# Filament structure and subcellular organization of the bacterial intermediate filament-like protein crescentin

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## ABSTRACT

The coiled coil protein crescentin is required for the crescent shape of the freshwater bacterium *Caulobacter crescentus* (*vibrioides*). Crescentin forms a filamentous structure on the inner, concave side of the curved cells. It has been proposed that crescentin is a homologue of eukaryotic intermediate filament (IF) proteins based on its ability to form filaments *in vitro*, the protein's length, sequence comparisons and the presence of a coiled coil discontinuity called the "stutter". Here, we have used electron cryomicroscopy (cryo-EM) to determine the structure of the full-length protein and its filament, exploiting a crescentin-specific nanobody. The filament is formed by two strands, related by two-fold symmetry that each consist of two dimers, resulting in an octameric assembly. Crescentin subunits form longitudinal contacts head-to-head and tail-to-tail, making the entire filament non-polar. Using *in vivo* site-directed cysteine crosslinking we demonstrated that contacts observed in the *in vitro* filament structure exist in cells. Electron cryotomography (cryo-ET) of cells expressing crescentin showed filaments on the concave side of the curved cells, close to the inner membrane, where they form a band. Comparison of our crescentin filament structure with current models of IF proteins and their filaments revealed similar coiled coil dimer formation as well as an absence of overall polarity. However, IF proteins form head-to-tail longitudinal contacts in contrast to crescentin and hence several inter-dimer contacts in IFs have no equivalents in crescentin filaments. We conclude that crescentin filaments are IF-like.


## SIGNIFICANCE STATEMENT

Crescentin is a coiled coil protein that is required for the crescent cell shape of bacteria such as *Caulobacter crescentus*. Crescentin has been proposed to be a member of the intermediate filament (IF) family of proteins, which form the third major class of cytoskeletal proteins in eukaryotes. To uncover the evolutionary relationship between crescentin and IF proteins, and the filaments they form, we have determined the three-dimensional structure of crescentin filaments by cryo-EM. This revealed the full-length structure of the parallel coiled coil dimer of crescentin and how dimers come together laterally and longitudinally, to form a non-polar, octameric filament. Comparing crescentin to IF proteins and their filaments revealed significant differences, especially in the longitudinal contacts, prompting its IF-like classification.

## INTRODUCTION

Fitness of bacteria in the environment is often dependent on their shape. It is therefore that most bacteria tightly control their shape, producing spheres (cocci), rods (bacilli), helices, filaments and irregular, appended as well as crescent shapes [1]. In many cases, shape is dependent on the stress-bearing cell wall. Hence shape determination is dependent on the ability to regulate cell wall synthesis and remodelling during vegetative growth, as well as during cell division [2].

*Caulobacter crescentus* (*vibrioides*) is crescent shaped. The Gram-negative freshwater bacterium has been studied extensively, and not only because its cells undergo a developmental transition from swarmer to stalked cells. The larger stalked cells are able to attach to surfaces via the stalk, while the swarmer cells are flagellated and motile. Only stalked cells replicate DNA and divide. Their cell division is asymmetric and leads to one swarmer cell and one stalked cell, closing the cell cycle [3]. Both cell types are crescent-shaped. It is thought that the crescent shape is advantageous for cell mobility in their aqueous environment, but it has recently been suggested that the crescent shape enables pili on the swarmer cells to attach to surfaces for biofilm formation [4].

Whatever the biological reason for its crescent shape, in *Caulobacter* it is dependent on the function of the protein crescentin (CreS) [5]. Immunofluorescence, functional fluorescent tagging, and sparse labelling with small amounts of additional (non-functional) CreS-GFP fusions showed crescentin to form a single filamentous structure on the inner (concave) surface of the cell crescent [5-7]. The proximity of the crescentin structure to the cell membrane was lost during treatment with the MreB inhibitor A22 or the cell wall inhibitors mecillinam and phosphomycin [6]. These and other experiments, including the discovery of a possible link to an organiser of vegetative cell wall synthesis, MreB, led to the proposal that crescentin filaments reduce cell wall synthesis on one side of the cell, leading to the crescent shape [6, 7]. Surprisingly, the heterologous expression of crescentin in *E. coli* leads to curved cells, suggesting a conserved or very simple mechanism of cell wall synthesis inhibition [6].

Crescentin levels in cells do not vary much during the cell cycle [7]. Crescentin filaments in cells are not dynamic and free molecules are added to the filamentous cellular structure rapidly, indicating strong cooperativity of assembly [7, 8]. Initial assembly starts with a cell-long thin structure that thickens as the cell progresses through the cell cycle. Re-arrangements of the crescentin structure occur during cell division, although it is not known how those are regulated or facilitated [7].

The discovery of crescentin as a regulator of cell shape attracted a lot of attention also because of a number of similarities to eukaryotic intermediate filament proteins (IFs) [5, 9]. IFs are the third major class of intracellular filaments in eukaryotes, in addition to microfilaments (actin) and microtubules (tubulin). IFs can be categorized into five major families and include two types of cytoplasmic keratins, vimentin and neurofilament, as well as nuclear lamins [10]. IFs have a well-

defined domain architecture, with a central α-helical “rod” domain driving the formation of parallel coiled coil dimers. The rod domain, which has been sub-divided into three coiled coil segments (coil 1A, 1B and 2) and two inter-connecting linkers (L1 and L12), is complemented primarily by disordered head and tail domains (nuclear lamins in contrast have folded Ig-fold tail domains). Cytoplasmic IFs have rod domains of about 308 residues, nuclear lamins of about 350 residues. All IF sequences have a “stutter” in the third rod segment that deviates from the regular coiled coil heptad repeat pattern. Despite many years of research there is still no complete structural description of any IF filament [11]. They are known to form tetramers through inter-dimer interactions mediated by their N-terminal regions of the rod domain (“A11” inter-dimer contact). Filaments are thought to contain tetramers as building blocks, at least for the cytoplasmic IFs, and three other types of inter-dimer contacts (“A22”, “A12”, and ”ACN") then lead to higher-order structures such as the prototypical 10 nm-thick mature fibres of many IF proteins [12].

Crescentin contains the heptad repeats needed to form an extended coiled coil dimer, and also a stutter in the C-terminal half. The rod domain of crescentin is longer than IF proteins, and it was demonstrated early on that crescentin forms very stable filaments *in vitro* [5]. Filament formation *in vitro* was robust to some domain deletions, except the entire central rod domain [9]. *In vivo*, filament formation and localization were more sensitive to modifications and in particular membrane attachment was shown to depend on the first 27 amino acids [6, 9]. Removal of the stutter also interfered with crescentin’s ability to curve cells [9].

Given the enigmatic relationship between crescentin and IFs, our lack of understanding how crescentin filaments form in cells and how they control cell shape, we set out to understand the crescentin filament structure. We employed electron cryomicroscopy (cryo-EM) on crescentin filaments bound by a newly developed megabody [13] to determine their structure, which revealed an “octameric” twisted and double-stranded filament without polarity. We used *in vivo* site-specific cysteine crosslinking to demonstrate that many, if not all features of the *in vitro* filament structure exist in crescentin filaments in cells. Electron cryotomography (cryo-ET) of *Caulobacter* cells expressing crescentin showed the filaments on the inner, concave side of cells, close to the inner membrane where they form a single, wide band. Comparison with current models of IF proteins revealed significant differences between their filament architectures.

115 **RESULTS**

116 **Development of a megabody for CreS structure determination**

117 In line with previously reported assembly properties of crescentin (CreS) and of many eukaryotic
118 IF proteins [5, 9, 12, 14-16], polymerization of purified, un-tagged *Caulobacter crescentus*
119 *(vibrioides)* CreS upon a decrease of pH *in vitro* led to CreS aggregates, bundles, or irregular
120 filaments that exhibited structural polymorphism. In some cases, cryo-EM micrographs of wild-type
121 (wt) CreS, namely $CreS_{wt,}$ polymerized at pH 7.0 showed unbundled filaments with a width of ~9
122 nm (**Fig. S1A**). Two-dimensional (2D) class averages revealed two intertwined strands with a
123 regular spacing of ~57 nm (distance between helical crossovers) (**Fig. S1B**). Nevertheless, the
124 smooth appearance of these filaments prevented determination of the register of individual
125 subunits along the filament axis.

126 To facilitate structure determination of CreS, we raised nanobodies (NBs) against purified $CreS_{wt}$
127 and selected NBs that bind to $CreS_{wt}$ polymers formed at low pH conditions (**Materials and**
128 **Methods**). Coiled coil prediction [17] and three-dimensional (3D) structure prediction by AlphaFold
129 2 (AF2) [18] **(Fig. S1C)** indicated that CreS contains a central, coiled coil "rod" domain (residues
130 80-444), flanked by an N-terminal segment (residues 1-79, although somewhat shorter in AF2
131 predictions, **Fig. S1C**) and a C-terminal segment (residues 445-457) (**Fig. 1A**). Given the
132 elongated nature of the CreS coiled coil dimer, we engineered an NB derivative, megabody (MB),
133 to act as a bulky and rigid marker bound to CreS. For each NB investigated, a MB was generated
134 by grafting the NB onto a circular permutant of the scaffold protein *E. coli* YgjK, as previously
135 described [13]. Among the MBs, megabody 13 (MB13), a derivative of nanobody 13 (NB13) binds
136 to both $CreS_{wt}$ and $CreS_{sat}$ at pH 8. $CreS_{sat}$ contains a stretch of three amino acids (Ser, Ala and
137 Thr; SAT), inserted before residue 406, to remove the "stutter" that locally disrupts the continuity
138 of the heptad-repeat coiled coil [9, 19] (**Figs. 1B-C and S1D-E**). Cryo-EM analysis revealed that
139 for both versions of CreS, purified CreS-MB13 complex at pH 8 polymerizes into regular,
140 unbundled filaments upon lowering the pH to 6.5 in the presence of detergents, such as CHAPS
141 (**Fig. 1D**). These filaments showed a regular spacing of ~57 nm between neighbouring "nodes",
142 where a node is a segment of the CreS filament decorated with multiple MB13 molecules (**Figs.**
143 **1E and S3A**).

144 For cryo-EM structure determination of CreS, we treated nodes as single particles, using a box
145 size of ~420 Å (henceforth referred to as "small box"), to obtain well-resolved reconstructions of
146 CreS bound with NB13 molecules (**Materials and Methods, Table S3, Fig. S2**). We next extended
147 the box size to ~960 Å (referred to as "large box") for the visualization of a more complete CreS
148 structure, which included regions that are distant from the NB13 binding site, which was mapped
149 to a segment near the C-terminus of CreS (**Materials and Methods, Fig. 2A, Table S3, Fig. S2**).
150 The best small box and large box reconstructions were of $CreS_{sat}$ complexed with NB13,
151 determined at nominal resolutions of 3.34 Å (3.79 Å against the fitted atomic model) and 4.11 Å

152 (4.75 Å against the fitted atomic model), respectively (**Fig. S3B-E, Table S3**). The register of
153 individual amino acids on $CreS_{sat}$ was assigned based on the binding site of NB13 on CreS and
154 the observed patterns of well-resolved side chain densities (**Materials and Methods, Figs. S3B**).
155 The resulting atomic models of $CreS_{sat}$ and NB13 allowed for interpretation of $CreS_{wt}$ maps,
156 determined at lower resolutions (4.36 Å -5.78 Å) (**Fig. S4, Table S3**).

157 **Architecture of CreS filaments**

158 In the presence of MB13, $CreS_{wt}$ and $CreS_{sat}$ similarly assemble into a "supertwist"-like filament,
159 where two intertwined strands, I and II, are related by a two-fold axis perpendicular to the filament
160 axis (**Fig. 2B-C**). When looking at the map down this two-fold axis, each strand consists of four
161 partial CreS dimers (dimers A, B, C, and D for strand I, and symmetry related dimers A', B', C' and
162 D' for strand II) within a viewing window of ~960 Å wide. Each dimer is a parallel, polar coiled coil
163 of CreS molecules. Strands I and II are held together via interactions between CreS dimers. Taking
164 strand I as an example, inter-dimer interactions are found between a pair of dimers near their N-
165 termini (A-C pair) or their C-termini (B-D pair) in the longitudinal direction (i.e., along the filament
166 axis) (**Fig. 2D**). Hence, the two-stranded CreS filament presented here lacks polarity, as is the
167 case for the individual strands on their own. This is reminiscent of eukaryotic intermediate filaments
168 (IFs), which are also known to be non-polar [20-23]. Furthermore, each pair of longitudinally
169 associated dimers are structurally similar to each other, especially at the regions close to the inter-
170 dimer interface near the N-termini or C-termini. Thus, every pair of longitudinally associated dimers
171 are related by a local pseudo two-fold axis perpendicular to the inter-strand two-fold axis mentioned
172 above (**Fig. 2D**). Each pair of laterally interacting dimers (A-B pair or C-D pair) alternate at a
173 crossover due to them twisting around each other, giving rise to a crossover distance of ~480 Å
174 along the strand (**Fig. 2D**).

175 Assembling a nearly complete CreS dimer (residues 31-446 for $CreS_{sat}$ or 31-443 for $CreS_{wt}$),
176 based on the observed partial dimers A and B, resulted in an expanded atomic model of the strand
177 (**Fig. 2E**). The partial dimer C is superimposable with the equivalent part of the expanded dimer B
178 upon a shift of 564 Å and a rotation of 78° (**Fig. 2F**). Likewise, the partial dimer D is superimposable
179 with the equivalent region of the expanded dimer A upon a shift of 559 Å and a rotation of 53° (**Fig.
180 2G**). These observations indicate that every strand has a regular spacing of ~560 Å, which
181 corresponds to the length of a CreS molecule in a coiled coil. As a result, these transformations
182 enable each strand and the two-stranded filament to propagate in a roughly linear fashion.

183 **Assembly of CreS filaments**

184 A detailed structural analysis revealed five different types of dimer-dimer interactions that result in
185 the observed CreS filament (**Fig. 3A**). Within each strand, there are both lateral and longitudinal
186 interactions between CreS dimers. Types 1 and 2 involve longitudinal inter-dimer interactions near
187 the C-termini (pairs B-D and B'-D', residues ~443-457, most of which are disordered in our cryo-
188 EM maps) and near the N-termini (pairs A-C and A'-C', residues ~31-83), respectively (**Fig. 3B**).

189 Type 3 represents lateral inter-dimer interactions (pairs A-B, C-D, A'-B', C'-D') (**Fig. 3B**). Dimer-
190 dimer interactions between the two strands are primarily along the lateral direction, including type
191 4 (pair B-B', antiparallel) and type 5 (pairs B-D' and B'-D, parallel) (**Fig. 3B**). Whereas relatively
192 short stretches of amino acids are responsible for longitudinal interaction types, all lateral
193 interaction types cover long inter-dimer binding interfaces.

