

1 **An oligomeric state-dependent switch in FICD regulates**  
2 **AMPylation and deAMPylation of the chaperone BiP**

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15 **Impact Statement**

16

17 Unique amongst known chaperones, the endoplasmic reticulum (ER)-localized  
18 Hsp70, BiP, is subject to transient inactivation under conditions of low ER stress  
19 by reversible, covalent modification – AMPylation. The enzyme responsible for  
20 this modification, FICD, is in fact a bifunctional enzyme with a single active site  
21 capable of both AMPylation and deAMPylation. Here we elucidate, by  
22 biochemical, biophysical and structural means, the mechanism by which this  
23 enzyme is able to switch enzymatic modality: by regulation of its oligomeric  
24 state. The oligomeric state-dependent reciprocal regulation of FICD activity is, in  
25 turn, sensitive to the ATP/ADP ratio. This allosteric pathway potentially  
26 facilitates the sensing of unfolded protein load in the ER and permits the  
27 transduction of this signal into a post-translational buffering of ER chaperone  
28 activity.

29 **Abstract**

30

31 AMPylation is an inactivating modification that matches the activity of the major  
32 endoplasmic reticulum (ER) chaperone BiP to the burden of unfolded proteins. A single  
33 ER-localised Fic protein, FICD (HYPE), catalyses both AMPylation and  
34 deAMPylation of BiP. However, the basis for the switch in FICD's activity is unknown.  
35 We report on the transition of FICD from a dimeric enzyme, that deAMPylates BiP, to  
36 a monomer with potent AMPylation activity. Mutations in the dimer interface or in  
37 residues tracing an inhibitory relay from the dimer interface to the enzyme's active site  
38 favour BiP AMPylation in vitro and in cells. Mechanistically, monomerisation relieves  
39 a repressive effect allosterically-propagated from the dimer interface to the inhibitory  
40 Glu234, thereby permitting AMPylation-competent binding of MgATP. Whereas, a  
41 reciprocal signal propagated from the nucleotide binding site, provides a mechanism  
42 for coupling the oligomeric-state and enzymatic activity of FICD to the energy status  
43 of the ER.

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45 (148 Words)

46

47 **Introduction**

48 In all domains of life, protein folding homeostasis is achieved by balancing the burden  
49 of unfolded proteins and the complement of chaperones. In the endoplasmic reticulum  
50 (ER) of animal cells, this match is facilitated by the unfolded protein response (UPR).  
51 In addition to well-recognized transcriptional and translational strands of the UPR  
52 (Walter & Ron, 2011), recent findings have drawn attention to the existence of rapid  
53 post-translational mechanisms that adjust the activity of the ER Hsp70 chaperone BiP.  
54 Best understood amongst these is AMPylation, the covalent addition of an AMP moiety  
55 from ATP onto a hydroxyl group-containing amino acid side chain.

56 AMPylation conspicuously occurs on Thr518 of BiP (Preissler *et al*, 2015b; Broncel *et*  
57 *al*, 2016; Casey *et al*, 2017). The resulting BiP-AMP is locked in a domain-coupled  
58 ATP-like state (Preissler *et al*, 2015b, 2017b; Witeska *et al*, 2017). Consequently, BiP-  
59 AMP has high rates of client protein dissociation (Preissler *et al*, 2015b). Moreover,  
60 the ATPase activity of BiP-AMP is resistant to stimulation by J-protein co-factors,  
61 which greatly reduces the chaperone's ability to form high-affinity complexes with its  
62 clients (Preissler *et al*, 2017b). AMPylation therefore serves to inactivate BiP. This  
63 modification is temporally dynamic and the levels of BiP-AMP respond to changes of  
64 the protein folding load in the ER.

65 Consistent with its inactivating character, BiP modification in cells is enhanced by  
66 inhibition of protein synthesis (Laitusis *et al*, 1999) or during recovery from ER stress;  
67 when BiP levels exceed the requirements of unfolded client proteins (Preissler *et al*,  
68 2015b). Conversely, as levels of ER stress increase, modification is reversed by  
69 deAMPylation, recruiting BiP back into the chaperone cycle (Laitusis *et al*, 1999;  
70 Chambers *et al*, 2012; Preissler *et al*, 2015b). Accordingly, BiP modification creates a  
71 readily-accessible pool of latent folding capacity that buffers both ER stress (through  
72 deAMPylation) and over-chaperoning (through AMPylation). These features may  
73 contribute to the observation whereby in the *Drosophila* visual system, loss of the  
74 ability to AMPylate BiP results in light-induced blindness (Rahman *et al*, 2012;  
75 Moehlman *et al*, 2018).

76 AMPylation of BiP is mediated by the ER-localised enzyme FICD (filamentation  
77 induced by cAMP domain protein, also known as HYPE) (Ham *et al*, 2014; Sanyal *et*  
78 *al*, 2015; Preissler *et al*, 2015b). FICD is the only known metazoan representative of a  
79 large family of bacterial Fic-domain proteins (Khater & Mohanty, 2015a). Fic proteins

80 contain a conserved active site motif, HPFx(D/E)GN(G/K)R<sub>1</sub>XXR<sub>2</sub>, and many possess  
81 a glutamate-containing inhibitory alpha helix ( $\alpha_{inh}$ ) responsible for auto-inhibition of  
82 their canonical AMPylation activity (Engel *et al*, 2012; Goepfert *et al*, 2013). FICD is  
83 a class II Fic protein (with its  $\alpha_{inh}$  N-terminal to its Fic domain) and an ER-localised  
84 type II, single-pass transmembrane protein, with a short cytoplasmic portion and a large  
85 luminal-facing catalytic domain (Worby *et al*, 2009; Bunney *et al*, 2014).

86 Crystal structures of FICD and other Fic domain proteins suggest that engagement of  
87 Glu234 (of the  $\alpha_{inh}$ ) with Arg374 (R<sub>2</sub> of the Fic motif) prevents binding of MgATP in  
88 a conformation conducive to catalysis (Engel *et al*, 2012; Goepfert *et al*, 2013; Bunney  
89 *et al*, 2014; Truttmann *et al*, 2016). Moreover, *in vitro*, modification of BiP by purified  
90 FICD requires mutation of Glu234; an observation suggesting that an AMPylation  
91 repressed state is favoured by wild-type FICD. Remarkably, the Fic domain of FICD is  
92 also responsible for BiP deAMPylation; an activity that depends on Glu234 (Preissler  
93 *et al*, 2017a; Casey *et al*, 2017) and magnesium (Veyron *et al*, 2019). These findings  
94 point to deAMPylation as the default activity of the bifunctional enzyme and implicate  
95 Glu234 in a functional switch between the two antagonistic activities of the Fic active  
96 site.

97 The Fic domain of human FICD forms a stable back-to-back asymmetric dimer via two  
98 dimerisation surfaces (Bunney *et al*, 2014; Truttmann *et al*, 2016) and a monomerising  
99 mutation in the dimer interface of *Drosophila* FICD did not block BiP deAMPylation  
100 *in vitro* (Casey *et al*, 2017). Nonetheless, distantly related bacterial enzymes hint at a  
101 possible regulatory role for Fic dimerisation: a mutation in *Clostridium difficile* Fic  
102 (CdFic) dimer interface increased auto-AMPylation (Dedic *et al*, 2016) and changes in  
103 oligomeric state affected the activity of the class III Fic protein from *Neisseria*  
104 *meningitidis* (NmFic) (Stanger *et al*, 2016).

105 Here we report on the biochemical and structural basis of an oligomeric state-dependent  
106 switch in FICD's activity, which is well suited to post-translationally regulate protein  
107 folding homeostasis in the ER.

108 **Results**

109 **Disrupting the FICD dimer favours BiP AMPylation**

110 Whilst the *FICD* gene is necessary for BiP AMPylation, over-expression of the wild-  
111 type FICD enzyme does not result in a detectable pool of BiP-AMP in cells (Preissler  
112 *et al*, 2015b). These findings were explained in terms of dominance of the  
113 deAMPylation activity of wild-type FICD, as observed in vitro (Preissler *et al*, 2017a).  
114 However, somewhere between low-level endogenous expression, which yields  
115 physiologically-regulated AMPylation, and over-expression, which precludes BiP-  
116 AMP accumulation, retrovirally-rescued *FICD*<sup>-/-</sup> cells were endowed with a measure of  
117 BiP AMPylation (Figure 1A and S1A-C). This finding points to a protein-dosage effect  
118 on wild-type FICD's activity and suggests that the enzymatic mode of (recombinant)  
119 FICD may be affected by its concentration in the ER.

120 Purified FICD forms a homodimeric complex in vitro (Bunney *et al*, 2014). Co-  
121 expression of reciprocally-tagged FICD confirmed that the wild-type protein forms  
122 homomeric complexes in cells that are disrupted by a previously characterised  
123 Leu258Asp mutation within the major dimerisation surface (Bunney *et al*, 2014)  
124 (Figure 1B). Unlike the wild-type dimerisation-competent enzyme, at a similar level of  
125 over-expression, the monomeric FICD<sup>L258D</sup> yielded a clear BiP-AMP signal in *FICD*<sup>-/-</sup>  
126 cells (Figure 1C). This pool was conspicuous even under basal conditions, in which  
127 wild-type cells have only a weak BiP-AMP signal, suggesting that the imposed  
128 monomeric state deregulated FICD's activity.

129 Together, these observations intimate that dynamic changes in the equilibrium between  
130 the monomer and dimer may contribute to a switch between FICD's mutually  
131 antagonistic activities – AMPylation and deAMPylation of BiP. Increasing its  
132 concentration by over-expression favours FICD dimerisation and thus perturbs such  
133 regulatory transitions. This could account for the observation that FICD  
134 overexpression, in unstressed wild-type cells, abolishes the small pool of BiP-AMP  
135 normally observed under basal conditions (Preissler *et al*, 2017b).

136 Size-exclusion chromatography (SEC) and analytical ultracentrifugation (AUC), with  
137 purified proteins, confirmed the stability of the FICD dimer (Figure 1D-E and S1D-G).  
138 These techniques also confirmed the strong disrupting effect of the Leu258Asp  
139 mutation (in the principal dimer surface) and revealed a weaker disrupting effect of a

140 Gly299Ser mutation (in the secondary dimer surface) ([Figure S1D-G](#)). AUC yielded a  
141 1.2 nM dimer dissociation constant ( $K_d$ ) of wild-type FICD and SEC indicated a  $K_d$  in  
142 the millimolar range for FICD<sup>L258D</sup> and a  $K_d$  of 9.5  $\mu$ M for FICD<sup>G299S</sup>. We therefore  
143 conclude that between 0.2  $\mu$ M and 5  $\mu$ M (concentrations at which the experiments that  
144 follow were performed) the wild-type protein is dimeric, FICD<sup>L258D</sup> is monomeric, and  
145 FICD<sup>G299S</sup> is partially monomeric.

146 In the presence of [ $\alpha$ -<sup>32</sup>P]-ATP both FICD<sup>L258D</sup> and FICD<sup>G299S</sup> established a pool of  
147 AMPylated, radioactive BiP in vitro [[Figure 1F](#); also observed in the *Drosophila*  
148 counterpart of FICD<sup>L258D</sup> (Casey *et al*, 2017)], whereas the wild-type enzyme did not,  
149 as previously observed (Preissler *et al*, 2015b, 2017a). BiP is a substrate for  
150 AMPylation in its monomeric, ATP-bound, domain-docked conformation (Preissler *et*  
151 *al*, 2015b, 2017b). These experiments were therefore performed with an ATPase-  
152 deficient, oligomerisation-defective, ATP-bound BiP mutant, BiP<sup>T229A-V461F</sup>. Thus, the  
153 BiP-AMP signal is a result of the concentration of substrate (unmodified and modified  
154 BiP) and the relative AMPylation and deAMPylation activities of the FICD enzyme.  
155 As expected, a strong BiP-AMP signal was elicited by the unrestrained AMPylation-  
156 active FICD<sup>E234G</sup> (which cannot deAMPylate BiP). FICD<sup>E234G-L258D</sup> gave rise to a  
157 similar, but reproducibly slightly weaker, BiP-AMP signal relative to FICD<sup>E234G</sup>.

## 158 **Monomerisation switches FICD's enzymatic activities**

159 The ability of the dimer interface FICD mutants to yield a detectable BiP-AMP signal  
160 in vitro agreed with the in vivo data and suggested a substantial change in the regulation  
161 of the enzyme's antagonistic activities – either inhibition of deAMPylation, de-  
162 repression of AMPylation, or a combination of both. To distinguish between these  
163 possibilities, we analysed the deAMPylation activities of the FICD mutants in an assay  
164 that uncouples deAMPylation from AMPylation. As previously observed, wild-type  
165 FICD caused the release of fluorescently labelled AMP from in vitro AMPylated BiP,  
166 whereas FICD<sup>E234G</sup> did not (Preissler *et al*, 2017a) ([Figure 2A](#)). FICD<sup>L258D</sup> and  
167 FICD<sup>G299S</sup> consistently deAMPylated BiP 2-fold slower than the wild-type ([Figure 2A](#)  
168 and [S2A](#)). The residual in vitro deAMPylation activity of FICD<sup>L258D</sup> and the absence  
169 of such activity in FICD<sup>E234G</sup> is consistent with the divergent effect of expressing these  
170 deregulated mutants on a UPR reporter in cells ([Figure S2B-C](#)).

171 The FICD-mediated BiP AMPylation/deAMPylation cycle converts the co-substrate  
172 ATP to the end products AMP and pyrophosphate (Preissler *et al*, 2017a). We exploited  
173 this feature to quantify enzymatic activity. FICD was incubated with [ $\alpha$ -<sup>32</sup>P]-ATP,  
174 either in the presence or absence of ATPase-deficient BiP<sup>T229A</sup>, and accumulation of  
175 radioactive AMP was measured by thin layer chromatography. Only background levels  
176 of AMP were generated by catalytically inactive FICD<sup>H363A</sup> or FICD<sup>E234G-H363A</sup> (Figure  
177 2B). The deregulated, deAMPylation-defective FICD<sup>E234G</sup> yielded a weak AMP signal  
178 that was not increased further by the presence of BiP, suggesting that the Glu234Gly  
179 mutation enables some BiP-independent ATP hydrolysis to AMP. Conversely, small  
180 but significant amounts of AMP were produced by wild-type FICD but in a strictly BiP-  
181 dependent fashion (Figure 2B-C and Figure S2D). These observations are consistent  
182 with a slow, FICD-driven progression through the BiP AMPylation/deAMPylation  
183 cycle indicating incomplete repression of wild-type FICD's AMPylation activity under  
184 these conditions. As expected, abundant BiP-dependent AMP production was observed  
185 in reactions containing AMPylation-active FICD<sup>E234G</sup> alongside deAMPylation-active  
186 wild-type FICD (Figure 2B, lane 11). Importantly, large amounts of AMP were also  
187 generated when BiP was exposed to FICD<sup>L258D</sup> and, to lesser extent, FICD<sup>G299S</sup> (Figure  
188 2C and S2D). Together, these observations suggest that the AMPylation activities of  
189 the monomeric FICD mutants are significantly enhanced relative to the wild-type,  
190 whilst their deAMPylation activities are more modestly impaired.

191 To directly assess the AMPylation activities of bifunctional FICDs we exploited the  
192 high affinity of the catalytically inactive FICD<sup>H363A</sup> for BiP-AMP, as a “trap” that  
193 protects BiP-AMP from deAMPylation (Figure 2D). To disfavour interference with the  
194 FICD enzyme being assayed we engineered the trap as a covalent disulfide linked dimer  
195 incapable of exchanging subunits with the active FICD being assayed. A cysteine  
196 (Ala252Cys) was introduced into the major dimerisation surface of the trap. To  
197 preclude aberrant disulphide bond formation, the single endogenous cysteine of FICD  
198 was also replaced (Cys421Ser). After purification and oxidation, this protein (s-  
199 sFICD<sup>A252C-H363A-C421S</sup>; the trap) formed a stable disulphide-bonded dimer (Figure S2E-  
200 F) that tightly bound BiP-AMP with fast association and slow dissociation kinetics  
201 (Figure S2G-H). Moreover, the binding of the trap to unmodified BiP was, in  
202 comparison, negligible (Figure S2G). We reasoned that adding the trap in excess to  
203 reactions assembled with BiP, ATP and FICD would sequester the BiP-AMP product

204 and prevent its deAMPylation, enabling the comparison of AMPylation rates in  
205 isolation from the deAMPylating activity.

206 In presence of the trap, wild-type FICD produced a detectable BiP-AMP signal; but not  
207 in the absence of the trap (compare [Figures 1F](#) and [2E](#)). Importantly, presence of the  
208 trap revealed that AMPylation of BiP was greatly accelerated by FICD monomerisation  
209 (> 19-fold compared to the wild-type) ([Figure 2E](#)). As expected, BiP AMPylation by  
210 FICD<sup>E234G</sup> was even faster.

211 If the enhanced AMPylation activity of the dimerisation-defective mutants, observed  
212 above, truly represents divergent enzymatic activities of different FICD oligomeric  
213 states, it should be possible to reveal this feature by diluting the wild-type enzyme to  
214 concentrations at which an appreciable pool of monomer emerges. In AMPylation  
215 reactions set up with [ $\alpha$ -<sup>32</sup>P]-ATP a detectable signal from radiolabelled BiP-AMP was  
216 noted at enzyme concentrations near the  $K_d$  of dimerisation (between 10 and 2.5 nM;  
217 [Figure 3A, left](#)). The inverse relationship of enzyme concentration to the BiP-AMP  
218 signal likely reflects the opposing activities and relative populations of AMPylation-  
219 biased FICD monomers and the deAMPylation-biased FICD dimers in each reaction.  
220 This counter-intuitive relationship of enzyme to product is resolved in the presence of  
221 the AMPylation trap; the BiP-AMP signal increased in a time- and enzyme  
222 concentration-dependent manner, as expected from a reaction which is proportional to  
223 the absolute concentration of monomeric enzyme ([Figure 3A, right](#)). In the presence of  
224 the trap the shift in the peak of the BiP-AMP signal, after 16 hours, towards lower  
225 concentrations of FICD, likely reflects incomplete protection of AMPylated BiP by the  
226 trap and its enhanced susceptibility to deAMPylation at higher concentrations of  
227 (dimeric) FICD.

228 If monomerisation significantly enhances AMPylation activity, constitutive FICD  
229 dimers that are unable to dissociate should have low AMPylation activity and fail to  
230 produce modified BiP even under dilute conditions. To test this prediction, we created  
231 a disulphide-linked wild-type FICD (s-sFICD<sup>A252C-C421S</sup>), which, after purification and  
232 oxidation, formed a covalent dimer ([Figure S3A](#)). Moreover, its SEC profile was  
233 indistinguishable from wild-type FICD or the cysteine-free counterpart, FICD<sup>C421S</sup>  
234 ([Figure S3B](#)). In the presence of the BiP-AMP trap, oxidised s-sFICD<sup>A252C-C421S</sup>  
235 produced significantly less AMPylated BiP than either wild-type or FICD<sup>C421S</sup> at  
236 similar concentrations ([Figure 3B, lane 8](#) and [S3C](#)).

237 Repression of AMPylation was imposed specifically by the covalent dimer, as non-  
238 oxidised FICD<sup>A252C-C421S</sup> elicited a conspicuous pool of BiP-AMP - more than the wild-  
239 type enzyme (Figure 3B, lane 9 and S3C) - an observation explained by the weakening  
240 of the FICD dimer imposed by the Ala252Cys mutation (Figure S1D-E). Similarly, in  
241 absence of the trap, the ability of pre-oxidised s-sFICD<sup>A252C-C421S</sup> to establish a pool of  
242 AMPylated BiP was greatly enhanced by diluting the enzyme into a buffer containing  
243 DTT. FICD<sup>C421S</sup>, by contrast, produced similar amounts of modified BiP under both  
244 non-reducing and reducing conditions (Figure 3C).

245 DeAMPylation activities of oxidised and non-oxidised FICD<sup>A252C-C421S</sup> were  
246 comparable and similar to wild-type FICD (Figure 3D-E, S2A and S3D), pointing to  
247 the integrity of these mutant enzymes. Together, these observations argue that covalent  
248 s-sFICD<sup>A252C-C421S</sup> dimers selectively report on the enzymatic characteristics of wild-  
249 type FICD in its dimeric state. This protein therefore serves to help validate the  
250 conclusion that a low concentration of wild-type FICD favours the formation of  
251 monomers, whose AMPylation activity is de-repressed, to promote BiP modification.

252 **An AMPylation-repressive signal is transmitted from the dimer interface to the**  
253 **active site**

254 The crystal structure of dimeric FICD suggests the existence of a hydrogen-bond  
255 network, involving the side-chains of Lys256 and Glu242, linking the dimer interface  
256 with the enzyme's active site, impinging on the AMPylation-inhibiting Glu234 (Figure  
257 4A). To test this notion, we mutated both putative dimer relay residues. FICD<sup>K256S</sup> and  
258 FICD<sup>E242A</sup> formed stable dimers, as assayed by SEC, with dimer  $K_d$  values under 400  
259 nM (Figure 4B and S1D-E). In vitro both mutants established a pool of modified BiP  
260 (Figure 4C and S4A). This remained the case even at FICD concentrations in which  
261 negligible amounts of monomer are predicted (2 and 10  $\mu$ M; Figure S4A). De-  
262 repression of AMPylation by these dimer relay mutations was also evidenced by the  
263 enhanced BiP-dependent AMP production, relative to wild-type FICD (Figure 4D),  
264 whilst deAMPylation activities were similar (Figure S2A and S4B). Combining the  
265 Lys256Ser and the monomerising Leu258Asp mutations (FICD<sup>K256S-L258D</sup>) further  
266 enhanced the BiP-AMP pool produced in vitro (Figure S4A), an observation only  
267 partially attributable to the concomitant decrease in the deAMPylation rate (Figure S2A  
268 and S4C). These observations suggest that residues connecting the dimer interface and

269 the active site contribute to repression of AMPylation and that mutating these residues  
270 uncouples a gain-of-AMPylation activity from the oligomeric state of FICD.

271 Transmission of a repressive signal via a network of intramolecular interactions is also  
272 supported by the correlation between de-repression of BiP AMPylation and the  
273 negative effect of various mutants on the global stability of FICD. Differential scanning  
274 fluorimetry (DSF) revealed an inverse relationship between the AMPylation activity  
275 and the melting temperature ( $T_m$ ) of FICD mutants (Figure 4E and S4D). These  
276 differences in flexibility were observed despite the fact that the DSF assays were  
277 conducted at relatively high protein concentrations (2  $\mu$ M) that would favour  
278 dimerisation of all but the most dimerisation-defective mutants.

279 Nucleotide binding stabilises all FICD variants (Figure S4D), a feature that is  
280 conspicuous in case of the AMPylation de-repressed FICD<sup>E234G</sup> (Bunney *et al*, 2014).  
281 However, monomerisation imposed by the Leu258Asp mutation, did not significantly  
282 increase ATP-induced stabilisation of FICD ( $\Delta T_m$ ) (Figure 4F and S4E). Interestingly,  
283 although AMPylation activity correlated with increased FICD flexibility this was not  
284 reflected in an appreciably altered propensity to bind ATP. This suggested that the  
285 variation in enzyme activity of different FICD mutants may arise not from variation in  
286 their affinity for nucleotide but from their particular mode of ATP binding. To explore  
287 this possibility, we set out to co-crystallise FICD variants with MgATP.

## 288 **Monomerisation favours AMPylation-competent binding of MgATP**

289 High-resolution X-ray crystal structures of monomeric and dimeric FICD were  
290 obtained in various nucleotide bound states (Table 1). The tertiary structure of the Fic  
291 domain of both the monomeric FICD<sup>L258D</sup> and the dimeric relay mutant FICD<sup>K256S</sup>  
292 deviated little from that of the nucleotide-free wild-type dimer structure (FICD:Apo;  
293 PDB: 4u04) (Figure 5A and S5A). Moreover, co-crystallisation of FICD<sup>L258D</sup>,  
294 FICD<sup>K256A</sup> or the wild-type dimer with ATP or an ATP analogue (AMPPNP) also  
295 resulted in no significant Fic domain conformational change from FICD:Apo (Figure  
296 5A and S5A). Accordingly, the greatest root-mean squared deviation (RMSD) between  
297 the Fic domain of the FICD:ATP structure and any other monomeric or dimer relay  
298 FICD structure is 0.53  $\text{\AA}$  (observed between FICD:ATP and FICD<sup>L258D</sup>:Apo; residues  
299 213-407). The only conspicuous change in global tertiary structure occurred in the TPR  
300 domain of FICD<sup>L258D</sup> co-crystallised with ATP or AMPPNP, in which the TPR domain

301 is flipped almost 180° from its position in other FICD structures (Figure 5A). Notably,  
302 in all FICD structures the  $\alpha_{inh}$  remains firmly juxtaposed to the core Fic domain.

303 When co-crystallised with MgATP or MgAMPPNP the resulting FICD structures  
304 contained clear densities for nucleotide (Figure 5B and S5B). The AMPylation-biased  
305 FICD mutants also contained discernible, octahedrally coordinated Mg<sup>2+</sup> ions (Figure  
306 5Bii-iii and S5B). As noted in other Fic AMPylases, this Mg<sup>2+</sup> was coordinated by the  
307  $\alpha$ - and  $\beta$ -phosphates of ATP/AMPPNP and Asp367 of the Fic motif (Xiao *et al*, 2010;  
308 Khater & Mohanty, 2015b; Bunney *et al*, 2014). Interestingly, in the dimeric wild-type  
309 FICD:ATP structure, crystallised in the presence of MgATP, there was no density that  
310 could be attributed to Mg<sup>2+</sup> (Figure 5Bi). The only possible candidate for Mg<sup>2+</sup> in this  
311 structure was a water density, located between all three phosphates, that fell in the Fic  
312 motif's anion-hole – a position incompatible with Mg<sup>2+</sup> coordination (Zheng *et al*,  
313 2017).

