

The SAS-5 N-terminal domain is a tetramer, with implications for centriole assembly in *C. elegans*

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The centriole is a conserved microtubule-based organelle essential for both centrosome formation and cilium biogenesis. It has a unique 9-fold symmetry and its assembly is governed by at least five component proteins (SPD-2, ZYG-1, SAS-5, SAS-6 and SAS-4), which are recruited in a hierarchical order. Recently published structural studies of the SAS-6 N-terminal domain have greatly advanced our understanding of the mechanisms of centriole assembly. However, it remains unclear how the weak interaction between the SAS-6 N-terminal head groups could drive the assembly of a closed ring-like structure, and what determines the stacking of multiple rings on top one another in centriole duplication. We recently reported that SAS-5 binds specifically to a very narrow region of the SAS-6 central coiled coil through its C-terminal domain (CTD, residues 391–404). Here, we further demonstrate by both static light scattering and small angle X-ray scattering that the SAS-5 N-terminal domain (NTD, residues 1–260) forms a tetramer. Specifically, we found that the tetramer is formed by SAS-5 residues 82–260, whereas residues 1–81 are intrinsically disordered. Taking these results together, we propose a working model for SAS-5-mediated assembly of the multi-layered central tube structure.

Introduction

Centrioles are the fundamental units for both centrosome formation and cilium biogenesis. *Caenorhabditis elegans* has become an important model organism in studying centrosome duplication and centriole assembly because of its experimental tractability and the availability of powerful genetic and genomic tools. Five proteins have been implicated in centriole biogenesis following work in *C. elegans*, namely ZYG-1,¹ SPD-2,^{2,3} SAS-4,^{4,5} SAS-5^{6,7} and SAS-6.^{7,8} Their structural and functional orthologs have also been identified in other species.^{9–16} All five of these proteins localize to centrioles and are required for centriole duplication. Depletion of any of them blocks centriole assembly.

Two research groups independently discovered the recruitment hierarchy of the five centriolar proteins during centriole duplication.^{17,18} Furthermore, it was reported previously that SAS-5 and SAS-6 physically interact with each other and are co-dependent for their centriolar localizations; disruption of their interaction leads to failure of centriole assembly.¹⁰ SAS-6 consists of a globular N-terminal head group, a central coiled coil and a flexible C-terminal tail. Recently reported crystal structures of the N-terminal head group of SAS-6 from various species suggest that nine SAS-6 coiled-coil-mediated homodimers can assemble through the interactions between neighboring pairs of head groups ($K_d \sim 60\text{--}100 \mu\text{M}$) into a ring-like structure reminiscent of the cartwheel-like central hub in mature centrioles.^{19,20} More

recently, cryoelectron tomographic studies of the exceptionally long basal body (derived from the mother centriole) of the flagellate *Trichonympha* revealed that the cartwheel structure is formed by a stack of rings, in which each ring contains nine SAS-6 dimers.²¹ However, it is not clear whether and how the relatively weak head-head interaction could faithfully drive the formation of the strict 9-fold symmetry of centrioles.^{22,23}

In contrast to SAS-6, SAS-5 has no detectable motifs. We have recently determined that the association between SAS-5 and SAS-6 is mediated by a specific interaction between the SAS-5 C-terminal domain (CTD, residues 391–404) and a narrow region of the SAS-6 central coiled coil.²³ To further investigate the function of SAS-5 in centriole assembly, we have cloned the SAS-5 N-terminal domain (NTD, residues 1–260) that is predicted to form a globular structure. We found that the N-terminal 81 residues of SAS-5 are intrinsically disordered, whereas the C-terminal part of the NTD, residues 82–260, forms a tetramer. Based on this data, we propose that SAS-5 plays a structural role in centriole assembly by connecting four SAS-6 homodimers in two neighboring rings of the central tube of the procentriole.