194 As mentioned in the **Introduction**, the central rod domains of eukaryotic IF proteins are generally
195 ~308 amino acids long, with the exceptions of nuclear lamins and invertebrate IF proteins (which
196 are ~350 amino acids long). It has been proposed to be divided into three coiled coil segments
197 with conserved lengths, 1A, 1B, and 2 [12], which are inter-connected by short linkers L1 and L12
198 (**Fig. S5A**). In contrast, CreS possesses a longer coiled coil rod domain (~364 amino acids), which
199 does not show equivalent linkers that disrupt the overall α-helical structure, based on our cryo-EM
200 maps (**Fig. S5A**). Both N-terminal and C-terminal segments of CreS are relatively short and do not
201 contain complex domains as seen in many eukaryotic IF proteins [24]. Furthermore, structural
202 comparisons of CreS filamentous assemblies with those of eukaryotic IF proteins are hindered by
203 a lack of atomic-level structural information concerning the assembly of eukaryotic IFs, despite
204 decades of research [21, 25-28]. Nevertheless, it has been established that the coiled coil dimer
205 of IF proteins functions as the basic subunit for IF assembly [12]. Four types of inter-dimer contacts,
206 A11, A12, A22, and ACN, have been proposed based on information gained primarily from X-ray
207 crystallographic studies of IF protein fragments and cross-linking mass spectrometry analyses of
208 IF protein oligomers [29-33] [20-23] [34]. These four contact types are shown in comparison to
209 those in CreS filaments in **Fig. S5B,** and their general lack of correspondence is elaborated on in
210 the **Discussion**. In particular, no counterparts of the ACN type (head-to-tail) are observed in our
211 *in vitro* CreS filament (**Fig. S5B)**. However, for eukaryotic IF proteins such as vimentin, it has been
212 proposed that four dimers laterally assemble into an octameric protofibril, of which multiple copies
213 come together in a symmetric way to produce the mature IF, which is often 10 nm wide [12, 26,
214 28, 35, 36]. The two-stranded CreS filament described here consists of four laterally associated
215 CreS dimers that arrange in an octameric manner very loosely reminiscent of that in the proposed
216 eukaryotic IF protofibril [26, 35] (**Fig. S5B, bottom**).

217 ***In vivo* crosslinking confirms crescentin filament architecture**

218 To address the *in vivo* relevance of the CreS filament structure determined *in vitro*, we probed
219 residue-residue contacts observed in the cryo-EM structure in *C. crescentus* cells using cysteine
220 cross-linking with a thiol-specific and cell permeable chemical cross-linker, bismaleimidoethane
221 (BMOE, spacer length ~8 Å). Guided by the CreS filament structure described above, we
222 introduced codons of pairs of cysteine residues into *creS*, which was expressed from a low-copy-
223 number plasmid under its native promoter in *C. crescentus* cells, in a *creS* deletion background
224 (**Materials and Methods)**. This yielded a protein level of CreS similar to the endogenous level in
225 wt strain CB15N (**Fig. S6A**). Cells expressing wt *creS* or a *creS* mutant this way had a cell

226 curvature comparable to wt strain, with two exceptions that will be discussed below (**Fig. S6B**).
227 We focused on residue pairs with a Cβ-Cβ distance of 6-12 Å at multiple inter-dimer interfaces in
228 both the $CreS_{sat}$ and $CreS_{wt}$ filament structures (**Table S4**).

229 For lateral dimer-dimer interactions (type 3, **Fig. 3**), we identified BMOE-dependent crosslinking
230 products for at least two residue pairs in each of three regions that are well separated along the
231 elongated inter-dimer interface (**Figs. 4 and 5**). In the absence of double-cysteine mutants, we
232 detected no signal or only weak background signal for wt CreS and single cysteine CreS mutants,
233 confirming that cross-linking was largely specific (**Fig. 4C**). The three lateral interaction regions
234 probed include "crossover" (A282C/D221C, K296C/A207C, and K296C/Q204C), "middle"
235 (T120C/R384C, A134C/K369C, and T131C/K369C), and "NC" (S34C/R419C and A37C/R419C),
236 where NC denotes interactions between the N-terminus and C-terminus proximal regions (**Figs. 4**
237 **and 5**).

238 For longitudinal interactions, we observed BMOE-dependent crosslinks between residue pairs
239 near the N-termini (E43C/S74C and E57C/E63C, region NN, type 2) and C-termini (M443C, region
240 CC, type 1), respectively (**Fig. 5**). Since all these inter-dimer interaction regions are most likely
241 important for the assembly of individual strands, these results indicate that the single-stranded
242 architecture observed *in vitro* likely acts as a structural unit for producing CreS assemblies *in vivo*.

243 **Subcellular organization of CreS filaments**

244 The ultrastructure of endogenous CreS assemblies at normal levels in *C. crescentus* has
245 previously been challenging to visualize [5-8, 37, 38]. We therefore imaged wt *C. crescentus* cells
246 where wt *creS* was moderately overexpressed using electron cryotomography (cryo-ET).
247 Tomographic reconstructions of these cells revealed an ~4 nm thick extended structure that lines
248 the inner cell membrane at a distance of ~ 5 nm and that spans a major portion of the cell's length
249 on the concave side of the cell (**Fig. 6A**). Consistent with this structure being CreS or CreS-
250 containing filaments, over-production of $creS_{\Delta N27}$, where the first 27 amino acids were truncated,
251 in a *creS* deletion background led to prominent bundles that detach from the inner cell membrane
252 (**Fig. 6B**). This N-terminal region is positively charged and had previously been shown to be
253 required for attachment of CreS to the inner cell membrane [6, 38]. Additionally, similar filamentous
254 densities were not detected in cryo-ET reconstructions of Δ*creS* cells (**Fig. 6C**). Furthermore, CTP
255 synthase (CtpS) is another filament-forming protein that localizes to the inner cell curvature in *C.*
256 *crescentus* [39]. CtpS is known to interact with CreS and acts as a regulator of cell curvature [39].
257 Over-production of wt *creS* in *ΔcreS* cells where *ctpS* expression was suppressed showed a
258 similarly extended structure lining the inner cell curvature (**Fig. 6D**). Thus, CreS can form
259 filamentous structures along the inner cell curvature in *Caulobacter* cells. This is in agreement with
260 the subcellular localization and low-resolution views of CreS as revealed by diffraction-limited
261 fluorescence microscopy [5-7].

262

263 **Functional requirements**

264 Having determined the assembly and subcellular organization of CreS, we next addressed the
265 molecular determinants of CreS-dependent cell curvature generation. Ectopic expression of wt
266 *creS* in a heterologous system, *E. coli* strain C41, produced curved cells that are normally straight,
267 as evidenced by phase contrast light microscopy and cryo-EM (**Fig. S7A, E, I and L**) [6].
268 Consistent with a role of N-terminal and C-terminal segments in CreS filament assembly, purified
269 $CreS_{rod}$ that contains only the rod domain failed to assemble into long filaments *in vitro*, and
270 overexpression of $creS_{rod}$ did not curve *E. coli* cells (**Fig. S7B, F, J and L**). Further supporting this
271 idea is that two double-cysteine mutations, E43C/S74C and E57C/E63C, in the aforementioned
272 NN region (longitudinal inter-dimer interaction type 2) caused cell straightening in *C. crescentus*
273 (**Fig. S6B**). Moreover, previous mutational analyses showed that deleting each of four important
274 regions for CreS assembly observed in our structure resulted in altered assembly properties as
275 well as partial or nearly complete loss of cell curvature in *C. crescentus* [9]. These include the rod
276 domain (inter-dimer interaction types 3, 4, 5), the NN region (type 2), residues 121-143 (intended
277 to correspond to the L1 linker region in eukaryotic IF proteins, types 3 and 5), and the C-terminal
278 segment (type 1).

279 Nevertheless, correct CreS assembly is not the only functional requirement. Similar to previous
280 findings using *C. crescentus* [6], deleting the first 27 amino acids, which are disordered in our cryo-
281 EM maps, or mutating all negatively charged arginine residues to alanine residues in this region
282 (N27RtoA) yielded straight *E. coli* cells without disrupting CreS filament assembly *in vitro* (**Fig.**
283 **S7C, D, G, H, K, and L**). Thus, the faithful assembly and subcellular organization of CreS on the
284 inner membrane are both indispensable for its function in bacterial cell shape determination.

## DISCUSSION

We have determined the complete structure of crescentin and of its filament (**Figs. 1-3**). To our knowledge, this has so far not been possible for any other IF or IF-like protein [11, 27]. Coiled coil-containing filaments are often refractory to structure determination by X-ray crystallography and also cryo-EM because of their elongated nature, the smoothness of the filaments, and considerable heterogeneity of the filaments formed. AlphaFold 2 predictions on coiled coil proteins are not as powerful as for globular proteins [18], which is also the case for crescentin (**Fig. S1C**). To circumvent these problems, we obtained a crescentin-binding nanobody that we converted into a megabody for cryo-EM structural studies. Because the megabody bound close to the C-terminus of crescentin and all N- and C-terminal ends in the crescentin filament are located in close proximity, this enabled us to obtain a reliable structure of that part at close to 3.3 Å resolution. Further along the filament, atomic modelling was less certain, but the maps clearly indicated that the rod domain of crescentin most likely forms a continuous coiled coil, without flexible linkers. Because we worried about the effect of the conditions used to obtain the filaments, including the use of the megabody, we used site-directed *in vivo* cysteine crosslinking to verify the obtained structure (**Figs. 4-5**). Given that we could not find any major discrepancies, we are confident that the obtained structure is a good representation of the structures crescentin forms in cells. Further supporting this notion is that mutations in a number of structurally important regions for CreS assembly identified here lead to an impaired or abolished cellular function of CreS, based on our and previous results (**Fig. S7**) [9].

Cryo-ET of cells overexpressing crescentin revealed filamentous structures on the concave sides of cells, close to the inner membrane (**Fig. 6**). Because of earlier misunderstandings [39], we demonstrated that the observed structures are not CTP synthase (CtpS). From these findings the question arises how the slowly twisting *in vitro* filaments correspond to the band-like appearance of crescentin in cells. While it is difficult to answer this without further and higher-resolution insights into the structure of the *in vivo* crescentin assemblies, it is important to note that it has been reported that when crescentin filamentous structures are released from the membrane through the inhibition of MreB or cell wall synthesis, the filaments form helical structures [6]. It seems plausible that membrane attachment removes some of the twisting that our *in vitro* structure revealed. The cryo-ET data shows crescentin to be close to the membrane (~5 nm in distance). We suggest that this distance could slow down or even stop elongasomes moving past as they circle the cells along their short axes to make cell wall [40-42].

We confirmed earlier results that the first 27 residues of crescentin are required for its membrane localization [6] (**Fig. 6**). Despite trying, we have been unable to demonstrate convincingly direct membrane binding of crescentin filaments to lipid membranes or liposomes *in vitro*. Given the above mentioned finding that the inhibition of cell wall synthesis or MreB removes crescentin from the membrane [6], it seems likely that membrane attachment, involving the first 27 residues of

322 crescentin, is mediated by another cellular component. One such component could be MreB [7],
323 although AlphaFold 2 is not able to predict a complex between crescentin and MreB in our hands.
324 Possibly also speaking against this idea is the finding that crescentin over-expression in *E. coli*
325 leads to curved cells [6]. If another component is needed to localize crescentin to the membrane,
326 then it must be present (and functional) in *E. coli* as well.

327 Our structure of crescentin and its filament enables us to compare it to what is known about
328 eukaryotic IF proteins and their filaments. It is important to point out again that there is still no
329 complete atomic structure of any IF protein and in particular of an IF filament [11, 26]. Given the
330 formidable technical difficulties with these proteins, considerable efforts have been spent using a
331 "divide and conquer" approach combining X-ray crystallography and crosslinking, to obtain
332 pseudo-atomic models of IF proteins and filaments. Comparing our structure with these models
333 reveals several important similarities and differences (**Fig. 3, Fig. S5**). Crescentin and most IFs
334 form long parallel coiled coil dimers with disordered domains at each end. Vimentin and other
335 cytoplasmic IFs have been shown to form tetramers, two dimers coming together via their coil1
336 domains (A11 contact) [20-23]. No such contact exists in the structure of the crescentin filament.
337 In fact, apart from IF's A12 contact there does not seem to be much similarity in the way dimers
338 pack into larger fibres between IFs and crescentin. Furthermore, the most important difference
339 between them is the longitudinal contacts. In crescentin, they are exclusively N-N (head-to-head)
340 and C-C (tail-to-tail), whereas all the current IF models assume ACN contacts that link dimers
341 head-to-tail. It is, however, important to point out that the experimental evidence for ACN contacts
342 is sparse. Their existence seems to be derived from nuclear lamins that form chains of head-to-
343 tail dimers instead of tetramers [34] and from the fact that it is difficult to build larger IF filament
344 models from A11 tetramers without ACN head-to-tail arrangements.

345 All IF proteins contain a stutter that inserts into the coiled coil-forming heptad repeat four extra
346 residues. This is thought to not interrupt the coiled coil *per se*, but to change the superhelical
347 arrangement such that the two helices run more parallel for a short distance. Crescentin also
348 contains a stutter (residues 406-409) and we have investigated the consequences of removing it
349 by reverting the repeat pattern back to regular heptad repeats by inserting three residues, Ser, Ala
350 and Thr (SAT), before position 406 [9]. While it has been reported that such a modification removes
351 crescentin's ability to curve cells [9], we have found little evidence that it changes its behaviour *in*
352 *vitro*. The structures of the WT and SAT versions are very similar, except that the SAT version is
353 slightly longer because the helices contain one almost complete extra helical turn. It will need
354 further work to investigate this in more detail. For example, the stutter might be needed to form
355 higher-order assemblies such as the ones seen in cells (**Fig. 6**).

356 Going forward it will be important to figure out how crescentin filaments locally modify and reduce
357 cell wall synthesis via the elongasome and how crescentin filaments localize to the inner
358 membrane since we could not reconstitute membrane binding *in vitro*. Because crescentin

filaments are thin and smooth, cellular tomography is unlikely to deliver directly how this is done. Genetic and *in vitro* approaches will have to be pushed further to provide answers.

There are a number of other IF-like proteins known in bacteria, such as Scy and Filp in Streptomyces [43]. Beyond these, other bacterial coiled coil rich proteins, whose evolutionary relationships to IFs are even more enigmatic, such as CrvA and CrvB in *V. cholerae* and CcfM in *M. gryphiswaldense,* share a similar cellular function with CreS in maintaining curved bacterial cell shapes [44, 45]. It will be important to investigate what structures these proteins form. Revealing the evolutionary relationships between IF and their bacterial counterparts will also require more certainty about the structures of IF filaments, an area of eukaryotic structural cell biology that needs urgent attention, perhaps using similar nanobody and cryo-EM approaches as pioneered here. But even with the dissimilarities unearthed, between the crescentin filament structure and the current model of IF filaments, it is intriguing that non-polar octameric filaments made out of long parallel coiled coil dimers characterize both set of proteins. It seems therefore appropriate to us to label crescentin as “IF-like”.

373 **MATERIALS AND METHODS**

374 ***Caulobacter crescentus* and *Escherichia coli* strains.** *C. crescentus* cells were grown in
375 peptone yeast extract (PYE) media at 30°C [46]. Antibiotics were used when required in liquid
376 (solid) media at the following concentrations: 1 (1) µg/mL and 1 (2) µg/mL for chloramphenicol and
377 oxytetracycline, respectively. For ectopic expression of wildtype (wt) *creS* and its variants in *C.*
378 *crescentus*, a parental strain as indicated was transformed with an appropriate plasmid, based on
379 either the low-copy-number pCT133 or medium-copy-number pCT155 vector (**Table S1**). To
380 generate these plasmids, the sequences of *creS* and their native promoter were amplified from
381 strain CB15N and then assembled into the pCT133 or pCT155 backbone using Gibson assembly
382 (New England Biolabs). For protein expression in *E. coli*, an appropriate expression plasmid (**Table**
383 **S1**) was electroporated into C41 (DE3) cells unless stated otherwise. Ampicillin and kanamycin
384 were used at 100 µg/mL and 50 µg/mL, respectively, when required. *E. coli* growth and induction
385 were both at 37°C unless stated otherwise.

