2 proteins from plants and structurally similar FtsZA from red algae (68). For FtsZ2, the measured GTPase activity was 10.6 ± 1.1 nmol GTP hydrolyzed/min/mg protein when 1 mM GTP was used, a result very close to a previously reported value of 12 ± 0.5 nmol GTP hydrolyzed/min/mg protein (13) confirming the validity of the GTPase assay. Our data suggested that tARC6 does not facilitate FtsZ2 assembly by decreasing GTPase activity of FtsZ2 (Fig. 6A). The slightly elevated GTPase activity measured in the assays may be simply due to the fact that in the presence of tARC6 there are more FtsZ2 assemblies and thus the total capacity to hydrolyze GTP is increased.

The FRAP experiment revealed that ARC6STROMAL had only a small effect on FtsZ2 dynamics. This was similar to the finding by TerBush et al., (37) that the difference of FtsZ2 fluorescence recovery in the presence and absence of ARC6STROMAL was not statistically significant. We have recently switched to a different data analysis approach, using the two binding states model, which shows a better fit to the recovery curves (68,69). For comparison, the old data (19,37) were reprocessed with new methods and the results are shown in Table 1 and Fig. S3. The new data as well as the reprocessed data show that coexpression of ARC6STROMAL with FtsZ2 has only a subtle effect on FtsZ2 turnover. This was somewhat surprising, given the stabilizing effect of ARC6 on FtsZ assembly in vivo (27) and in our in vitro assays reported here. Nevertheless, a similar lack of effect on FtsZ turnover in the absence of FtsZ-regulating proteins was noted in bacteria with null mutations in ZapA (16) and with the gain-of-function allele ftsA* (70).

Dynamics of FtsZ2 aside, the more significant result is that when ARC6STROMAL was coexpressed with the GTPase deficient FtsZ2D322A, ARC6STROMAL exhibited a drastically increased size of the more mobile fraction c_m and showed overall accelerated recovery. This indicates that the ARC6STROMAL-FtsZ2 interaction is transient and that ARC6STROMAL has low affinity for GTP-bound FtsZ (Fig. 4F and Table 1). This is in agreement with the light scattering data where FtsZ2 assembly was not affected by tARC6 when the assembly was induced by addition of GpCpp, but was enhanced (compared to the control reactions without tARC6) when GDP was also present to promote disassembly (Fig. 5B).

Experiments with GDP-induced disassembly of FtsZ2 filaments clearly showed that after addition of excess GDP, equilibrium between assembly and disassembly of FtsZ2 filaments was shifted significantly toward disassembly (Fig. 5A), but the presence of tARC6 rendered FtsZ filaments more resistant to such disassembly and produced curved and twisted FtsZ2 filaments. This morphology was similar to longitudinaly bent and curved protofilaments of GDP-bound *Mycobacterium tuberculosis* FtsZ (71) and to helically curved GDP-bound FtsZ filaments in *Methanococcus jannaschii* (54). Our data are consistent with a model where tARC6 interacts with GDP-containing FtsZ2 filaments and filament bundles and prevents dissociation of GDP-bound FtsZ2 from the filaments. A recent
study of prokaryotic division proteins ZipA and FtsA*(a hyperactive FtsA mutant), have shown stabilizing effects on FtsZ polymers assembled in the presence of GDP (72).