Results and Discussion

The N-terminal domain of SAS-5 is composed mostly of helical structures. We recently reported that SAS-5 binds specifically to the central region of the SAS-6 coiled coil via its CTD.²³

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We performed further bioinformatic analysis of SAS-5 to find out its overall domain arrangement and structure compositions. GlobPlot V2.3,²⁴ which was employed to predict intrinsic disorder and globularity of SAS-5, suggested that the N-terminal region of SAS-5 (residues 1–260) forms a folded structure whereas the C-terminal one third of the protein (residues 261–404) is mostly disordered (Fig. 1A). Secondary structure predictions by PSIPRED V3.3²⁵ suggested that the SAS-5 NTD is composed mostly of helical structures, with residues 1–81 less ordered than the rest in this domain (Fig. 1B). Primary sequence alignment of SAS-5 from three *Caenorhabditis* species also demonstrated that the N-terminal 81 residues are less conserved than the central and the C-terminal regions (Fig. 1C). To investigate the structure of SAS-5, we cloned the SAS-5 NTD into an expression vector that adds a cleavable maltose binding protein (MBP) tag plus a 10 × His tag to the N terminus of the protein (Fig. 1D). The SAS-5 NTD was eluted at 57 ml on the Superdex-200 16/60 column (GE Healthcare) after removal of the tag and was relatively pure (Fig. 1E and F). We then subjected the purified protein to circular dichroism (CD) experiments to measure the secondary structures and to measure the melting temperature (*T*_m) of the SAS-5 NTD. Consistent with the secondary structural predictions, we found that the protein is predominantly α-helical, with a far-UV CD spectrum of the protein displaying two characteristic negative peaks at 209 nm and 223 nm (Fig. 1G). *T*_m was measured to be ~50°C (Fig. 1H), indicating that the SAS-5 NTD indeed forms a folded domain.

The SAS-5 N-terminal domain forms a tetramer. Since the elution volume of a protein in gel filtration chromatography is determined not only by the molecular weight (M.W.) but also by the overall shape of the molecule, it is not advisable to evaluate the oligomeric state of a protein using gel filtration chromatography. We performed static light scattering (SLS) experiments to assess the oligomeric state of SAS-5. The result suggested that the SAS-5 NTD has an M.W. of 120–160 kDa (Fig. 1I), and could be either a tetramer (31 × 4 = 124 kDa) or a pentamer (31 × 5 = 155 kDa). To further verify the oligomeric state of the SAS-5 NTD, we recloned residues 1–260 of SAS-5 into KiM2a, a pMAL-derived vector that adds an MBP tag to the N terminus of the target protein (Fig. 2A). To minimize the flexibility of the linker, we used only two alanines (AA) to connect the tag and SAS-5. The fusion protein was overexpressed in *Escherichia coli* strain BL21(DE3) and pure protein eluted from an amylose column was applied onto a Sephacryl S-400 16/60 column (GE Healthcare). The MBP-AA-SAS5-NTD was eluted at 70.4 ml with a symmetric elution peak and the protein seemed of high purity on an SDS-PAGE gel (Fig. 2B). SLS experiments showed that it has an M.W. of 290 ± 10 kDa, suggesting that the protein forms a tetramer (Fig. 2C).

To assess the overall conformation of the SAS-5 tetramer, we performed further small angle X-ray scattering (SAXS) experiments using the MBP-AA-SAS5-NTD purified after Sephacryl S-400 16/60 chromatography. The ab initio model showed a radiated structure with a body and four arms, which confirmed the tetrameric form of the SAS-5 NTD (Fig. 2D). Both GlobPlot V2.3²⁴ and PSIPRED V3.3²⁵ predicted an intrinsically disordered

stretch at the N-terminus of SAS-5 (Fig. 1A and B). This N-terminal region is consistently less conserved than the rest of the protein (Fig. 1C). To verify this prediction, we cloned residues 1–81 of SAS-5 into pET29a (Novagen) with a C-terminal 6 × His tag. SLS results indicated that the protein forms a monomer in solution (Fig. 2E). CD experiments confirmed that it contains mostly random coils (Fig. 2F). The ab initio model of the residues 1–81 of SAS-5 by SAXS further demonstrated that it forms an extended structure of 10–15 nm in length (Fig. 2G). Conversely, another construct with residues 82–260 fused to the MBP tag still forms a tetramer as verified by CD experiments (Fig. 2H). Therefore, the four elongated “arms” in the ab initio model of the MBP-AA-SAS5-NTD are the N termini of the four SAS-5 molecules, whereas the “body” represents the tetramer formed by residues 82–260 of SAS-5 (Fig. 2D). At the tip of each “arm” was a spherical structure which is the globular MBP tag (Fig. 2D).