386 For imaging, *C. crescentus* strains were grown overnight in PYE medium with the appropriate
387 antibiotics, sub-cultured, and grown to mid-log phase. Glucose was used at a concentration of
388 0.2 % (w/v) to suppress protein expression driven by the leaky, xylose-inducible promoter. *E. coli*
389 strains over-expressing CreS constructs were grown overnight in Luria-Bertani (LB) media with
390 ampicillin to stationary phase, sub-cultured, grown to $OD_{600\ nm}$ ~0.2, and induced by 0.05 mM
391 IPTG for ~2.5 h.

392 **Crescentin expression and production.** The amino acid sequences of the proteins used in this
393 study are listed in **Table S2**. Non-tagged wt crescentin ($CreS_{wt}$, GenBank accession identifier:
394 ACL97278.1) was produced in *E. coli* BL21 (DE3) cells carrying a pET29a-based plasmid for
395 $CreS_{wt}$ expression (strain CJW1659, C. Jacobs-Wagner, Stanford University, USA) [9]. To produce
396 a "stutter mutant", $CreS_{sat}$, that has a three amino acid insertion (Ser, Ala and Thr, SAT) before
397 residue 406 to remove the stutter, strain CJW2045 (C. Jacobs-Wagner) was used [9]. Other non-
398 tagged CreS constructs included $CreS_{rod}$ (amino acids 80-444), $CreS_{\Delta N27}$ (amino acids 28-457),
399 and $CreS_{N274RtoA}$ (all Arg mutated to Ala in amino acids 1-27). These were constructed using
400 Gibson assembly (New England Biolabs) and employing pFE127 as a parental plasmid, which is
401 a pHis17-based plasmid for expressing CreS-His, full length CreS followed by GSHHHHHH. The
402 expression and purification of all non-tagged CreS proteins followed the same procedures below.
403 All purification steps were carried out at 4°C. *E. coli* cells were grown in 2×TY media with
404 kanamycin or ampicillin to reach an $OD_{600\ nm}$ of ~0.6 before 1 mM IPTG was added to induce
405 protein expression for 4 h. Cells were then spun down, and pellets were lysed by sonication in 50
406 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2, 0.1% (v/v) Triton X-100, supplemented with
407 lysozyme, DNase, RNase, and protease inhibitor cocktail (Roche). After centrifugation at 45,000
408 ×g for 40 min, the supernatant was incubated with 30 g/L of Avicel PH-10 cellulose microspheres
409 (Sigma-Aldrich) overnight [47]. The microspheres were washed with 50 mM sodium phosphate

410 buffer, 1 M NaCl, pH 7.2, followed by elution with 50 mM sodium phosphate buffer pH 7.2, 150
411 mM NaCl, 40% (w/v) glucose. The eluate was dialyzed against 50 mM CHES, pH 10, and
412 concentrated before being injected onto a HiLoad 16/600 Superdex 200 PG column (Cytiva) for
413 gel filtration in 5 mM Tris-HCl, pH 8. Peak fractions corresponding to CreS were pooled and
414 concentrated using an Amicron Ultra-15 centrifugal filter (30 kDa molecular weight cutoff [MWCO],
415 Millipore) to a final concentration of ~9 g/L.

416 To purify CreS-His, cells were lysed by sonication in 50 mM Tris-HCl, 300 mM NaCl, 2 mM tris(2-
417 carboxyethyl)phosphine (TCEP), pH 8 (buffer A), supplemented with lysozyme, DNase, RNase,
418 and protease inhibitor cocktail (Roche). The lysate was cleared by centrifugation at 75,000 ×g for
419 1 h and filtered before being loaded onto a 5 mL HisTrap HP column (Cytiva). After washing with
420 increased concentrations (0, 20, 50, and 80 mM) of imidazole in buffer A in a stepwise manner,
421 proteins were eluted with 500 mM imidazole in buffer A. Upon concentration, the eluate was further
422 purified via size exclusion chromatography as above, and protein fractions were treated in the
423 same way as mentioned above.

424 **Expression and production of NB13 and MB13.** Camelid nanobodies (NBs) were raised and
425 selected against purified $CreS_{wt}$ (in 5 mM Tris-HCl, pH 8) through the commercial service provided
426 by the VIB Nanobody Core, Vrije Universiteit Brussel, Belgium. Twenty five of 97 NB clones
427 delivered were chosen based on their sequence diversity and antigen binding capacity for another
428 round of selection against CreS filaments at both pH 7 and pH 6.5, using an enzyme-linked
429 immunosorbent assay (ELISA). To identify suitable NB clones for biochemical and structural
430 studies, purified NB and megabodies (MB, a large nanobody derivative described below [13]) were
431 produced for each of the top eight clones selected based on the ELISA data. The binding properties
432 of each protein to purified CreS were examined using size exclusion chromatography (pH 8) and
433 negative staining electron microscopy (pH 7 and pH 6.5). The final selection was clone No. 13,
434 whose megabody, MB13, together with CreS, forms a stable complex at pH 8 that assembles into
435 single, uniform filaments *in vitro* upon acidification.

436 To produce the nanobody version of clone No. 13, namely NB13, the non-suppressor *E. coli* strain
437 WK6 [48] was transformed with a phagemid vector pMECS containing the PelB leader sequence,
438 followed by the NB13 sequence and followed by an HA tag and a 6×His tag. For periplasmic
439 expression of the megabody of NB13, MB13, with a C-terminal 6×His tag, a pET-22b-based
440 plasmid was generated using Gibson assembly (New England Biolabs). In this construct, NB13
441 was grafted onto a circular permutant of the scaffold protein YgjK (*E coli* K12 glucosidase, 86 kDa)
442 as previously described [13]. The resultant plasmid was electroporated into C41 (DE3) cells. For
443 both NB13 and MB13, cells were grown in Terrific Broth with ampicillin, 2 mM $MgCl_2$ and 0.1%
444 (w/v) glucose until $OD_{600\ nm}$ ~0.6. Protein expression was induced at 25°C for ~19 h with 1 mM
445 IPTG. All purification steps were carried out at 4°C unless stated otherwise. Cell pellets were
446 treated with 0.2 M Tris pH 8, 0.5 mM EDTA, 0.5 M sucrose (TES) on ice for 1 h, and four times

447 diluted TES on ice for another 1.5 h. After clearance by centrifugation at 50,000 ×g for 30 min, the
448 resultant periplasmic extract was loaded onto a 5 mL HisTrap HP column (Cytiva). The column
449 was washed sequentially with a) 20 mM Tris pH 8, 900 mM NaCl, b) 20 mM imidazole in buffer B
450 (20 mM Tris-HCl, 50 mM NaCl, pH 8), c) 50 mM imidazole in buffer B, and eluted with 500 mM
451 imidazole in buffer B. For NB13, the eluate was sufficiently pure and concentrated using an
452 Amicron Ultra-15 centrifugal filter (3 kDa MWCO, Millipore) to a final concentration of ~14 g/L. For
453 MB13, the eluate was concentrated, and further purified via gel filtration using a Superdex 200
454 Increase 10/300 GL column (Cytiva), equilibrated in 10 mM Tris-HCl, 20 mM NaCl, pH 8. Fractions
455 corresponding to MB13 were pooled and concentrated using a Vivaspin Turbo 15 centrifugal filter
456 (50 kDa MWCO) to a final concentration of ~36 g/L. Purified proteins were flash frozen in liquid
457 nitrogen and stored at -80°C.

458 **Electron microscopy of negatively stained samples.** Purified CreS proteins were diluted into
459 25 mM PIPES, pH 6.5 to reach a concentration of ~0.2 g/L, and incubated at room temperature
460 (RT) for 15 min to allow polymerization. The resultant sample was applied onto 400-mesh
461 continuous carbon film copper grids (Electron Microscopy Sciences), which were then stained with
462 2% (w/v) uranyl acetate. Grids were imaged using a Tecnai 12 electron microscope (Thermo Fisher
463 Scientific, TFS) equipped with an Orius CCD camera and operated at 120 kV.

464 **Single particle cryo-EM sample preparation.** Purified $CreS_{wt}$ or $CreS_{sat}$ were incubated with
465 MB13 at a molar ratio of 1:4 in 5 mM Tris-HCl pH 8 at 4°C overnight. The resultant mixture was
466 applied onto a Superose 6 Increase 3.2/300 gel filtration column (Cytiva) in 5 mM Tris-HCl pH 8.
467 Fractions B9 to B5, corresponding to the CreS-MB13 complex (**Fig. 1, Fig. S1**), were pooled and
468 concentrated using a Vivaspin 500 centrifugal filter (100 kDa MWCO). The sample was diluted into
469 25 mM PIPES pH 6.5 to allow for polymerization at RT for 15 min, and then treated with CHAPS
470 through the addition of a 1% (w/v) CHAPS stock solution to reach a final concentration of 0.05%
471 (w/v) at RT for 5 min. This procedure consistently yielded uniform, single CreS filaments in complex
472 with MB13.

473 For cryo-EM grid preparation, sample aliquots of 3 µL were applied onto UltrAuFoil R2/2 grids (300
474 mesh, Quantifoil). Grids were blotted for 1 s at 10°C with 100% relative humidity and immediately
475 plunge frozen into liquid ethane using a Vitrobot Mark IV system (TFS). Movies of MB13-decorated
476 CreS filaments embedded in vitreous ice were collected at liquid nitrogen temperature using a
477 Titan Krios transmission electron microscope, operated at 300 kV and using the programme EPU
478 (TFS). For dataset $CreS_{sat}$, movies were collected at a nominal magnification of 81,000× in super
479 resolution mode using a K3 direct electron detector (Gatan), resulting in a super resolution pixel
480 size of 0.53 Å/pixel on the specimen level. The total exposure was 53 $e^-/Å^2$ in 2.4 s with a dose
481 rate of 25 $e^-$/pixel/s, a frame rate of 60 ms, and a defocus range of 0.4-2.6 µm. For dataset $Cres_{wt}$,
482 movies were collected at a nominal magnification of 75,000× using a Falcon 4 camera (TFS),
483 giving a physical pixel size of 1.08 Å/pixel on the specimen level. The total exposure was 34 $e^-/Å^2$

484 in 10 s with a dose rate of 4 $e^-$/pixel/s, a frame rate of 4 ms (EER - electron event representation),
485 and a defocus range of 0.4-4.0 μm. Data collection statistics have been summarized in **Table S3**.

486 **Image processing.** The image processing procedures for datasets $CreS_{wt}$ and $CreS_{sat}$ were
487 essentially the same except for slight differences in pixel size and box size (see **Table S3** for
488 details). The following description focuses on dataset $CreS_{sat}$. Movies were corrected for inter-
489 frame motions using MotionCor2 [49]. Aligned frames were summed and down-sampled to
490 produce individual micrographs (1.06 Å/pixel) that were used for estimation of contrast transfer
491 function (CTF) parameters using CTFFIND4 [50]. Nearly every single filament on the micrographs
492 (e.g., **Fig. 1B**) showed a regular spacing of ~60 nm between neighbouring "nodes", of which each
493 represents a segment of the CreS filament decorated with MB13 molecules. About 870,000
494 particles centred on individual nodes were picked from all micrographs that had been denoised for
495 picking purposes using a neural network model pre-trained in Topaz [51, 52].

496 The following steps involved the alternate use of Relion 3.1 [53] and cryoSPARC [54]. Particles
497 were first extracted with a box size of 100 pixels and 4.24 Å pixel size (Fourier cropping throughout
498 the processing). About 690,000 particles were retained after reference-free 2D classification where
499 low-quality particles were identified and removed. Six *ab initio* 3D references were then generated,
500 and the best resolved reconstruction was used as a starting reference for global particle alignments.
501 Particles were subsequently re-extracted with a box size of 200 pixels and 2.12 Å pixel size.
502 Heterogenous refinement in 3D with four classes resulted in one class of particles that showed
503 partial occupancy of MB13 molecules and those were discarded. Visual inspection of the map
504 reconstructed using the remaining ~600,000 particles suggested the presence of a two-fold
505 symmetry axis perpendicular to the filament axis. Thus, the poses of these particles were then
506 refined where C2 symmetry was imposed. Next, to produce high resolution reconstructions,
507 particles were re-centred and re-extracted with 1.325 Å pixel size and with a box size of 320 pixels,
508 hereafter referred to as "small box" (SB). In parallel, given that a box size of 400 Å is not sufficient
509 to reveal the structure of a complete CreS molecule, another set of particle images were extracted
510 with 1.59 Å pixel size and a box size of 600 pixels, hereafter referred to as "large box" (LB). The
511 following procedures were applied independently to both sets of particle images. After 3D
512 refinement and Bayesian polishing [55], the densities of scaffold proteins (i.e., YgjK) were
513 subtracted from original particle images. The resultant particle images, which essentially represent
514 nanobody-bound CreS filament segments were subjected to local refinement with C2 symmetry,
515 yielding the reconstruction SAT-SB-C2 for set SB (SAT-LB-C2 for set LB, see **Table S3**). To
516 improve map resolutions, particles were expanded by the C2 symmetry [56], and the densities
517 corresponding to one of two symmetry-related copies were subtracted from images. Subsequent
518 local refinement with C1 symmetry led to a higher resolution reconstruction, SAT-SB-C1 for set
519 SB (SAT-LB-C1 for set LB). The resolutions of reconstructions were estimated based on two
520 methods. One was the Fourier Shell Correlation (FSC) between two independently calculated half
521 maps (Gold Standard FSC) using an FSC cut-off of 0.143 [57]. The other was a model-map FSC

522 between the final EM map and a map computed based on an atomic model (described below),
523 built into and refined against the EM map, using an FSC cut-off of 0.5 [58]. All maps were
524 sharpened using a deep learning-based programme, DeepEMhancer [59]. Local resolution was
525 assessed using Relion [53].

526 **Atomic model building and refinement.** The reconstructions SAT-SB-C1 and SAT-LB-C1
527 showed a structure consisting of four partial CreS coiled coil dimers (i.e., two pairs of longitudinally
528 or laterally associated partial dimers) (**Fig. 2**). The two partial dimers within each longitudinal pair
529 are related by pseudo two-fold symmetry and are held together by interactions between the C-
530 termini or between the N-termini. Thus, each lateral pair is formed by two segments, the N- and C-
531 segments, with their N-terminus and C-terminus oriented towards the pseudo two-fold symmetry
532 axis, respectively. For each segment, we computationally determined the amino acid register by
533 aligning the observed map density with the amino acid sequence of $CreS_{sat}$ using a previously
534 described approach [60]. In this process, we focused on a fragment of ~20 amino acids that
535 showed the most prominent side chain densities in SAT-SB-C1. Briefly, every amino acid position
536 in the fragment was assigned a number among 0-6 based on the estimated size of the side chain
537 density by visual inspection. The resultant sequence of numbers was searched against that of the
538 entire amino acid sequence of $CreS_{sat,}$ according to a scoring matrix, where a mismatch of a small
539 residue in the sequence with the observed large side chain density at a given position was
540 penalized. The amino acid register determined *in silico* this way was further supported by several
541 lines of evidence. First, the resultant amino acid sequence fitted well into the observed side chain
542 density (e.g., **Fig. S3**), as judged by visual inspection. Second, the binding region of NB13 on CreS
543 was mapped to residues 413-433 (**Fig. 2A**), consistent with the assigned amino acid register for
544 the C segment. Third, whole cell cysteine crosslinking revealed residue-residue contacts that were
545 consistent with the atomic model of the single-stranded structure (**Figs. 4-5**). In particular, laterally
546 associated inter-dimer residue-residue contacts supported the assigned amino acid register for the
547 N segment (**Fig. 4**).

