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Report

Septin Stability and Recycling during Dynamic Structural Transitions in Cell Division and Development

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Summary

Septins are conserved proteins found in hetero-oligomeric complexes that are incorporated into distinct structures during cell division and differentiation; yeast septins Cdc3, Cdc10, Cdc11, and Cdc12 form hetero-octamers and polymerize into filaments, which form a "collar" at the mother-bud neck [1]. Posttranslational modifications, nucleotide binding, and protein-protein and protein-lipid interactions influence assembly and disassembly of septin structures [2], but whether individual septins are used repeatedly to build higher-order assemblies was not known. We used fluorescence-based pulse-chase methods to visualize the fate of pre-existing (old) and newly synthesized (new) molecules of two septins, Cdc10 and Cdc12. They were recycled through multiple mitotic divisions, and old and new molecules were incorporated indistinguishably into the collar. Likewise, old and new subunits intermixed within hetero-octamers, indicating that exchange occurs at this organizational level. Remarkably, in meiosis, Cdc10 made during vegetative growth was reutilized to build sporulation-specific structures and reused again during spore germination for budding and during subsequent mitotic divisions. Although Cdc12 also persisted during sporulation, it was excluded from septin structures and replaced by another subunit, Spr3; only new Cdc12 populated the collar of germinating spores. Thus, mechanisms governing septin incorporation are specific to each subunit and to the developmental state of the cell.

Results and Discussion

To monitor the fate of Cdc12 in time and space in live cells, we constructed a functional Cdc12-AGT chimera. The AGT tag, commercially available as the SNAP-tag, is a domain of human O^6-alkylguanine-DNA alkyltransferase that can covalently couple to benzylguanine (BG) and its derivatives [3]. Cdc12-AGT was fluorescently labeled in vivo via its reaction with fluorophore-conjugated BG-related molecules (either BG-DAF or TMR-*Star*) taken up from the culture medium. Despite a steady-state abundance similar to untagged Cdc12, as judged by immunoblotting of cell extracts (Figure S1, available online), efficient labeling of plasmid-expressed Cdc12-AGT was observed (Figure S1 and Table S1) only when the endogenous *CDC12* gene was absent and extrusion of these dyes was prevented by removal of the genes (*PDR5*, *SNQ2*, and *YOR1*) encoding three different plasma-membrane efflux pumps [4]. For pulse-chase analysis, the *cdc12*Δ *pdr5*Δ *snq2*Δ *yor1*Δ strain expressing Cdc12-AGT was incubated with a fluorescent BG derivative for 30 min (approximately one-third of a cell cycle), washed, and followed through multiple subsequent mitotic divisions in the absence of label. Fluorescent Cdc12-AGT localized to the mother-bud neck as a ring in unbudded and newly budded cells, as an hourglass-shaped collar in cells with medium-sized buds, and as a pair of split rings in large-budded cells (Figure 1 and Figure S1), patterns indistinguishable from those of Cdc12-GFP [5] (Figure S1) and consistent with localization of native Cdc12 determined by indirect immunofluorescence and electron microscopy [6]. Cdc12-AGT labeled during the 30 min pulse was still readily detectable at the bud neck after more than five cell doublings (Figure 1A) and became undetectable only after approximately eight divisions (Figure 1A). The rate of this observed decrease in the level of the fluorescent signal is fully compatible with simple dilution by newly synthesized, unlabeled Cdc12-AGT during the course of each division, rather than with active removal of the older molecules by degradation. Consistent with our findings, the "decay" rate reported for Cdc3 is also well explained by simple growth dilution (see Supplemental Discussion). Also in accord with our results, it has been reported that Cdc3, Cdc10, Cdc11, and Cdc12 molecules labeled with ^{15}N and then chased were not degraded, although these were followed for only one cell cycle [7].

