Ca^{2+}-inducible phase separation of centrins in proliferating malaria parasites

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Abstract

Centrins are universally involved in eukaryotic cell division, but their precise mode of function remains unclear. They are small calcium-binding proteins and a conserved component of microtubule organizing centers (MTOCs) that organize the mitotic spindles. Malaria-causing parasites have a particularly divergent acentriolar MTOC, which incorporates several essential centrins. Here, we reveal calcium-inducible liquid-liquid phase separation as a principle of assembly for *Plasmodium* and human centrins. We define the disordered N-terminus and calcium-binding as essential features for reversible biomolecular condensation and show liquid-like dynamics in vivo using live-cell STED microscopy. Inducible protein overexpression revealed concentration-dependent formation of centrin assemblies with condensate-like properties. Our study thereby provides a model for centrosome assembly in malaria-causing parasites and suggests a novel mode of centrin accumulation at eukaryotic MTOCs.

Introduction

Malaria-causing parasites are divergent unicellular eukaryotes and still kill over 600.000 people annually (*1*). To proliferate in the blood of their human host they use an unconventional cell division mode called schizogony, during which multiple asynchronous rounds of nuclear divisions are followed by the budding of daughter parasites (*2*, *3*). Nuclear multiplication requires formation and duplication of the parasite MTOC, also called centriolar plaque, which significantly differs from the highly structured spindle pole body of yeast and the centriole-containing mammalian centrosome. Centriolar plaques consist of an amorphous chromatin-free intranuclear compartment, which connects through the nuclear envelope to a protein dense extranuclear compartment, where centrin localizes (*4*, *5*). Centrins have various proposed functions and are one of the most widely conserved component of eukaryotic MTOCs (*6*, *7*). In yeast, they localize to the half-bridge of spindle pole bodies, where they are required for duplication. In mammalian cells, centrins are found inside the centrioles and are implicated in the function of centrosomes (*8*, *9*). Centrins contain four EF-hand (EFh) domains that can chelate calcium with high affinity, causing conformational changes (*10*, *11*). Calcium-dependent self-interaction of centrins has been documented in a range of eukaryotes including for human centrin 2, HsCen2 (*10*, *12–15*), although the nature of this interaction remains unclear. While yeasts encode one centrin (*16*), mammals have four centrins but only HsCen2 and 3 are directly associated with the centrosome (*17*, *18*). The centrin protein family in *Plasmodium* spp. has also expanded to four members (Fig. 1A), of which three are likely essential (*19–21*). Their relocalization from the cytoplasm to the centriolar plaque upon schizogony has been suggested (*5*, *16*, *21*). Which mechanisms drive centrin accumulation in the context of divergent centrosomes is unclear.

Results

To test the localization of PfCen1-4, we episomally expressed GFP-tagged proteins in cultured *P. falciparum* blood stage parasites (Fig. S1), as parasites did not tolerate endogenous tagging (*21*). PfCen1-4 localized to the centriolar plaque (Fig. 1B) and Stimulated Emission Depletion (STED) microscopy showed heterogenous shapes of the fluorescent signal. To address whether centrin might be dynamically reorganized within this diffraction-limited region we implemented live cell STED using a parasite line expressing PfCen1 tagged with Halo (Fig. S1) and labeled with the MaP-SiR-Halo fluorogenic dye (*22*) (Fig. 1C-D). This either revealed 'wobbling' droplet-like shapes (Mov S1), or structures which seemed to stretch and retract along a defined axis (Mov S2), as the difference in aspect ratio suggest (Fig. 1C-D). Those dynamics were reminiscent of biomolecular condensates (*23*), concentrated protein droplets that form by liquid-liquid phase separation (LLPS), which has emerged as a principle explaining the formation of membrane-less organelles, including centrosomes (*24*).