548 To interpret the highest resolution reconstruction SAT-SB-C1, we predicted the coiled coil dimeric
549 structures for the N segment and C segment based on their estimated length using CCFold [61].
550 A homology model of NB13 was generated using SWISS-MODEL [62] with the structure of a
551 nanobody (NB-ALFA) against the ALFA-tag (PDB entry 6I2G) as a template. These starting atomic
552 models were fitted into the cryo-EM map in Chimera [63], followed by manual model re-building in
553 Coot [64]. In this process, the orientation of NB13 was initially determined based on the β-strands
554 identified in the map and was similar to that of NB-ALFA when complexed with the ALFA-tag that
555 forms an α-helix. The atomic coordinates were subsequently refined against the map in real space
556 using Phenix [65, 66], where secondary structure restraints were applied. Multiple cycles of manual
557 model rebuilding and real space refinement improved the fitting of the model into the density map
558 and model geometry. To interpret the reconstruction SAT-LB-C1, we expanded the atomic model
559 for SAT-SB-C1 by fitting predicted structures of the remaining regions of $CreS_{sat}$. In local regions

where the map resolution was not sufficient for an unambiguous secondary structural assignment between a helix and a loop, we assumed that it was helical according to coiled coil and secondary structure predictions. The expanded atomic model was rebuilt and refined using the same procedures described above. For each of the C2 reconstructions, SAT-SB-C2 and SAT-LB-C2, the atomic model for their C1 counterpart and the C2 symmetry related copy were fitted separately into the map as rigid bodies, and then combined into a single atomic model. Per-atom real space refinement of the resultant atomic model was not performed due to the low-resolution nature of these reconstructions compared with their respective C1 counterparts.

The omission of three-amino acids (SAT) in $CreS_{wt}$ with respect to $CreS_{sat}$ produces a stutter that locally disrupts the continuity of the dimeric heptad-repeat coiled coil [67]. Thus, it is reasonable to assume that the preceding and later segments along the coiled coil have little structural changes upon introduction of the stutter. In line with this, we found that the overall arrangement of $CreS_{wt}$ dimers in the filament is similar to that observed for $CreS_{sat}$ (**Fig. 2**). We determined the amino acid register for the C segment using NB13 molecules as a spatial reference and for the N segment by assuming that $CreS_{wt}$ and $CreS_{sat}$ share the same lateral, inter-dimer interactions between the N-proximal region and C-proximal region. To interpret the reconstruction WT-SB-C1, we used the $CreS_{sat}$ structure as a starting atomic model and replaced the segment corresponding to the stutter region in $CreS_{wt}$ with a sequence adjusted, stutter-containing fragment in vimentin (PDB entry 1GK4). The resultant atomic model was subjected to multiple rounds of model rebuilding and real space refinement against the map, as mentioned above. For the reconstruction WT-LB-C1, the atomic model for WT-SB-C1 was expanded with appropriate segments from the $CreS_{sat}$ structure, followed by model rebuilding and refinement. The atomic model for each of the two C2 reconstructions, WT-SB-C2 and WT-LB-C2, was generated using the aforementioned rigid body fitting approach.

The final atomic models were geometrically validated based on the criteria of MolProbity [68]. Model statistics have been summarized in **Table S3**. All figures were generated using Pymol (https://pymol.org/) or Chimera [63].

**Electron cryotomography (cryo-ET).** *C. crescentus* cells were pelleted at 8,000 ×g for 2 min and resuspended in PYE media to reach an $OD_{600\ nm}$ of ~5. The resuspension was mixed with protein A-conjugated 10 nm gold fiducials (BBI Solutions). Aliquots of 2.5 µL sample were applied onto freshly glow-discharged Quantifoil R 3.5/1 Cu/Rh (200 mesh) grids, followed by blotting for 2-3 s and plunge freezing into liquid ethane using a Vitrobot Mark IV system (TFS). Specimens were imaged using a Titan Krios transmission electron microscope (TFS), operated at 300 kV and equipped with a BioQuantum imaging filter (Gatan) and a K3 camera (Gatan). Tilt series were collected from -60° to 60° in 2° increments at a nominal magnification of 19,500× with a pixel size of 3.84 Å /pixel using SerialEM [69]. The total exposure was ~180 $e^-/Å^2$ with a defocus of 8-10 µm.

596 Tilt series were aligned automatically using Batchruntomo in IMOD [70, 71], and tomograms were
597 reconstructed using the SIRT algorithm in TOMO3D [72].

598 **Mapping of the NB13 binding site on crescentin.** To map the binding site of NB13 on CreS,
599 CreS-His variants were constructed for ectopic expression in C41 (DE3) cells by introducing either
600 a truncation or a substitution into the parental pFE127 plasmid via site-directed PCR-based
601 mutagenesis (KLD enzyme mix, New England Biolabs). Protein expression was induced for 4 h at
602 37°C by adding 1 mM IPTG when cell cultures reached $OD_{600\ nm}$ ~0.5 in LB with ampicillin. Cell
603 pellets were washed twice with cold PBS and lysed using CelLytic Express (Sigma-Aldrich) with
604 protease inhibitor cocktail (Roche) at RT for 20 min. Purified CreS controls and equivalent $OD_{600}$
605 $_{nm}$ units of cleared cell lysates by centrifugation were resolved on a 4-20% SDS-polyacrylamide
606 gel (Bio-Rad). In this step, each biological sample was split into two halves, and the two halves
607 were loaded separately on the same gel. For Western blotting analysis, one half was
608 immunoblotted with NB13 (7 µg/mL) followed by washing and probing with α-HA-peroxidase
609 (1:1000, Roche), whereas the other half was probed with α-His-peroxidase only (1:4000, TFS).

610 **Whole cell cysteine crosslinking.** *C. crescentus* strains harbouring a low-copy-number plasmid
611 for expressing CreS or its mutants were grown overnight in PYE with oxytetracycline, sub-cultured,
612 and grown to $OD_{600\ nm}$ 0.5-0.6. Control strains, CB15N and CB15N Δ*creS* were grown in the same
613 way without antibiotics. About 1.2 $OD_{600\ nm}$ units of cells were spun down at 8,000 ×g for 3 min,
614 and then kept on ice for the following steps. Pellets were washed with cold PBS and resuspended
615 in 80 µL of cold PBS, followed by a 10 min reaction upon addition of 2 µL DMSO or
616 bis(maleimido)ethane (BMOE, final concentration 0.5 mM, ~8 Å arm length, TFS) in DMSO. The
617 reaction was quenched by adding 1 µL of β-mercaptoethanol (BME, stock ~2.3 mM). Cells were
618 then lysed using CelLytic Express (Sigma-Aldrich), supplemented with protease inhibitor cocktail
619 (Roche) at RT for 15 min. The suspension was incubated at 70°C for 5 min in the presence of LDS
620 loading buffer supplemented with 4% (v/v) BME. Samples equivalent to 0.1 $OD_{600\ nm}$ units of cells
621 were resolved on a 4-20% SDS-polyacrylamide gel (Bio-Rad), and immunoblotted with purified
622 NB13 (16 µg/mL), followed by washing and then probing with α-HA-peroxidase (1:1000, Roche).

623 **Light microscopy** Cells grown in conditions described above were imaged upon immobilization
624 on 1% agarose pads. Images were acquired using a Nikon Eclipse Ti2 microscope equipped with
625 a Neo sCMOS camera (Andor) and a Nikon Plan APO DIC objective (100×, numerical aperture
626 1.40). Cell segmentation and cell curvature analysis were performed using MicrobeJ [73] as a
627 plugin in ImageJ [74]. The number of cells analysed was between 114 and 519 for *C. crescentus*
628 strains, and between 140 and 293 for *E. coli* strains. For each strain, mean cell curvature ($\mu m^{-1}$)
629 and standard deviation ($\mu m^{-1}$) were computed and presented.

**DATA AVAILABILITY:** The atomic coordinates derived using reconstructions SAT-SB-C2, SAT-SB-C1, SAT-LB-C2, SAT-LB-C1, WT-SB-C2, WT-SB-C1, WT-LB-C2, and WT-LB-C1 have been deposited with the Protein Data Bank (accession numbers 8AFH, 8AFE, 8AJB, 8AHL, 8AFM, 8AFL, 8AIX, 8AIA). The cryo-EM maps of SAT-SB-C2, SAT-SB-C1, SAT-LB-C2, SAT-LB-C1, WT-SB-C2, WT-SB-C1, WT-LB-C2, and WT-LB-C1 have been deposited with the Electron Microscopy Data Bank (accession numbers EMD-15398, EMD-15395, EMD-15476, EMD-15446, EMD-15402, EMD-15401, EMD-15473, EMD-15465). Table S3 summarises details.

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824

825 **FIGURES**

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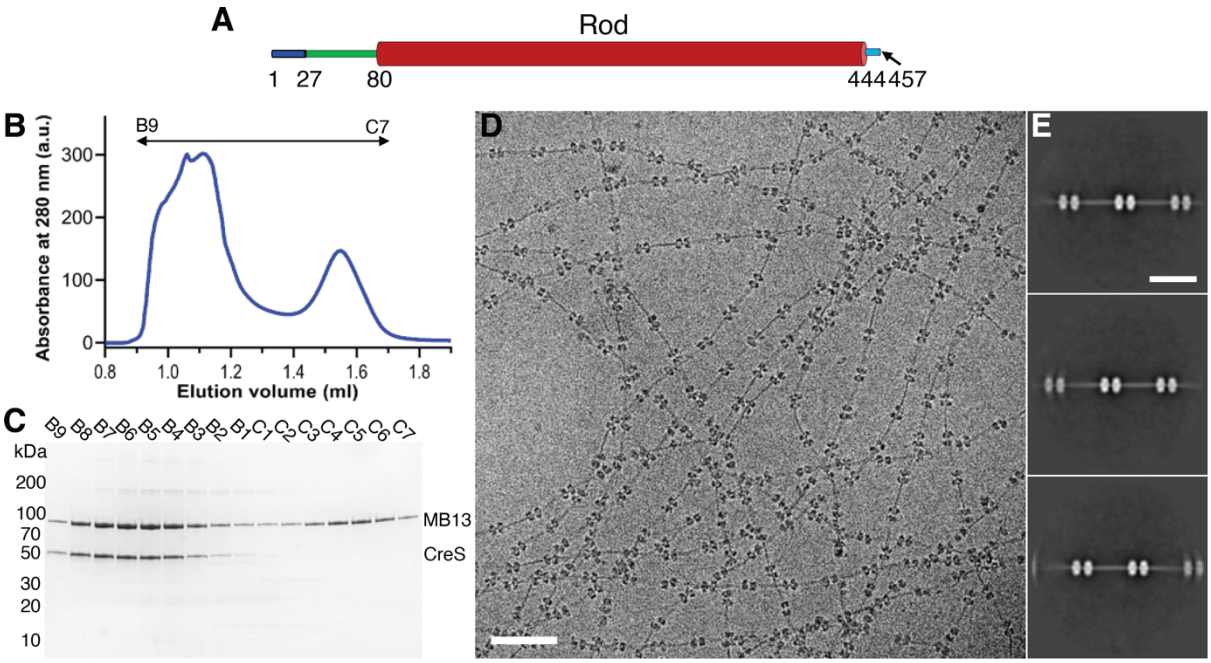


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829 **Figure 1. Production and characterization of a CreS-specific megabody (MB) for structure**
830 **determination of CreS filaments. (A)** Domain organization of full length CreS. The rod domain
831 was defined based on coiled coil prediction [17] and structure prediction using AlphaFold 2 [18]
832 (see **Fig. S1C**). **(B**) Size exclusion chromatography (SEC) profile of $CreS_{sat}$ (a mutant version that
833 has a three amino acid insertion [Ser, Ala and Thr, SAT] before position 406), preincubated with
834 MB13 at 1:4 molar ratio at pH 8. See **Fig. S1D-E** for SEC profile of $CreS_{wt}$ mixed with MB13. (**C**)
835 SDS-PAGE analysis of SEC fractions. Fractions B9 to B5 were pooled and used for EM studies.
836 (**D**) A typical cryo-EM image showing single CreS filaments in complex with MB13 that are formed
837 at pH 6.5. (**D**) Reference-free 2D classification of these filaments reveals a regular spacing of ~60
838 nm between neighbouring “nodes” along the filament. The nodes are caused by MB binding.

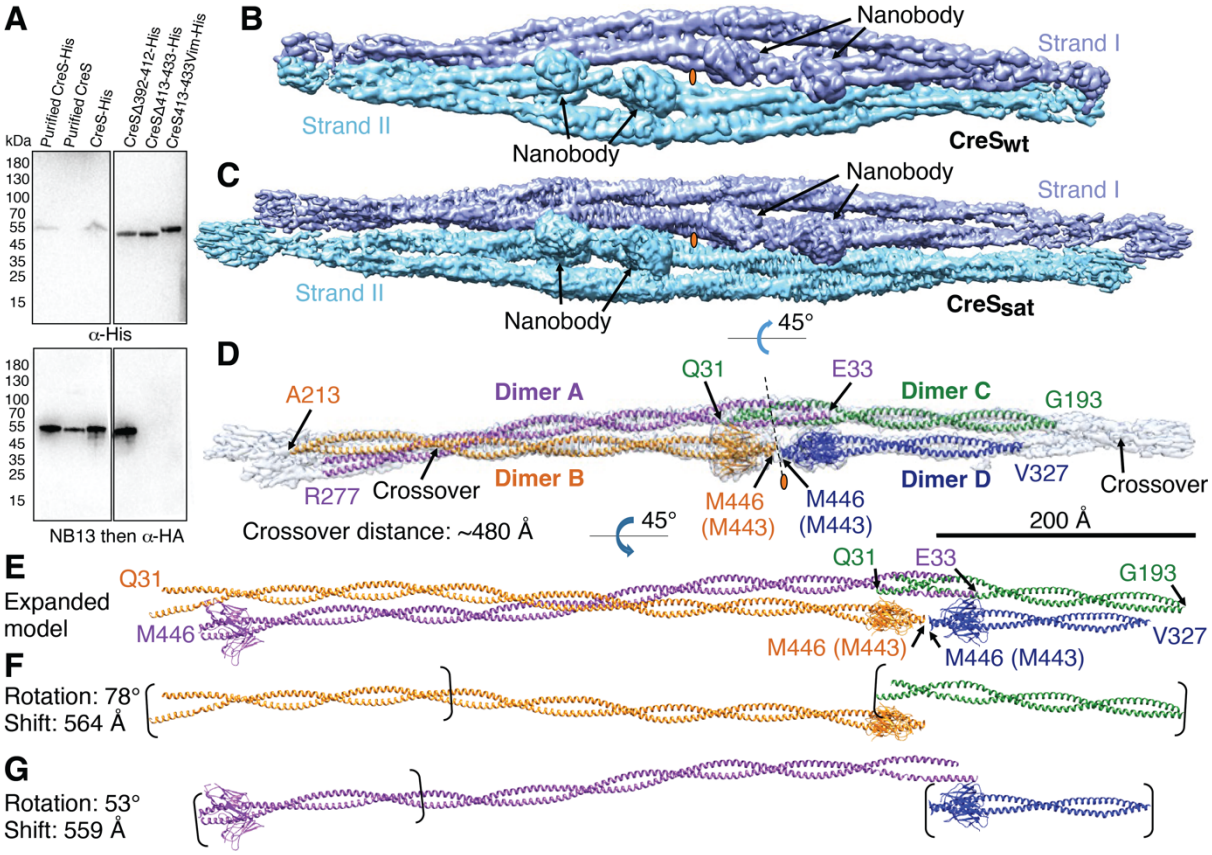


**Figure 2. Architecture and assembly of CreS filaments.** (**A**) Mapping of the CreS-specific nanobody (NB13) binding region on CreS *in vitro*. All samples except for purified proteins as indicated were cell lysates. The samples were immunoblotted with α-His for analysis of protein expression and with NB13 followed by α-HA for analysis of NB13 binding to CreS. Cryo-EM shows that $CreS_{wt}$ (**B**) and $CreS_{sat}$ (**C**) filaments share a common architecture formed by two strands (shown in cyan and blue) that twist around each other. (**D**) Each strand, with roughly the length of an asymmetric unit shown, consists of two CreS parallel coiled coil dimers that are held together via inter-dimer interactions. (**E**) The expanded atomic model reveals the structure of nearly complete CreS molecules, mostly missing N-terminal residues before Q31 that are presumably disordered. (**F-G**) Transformation of individual CreS dimers along the filament axis in a roughly linear fashion produces the observed single-stranded filamentous assembly. Panels **B-G** are drawn to scale (black scale bar: 200 nm). The positions of pseudo two-fold symmetry axes are indicated by an orange oval. All CreS atomic models shown are of $CreS_{sat}$ throughout all figure panels unless otherwise stated. In these cases, residues are numbered based on $CreS_{sat,}$ whereas residue numbers in parentheses are according to $CreS_{wt.}$