What structural roles could SAS-5 play in *C. elegans* centriole assembly? We recently reported that the association of SAS-5 and SAS-6 is mediated by a robust interaction (*K*_d = 3 μM) between the SAS-5 CTD and a central segment in the SAS-6 coiled coil (Fig. 3A).²³ It was also shown recently that the *Trichonympha* basal body, which is derived from the mother centriole, contains a stack of rings formed by SAS-6.²¹ Putting these observations together in the context of centriole assembly, we wish to propose a hypothetical working model for how SAS-5 may connect neighboring rings in the stacking assembly of the central tube (Fig. 3B). In this model, each SAS-5 anchors its CTD onto the SAS-6 coiled coil, and the SAS-5 NTDs form a tetramer that bridges the four SAS-6 homodimers bound to the SAS-5 CTDs. Two of the four SAS-6 dimers belong to one ring (layer N) and the other two to the neighboring ring (layer N+1). In this way, the multi-layered cartwheel assembly mediated by SAS-5 would strengthen interactions both within the same ring and across neighboring rings formed by SAS-6 (Fig. 3B).

Notably, the N-terminal flexible part of SAS-5 (residues 1–81), which is 10–15 nm as demonstrated by the SAXS experiments (Fig. 2G), would be freely movable in our assembly model. This would allow the SAS-5 N terminus to reach the head group of SAS-6. An important test of this model is whether the SAS-5 N-terminal part directly interacts with the SAS-6 head groups or it has other uncharacterized functions in centriole duplication. This will be investigated in future work.

Materials and Methods

Cloning, protein expression and purification. Sequences encoding SAS-5 residues 1–260, 1–81 and 82–260 were all amplified by PCR from *C. elegans* cDNA and cloned into pET29a (Novagen), MalpET and/or KiM2a. MalpET and KiM2a are both pMAL derived vectors with the former adding both MBP and 10 × His tags (cleavable by TEV protease) and the latter adding only MBP tag plus a two-Ala linker to the N terminus of SAS-5. All recombinant proteins were expressed in *E. coli* BL21 (DE3) cells. Cells were grown at 37°C. At an OD₆₀₀ of 0.6–0.8, cultures were

