

1 **Heimdallarchaea encodes profilin with eukaryotic-like actin regulation and polyproline**  
2 **binding**  
3

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## 4 Abstract

5 The evolutionary events which led to the first eukaryotic cell are still controversial<sup>1-4</sup>. The  
6 Asgard genome encodes a variety of eukaryotic signature proteins previously unseen in  
7 prokaryotes. Functional and structural characterization of these proteins is beginning to shed  
8 light on the complexity and pedigree of the ancestral eukaryotic cell<sup>5,6</sup>. In eukaryotes, the key  
9 cytoskeletal protein actin is important for diverse cellular processes such as membrane  
10 remodeling and cell motility<sup>7</sup>. Dynamic polymerization of actin provides both structure and  
11 generates the force which drives motility and membrane remodeling. These processes demand  
12 rapid filament assembly and disassembly on microsecond timescales. In eukaryotes, a variety of  
13 highly adapted proteins including gelsolin, profilin, VASP, ARP2/3 and signaling molecules  
14 (Phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>)) are crucial for organizing cellular cytoskeleton  
15 dynamics. Amongst others, the Asgard genomes encode predicted putative profilin homologues  
16 that regulate eukaryotic actin polymerization *in vitro*<sup>5,8</sup>. Interestingly, Asgard profilins appear to  
17 be regulated by PIP<sub>2</sub>, but not by polyproline rich motifs which are important for recruitment of  
18 actin:profilin complexes in eukaryotes<sup>5,9</sup>. These findings indicate that the Asgard archaea may  
19 have possessed analogous membrane organization to present-day eukaryotes, but that  
20 polyproline-mediated profilin regulation may have emerge later in the eukaryotic lineage<sup>5</sup>. Here,  
21 we show that Heimdallarchaeota, a candidate phylum within the Asgard superphylum, encodes a  
22 putative profilin (heimProfilin) that interacts with PIP<sub>2</sub> and is regulated by polyproline motifs,  
23 implicative of an origin predating the rise of the eukaryotes. Additionally, we provide evidence  
24 for a novel regulatory mechanism whereby an extended N-terminal loop abolishes PIP<sub>2</sub> and  
25 polyproline interactions. Lastly, we provide the first evidence for actin polymerization of an  
26 Asgard actin homologue. In context, these findings provide further evidence for the existence of  
27 a complex cytoskeleton already in Last eukaryotic common ancestor (LECA).

28

## 29 Results and Discussion

30 The recent discovery of the Asgard superphylum represents a major breakthrough in the study of  
31 eukaryogenesis<sup>1,8</sup>. While the Asgard phyla are predicted to encode a large number of eukaryotic  
32 signature proteins (ESPs), only a limited knowledge is available on the actual eukaryotic-like  
33 function of these Asgard genes<sup>5,10</sup>. To verify that Heimdallarchaeota encode a *bona fide* profilin,  
34 we determined the 3D protein structure using nuclear magnetic resonance (NMR). Several

35 profilin structures from the Asgard superphylum including Loki profilin-1, Loki profilin-2 and  
36 Odin profilin have been determined previously by X-Ray crystallography both individually and  
37 bound to rabbit actin<sup>5</sup>. However, there are considerable phylogenetic differences separating the  
38 known Asgard phyla, and Heimdallarchaeota is currently thought to be more closely related to  
39 eukaryotes than any other Asgard phyla<sup>8</sup>. Nevertheless, sequence conservation amongst the  
40 Asgard profilin homologues is relatively low and identity is mostly established through structural  
41 homology. At a first glance, our NMR structure depicts a typical profilin fold, with seven strands  
42 interlinked by loops connecting four helices (Fig. 1). However, the orientation, positions and  
43 length of the helices and loops differ dramatically compared with Loki profilin-1 and canonical  
44 eukaryotic profilins. Detailed structural comparison reveals that Heimdallarchaeota profilin  
45 (heimProfilin) is divergent from the Loki profilin-1 (root mean squared deviation (RMSD) >  
46 3.75 Å (Supplementary Fig. 1). Notably, differences include the formation of an additional helix  
47 between residues H123-S129, the re-orientation of the N-terminal helix (residues S27-Q35) to an  
48 open conformation, a shorter Loki-loop, the absence of a helix between residues G72-P75 and  
49 the presence of a long disordered N-terminal loop (residues 1-20) (Fig. 1c). These structural  
50 differences indicate that despite the overall profilin fold, the heimProfilin differs to the  
51 eukaryotic and the recently determined Loki profilins.