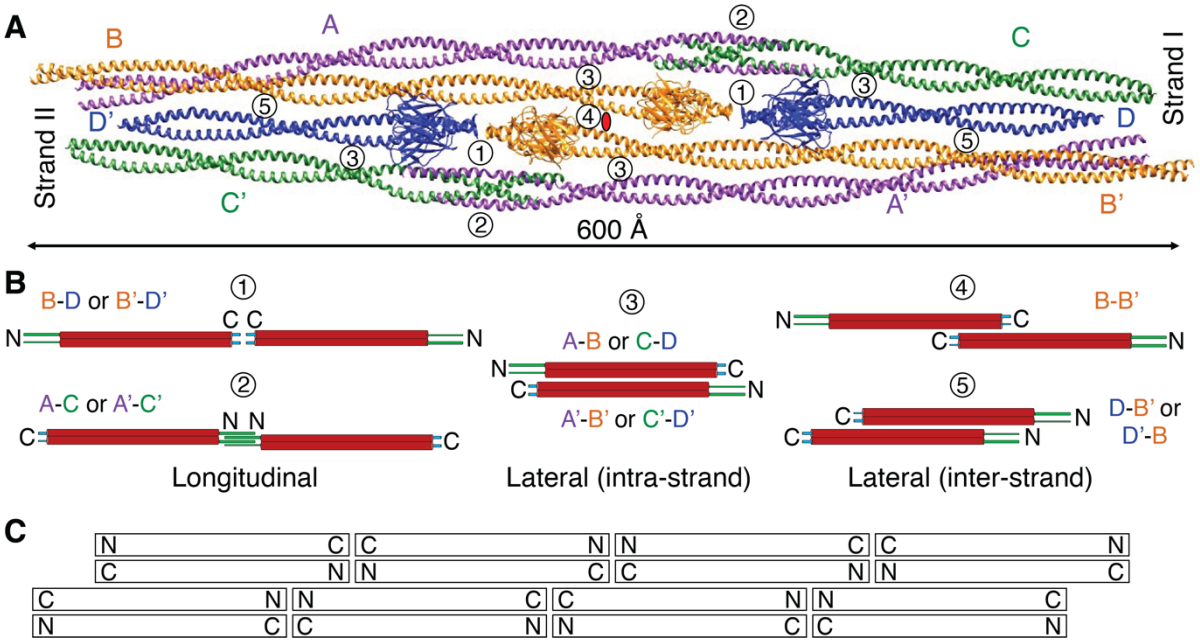


**Figure 3. Contacts between CreS coiled coil dimers**. (**A**) CreS coiled coil dimers assemble into the two-stranded filament structure via a multitude of longitudinal and lateral inter-dimer interactions that can be classified into five types. (**B**) Schematic representation of the five types of observed dimer-dimer interactions. **(C)** Schematic of the octameric CreS filament, highlighting significant differences to current intermediate filament (IF) models (see also **Fig. S5**).

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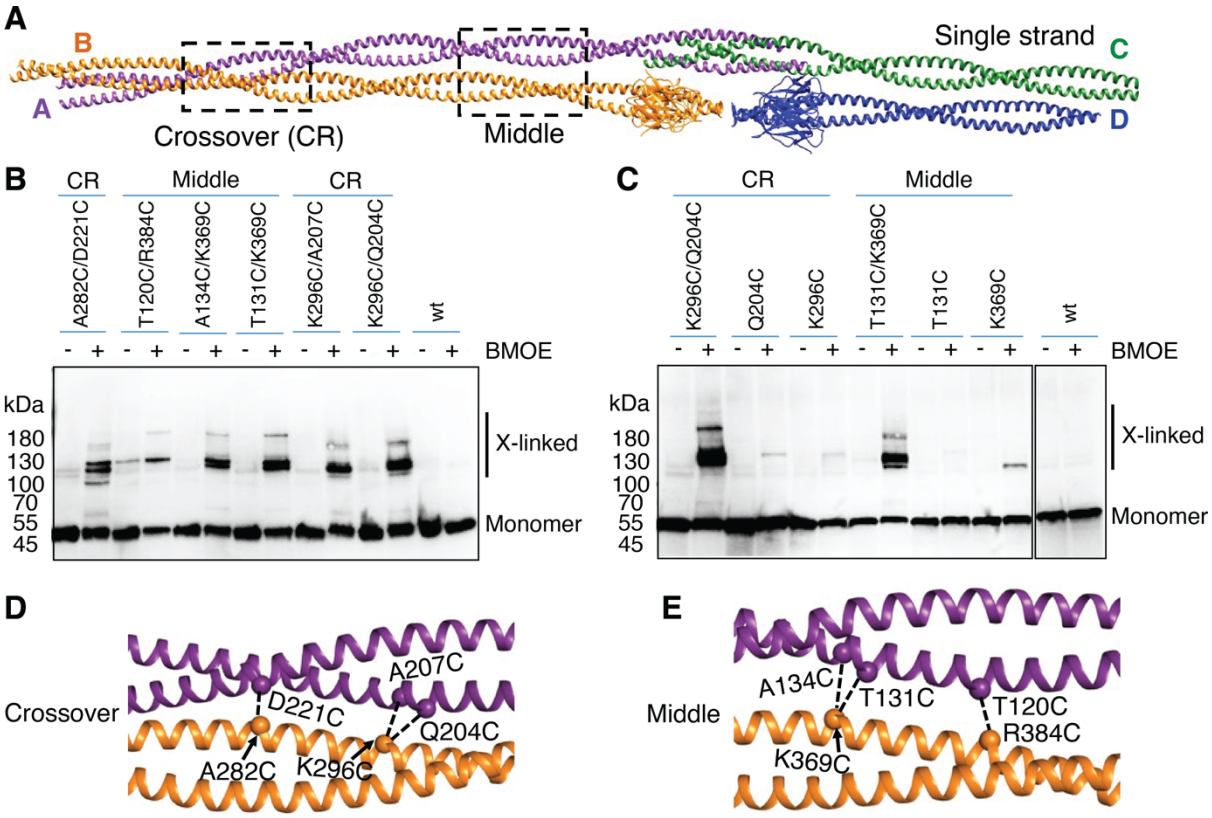


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866 **Figure 4. Structure-guided probing of *in vivo* CreS assembly, with a focus on lateral**
867 **interactions. (A)** The single-stranded filament structure of CreS. Dashed rectangles outline the
868 regions in which cysteine substitutions were introduced for *in vivo* crosslinking. (**B-C**) BMOE-
869 dependent cysteine crosslinking of Δ*creS C. crescentus* cells carrying a low-copy-number plasmid
870 expressing *creS* or its cysteine mutants. Reaction products were subjected to Western blotting
871 analysis with a CreS-specific nanobody, NB13. (**D-E**) A close-up view of the regions Crossover
872 (CR, **D**) and Middle (**E**). Cα atoms are shown as spheres. Dashed lines indicate residue-residue
873 pairs probed in **B** and **C**.

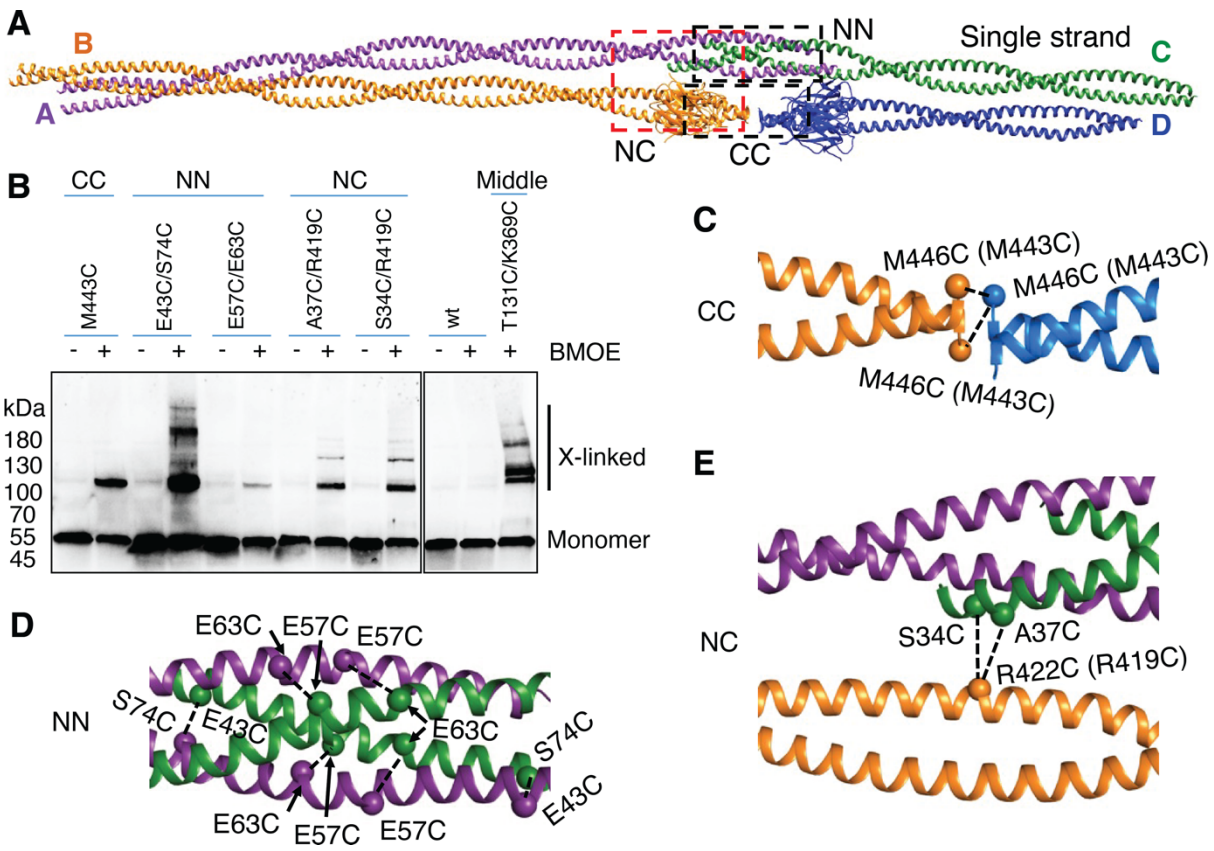



**Figure 5. Structure-guided probing of *in vivo* CreS assembly, with a focus on longitudinal interactions. (A)** The single-stranded structure of CreS. Dashed rectangles outline the regions where cysteine substitutions were introduced for *in vivo* crosslinking. (**B**) BMOE-mediated cysteine crosslinking of Δ*creS C. crescentus* cells carrying a low-copy-number plasmid expressing *creS* or its cysteine mutants. Reaction products were analysed as described in **Fig. 4B**. (**C-E**) A close-up view of the regions CC (**C**), NN (**D**), and NC (**E**). Cα atoms are shown as spheres. Dashed lines indicate residue-residue pairs probed in **B**. As in **Fig. 1**, residues in **C** and **E** are numbered based on $CreS_{sat,}$ whereas residue numbers in parentheses are according to $CreS_{wt}$.

