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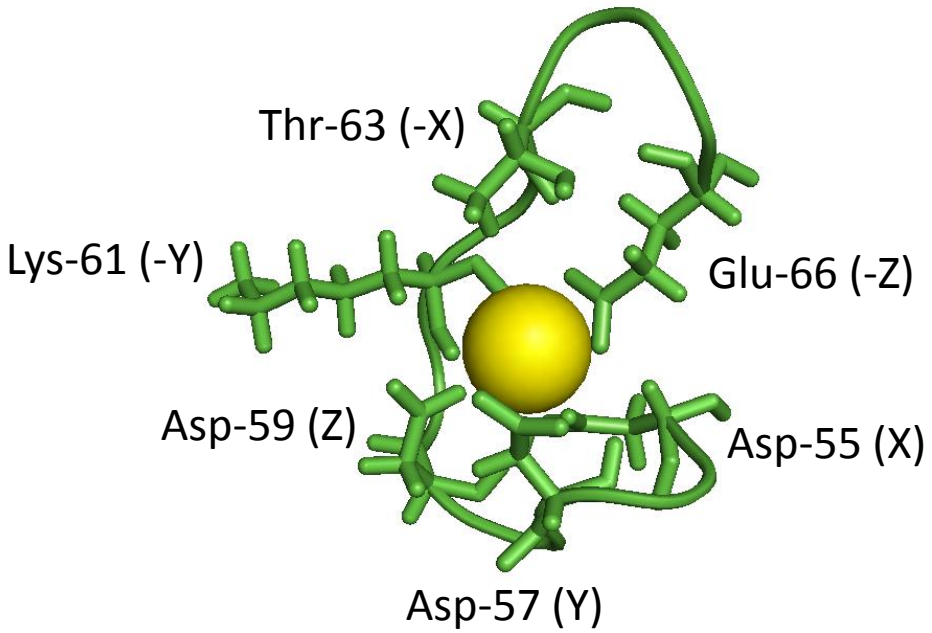
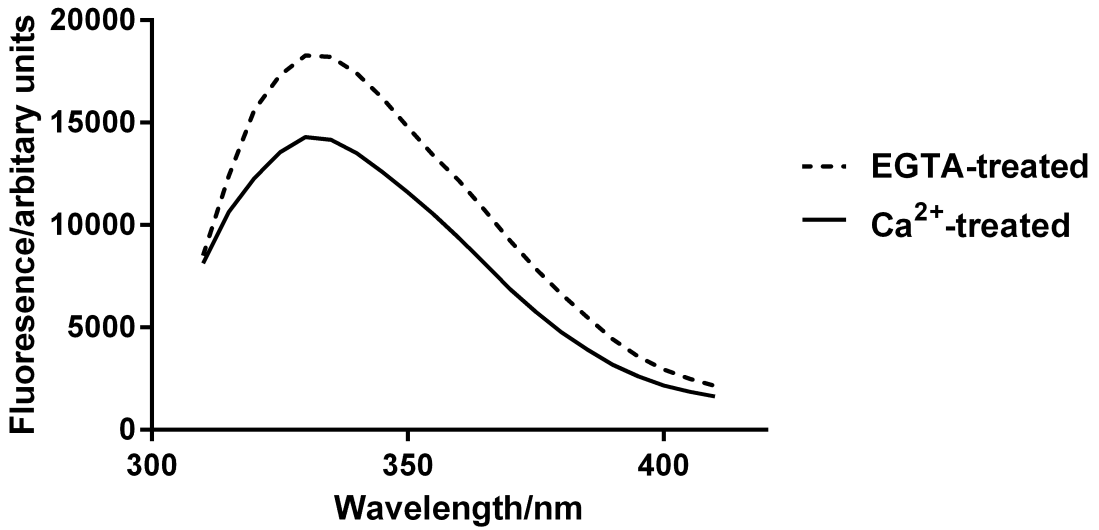
Abstract: FH22 has been previously identified as a calcium-binding protein from the common liver fluke, Fasciola hepatica. It is part of a family of at least four proteins in this organism which combine an EF-hand containing N-terminal domain with a C-terminal dynein light chain-like domain. Here we report further biochemical properties of FH22, which we propose should be renamed FhCaBP1 for consistency with other family members. Molecular modelling predicted that the two domains are linked by a flexible region and that the second EF-hand in the N-terminal domain is most likely the calcium ion binding site. Native gel electrophoresis demonstrated that the protein binds both calcium and manganese ions, but not cadmium, magnesium, strontium, barium, cobalt, copper(II), iron (II), nickel, zinc, lead or potassium ions. Calcium ion binding alters the conformation of the protein and increases its stability towards thermal denaturation. FhCaBP1 is a dimer in solution and calcium ions have no detectable effect on the protein's ability to dimerise. FhCaBP1 binds to the calmodulin antagonists trifluoperazine and chlorpromazine. Overall, the FhCaBP1's biochemical properties are most similar to FhCaBP2 a fact consistent with the close sequence and predicted structural similarity between the two proteins.