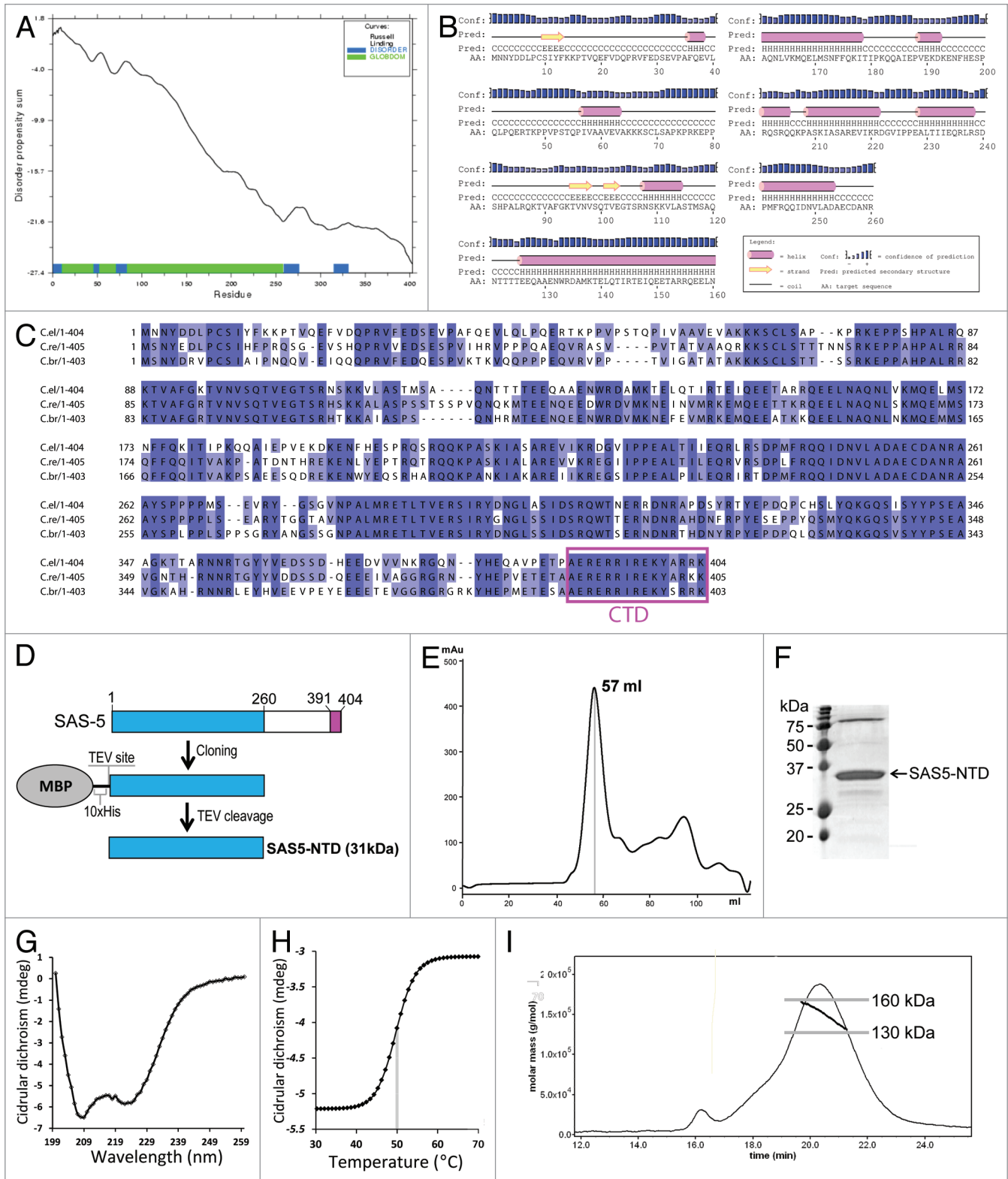


Figure 1. For figure legend, see page 4.

cold shocked on ice for 10–15 min and then shifted to 18°C. Protein induction was done overnight with 0.5 mM of isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma Aldrich). Cells were

harvested and resuspended in cold lysis buffer containing 20 mM TRIS-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole and 5% (v/v) glycerol.

Figure 1. Bioinformatic analyses and biophysical characterizations of SAS-5. **(A)** Disorder-globularity prediction plot of SAS-5 generated by Globplot-2 v2.3 using default setups.²⁴ Residues 1–260 were predicted to form a folded domain (green blocks), whereas residues 261–404 are mostly disordered. **(B)** Secondary structure prediction of SAS-5 residues 1–260 by PSIPRED v3.3.²⁵ This region contains mostly α helices with residues ~1–90 being less ordered than the rest. **(C)** Primary sequence alignment of SAS-5 from three *Caenorhabditis* species. *C.el*, *Caenorhabditis elegans*; *C.re*, *C. remanei*; *C.br*, *C. briggsae*. Boxed is the CTD that binds specifically to the SAS-6 coiled coil.²³ **(D)** Domain arrangement and expression construct of SAS-5. The SAS-5 NTD was cloned into MalpET for overexpression in bacteria. Fusion protein purified by Ni-HiTrap was incubated with TEV protease to remove the MBP-10 \times His tag. **(E)** Elution profile of the SAS-5 NTD on the Superdex-200 16/60 gel filtration column (GE Healthcare). Target protein was eluted at ~57 ml. **(F)** Purified SAS-5 NTD on a 12% (v/v) SDS-PAGE. **(G)** A far-UV CD spectrum of purified SAS-5 NTD suggested the protein is predominantly helical. **(H)** Melting temperature of the SAS-5 NTD was determined to be 50°C by CD at the wavelength of 209 nm. **(I)** The SLS result of purified SAS-5 NTD suggested it forms either a tetramer or a pentamer.