314 Alignment of the nucleotide-bound structures revealed that ATP or AMPPNP were  
315 bound very differently by the wild-type dimer and the AMPylation-biased monomeric  
316 or dimer relay FICD mutants (Figure 5C and S5C). Concordantly, the RMSD of ATP  
317 between the wild-type FICD and monomeric FICD<sup>L258D</sup> was 2.17 Å (and 2.23 Å for  
318 FICD<sup>K256A</sup>'s ATP). As previously observed in other ATP-bound Fic proteins that  
319 possess an inhibitory glutamate, the nucleotide in FICD:ATP was in an AMPylation  
320 non-competent conformation (Engel *et al*, 2012; Goepfert *et al*, 2013) that is unable to  
321 coordinate Mg<sup>2+</sup>; an essential ion for FICD-mediated AMPylation (Ham *et al*, 2014).  
322 Moreover, the position of the ATP  $\alpha$ -phosphate precludes in-line nucleophilic attack  
323 (by the hydroxyl group of BiP's Thr518) due to the proximity of the flap residue Val316  
324 (Figure 5C and S5D). Furthermore, an attacking nucleophile in-line with Pa-O3 $\alpha$   
325 would be at a considerable distance from the catalytic His363 (required to deprotonate  
326 Thr518's hydroxyl group) (Figure 5Bi, 5C and S5D).

327 By contrast, in the active sites of FICD<sup>K256A</sup> or FICD<sup>L258D</sup> MgATP and MgAMPPNP  
328 assumed AMPylation-competent conformations: their  $\alpha$ -phosphates were in the  
329 canonical position (Figure S5E), as defined by AMPylation-active Fic proteins lacking  
330 inhibitory glutamates (Xiao *et al*, 2010; Engel *et al*, 2012; Goepfert *et al*, 2013; Bunney  
331 *et al*, 2014). As a result, in-line nucleophilic attack into the  $\alpha$ - $\beta$ -phosphoanhydride bond

332 of ATP would not be sterically hindered and the Nε2 of His363 would be well  
333 positioned for general base catalysis ([Figure 5C](#) and [S5C-D](#)).

334 The presence of ATP in both dimeric wild-type FICD and monomeric FICD<sup>L258D</sup>  
335 (although in different binding modes) is consonant with the DSF data ([Figure 4F](#) and  
336 [S4E](#)). Apart from Glu234, the residues directly interacting with ATP are similarly  
337 positioned in all structures (maximum RMSD 0.83 Å). However, considerable  
338 variability is observed in Glu234, with an RMSD of 4.20 Å between monomeric and  
339 dimeric wild-type ATP structures, which may hint at the basis of monomerisation-  
340 induced AMPylation competency. In ATP-bound structures the inhibitory glutamate is  
341 displaced from the respective apo ground-state position, in which it forms an inhibitory  
342 salt-bridge with Arg374: R<sub>2</sub> of the Fic motif ([Figure S6A](#)). However, the displacement  
343 of the Glu234 side chain observed in the FICD:ATP structure (from its position in  
344 FICD:Apo; PDB 4u0u) would be insufficient for AMPylation-competent binding of the  
345 γ-phosphate of an ATP/AMPPNP (see distances i and ii, [Figure 5C](#) and [S5C](#)). This  
346 steric clash is relieved by the side chain conformations observed in the AMPylation-  
347 competent structures (see iii and iv, [Figure 5C](#) and [S5C](#)).

348 The findings above suggest that the AMPylation-biased FICD mutants attain their  
349 ability to competently bind MgATP by increased flexibility at the top of the  $\alpha_{inh}$  and by  
350 extension through increased Glu234 dynamism. It is notable that all the nucleotide  
351 triphosphate-bound FICDs crystallised with intact dimer interfaces ([Figure S6A](#) and  
352 [B](#)). Moreover, with the exception of direct hydrogen bonds to mutated Lys256 side  
353 chains, in all FICD crystals the putative dimer relay hydrogen-bond network was  
354 maintained ([Figure S6A](#)). It seems likely that much of the monomerisation-linked  
355 conformational flexibility that facilitates binding of MgATP in solution cannot be  
356 trapped crystallographically. Nonetheless, comparing B-factors across the nucleotide  
357 triphosphate-bound FICD structures is informative: despite similar crystal packing  
358 ([Figure S6B](#)) the average residue B-factors, both in the dimerisation interface and near  
359 Glu234, positively correlated with the AMPylation activities of the respective mutants  
360 ([Figure S7](#)).

### 361 **ATP is an allosteric modulator of FICD**

362 Given the conspicuous difference in the ATP binding modes observed between  
363 AMPylation-competent FICD mutants and the AMPylation-incompetent wild-type

364 dimeric FICD, we were intrigued by the possibility that ATP may modulate other  
365 aspects of FICD enzymology and regulation.

366 In order to explore the effects of nucleotide on the different pre-AMPylation complexes  
367 formed between either dimeric or monomeric FICD and its co-substrate, ATP-bound  
368 BiP, we utilised BioLayer Interferometry (BLI). Biotinylated, client-binding-impaired,  
369 ATPase-defective BiP<sup>T229A-V461F</sup> was made nucleotide free (Apo) and immobilised on  
370 a streptavidin biosensor. Its interactions with catalytically inactive, dimeric FICD<sup>H363A</sup>  
371 or catalytically inactive, monomeric FICD<sup>L258D-H363A</sup> were measured in the presence  
372 and absence of nucleotides. The binding of both monomeric and dimeric FICD to  
373 immobilised BiP was greatly enhanced by the pre-saturation of BiP with ATP (Figure  
374 6A and S8A). This is consistent with ATP-bound BiP as the substrate for FICD-  
375 mediated AMPylation (Preissler *et al*, 2015b). Moreover, the binding signal produced  
376 by immobilised, ATP-bound BiP interacting with monomeric FICD<sup>L258D-H363A</sup>:Apo was  
377 significantly stronger than that produced from the corresponding dimeric  
378 FICD<sup>H363A</sup>:Apo analyte (Figure 6A). In contrast, AMPylated BiP bound more tightly to  
379 dimeric FICD<sup>H363A</sup> than to monomeric FICD<sup>L258D-H363A</sup> (forming a pre-deAMPylation  
380 complex, Figure S2G). These findings align with the role of dimeric FICD in  
381 deAMPylation and the monomer in AMPylation.

382 Interestingly, in presence of magnesium bound nucleotide (either MgATP or MgADP)  
383 the FICD<sup>H363A</sup> interaction with ATP-bound BiP was weakened (Figure 6A). This effect  
384 was considerably more pronounced for monomeric FICD<sup>L258D-H363A</sup>. To quantify the  
385 effect of FICD monomerisation on the kinetics of pre-AMPylation complex  
386 dissociation, BLI probes preassembled with biotinylated, ATP-bound BiP and either  
387 apo dimeric FICD<sup>H363A</sup> or apo monomeric FICD<sup>L258D-H363A</sup> were transferred into  
388 otherwise identical solutions  $\pm$  ATP (schematised in Figure S8B). The ensuing  
389 dissociations fit biphasic exponential decays and revealed that ATP binding to FICD  
390 accelerated the dissociation of monomeric FICD<sup>H363A</sup> more than dimeric FICD<sup>H363A</sup>  
391 (Figure 6B and S8C). The effect of ATP was noted on both the slow dissociation phase  
392 of the monomer ( $k_{off,slow}$ ; Figure 6C-D) and on the percentage of dissociation attributed  
393 to the fast phase (%Fast; Figure 6D and S8D). The effect of ATP on the dissociation  
394 kinetics of the FICD<sup>L258D-H363A</sup>/BiP:ATP complex, measured under conditions of  
395 effectively infinite dilution, argues against a simple one-site competition between ATP-

396 bound BiP and ATP for the Fic domain active site. Instead, these observations are better  
397 explained as allosteric modulation of monomeric FICD by ATP.

398 The structural data indicates that FICD's oligomeric state can impact significantly on  
399 the mode of ATP binding, and [Figure 6B](#) indicates an allosteric effect of nucleotide  
400 binding on FICD. Together these observations suggested bi-directional intramolecular  
401 signalling from the dimer interface to the nucleotide-binding active site and therefore  
402 the possibility that ATP binding in FICD's active site may also influence the oligomeric  
403 state of the protein. To investigate this hypothesis, hetero-dimers of N-terminally  
404 biotinylated FICD<sup>H363A</sup> assembled with non-biotinylated FICD<sup>H363A</sup> were loaded onto  
405 a BLI streptavidin biosensor. The dissociation of non-biotinylated FICD<sup>H363A</sup> from its  
406 immobilised partner was then observed by infinite dilution into buffers varying in their  
407 nucleotide composition ([Figure 6E](#) and [S8E](#), schematised in [Figure 6F](#)). ATP but not  
408 ADP induced a 3-fold increase in the dimer off rate ([Figure 6G](#)). This is suggestive of  
409 a mechanism whereby changing ATP/ADP ratios in the ER may modulate the  
410 oligomeric state of FICD.

411

412 **Discussion**

413 This study addresses a key process in the post-translational UPR by which bifunctional  
414 FICD switches between catalysis of BiP AMPylation and deAMPylation, in order to  
415 match the folding capacity of the ER to the burden of unfolded proteins independently  
416 of changes in gene expression. The high affinity of FICD protomers for each other  
417 specifies the presence of principally dimeric FICD in the ER, shown here to restrict the  
418 enzyme to deAMPylation. This is the dominant mode of FICD both in vitro and in cells  
419 under basal conditions (Preissler *et al*, 2017a; Casey *et al*, 2017). However, establishing  
420 a pool of monomeric FICD unmasks its potential as a BiP AMPylase and enfeebles  
421 deAMPylation. The structural counterpart to this switch is the mode by which MgATP,  
422 the AMPylation reaction's co-substrate, is productively engaged in the active site of the  
423 monomeric enzyme. Our studies suggest that monomerisation relieves the repression  
424 imposed on FICD AMPylation by weakening a network of intramolecular contacts. In  
425 the repressed state these contacts propagate from the dimer interface to the enzyme's  
426 active site and stabilise a conserved inhibitory residue, Glu234, to block AMPylation-  
427 competent binding of MgATP (Figure 7).

428 Our observations of a biphasic FICD concentration-dependent rescue of BiP  
429 AMPylation in *FICD*<sup>-/-</sup> cells and the conspicuous ability of the monomerising  
430 Leu258Asp mutation to establish a modified BiP pool in *FICD*<sup>-/-</sup> cells, all support an  
431 oligomeric state-dependent switch as a key contributor to FICD regulation in vivo. This  
432 case is further supported by the divergent enzymatic properties of monomeric mutants  
433 and enforced disulphide-linked dimers in vitro, and by measurements of the enzymatic  
434 activity of wild-type FICD in concentration regimes above and close to the dimerisation  
435 *K<sub>d</sub>*. Complete monomerisation resulted in a 19-fold increase in AMPylation activity and  
436 a 2-fold decrease in deAMPylation activity. The concordance between monomeric  
437 FICD<sup>L258D</sup>, dimerisation-defective mutants, and mutants in the repressive relay from  
438 the dimer interface to the active site gives confidence in the validity of the biophysical  
439 and structural insights provided by the mutants.

440 The inverse correlation observed between the thermal stability of FICD mutants and  
441 their AMPylation activity, supports a role for enhanced flexibility in enabling the  
442 enzyme to attain the conformation needed for catalysis of this reaction – a role clarified  
443 by the crystallographic findings (see below). The biophysical assays also suggest that  
444 monomeric FICD is more allosterically sensitive to ATP binding, as it exhibits a

445 pronounced nucleotide-dependent reduction in the affinity for its co-substrate, ATP-  
446 bound BiP. The observation that ATP significantly accelerated the dissociation of  
447 monomeric, nucleotide-free FICD from ATP-bound BiP suggests that this feature of  
448 the monomer is mediated allosterically (not by enhanced susceptibility of a destabilised  
449 protein to co-substrate competition for the same active site). The lower affinity of  
450 monomeric FICD for its BiP:ATP co-substrate, in the context of a quaternary pre-  
451 AMPylation complex, conspicuously distinguishes it from the dimer and is a feature  
452 that may also enhance AMPylation rates: ground-state destabilisation has been  
453 demonstrated in a number of enzymes as a means of catalytic rate enhancement, by  
454 reducing the otherwise anti-catalytic tight binding of an enzyme to its substrate  
455 (Andrews *et al*, 2013; Ruben *et al*, 2013).

456 A structure of the quaternary pre-AMPylation complex, that could inform our  
457 understanding of the features of the monomeric enzyme, does not exist. Nevertheless,  
458 important insights into the effect of monomerisation were provided by structures of  
459 FICD and its nucleotide co-substrate. Dimeric wild-type FICD binds ATP (without  
460 magnesium) in an AMPylation incompetent mode. This is consistent with all other  
461 inhibitory glutamate containing Fic structures crystallised with ATP or ATP analogues  
462 (Engel *et al*, 2012; Goepfert *et al*, 2013). In stark contrast, we have discovered that  
463 despite the presence of an inhibitory glutamate, monomerisation, or mutations in  
464 residues linking the dimer interface to Glu234, permit the binding of ATP with  
465 magnesium in a conformation competent for AMPylation.

466 Our studies suggest that the disparity in FICD's ATP binding modes stems from a  
467 monomerisation-induced increase in Glu234 flexibility (mediated by weakening of the  
468 dimer relay). This increase in flexibility is reflected in relatively subtle changes in the  
469 Glu234 side chain position, B-factor increases in the respective crystal structures, and  
470 markedly lower melting temperature of FICD<sup>K256A</sup> and FICD<sup>L258D</sup> relative to the wild-  
471 type dimer.

472 It seems likely that in solution monomerisation allows greater flexibility in this dimer  
473 relay network, facilitating motion and possibly unfolding at the top of the Glu234  
474 containing  $\alpha$ -helix ( $\alpha_{inh}$ ). Such considerations could explain the comparatively small  
475 differences in the position of Glu234, but stark differences in nucleotide conformation,  
476 observed between the dimeric wild-type and monomeric or dimer relay mutant

477 structures. That is to say, in solution the mutants exhibit sufficiently increased Glu234  
478 dynamics to permit binding of MgATP in a catalytically competent mode. However,  
479 the crystallisation process quite possibly favours rearrangements, including  $\alpha_{inh}$   
480 refolding and crystallographic reconstitution of the dimer interface, and convergence  
481 towards a low energy state (the one stabilised in solution by dimerisation). This then  
482 outweighs the energetic penalty of the resulting (crystallographically-induced)  
483 electronically or sterically strained carboxylate-carboxylate (Glu234-Glu263) or  
484 glutamate-phosphate contacts (Figure 5C and S5C). Crystallisation may therefore  
485 facilitate the apparent convergence of mutant FICD Glu234 conformations towards that  
486 imposed in solution by the dimer. By contrast, dimeric wild-type FICD is never able to  
487 bind MgATP competently, either in solution or in crystallo, due to its unperturbed  
488 allosteric dimer relay and consequently inflexible Glu234.

489 Oligomerisation state-mediated regulation of AMPylation is not unique to FICD.  
490 Tetramerisation of bacterial NmFic antagonises auto-AMPylation and AMPylation of  
491 its substrate, DNA gyrase (Stanger *et al*, 2016). Though the surfaces involved in  
492 oligomerisation of this class III Fic protein are different from that of FICD, these two  
493 repressive mechanisms converge on the state of their  $\alpha_{inh}s$ . As such, divergent Fic  
494 proteins potentially exploit, for regulatory purposes, an intrinsic metastability of this  
495 structurally conserved inhibitory  $\alpha$ -helix (Garcia-Pino *et al*, 2008). Interestingly, the  
496 more extensive dimerisation surface of FICD (which contains Leu258 and is situated  
497 at the boundary of the Fic domain core and the N-terminal Fic domain extension) also  
498 acts as a structurally conserved dimer interface in other class II bacterial Fic proteins:  
499 CdFic (Dedic *et al*, 2016) and *Bacteroides thetaiotaomicron* (BtFic; PDB: 3cuc), but  
500 not in the monomeric *Shewanella oneidensis* Fic (SoFic) protein (Goepfert *et al*, 2013).  
501 Moreover, a His57Ala mutation in dimeric CdFic (which is structurally equivalent to  
502 FICD<sup>K256A</sup>) causes increased solvent accessibility and auto-AMPylation of a region  
503 homologous to the loop linking FICD's Glu242-helix and the  $\alpha_{inh}$  (Dedic *et al*, 2016).  
504 Despite differences in detail, these findings suggest the conservation of a repressive  
505 relay from the dimer interface to the active site of dimeric Fic proteins.

506 Our biophysical observations also suggest a reciprocal allosteric signal propagated  
507 from FICD's nucleotide binding site back to the dimer interface; enhanced dimer  
508 dissociation was induced by ATP but not ADP. Consequently, it is tempting to

509 speculate that FICD's oligomeric state and hence enzymatic activity might be regulated  
510 by the ADP/ATP ratio in the ER. Under basal conditions, low ADP concentrations  
511 allow ATP to bind both the monomeric and dimeric pools of FICD, shifting the  
512 equilibrium towards the monomer and favouring BiP AMPylation. Stress conditions  
513 may increase ADP concentration in the ER (perhaps by increased ER chaperone  
514 ATPase activity). This increase would be proportionally much greater than the  
515 concomitant decrease in [ATP] (in terms of respective fold changes in concentration).  
516 The increased [ADP] would therefore be able to effectively compete with ATP for the  
517 monomer-dimer FICD pools and thereby shift the equilibrium back towards the BiP de-  
518 AMPylating FICD dimer.

519 The regulation of BiP by FICD-mediated AMPylation and deAMPylation provides the  
520 UPR with a rapid post-translational strand for matching the activity of a key ER  
521 chaperone to its client load. The simple biochemical mechanism proposed here for the  
522 requisite switch in FICD's antagonistic activities parallels the regulation of the UPR  
523 transducers, PERK and IRE1, whose catalytically-active conformation is strictly linked  
524 to dimerisation (Dey *et al*, 2007; Lee *et al*, 2008). A simple correlation emerges,  
525 whereby ER stress favours dimerisation of UPR effectors, activating PERK and IRE1  
526 to regulate gene expression and the FICD deAMPylyase to recruit BiP into the chaperone  
527 cycle (possibly through an increased ER ADP/ATP ratio). Resolution of ER stress  
528 favours the inactive monomeric state of PERK and IRE1 and, as suggested here, the  
529 AMPylation-competent monomeric FICD ([Figure 7](#)).

530

531 **Accession Numbers**

532 The FICD crystal structures have been deposited in the PDB with the following  
533 accession codes: 6i7g (FICD:ATP), 6i7h (FICD<sup>K256S</sup>:Apo), 6i7i (FICD<sup>K256A</sup>:MgATP),  
534 6i7j (FICD<sup>L258D</sup>:Apo), 6i7k (FICD<sup>L258D</sup>:MgATP), and 6i7l (FICD<sup>L258D</sup>:MgAMP-PNP).

535

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545

546 **Author contributions**

547 L.A.P. co-led and conceived the project, designed and conducted the biophysical  
548 experiments, analysed and interpreted the data, purified and crystallised proteins,  
549 collected, analysed and interpreted the X-ray diffraction data, and wrote the manuscript.  
550 C.R. designed, conducted and interpreted the *in vivo* experiments and contributed to  
551 revising the manuscript. Y.Y. supervised crystallisation efforts as well as the collection  
552 and processing of the X-ray diffraction data, contributed to analysis and interpretation  
553 of the structural data and to revising the manuscript. L.N. contributed to the *in vivo*  
554 experiments. S.H.M. conducted the AUC experiments and analysed the AUC data, and  
555 contributed to revising the manuscript. R.J.R. contributed to analysis and interpretation  
556 of the structural data and to revising the manuscript. D.R. conceived and oversaw the  
557 project, interpreted the data, and wrote the manuscript. S.P. co-led and conceived the  
558 project, designed and conducted the biochemical experiments, analysed and interpreted  
559 the data, and wrote the manuscript.

560

561

562 **Declaration of interests**

563 We declare no conflicts of interests.

564

565 **Figure legends**

566 **Figure 1**

567 **Monomeric mutant FICD promotes BiP AMPylation**

568 **A)** Immunoblot of endogenous BiP resolved by native-PAGE from lysates of CHO-K1  
569 S21 wild-type (wt) or *FICD*<sup>-/-</sup> cells either transiently overexpressing wild-type FICD  
570 (high expression level; Hi) or mutant FICD<sup>E234G</sup> (E/G) or stably expressing recombinant  
571 wild-type FICD (low expression level; Lo). The cells in lanes 1-4 were mock  
572 transfected. Where indicated cells were exposed to cycloheximide (CHX; 100 µg/mL)  
573 for 3 h before lysis. Unmodified ('A') and AMPylated ('B') monomeric and oligomeric  
574 (II and III) forms of BiP are indicated. Immunoblots of the same samples resolved by  
575 SDS-PAGE report on FICD, total BiP and eIF2 $\alpha$  (loading control). Data representative  
576 of four independent experiments are shown. See [Figure S1B-C](#).

577 **B)** Wild-type FICD forms homomeric complexes in vivo. Immunoblots of  
578 orthogonally-tagged wild-type and Leu258Asp mutant FICD in the input cell lysate and  
579 following recovery by pull-down with streptavidin (recognizing the AviTag) or anti-  
580 FLAG antibody. Proteins were detected with fluorescently-labelled streptavidin  
581 (StrepIR800) or FLAG antibody. Data representative of three independent experiments  
582 are shown.

583 **C)** Immunoblot of endogenous BiP from transfected CHO-K1 S21 *FICD*<sup>-/-</sup> cells (as in  
584 A). Note that cells expressing monomeric FICD<sup>L258D</sup> accumulate AMPylated BiP. Data  
585 representative of three independent experiments are shown.

586 **D)** Size-exclusion chromatography (SEC) analysis of wild-type and mutant FICD  
587 proteins (each at 20 µM). The elution times of protein standards are indicated as a  
588 reference. Note that the Leu258Asp mutation monomerises FICD, while Gly299Ser  
589 causes partial monomerisation. See [Figure S1D-E](#).

590 **E)** Comparison of the signal-averaged sedimentation coefficients of wild-type (red) and  
591 monomeric mutant FICD<sup>L258D</sup> (blue), as measured by analytical ultracentrifugation. A  
592 fit for monomer-dimer association (solid red line), constrained using the average value  
593 for the monomeric protein (dashed line, 2.82 S,  $S_{w,20} = 3.02$  S), yielded a  $K_d$  of 1.2 nM  
594 with a 95% confidence interval between 1.1 to 1.4 nM and a value of 4.08 S for the  
595 dimer ( $S_{w,20} = 4.36$  S). The fitted data points are from three independent experiments.  
596 See [Figure S1F-G](#).

597 **F)** Autoradiograph of BiP, AMPylated in vitro by the indicated FICD derivatives, with  
598 [ $\alpha$ -<sup>32</sup>P]-ATP as a substrate and resolved by SDS-PAGE. Proteins in the gel were  
599 visualized by Coomassie staining. A representative result of three independent  
600 experiments is shown.

601  
602

603 **Figure 2**

604 **Monomerising mutations de-repress FICD's AMPylation activity**

605 **A)** Monomerising FICD mutations inhibit deAMPylation. Shown is a representative  
606 plot of data points and fit curves of the time-dependent deAMPylation of a fluorescent  
607  $\text{BiP}^{\text{V461F}}\text{-AMP}^{\text{FAM}}$  by the indicated FICD proteins (at 7.5  $\mu\text{M}$ ) as detected by a change  
608 in fluorescence polarisation (FP). DeAMPylation rates calculated from independent  
609 experiments are given in [Figure S2A](#).

610 **B-C)** Dimer interface mutants both AMPylate and deAMPylate BiP. Shown are  
611 representative autoradiographs of thin layer chromatography (TLC) plates revealing  
612 AMP produced from reactions containing  $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$  and the indicated FICD  
613 enzymes in the presence or absence of the co-substrate BiP (arrow indicates direction  
614 of nucleotide migration). The radioactive signals were quantified and the AMP signals  
615 were normalised to the total nucleotide signal in each sample. Plotted below are mean  
616 values  $\pm$  SD from at least three independent experiments. Unpaired t-tests were  
617 performed. See [Figure S2D](#).

618 **D)** Cartoon depicting sequestration of AMPylated BiP by a covalently linked,  
619 disulphide-stapled, s-sFICD<sup>A252C-H363A-C421S</sup> dimer (trap). See [Figure S2E-H](#).

620 **E)** Detection of the time-dependent accumulation of AMPylated  $\text{BiP}^{\text{T229A-V461F}}$  in  
621 radioactive reactions, containing  $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$  and the indicated FICD proteins, in the  
622 presence of excess trap. At the specified time-points samples were taken and analysed  
623 by SDS-PAGE. The autoradiograph ( $^{32}\text{P}$ ) illustrates the radioactive signals, which  
624 represent AMPylated BiP; proteins were visualized by Coomassie staining. The  
625 radioactive signals were quantified and presented in the graph below. Mean values  $\pm$   
626 SD of three independent experiments are shown.