52  
53 The extended N-terminal loop in heimProfilin is completely absent in Loki profilin and  
54 eukaryotic profilin. To investigate the loop's function, we cloned and expressed a truncated form  
55 of heimProfilin which we called  $\Delta$ N-heimProfilin which lacked the extended N-terminal loop  
56 (residues 1-23). The overall fold of the  $\Delta$ N-heimProfilin was similar to that of the heimProfilin  
57 as judged from NMR backbone <sup>15</sup>N-<sup>1</sup>H and <sup>13</sup>C $\alpha$  chemical shifts (Supplementary Fig. 2). To  
58 further investigate the functions of both profilins, we allowed rabbit actin to polymerize in the  
59 presence of heimProfilin or  $\Delta$ N-heimProfilin and observed the resulting filament network with  
60 Airyscan super-resolution microscopy. In these experiments heimProfilin was able to inhibit  
61 filament network formation in a concentration dependent manner (Fig. 2a). In contrast,  $\Delta$ N-  
62 heimProfilin did not alter the filament network (Fig. 2a). To verify these results, we followed the  
63 polymerization dynamics of pyrene labeled rabbit muscle actin in the presence of heimProfilin or  
64  $\Delta$ N-heimProfilin. In line with the microscopy data, we found that heimProfilin was able to  
65 modulate rabbit actin polymerization in a concentration dependent manner (Fig. 2b). Conversely,

66  $\Delta$ N-heimProfilin did not alter rabbit actin polymerization (Fig. 2c). However, sedimentation  
67 assay data showed that both heimProfilin and  $\Delta$ N-heimProfilin were able to interact with rabbit  
68 actin (Supplementary Fig. 3d, e). These results indicate that the N-terminal loop is essential for  
69 regulation of actin polymerization dynamics, but not for actin binding and is indicative of the  
70 functional role for the extended N-terminal loop previously unseen in eukaryotic or Asgard  
71 profilin homologues. To further verify these results, we first cloned, expressed and purified an  
72 actin homologue from Heimdallarchaeota. We used two variants of Heimdall actin; a full length  
73 (heimActin) and a truncation mutant form ( $\Delta$ C-heimActin) where the last 35 C-terminal amino  
74 acids, crucial for polymerization, had been deleted<sup>7,11</sup>. Electron microscopy showed that purified  
75 heimActin could form thin filaments while the truncation mutant could not (Fig. 2d) and that the  
76 heimActin was more active in ATPase assay than the  $\Delta$ C-heimActin (Fig. 2e). We then compared  
77 the sedimentation profiles of the heimActin and  $\Delta$ C-heimActin in presence of heimProfilin and  
78  $\Delta$ N-heimProfilin. While both heimProfilin and  $\Delta$ N-heimProfilin were seen to interact with  
79 heimActin and  $\Delta$ C-heimActin respectively, we observed that only heimProfilin was able to  
80 modulate the polymerization of heimActin (Fig. 2f-h) corroborating the above polymerization  
81 modulation of rabbit actin by heimProfilin. This finding indicates that the Heimdallarchaeota  
82 possess profilins which are able to regulate both heimActin as well as eukaryotic actin  
83 polymerization.