Importantly, regardless of the number of generations after labeling, as long as fluorescence could be visualized, the signal was roughly equivalent in every cell in the population (data not shown; see Figure S2D), indicating that Cdc12-AGT molecules were partitioned into mother and daughter approximately equally. If, for example, old Cdc12-AGT molecules were preferentially localized to the mother side of the bud neck, as proposed by others (see Supplemental Discussion), the fluorescent signal ought to be brightest in the oldest mothers, and the buds of the newest daughters ought to display little or no signal, a pattern we did not observe. Nonetheless, to definitively determine whether old and new septin molecules are segregated within the otherwise symmetrical structures that contain them, we devised and applied a dual pulse-labeling procedure to differentially label pre-existing and newly made septin molecules. Cells expressing Cdc12-AGT were first tagged with a fluorescent BG derivative of one color, washed, chased for two divisions in the absence of label, and then pulse-labeled with an equally reactive fluorescent BG of another color. Essentially every available Cdc12-AGT molecule was fluorescently marked during exposure to the first label because, in the absence of a chase, no labeling with the second label was detectable (Figure S1B). We determined empirically that a chase of approximately two generations allowed for synthesis of a sufficient amount of new Cdc12-AGT molecules to achieve readily detectable labeling with the second dye, while retaining a readily detectable level of the Cdc12-AGT molecules labeled with the first dye. Using these conditions, we found that, regardless of whether Cdc12-AGT was labeled first with BG-DAF and later with TMR-*Star* or vice versa, the pattern of localization of old and new molecules was indistinguishable at both the center and the outer edges of the septin collar at the bud neck

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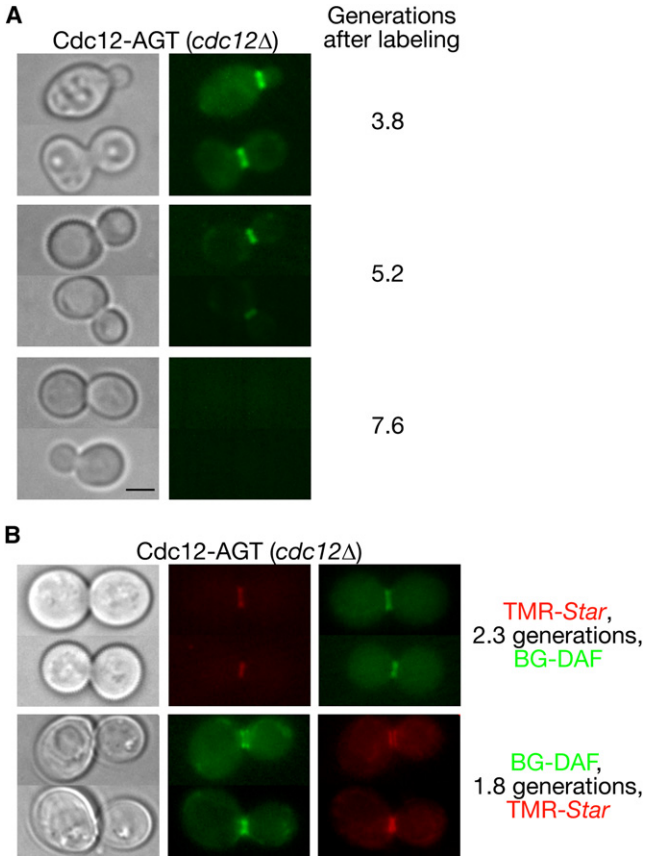

Figure 1. Cdc12-AGT Molecules Persist through Multiple Cell Divisions with Negligible Turnover and Colocalize with Newly Synthesized Cdc12-AGT

(A) Cells (*pdr5*Δ *snq2*Δ *yor1*Δ *cdc12*Δ) carrying YCp*CDC12-AGT* were exposed to 5 μM BG-DAF for 30 min at 30°C, washed thoroughly, incubated in fresh medium for 30 min at 30°C, washed again, and then resuspended in fresh medium and examined by transmitted light (left) or fluorescence microscopy (right) after undergoing the indicated number of doublings.

(B) Cells labeled with TMR-*Star* (upper panels) or BG-DAF (lower panels) and incubated for the indicated number of doublings as in (A) were labeled for 30 min with either BG-DAF (upper panels) or TMR-*Star* (lower panels) before viewing with appropriate filters. Representative cells are shown. The scale bar represents 2 μm.

(Figure 1B). Thus, at this resolution, new Cdc12-AGT and old molecules inherited from prior divisions were incorporated equivalently and isotropically.