627

628 **Figure 3**

629 **Monomerisation by dilution enhances the AMPylation activity of wild-type FICD**

630 **A)** Autoradiographs of in vitro reactions containing varying concentration of wild-type  
631 FICD protein and fixed concentrations of BiP<sup>T229A-V461F</sup> and [ $\alpha$ -<sup>32</sup>P]-ATP as co-  
632 substrates, resolved by SDS-PAGE after the indicated incubation times. The proteins  
633 were visualized by Coomassie staining of the gel (bottom). The reactions shown on the  
634 right were performed in the presence of an excess of s-sFICD<sup>A252C-H363A-C421S</sup> (trap) to  
635 delay de-modification of BiP. Representative gels are shown, and similar results were  
636 observed in three independent experiments.

637 **B)** As in (A) but with 0.2  $\mu$ M of the indicated FICD variant. The radioactive signals  
638 were detected by autoradiography, quantified, and normalised to the signal in lane 6.  
639 The mean radioactive signals  $\pm$  SD from three independent experiments are given. The  
640 proteins were visualized by staining with Coomassie. See [Figure S3A-B](#).

641 **C)** As in (A) but with dilutions of FICD<sup>C421S</sup> or covalently linked s-sFICD<sup>A252C-C421S</sup>.  
642 Reactions were preceded by a 16 h incubation of FICD in presence or absence of the  
643 reducing agent (DTT). Representative gels are shown of three independent  
644 experiments. See [Figure S3C](#).

645 **D)** Forced dimerisation does not significantly alter deAMPylation rates. Time-  
646 dependent deAMPylation of fluorescent BiP<sup>V461F</sup>-AMP<sup>FAM</sup> by the indicated FICD  
647 proteins (at 7.5  $\mu$ M) assayed by fluorescence polarisation (as in [Figure 2A](#)). A  
648 representative experiment (data points and fit curves) is shown and rates are given in  
649 [Figure S2A](#). See [Figure S3D](#).

650 **E)** Representative autoradiograph of thin layer chromatography (TLC) plates revealing  
651 AMP produced from reactions containing [ $\alpha$ -<sup>32</sup>P]-ATP and the indicated FICD  
652 enzymes in the presence of the co-substrate BiP. AMP signals were normalised to the  
653 total nucleotide signal in each sample and the graph below plots mean values  $\pm$  SD from  
654 at least three independent experiments.

655

656 **Figure 4**

657 **Residues connecting the FICD dimer interface with the inhibitory  $\alpha$ -helix stabilise**  
658 **FICD and repress AMPylation**

659 **A)** Ribbon diagram of the FICD dimer interface with monomers in purple and blue  
660 ribbons (PDB:6i7g). Residues involved in a H-bond network linking the dimer interface  
661 to the  $\alpha_{inh}$  (as well as Gly299 and Glu234) are shown as green sticks. Sub-3.50 Å  
662 hydrogen bonds made by Asn236, Leu238 and Lys256 are depicted as dotted cyan  
663 lines.

664 **B)** Size-exclusion chromatography elution profile of wild-type and mutant FICD  
665 proteins (each at 20  $\mu$ M). Protein absorbance at 280 nm is plotted against elution time.  
666 The elution times of protein standards are indicated as a reference.

667 **C)** Radioactive in vitro AMPylation reactions containing the indicated FICD proteins,  
668 [ $\alpha$ -<sup>32</sup>P]-ATP, and BiP<sup>T229A-V461F</sup> were analysed by SDS-PAGE. The radioactive BiP-  
669 AMP signals were detected by autoradiography and proteins were visualized by  
670 Coomassie staining of the gel. See [Figure S4A](#).

671 **D)** Representative autoradiograph of thin layer chromatography (TLC) plates revealing  
672 AMP produced from reactions containing [ $\alpha$ -<sup>32</sup>P]-ATP and the indicated FICD  
673 enzymes in the presence of the co-substrate BiP. The radioactive signals were  
674 quantified and the AMP signals were normalised to the total nucleotide signal in each  
675 sample. The graph shows mean AMP values  $\pm$  SD from three independent experiments.

676 **E)** Melting temperatures ( $T_m$ ) of the indicated FICD mutants (at 2  $\mu$ M) were measured  
677 by differential scanning fluorimetry (DSF). Shown is the mean  $T_m \pm$  SD of three  
678 independent experiments. The inset shows melt curves with their negative first  
679 derivatives from a representative experiment. See [Figure S4D](#).

680 **F)** A plot of the melting temperature of the indicated FICD proteins in absence (Apo)  
681 or presence of nucleotides. Shown are the mean  $T_m$  values  $\pm$  SD of three independent  
682 DSF experiments. Monomeric FICD<sup>L258D</sup> (mFICD) and FICD<sup>L258D-E234G</sup> (mFICD<sup>E/G</sup>)  
683 as well as dimeric wild-type FICD (dFICD) and FICD<sup>E234G</sup> (dFICD<sup>E/G</sup>) were tested.  
684 ADP and ATP concentrations in mM are given in parentheses. See [Figure S4E](#) for  $K_{1/2}$   
685 quantification.

686 **Figure 5**

687 **Monomeric FICD binds ATP in an AMPylation-competent conformation**

688 **A)** Monomerisation does not result in large conformational changes in FICD. Shown is  
689 the alignment, from residues 213-407, of FICD molecules in the asymmetric unit.  
690 Monomeric FICD<sup>L258D</sup> and dimeric wild-type FICD  $\pm$  ATP, are coloured as indicated.  
691 Glu234, ATP (and Mg, where applicable), are shown as sticks (or green spheres). The  
692 inhibitory alpha helix ( $\alpha_{inh}$ ) and gross domain architecture is annotated. Note the only  
693 significant deviation in tertiary structure is the flipping of the TPR domain in the  
694 FICD<sup>L258D</sup>:ATP structure. The FICD:Apo structure is from PDB: 4U0U. See [Figure S5A](#).

696 **B)** Cocrystallisation of FICD variants with MgATP results in electron densities for  
697 nucleotide and the inhibitory Glu234. Unbiased polder (OMIT) maps for ATP ( $\pm$  Mg)  
698 and Glu234 are shown as blue and purple meshes, respectively. **(i)** The wild-type dimer  
699 FICD structure displays a lack of density corresponding to a Mg<sup>2+</sup> ion. The ATP density  
700 is contoured at 3.5  $\sigma$  and the Glu234 at 5.0  $\sigma$ . **(ii)** The dimeric dimer relay mutant  
701 FICD<sup>K256A</sup> displays a clear MgATP density up to and including the  $\gamma$ -phosphate  
702 phosphorous atom. The ATP density and Glu234 densities are both contoured at 3.0  $\sigma$ .  
703 **(iii)** Monomeric FICD<sup>L258D</sup> shows a clear MgATP density. The ATP density is  
704 contoured at 3.0  $\sigma$  and the Glu234 at 5.0  $\sigma$ . All residues and water molecules interacting  
705 with ATP ( $\pm$  Mg) are shown as sticks and coloured by heteroatom. Mg<sup>2+</sup> coordination  
706 complex pseudo-bonds are shown in purple dashed lines. See [Figure S5B](#).

707 **C)** Unlike the monomeric or the dimer relay FICD mutants, dimeric wild-type FICD  
708 binds ATP in a configuration that would prevent BiP substrate AMPylation. The  
709 position of the  $\alpha$ -phosphate in the FICD:ATP structure would preclude in-line  
710 nucleophilic attack (see [Figure S5C-D](#)). The left panel represents the superposition of  
711 the structures in the upper panel of **(B)**, with ATP interacting residues shown as sticks  
712 and annotated. Only Glu234 deviates significantly in sidechain position. Note,  
713 however, that the FICD:ATP His363 sidechain is also flipped, forming a hydrogen bond  
714 to a ribose interacting water (see *Bi*). Mg<sup>2+</sup> and ATP are coloured to match the  
715 corresponding ribbons. Active site waters are omitted for clarity. Distances are  
716 indicated by dashed black lines. The inset is a blow-up displaying distances *i-iv* between  
717 the  $\gamma$ -phosphates and Glu234 residues. Note, distances *i* and *ii* are derived from the  $\gamma$ -

718 phosphate and Glu234 of different superimposed structures. Distances between  
719 Val316(C $\gamma$ 1) and the corresponding P $\alpha$  are shown in the right-hand side panel. See  
720 [Figures S6-7](#).

721

722 **Figure 6**

723 **ATP destabilises the pre-AMPylation complex and the FICD dimer**

724 **A)** BioLayer interferometry (BLI) derived association and dissociation traces of  
725 monomeric FICD<sup>L258D-H363A</sup> (mFICD<sup>H363A</sup>) or dimeric FICD<sup>H363A</sup> (dFICD<sup>H363A</sup>) from  
726 immobilized biotinylated BiP<sup>T229A-V461F</sup> in absence or presence of nucleotides. Unless  
727 indicated (\*) BiP was saturated with ATP before exposure to FICD variants. A  
728 representative experiment of three independent repetitions is shown. See [Figure S8A-B](#).

730 **B)** BLI dissociation traces of proteins as in (A). At t = 0 a pre-assembled complex of  
731 immobilised, ATP-saturated BiP and the indicated FICD proteins (associated without  
732 ATP) were transferred into a solution without or with ATP, as indicated. A  
733 representative experiment is shown and the biphasic dissociation kinetics are quantified  
734 in (C) and (D). Full association and dissociation traces are shown in [Figure S8C](#).

735 **C)** Graph of the slow dissociation rates ( $k_{off,slow}$ ) of monomeric FICD from BiP:ATP as  
736 shown in (B). Bars represent mean values  $\pm$  SD of three independent experiments.

737 **D)** The ATP-induced fold change in the percentage of the dissociation phase attributed  
738 to a fast dissociation (%Fast),  $k_{off,fast}$ , and  $k_{off,slow}$  derived from the data represented in  
739 (B). Bars show mean values  $\pm$  SD of three independent experiments. See [Figure S8D](#).

740 **E)** BLI dissociation traces of the FICD dimer at different nucleotide concentration. At  
741 t = 0 the species on the biosensor is a heterodimer of N-terminally biotinylated and an  
742 exchangeable, non-biotinylated FICD. Dissociation was conducted  $\pm$  ligands (5 mM),  
743 as indicated. A representative experiment of four independent repeats, with mono-  
744 exponential fits are shown. See [Figure S8E](#) for raw data.

745 **F)** Cartoon schematic of the BLI assay workflow used to derive data presented in (E)  
746 and [Figure S8E](#).

747 **G)** Quantification of the off rates derived from (E). ATP, but no ADP, significantly  
748 increases the dimer dissociation rate [\*\*: p < 0.01, by Tukey test; n.s.: not  
749 significant]. Data shown is the mean  $\pm$  SD of four independent experiments.

750 **Figure 7**

751 A proposed model of an oligomerisation state-dependent switch in FICD bifunctional  
752 active site. Under conditions of ER-stress the dimeric form FICD is favoured (right  
753 hand side). Dimeric FICD cannot bind ATP in an AMPylation competent mode but can  
754 efficiently catalyse deAMPylation of BiP-AMP (thereby remobilising BiP back into the  
755 chaperone cycle). A decrease in unfolded protein load in the ER, possibly associated  
756 with a decreased ER ADP/ATP ratio, shifts the FICD monomer-dimer equilibrium  
757 towards monomeric FICD. Monomeric FICD can bind MgATP in an AMPylation  
758 competent conformation and, as such, AMPylate and inactivate surplus BiP.

759 **Table 1:** Data Collection and refinement statistics. Values in parentheses correspond to the highest-resolution shell, with the following exceptions:  
760 \*The number of molecules in the biological unit is shown in parentheses; \*\*MolProbity percentile score is shown in parentheses (100<sup>th</sup> percentile  
761 is the best among structures of comparable resolutions, 0<sup>th</sup> percentile is the worst).  
762

	<b>FICD:ATP</b>	<b>FICD<sup>K256S</sup>:Apo</b>	<b>FICD<sup>K256A</sup>:MgATP</b>	<b>FICD<sup>L258D</sup>:Apo</b>	<b>FICD<sup>L258D</sup>:MgATP</b>	<b>FICD<sup>L258D</sup>:MgAMP -PNP</b>
<b>Data collection</b>						
Synchrotron stations	DLS I04	DLS I04	DLS I03	DLS I04	DLS I03	DLS I03
Space group	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2	<i>P</i> 22 <sub>1</sub> 2 <sub>1</sub>	<i>P</i> 22 <sub>1</sub> 2 <sub>1</sub>	<i>P</i> 3 <sub>1</sub> 21	<i>P</i> 6 <sub>4</sub> 22	<i>P</i> 6 <sub>4</sub> 22
Molecules in a.u.*	2 (2)	1 (2)	1 (2)	1 (1)	1 (1)	1 (1)
a,b,c; Å	77.67, 107.65, 132.60	43.82, 76.51, 131.97	41.90, 73.98, 134.04	118.14, 118.14, 79.55	186.84, 186.84, 76.84	186.36, 186.36, 77.10
α, β, γ; °	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 120.00	90.00, 90.00, 120.00	90.00, 90.00, 120.00
Resolution, Å	83.58-2.70 (2.83-2.70)	65.99-2.25 (2.32-2.25)	134.04-2.32 (2.41- 2.32)	62.80-2.65 (2.72- 2.65)	93.42-2.54 (2.65- 2.54)	93.18-2.31 (2.39- 2.31)
R <sub>merge</sub>	0.163 (0.717)	0.109 (0.385)	0.107 (0.636)	0.176 (0.856)	0.167 (1.009)	0.071 (0.611)
<I/σ(I)>	19.2 (1.8)	6.8 (2.4)	5.6 (1.0)	8.6 (2.2)	13.0 (2.5)	10.3 (1.8)
CC1/2	0.999 (0.720)	0.993 (0.547)	0.995 (0.567)	0.996 (0.549)	0.999 (0.503)	0.998 (0.523)
No. of unique reflections	31293 (4091)	21825 (1978)	18543 (1712)	18963 (1380)	26617 (3188)	34573 (3351)
Completeness, %	100.0 (100.0)	99.9 (99.5)	99.4 (97.3)	100.0 (100.0)	100.0 (100.0)	99.4 (99.1)
Redundancy	6.4 (6.5)	4.4 (4.4)	3.7 (3.7)	9.7 (10.0)	16.1 (16.5)	4.6 (4.6)
<b>Refinement</b>						
R <sub>work</sub> /R <sub>free</sub>	0.280 / 0.319	0.208 / 0.259	0.282 / 0.325	0.228 / 0.283	0.232 / 0.252	0.214 / 0.251
No. of atoms (non-H)	5650	2851	2731	2951	2828	2940
Average B-factors, Å <sup>2</sup>	55.3	42.5	54.6	50.9	58.2	56.4
RMS Bond lengths, Å	0.002	0.003	0.003	0.003	0.002	0.003

RMS Bond angles, $^{\circ}$	1.142	1.180	0.763	1.222	1.127	1.170
Ramachandran favoured region, %	96.5	98.5	98.2	97.9	98.5	99.4
Ramachandran outliers, %	0	0	0	0	0	0
MolProbity score**	1.33 (100 <sup>th</sup> )	0.86 (100 <sup>th</sup> )	0.74 (100 <sup>th</sup> )	0.99 (100 <sup>th</sup> )	0.97 (100 <sup>th</sup> )	0.99 (100 <sup>th</sup> )
PDB code	6i7g	6i7h	6i7i	6i7j	6i7k	6i7l

763

764 **Figure S1**

765 **Low-level expression facilitates AMPylation in vivo and FICD mutations are able**  
766 **to disrupt the tight dimer formed in solution.**

767 **A)** Schematic representation of the domain organization of FICD and the shorter protein  
768 fragment used for in vitro experiments. The transmembrane domain (blue), the TPR  
769 domain (orange), the  $\alpha$ -helical linker (green), the Fic domain (purple) and the core Fic  
770 domain (deep purple) including the active site motif are indicated.

771 **B-C)** Characterization of CHO-K1 *FICD*<sup>-/-</sup> UPR reporter clones stably expressing wild-  
772 type FICD. **(B)** Flow cytometry analysis of CHO-K1 *FICD*<sup>-/-</sup> UPR reporter clones  
773 stably-expressing mCherry and FICD. Clones were selected based on mCherry signal,  
774 assuming a direct correlation with FICD expression levels. **(C)** Immunoblot of  
775 endogenous BiP from CHO-K1 *FICD*<sup>-/-</sup> clones shown in *(B)* exposed to cycloheximide  
776 as in [Figure 1A](#). Note that only clone 10, with an intermediate mCherry signal, showed  
777 detectable accumulation of AMPylated BiP.

778 **D-E)** Size-exclusion chromatography (SEC) analysis of wild-type and mutant FICD  
779 proteins. **(D)** SEC elution profiles with FICD proteins at the indicated concentrations.  
780 Black dots mark the position of the elution peaks. Dotted lines mark the approximate  
781 elution peak times for dimeric (10.2 min) and monomeric (11.4 min) FICD,  
782 respectively. **(E)** Plot of the elution peak times from *(D)* as a function of protein  
783 concentration. With the exception of FICD<sup>G299S</sup> (\*; a mutation that shifts the elution  
784 time relative to the monomer) best-fit monomer-dimer association curves are shown  
785 with the top plateau constrained to the monomer elution time (11.4 min). Approximate  
786 dimerisation  $K_d$ s were derived and are shown in the figure key for the different partially  
787 monomerising mutants (with 95% confidence intervals). Note that FICD<sup>L258D</sup> eluted as  
788 a monomer and wild-type FICD principally as a dimer at all concentrations tested (0.2-  
789 50  $\mu$ M). Conversely, FICD<sup>G299S</sup> and non-oxidized FICD<sup>A252C-C421S</sup> formed much  
790 weaker dimers. As in *(D)* the monomer and dimer elution times are represented by  
791 dotted (horizontal) lines.

792 **F-G)** Analysis of FICD by analytical ultracentrifugation. Overlays of  $c(s)$  distributions  
793 of **(F)** wild-type FICD and **(G)** FICD<sup>L258D</sup> are shown in units of experimental  $s$ -values.  
794 A signal-weighted isotherm for the wild-type protein ([Figure 1E](#)) was generated from  
795 integration of the titration series distributions.

796 **Figure S2**

797 **Monomerisation inhibits deAMPylation and markedly stimulates FICD**  
798 **AMPylation activity.**

799 **A)** Summary of deAMPylation rates of wild-type and mutant FICD proteins. Shown  
800 are deAMPylation rates of BiP<sup>V461F</sup>-AMP<sup>FAM</sup> by the indicated FICD proteins (at 0.75  
801  $\mu$ M or 7.5  $\mu$ M) as detected by a change in fluorescence polarisation. Mean values  $\pm$  SD  
802 of the normalized raw data fitted to a single-exponential decay function of at least four  
803 independent measurements are presented.

804 **B-C)** The effect of FICD overexpression on a UPR reporter. **(B)** Flow cytometry  
805 analysis of wild-type and *FICD*<sup>-/-</sup> CHO-K1 *CHOP*::*GFP* UPR reporter cells transfected  
806 with plasmids encoding wild-type or the indicated FICD derivatives and a mCherry  
807 transfection marker. Shown are the median values  $\pm$  SD of the GFP fluorescence signal  
808 of mCherry-positive cells from three independent experiments (fold change relative to  
809 wild-type cells transfected with a plasmid encoding mCherry alone). Note that only  
810 Glu234Gly-containing, deAMPylation-deficient FICDs activate the reporter. **(C)** Flow  
811 cytometry raw data of a representative experiment quantified in *(B)*.

812 **D)** AMP production by FICD dimer interface or relay mutants is BiP dependent. AMP  
813 production in the presence of [ $\alpha$ -<sup>32</sup>P]-ATP was measured by TLC and autoradiography  
814 (as in [Figure 2B](#)). Plotted below are mean AMP values  $\pm$  SD (n = 3).

815 **E-G)** Characterization of covalently linked s-sFICD<sup>A252C-H363A-C421S</sup> dimers – a trap for  
816 BiP-AMP. **(E)** Coomassie-stained, SDS-PAGE gel of the indicated FICD proteins. **(F)**  
817 Size-exclusion chromatography elution profiles of wild-type FICD and covalently  
818 linked s-sFICD<sup>A252C-H363A-C421S</sup> (trap) dimers at 20  $\mu$ M, as in [Figure 1D](#). Note that the  
819 oxidised trap elutes, like the wild-type FICD, as a dimer. **(G)** BioLayer interferometry  
820 (BLI) derived association and dissociation traces of the indicated FICD proteins (in  
821 solution) from immobilized AMPylated (BiP-AMP) or unmodified BiP. The trap (s-  
822 sFICD<sup>A252C-H363A-C421S</sup>) and FICD<sup>H363A</sup> had indistinguishable tight interaction with BiP-  
823 AMP (with low off rates). The interaction of BiP-AMP with monomeric FICD<sup>L258D-</sup>  
824 <sup>H363A</sup> was more transient. The interaction between these FICD variants and unmodified  
825 BiP was further diminished.

826 **H)** Sequestration of AMPylated BiP by trap FICD analysed by SEC. Elution profiles  
827 of in vitro AMPylation reactions containing the indicated components in the presence

828 or absence of covalently linked s-sFICD<sup>A252C-H363A-C421S</sup> (trap) dimers to sequester the  
829 AMPylated BiP product. Note that the trap forms a stable complex with BiP when  
830 AMPylated by monomeric FICD<sup>L258D</sup>. An early eluting species, representing a stable  
831 complex between modified BiP and trap, only occurs in the reaction containing  
832 AMPylation-active, monomeric FICD<sup>L258D</sup> and ATP (bottom right panel, pink trace).  
833 Here, BiP-mediated ATP hydrolysis and substrate interactions were discouraged by use  
834 of a BiP<sup>T229A-V461F</sup> double mutant.

835

836 **Figure S3**

837 **Non-disulphide-linked FICD<sup>A252C-C421S</sup> shows enhanced AMPylation activity.**

838 **A)** Coomassie-stained, non-reducing SDS-PAGE gel of the indicated FICD proteins.  
839 **B)** Size exclusion chromatography (SEC) elution profiles of FICD proteins injected at  
840 a concentration of 20  $\mu$ M. Protein absorbance at 280 nm is plotted against elution time.  
841 The elution times of protein standards are indicated as a reference. Note that wild-type  
842 FICD, FICD<sup>C421S</sup>, and oxidised s-sFICD<sup>A252C-C421S</sup> co-elute as dimers. See [Figure S1D-E](#).

844 **C)** Radioactive in vitro AMPylation reactions were performed as in the right hand side  
845 panel of [Figure 3A](#), that is with the indicated FICD proteins under non-reducing  
846 conditions in presence of covalently linked s-sFICD<sup>A252C-H363A-C421S</sup> dimers (trap). Note  
847 that the accumulation of modified BiP correlates with the FICD concentration. Less  
848 modified BiP was produced by covalently-linked, oxidised s-sFICD<sup>A252C-C421S</sup> dimers,  
849 whereas more AMPylated BiP was generated in reactions containing non-oxidised  
850 FICD<sup>A252C-C421S</sup>. The trap, present at 5  $\mu$ M, co-migrates with the indicated FICD  
851 enzyme and dominates the signal in the Coomassie stained gel (FICD/trap).

852 **D)** Time-dependent in vitro deAMPylation of fluorescent BiP<sup>V461F</sup>-AMP<sup>FAM</sup> by the  
853 indicated FICD proteins (at 7.5  $\mu$ M) assayed by fluorescence polarisation (as in [Figure](#)  
854 [2A](#)). A representative experiment (data points and fit curves) is shown and  
855 deAMPylation rates are presented in [Figure S2A](#). Note that non-oxidised FICD<sup>A252C-</sup>  
856 <sup>C421S</sup> has very similar deAMPylation kinetics to the wild-type protein. This contrasts  
857 with the oxidised form which displays a slight increase in deAMPylation rate ([Figure](#)  
858 [3D and S2A](#)).

859

860 **Figure S4**

861 **FICD dimer relay mutants produce a pool of AMPylated BiP in vitro, and FICD**  
862 **AMPylation activity correlates with increased flexibility.**

863 **A)** Radioactive in vitro AMPylation reactions with the indicated FICD proteins at the  
864 indicated concentrations, [ $\alpha$ -<sup>32</sup>P]-ATP, and BiP<sup>T229A-V461F</sup> were analysed by SDS-  
865 PAGE. The radioactive signals were detected by autoradiography and proteins were  
866 visualised by Coomassie staining. Note the enhanced production of AMPylated BiP in  
867 the presence of dimer relay mutants, FICD<sup>K256S</sup> and FICD<sup>E242A</sup>, relative to the wild-type  
868 protein and a further increase in the production of AMPylated BiP by the monomeric  
869 FICD<sup>K256S-L256D</sup> double mutant relative to the monomeric FICD<sup>L258D</sup>. Also note the  
870 auto-AMPylation signals of the monomeric FICDs at high enzyme concentration.

871 **B-C)** In vitro deAMPylation of fluorescent BiP<sup>V461F</sup>-AMP<sup>FAM</sup> by the indicated FICD  
872 proteins (at 7.5  $\mu$ M) measured by fluorescence polarisation. A representative  
873 experiment (data points and fit curves) is shown and rates are presented in [Figure S2A](#).  
874 Note the impaired deAMPylation activity of the monomeric FICD<sup>K256S-L256D</sup> double  
875 mutant in (C).