84 To further investigate the interaction between heimProfilin and heimActin and to see which resi-  
85 dues are required for the interaction, we turned to nuclear magnetic resonance (NMR) spectros-  
86 copy and performed binding titrations between heimProfilin, heimActin and their respective mu-  
87 tants. We observed chemical shift changes for the interaction between both heimProfilin and  $\Delta$ N-  
88 heimProfilin with heimActin, in line with the pyrene polymerization and sedimentation assays  
89 (Fig. 2 and Supplementary Fig. 4).  $\Delta$ N-heimProfilin exhibited stronger chemical shift changes  
90 than heimProfilin in the presence of heimActin when comparing similar amino acid residues at  
91 similar concentrations, indicating the N-terminal loop might modulate binding in a manner un-  
92 seen in the sedimentation assays. We also observed that the interaction between  $\Delta$ N-  
93 heimProfilin and  $\Delta$ C-heimActin was modest (few residues exhibiting small chemical shift  
94 changes) in line with the sedimentation assay (Supplementary Fig. 5). From the NMR titrations,  
95 we were able to map the site of interaction (Supplementary Fig. 1 and 4), which corresponded to

96 the following residues in heimProfilin: N31, W59 and W59 side-chain, S64, Q69, W70, F84,  
97 G103, G104, I106, N113, T127, E139. For binding experiments with  $\Delta$ C-heimActin, we ob-  
98 served chemical shift changes for the following residues in  $\Delta$ N-heimProfilin: Y26, Y41, I51,  
99 W59, G63, Q69, M71, G104, N113, G130. For comparison, the residues responsible for actin  
100 interaction in eukaryotes are shown on the sequence alignment for both archaeal and eukaryotic  
101 profilin (Supplementary Fig. 1). Together these results indicate that the Heimdallarchaeota  
102 profilin is functional and is consistent with dynamic barbed end binding of actin.

103  
104 Polyproline binding from the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family  
105 of proteins is important for nucleation and elongation of actin filaments<sup>12</sup>. To verify if  
106 heimProfilin binds to polyproline we performed binding experiments both by NMR spectroscopy  
107 and Isothermal titration calorimetry (ITC), using heimProfilin and  $\Delta$ N-heimProfilin. We  
108 observed a moderate binding of  $\Delta$ N-heimProfilin to polyproline with an affinity constant of ~  
109 200  $\mu$ M and a very weak binding for the heimProfilin with affinity constant in the higher micro  
110 molar range (Fig. 3). Titration by NMR reveals that the residues responsible for polyproline  
111 binding were K22, G49, Y52, W53 and W53 side-chain, I106, A111, A145, F147 and Q148  
112 (Supplementary Fig. 1). Revisiting the structure of heimProfilin and comparing it with that of  
113 eukaryotic profilin reveals that the N-terminal helix is orientated upwards creating a pocket  
114 which allows the polyproline motif to bind in an “L-like” fashion as compared to the human  
115 profilin-polyproline binding (Supplementary Fig. 1). These striking observations explains the  
116 reason why Loki profilin-1, -2, and Odin profilins could not interact with polyproline motifs,  
117 whereas heimProfilin could. Structural data from Loki profilin-1, -2, and Odin profilins indicated  
118 that their N- and C-terminal helices parallel and are more close to each other making this type of  
119 interaction highly unlikely<sup>5</sup>. These results suggest that, contrarily to what was previously  
120 thought, polyproline binding (to profilin) could have emerged before the split between the  
121 Asgard and eukaryotic lineages. This pose the question, why do profilin from Loki and Odin do  
122 not possess the N-terminal loop extension? One explanation could be that some Asgard lost this  
123 loop, or conversely, that Heimdallarchaea acquired the loop independently by convergent  
124 evolution, or through horizontal gene transfer. We performed a blast search and alignment  
125 analysis of all profilins deposited in NCBI in an attempt to identify any indication of additional