**Dimerization of ARC6, 3D Reconstruction**—The results of recombinant protein purification and single particle analysis presented here indicate that tARC6, as well as ARC6ΔJ, form dimers in vitro. Dimerization was also confirmed with the full length mature form of ARC6 (aa 89-801) (Fig 1D). This is in agreement with a previous report where the stromal portion of ARC6, similar to tARC6 used here, was shown to self-interact in chloroplasts in vivo (31). The cyanobacterial orthologue of ARC6, Ftn2, also self-interacts (73). On the other hand, a recent study reported the recombinant N-terminal, stromal portion of ARC6 (aa 76-618) to be monomeric and implied that dimerization of ARC6 is induced through interaction of its CTD with PDV2 protein dimers in the intermembrane space of the chloroplast envelope (36). The dimeric versus monomeric form of the recombinant ARC6 may be attributed to salt concentration during gel filtration: 100 mM used here versus 200 mM in the report by Wang et al. (36). In our experiments, when the purified tARC6 was concentrated and equilibrated in a buffer containing higher salt concentration (up to 500 mM NaCl) and subjected to a second round of gel filtration, it showed a shift towards the monomeric form, while under low salt conditions (100 mM NaCl) tARC6 was exclusively in dimeric form (unpublished data).

We agree with Wang et al., (36) that the dimers represents the active form of ARC6 and speculate that since the ionic strength of chloroplast stroma under physiological conditions (~200 mM) (74) is between the values mentioned above, ARC6 may exist in vivo as a mixture of dimers and monomers. Future experiments should investigate whether tARC6 monomers have any activity towards FtsZ2 assembly or stabilization and investigate the stoichiometry of ARC6 monomers and dimers in vivo.

ARC6 includes a conserved region previously designated as the J-like domain, missing a key residue common to canonical J domains (27) and therefore unlikely to function as a typical J-domain protein and interact with HSP70 chaperone proteins (35). Here we have confirmed that the J-like domain is not required for dimerization or stabilization of FtsZ2 filaments by tARC6, nor is it required for ARC6 interaction with the bacterial division machinery (Fig. 1C). On the other hand, in the cyanobacterial ARC6 homologue ZipN the J-like domain is essential for correct localization to the septum of *Synechocystis* sp. PCC 6803 in vivo and for binding of ZipN to FtsZ in vitro (75). This difference may be due to the evolution of both the protein sequence and protein-protein interactions after endosymbiosis.

In the 3-D reconstruction of tARC6 to ~2 nm resolution, tARC6 assumes a shape that can roughly be described as a horseshoe with each of the arms corresponding to a monomer that appear to be joined together at the apex of the horseshoe. The distal ends of the arms versus the apex region are at opposite ends of the molecule and are likely to house the C- and N-terminus, respectively. The 3D reconstruction of ARC6 revealed a density distribution that concurs with this idea. In this model, crystal structure of the CTD of ARC6 fit well into the two small densities while the reconstruction of tARC6 showed a good match with the U-shaped bottom portion of the ARC6 density map. The 3D reconstructions of tARC6 and ARC6 have confirmed their dimeric forms and provided clues for dimerization of ARC6 as shown in Fig.7C. We propose that the stromal portion of ARC6 establishes its dimeric form. Dimerization brings the CTDs of ARC6 close to each other thereby enabling the interaction with the C-termini of two PDV2 molecules that are present as a dimer (36) to form ARC6-PDV2 dimer-dimer complexes. In the stroma, the two FtsZ-binding domains in the ARC6 dimer would each bind adjacent GDP-containing FtsZ2 molecules in the FtsZ filament. It should be noted that the current experiments have been conducted with FtsZ2 filaments, which is the major point of interaction of ARC6. Future studies should include the other FtsZ protein, FtsZ1. In chloroplasts as well as *in vitro*, FtsZ2 and FtsZ1 coassemble and form heteropolymers (14,19,37).

**EXPERIMENTAL PROCEDURES**

*ARC6 and FtsZ2 Constructs for Expression in E. coli*—The FtsZ2 and Arc6 sequences and proteins used in this work are of the model plant
Arabidopsis thaliana. There, the FtsZ1 family has a single member AtFtsZ1-1 while the FtsZ2 family has two proteins, AtFtsZ2-1 and AtFtsZ2-2. Since AtFtsZ2-2 is redundant with AtFtsZ2-1 [25], only AtFtsZ2-1 is used and referred to as FtsZ2 henceforth.