The five septins in mitotic cells (Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7) are homologous and colocalize at the bud neck [1]. Cdc10 is the smallest and, unlike the others, lacks a C-terminal extension with a recognizable coiled-coil domain, but it is nonetheless important for the integrity and stability of higher-order septin structures in vivo and in vitro [8, 9]. To determine whether the behavior we observed for Cdc12-AGT could be generalized, we performed analogous experiments in *pdr5*Δ *snq2*Δ *yor1*Δ cells carrying at the endogenous *CDC10* locus an integrated functional *CDC10-AGT* fusion, which was expressed at a level similar to untagged Cdc10 (Figure S2A). Upon in vivo labeling with BG-DAF (not shown) or TMR-*Star* (Figure S2B), Cdc10-AGT was readily detectable at the bud neck in a pattern equivalent to that previously reported for a functional Cdc10-GFP fusion [10]. After pulse-labeling, subsequent diminution of the signal occurred with kinetics consistent with division-dependent dilution, and Cdc10-AGT were partitioned into mother and daughter in approximately equal amounts (Figure S2D), all as observed for Cdc12-AGT. Likewise, when these cells were subjected to the sequential two-color labeling regimen, old and new Cdc10-AGT molecules were incorporated equivalently into the septin collar (Figure S2C, top).

Although the majority of the Cdc10-AGT molecules were tagged with the first label (TMR-*Star*) during our standard exposure time, some labeling with the second label (BG-DAF) was detectable even in the absence of a chase (Figure S2A), suggesting that labeling of Cdc10-AGT is somewhat slower than that of Cdc12-AGT. Given that we have recently demonstrated that a Cdc10 doublet occupies the central position in every rod-like septin hetero-octamer, whereas Cdc12 occupies the penultimate position at each end of the rod [11], it is possible that in this context, the AGT domain attached to the short Cdc10 C terminus is somewhat less accessible than when attached to the extended C-terminal tail of Cdc12. However, accessibility of Cdc10-AGT and Cdc12-AGT to labeling was not dependent on the nature of the septin structure (i.e., single ring, collar, or split ring) at the time of labeling because septin structures diagnostic of every cell-cycle stage containing either Cdc10-AGT or Cdc12-AGT were labeled upon exposure of cells to the fluorescent dye for just 30 min, a period significantly shorter than a full cell cycle (~90–120 min; data not shown).

Yeast septins are phosphorylated and SUMOylated, modifications correlated with the transitions that septin-containing structures undergo during cell-cycle progression [1]. Some of these modifications and certain septin-associated proteins are found preferentially on one side or the other of the bud neck [12, 13]. Given that our pulse-chase analysis revealed that Cdc10-AGT and Cdc12-AGT are extremely stable and that pre-existing molecules inherited from a previous division are incorporated indistinguishably from the newly made molecules, the striking cell-cycle-dependent changes in the state of septin organization cannot arise from wholesale removal of old molecules and resynthesis of new septin monomers. Rather, our results demand that periodic changes in the supramolecular architecture of septin-containing structures must be driven by cell-cycle-dependent posttranslational modifications that are erased and reimposed with each cell division, as originally suggested by Dobbelaere et al., who used a different and less direct approach [14]. Likewise, observed asymmetries in the association of other bud-neck-localized proteins must be dictated by additional cues and processes aside from the septins themselves.

The nature of the tag itself or the procedure used to study the septin-AGT chimeras could influence septin stability and contribute to the uniform inheritance we observed. To rule out this possibility, we fused the same septins to different tags and devised an independent approach to monitor the persistence of old septins and the fate of newly made septins. This method, which used the *HO* promoter to drive septin expression in a haploid-, cell-cycle-, and mother-cell-specific manner (see Supplemental Data), corroborated the finding that new and old septins are incorporated simultaneously into the filaments in the septin collar at the bud neck. Thus, septins must be recruited from a common pool generated, for old septins, by recruitment from previously existing structures and, for new septins, by their de novo synthesis.

Our finding that old and new septin molecules colocalize may explain the results of fluorescence recovery after photobleaching (FRAP) experiments, which demonstrated rapid