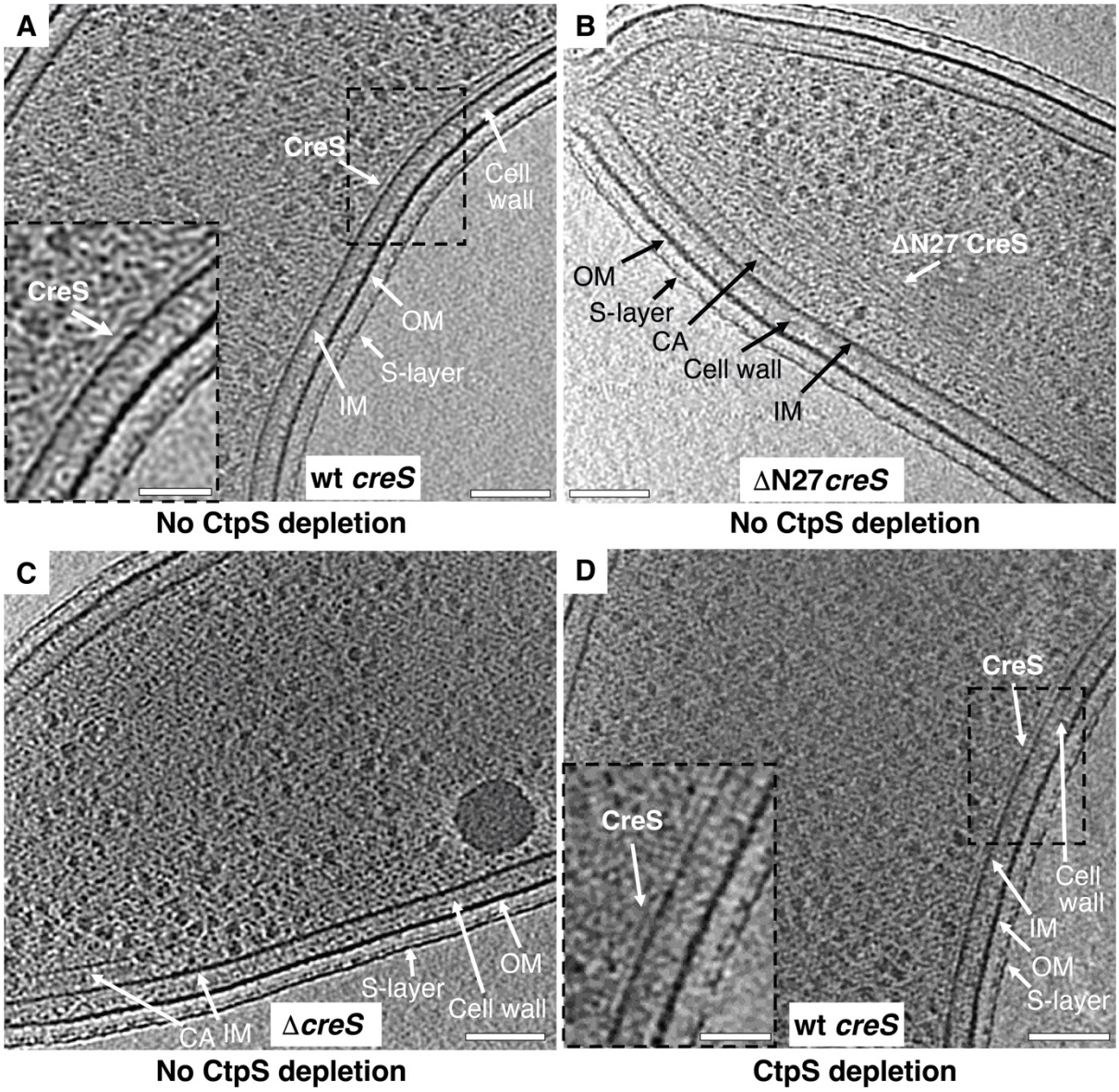
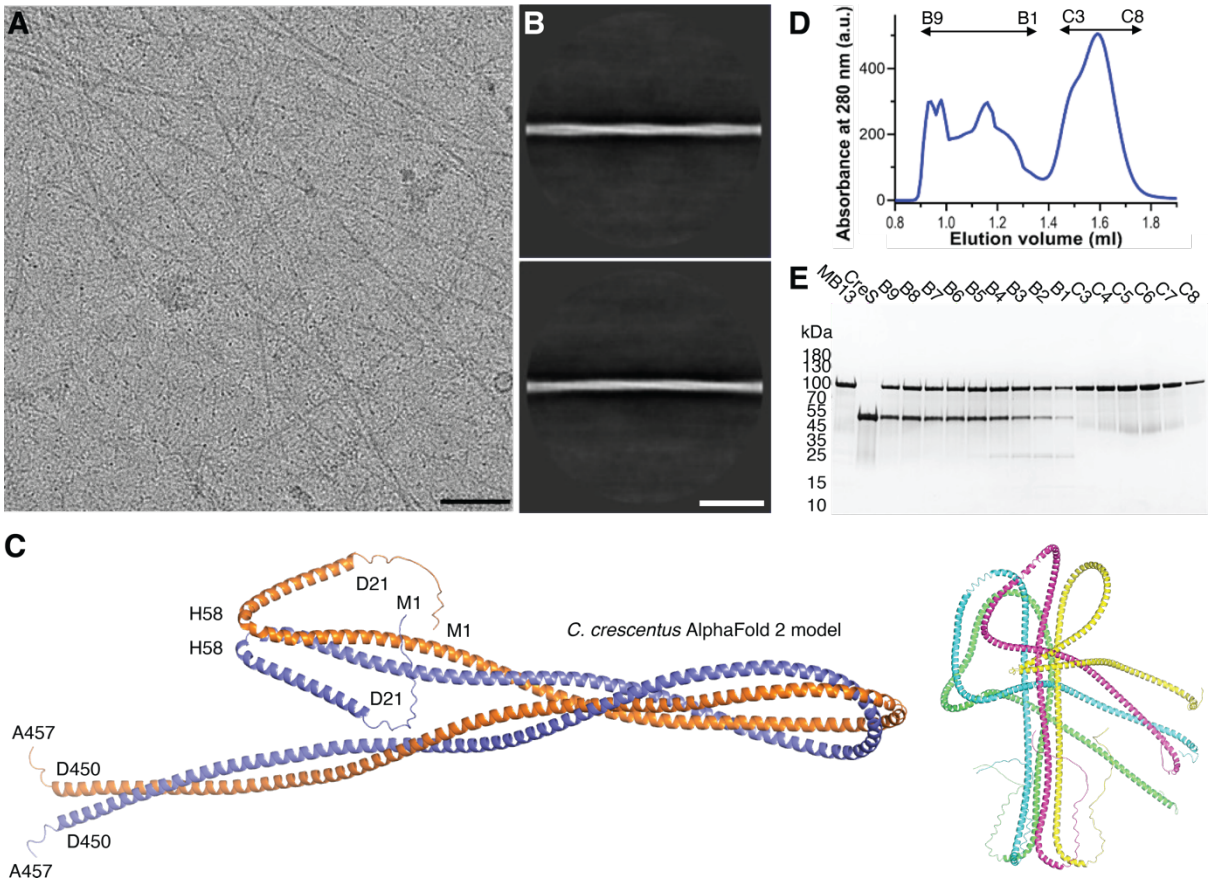



**Figure 6. Subcellular organization of CreS filaments in cells.** Tomographic slices through wild-type (wt) *C. crescentus* cells with wt *creS* overexpression (**A**, strain CJW1430), Δ*creS* cells with ΔN27 *creS* overexpression (**B**, strain YL006), Δ*creS* cells (**C**, strain LS2813), and Δ*creS* cells with both CtpS depletion and wt *creS* overexpression (**D**, strain YL062). Scale bar: 100 nm. The insets (scale bar: 200 nm) in **A** and **D** show a close-up view of the area outlined by the dashed rectangle. IM: inner cell membrane; CA: chemosensory array; OM: outer membrane.

892 **SUPPLEMENTARY FIGURES**

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895 **Figure S1.** CreS$_{wt}$ assembles into two-stranded filaments *in vitro*. (**A**) Cryo-EM micrograph of
896 single filaments of CreS$_{wt}$ formed at pH 7. (**B**) Two-dimensional (2D) class averages show the
897 presence of two intertwined strands. Scale bar: 100 nm (**A**) and 50 nm (**B**). Megabody MB13 forms
898 a complex with CreS$_{wt}$. **(C)** Left: annotated AlphaFold 2 model of CreS dimer. Right: AlphaFold 2
899 model of CreS tetramer. (**D**) Size exclusion chromatography (SEC) profile of CreS$_{wt}$, preincubated
900 with an excess of MB13 (1:4 molar ratio) at pH 8. (**E**) SDS-PAGE analysis of SEC fractions.
901 Fractions B9 to B5 were pooled and used for EM analysis. MB13 top, CreS$_{wt}$ bottom bands.

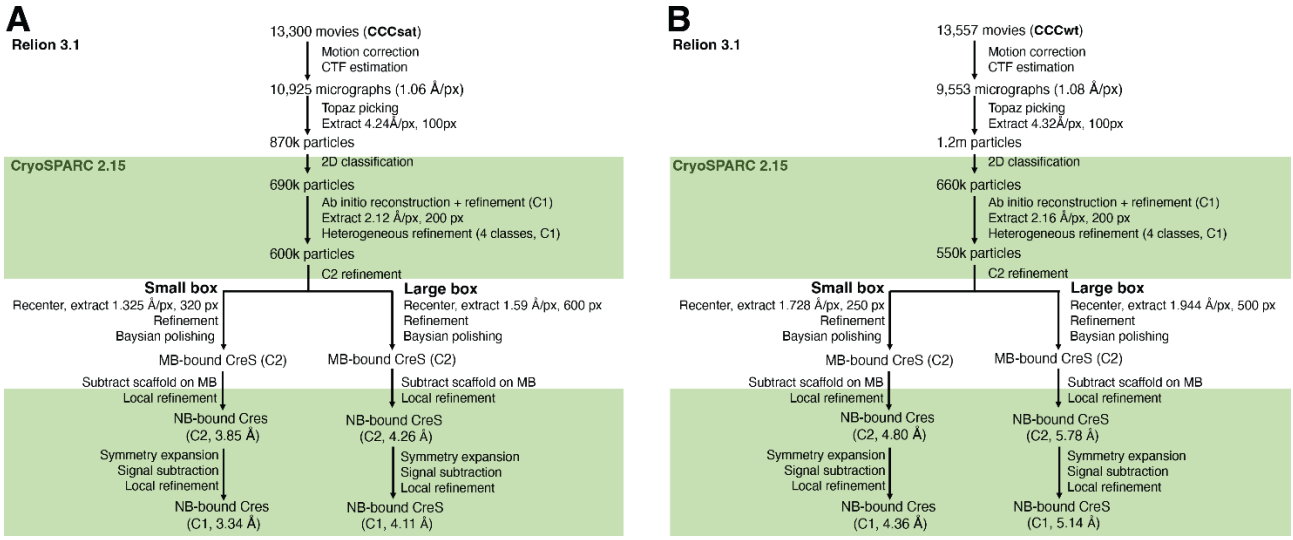


**Figure S2**. Cryo-EM structure determination of CreS. Image processing workflow used for $CreS_{sat}$ (**A**) and $CreS_{wt}$ (**B**).

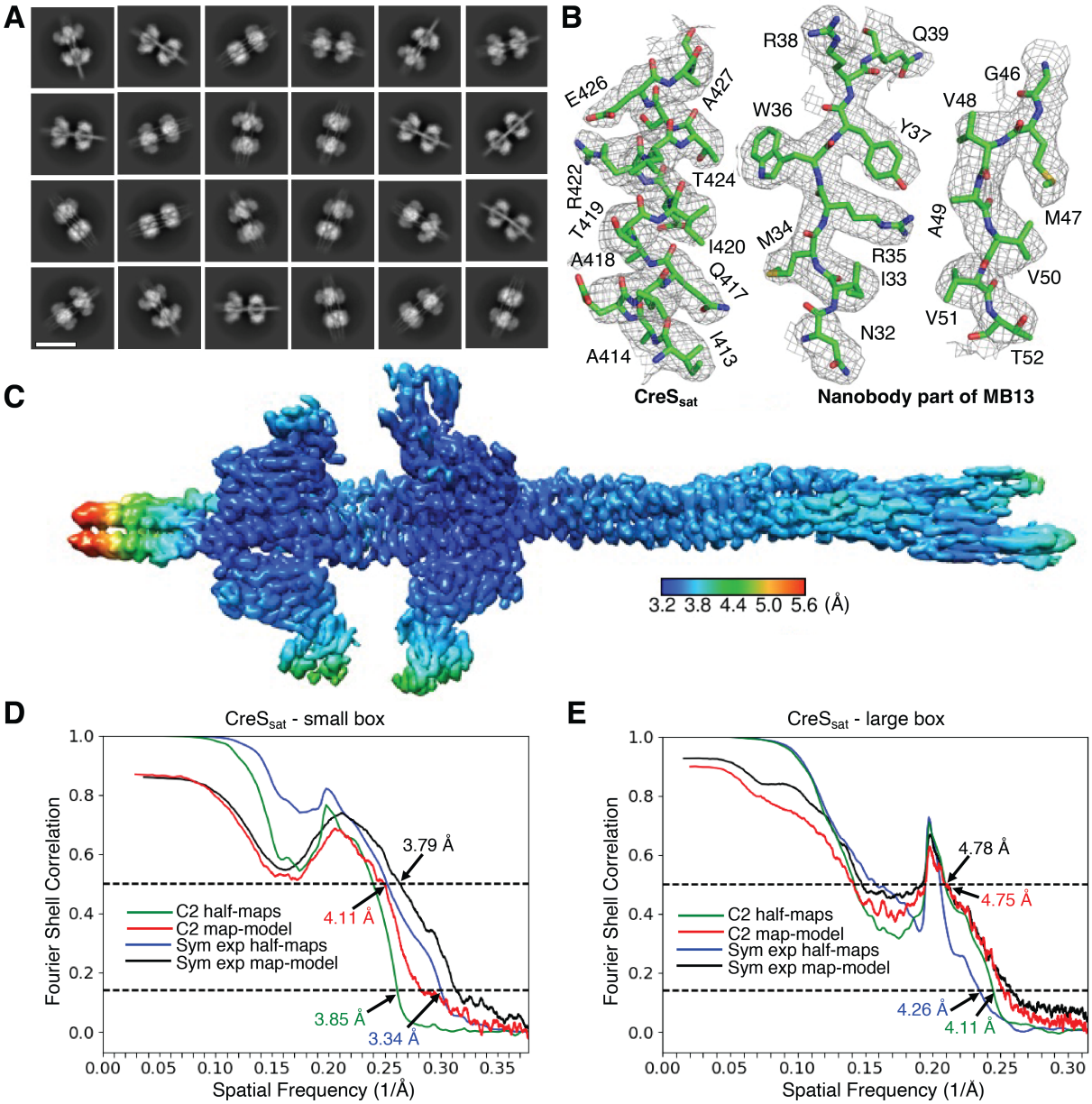


**Figure S3.** Cryo-EM analysis of $CreS_{sat}$ filaments in complex with megabody MB13. Related to **Figure 2**. (**A**) 2D class averages of individual nodes formed by MB13 and $CreS_{sat}$ in different orientations. (**B**) Typical cryo-EM map densities for $CreS_{sat}$ and MB13, with the fitted atomic model superimposed. (**C**) Local resolution of the symmetry-expanded map reconstructed with a box size of 424 Å. FSC-based resolution estimation for maps reconstructed using a box size of 424 Å (**D**, small box) and 960 Å (**E**, large box).

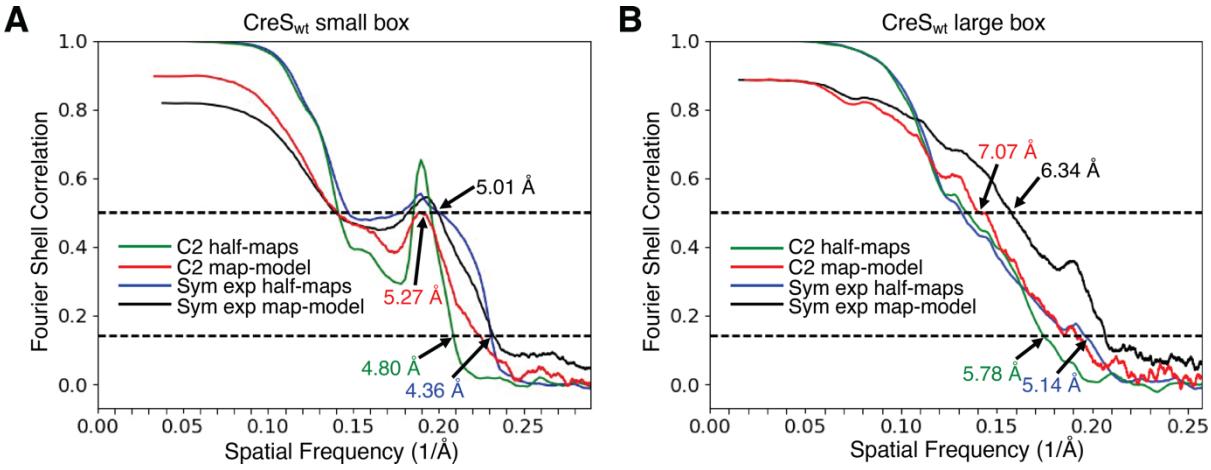


**Figure S4.** Cryo-EM analysis of $CreS_{wt}$ filaments in complex with megabody MB13. Related to **Fig. 2.** Resolution estimation for maps reconstructed using a box size of 432 Å (**A**, small box) and 972 Å (**B**, large box) based on FSC curves.