Constructs for expression in Escherichia coli (E. coli) were created by PCR amplification from cDNA templates using specific primers with engineered restriction sites as listed in Table S1, and cloning the amplified fragments into the corresponding restriction sites in Novagen pET28a vector (EMD Millipore, MA, USA). All proteins contain N-terminal 6xHis tag. The constructs are outlined in Fig. 1A and do not include the predicted chloroplast transit sequences: The mature form of ARC6 (ARC6, amino acid residues 89-801; primers tA6_Fv2 and tA6_R), the truncated stromal portion of ARC6 containing the entire N-terminal conserved region (tARC6; aa 89-519; primers tA6_F and tA6_R), the conserved N-terminal region of ARC6 without the domain formerly designated as J-like (27) (tARC6ΔJ; aa 159-519; primers tA6_F-2N and tA6_R), the truncated form of FtsZ2 missing both the non-conserved N-terminal region and the conserved C-terminal mature form of FtsZ2-1 (FtsZ2; aa 49-478; henceforth. tA6_R-2N), the second half of the N-terminal conserved region of ARC6 (tARC6ΔN; aa 89-310; primers tA6_F and tA6_R-2N), the first half of the N-terminal conserved region of ARC6 (tARC6ΔC; aa 89-310; primers tA6_F and tA6_R), the full length mature form of FtsZ2-1 (FtsZ2; aa 49-478; primers fFt2_F and fFt2_R) and the truncated form of FtsZ2 missing both the non-conserved N-terminal region and the conserved C-terminal peptide(FtsZ2ΔNC; aa 118-424; primers Ft2_F and Ft2_R). Constructs were confirmed by sequencing and introduced to E. coli Rosetta (DE3) pLysS strain for expression.

Purification of FtsZ2 and ARC6 Recombinant Proteins— Cultures were grown at 37°C to OD_{600}=0.4 and induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG; RPI, Mount Prospect, IL) to 0.5 mM final concentration. Cells were then kept on an orbital shaker at 18°C overnight, harvested by centrifugation and lysed by French Press at 20,000 psi in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, PMSF). The Lysate was centrifuged at 12,000 x g for 1 hour at 4°C. Proteins were purified by Ni-NTA column using the lysis buffer without PMSF, eluted with 20 mM Tris-HCl pH 8.0 buffer containing 100 mM NaCl and 500 mM imidazol. Fractions containing the recombinant protein were pooled, concentrated using an Amicon Ultra spin column (10 kDa cutoff; MilliporeSigma). The protein was equilibrated in 50mM HEPES, pH 7.5, 100 mM KCl and then further purified by SEC650 or Superdex-6 gel permeation column on an NGC Medium-Pressure Chromatography Systems (BioRad). Elution volumes were calibrated using Gel Filtration Chromatography Standard (BioRad). Purity of the fractions was confirmed by SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined by absorbance measured at 280 nm.

Light Scattering Assay of FtsZ2 Assembly and Disassembly—Assembly of FtsZ2 or FtsZ2ΔNC (2 µM) in polymerization buffer (50mM HEPES, pH 7.5, 100 mM KCl and 1 mM MgSO$_4$) in the absence/presence of 2 µM or 3 µM tARC6 or tARC6ΔJ was initialized by addition of 1 µL of nucleotides from the concentrated stock to the final concentration of 1 mM GTP or 0.1 mM GpCpp. The final volume of assembly reactions was 100 µL. Assembly kinetics was monitored continuously for 5 min by 90° light scattering in a fluorimeter (Hitachi F-4500 FL Spectrophotometer) with both the excitation and emission wavelengths set at 350 nm and a slit width of 5 nm. Polymerization was performed in a thermostatically controlled fluorimeter cuvette at 25 °C, with temperature maintained by a circulating water bath. Disassembly was performed as previously described (51) with modifications: First, polymerization of FtsZ2 (2 µM) in polymerization buffer with or without tARC6 (2 µM) was initiated by addition of 0.1 mM GTP and the assembly was allowed to proceed at 25 °C for 5 minutes. GDP was then added to a final concentration of 3 mM to induce the disassembly of FtsZ2 filaments and light scattering was monitored for additional 5 minutes. The initial rates of assembly or disassembly were estimated from the initial linear part of the light scattering plot where correlation coefficient was higher than 0.95.