126 structural elements upstream from the known start position of all other profilins to verify that the  
127 genes were correctly annotated. Interestingly, we observed that several profilins, mainly from the  
128 Thorarchaeota (TF12995.1, TFG09823.1, TFG30347.1, TFF94849.1, RLI55859.1), contained  
129 sequences with N-terminal extension ranging between 5 and 22 residues, upstream of their  
130 previous designated start position (Supplementary Fig. 6). In attempt to see if these thorProfilin  
131 retain the profilin fold and if they also possesses the N-terminal loop, we modeled the 3D  
132 structure for one of the thorProfilin, TF12995.1, which contains 22 amino acids upstream the  
133 known start position using the online software RaptorX<sup>13</sup>. Indeed, we found that the overall fold  
134 of the predicted structure matches our 3D structure determined for heimProfilin with an  
135 additional N-terminal extension (Supplementary Fig. 7). This indicates that these N-terminal  
136 extensions are present in other archaea and could potentially play similar roles as observed for  
137 heimProfilin albeit maybe not to the same extent.

138 In eukaryotes membrane phospholipids, particularly Phosphatidylinositol-4,5-bisphosphate  
139 (PIP<sub>2</sub>), regulate the activities of many actin binding proteins including profilin, cofilin, ezrin,  
140 Dia2, N-WASP and meosin<sup>14</sup>. It should be noted that Asgard archaea likely do not possess simi-  
141 lar eukaryotic membrane architecture. However, they do express membranes with lipids that  
142 have similar features. For example, some archaea lipids have similar inositol head groups but  
143 varied tails known as archaeols<sup>15</sup>. Therefore, we verified whether heimProfilin is able to interact  
144 with PIP<sub>2</sub> by monitoring changes in NMR chemical shifts upon addition of PIP<sub>2</sub> into a solution of  
145 heimProfilin or ΔN-heimProfilin. We observed that while ΔN-heimProfilin interacted with PIP<sub>2</sub>,  
146 no interaction was observed for the heimProfilin (Fig. 4). The residues responsible for phospho-  
147 lipid binding in the Asgard superphyla have not been mapped before and were only speculated  
148 from surface charge distribution<sup>5</sup>. These NMR titration experiments gave us a perfect opportuni-  
149 ty to mapped this binding site. The following residues were observed to display chemical shift  
150 perturbation upon addition of PIP<sub>2</sub>: S27, D28, L30, N31, Q35, S36, V43, G49, N99, K110,  
151 A111, F117, L118, S119, E139, I140, M142, M143, K146, F147, Q148. Although, the change in  
152 chemical shift of specific residue does not mean a direct interaction of that residue, we observed  
153 that all the residues displaying large changes in chemical shift were located on the same surface  
154 of the protein (Fig. 4). This strongly indicates the binding interphase for PIP<sub>2</sub>. We also investi-  
155 gated the potential interaction of inositol trisphosphate (IP<sub>3</sub>), a second messenger signaling mol-

156 eucle resulting from the hydrolysis of PIP<sub>2</sub>. However, we did not observe any interaction for IP<sub>3</sub>  
157 with either heimProfilin or the ΔN-heimProfilin (Fig. 4e, f and h). Taken together, these observa-  
158 tions indicate that, i) Asgard profilins interact with phospholipids, implicating phospholipids in  
159 Asgard archaea actin modulation and ii) profilin from Heimdallarchaeota possess an extended N-  
160 terminal loop involved in actin polymerization regulation (Supplementary Fig. 8a). Interestingly,  
161 deleting this loop enhanced binding to both PIP<sub>2</sub> and polyproline motifs; two important partners  
162 in modulation of actin cytoskeleton dynamics in eukaryotes. In addition, we observed from se-  
163 quence analysis that other profilins possess potential N-terminal extensions that could play a role  
164 in modulating actin polymerization, indicating that polyproline-mediated regulation could pre-  
165 date the Asgard-Eukarya split. We propose a model where modification of the extended N-  
166 terminal loop, or interaction with third-party proteins, causes the heimProfilin protein to behave  
167 similarly to ΔN-heimProfilin. This inhibits modulation of actin polymerization dynamics but  
168 allows for PIP<sub>2</sub> and polyproline interactions. Concurring or subsequent demodification would  
169 flip heimProfilin to an actin modulating state, allowing for actin polymerization regulation. Mod-  
170 ification or interaction with third-party proteins would then be able to reset profilin to the first  
171 step of the cycle (Supplementary Fig. 8b). In conclusion, this study suggests that Asgard archaea  
172 encode a complex cytoskeleton functionally analogous to major eukaryotic cytoskeletal charac-  
173 teristics. Moreover, Heimdallarchaeota expresses profilins that are potentially regulated by phos-  
174 pholipid binding and polyproline interaction, something which was long thought to be eukaryot-  
175 ic-specific, and previously not observed in other Asgard archaea.