876 **D)** DSF  $T_m$  analysis of wild-type (wt) and mutant FICD proteins in absence (Apo) or  
877 presence of ATP or ADP. Nucleotide concentrations are given in parentheses. Non-  
878 oxidised and oxidised forms of FICD<sup>A252C-C421S</sup> were assayed in buffer lacking reducing  
879 agent (which did not affect the  $T_m$  of wild-type FICD; not shown). Shown are the mean  
880  $T_m$  values  $\pm$  SD from three independent experiments. Note that FICD<sup>K256A</sup> is more  
881 stable than FICD<sup>K256S</sup> but less than wild-type FICD. Furthermore, the stabilities of  
882 oxidised and non-oxidised FICD<sup>C421S-A252C</sup> relative to the wild-type correlate inversely  
883 with their AMPylation activities ([Figure 3B](#)). The same data for the wild-type FICD,  
884 FICD<sup>E242A</sup>, FICD<sup>G299S</sup>, FICD<sup>L258D</sup> and FICD<sup>K256S-L258D</sup> in the Apo state are presented in  
885 [Figure 4E](#).

886 **E)** Plot of the increase in FICD melting temperature ( $\Delta T_m$ ) against ATP concentration  
887 as measured by DSF (derived from [Figure 4F](#)). Note the similarity in the plot of  
888 FICD<sup>L258D</sup> (mFICD) and the wild-type dimer (dFICD); mFICD  $K_{1/2}$  2.5  $\pm$  0.6 mM and  
889 dFICD  $K_{1/2}$  3.2  $\pm$  0.3 mM. Shown are mean  $\Delta T_m$  values  $\pm$  SD of three independent  
890 experiments with the best fit lines for a one site binding model.

891 **Figure S5**

892 **Monomerisation allows ATP to bind to FICD in a mode conducive to BiP**  
893 **AMPylation.**

894 **A)** Mutation of the dimer relay residue Lys256 does not result in large conformational  
895 changes in FICD. Shown is the alignment (residues 213-407) of the molecules in the  
896 asymmetric unit. Structures are coloured as indicated. Glu234, ATP and Mg (where  
897 applicable), are shown as sticks. The inhibitory alpha helix ( $\alpha_{inh}$ ) and gross domain  
898 architecture is annotated. The FICD:Apo structure is from PDB: 4U0U.

899 **B)** Electron density of both MgAMPPNP and the inhibitory Glu234, from monomeric  
900 FICD<sup>L258D</sup> co-crystallized with MgAMPPNP. Unbiased polder (OMIT) maps are  
901 shown in blue and purple meshes, contoured at 3.0 and 5.0  $\sigma$ , respectively. All residues  
902 and water molecules interacting with MgAMPPNP are shown as sticks and coloured  
903 by heteroatom. Mg<sup>2+</sup> coordination complex pseudo-bonds are shown in purple dashed  
904 lines.

905 **C)** Unlike wild-type FICD, monomeric FICD<sup>L258D</sup> binds ATP and ATP analogues in  
906 an AMPylation competent conformation. The indicated structures and distances are  
907 shown as in [Figure 5C](#), with ATP interacting residues shown as sticks and annotated.  
908 The position of the  $\alpha$ -phosphate relative to Val316 in the FICD:ATP structure (see  
909 distances in right hand side panel) would preclude in-line nucleophilic attack (see D-  
910 E). The inset is a blow-up displaying distances *i-iv* between the  $\gamma$ -phosphates and  
911 Glu234 residues. A potentially significant difference in the Glu234 position between  
912 the FICD<sup>L258D</sup>:MgAMPPNP and FICD:ATP structures is apparent: hypothetical  
913 distance *ii* (2.68 Å, between Glu234 of FICD:ATP and AMPPNP  $\gamma$ -phosphate of  
914 FICD<sup>L258D</sup>) is less favourable than the observed distance *iii* (2.94 Å, between the  
915 AMPPNP  $\gamma$ -phosphate and Glu234 of FICD<sup>L258D</sup>). Note, His363 of FICD:ATP is in a  
916 non-optimal flip state to facilitate general base catalysis (see [Figure 5B](#)).

917 **D) (i)** The mode of ATP binding in wild-type dimeric FICD sterically occludes the  
918 nucleophilic attack required for AMPylation. Shown are semi-opaque 3 Å centroids  
919 centred on P $\alpha$  and Val316 (C $\gamma$ 1). The putative BiP Thr518 nucleophile (depicted by the  
920 cross) is positioned in-line with the scissile phosphoanhydride (parallel to the plane of  
921 the paper) and 3 Å from P $\alpha$ . This nucleophile position lies within the Val316 centroid  
922 (indicating a steric clash). For clarity, the FICD:ATP structure is overlaid with a thin

923 slice of the FICD:ATP structure in the plane of the  $\text{P}\alpha$ -O $3\alpha$  bond. (*ii*) In the monomeric  
924 AMPylation-competent FICD<sup>L258D</sup>:ATP structure the nucleophile lies outside the  
925 Val316 centroid in proximity to His363 (the general base).

926 **E)** The ATP  $\alpha$ -phosphates of monomer or dimer relay mutants are in the same position  
927 as that competently bound to the AMPylation unrestrained dimeric FICD<sup>E234G</sup>. Shown  
928 are all AMPylation competent MgATP structures overlaid as in (*C*) and [Figure 5C](#). The  
929 dimeric FICD<sup>E234G</sup>:MgATP (dark blue, PDB: 4U07) is also included as a reference for  
930 an active AMPylating enzyme.

931

932 **Figure S6**

933 **FICD crystallographic packing and dimer interface.**

934 **A)** The hydrogen bonding network connecting the dimer interface and enzyme active  
935 site is maintained in the crystal structures of monomeric and Lys256 mutant FICDs. An  
936 alignment of the hydrogen bond network linking the dimer interface to Glu234 in the  
937 indicated structures is displayed (in the same view as [Figure 4A](#)). H-bonds are shown  
938 in blue dashed lines. Where indicated, single molecules from the asymmetric unit  
939 (underlined) are displayed with their respective symmetry mates (Sym1). Note, the  
940 side-chains of Asp258 and (Sym1)Arg250 of the monomeric FICD<sup>L258D</sup> (cococrystallised  
941 with nucleotide) form a crystallographically induced inter-molecular H-bond (magenta  
942 dashed line). The salt-bridges between the Glu234 and the Fic motif Arg374 (magenta  
943 dashed lines) in the FICD<sup>L258D</sup>:Apo and FICD<sup>K256S</sup>:Apo structures, observed in other  
944 inhibitory glutamate-containing Fic crystal structures, are also shown.

945 **B)** Dimer interface contacts are imposed crystallographically, and crystal packing  
946 around the  $\alpha_{inh}$  is similar in all FICD structures. FICDs with similar crystal packings are  
947 grouped into panels (*i-iv*). The inhibitory alpha helix ( $\alpha_{inh}$ ) is denoted with an asterisk  
948 (\*) and Glu234s are shown as sticks. The wild-type dimeric FICD:Apo structure  
949 (FICD:Apo; PDB:4U0U) is provided in all panels for reference. Where a single FICD  
950 molecule constituted the asymmetric unit, symmetry mates within 4 Å of its dimer  
951 interface (Sym1) or 4 Å of its inhibitory helix region (Sym2/3) are also displayed. Note  
952 that crystals of the Lys256 mutants (*ii*) contain a single molecule in their asymmetric  
953 unit but are packed as dimers, crystallographically reconstituting the dimeric biological  
954 unit. The asymmetric unit of FICD<sup>L258D</sup> bound to ATP (or an ATP analogue) (*iv*)  
955 contains a single molecule and thus corresponds to the biological unit of this  
956 monomeric protein. However, packing against its symmetry mates (Sym1),  
957 crystallographically reconstitutes a dimer interface that is highly similar, but not  
958 identical, to that observed in the wild-type protein (see Asp258 and (Sym1)Arg250 in  
959 [S6A](#), above). Sym2 in (*iv*) serves to highlight the replacement of the flipped out TPR  
960 domain with the flipped out TPR domain from a symmetry mate. In (*iv*) there are no  
961 crystal contacts in the vicinity of the  $\alpha_{inh}$ .

962

963 **Figure S7**

964 **AMPylation activity correlates with enhanced flexibility of the dimer interface**  
965 **and Glu234.**

966 The residue average B-factors, for the four FICD complexes cocrystallised with ATP,  
967 are shown [in (*i-iv*)] with a cold to hot colour code. They display a trend of increasing  
968 B-factors in the dimer interface and in the inhibitory glutamate region. This increase in  
969 B-factor is indicative of increasing flexibility and correlates with greater AMPylation  
970 activity of the corresponding FICD. All of these structures have almost identical dimer  
971 packing in their respective crystals and limited crystal contacts around the inhibitory  
972 helix (see [Figure S6](#)). Note, structure averaged B-factors are comparable (see [Table 1](#)).  
973 For clarity, the TPR domain (up to residue 182) is not shown.

974

975 **Figure S8**

976 **ATP negatively modulates pre-AMPylation complex and FICD dimer stability.**

977 **A)** Immobilised BiP responds allosterically to, is saturated by and retains ATP for the  
978 duration of BLI kinetic assays. BLI traces of the interaction between FICD<sup>L258D-H363A</sup>  
979 and immobilised biotinylated BiP<sup>T229A-V461F</sup> in different nucleotide states. Before  
980 exposure to FICD<sup>L258D-H363A</sup> immobilised BiP:Apo was subjected to two consecutive  
981 incubation steps (activation and wash) in the presence or absence of ATP as indicated.  
982 FICD association and dissociation steps (shown) were then conducted in a nucleotide  
983 (Nt.)-free solution. Note that BiP only interacts with FICD<sup>L258D-H363A</sup> when pre-  
984 saturated with ATP. Importantly, ATP pre-bound BiP retains its affinity for FICD<sup>L258D-</sup>  
985 <sup>H363A</sup> even if subsequently washed in a buffer lacking ATP (compare red and green  
986 traces). Thus BiP retains its bound ATP for the duration of the kinetic experiment,  
987 experimentally uncoupling the effect of nucleotide on the FICD analyte from its effect  
988 on the immobilised BiP ligand.

989 **B)** Cartoon schematic of the BLI assays presented in [Figures 6A-B](#). The pre-  
990 AMPylation complex is formed between the immobilised BiP:ATP ‘ligand’ and the  
991 FICD ‘analyte’.

992 **C)** The BLI association and dissociation traces from [Figure 6B](#) are shown. The  
993 immobilised biotinylated BiP<sup>T229A-V461F</sup> was saturated with ATP and then exposed to  
994 nucleotide-free FICDs. Dissociation was performed in absence or presence of ATP, as  
995 indicated. [mFICD<sup>H363A</sup>: FICD<sup>L258D-H363A</sup>; dFICD<sup>H363A</sup>: FICD<sup>H363A</sup>].

996 **D)** Quantification of the biphasic exponential decay fitting of dissociation traces shown  
997 in [Figure 6B](#). Relative ATP-induced changes of these kinetic parameters are given in  
998 [Figure 6D](#). Shown are mean values  $\pm$  SD from three independent experiments. Note the  
999 greater relative contribution of fast dissociation of mFICD in presence of ATP versus  
1000 absence.

1001 **E)** Representative BLI traces of an FICD dimer dissociation experiment plotted in  
1002 [Figure 6E](#). The legend indicates the form of unlabelled FICD incubated with the N-  
1003 terminally biotinylated FICD (at a 100-fold molar excess, prior to biosensor loading)  
1004 and also the ligand present in the dissociation buffer (at 5 mM) if applicable. Note,  
1005 probes loaded with biotinylated FICD incubated with mFICD<sup>H363A</sup> act as controls for

FICD monomerisation

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1006 non-specific association and dissociation signals, these were subtracted from the  
1007 respective dFICD<sup>H363A</sup> traces in [Figure 6E](#).

1008 **Table S1**

1009 Crystallisation conditions. Where applicable the crystallisation conditions (and seed dilution) of the crystals used for micro-seeding are also shown.  
 1010 Note, PEG percentage is given in w/v and EtOH percentage in v/v.

Dataset	PDB Code	Crystallisation Condition ( <i>Protein:Seeds:Well Solution (nl)</i> )	Seed Protein	Seed Crystal Conditions ( <i>Seed Dilution</i> )
FICD:ATP	6i7g	0.1 M Tris pH 7.5; 20% PEG 300; 5% PEG8K; 10% Glycerol (150:50:100)	FICD	0.2 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1 M NaCacodylate, 30% PEG 8000 (1/3)
FICD <sup>K256S</sup> :Apo	6i7h	0.1 M Tris pH 8.5; 0.05 M MgCl <sub>2</sub> ; 40% EtOH (200:0:100)	N/A	N/A
FICD <sup>K256A</sup> :MgATP	6i7i	0.1 M Bis-Tris pH 6.5; 0.2 M MgCl <sub>2</sub> ; 25% PEG3350 (100:25:100)	FICD <sup>K256A</sup>	0.1 M Na <sub>3</sub> Citrate pH 5.5, 40% PEG 600 (1/10)
FICD <sup>L258D</sup> :Apo	6i7j	0.1 M Tris pH 8.5; 2.0 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (150:50:100)	FICD <sup>L258D</sup>	0.1 M Tris pH 8.5, 0.2 M Li <sub>2</sub> SO <sub>4</sub> , 40% PEG 4000 (1/2)
FICD <sup>L258D</sup> :MgATP	6i7k	1.0 M NaCl; 10% EtOH (150:50:200)	FICD <sup>L258D-H363A</sup>	0.1 M HEPES pH 7.5, 1 M NaOAc (1/100)
FICD <sup>L258D</sup> :MgAMP-PNP	6i7l	1.5 M NaCl; 10% EtOH (150:50:200)	FICD <sup>K256A</sup>	0.1 M Na <sub>3</sub> Citrate pH 5.5, 40% PEG 600 (1/500)

1011

1012 **Table S2**

1013 List of plasmids used, their lab names, description, their corresponding label and references.

1014 **Materials and Methods**

1015

1016 **Plasmid construction**

1017 The plasmids used in this study have been described previously or were generated by  
1018 standard molecular cloning procedures and are listed in [Table S2](#).

1019

1020 **Cell lines**

1021 All cells were grown on tissue culture dishes or multi-well plates (Corning) at 37 °C  
1022 and 5% CO<sub>2</sub>. CHO-K1 cells (ATCC CCL-61) were phenotypically validated as proline  
1023 auxotrophs and their *Cricetulus griseus* origin was confirmed by genomic sequencing.  
1024 *CHOP::GFP* and *XBPIs::Turquoise* reporters were introduced sequentially under  
1025 G418 and puromycin selection to generate the previously-described derivative CHO-  
1026 K1 S21 clone (Sekine *et al*, 2016). The cells were cultured in Nutrient mixture F-12  
1027 Ham (Sigma) supplemented with 10% (v/v) serum (FetalClone II; HyClone), 1 x  
1028 Penicillin-Streptomycin (Sigma), and 2 mM L-glutamine (Sigma). The CHO-K1 *FICD*  
1029 <sup>-/-</sup> cell line used in this study was described previously (Preissler *et al*, 2015b). HEK293T  
1030 cells (ATCC CRL-3216) were cultured in Dulbecco's Modified Eagle's Medium  
1031 (Sigma) supplemented as described above. Cell lines were subjected to random testing  
1032 for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit  
1033 (Lonza).

1034 Experiments were performed at cell densities of 60-90% confluence. Where indicated,  
1035 cells were treated with cycloheximide (Sigma) at 100 µg/ml diluted with fresh, pre-  
1036 warmed medium and then applied to the cells by medium exchange.

1037

1038 **Mammalian cell lysates**

1039 Cell lysis was performed as described in (Preissler *et al*, 2015a) with modifications. In  
1040 brief, mammalian cells were cultured on 10 cm dishes and treated as indicated and/or  
1041 transfected using Lipofectamine LTX with 5 µg plasmid DNA, and allowed to grow  
1042 for 24 to 40 h. Before lysis, the dishes were placed on ice, washed with ice-cold PBS,  
1043 and cells were detached in PBS containing 1 mM ethylenediaminetetraacetic acid

1044 (EDTA) using a cell scraper. The cells were sedimented for 5 min at  $370 \times g$  at  $4^{\circ}\text{C}$   
1045 and lysed in HG lysis buffer [20 mM HEPES-KOH pH 7.4, 150 mM NaCl, 2 mM  
1046 MgCl<sub>2</sub>, 10 mM D-glucose, 10% (v/v) glycerol, 1% (v/v) Triton X-100] containing  
1047 protease inhibitors (2 mM phenylmethylsulphonyl fluoride (PMSF), 4 µg/ml pepstatin,  
1048 4 µg/ml leupeptin, 8 µg/ml aprotinin) with 100 U/ml hexokinase (from *Saccharomyces*  
1049 *cerevisiae* Type F-300; Sigma) for 10 min on ice. The lysates were cleared for 10 min  
1050 at  $21,000 \times g$  at  $4^{\circ}\text{C}$ . Bio-Rad protein assay reagent (BioRad) was used to determine  
1051 the protein concentrations of lysates. For analysis by SDS-PAGE, SDS sample buffer  
1052 was added to the lysates and proteins were denatured by heating for 10 min at  $70^{\circ}\text{C}$   
1053 before separation on 12.5% SDS polyacrylamide gels. To detect endogenous BiP by  
1054 native-PAGE the lysate samples were loaded immediately on native gels (see below).

1055

### 1056 **Native polyacrylamide gel electrophoresis (native-PAGE)**

1057 Non-denaturing native-PAGE was performed as described (Preissler *et al*, 2015a).  
1058 Briefly, Tris-glycine polyacrylamide gels (4.5% stacking gel and a 7.5% separation gel)  
1059 were used to separate proteins from mammalian cell lysates to detect BiP monomers  
1060 and oligomers. The separation was performed in running buffer (25 mM Tris, 192 mM  
1061 glycine, pH ~8.8) at 120 V for 2 h. Afterwards, the proteins were transferred to a  
1062 polyvinylidene difluoride (PVDF) membrane in blotting buffer (48 mM Tris, 39 mM  
1063 glycine; pH ~9.2) supplemented with 0.04 (w/v) SDS for 16 h at 30 V for  
1064 immunodetection. The membrane was washed for 20 minutes in blotting buffer  
1065 (without SDS) supplemented with 20% (v/v) methanol before blocking. Volumes of  
1066 lysates corresponding to 30 µg of total protein were loaded per lane to detect  
1067 endogenous BiP from cell lysates by immunoblotting.

1068

### 1069 **Streptavidin pull-down and FLAG immunoprecipitation**

1070 To analyse the formation of FICD dimers *in vivo* (Figure 1B), CHO-K1 cells were  
1071 transfected with 4 µg plasmid DNA encoding His<sub>6</sub>-AviTag-FICD (UK 2275) or His<sub>6</sub>-  
1072 AviTag-FICD<sup>L258D</sup> (UK 2319) and FLAG-FICD (UK 2276) or FLAG-FICD<sup>L258D</sup> (UK  
1073 2318), and 4 µg plasmid DNA encoding BirA (in order to keep the final amount of  
1074 plasmid DNA the same, an empty pCEFL plasmid was used; Table S2) as described  
1075 above. 24 h before lysis the medium was exchanged to medium containing 50 µM

1076 Biotin (Molecular Probes). For streptavidin pull-down of His<sub>6</sub>-AviTag-FICD, CHO-K1  
1077 cells were transfected and allowed to grow for approximately 40 h before lysis in lysis  
1078 buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v)  
1079 glycerol] supplemented with protease inhibitors. The lysates were cleared twice,  
1080 normalized and equal volumes of the lysates were incubated with 50 µl Dynabeads  
1081 (MyOne Streptavidin C1, Life Technologies) for 60 to 90 min at 4 °C, rotating. The  
1082 beads were then recovered by centrifugation for 1 min at 200 × g and by placing the  
1083 tube in a magnetic separation stand. They were then washed three times at 25 °C with  
1084 RIPA buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5%  
1085 (v/v) sodium deoxycholate, 0.1% (v/v) SDS] supplemented with protease inhibitors.  
1086 Bound proteins were eluted in 25 µl urea sample buffer [8 M urea, 1.36% (v/v) SDS,  
1087 12% (v/v) glycerol, 40 mM Tris-HCl pH 6.8, 0.002% (w/v) bromophenol blue, 100  
1088 mM DTT] and heating for 10 min at 70 °C. Equal volumes of the samples were loaded  
1089 on a 12.5% SDS polyacrylamide gel and His<sub>6</sub>-AviTag-FICD and FLAG-FICD were  
1090 detected by immunoblotting. Samples of the normalized lysates (60 µg) were loaded as  
1091 an ‘input’ control.

1092 For the reciprocal experiment, FLAG M2 immunoprecipitation of FLAG-FICD, equal  
1093 volumes of the cleared and normalized lysates were incubated with 20 µl of Anti-FLAG  
1094 M2 affinity gel (Sigma) for 60 to 90 min at 4 °C, rotating. The beads were then  
1095 recovered by centrifugation for 1 min at 5,000 × g and washed three times with RIPA  
1096 buffer. The proteins were eluted in 35 µl 2 × SDS sample buffer (without DTT) for 10  
1097 min at 70 °C. The beads were then sedimented and the supernatants were transferred to  
1098 new tubes to which 50 mM DTT was added. Equal sample volumes were analysed by  
1099 SDS-PAGE and immunoblotting as described above.

1100

## 1101 **Immunoblot analysis**

1102 After separation by SDS-PAGE or native-PAGE (see above) the proteins were  
1103 transferred onto PVDF membranes. The membranes were blocked with 5% (w/v) dried  
1104 skimmed milk in TBS (25 mM Tris-HCl pH 7.5, 150 mM NaCl) and incubated with  
1105 primary antibodies followed by IRDye fluorescently labelled secondary antibodies (LI-  
1106 COR). The membranes were scanned with an Odyssey near-infrared imager (LI-COR).  
1107 Primary antibodies and antisera against hamster BiP [chicken anti-BiP (Avezov *et al.*,

1108 2013)], eIF2 $\alpha$  [mouse anti-eIF2 $\alpha$  (Scorsone *et al*, 1987)], FICD [chicken anti-FICD  
1109 (Preissler *et al*, 2015b)], monoclonal anti-FLAG M2 (Sigma), and IRDye 800CW  
1110 Streptavidin (LI-COR) were used.

1111

1112 **Flow cytometry**

1113 FICD (wild-type and mutants) over-expression-dependent induction of unfolded  
1114 protein response signalling was analysed by transient transfection of wild-type and  
1115 *FICD*<sup>-/-</sup> *CHOP*::*GFP* CHO-K1 UPR reporter cell lines with plasmid DNA encoding  
1116 the FICD protein and mCherry as a transfection marker, using Lipofectamine LTX as  
1117 described previously (Preissler *et al*, 2015b). 0.5  $\mu$ g DNA was used in [Figure S2B-C](#) to  
1118 transfect cells growing in 12-well plates. 40 h after transfection the cells were washed  
1119 with PBS and collected in PBS containing 4 mM EDTA, and single cell fluorescent  
1120 signals (20,000/sample) were analysed by dual-channel flow cytometry with an  
1121 LSRIFortessa cell analyser (BD Biosciences). GFP and mCherry fluorescence was  
1122 detected with excitation laser 488 nm, filter 530/30, and excitation laser 561, filter  
1123 610/20, respectively. Data were processed using FlowJo and median reporter (in Q1  
1124 and Q2) analysis was performed using Prism 6.0e (GraphPad).

1125

1126 **Production of VSV-G retrovirus in HEK293T cells and infection of CHO-K1 cells**

1127 In an attempt to establish BiP AMPylation in *FICD*<sup>-/-</sup> cells ([Figure 1A](#)), cells were  
1128 targeted with retrovirus expressing FICD (incorporating the naturally-occurring  
1129 repressive uORF found in its cDNA) and mCherry. HEK293T cells were split onto 6  
1130 cm dishes 24 h prior to co-transfection of pBABE-mCherry plasmid encoding FICD  
1131 (UK 1939; [Table S2](#)) with VSV-G retroviral packaging vectors, using TransIT-293  
1132 Transfection Reagent (Mirus) according to the manufacturer's instructions. 16 h after  
1133 transfection, medium was changed to medium supplemented with 1% (w/v) BSA  
1134 (Sigma). Retroviral infections were performed following a 24 h incubation by diluting  
1135 0.45  $\mu$ m filter-sterilized cell culture supernatants at a 1:1 ratio into CHO-K1 cell  
1136 medium supplemented with 10  $\mu$ g/ml polybrene (8 ml final volume) and adding this  
1137 preparation to *FICD*<sup>-/-</sup> CHO-K1 cells (1 x 10<sup>6</sup> cells seeded onto 10 cm dishes 24 h prior  
1138 to infection). Infections proceeded for 8 h, after which viral supernatant was replaced  
1139 with fresh medium. 48 h later, the cells were split into four 10 cm dishes. Five days

1140 after transfection, single cells were sorted according to their mCherry intensity.  
1141 Selected clones were expanded and analysed by flow cytometry (to assess mCherry  
1142 intensity) and native-PAGE (to check for BiP AMPylation).