Sedimentation Assay—FtsZ2 and ARC6 proteins were pre-centrifuged at 29,700 g for 10 minutes at RT using SIGMA 3-18K Centrifuge (Sigma
Laborzentrifugen HmbH, Osterode am Harz, Germany), to remove aggregates and the supernatants were used for the assay. 2 µM FtsZ2 was polymerized in the absence or presence of 1, 2 or 3 µM tARC6 as described above and centrifuged again using the same conditions. Supernatants were transferred to clean tubes and the pellets were resuspended in the polymerization buffer (50mM HEPES, pH 7.5, 100 mM KCl and 1 mM MgCl₂) of the same volume as that of the supernatant. To five volumes of the supernatant or the resuspended pellet, one volume of the 6x sample buffer (0.375M Tris pH 6.8, 12% w/v SDS, 60% v/v glycerol, 0.6 M DTT, 0.06% w/v bromophenol blue) was added. Samples were then heated at 95°C for 10 min and 20 µL was loaded on 15% SDS-PAGE gel. Gels were stained by Coomassie Brilliant Blue G-250.

Electron Microscopy and Image Analysis of FtsZ Filaments—Assembly mixtures from light scattering assays were used for negative stain and electron microscopy. Carbon-coated grids were freshly glow-discharged and 5 µL of the reaction mixtures were adsorbed for approximately 10 seconds, washed briefly in water and negatively stained with a 2% (w/v) aqueous solution of uranyl acetate (pH 4.5). Specimens were observed in a JEOL 1200 EX transmission electron microscope operated at an acceleration voltage of 100 kV. Electron micrographs were recorded at calibrated magnifications using a 3k slow-scan CCD camera (model 15C, SIA) and processed and analyzed using ImageJ software (76).

GTPase Activity—GTPase activity was measured using BIOMOL® GREEN Reagent (Enzo Life Sciences, Farmingdale, NY) in 96-well flat bottom plates in 50 µL reactions containing 2 µM FtsZ in the polymerization buffer, at 25°C. Absorbance at 620 nm was measured using a Varioskan LUX microplate reader (ThermoFisher, Waltham, MA). Data were plotted using Microsoft Excel and corrected against a sample containing no protein. Background from the buffer and proteins was also measured and subtracted.

Constructs and Expression of Fluorescently Tagged FtsZ2 and ARC6STROMAL in S. pombe—FtsZ2-eCFP and the GTPase activity-deficient FtsZ2D322A-eCFP constructs were described in TerBush and Osteryoung (19). Stromal portion of ARC6 (ARC6STROMAL, aa 68-614) which is similar to tARC6 construct (aa 89-519) used in this study, fused with mVenus fluorescent protein, ARC6STROMAL-mVenus, and yeast transformation and selection on auxotrophic media were as described in TerBush et al. (37). Yeast strains were streaked for isolation on appropriate auxotrophic PMG medium with 15 µM thiamine to repress expression. Plates were incubated at 30-32°C for 3-5 days and individual colonies were picked and used to inoculate 3 mL of selective liquid PMG culture without thiamine to induce expression of the fusion proteins. These cultures were allowed to grow at 32°C on an orbital shaker set to 250 rpm for ~40 h to reach steady state conditions for filament assembly and turnover.