Cells were broken using an EmulsiFlex-C3 homogenizer (Avestin) and cell lysate was cleared by centrifugation at 30,000 \times g for 30 min. The supernatant was filtered (0.4- μ m) and loaded onto a gravity amylose column (for MBP-AA-SAS-5 aa1-260/aa82-260) or a Ni-HiTrap column from GE Healthcare (for MBP-10 \times His-SAS-5 aa1-260 and SAS-5 aa1-81_6 \times His) pre-equilibrated in the same lysis buffer. The amylose column was washed with 10 \times column volume (cv) of buffer containing 20 mM HEPES-NaOH (pH 7.2), 300 mM NaCl, 15 mM β -mercaptoethanol and 25% (v/v) glycerol; bound protein was eluted using the same buffer containing 50 mM maltose. Eluted protein was concentrated using centrifugal filters (Amicon, MWCO 10-kDa) and loaded onto a Sephacryl S-400 16/60 column (GE Healthcare) pre-equilibrated with 20 mM TRIS-HCl (pH 8.0), 100 mM NaCl and 5% (v/v) glycerol. Fractions containing target protein were pooled for SLS and SAXS experiments (Fig. 2B). For MBP-10 \times His-SAS-5 and SAS-5 aa1-81_6 \times His, bound proteins on the Ni-HiTrap column were eluted using the lysis buffer containing a linear gradient concentration of imidazole (20–500 mM, 20 \times cv). The N-terminal MBP-10 \times His tag in MBP-10 \times His-SAS-5 was removed by incubating purified protein with 1% (w/w) of TEV protease while dialyzing against 2–4 L of the same lysis buffer overnight at 4°C. The dialyzed protein sample was passed through a Ni-HiTrap column to remove both released MBP-10 \times His tag and uncut fusion protein. Flow-through was concentrated and further purified using a Superdex-200 16/60 column (GE Healthcare) pre-equilibrated with 20 mM TRIS-HCl (pH 8.0), 100 mM NaCl and 5% (v/v) glycerol. Fractions containing the SAS-5 NTD (elution volume ~57 ml) were pooled and concentrated to 5–10 mg/ml for later experiments (Fig. 2F).

Static light scattering (SLS). The SLS studies were performed on an Agilent 1260 Infinity LC System (Agilent Technologies). The liquid chromatography equipment consists of an HPLC system (Agilent Technologies) connected in series with a triple-angle laser light scattering detector (miniDAWN TREOS), a UV detector at 280 nm (Agilent technologies) and a refractive index detector (RI-101, Shodex). Fifty to 100 μ l of protein samples (~1–5 mg/ml) were eluted from a Superdex-200 10/300 GL column (GE Healthcare) at a flow rate of 0.5 ml per min.

Data analysis was performed using the Astra software (Wyatt technology).

Circular dichroism (CD). CD measurements were performed on a Chirascan™-plus unit (AppliedPhotophysics) with a 0.5-mm pathlength cuvette. Protein samples of 0.2 mg/ml were used in both wavelength scan and melting temperature measurement (λ = 209 nm). Data points were exported and curves were generated using Windows Excel-2013 (Microsoft).