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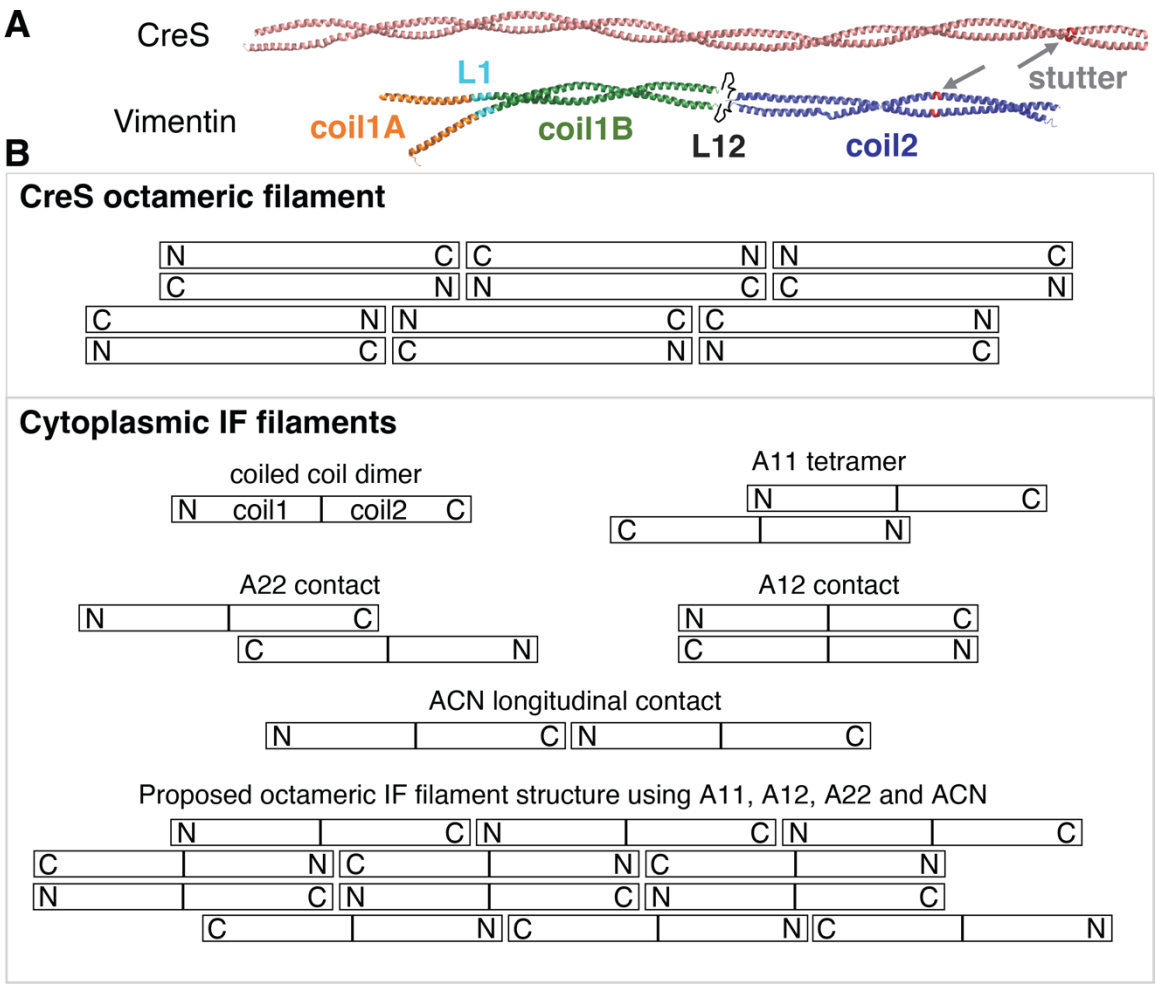


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923 **Figure S5**. Structural comparison of CreS with eukaryotic IF proteins. (**A**) To-scale comparison of
924 the atomic model of a CreS dimer (upper) with the composite atomic model of a vimentin dimer
925 (lower). The IF structure shown is a hybrid model assembled from crystal structures of vimentin
926 fragments (PDB entries 1GK4, 3S4R, 3TRT, 3UF1, and 1GK6), similar to what was reported [21].
927 (**B**) Comparison of crescentin filaments (upper box, same as **Fig. 3C**) with current models of
928 cytoplasmic IF protein filaments (lower box). IF proteins also form parallel and extended coiled coil
929 dimers. Many IF proteins have been shown to form staggered and antiparallel "A11" tetramers, in
930 which two dimers come together via their coil1 rod segments. IF polymers have also been shown,
931 mostly by cross-linking studies, to contain staggered A22 and flush A12 contacts. End-to-end
932 contacts are believed to be via N- and C-terminal ends coming together, head-to-tail ACN contacts.
933 There is little experimental evidence for ACN contacts in cytoplasmic IF filaments, but it is difficult
934 to build gap-free models using the other contacts, without ACN contacts. The current model of an
935 IF "octameric" protofilament is shown at the bottom [11]. The most striking difference to the
936 structure of crescentin filaments discovered here is that all longitudinal contacts are head-to-tail
937 (N-C) in IFs (ACN), and not N-N and C-C head-to-head and head-to-tail, as in crescentin. Both

filaments lack overall polarity and one inter-dimer contact is similar, A12 and interaction type 3 **(Fig. 3A & C)**, whereas the others are different. For example, there is no parallel inter-dimer contact in the IF model, as in crescentin's interaction type 5 **(Fig. 3A & C)**. Furthermore, A11 and A22 in IF filaments are more substantial than the somewhat related contact types 2 (N-N) and 4 (C-C overlap) in crescentin, respectively (**Fig. 3A & C)**. While somewhat related in overall architecture, the way the dimers come together to form filaments can be described as significantly different.

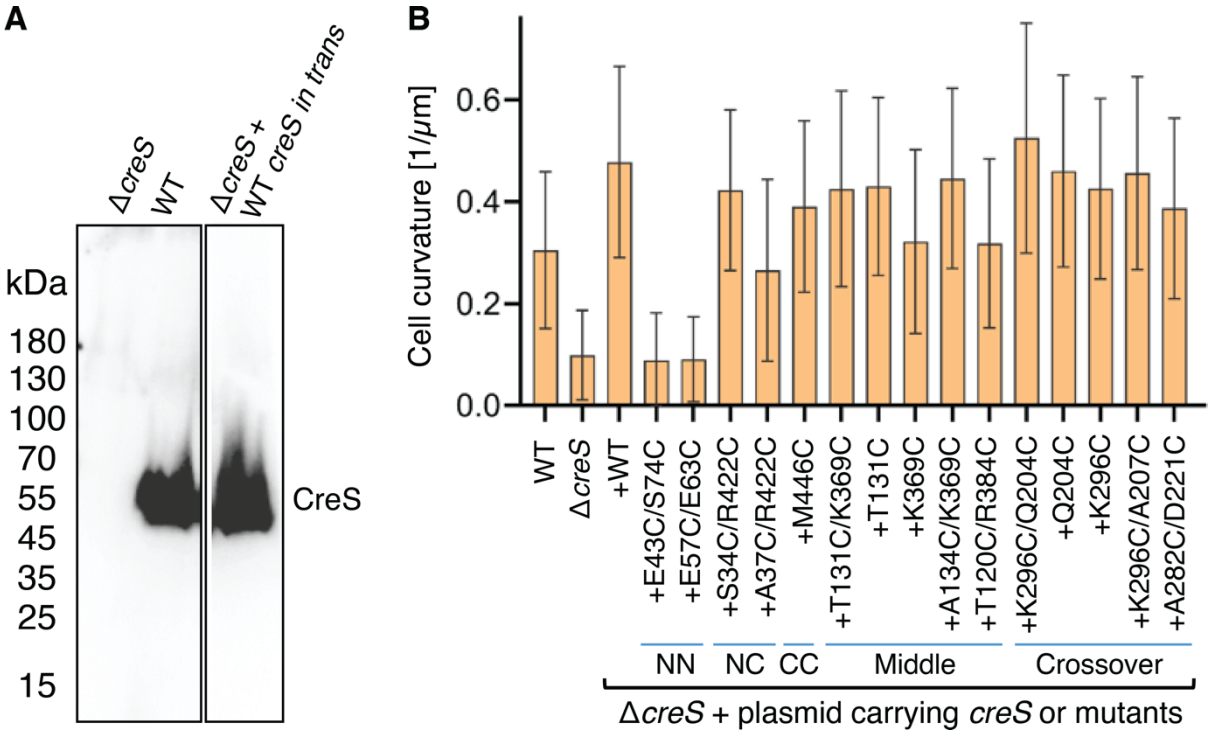


**Figure S6.** Characterization of *C. crescentus* cells carrying a *creS*-expressing plasmid. (**A**) Western blotting analysis of CreS protein levels in *C. crescentus* strains. Production of CreS from a low-copy-number plasmid under its native promoter in a *creS* deletion background yields a similar protein level as that in the wt strain. (**B**) Quantification of cell curvature of *C. crescentus* strains used for cysteine crosslinking experiments (as in **Figs 4-5**). The mean curvature values are shown, and error bars represent standard deviations. The number of cells analysed for each strain was between 114 and 591.

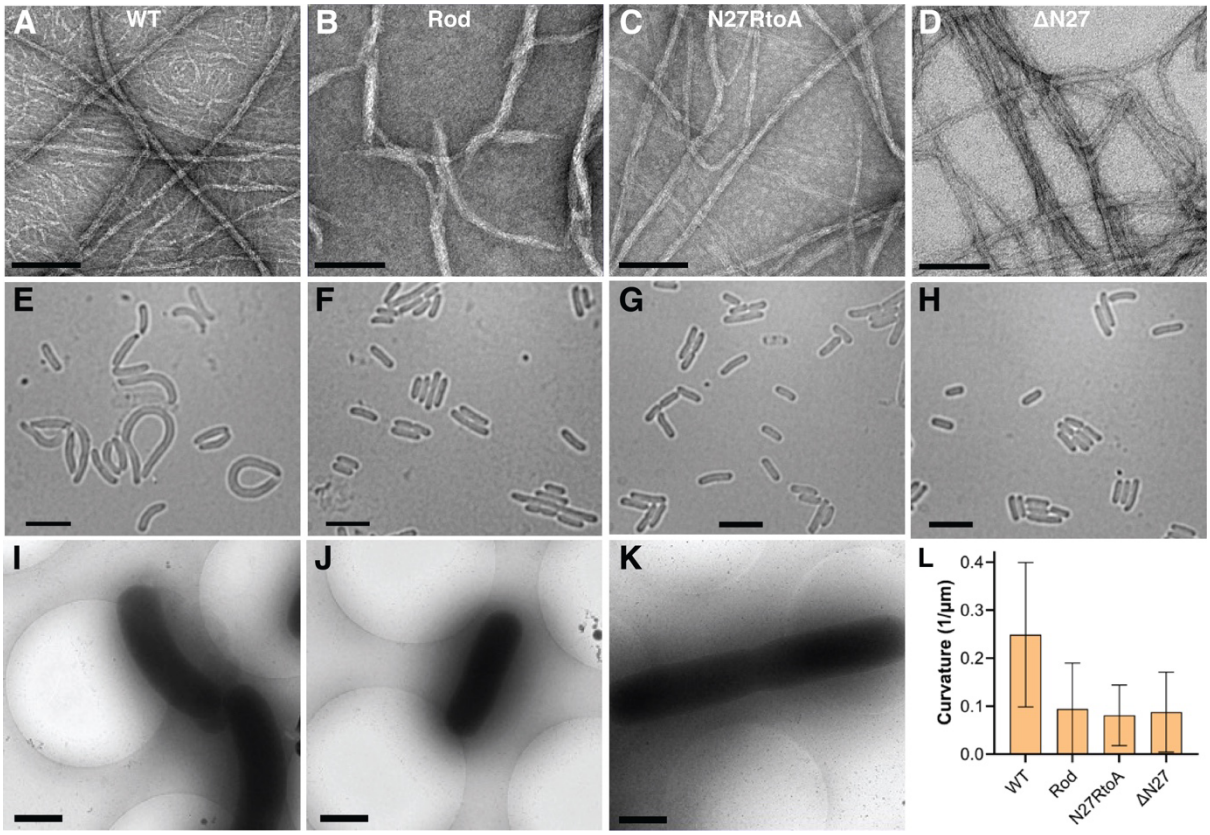


**Figure S7. The N-terminal residues 1-27 of CreS are required for its cell shape function in *E. coli*.** (**A-D**) EM micrographs of negatively stained filaments formed *in vitro* using purified $CreS_{wt}$ (**A**), $CreS_{rod}$ (**B**), $CreS_{N27RtoA}$, where all Arg residues were substituted with an Ala in the N-terminal 27 amino acids (**C**), and $CreS_{\Delta N27}$ (**D**). Scale bar: 100 nm. (**E-J**) Typical phase contrast images of *E. coli* cells overexpressing $CreS_{wt}$ (**E**), $CreS_{rod}$ (**F**), $CreS_{N27RtoA}$ (**G**), and $CreS_{\Delta N27}$ (**H**). Scale bar: 5 μm. (**I-K**) Typical cryo-EM images of *E. coli* cells overexpressing $CreS_{wt}$ (**I**), $CreS_{rod}$ (**J**), and $CreS_{N27RtoA}$ (**K**). Scale bar: 1 μm. (**L**) Quantification of cell curvature based on phase contrast images (corresponding to panels **E-J**). Shown are the mean curvature values with error bars representing standard deviations. The numbers of cells analysed for each strain were between 140 and 293. Protein expression was induced by 0.05 mM IPTG for ~2.5 h.

967 **SUPPLEMENTARY TABLES**

968 **Table S1. Strains and plasmids**

| Name | Description/Genotype | Source |
|---|---|---|
| **Strains** | | |
| *E. coli* | | |
| BL21 (DE3) | F– *ompT hsdSB (rB- mB-) gal dcm* (DE3) | NEB |
| C41 (DE3) | F– *ompT hsdSB (rB- mB-) gal dcm* (DE3) | Sigma-Aldrich |
| WK6 | F' *lacIq* Δ(*lacZ*)M15 *proA+B+* Δ(*lac-proAB*) *galE rpsL* | VIB |
| CJW1659 | BL21 (DE3) expressing $CreS_{wt}$ (non-tagged) | [1] |
| CJW1661 | C41 (DE3) expressing $CreS_{wt}$ (non-tagged) | [1] |
| CJW2045 | BL21 (DE3) expressing $CreS_{sat}$ (insertion of three amino acids (Ser, Ala, SAT, at position 406) | [1] |
| *C. crescentus (vibrioides)* | | |
| CB15N | Synchronizable strain of CB15 | [2] |
| LS2813 | CB15N Δ*creS* | [3] |
| ZG216 | CB15N Δ*creS ctpS*1-400 $P_{xylX}$-*ctpS* (*ctpS* truncated to first 400 bp and another *ctpS* under a xylose inducible promoter on chromosome) | [4] |
| CJW1430 | CB15N + pJS14-based medium-copy-number plasmid for expressing CreS under its native promoter | [5] |
| YL010 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{wt}$ | This study |
| YL006 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{\Delta N27}$ | This study |
| YL012 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{M443C}$ | This study |
| YL017 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{E43C+S74C}$ | This study |
| YL018 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{E57C+E63C}$ | This study |
| YL019 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{R419C+S34C}$ | This study |
| YL020 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{R419C+A37C}$ | This study |
| YL022 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{K369C+T131C}$ | This study |
| YL023 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{K369C+A134C}$ | This study |
| YL024 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{R384C+T120C}$ | This study |
| YL025 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{K296C+Q204C}$ | This study |
| YL026 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{K296C+A207C}$ | This study |
| YL027 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{A282C+D221C}$ | This study |
| YL031 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{Q204C}$ | This study |
| YL032 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{K296C}$ | This study |
| YL033 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{T131C}$ | This study |
| YL034 | CB15N Δ*creS* + pCT133-$P_{cres}$-$CreS_{K369C}$ | This study |
| YL062 | ZG216 + pCT155-$P_{cres}$-$CreS_{WT}$ | This study |
| **Plasmids** | | |
| pCT155-$P_{van}$-*3xM2-didA* | pCT155-based parental plasmid for cloning (medium copy number, pJS14-based, $chlor^R$) | [6] |
| pCT133-$P_{van}$-*3xM2-didA* | pCT133-based parental plasmid for cloning (low copy number, pMR20-based, $tet^R$) | [6] |
| pHis17-CreSΔN27 | pHis17-based plasmid for expressing $CreS_{\Delta N27}$ (residues 28-457) | This study |
| pHis17-CreSN274RtoA | pHis17-based plasmid for expressing $CreS_{N27RtoA}$ (all Arg mutated to Ala in residues 1-27) | This study |
| pHis17-CreSRod | pHis17-based plasmid for expressing $CreS_{Rod}$ (residues 80-444) | This study |
| pFE127 | pHis17-based plasmid for expressing CreS-His ($CreS_{wt}$ followed by GSHHHHHH) | This study |