Fluorescence Microscopy and FRAP Analysis—S. pombe cells grown in liquid selective PMG media were imaged by differential interference contrast (DIC) and fluorescence microscopy, using Leica DMRA2 microscope (Leica, Wetzlar, Germany) with an HCX PL FLUOTAR 100X/1.3 oil-immersion objective and a Retiga Exi CCD camera (QImaging, Burnaby, BC, Canada). A drop of liquid culture was pipetted onto a glass poly-L-lysine coated slide and covered with a No. 1.5 coverslip. All imaging was performed at room temperature. Image of ARC6STROMAL-mVenus expressed alone (Fig. 4A) was a single image, since this protein adopted a diffuse localization. All other images were acquired as Z stacks with 0.5 µm increments. Z-stacks were subsequently de-blurred using nearest neighbor deconvolution at 70% haze removal using Image-Pro 7.0 software (Media Cybernetics). Additional image processing and analysis for epifluorescence micrographs was performed using Fiji software (77) as follows. Maximum intensity projections were pseudocolored green for ARC6 and magenta for FtsZ2 fluorescent signals. In coexpression overlays, colocalized fluorescence signals are shown in white. Colocalization of fluorescence signals in coexpressing strains was quantified by creating a composite image of the two fluorescence signals from a de-blurred Z stack, cropping the image to contain only the single cell being analyzed, unmerging the 2 channels and using the Coloc2 plugin within Fiji to calculate a Pearson’s Correlation Coefficient (PCC) (78), and
averaging all PCC values for each coexpression strain, ± SEM.

Fluorescence Recovery After Photobleaching (FRAP) experiments were performed at room temperature on a Fluoview FV1000 laser-scanning confocal microscope (Olympus America, Waltham, MA) with a Plan FLN 60X/1.42 oil-immersion objective with zoom and scan size resulting in pixel size 0.06 µm, as described in (37). Photobleaching of the region of interest (ROI) was performed for 20 ms at a laser intensity set so that one-half to two-thirds of the fluorescence signal was bleached. mVenus and eCFP fusion constructs were excited and bleached with a 515 and 458 nm laser, respectively. All time-course imaging for FRAP experiments were performed with 10 s intervals between time points. Raw FRAP data was processed to produce photobleaching-corrected and normalized recovery datasets (79). All datasets for each fluorescent fusion were averaged together to produce the average recovery dataset. Standard error of the mean (SEM) was calculated for normalized recovery at each time point. This average FRAP recovery dataset was used for curve-fitting using pro Fit software (QuantumSoft), where the data were fit to the two unbinding states equation from the FRAPAnalyser software (http://actinsim.uni.lu/eng/Downloads): 

\[ f(t) = (1 - r)(1 - c_{eq1}e^{-koff1t} - c_{eq2}e^{-koff2t}) \]

where \( koff1 \) and \( koff2 \) are the two unbinding constants for each of the two states, \( c_{eq1} \) and \( c_{eq2} \) are the fractions of bound molecules, and \( r \) accounts for the effect of non-complete recovery (80).

Single Particle Analysis and 3D Reconstruction—Specimens for TEM were prepared and negatively stained according to Valentine et al., (81) using freshly purified recombinant proteins and an aqueous solution of 2% (w/v) uranyl acetate (pH 4.2). Specimens were observed in a JEOL 1200 EX TEM operated at an accelerating voltage of 100 kV. Images were captured at a calibrated magnification using a CCD camera (model 15C, SIA, Duluth, GA) and Maxim DL imaging software, with pixel size corresponding to 0.51 nm. Suitable micrographs (absence of drift, astigmatism, etc.) were selected, and the Boxer program in EMAN 1.9 was used to hand pick tARC6 and ARC6 particles (n=4121 and 4215, respectively) from micrographs with similar defocus values. Particles were further processed by bandpass filtering to remove spatial frequencies below 11 Å and above 100 Å. Reference-free classification were performed by multivariate statistical analysis and hierarchical ascendant classification within the framework of the EMAN 1.9 software package (82). Initial models were generated by cross common lines Euler search performed on class averages and angular refinement iteration was stopped when no further improvement in the statistics was observed. Refinements were repeated using the same starting model and C1 or C2 symmetry to check for symmetry artefacts. Both C1 and C2 yielded similar models, the C2 symmetry is shown in results. Resolution was estimated by Fourier shell correlation (FSC) using the 0.5 criterion (83). 3D reconstructions were manipulated and visualized using the UCSF Chimera software package (84). FtsZ2 model was generated in the Phyre2 server (85) with Mycobacterium tuberculosis FtsZ (pdb accession code 1RLU) as a template. The model of an FtsZ2 protofilament was created manually based on known FtsZ protofilament structure from Methanocaldococcus jannaschii (pdb accession code 1W59) (86) and visualized using PyMOL (http://www.pymol.sourceforge.net). The single helix corresponding to the transmembrane domain of ARC6 (residues 619-638) was also generated in PyMOL.