Small angle X-ray scattering (SAXS). SAXS data for the MBP-tagged SAS-5 NTD and the SAS-5 aa1-81_6 \times His were collected following standard procedures on the BM29 BioSAXS beamline of the ESRF at Grenoble, France using a robotic sample changer and a Pilatus 1M detector. The scattering patterns were measured at the solute concentrations of 1, 4 and 8 mg/ml using a sample detector distance of 2.841 m and a wavelength of λ = 1.55 Å. To monitor for radiation damage, ten one-second exposures were collected for each protein sample and no radiation effect was observed. The data were normalized to the intensity of the transmitted beam, and the scattering of the buffer was subtracted. Data collection, processing and analysis are performed automatically using the beamline software BsxCuBE with help of the beamline scientist Dr. Adam Round.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author's Contributions

ES, RQ and GD designed the experiments and performed data analyses. ES, RQ and JL performed experiments. GD supervised the project and wrote the manuscript.

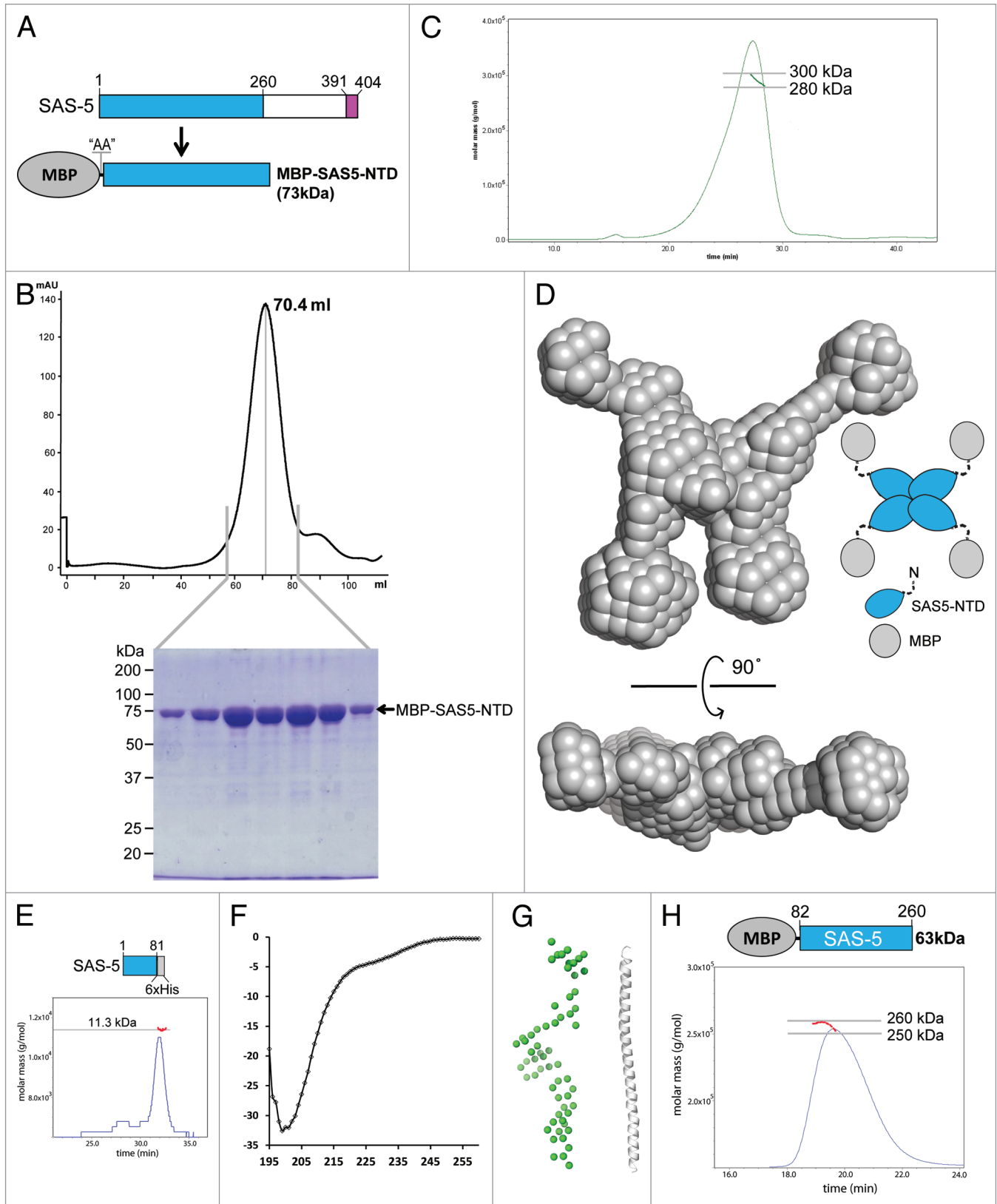


Figure 2. For figure legend, see page 6.