1143

1144 **Protein purification**

1145 FICD

1146 Wild-type and mutant human FICD proteins (aa 104-445) were expressed as His<sub>6</sub>-Smt3  
1147 fusion constructs in T7 Express *lysY/I<sup>q</sup>* (NEB) *E. coli* cells. The cells were grown in LB  
1148 medium (usually 6 l per construct) containing 50 µg/ml kanamycin at 37 °C to an  
1149 optical density (OD<sub>600nm</sub>) of 0.6 and then shifted to 18 °C for 20 min, followed by  
1150 induction of protein expression with 0.5 mM isopropylthio β-D-1-galactopyranoside  
1151 (IPTG). The cultures were further incubated for 16 h at 18 °C, harvested, and lysed with  
1152 a high-pressure homogenizer (EmulsiFlex-C3; Avestin) in buffer A [25 mM Tris-HCl  
1153 pH 8.0, 500 mM NaCl, 40 mM imidazole, 1 mM MgCl<sub>2</sub>, 0.1 mM tris(2-  
1154 carboxyethyl)phosphine (TCEP)] containing protease inhibitors [2 mM PMSF, 4 µg/ml  
1155 pepstatin, 4 µg/ml leupeptin, 8 µg/ml aprotinin], 0.1 mg/ml DNaseI, and 20 µg/ml  
1156 RNaseA. The lysates were centrifuged for 30 min at 45,000 × g and incubated with 1  
1157 ml of Ni-NTA agarose (Qiagen) per 1 l expression culture, for 30 min rotating at 4 °C.  
1158 Afterwards, the beads were transferred to a gravity-flow Econo column (49 ml volume;  
1159 BioRad), washed with five column volumes (CV) buffer A without MgCl<sub>2</sub> and buffer  
1160 B (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM imidazole, 0.1 mM TCEP). The  
1161 beads were further washed sequentially with buffer B sequentially supplemented with  
1162 (i) 1 M NaCl, (ii) 10 mM MgCl<sub>2</sub> + 5 mM ATP and (iii) 0.5 M Tris-HCl pH 8.0 [each 5  
1163 CV], followed by 2 CV TNT-Iz10 (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM  
1164 TCEP, 10 mM imidazole). Proteins were eluted by on-column cleavage with 1.5 µg/ml  
1165 Ulp1 protease carrying a C-terminal StrepII-tag [Ulp1-StrepII (UK 1983)] in 1 bed  
1166 volume TNT-Iz10 overnight at 4 °C. The eluate was collected, retained cleavage  
1167 products were washed off the beads with TNT-Iz10, and all fractions were pooled. The  
1168 total eluate was diluted 1:2 with 25 mM Tris-HCl pH 8.0 and further purified by anion  
1169 exchange chromatography using a 6 ml RESOURCE Q column (GE Healthcare)  
1170 equilibrated in 95% AEX-A (25 mM Tris-HCl pH 8.0, 25 mM NaCl) and 5% AEX-B  
1171 (25 mM Tris-HCl, 1 M NaCl). Proteins were eluted by applying a gradient from 5-30%

1172 AEX-B in 20 CV at 3 ml/min. Fractions of elution peaks (absorbance at 280 nm,  $A_{280\text{nm}}$ )  
1173 corresponding to monomeric or dimeric FICD were pooled and concentrated using 30  
1174 kDa MWCO centrifugal filters (Amicon Ultra; Merck Millipore) in the presence of 1  
1175 mM TCEP. The proteins were then subjected to size-exclusion chromatography using  
1176 a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) equilibrated in SEC  
1177 buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl). Peaks corresponding to monomeric  
1178 or dimeric FICD were supplemented with 1 mM TCEP, concentrated ( $> 120 \mu\text{M}$ ), and  
1179 frozen in aliquots.

1180 BiP

1181 Mutant Chinese hamster BiP proteins with an N-terminal His<sub>6</sub>-tag were purified as  
1182 described before with modifications (Preissler *et al*, 2017b). Proteins were expressed  
1183 in M15 *Escherichia coli* (*E. coli*) cells (Qiagen). The bacterial cultures were grown in  
1184 LB medium supplemented with 100  $\mu\text{g}/\text{ml}$  ampicillin and 50  $\mu\text{g}/\text{ml}$  kanamycin at 37  
1185 °C to an  $OD_{600\text{nm}}$  of 0.8 and expression was induced with 1 mM IPTG. The cells were  
1186 further grown for 6 h at 37 °C, harvested and lysed in buffer C [50 mM Tris-HCl pH 8,  
1187 500 mM NaCl, 1 mM MgCl<sub>2</sub>, 10% (v/v) glycerol, 20 mM imidazole] containing 0.1  
1188 mg/ml DNaseI and protease inhibitors. The lysates were cleared for 30 min at 45,000  
1189  $\times g$  and incubated with 1 ml of Ni-NTA agarose (Quiagen) per 1 l of expression culture,  
1190 for 2 h rotating at 4 °C. Afterwards, the matrix was transferred to a gravity-flow Econo  
1191 column (49 ml volume; BioRad) and washed with buffer D [50 mM Tris-HCl pH 8.0,  
1192 500 mM NaCl, 10% (v/v) glycerol, 30 mM imidazole], buffer E [50 mM Tris-HCl pH  
1193 8.0, 300 mM NaCl, 10 mM imidazole, 5 mM  $\beta$ -mercaptoethanol], and buffer F  
1194 sequentially supplemented with (i) 1 M NaCl, (ii) 10 mM MgCl<sub>2</sub> + 3 mM ATP, (iii) 0.5  
1195 M Tris-HCl pH 7.4, or (iv) 35 mM imidazole. The BiP proteins were then eluted with  
1196 buffer F [50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM  $\beta$ -mercaptoethanol, 250 mM  
1197 imidazole], dialyzed against HKM (50 mM HEPES-KOH pH 7.4, 150 mM KCl, 10  
1198 mM MgCl<sub>2</sub>) and concentrated with 30 kDa MWCO centrifugal filters. The proteins  
1199 were flash-frozen in aliquots and stored at -80 °C.

1200 GST-TEV-BiP constructs were purified like His<sub>6</sub>-Smt3-FICD, above, with minor  
1201 alterations. Purification proceeded without the inclusion of imidazole in the purification  
1202 buffers. Cleared lysates were supplemented with 1 mM DTT and incubated with GSH-  
1203 Sepharose 4B matrix (GE Healthcare) for 1 h at 4 °C. 2 CV of TNT(0.1) (25 mM Tris-

1204 HCl pH 8.0, 150 mM NaCl, 0.1 mM TCEP) was used as a final wash step before elution.  
1205 GST-TEV-BiP was eluted with 10 mM HEPES-KOH pH 7.4, 20 mM Tris-HCl pH 8.0,  
1206 30 mM KCl, 120 mM NaCl, 2 mM MgCl<sub>2</sub> and 40 mM reduced glutathione. The eluate  
1207 was cleaved with TEV protease (1/200 w/w; UK 759), whilst dialysing into TN plus 1  
1208 mM DTT, for 16 h at 4 °C. Uncleaved BiP was depleted by incubation, for 1 h at 4 °C,  
1209 with GSH-Sepharose 4B matrix (1 ml per 5 mg of protein). The flow through was  
1210 collected. Retained, cleaved material was washed from the matrix with 5 CV of  
1211 TNT(0.1). All the cleaved, non-bound material was pooled. In order to AMPylate BiP,  
1212 the cleaved product was combined with 1/50 (w/w) GST-TEV-FICD(45-458)<sup>E234G</sup> (UK  
1213 1479; purified like the GST-TEV-BiP without TEV cleavage steps). The AMPylation  
1214 reaction was supplemented with 10 mM MgATP (10 mM MgCl<sub>2</sub> + 10 mM ATP), and  
1215 incubated for 16 h at 25 °C. GST-TEV-FICD was then depleted by incubation with  
1216 GSH-Sepharose 4B matrix, as above. Proteins were concentrated to > 200 μM. Aliquots  
1217 were snap-frozen in liquid nitrogen and stored at -80 °C.

1218 When required, protein samples were validated as being nucleotide free (Apo) by their  
1219 A<sub>260/280</sub> ratio and reference to IP-RP-HPLC analysis as conducted in (Preissler *et al.*,  
1220 2017a).

### 1221 Formation of disulphide-linked FICD dimers

1222 Expression and purification of disulphide-linked dimers [of FICD<sup>A252C-C421S</sup> (UK 2219)  
1223 and FICD<sup>A252C-H363A-C421S</sup> (trap; UK 2269)] was performed as described above with  
1224 some alterations. After the affinity chromatography step, on-column cleavage was  
1225 performed in TNT-Iz10 containing 1.5 μg/ml Upl1-StrepII and the retained cleavage  
1226 products were washed off the beads with TN-Iz10 (25 mM Tris-HCl pH 8.0, 150 mM  
1227 NaCl, 10 mM imidazole) in the absence of reducing agent. The pooled eluate was  
1228 concentrated and diluted 1:4 with TN-Iz10. To allow for efficient disulphide bond  
1229 formation the samples were supplemented with 20 mM oxidized glutathione and  
1230 incubated overnight at 4 °C. Afterwards, the protein solutions were diluted 1:2 with 25  
1231 mM Tris-HCl pH 8.0 and further purified by anion-exchange and size-exclusion  
1232 chromatography. The final preparations were analysed by non-reducing SDS-PAGE to  
1233 confirm quantitative formation of covalently linked dimers (> 95%). Cysteine-free  
1234 FICD<sup>C421S</sup> (UK 2161) was purified according to the same protocol. A separate  
1235 preparation of non-disulphide-bonded FICD<sup>A252C-C421S</sup> (UK 2219), which was not

1236 subjected to oxidation with glutathione, was used in control experiments (Figure 3B,  
1237 S3A and C).

1238 **In vitro AMPylation**

1239 Standard radioactive in vitro AMPylation reactions were performed in HKMC buffer  
1240 (50 mM HEPES-KOH pH 7.4, 150 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>) containing  
1241 40 μM ATP, 0.034 MBq [ $\alpha$ -<sup>32</sup>P]-ATP (EasyTide; Perkin Elmer), 0.2 μM FICD, and 1.5  
1242 μM ATP-hydrolysis and substrate-binding deficient BiP<sup>T229A-V461F</sup> (UK 1825) in a final  
1243 volume of 15 μl. Where indicated, samples contained 5 μM s-sFICD<sup>A252C-H363A-C421S</sup>  
1244 (UK 2269, trap) to sequester modified BiP. The reactions were started by addition of  
1245 nucleotides. After a 20 min incubation at 25 °C the reactions were stopped by addition  
1246 of 5 μl 4 × SDS sample buffer and denaturation for 5 min at 75 °C. The samples were  
1247 applied to SDS-PAGE and the gels were stained with Coomassie (InstantBlue;  
1248 expedeon). The dried gels were exposed to a storage phosphor screen and radioactive  
1249 signals were detected with a Typhoon biomolecular imager (GE Healthcare). Signals  
1250 were quantified using ImageJ64 software (NIH).

1251 The reactions to analyse AMPylation at elevated concentrations (2 or 10 μM; Figure  
1252 S4A) contained 2 μM BiP<sup>T229A-V461F</sup>, 80 μM ATP and 0.034 MBq [ $\alpha$ -<sup>32</sup>P]-ATP in a final  
1253 volume of 15 μl. The reactions were stopped after 5 min incubation at 25 °C.

1254 Time course experiments (Figure 2E) were performed likewise but reactions contained  
1255 40 μM ATP, 0.136 MBq [ $\alpha$ -<sup>32</sup>P]-ATP, 0.3 μM FICD, 2 μM BiP<sup>T229A-V461F</sup>, and 5 μM  
1256 trap in a final volume of 60 μl. The reactions were incubated at 30 °C and samples (15  
1257 μl) were taken at different time intervals and processed as described above.

1258 To study the effect of the concentration of wild-type FICD protein on its ability to  
1259 establish a pool of AMPylated BiP (Figure 3A), final reactions were setup with 400 μM  
1260 ATP, 0.049 MBq [ $\alpha$ -<sup>32</sup>P]-ATP, 2.5 nM to 400 nM FICD (UK 2052) and 5 μM BiP<sup>T229A-</sup>  
1261 <sup>V461F</sup>, without or with 5 μM trap in a final volume of 15 μl. The reactions were pre-  
1262 incubated for 2 h before addition of nucleotides. After 2 and 16 h incubation with  
1263 nucleotides at 25 °C (as indicated) samples (5 μl) were taken and denatured by heating  
1264 in SDS sample buffer for analysis.

1265 To compare the activity of disulphide-bonded FICD under non-reducing and reducing  
1266 conditions (Figure 3C) s-sFICD<sup>A252C-C421S</sup> protein (UK 2219) was pre-incubated 16 h at

1267 25 °C without or with 10 mM DTT and a sample was analysed by non-reducing SDS-  
1268 PAGE after denaturation in SDS sample buffer containing 40 mM N-ethylmaleimide  
1269 (NEM). Afterwards, AMPylation reactions (15  $\mu$ l final volume) were set up with 400  
1270  $\mu$ M ATP, 0.049 MBq [ $\alpha$ -<sup>32</sup>P]-ATP, 2.5 nM to 400 nM s-sFICD<sup>A252C-C421S</sup>, and 5  $\mu$ M  
1271 BiP<sup>T229A-V461F</sup> in the presence or absence of 5 mM DTT. Samples were incubated for  
1272 16 h at 25 °C and 5  $\mu$ l was taken and processed for analysis by reducing SDS-PAGE as  
1273 described above. Parallel reactions performed with cysteine-free FICD<sup>C421S</sup> (UK 2161),  
1274 which underwent the same purification and oxidation procedure, served as a control.  
1275 The experiment presented in [Figure S3C](#) was performed accordingly under non-  
1276 reducing conditions, but the reactions were incubated for 2 h at 25 °C and in the  
1277 presence of 5  $\mu$ M trap.

1278

### 1279 **Coupled in vitro AMPylation/deAMPylation reactions**

1280 To measure AMPylation-/deAMPylation-dependent AMP production by FICD  
1281 proteins reactions were set up in HKM buffer containing 250  $\mu$ M ATP, 0.0185 MBq  
1282 [ $\alpha$ -<sup>32</sup>P]-ATP, 3 mM TCEP, 5  $\mu$ M ATP-hydrolysis-deficient BiP<sup>T229A</sup> (UK 838), and 2  
1283  $\mu$ M FICD proteins in a final volume of 30  $\mu$ l. The reactions were started by addition of  
1284 nucleotides and incubated for 2 h at 30 °C. Afterwards, 2  $\mu$ l were spotted onto a thin  
1285 layer chromatography (TLC) plate (PEI Cellulose F; Merck Millipore) pre-spotted with  
1286 2  $\mu$ l of nucleotide mix containing AMP, ADP, and ATP (each at 3.5 mM). The TLC  
1287 plate was developed with 400 mM LiCl and 10% (v/v) acetic acid as a mobile phase  
1288 and the dried plates were exposed to a storage phosphor screen. The signals were  
1289 detected with a Typhoon biomolecular imager and quantified using ImageJ64.

1290

### 1291 **DeAMPylation measured by fluorescence polarisation (FP)**

1292 Measurement of deAMPylation kinetics was performed as described previously  
1293 (Preissler *et al*, 2017a) with modifications. The probe (BiP<sup>V461F</sup> modified with  
1294 fluorescent, FAM-labelled AMP; BiP<sup>V461F</sup>-AMP<sup>FAM</sup>) was generated by pre-incubating  
1295 FICD<sup>E234G</sup> at 25  $\mu$ M in HKM buffer with 200  $\mu$ M ATP-FAM [N<sup>6</sup>-(6-amino)hexyl-  
1296 adenosine-5'-triphosphate; Jena Bioscience] for 10 min at 30 °C, followed by addition  
1297 of 25  $\mu$ M His<sub>6</sub>-tagged BiP<sup>V461F</sup> (UK 182) to a final volume of 50  $\mu$ l, and further  
1298 incubation for 2 h at 30 °C. Afterwards, the reaction was diluted with 950  $\mu$ l of HKMG-

1299 Iz20 [50 mM HEPES-KOH pH 7.4, 150 mM KCl, 10 mM MgCl<sub>2</sub>, 5% (v/v) glycerol,  
1300 20 mM imidazole] and BiP proteins were bound to 80 µl Ni-NTA agarose beads  
1301 (Qiagen) for 30 min at 25 °C in the presence of 0.01% Triton X-100. Following several  
1302 wash steps in the same buffer proteins were eluted in HKMG-Iz250 [50 mM HEPES-  
1303 KOH pH 7.4, 150 mM KCl, 10 mM MgCl<sub>2</sub>, 5% (v/v) glycerol, 250 mM imidazole],  
1304 flash-frozen in aliquots, and stored at -80 °C.  
  
1305 DeAMPylation reactions were performed in FP buffer [50 mM HEPES-KOH pH 7.4,  
1306 150 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.1% (v/v) Triton X-100] in 384-well  
1307 polysterene microplates (black, flat bottom, µCLEAR; greiner bio-one) at 30 °C in a  
1308 final volume of 30 µl containing trace amounts of fluorescent BiP<sup>V461F-AMP-FAM</sup> probe  
1309 (17 nM) and FICD proteins (0.75 or 7.5 µM). Fluorescence polarisation of FAM ( $\lambda_{\text{ex}} =$   
1310 485 nm,  $\lambda_{\text{em}} = 535$  nm) was measured with an Infinite F500 plate reader (Tecan). Fitting  
1311 of the raw data to a single-exponential decay function was done using Prism 6.0e  
1312 (GraphPad).

1313

#### 1314 **Analytical size-exclusion chromatography**

1315 Analytical size-exclusion chromatography (SEC) was performed as described  
1316 previously (Preissler *et al*, 2015a). Purified FICD proteins were adjusted to 20 µM in  
1317 HKMC buffer (50 mM HEPES-KOH pH 7.4, 150 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM  
1318 CaCl<sub>2</sub>) and incubated at 25 °C for at least 20 min before injection. From each sample  
1319 10 µl was injected onto a SEC-3 HPLC column (300 Å pore size; Agilent Technologies)  
1320 equilibrated with HKMC at a flow rate of 0.3 ml/min. Runs were performed at 25 °C  
1321 and A<sub>280nm</sub> absorbance traces were recorded. Protein standards (Bio-Rad, cat. no. 151–  
1322 1901) were run as size references and the elution peaks of γ-globulin (158 kDa),  
1323 ovalbumin (44 kDa), and myoglobin (17 kDa) are indicated. For dimer SEC studies  
1324 in [Figure S1D-E](#), the FICD proteins were incubated for 16 h at 25 °C before injection.  
1325 To investigate capture of AMPylated BiP by s-sFICD<sup>A252C-H363A-C421S</sup> (UK 2269, trap),  
1326 by SEC ([Figure S2H](#)), in vitro AMPylation reactions containing different combinations  
1327 of 20 µM BiP<sup>T229A-V461F</sup> (UK 1825), 10 µM trap, and 3 µM FICD<sup>L258D</sup> (UK 2091) were  
1328 performed in HKMC (supplemented with 2 mM ATP when indicated) and incubated  
1329 for 1.5 h at 30 °C before injection.

1330

1331 **Fluorescence detection system sedimentation velocity analytical**  
1332 **ultracentrifugation (FDS-SV-AUC)**

1333 Bacterial expression and purification of FICD proteins carrying an N-terminal cysteine  
1334 for site-specific labelling (FICD<sup>NC</sup>, UK 2339, and FICD<sup>L258D-NC</sup>, UK 2367) was  
1335 performed as described above with the following alterations: Cells were lysed in the  
1336 presence of 5 mM  $\beta$ -mercaptoethanol and the eluate pool after affinity chromatography  
1337 and on-column cleavage was supplemented with 5 mM DTT and diluted 1:2 with 25  
1338 mM Tris-HCl pH 8.0 containing 0.2 mM TCEP. The subsequent anion-exchange  
1339 chromatography step was performed with buffer solutions AEX-A and AEX-B  
1340 supplemented with 0.2 mM TCEP. Afterwards, the peak fractions corresponding to the  
1341 dimeric form of FICD were pooled and concentrated. The protein at 200  $\mu$ M was  
1342 labelled in 150  $\mu$ l with 600  $\mu$ M Oregon Green 488-iodoacetamide in the presence of  
1343 0.5 mM TCEP and 0.1 mM EDTA for 16 h at 4 °C. The reaction was quenched with 2  
1344 mM DTT for 10 min at 25 °C. Afterwards the sample was passed through a CentriPure  
1345 P2 desalting column (emp) equilibrated in SEC buffer containing 0.2 mM TCEP. The  
1346 eluate was applied to size-exclusion chromatography using a Superdex 200 10/300 GL  
1347 column (GE Healthcare) in the presence of 0.2 mM TCEP. The fractions of the  $A_{280\text{nm}}$   
1348 peak, corresponding to dimeric FICD, were pooled and the concentration of TCEP was  
1349 adjusted to 1 mM. The proteins were concentrated and frozen in aliquots. The protein  
1350 concentration was determined after denaturing the proteins with 6 M guanidine  
1351 hydrochloride by measuring absorbance at 280 nm and 496 nm with a NanoDrop  
1352 Spectrophotometer (Thermo Fisher Scientific). The concentration was calculated using  
1353 the following equation:

1354 Protein concentration (M) =  $[A_{280\text{nm}} - (A_{496\text{nm}} \times 0.12)]/\varepsilon$

1355 Where 0.12 is the correction factor for the fluorophore's absorbance at 280 nm, and  $\varepsilon$   
1356 is the calculated molar extinction coefficient of FICD ( $29,340 \text{ cm}^{-1}\text{M}^{-1}$ ). The labelling  
1357 efficiency of the FICD<sup>NC</sup> preparation was 74% as calculated based on the  $A_{496\text{nm}}$  value  
1358 and assuming an extinction coefficient for Oregon Green 488 of  $70,000 \text{ cm}^{-1}\text{M}^{-1}$ . The  
1359 labelling efficiency of the monomeric FICD<sup>L258D-NC</sup> control preparation was 9.6%.  
1360 Labelling of the endogenous cysteine residue (Cys421) of wild-type FICD was very  
1361 inefficient (< 1%) and thus considered negligible.

1362 Samples of Oregon Green-labelled FICD in 50 mM HEPES-KOH pH 7.4, 150 mM  
1363 KCl, 10 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.3 mM TCEP, 0.1 % Tween-20, 0.15 mg/ml BSA  
1364 (Sigma), ranging in concentration from 1.6 μM to 31 pM, were centrifuged at 45,0000  
1365 rpm at 20 °C in an An50Ti rotor using an Optima XL-I analytical ultracentrifuge  
1366 (Beckmann) equipped with a fluorescence optical detection system (Aviv Biomedical)  
1367 with fixed excitation at 488 nm and fluorescence detection at > 505 nm. Data were  
1368 processed and analysed using SEDFIT 15 and SEDPHAT 13b (Schuck, 2003)  
1369 according to the published protocol for high-affinity interactions detected by  
1370 fluorescence (Chaturvedi *et al*, 2017). Data were plotted with Prism 6.0e (GraphPad)  
1371 or GUSSI (Brautigam, 2015).

1372

1373 **Differential Scanning Fluorimetry (DSF)**

1374 DSF experiments were performed on an ABI 7500 qPCR machine (Applied  
1375 Biosciences). Experiments were carried out in 96-well qPCR plates (Thermofisher),  
1376 with each sample in technical triplicate and in a final volume of 20 μl. Protein was used  
1377 at a final concentration of 2 μM, ligands at the concentration indicated in the figure  
1378 legend (2.5-20 mM), and SYPRO Orange (Thermofisher) dye at a 10x concentration in  
1379 a buffer of HKM plus 1 mM TCEP (unless otherwise specified). For the ATP titration  
1380 (Figure 4F and S4E), the DSF buffer was supplemented with an additional 15 mM  
1381 MgCl<sub>2</sub> (25 mM total MgCl<sub>2</sub>). Fluorescence of the SYPRO Orange dye was monitored  
1382 over a temperature range of 20-95 °C using the VIC filter set. Data was then analysed  
1383 in Prism 7.0e (GraphPad), with melting temperature calculated as the global minimums  
1384 of the negative first derivatives of the relative fluorescent unit melt curves (with respect  
1385 to temperature).

1386

1387 **Bio-layer interferometry (BLI)**

1388 In vitro biotinylation

1389 Ligands for BLI were generated from the tag cleaved forms of unmodified or  
1390 AMPylated GST-TEV-AviTag-haBiPV461F(19-654) (UK 2043) and GST-TEV-  
1391 AviTag-haBiP(28-635)<sup>T229A-V461F</sup> (UK 2331). Biotinylation was conducted in vitro on  
1392 100 μM target protein, with 200 μM biotin (Sigma), 2 μM GST-BirA (UK 1801) in a  
1393 buffer of 2 mM ATP, 5 mM MgCl<sub>2</sub>, 25 mM Tris-HCl pH 8.0, 150 mM NaCl and 1

1394 mM TCEP. The reaction mixture was incubated for 16 h at 4 °C. Excess biotin was  
1395 removed by size-exclusion chromatography on a S200 10/300 GL column (GE  
1396 Healthcare) with a distal 1 ml GSTrap 4B (GE Healthcare), connected in series. The  
1397 ligand was confirmed as being > 95% biotinylated as judged by streptavidin gel-shift.  
1398 In the case of Biotinylated-AviTag-haBiP(28-635)<sup>T229A-V461F</sup> this protein was also  
1399 made nucleotide free by the addition of 2 U CIP (NEB) per mg of BiP, plus extensive  
1400 dialysis into TN buffer with 1 mM DTT and 2 mM EDTA (dialysed with several  
1401 dialysate changes, for 2 days at 4 °C). The protein was then purified by anion  
1402 exchange chromatography on a MonoQ 5/50 GL column (GE Healthcare) using  
1403 buffers AEX-A and AEX-B with a gradient of 7.5-50% B over 20 CV at a flow rate  
1404 of 1 ml/min. The protein was concentrated using a 30 kDa MWCO centrifugal filters  
1405 (Amicon Ultra; Merck Millipore) and then gel filtered, as above, but into an HKM  
1406 buffer. Fractions were pooled and supplemented with 1 mM TCEP. All proteins after  
1407 biotinylation and purification were concentrated to > 20 µM, flash-frozen in small  
1408 aliquots and stored at -80 °C.