Colocalization of GFP-tARC6 on FtsZ2 Polymers in vitro—FtsZ2 (4 µM) was polymerized in the presence of 4 µM GFP-tARC6 in the assembly buffer, pH 7.5, at 25 °C for 5 min. A drop of the reaction mixture was applied to a glass slide, covered with 0.17 mm coverglass and the filaments were visualized by phase-contrast and fluorescence microscopy, using Zeiss Axioptph microscope, Plan Apochromat 60x/1.4 oil immersion objective and appropriate fluorescence filter set (excitation 465-490 nm. Emission 500-550 nm). Images were captured on a Coolsnap cf (Photometrics, Tucson, AZ) CCD camera.
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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.
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Table 1. FRAP analysis of FtsZ2, FtsZ2\textsubscript{D322A} and ARC6\textsubscript{STROMAL} in single and coexpression strains.

Results from two binding states model. Parameters are: the unbinding rate constants for two binding states ($k_{off1}$,$k_{off2}$), fractions of bound molecules ($c_{eq1}$, $c_{eq2}$), parameter ($r$) accounts for the effect of non-complete recovery (80). * FRAP data from (19) reprocessed with new methods, from (68). ** FRAP data from (37) reprocessed with new methods.

| Protein                     | $r$  | $c_{eq1}$ | $c_{eq2}$ | $k_{off1}$ | $k_{off2}$ | Recovery @ 250 s | $n$ |
|-----------------------------|------|-----------|-----------|------------|------------|------------------|----|
| FtsZ2-eCFP                  | 0.21 | 0.14      | 0.87      | 0.11       | 1.2e\textsuperscript{-3} | 29%             | 11 |
| FtsZ2-eCFP + ARC6\textsubscript{STROMAL}-mVenus | 0.50 | 0.17      | 0.83      | 6.7e\textsuperscript{-2} | 3.7e\textsuperscript{-3} | 34%             | 10 |
| FtsZ2\textsubscript{D322A}-eCFP | -56.65 | 7.8e\textsuperscript{-4} | 0.99      | 2.6e\textsuperscript{-2} | 1.6e\textsuperscript{-6} | 8%              | 10 |
| FtsZ2\textsubscript{D322A}-eCFP + ARC6\textsubscript{STROMAL}-mVenus | -3.57 | 8.1e\textsuperscript{-3} | 0.99      | 0.17       | 3.3e\textsuperscript{5} | 7%              | 12 |
| FtsZ2-eCFP*                 | 0.64 | 8.8e\textsuperscript{-2} | 0.91      | 0.20       | 6.1e\textsuperscript{-3} | 29%             | 10 |
| FtsZ2\textsubscript{D322A}-eCFP** | 0.89 | 7.7e\textsuperscript{-2} | 0.92      | 0.19       | 3.3e\textsuperscript{-3} | 6%              | 10 |
| FtsZ2-eCFP + ARC6\textsubscript{STROMAL}-mVenus** | 0.65 | 0.12      | 0.88      | 0.35       | 3.8e\textsuperscript{-3} | 25%             | 11 |
| FtsZ2-eCFP**                | 0.47 | 0.85      | 0.15      | 0.21       | 5.7e\textsuperscript{-3} | 44%             | 14 |
FIGURE LEGENDS