Figure 2. The SAS-5 NTD forms a tetramer with residues 1–81 being intrinsically disordered. (A) Expression construct of the SAS-5 NTD fused to MBP with a two-Ala linker. (B) Elution profile of the MBP-tagged SAS-5 NTD on the Sephacryl S-400 16/60 gel filtration column (GE Healthcare); fractions were checked on a 12% (v/v) SDS-PAGE gel. (C) An SLS profile of purified MBP-tagged SAS-5 NTD suggested a tetrameric form of the protein ($73 \times 4 = 292$ kDa). (D) Two different views of the ab initio low resolution shape reconstructed by GASBOR. A hypothetical model of the complex (right) showed that the SAS-5 NTD forms a tetramer with the N-terminal stretch of residues being flexible as predicted in Figure 1A and B. The blobs at the tips of the four arms are the MBP moieties. (E) An SLS profile of purified SAS-5 residues 1–81 suggested it forms a monomer in solution. (F) A far-UV CD spectrum of purified SAS-5 residues 1–81 suggested the protein contains mostly random coils. (G) Green beads show the ab initio low resolution shape of SAS-5 residues 1–81 reconstructed by GASBOR. For comparison, a 70-residue α helix, which is ~ 10 nm long, is shown on the right. (H) An SLS profile of the MBP-tagged SAS-5 residues 82–260 suggested this part of the protein forms a tetramer ($63 \times 4 = 252$ kDa).

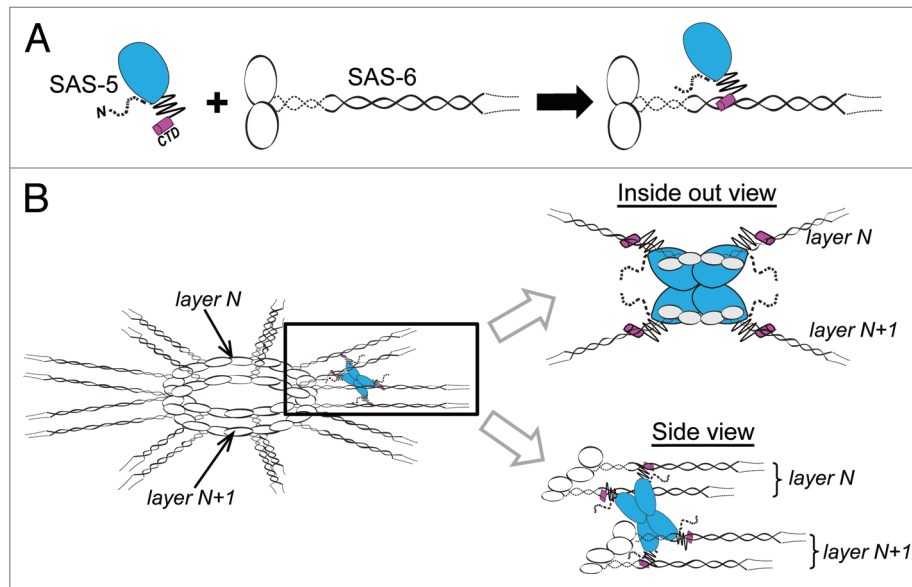


Figure 3. A hypothetical model of how the tetrameric SAS-5 NTD might strengthen the assembly of the central hub in centriole assembly. (A) Schematic drawing of the SAS-5/SAS-6 complex reported previously.²³ (B) A working model showing how the tetrameric SAS-5 complex could connect four SAS-6 dimers in two neighboring rings during central hub assembly.

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