176

## 177 **Figure Legends**

178 **Figure 1. Heimdallarchaeota encodes profilin with extended structures.** **a**, Schematic of the  
179 structure of heimProfilin. The orientations of the N- and C-terminal helices are displayed. In  
180 addition, the helix between residues H123-S129 is also shown. **b**, Reorientation of the structure  
181 in **a** by 90° to show the extended N-terminal loop between residues 1-124. **c**, Schematic of  
182 heimProfilin showing notable differences in structural elements to that of human profilin **d** and **e**,  
183 Schematics of human profilin-1 are also displayed for comparison. Note that the N-terminal helix  
184 in heimProfilin is reoriented by almost 60° as compared to the human profilin-1. The human  
185 profilin does not harbor the N-terminal extension and the N-terminal helix is slightly longer.

186

187 **Figure 2. Heimdallarchaeota encodes actin (heimActin) that polymerizes and is modulated**  
188 **by its heimProfilin. a,** Airyscan super-resolution microscopy of rabbit actin in presence of  
189 different concentrations of heimProfilin; 0  $\mu\text{M}$ , 128  $\mu\text{M}$  and 256  $\mu\text{M}$ , or 256  $\mu\text{M}$  of  $\text{N}\Delta$ -  
190 heimProfilin. **b,** Pyrene-polymerization profiles of 2  $\mu\text{M}$  rabbit actin (10% pyrene-labeled) alone  
191 (pink) or with different concentrations of heimProfilin; 19  $\mu\text{M}$  (black), 48  $\mu\text{M}$  (grey), 93  $\mu\text{M}$   
192 (olive), 137  $\mu\text{M}$  (red), 200  $\mu\text{M}$  (green), 250  $\mu\text{M}$  (blue) and 280 $\mu\text{M}$  (magenta). **c,** Pyrene-  
193 polymerization profiles of 1  $\mu\text{M}$  of rabbit actin (10% pyrene-labeled) alone (pink) or with  
194 different concentrations of  $\Delta\text{N}$ -heimProfilin; 20  $\mu\text{M}$  (black), 50  $\mu\text{M}$  (grey), 100  $\mu\text{M}$  (olive), 150  
195  $\mu\text{M}$  (red), 200  $\mu\text{M}$  (green), 250  $\mu\text{M}$  (blue). **d,** Electron microscopy (EM) images of heimActin  
196 forming thin, uniform filamentous polymers. **e,** Phosphate released during polymerization of  
197 heimActin (blue) or  $\Delta\text{C}$ -heimActin (red) as a function of temperature. **f,** Sedimentation assay for  
198 heimActin alone and with increasing concentrations of heimProfilin. **g,** Same as in f) but with  
199 heimActin and  $\Delta\text{N}$ -heimProfilin. Here, both  $\Delta\text{N}$ -heimProfilin and heimActin appear in the  
200 soluble fraction but not in the pellet, indicating that  $\Delta\text{N}$ -heimProfilin do not increase the  
201 polymerization of heimActin. **h,** Presence of heimProfilin increases the polymerization of  $\Delta\text{C}$ -  
202 heimActin.