Figure 1. Arc6 and FtsZ2 constructs, expression phenotypes and protein purification. A, ARC6 structural elements. TP: transit peptide; J-like: J-like domain, TM: transmembrane domain; PDV2:PDV2-binding domain (679-774). Constructs encode the full length mature form of ARC6 (89-801), stromal portion tARC6 (89-519), tARC6ΔJ (157-519) lacking the J-like domain, tARC6ΔC (89-310), tARC6ΔN (310-519), GFP-tagged tARC6 and mVenus-tagged stromal portion of ARC6, ARC6STROMAL. B, FtsZ2 structural elements. TP: transit peptide; CTP: conserved C-terminal peptide of FtsZ2 (463-478). Constructs encode full length mature form of FtsZ2 (49-476), FtsZ2ΔNC (118-424) lacking both the C- and N-terminal regions, and the eCFP-tagged version of FtsZ2 and of the GTPase-deficient mutant version of FtsZ2, FtsZ2D322A. C, phase contrast images of E. coli carrying the expression vectors before (left panels) and after (right panels) overnight growth at 18 °C in the presence of 0.5 mM IPTG. Scale bar corresponds to 20 µm. D, gel permeation chromatography (GPC) purification of tARC6, FtsZ2 and FtsZ2ΔNC on SEC 650 column (top row) and of ARC6 and tARC6ΔJ purification on Superdex-6 column (bottom row), showing a single peak with relative molecular mass Mr corresponding to the dimeric form of the protein. The rightmost panes show elution volume calibration on each column. Standards and their Mr: 1, thyroglobulin (670 kDa); 2, bovine γ-globulin (158 kDa); 3, chicken ovalbumin (44 kDa), 4: equine myoglobin (17 kDa).

Figure 2. tARC6 facilitates FtsZ2 assembly through interaction. A, B, light scattering assay of FtsZ2 (A) or FtsZ2ΔNC (B) in the absence/presence of tARC6. C, light scattering assay of FtsZ2 assembly in the absence/presence of tARC6ΔJ. Results from representative experiments are shown. Number of replicates is given below. D, the initial rate of assembly of FtsZ2 or FtsZ2ΔNC in the presence of equimolar amount tARC6 or tARC6ΔJ, normalized to control reactions without tARC6 or tARC6ΔJ. Error bars represent standard deviation; n=10 (FtsZ2+tARC6), n=5 (FtsZ2ΔNC+tARC6), n=6 (FtsZ2+ tARC6ΔJ). ** indicates significant difference from the control reactions without the respective ARC6 protein construct (p < 0.01, Student’s t-test). E, F, sedimentation assay FtsZ2 (E) or FtsZ2ΔNC (F) copelleting with tARC6 after assembly at FtsZ:tARC6 molar ratios as indicated. The spliced images in (E) are all from the same gel. M, Molecular mass markers; P, pellet; S, supernatant.

Figure 3. Effect of ARC6 on FtsZ2 filament morphology. A, B, FtsZ2 filament bundles in the absence (A) and presence (B) of equimolar amount of tARC6. Insets show the same samples at higher magnification. Scale bar corresponds to 1 µm and to 100 nm in the inset, respectively. C, filaments and higher order structures formed by bacterial FtsZ in the presence of SepF or FzlA. Micrographs adapted from (47) and (49). Scale bars correspond to 100 nm. D, width and E, length of FtsZ2 filament bundles in the absence or presence of tARC6.