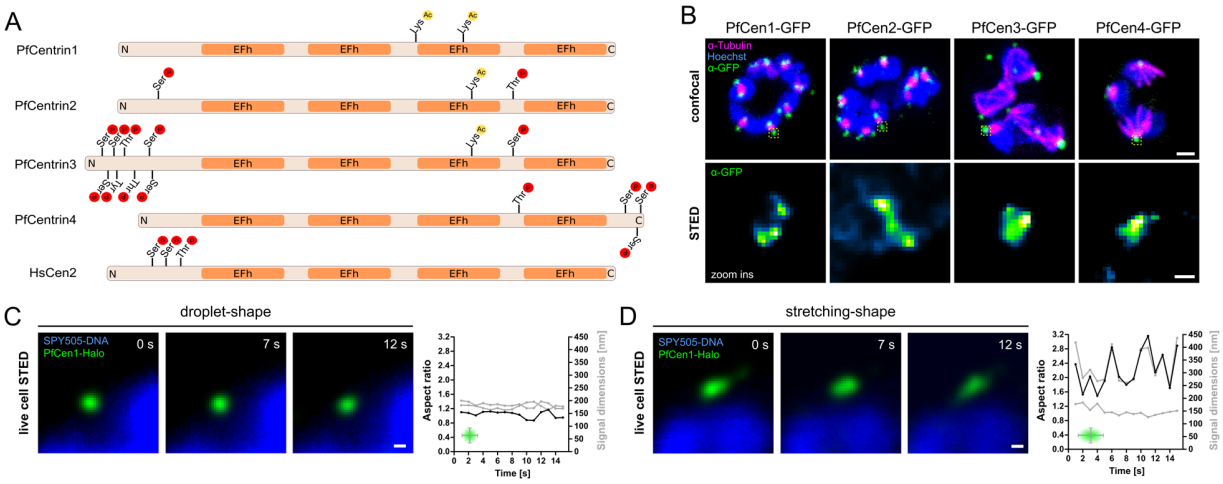

Fig. 1. PfCen1-4 localize to centriolar plaque and can display liquid-like dynamics. (**A**) Schematic of PfCen1-4 and HsCen2 indicating reported post-translational modifications and EFh domains. (**B**) Immunofluorescence staining of tubulin and PfCen1-4-GFP in parasite strains. DNA stained with Hoechst. Maximum intensity projections (MIP). Scale bars; confocal, 1 µm, STED, 100 nm. (**C-D**) STED time lapse of centriolar plaque region of parasites expressing PfCen1-Halo labeled with MaP-SiR-Halo dye. DNA stained with SPY505-DNA. Scale bar, 100nm. Quantification of ratio (black line) between height and width (grey lines) of the PfCen1 signal.

To test whether centrins can phase-separate, we expressed PfCen1-4 and HsCen2 in *E. coli* (Fig. S2) and imaged concentrated recombinant protein solutions. After addition of calcium, we observed rapid droplet formation for PfCen1, PfCen3, and HsCen2 (Fig. 2A, Mov S3-5). The droplets fused and showed surface wetting, both hallmarks of LLPS (*25*). Addition of EDTA led to instant dissolution of the droplets except for PfCen3 where some non-coalescing droplets remained (Fig. S3, Mov S6). To quantify centrin phase separation kinetics, we measured the turbidity of the solutions by light scattering (Fig. 2B). Upon calcium addition we observed a rapid increase in turbidity that was caused by protein droplet formation (Fig. S4A) in PfCen1, PfCen3, and HsCen2 solutions (Fig. 2B). Addition of Mg^{2+} as an alternative bivalent cation demonstrated that the effect was calcium-specific (Fig. S4B). GFP-tagging of PfCen1 did not disrupt LLPS (Fig. S4C). Since PfCen3 condensation was not completely reversible, we added EDTA at earlier timepoints and found that the irreversible fraction increased over time (Fig. S5A), suggesting a maturation of the biomolecular condensate towards a more solid or gel-like state (*26*). In contrast, maturation in PfCen1 condensates was not observed within the first 3 hours after induction (Fig. S5B).

A common feature of phase-separating proteins are intrinsically disordered regions (IDRs) (*23*). Indeed, IUPred3 showed high IDR probability values within the N-termini of PfCen1, PfCen3, and HsCen2, but not for PfCen2 and PfCen4 (Fig. S6A). For centrins from several distant eukaryotes 'polymer-like' behavior was previously noted (*12–14*). Analysis for IDRs in centrins from a range of eukaryotes suggests that most of those species have at least one IDR-containing centrin (Fig. S6B). We therefore tested LLPS after deleting the IDR-containing N-terminus from PfCen1. This strongly reduced LLPS and we merely detected some aggregation (Fig. 2C). LLPS was not rescued by replacement with the IDR-free N-terminus of PfCen4, neither was the N-terminus of PfCen1 sufficient to confer LLPS to PfCen4. To test the role of EFh domains we mutated four critical aspartate residues in the Ca^{2+}-binding pocket, which abolished LLPS (Fig. 2C). This suggests that active EFh-domains and a disordered N-terminus are essential but not sufficient for calcium-induced phase separation of centrins.