203  
204 **Figure 3. Heimdallarchaeota profilin interacts with polyproline.** Isothermal titration  
205 calorimetric (ITC) binding measurements between heimProfilin **a)** or  $\Delta\text{N}$ -heimProfilin **b)** with  
206 polyproline motifs of VASP (PPPAPPLPAAQ). The heimProfilin showed weaker binding  
207 strength compared to the  $\Delta\text{N}$ -heimProfilin which had a  $K_D$  of  $\sim 200\mu\text{M}$ . **c and d,** Nuclear  
208 magnetic resonance (NMR)  $^1\text{H}$ - $^{15}\text{N}$  chemical shifts for the binding reaction of heimProfilin and  
209  $\Delta\text{N}$ -heimProfilin with polyproline (VASP) respectively. **e and f,** Expansions from c) and d)  
210 showing the movement of some interacting residues as the concentration of polyproline  
211 increases.

212  
213 **Figure 4. HeimProfilin N-terminal extension is important for interaction with**  
214 **phospholipids. a,** Overlay  $^1\text{H}$ - $^{15}\text{N}$  TROSY-HSQC of  $\Delta\text{N}$ -heimProfilin (400  $\mu\text{M}$ ) with  
215 increasing concentrations of Phosphatidylinositol-4,5-biphosphate ( $\text{PIP}_2$ ); 0  $\mu\text{M}$  (red) 150  $\mu\text{M}$   
216 (cyan), 300  $\mu\text{M}$  (magenta), 600  $\mu\text{M}$  (green), 1200  $\mu\text{M}$  (yellow), 2100  $\mu\text{M}$  (blue). **b,** Schematic of



217 heimProfilin with the interacting residues color coded. The interaction site appears to be located  
218 between the N- and C-terminal helix. **c-d**, Expansion of a) showing a few residues belonging to  
219 the b-strand residues F117-W120 and residues Q110-A111 are also strongly involved in the  
220 interaction. **e**, Overlay  $^1\text{H}$ - $^{15}\text{N}$  TROSY-HSQC of  $\Delta\text{N}$ -heimProfilin (300  $\mu\text{M}$ ) with increasing  
221 concentrations of D-myo-inositol-1,4,5-triphosphate ( $\text{IP}_3$ ); 0  $\mu\text{M}$  (red) 150  $\mu\text{M}$  (cyan), 300  $\mu\text{M}$   
222 (magenta), 600  $\mu\text{M}$  (green), 1200  $\mu\text{M}$  (yellow), 2100  $\mu\text{M}$  (blue). **f**, Similar to a) but with  
223 heimProfilin and  $\text{PIP}_2$ . **g**, Similar to e) but with heimProfilin interacting with  $\text{IP}_3$ . No chemical  
224 shift changes were observed for heimProfilin-  $\text{IP}_3$  interaction. **h**, Expansion of a region in f).