1409 Kinetic experiments

1410 All BLI experiments were conducted on the FortéBio Octet RED96 System (Pall  
1411 FortéBio) in a buffer basis of HKM plus 0.05% Triton X-100 (HKMTx). Nucleotide  
1412 was added as indicated. Streptavidin (SA)-coated biosensors (Pall FortéBio) were  
1413 hydrated in HKMTx for at least 30 min prior to use. All BLI experiments were  
1414 conducted at 30 °C with the experimental steps as indicated in the text. BLI reactions  
1415 were prepared in 200 µl volumes in 96 well microplates (greiner bio-one, cat. no.  
1416 655209). Ligand loading was performed for 300 to 600 s at a shake speed of 1000  
1417 rpm until a binding signal of 1 nm was reached. The immobilised ligand sensor was  
1418 then baselined in assay solution for at least 200 s. For kinetic experiments with  
1419 biotinylated-AviTag-haBiP(28-635)<sup>T229A-V461F</sup>:Apo [BiP<sup>T229A-V461F</sup>:Apo (UK 2331)]  
1420 loaded on the tip, a 10 Hz acquisition rate was used and the baseline, association and  
1421 dissociation steps were conducted at a 400 rpm shake speed. Preceding the baseline  
1422 step biotinylated BiP<sup>T229A-V461F</sup>:Apo was also activated with or without ATP (2 mM  
1423 unless otherwise stated), as indicated, for 300 s at a 1000 rpm shake speed. In these  
1424 experiments FICD analyte association or dissociation steps were conducted in the  
1425 presence or absence of nucleotide, as indicated, with ATP at 8 mM and ADP at 2  
1426 mM. These concentrations were chosen in an attempt to saturate either monomeric or

1427 dimeric FICD with the respective nucleotide [ $K_d$  of MgADP for wild-type FICD is  
1428 1.52  $\mu$ M by ITC (Bunney *et al*, 2014);  $K_{1/2}$  of ATP induced FICD  $T_m$  shift in the low  
1429 mM range] and/or to make ATP binding non-rate limiting. In [Figure S8A](#), as a control  
1430 for the absence of substantial ATP dissociation from BiP, between the activation and  
1431 baseline step an additional 1500 s BiP wash ( $\pm$  ATP) was included, as indicated.

1432 Other BLI experiments were conducted with all steps at a 1000 rpm shake speed with  
1433 a 5 Hz acquisition rate. All association-dissociation kinetics were completed in  $\leq$   
1434 1500 s. Data was processed in Prism 7.0e (GraphPad). Note, the FICD variants used  
1435 as analytes in all BLI experiments were catalytically inactive His363Ala variants  
1436 (used at 250 nM).

1437 In the dimer dissociation BLI experiments biotinylated AviTag-FICD(104-458)<sup>H363A</sup>  
1438 (UK 2422) was diluted to 3 nM and incubated for 10 min at 25 °C with either dimeric  
1439 FICD<sup>H363A</sup> or monomeric FICD<sup>L258D-H363A</sup> (at 300 nM) in HKMTx. After this  
1440 incubation period the streptavidin biosensors were loaded until those immobilising  
1441 hetero-labelled dimers (biotinylated AviTag-FICD(104-458)<sup>H363A</sup> with FICD<sup>H363A</sup>)  
1442 were loaded to a 1 nm displacement. Dissociation was initiated by dipping in HKTx  
1443 buffer (50 mM HEPES-KOH pH 7.4, 150 mM KCl and 0.05% Triton X-100)  $\pm$   
1444 nucleotide at 5 mM, as indicated. Data was processed by subtracting the respective  
1445 monomer incubated biotinylated FICD tip from the dimeric hetero-labelled dimer  
1446 dissociation, followed by fitting of the corrected dissociation to mono-exponential  
1447 decay using Prism 7.0e (GraphPad).

1448

#### 1449 **Protein crystallization and structure determination**

1450 FICD proteins were purified as above in *Protein Purification* but gel filtered into a  
1451 final buffer of 10 mM Tris-HCl pH 8.0, 150 mM NaCl and 1 mM TCEP [T(10)NT].  
1452 Proteins were diluted to 9 mg/ml in T(10)NT prior to crystallisation, via sitting drop  
1453 vapour diffusion. For structures containing ATP, final diluted protein solutions were  
1454 supplemented with MgATP (from a pH 7.4, 100 mM stock solution) to a final  
1455 concentration of 10 mM. A drop ratio of protein solution to crystallisation well  
1456 solution of 200:100 nl was used. Where applicable crystals were obtained by  
1457 microseeding (D'Arcy *et al*, 2007), from conditions provided in [Table S1](#). In these  
1458 instances, a drop ratio of protein solution to water-diluted seeds to crystallisation well

1459 solution of 150:50:100 nl was used. The best diffracting crystals were obtained in  
1460 crystallisation conditions detailed in [Table S1](#).  
1461 Diffraction data were collected from the Diamond Light Source, and the data  
1462 processed using XDS (Kabsch, 2010) and the CCP4 module Aimless (Winn *et al*,  
1463 2011; Evans & Murshudov, 2013). Structures were solved by molecular replacement  
1464 using the CCP4 module Phaser (McCoy *et al*, 2007; Winn *et al*, 2011). For the  
1465 FICD<sup>L258D</sup>:Apo and FICD:ATP structures the human FICD protein (FICD:MgADP)  
1466 structure 4U0U from the Protein Data Bank (PDB) was used as a search model.  
1467 Subsequent molecular replacements used the solved FICD<sup>L258D</sup>:Apo structure as a  
1468 search model. Manual model building was carried out in COOT (Emsley *et al*, 2010)  
1469 and refined using refmac5 (Winn *et al*, 2003). Metal binding sites were validated  
1470 using the CheckMyMetal server (Zheng *et al*, 2017). Polder (OMIT) maps were  
1471 generated by using the Polder Map module of Phenix (Liebschner *et al*, 2017; Adams  
1472 *et al*, 2010). Structural figures were prepared using UCSF Chimera (Pettersen *et al*,  
1473 2004) and PyMol (Schrödinger, LLC, 2015).

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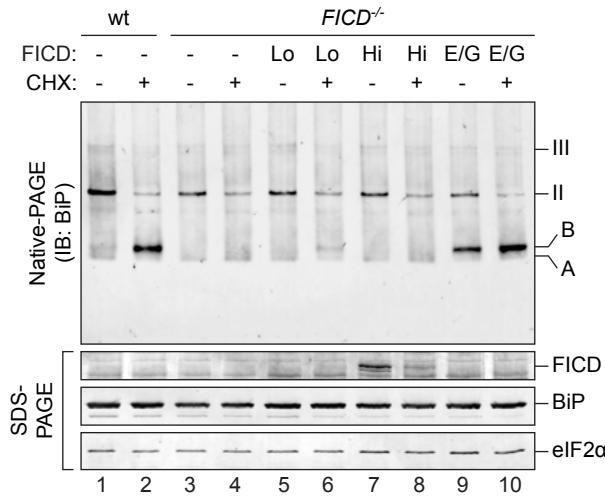
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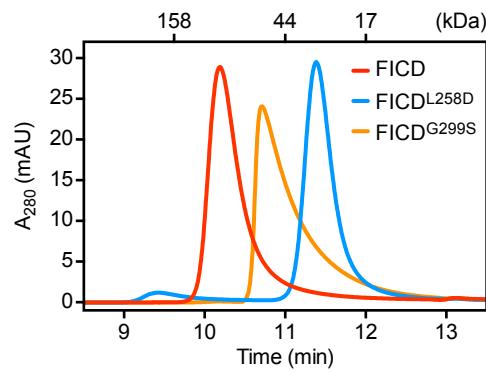
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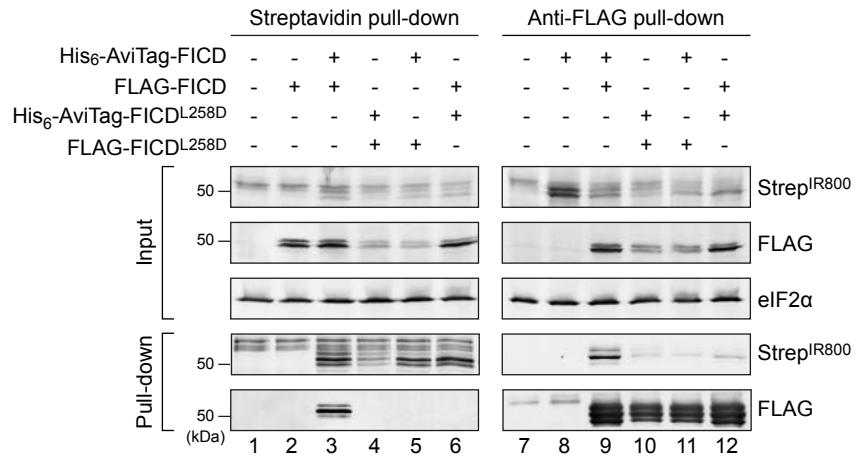
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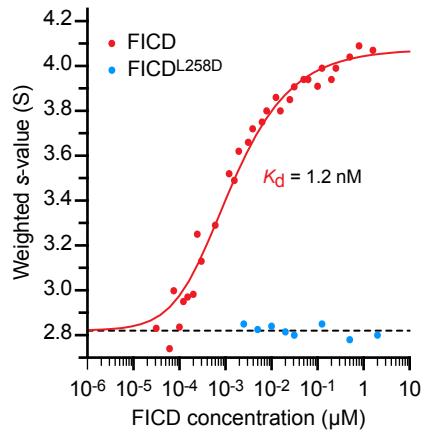
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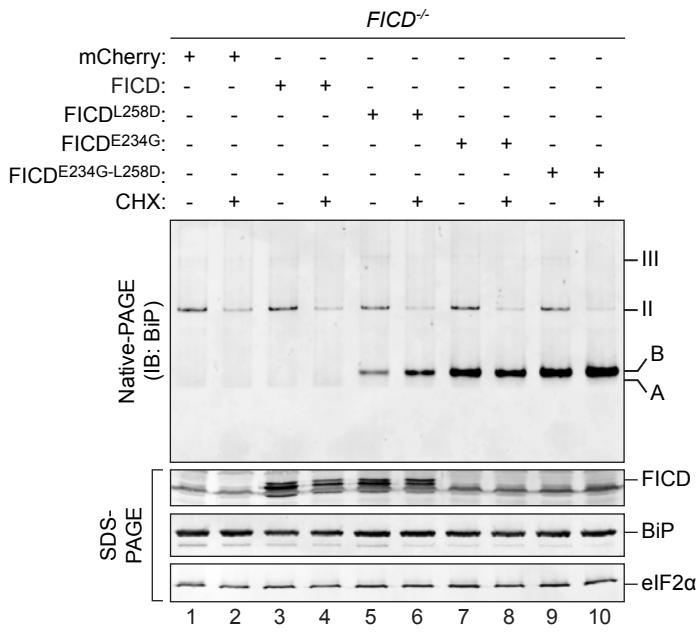
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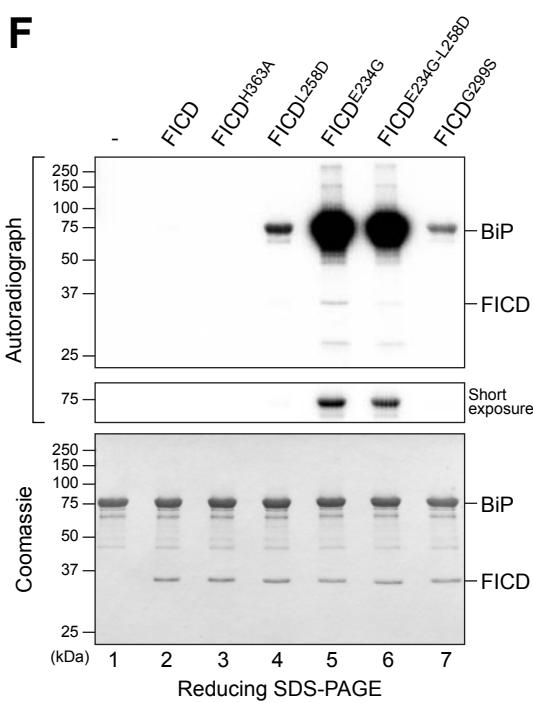
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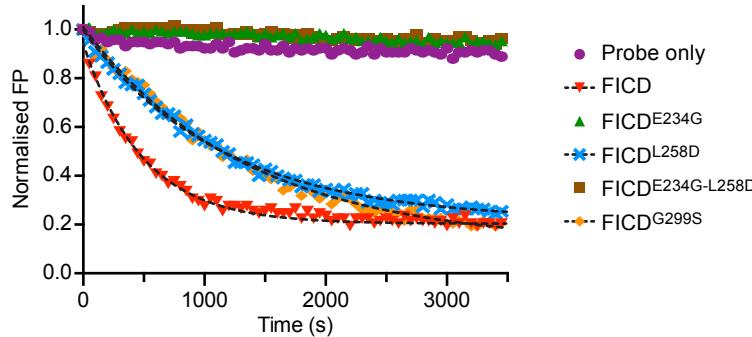


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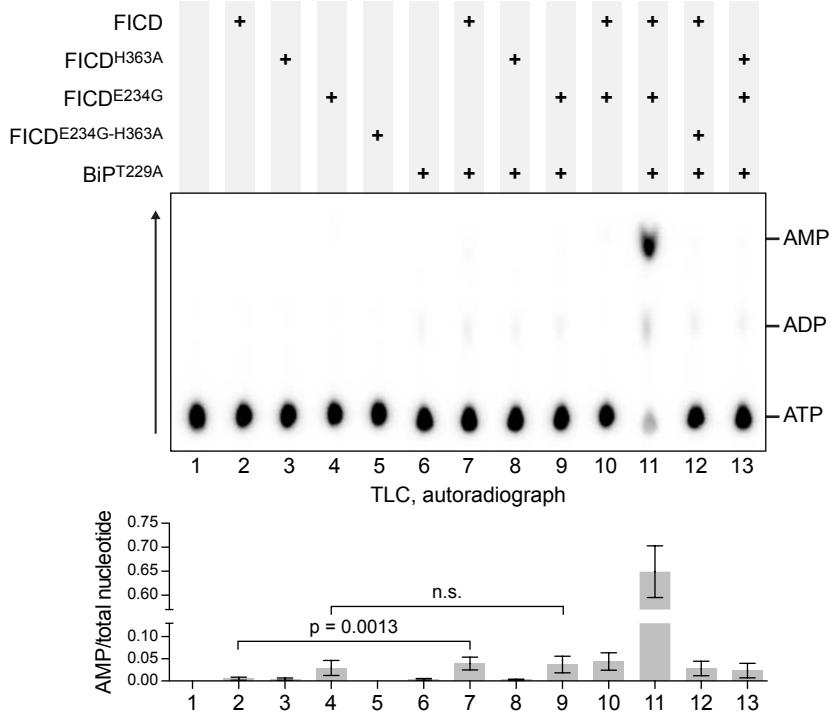


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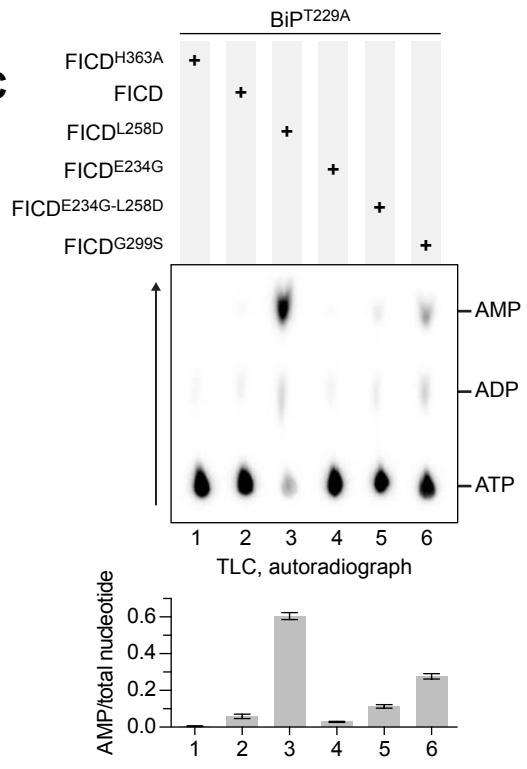
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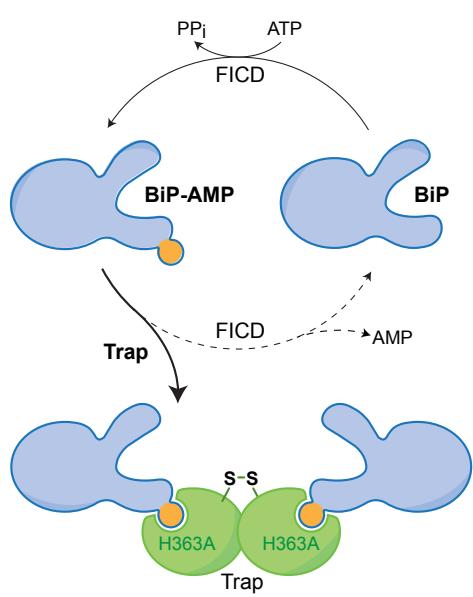
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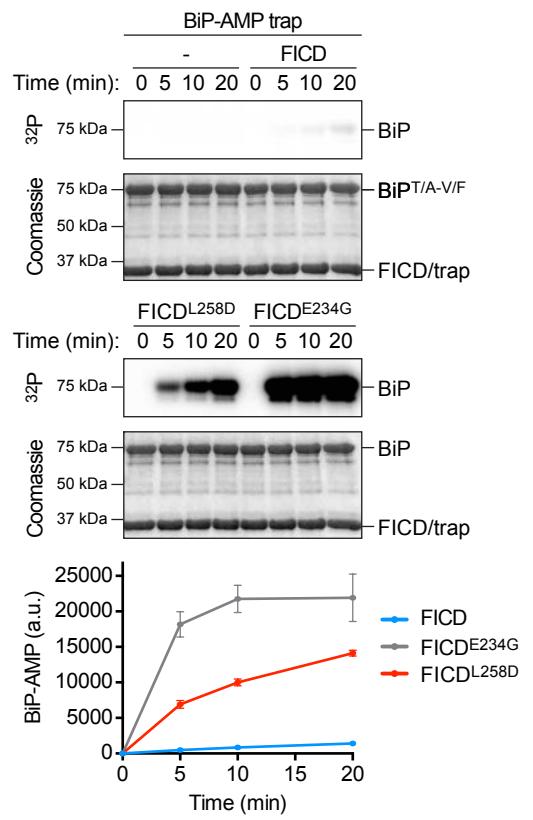
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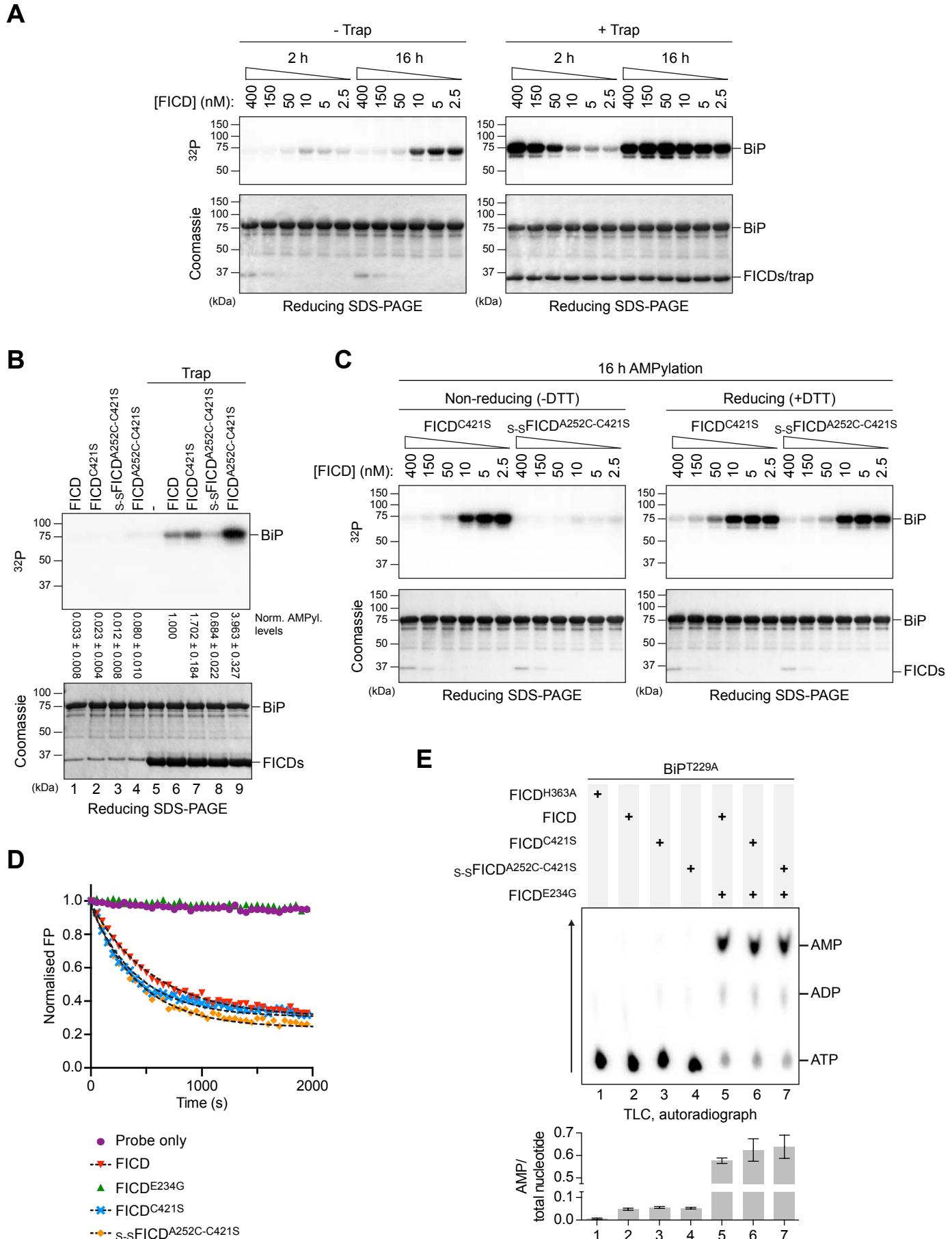
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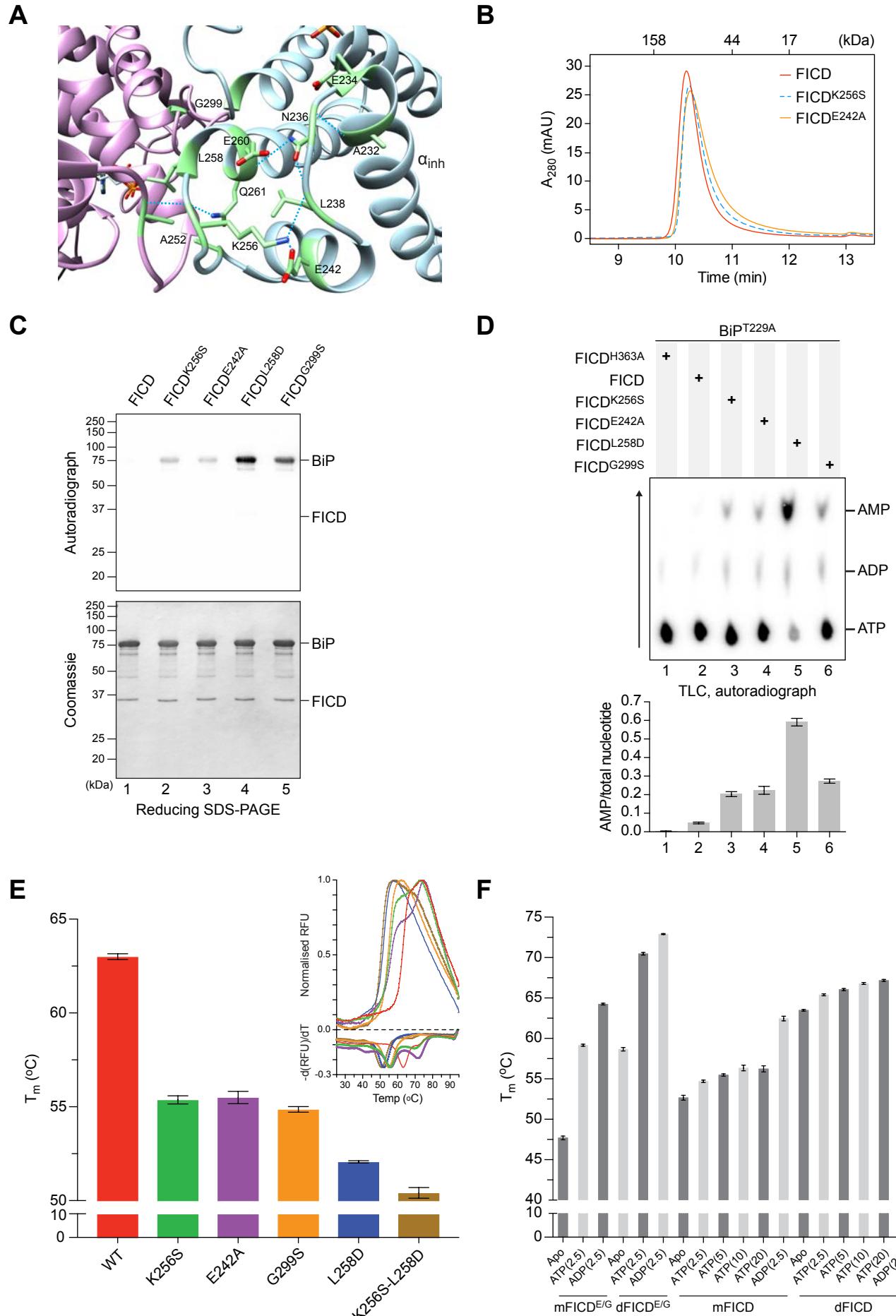
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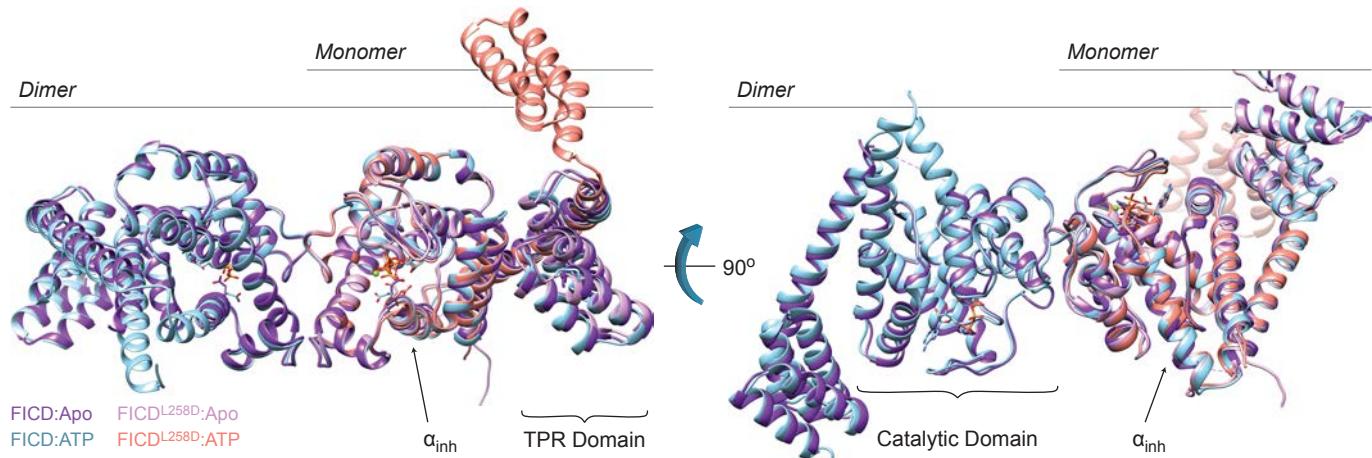