Figure 4. Filament assembly and subunit exchange in S. pombe. A-E, Epifluorescence micrographs of A, ARC6STROMAL-mVenus, B, FtsZ2-eCFP, C, FtsZ2D322A-eCFP, D, ARC6STROMAL-mVenus coexpressed with FtsZ2-eCFP, and E, ARC6STROMAL-mVenus coexpressed with FtsZ2D322A-eCFP. Fluorescent signals from ARC6STROMAL-mVenus are pseudo colored green, while those of FtsZ2-eCFP and FtsZ2D322A-eCFP are pseudo colored magenta. The white color in merged images represents regions where the two fluorescence signals overlap and colocalize. White lines denote cell boundaries. Scale bar represents 5
μm. F, FRAP analysis of FtsZ2-eCFP and FtsZ2_{D322A}-eCFP expressed alone and with ARC6_{STROMAL}-mVenus. Graphs of the normalized fluorescence recovery vs time (s) are shown on top. The residuals of the fit shown below each recovery plot are well within 0.10 normalized recovery units (10%). Data are normalized to the pre-bleach fluorescence intensity (1 on the y-axis) and the fluorescence intensity at the time of photobleaching (0 on the y-axis). n represents the number of independent FRAP experiments performed. Error bars represent SEM at each time point.

Figure 5. Stabilization of FtsZ2 filaments by ARC6 under disassembly condition. A, light scattering assay of FtsZ2 disassembly in the absence/presence of tARC6. After 5 min assembly in the presence of GTP, disassembly was initiated by the addition of excess GDP. B, rate of disassembly in FtsZ2+tARC6 reactions relative to FtsZ2-alone reactions, from five pairwise comparisons. Error bar = S.D. ** indicates significant difference from the FtsZ2-alone control reactions (p < 0.01, Student’s t-test). C, D, electron micrograph of the FtsZ2-only (C) and FtsZ2 + tARC6 (D) reactions after disassembly. Insets show lower-magnification view of the assembled FtsZ filament bundles before disassembly. Scale bar corresponds to 100 nm and 1 μm in the insets, respectively.

Figure 6. GTPase activity of FtsZ2, effect of ARC6 and nucleotide on FtsZ2 assembly. A, GTPase activity of FtsZ2 at increasing concentration of GTP in the absence/presence of equimolar amount (2 μM) of tARC6. Assay for each GTP concentration was repeated four to seven times. * indicates significant difference from the FtsZ2-alone control reactions (p < 0.05, Student’s t-test). B, light scattering assay of FtsZ2 polymerization in the absence/presence of equimolar amount (2 μM) of tARC6 in reactions containing 0.1 mM GpCpp or 0.1 mM GpCpp + 0.2 mM GDP. Addition of GDP serves to induce slow disassembly of FtsZ. Representative plot is shown, the assay was repeated six times with similar results. C, comparison of the rates of FtsZ2 assembly in reactions containing GpCpp + GDP; Rates in FtsZ2+tARC6 reactions are expressed relative to reactions with FtsZ2 alone. n=7; ** indicates significant difference from the FtsZ2-alone control reactions (p < 0.01, Student’s t-test). Error bars = S.D.

Figure 7. Single particle analysis and 3D reconstruction of tARC6 and ARC6, model of ARC6 membrane topology and FtsZ2 protofilament binding. A, gallery of representative reference-free class averages of tARC6 (top row) and corresponding back projections from the final 3D reconstructions under similar orientations (bottom row). B, 3D reconstruction of a tARC6 dimer. C, 3D reconstruction of tARC6 dimer (pale yellow) superimposed on the full-length mature form, ARC6, dimer density map (black mesh). Crystal structures of ARC6 C-terminal domain, CTD, (magenta) (36) are fitted into the top part of ARC6 dimer density map. Two single helices corresponding to the transmembrane domain, TM, of ARC6 (black) are located between the CTDs of ARC6 and the tARC6 dimer reconstruction. Each molecule of the stroma-localized portion of ARC6 dimer is depicted interacting with the flexible C-terminus of adjacent FtsZ2 molecules in a protofilament. The C-terminus of FtsZ2 contains the ARC6-binding C-terminal peptide (CTP). IEM: Inner Envelope membrane; IMS: Intermembrane Space. Scale bar corresponds to 5 nm in A.
The Chloroplast Division Protein ARC6 Acts to Inhibit Disassembly of GDP-bound FtsZ2
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