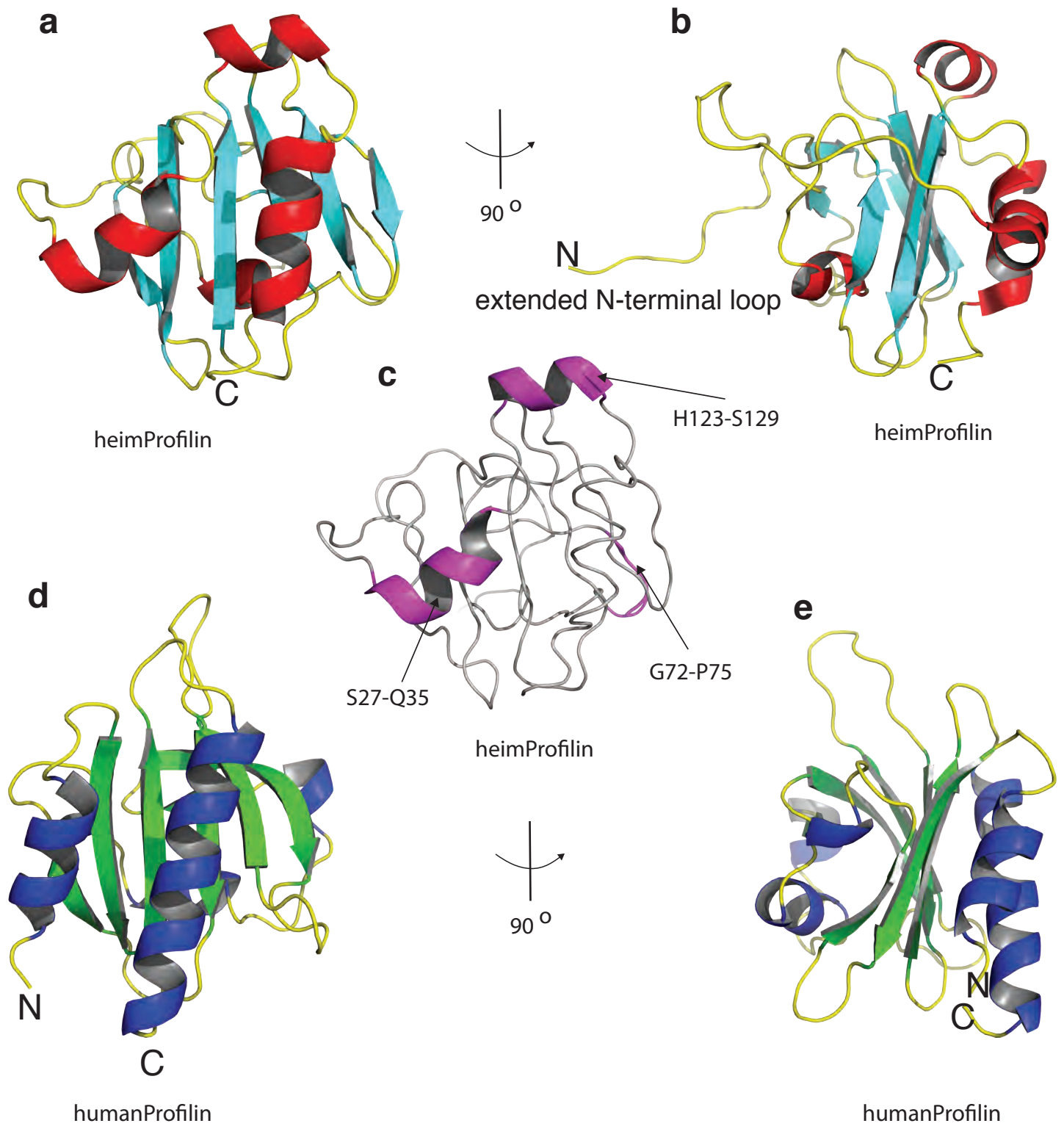
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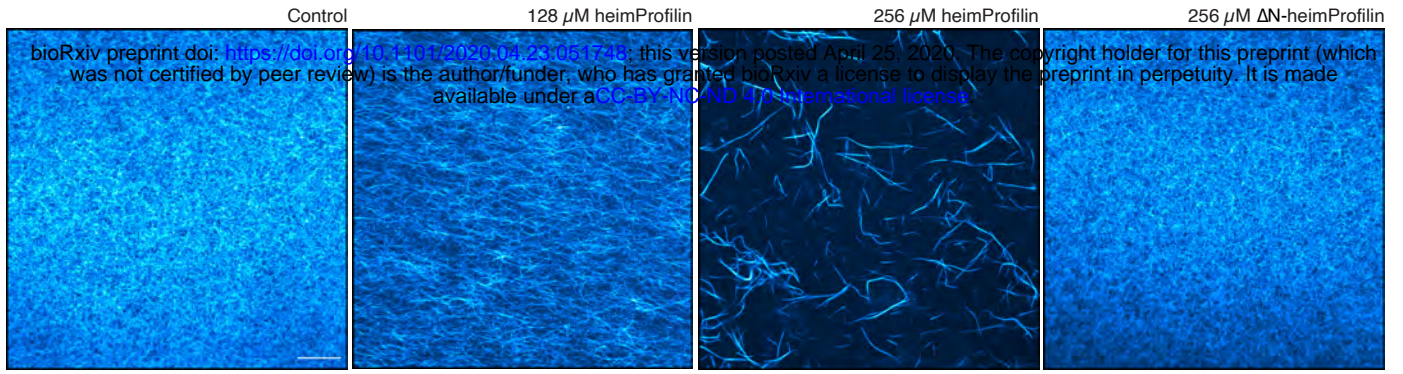
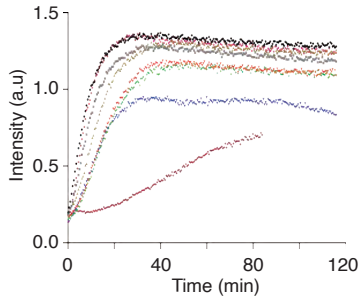
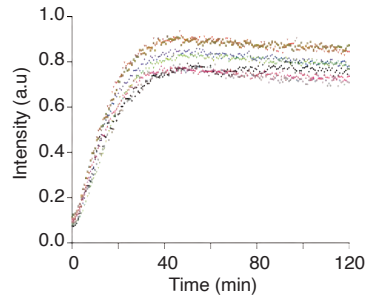
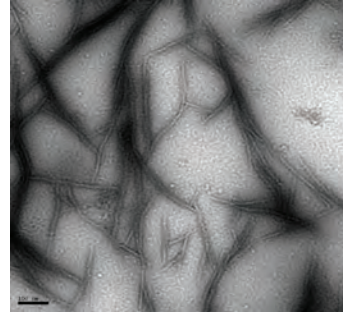
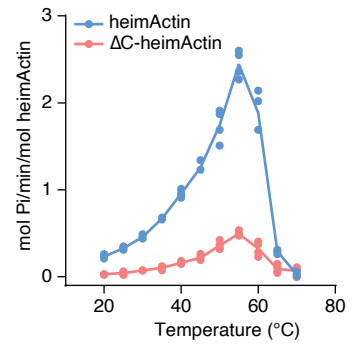
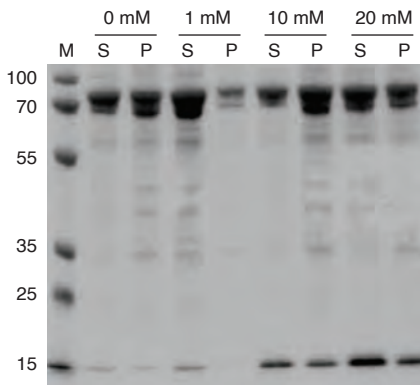
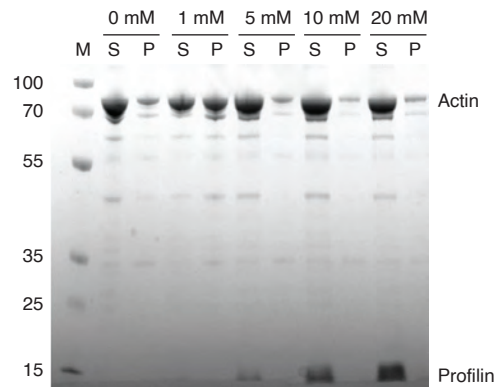
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261 **Author's contribution:** S. S, F. H, S. R. H, A-C. L and C. C conceived the project. S. S, S. R. H  
262 and FH cloned, expressed and purified all proteins. SS and SRH performed the pyrene and  
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264 electron microscopy. C. C performed all NMR and ITC experiments. C. C wrote the paper with  
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