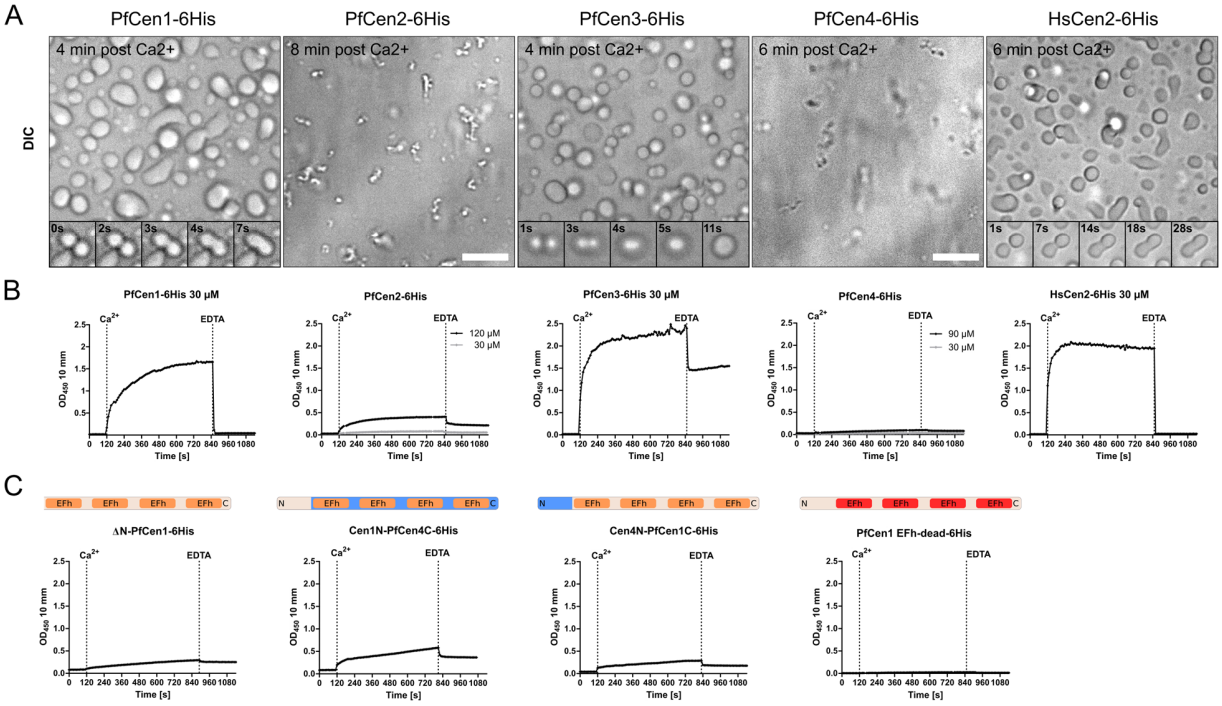

Fig. 2. Centrins require disordered N-terminus and calcium-binding for LLPS. (**A**) Widefield image of concentrated recombinant centrin solutions after calcium addition. Scale bars, 10 µm. Inlays show time lapse images of droplet fusion events. (**B**) Turbidity measurements in recombinant centrin solutions at 30 µM (and higher concentration where indicated) during addition of calcium followed by EDTA. (**C**) Turbidity measurement of various PfCen1 mutants at 30 µM.

Since phase separation is concentration-dependent we aimed to increase centrin levels to test whether we can induce additional formation of condensates in parasites. To exert better control over the expression levels of PfCen1-GFP, than with the classical overexpression vector (Fig. S1, S7A), we designed a novel *P. Falciparum* Inducible Overexpression (pFIO) plasmid to be transfected in a DiCre recombinase expressing acceptor strain (Fig. 3A). Upon addition of rapamycin the dimerized recombinase excises the first open reading frame placing the gene of interest in front of the active promoter. We designed a version with a medium strength, *hsp70* promoter fragment (pFIO) and a stronger, *hsp86* promoter (pFIO+) and confirmed a strong increase in median expression levels upon induction (Fig. S7B). In centrin-overexpressing cells we frequently observed accumulations of PfCen1-GFP signal that were not associated