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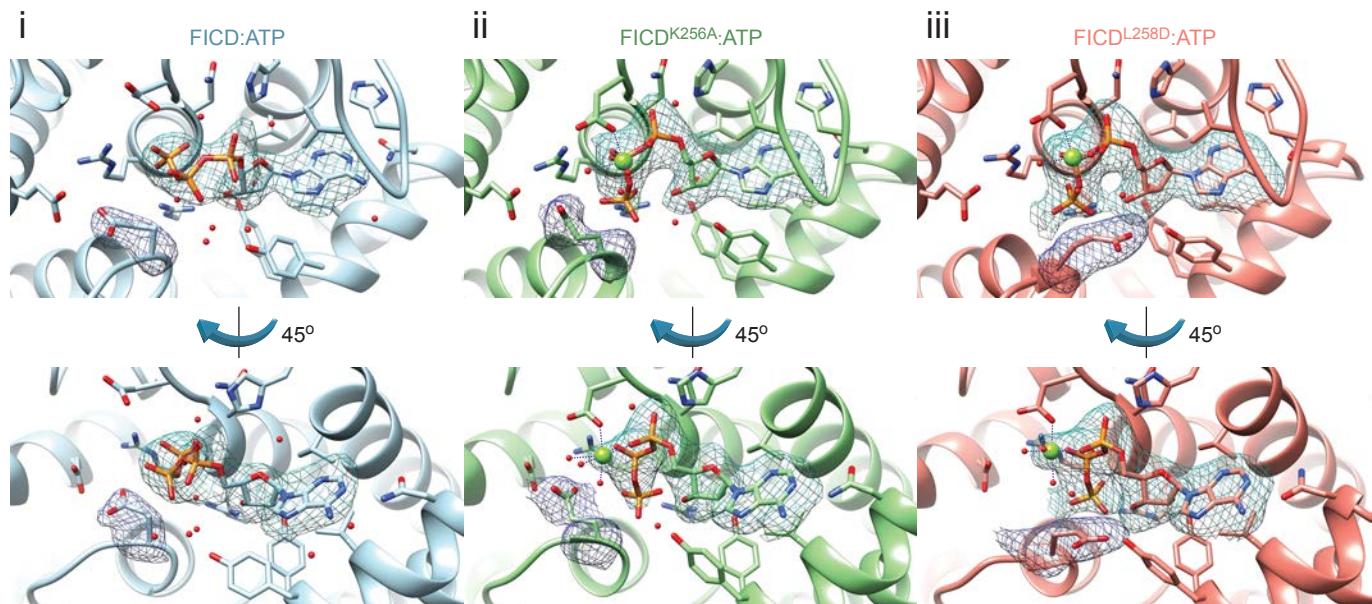


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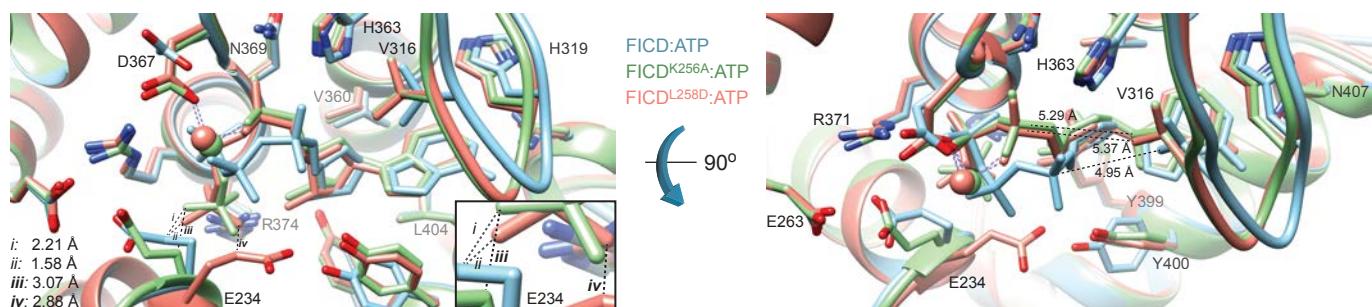
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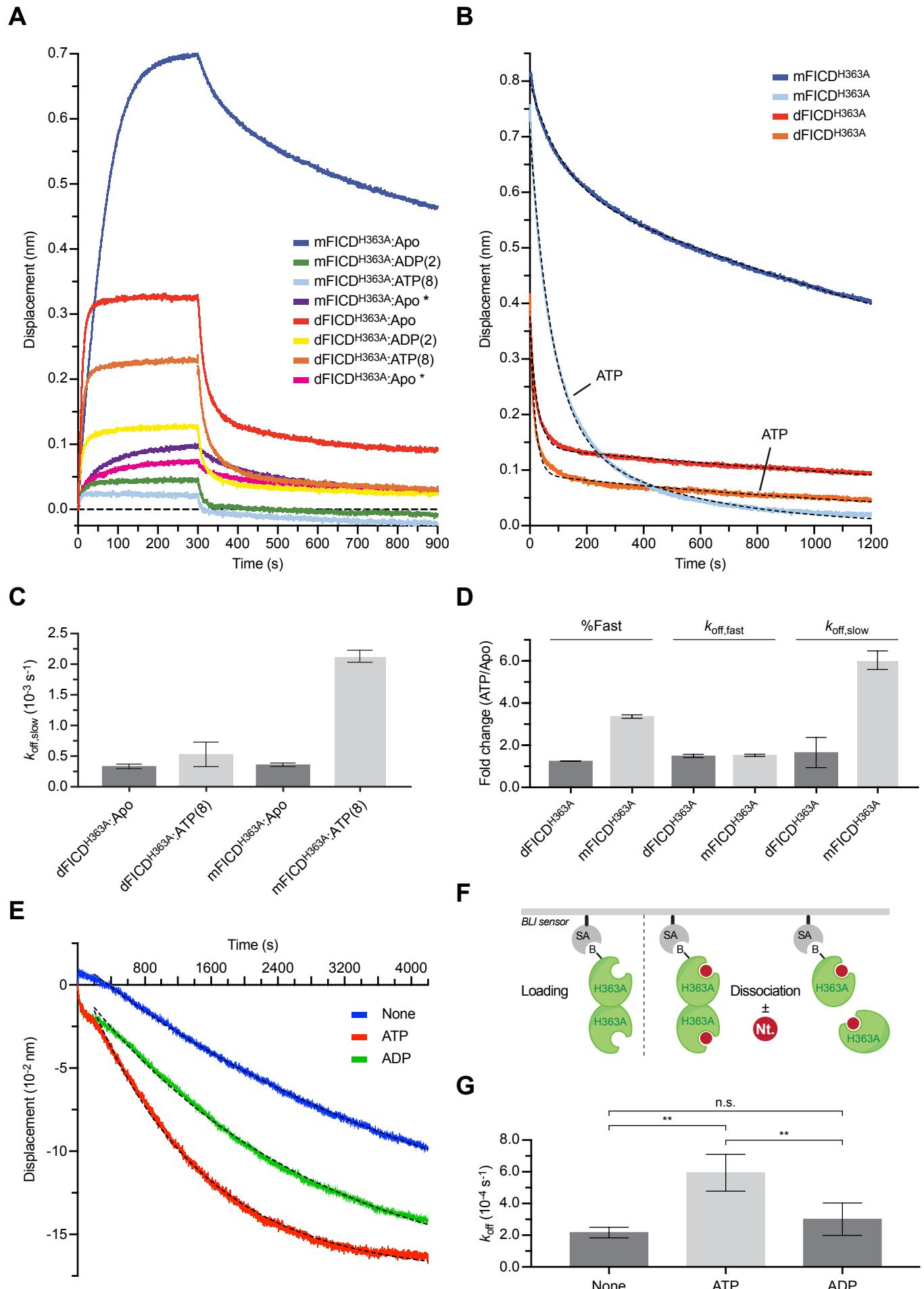
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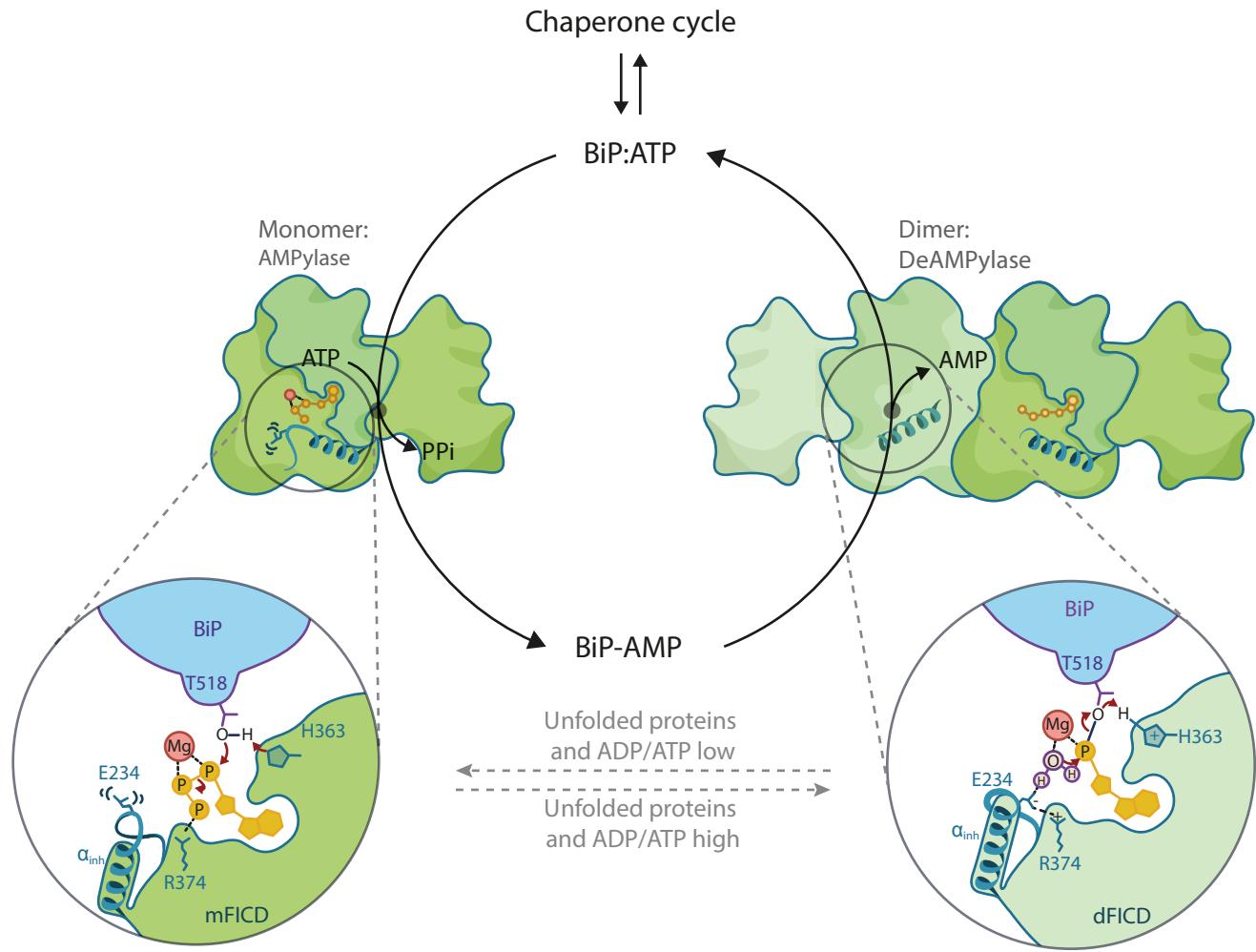
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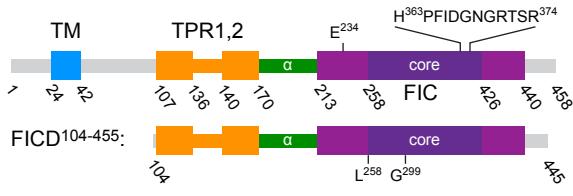


**Figure 7**

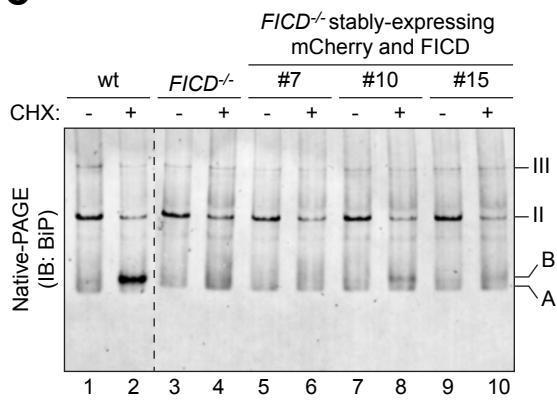


## Figure S1

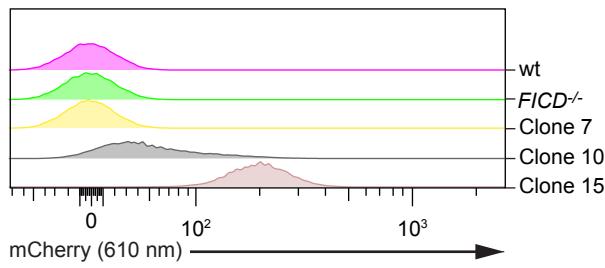
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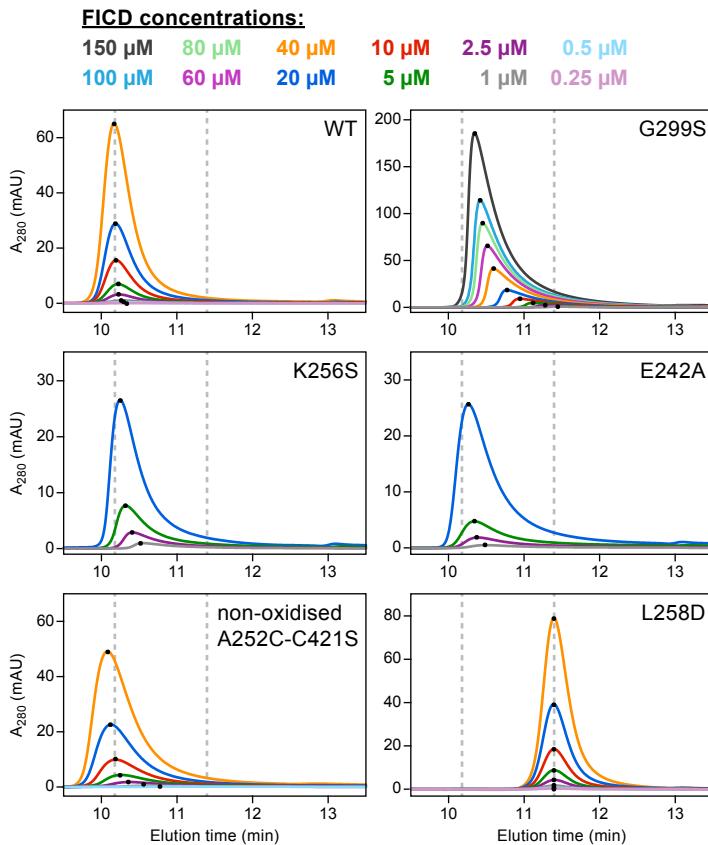
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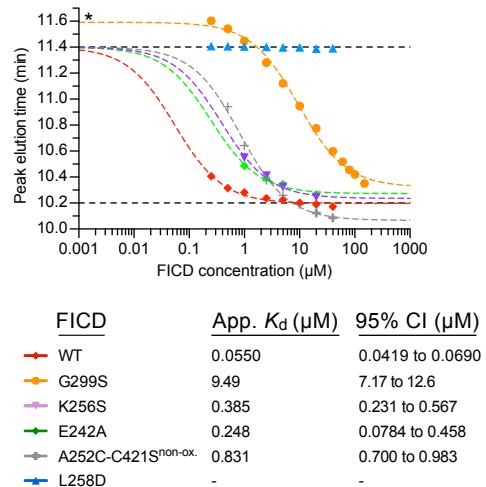
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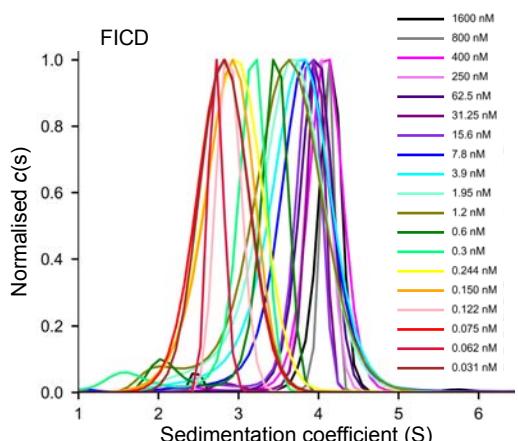
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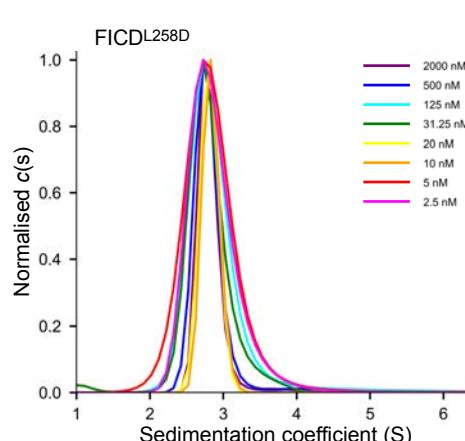
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**F**

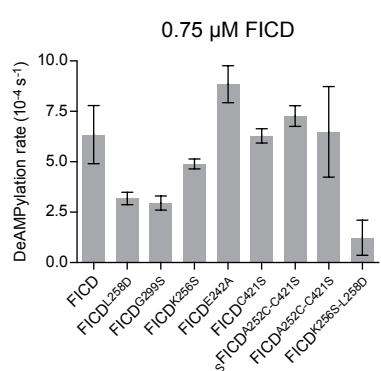


**G**

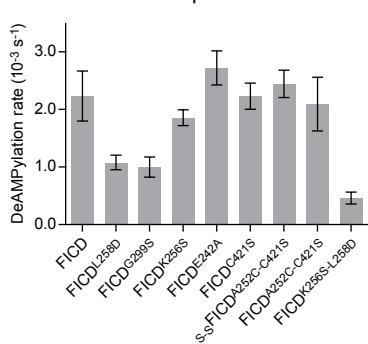


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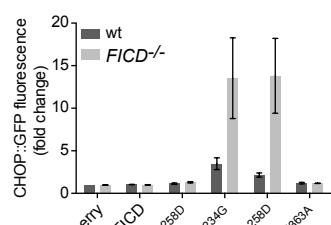
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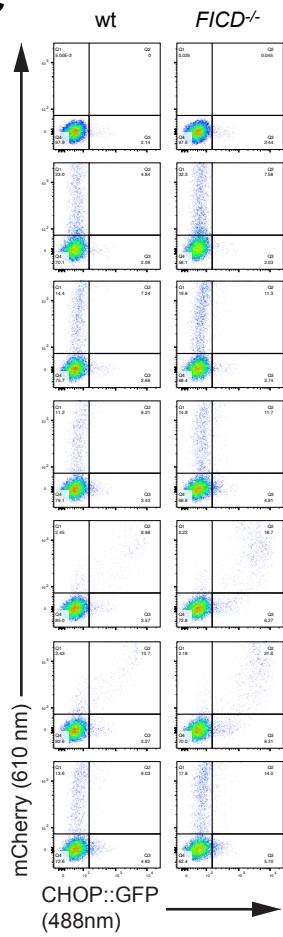
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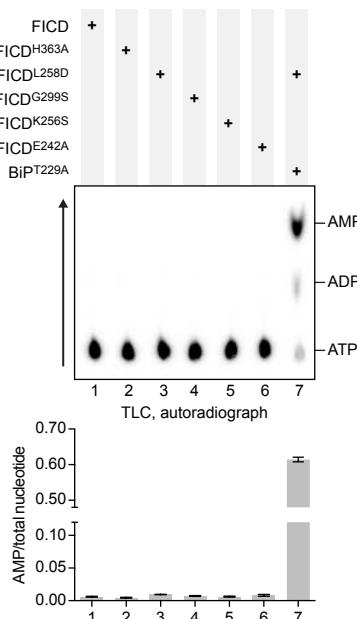
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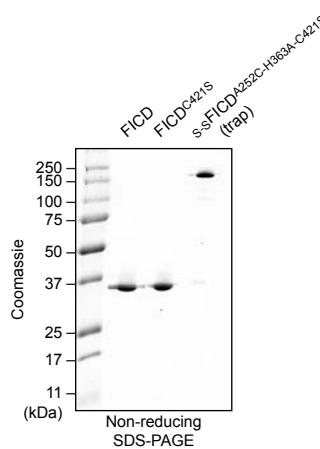
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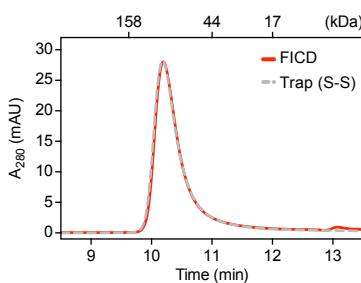
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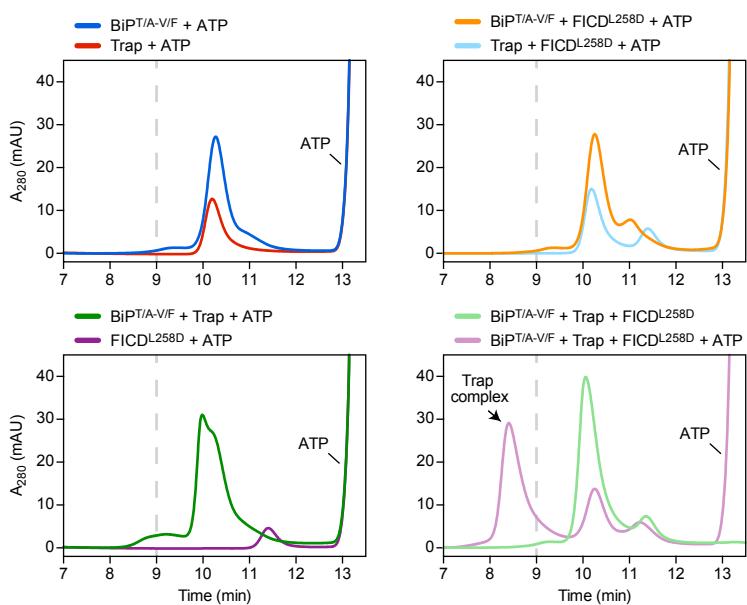
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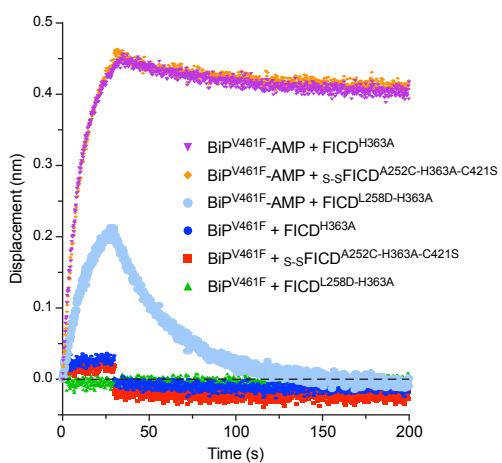
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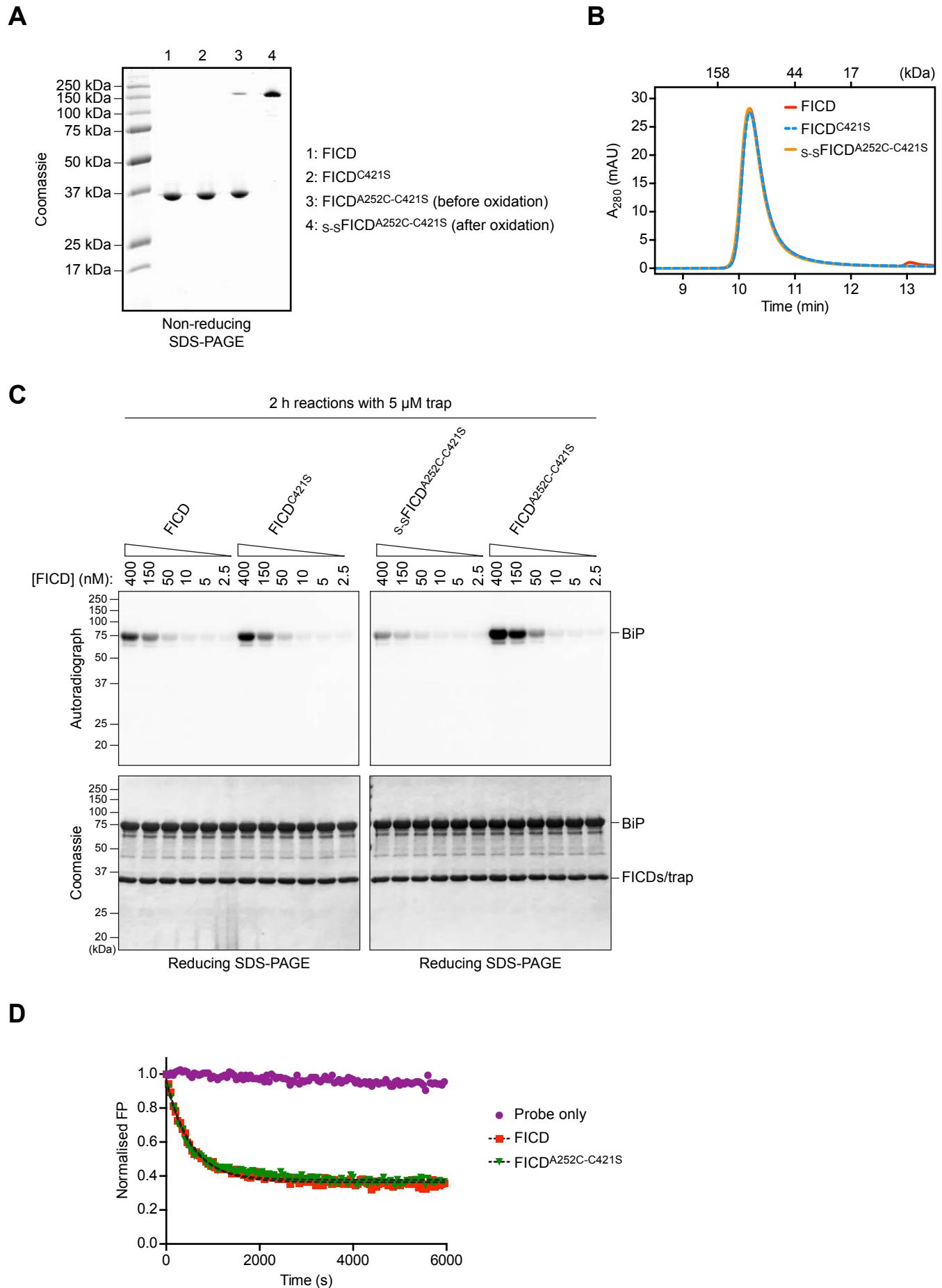
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**G**