| | | |
|---|---|---|
| pHis17-CreSΔ392-412-His | pHis17-based plasmid for expressing $CreS_{\Delta 392-412}$-His (residues 392-412 deleted) | This study |
| pHis17-CreSΔ413-433-His | pHis17-based plasmid for expressing $CreS_{\Delta 413-433}$-His (residues 413-433 deleted) | This study |
| pHis17-CreS413-433Vim-His | pHis17-based plasmid for expressing $CreS_{413-433Vim}$-His (residues 413-433 replaced by the homologous region in human vimentin) | This study |
| pMECS-NB13-HA-His | pMECS-based phagemid for expressing NB13-HA-His, a CreS-specific nanobody followed an HA tag and a 6x His tag | This study (provided by VIB) |
| pET-22b-MB13-His | pET-22b based plasmid for expressing MB13-His, a CreS-specific megabody with a C-terminal a 6x His tag | This study |
| pCT133-$P_{cres}$-$CreS_{wt}$ | pCT133 based plasmid for expressing $CreS_{wt}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{\Delta N27}$ | pCT133 based plasmid for expressing $CreS_{\Delta N27}$ (aa 1-27 deleted) under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{M443C}$ | pCT133 based plasmid for expressing $CreS_{M443C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{E43C+S74C}$ | pCT133 based plasmid for expressing $CreS_{E43C+S74C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{E57C+E63C}$ | pCT133 based plasmid for expressing $CreS_{E57C+E63C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{R419C+S34C}$ | pCT133 based plasmid for expressing $CreS_{R419C+S34C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{R419C+A37C}$ | pCT133 based plasmid for expressing $CreS_{R419C+A37C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{K369C+T131C}$ | pCT133 based plasmid for expressing $CreS_{K369C+T131C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{K369C+A134C}$ | pCT133 based plasmid for expressing $CreS_{K369C+A134C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{R384C+T120C}$ | pCT133 based plasmid for expressing $CreS_{R384C+T120C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{K296C+Q204C}$ | pCT133 based plasmid for expressing $CreS_{K296C+Q204C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{K296C+A207C}$ | pCT133 based plasmid for expressing $CreS_{K296C+A207C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{A282C+D221C}$ | pCT133 based plasmid for expressing $CreS_{A282C+D221C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{Q204C}$ | pCT133 based plasmid for expressing $CreS_{Q204C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{K296C}$ | pCT133 based plasmid for expressing $CreS_{K296C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{T131C}$ | pCT133 based plasmid for expressing $CreS_{T131C}$ under its native promoter | This study |
| pCT133-$P_{cres}$-$CreS_{K369C}$ | pCT133 based plasmid for expressing $CreS_{K369C}$ under its native promoter | This study |
| pCT155-$P_{cres}$-$CreS_{WT}$ | pCT155 based plasmid for expressing $CreS_{wt}$ under its native promoter | This study |

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**Table S2. Amino acid sequences of proteins used in this study**

**CreS *Caulobacter crescentus* crescentin** (GenBank accession identifier: ACL97278.1)

MRLLSKNSRETKNGKPTVLGDEARAEAMQHQIESTQAIGQRYETIHGGLDSIGRVMEHLKAIEPLIAEIRG
PVSQEFEARRAEHAELIAVRANLDQAQRQIALIQAEEREVSARLAAAETALGESDARRQTQDAALEDNALE
IDRLRNALLQSDLKVSSLDASLRDATARIEHLVQDVEGLRVQAQDIDARRGDAEAALARANQDNALLGEEA
ATLKKRVDQAGLDLARLSRIETDLEAQLAAERARVQAVENALAAHQADSGRTIRGLESQVEANRAEISALQ
TRLETATGRADKLEEMNGQISARLADSSAQQKAVERRAGDLNVALERALDRIRALEEEADGLRQRHAGVDT
ARATAIERADQLAKSAVAQEKALKRAEERAQQLRARLDAMQEAQDQVRRDHEAKIAELQATIERLTSEAAL
AEGALEAARRDRSRLQMALLGASDGDVAASA

**CreS stutter mutant (SAT insertion before 406)**

MRLLSKNSRETKNGKPTVLGDEARAEAMQHQIESTQAIGQRYETIHGGLDSIGRVMEHLKAIEPLIAEIRG
PVSQEFEARRAEHAELIAVRANLDQAQRQIALIQAEEREVSARLAAAETALGESDARRQTQDAALEDNALE
IDRLRNALLQSDLKVSSLDASLRDATARIEHLVQDVEGLRVQAQDIDARRGDAEAALARANQDNALLGEEA
ATLKKRVDQAGLDLARLSRIETDLEAQLAAERARVQAVENALAAHQADSGRTIRGLESQVEANRAEISALQ
TRLETATGRADKLEEMNGQISARLADSSAQQKAVERRAGDLNVALERALDRIRALEEEADGLRQRHAGVDT
ARATAIERADQLAKSAVAQEKALKRAEERAQQLRARLDAMQEAQDQVRRDSATHEAKIAELQATIERLTSE
AALAEGALEAARRDRSRLQMALLGASDGDVAASA

**Nanobody NB13 (HA-tag, 6×His-tag)**

QVQLQESGGGLVQTGGSLRLSCASSRSIDGINIMRWYRQAPGKQRGMVAVVTGWGSTNYVDSVKGRFIISR
DSAKDTVYLQMNNLKPEDTAVYSCNAIYRGSEYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH

**Megabody MB13 (NB13, YgjK, 10×His-tag)**

EVQLQESGGGLVYKEETQSGLNNYARVVEKGQYDSLEIPAQVAASWESGRDDAAVFGFIDKEQLDKYVANG
GKRSDWTVKFAENRSQDGTLLGYSLLQESVDQASYMYSDNHYLAEMATILGKPEEAKRYRQLAQQLADYIN
TCMFDPTTQFYYDVRIEDKPLANGCAGKPIVERGKGPEGWSPLFNGAATQANADAVVKVMLDPKEFNTFVP
LGTAALTNPAFGADIYWRGRVWVDQFWFGLKGMERYGYRDDALKLADTFFRHAKGLTADGPIQENYNPLTG
AQQGAPNFSWSAAHLYMLYNDFFRKQASGGGSGGGGSGGGGSGNADNYKNVINRTGAPQYMKDYDYDDHQR
FNPFFDLGAWHGHLLPDGPNTMGGFPGVALLTEEYINFMASNFDRLTVWQDGKKVDFTLEAYSIPGALVQK
LTAKDVQVEMTLRFATPRTSLLETKITSNKPLDLVWDGELLEKLEAKEGKPLSDKTIAGEYPDYQRKISAT
RDGLKVTFGKVRATWDLLTSGESEYQVHKSLPVQTEINGNRFTSKAHINGSTTLYTTYSHLLTAQEVSKEQ
MQIRDILARPAFYLTASQQRWEEYLKKGLTNPDATPEQTRVAVKAIETLNGNWRSPGGAVKFNTVTPSVTG
RWFSGNQTWPWDTWKQAFAMAHFNPDIAKENIRAVFSWQIQPGDSVRPQDVGFVPDLIAWNLSPERGGDGG
NWNERNTKPSLAAWSVMEVYNVTQDKTWVAEMYPKLVAYHDWWLRNRDHNGNGVPEYGATRDKAHNTESGE
MLFTVKKDSLRLSCASSRSIDGINIMRWYRQAPGKQRGMVAVVTGWGSTNYVDSVKGRFIISRDSAKDTVY
LQMNNLKPEDTAVYSCNAIYRGSEYWGQGTQVTVSSGENLYFQGSHHHHHHHHHH

1006 **Table S3. Cryo-EM data collection and processing statistics**

| | **$CreS_{sat}$** | | | | **$CreS_{wt}$** | | | |
|---|---|---|---|---|---|---|---|---|
| | Small box | | Large box | | Small box | | Large box | |
| | SAT-SB-C2 | SAT-SB-C1 | SAT-LB-C2 | SAT-LB-C1 | WT-SB-C2 | WT-SB-C1 | WT-LB-C2 | WT-LB-C1 |
| EMDB ID | EMD-15398 | EMD-15395 | EMD-15476 | EMD-15446 | EMD-15402 | EMD-15401 | EMD-15473 | EMD-15465 |
| PDB ID | 8AFH | 8AFE | 8AJB | 8AHL | 8AFM | 8AFL | 8AIX | 8AIA |
| **Data collection and processing** | | | | | | | | |
| Microscope | Titan Krios IV (eBIC) | | | | Titan Krios II (MRC LMB) | | | |
| Accelerating voltage (kV) | 300 | | | | 300 | | | |
| Camera | Gatan K3 | | | | Falcon IV | | | |
| Micrographs (collected/selected) | 13330 / 10925 | | | | 13557 / 9553 | | | |
| Nominal magnification | 81000x | | | | 75000x | | | |
| Pixel size (Å/pixel) | 0.53[a] | | | | 1.08 | | | |
| Dose rate ($e^-$/pixel/s) | 25 | | | | 4 | | | |
| Total exposure ($e^-/Å^2$) | 53 | | | | 34 | | | |
| Frame rate (ms) | 60 | | | | 4 (EER - electron event representation) | | | |
| No. of frames | 40 | | | | 2410 | | | |
| Defocus (µm) | 0.4-2.6 | | | | 0.4-4.0 | | | |
| No. of particels (extraction) | 869812 | | | | 1211745 | | | |
| No. of particels (reconstruction) | 599993 | 1199985 | 585252 | 1170504 | 550575 | 1101149 | 550574 | 1101148 |
| Map resolution (half-map)[b] (Å) | 3.85 | 3.34 | 4.26 | 4.11 | 4.8 | 4.36 | 5.78 | 5.14 |
| Map resolution (model-map)[c] (Å) | 4.11 | 3.79 | 4.78 | 4.75 | 5.27 | 5.01 | 7.07 | 6.34 |
| Pixel size (reconstruction, Å/px) | 1.325 | 1.325 | 1.59 | 1.59 | 1.728 | 1.728 | 1.944 | 1.944 |
| Box size (reconstruction, px) | 320 | 320 | 600 | 600 | 250 | 250 | 500 | 500 |
| Point group symmetry | C2 | C1 | C2 | C1 | C2 | C1 | C2 | C1 |
| **Model Statistics** | | | | | | | | |
| Model-map correlation coeffcient[d] - $CC_{volume}/CC_{mask}$ | 0.70/0.66 | 0.70/0.685 | 0.64/0.55 | 0.70/0.64 | 0.64/0.60 | 0.66/0.63 | 0.69/0.67 | 0.71/0.67 |
| No. of atoms | | | | | | | | |
| Protein | 13578 | 6789 | 30112 | 15056 | 7846 | 3923 | 21930 | 10965 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| R.m.s deviations[e] | | | | | | | | |
| Bond lengths (Å) | 0.006 | 0.006 | 0.007 | 0.006 | 0.005 | 0.004 | 0.007 | 0.007 |
| Bond angles (°) | 0.981 | 0.677 | 1.251 | 1.019 | 0.995 | 0.723 | 1.265 | 1.072 |
| Ramachadran plot[e] | | | | | | | | |
| Favored (%) | 95.1 | 95.1 | 95.2 | 95.2 | 91.6 | 91.6 | 89.2 | 89.2 |
| Allowed (%) | 4.9 | 4.9 | 4.8 | 4.8 | 8.4 | 8.4 | 10.8 | 10.8 |
| Outliers (%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Rotamer outliers[e] | 0.00 | 0.00 | 0.13 | 0.13 | 0.25 | 0.25 | 0.09 | 0.09 |
| Clash score[e] | 14.81 | 13.85 | 29.63 | 29.56 | 17.91 | 18.17 | 48.71 | 48.51 |
| Molprobity score[e] | 2.02 | 1.99 | 2.29 | 2.29 | 2.25 | 2.26 | 2.74 | 2.73 |
| Cβ outliers[e] | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

[a] Super resolution pixel size. The physical pixel size is 1.06 Å/pixel.

[b] Based on FSC between two independently calculated half maps (FSC cutoff 0.143) [7, 8].

[c] Based on model-map FSC between the cryo-EM map and a map calculated based on the atomic model specifying the cryo-EM map resolution (FSC cutoff 0.5) [7].

[d] Atomic coordinates were refined against the cryo-EM map in real space using Phenix [9]. $CC_{mask}$ and $CC_{volume}$ are defined as in [10].

[e] Based on the criteria of MolProbity [11].

1018 **Table S4. Comparison of residue-residue contacts between $CreS_{sat}$ and $CreS_{wt}$.**

| Region | Type | Residue A[a] | Residue B[a] | $CreS_{wt}$[b] (Å) | $CreS_{sat}$[b] (Å) |
|---|---|---|---|---|---|
| Middle | Inter-dimer, lateral | K369 | T131 | 8 (9) | 8 (9) |
| Middle | Intra-dimer | K369 | K369 | 17 (15) | 16 (14) |
| Middle | Intra-dimer | T131 | T131 | 16 (15) | 15 (14) |
| Middle | Inter-dimer, lateral | K369 | A134 | 7 (10) | 8 (10) |
| Middle | Inter-dimer, lateral | R384 | T120 | 8 (10) | 8 (9) |
| Crossover | Inter-dimer, lateral | K296 | Q204 | 11 (11) | 10 (11) |
| Crossover | Intra-dimer | K296 | K296 | N/A[c] | 16 (14) |
| Crossover | Intra-dimer | Q204 | Q204 | 17 (15) | 15 (14) |
| Crossover | Inter-dimer, lateral | K296 | A207 | 8 (10) | 7 (9) |
| Crossover | Inter-dimer, lateral | A282 | D221 | N/A[c] | 6 (6) |
| NN | Inter-dimer, longitudinal | E43 | S74 | 12 (13) | 7 (7) |
| NN | Inter-dimer, longitudinal | E57 | E63 | 12 (14) | 8 (9) |
| NN | Intra-monomer | E57 | E63 | 11 (10) | 12 (11) |
| CC | Inter-dimer, longitudinal | M443 (446) | M443 (446) | N/A[c] | 12 (10) |
| CC | Intra-dimer | M443 (446) | M443 (446) | 18 (16) | 18 (17) |
| NC | Inter-dimer, longitudinal | R419 (422) | S34 | N/A[c] | 12 (12) |
| NC | Inter-dimer, longitudinal | R419 (422) | A37 | N/A[c] | 10 (9) |

1019
1020 [a] Based on $CreS_{wt}$ numbering. Values in parentheses represent residue number based on $CreS_{sat}$
1021 numbering.
1022
1023 [b] Cβ-Cβ distance between two residues. Values in parentheses represent the Cα-Cα distance. For
1024 a given pair, the distance shown is the shortest among all equivalent pairs related by pseudo
1025 symmetry.
1026
1027 [c] At least one of the two residues is absent in the atomic model due to disorder or an ambiguity in
1028 model building.

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