with any spindle structure or nucleus (Fig. 3B). Those Extra-Centrosomal Centrin Accumulations (ECCAs) occurred in 48% (n=54) of cells carrying the medium strength promoter and in 97% (n=60) of cells with the strong promoter. The number of ECCAs correlated positively with total cell fluorescence intensity (Fig. S8). ECCAs and centrosomal centrin foci were negative for the protein aggregate stain Proteostat, further supporting the notion that they are biomolecular condensates (Fig. 3C) (*27*). Time-lapse microscopy of PfCen1-GFP showed a homogenous cytoplasmic distribution prior to assembly into ECCAs or MTOC foci (Mov S7). PfCen1-GFP coalescence into ECCAs and at the centriolar plaque occurred prematurely and timing was concentration dependent (Fig. 3D) (*5*). The increase in intracellular calcium associated with schizogony could promote timely centrin accumulation at the MTOC (*28*). ECCAs often formed at the centriolar plaque before detaching, which further suggests an involvement of schizogony-specific nucleation factors (Mov S8). Centrin accumulation by biomolecular condensation predicts that once initiated, additional centrin would only accrue in the condensate fraction. Machine-learning-based image analysis indeed showed that PfCen1-GFP concentration in the cytoplasm remained stable, while the fraction of PfCen1-GFP in foci increased (Fig. 3E). The partition coefficient between cytoplasmic and foci fraction was stable around 4.0 until it slightly increased to 5.3 after spindle formation (Fig. S9). At the end of schizogony centrin foci are dissolved into the cytoplasm (Fig. 3E). Taken together these observations argue for a regulated phase separation as the mechanism of centrin assembly. To test the significance of the IDR *in vivo* (Fig. 2B, C), we overexpressed the N-terminal deletion mutant, observing ECCAs in only 31% (n=55) of cells (Fig. 3F). The EFh-dead PfCen1 mutant (Fig. 2C), lacked any discernible formation of foci in vivo (Fig. 3G), suggesting a critical role for Ca^{2+} responsiveness for targeting and condensation. Remarkably, HsCen2-GFP expressed in *P. falciparum* also accumulated at the centriolar plaque, indicating that they might co-condensate with the endogenous centrins through an evolutionarily conserved mechanism (Fig. 3H).

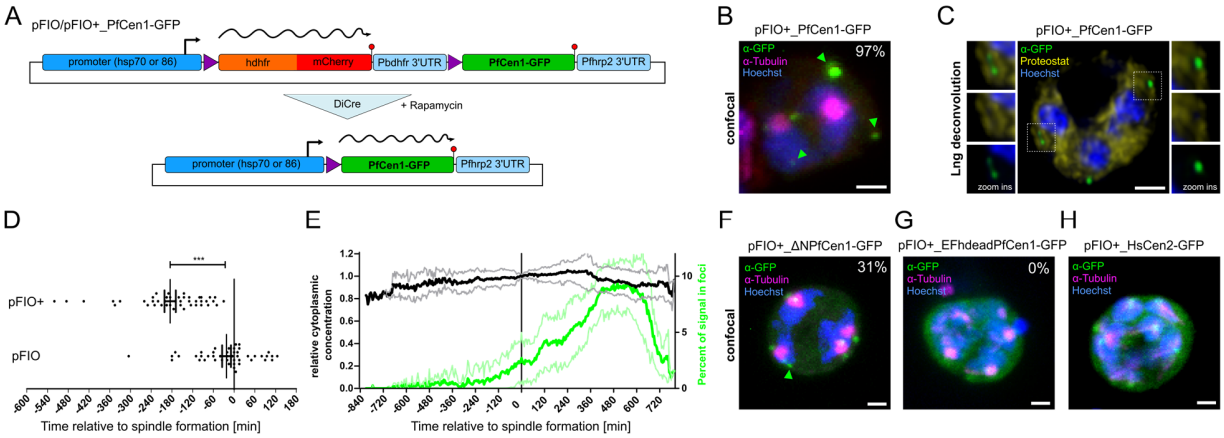

Fig. 3. PfCen1 displays condensate-like properties in cells. (**A**) Schematic of pFIO plasmid during DiCre-dependent recombination. (**B**) Immunofluorescence staining of tubulin and GFP in parasites overexpressing PfCen1-GFP (pFIO+). Percentage of cells containing ECCAs (arrows) indicated. (**C**) Proteostat and PfCen1-GFP live cell staining. (**D**) Quantification of timepoint of PfCen1-GFP foci appearance relative to mitotic spindle formation detected by SPY555-tubulin in movies with pFIO/pFIO+. (**E**) Normalized mean cytoplasmic PfCen1-GFP fluorescence intensity over time and share of PfCen1-GFP fluorescence signal contained within foci with standard deviation (n = 33) (**F**) as for (B) in cells overexpressing PfCen1-GFP lacking N-terminus. (**G**) EFh-dead mutant. (**H**) HsCen2-GFP. All images are MIP. DNA stained by Hoechst. Scale bars; 1 µm.

Conclusion

Centrins are conserved in all analyzed eukaryotes and implicated in a wide range of processes, which require their assembly (*10*). Our study in a highly divergent parasite reveals calcium-regulated phase separation as a new centrin assembly principle. Phase condensation can further explain how the centriolar plaque remains 'fluid' enough to allow splitting during subsequent nuclear division cycles (*5*, *29*). Biomolecular condensation and maturation have been ascribed to various centrosomal proteins (*24*), but remains controversial as the dominant principle for biogenesis of membraneless organelles (*30*). Our study suggests a combination of cell cycle-regulated nucleation with LLPS as a driver for the accumulation of centrosomal proteins.

Materials and Methods

Materials and methods section including movie links can be found in the supplemental information file.

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Authors declare that they have no competing interests.

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All data are available in the main text or the supplementary information.