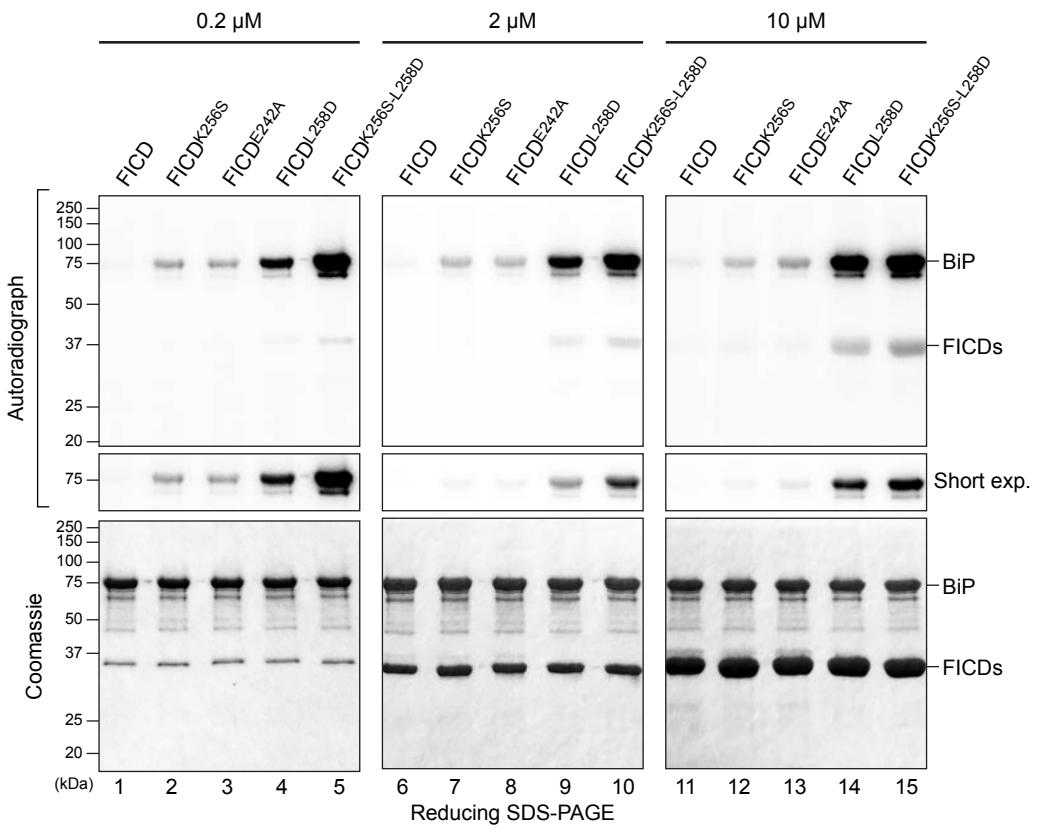


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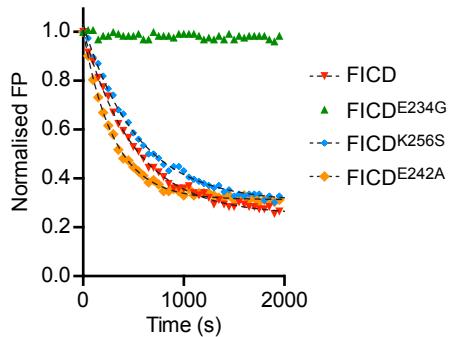


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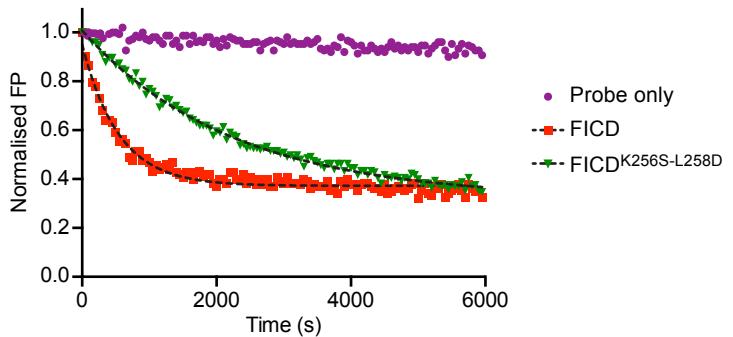
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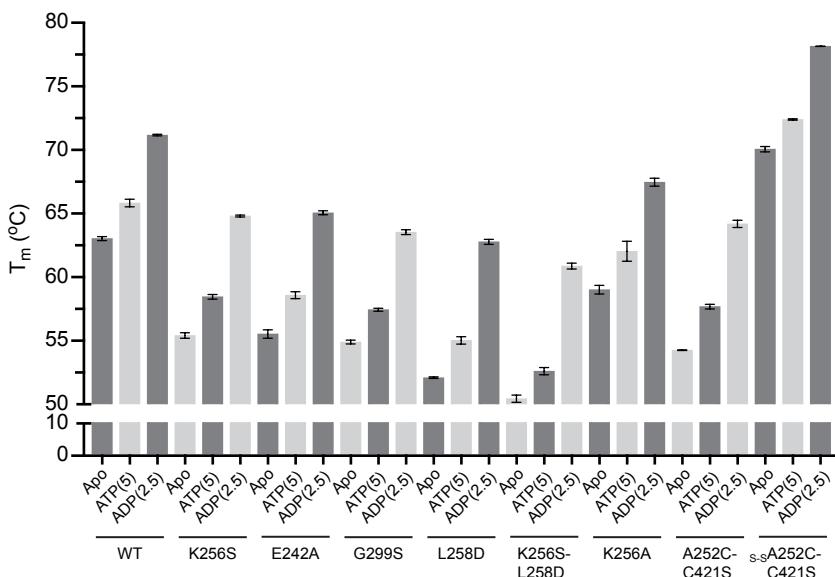
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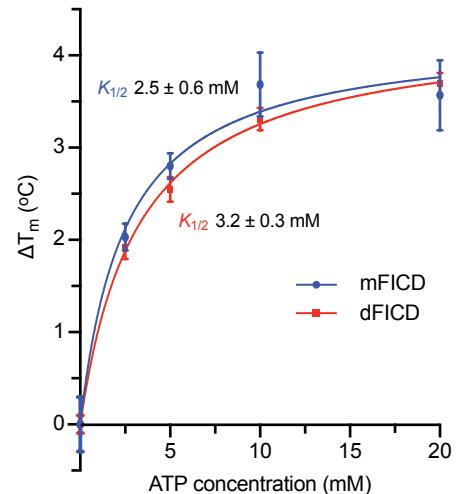
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**D**

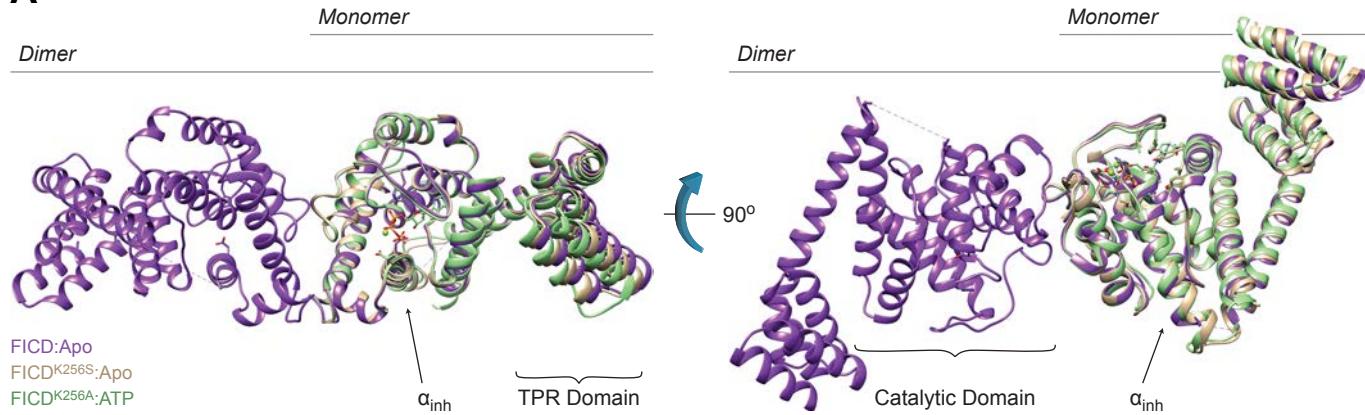


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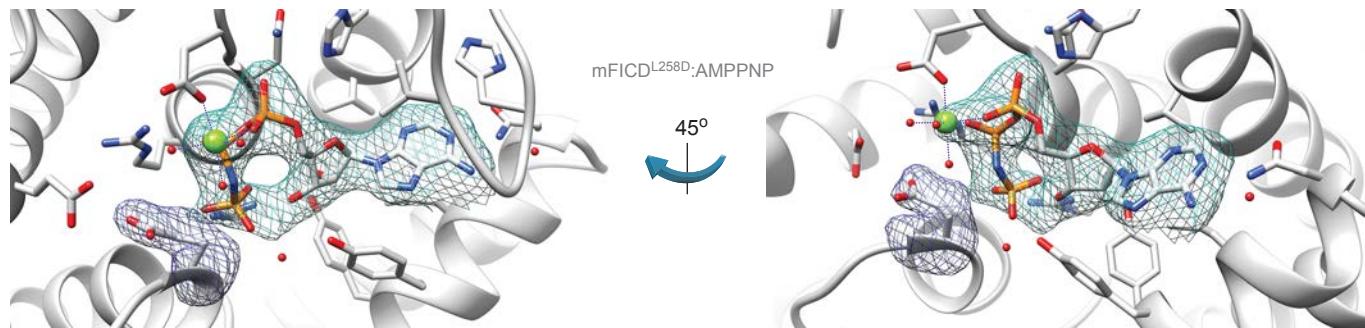


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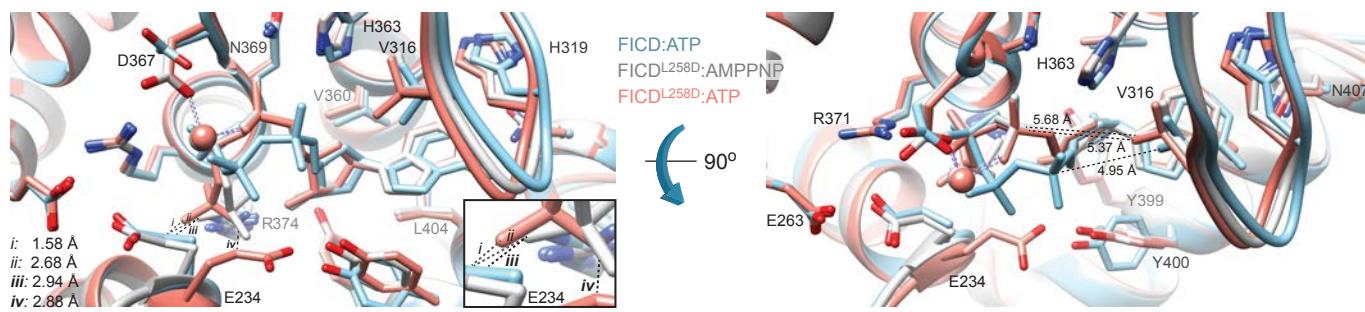
**A**



**B**



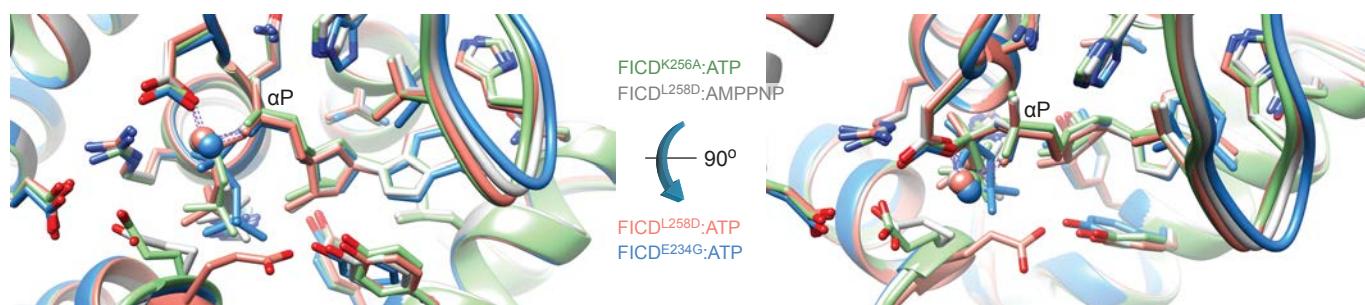
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**D**

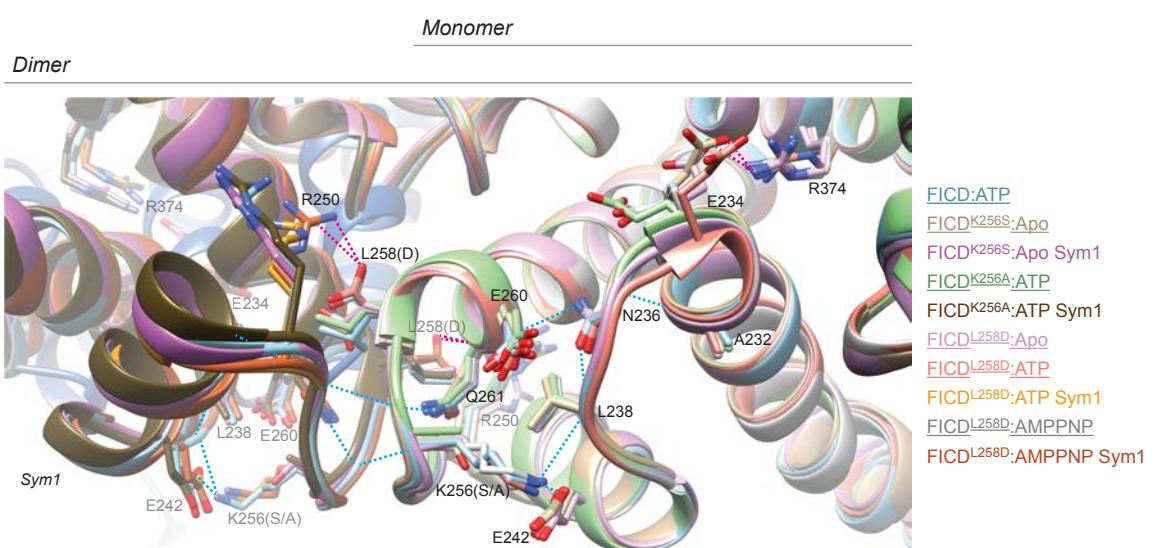


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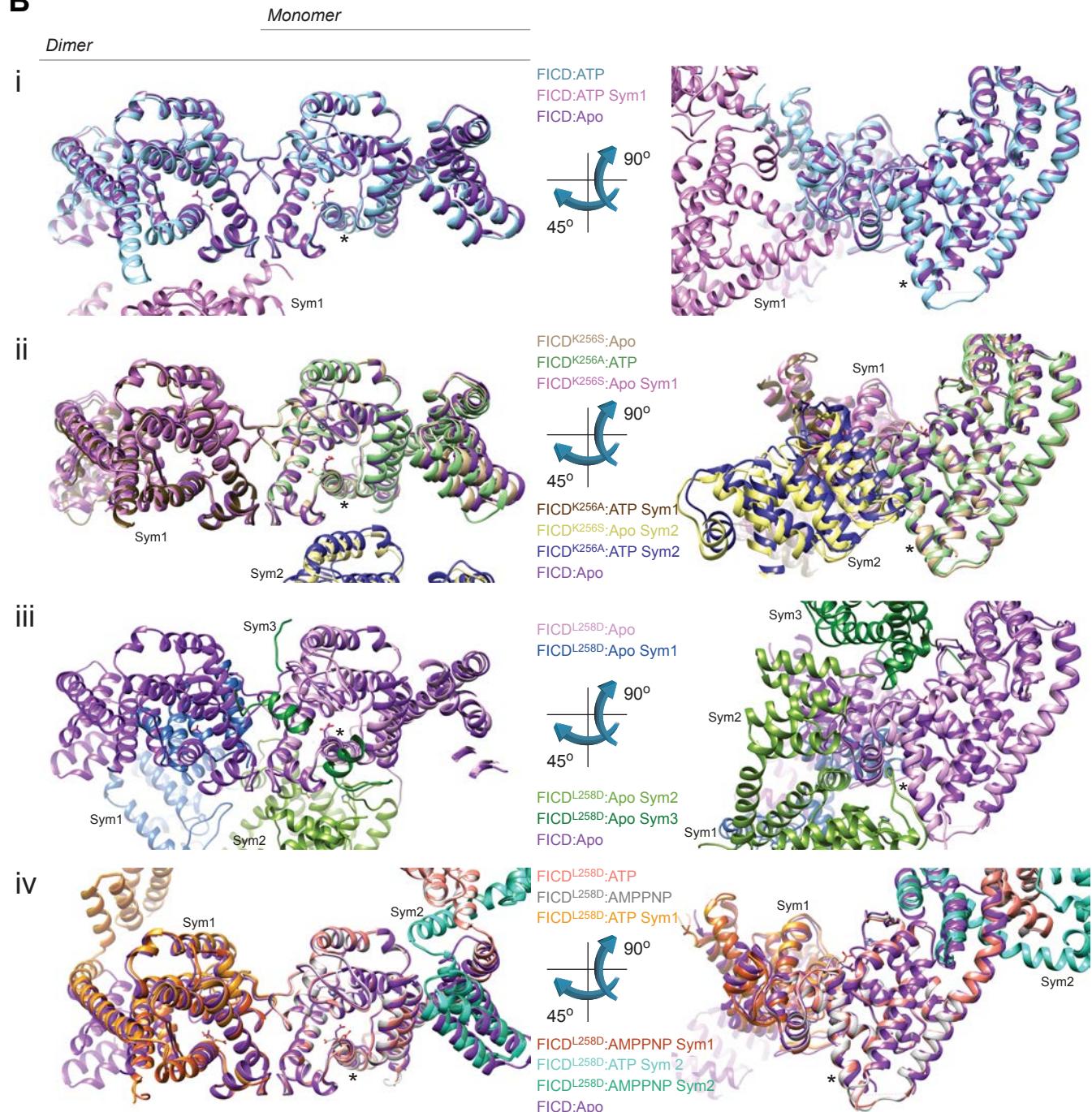


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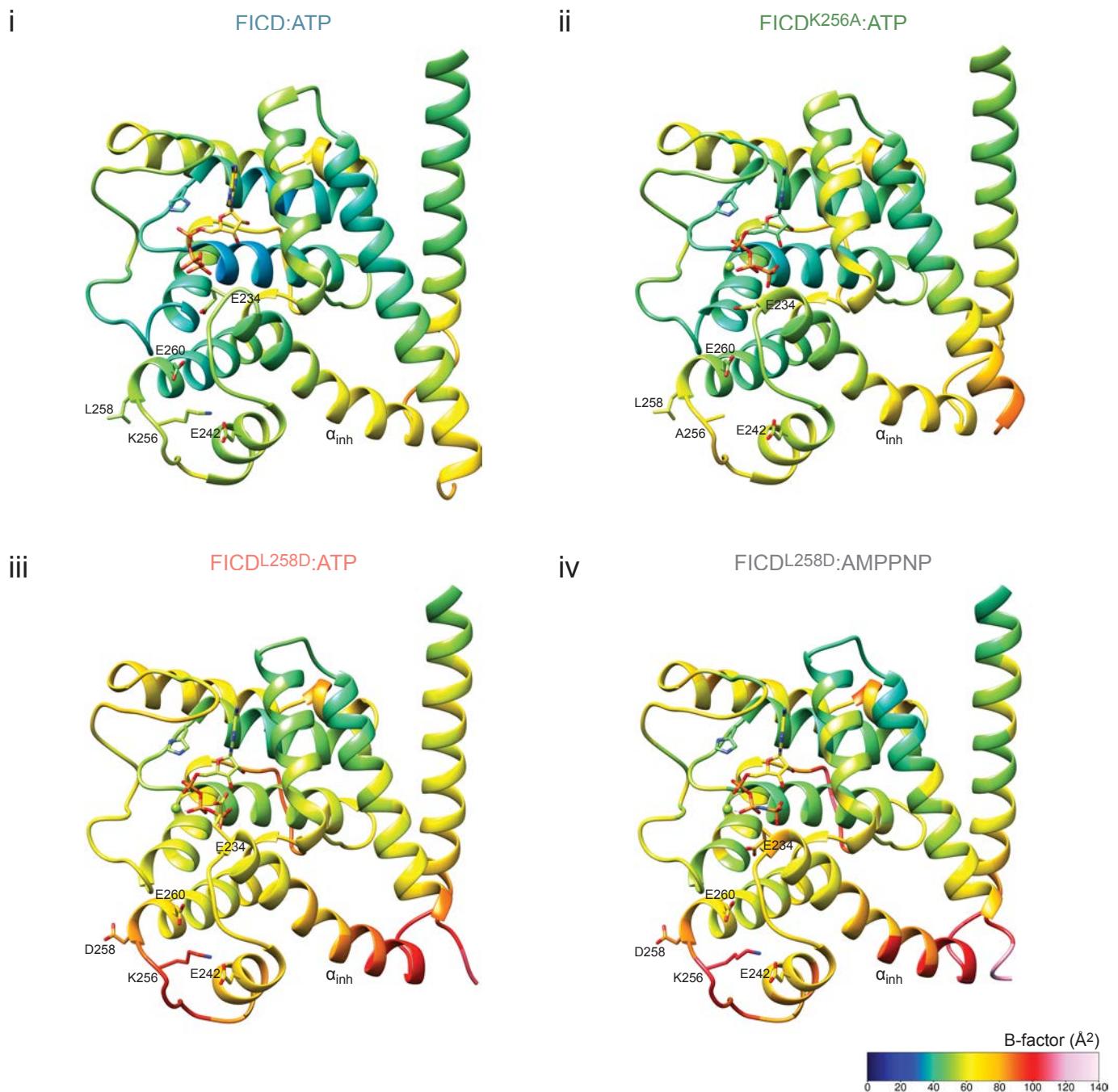
**A**



**B**



## Figure S7



## Figure S8