Graphical Abstract





Highlights (for review)

- FH22 (FhCaBP1) has an EF-hand domain and a dynein light chain-like domain
- FhCaBP1 binds calcium and manganese ions
- Calcium binding alters FhCaBP1's conformation and stability
- FhCaBP1 is a homodimer
- FhCaBP1 binds to the drugs trifluoperazine and chlorpromazine

FhCaBP1 (FH22): a *Fasciola hepatica* calcium-binding protein with EF-hand and dynein light chain domains

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Abstract

FH22 has been previously identified as a calcium-binding protein from the common liver fluke, *Fasciola hepatica.* It is part of a family of at least four proteins in this organism which combine an EF-hand containing N-terminal domain with a C-terminal dynein light chain-like domain. Here we report further biochemical properties of FH22, which we propose should be renamed FhCaBP1 for consistency with other family members. Molecular modelling predicted that the two domains are linked by a flexible region and that the second EF-hand in the N-terminal domain is most likely the calcium ion binding site. Native gel electrophoresis demonstrated that the protein binds both calcium and manganese ions, but not cadmium, magnesium, strontium, barium, cobalt, copper(II), iron (II), nickel, zinc, lead or potassium ions. Calcium ion binding alters the conformation of the protein and increases its stability towards thermal denaturation. FhCaBP1 is a dimer in solution and calcium ions have no detectable effect on the protein's ability to dimerise. FhCaBP1 binds to the calmodulin antagonists trifluoperazine and chlorpromazine. Overall, the FhCaBP1's biochemical properties are most similar to FhCaBP2 a fact consistent with the close sequence and predicted structural similarity between the two proteins.

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Introduction

The common liver fluke, *Fasciola hepatica* expresses at least four members of a family of unusual calcium binding proteins (Russell and Timson, 2014). These proteins combine an N-terminal EF-hand domain and a C-terminal dynein light chain-like (DLC-like) domain. Similar proteins are found in other species from the phylum Platyhelminthes (Thomas and Timson, 2016). The best characterised of these are the tegumental allergen like (TAL) proteins from *Schistosoma mansoni* (Fitzsimmons, et al., 2012, Francis and Bickle, 1992, Hoffmann and Strand, 1997, Jeffs, et al., 1991, Mohamed, et al., 1998, Santiago, et al., 1998). This species expresses 13 family members, some of which have been shown to elicit IgE-mediated immune responses in the host (Fitzsimmons, et al., 2012, Fitzsimmons, et al., 2007, Webster, et al., 1997). Proteins from this family have also been identified and characterised from other species in the genus *Schistosoma*, from the Chinese liver fluke *Clonorchis sinensis* (Huang, et al., 2007, Kim, et al., 2012, Senawong, et al., 2012, Subpipattana, et al., 2012, Vichasri-Grams, et al., 2006), the giant liver fluke *Fasciola gigantica* and the carcinogenic liver fluke, *Opisthorchis viverrini* (Fitzsimmons, et al., 2004, Li, et al., 2000, Santiago, et al., 1998, Waine, et al., 1994, Xu, et al., 2014, Zhang, et al., 2012).

While many of the proteins have been localised to the tegument of the worm, their *in vivo* functions have not been elucidated (Fitzsimmons, et al., 2012, Havercroft, et al., 1990, Huang, et al., 2007, Kim, et al., 2012, Lopes, et al., 2009, Mohamed, et al., 1998, Subpipattana, et al., 2012, Xu, et al., 2014, Zhang, et al., 2012). From the available data it is evident that, despite clear sequence similarities, differences exist between the proteins at the biochemical level. For example, while the EF-hand domain is a well-characterised calcium ion binding structure, at least one *S. mansoni* TAL protein (SmTAL3; Sm20.8; UniProt: **P91804**) does not bind calcium (Mohamed, et al., 1998, Thomas, et al., 2015). In *F. hepatica* the four proteins from this family are FH22 (Uniprot: **O46121**), FhCaBP2 (**A0A0B5GUS3**), FhCaBP3 (**K7WKP9**) and FhCaBP4 (**I6U578**). Of these, FH22 was discovered almost two decades ago and shown to interact with calcium ions. Since then, no further work has been

reported on this protein (Ruiz de Eguino, et al., 1999). FhCaBP2 binds calcium and manganese ions through the second EF-hand motif in the N-terminal domain. It forms a homodimer and calcium ion binding has no effect on dimer formation (Nguyen, et al., 2016, Thomas and Timson, 2015). FhCaBP3 also interacts with calcium and manganese ions (Banford, et al., 2013). However in this case, calcium ion binding promotes the monomeric over the dimeric state (which is favoured in the absence of these ions). Interestingly, in FhCaBP4 the situation is reversed: calcium ion binding promotes dimerization (Orr, et al., 2012). All three of these proteins interact with calmodulin antagonists and related drugs; however, the range of drugs which binds varies between the proteins, demonstrating that they can be distinguished pharmacologically (Banford, et al., 2013, Orr, et al., 2012, Thomas and Timson, 2015, Thomas and Timson, 2016).

Despite uncertainties about the role of these proteins, their tegumental location and widespread importance of calcium signalling in eukaryotes suggests that these proteins may have potential as drug or vaccine targets. The increasingly widespread resistance to the drug of choice for treating *Fasciola spp.* infections (triclabendazole) underlies the urgent need to explore a diverse range of potential, novel targets for future drug discovery (Brennan, et al., 2007, Cabada, et al., 2016, Gil, et al., 2014, Winkelhagen, et al., 2012). A key initial step in this process is the biochemical characterisation of the protein concerned, which was the aim of the work presented in this paper. We present the biochemical characterisation of FH22 (including predicted three-dimensional structure, ion binding, oligomerisation properties and interactions with calmodulin antagonists), building on previously reported work on this protein and complementing our own work on other family members (Ruiz de Eguino, et al., 1999)(Banford, et al., 2013, Nguyen, et al., 2016, Orr, et al., 2012, Thomas and Timson, 2015). We report data on the protein's ion and drug binding properties, predicted structure and dimerization. For consistency with other family members (and the conventions applied in *F. gigantica*), we propose that FH22 should be renamed FhCaBP1.

Materials and Methods

Bioinformatics and molecular modelling

Physical properties of the protein were estimated using ProtParam as implemented in ExPASY (Gasteiger, et al., 2005). An initial molecular model was generated using Phyre2 (Krieger, et al., 2009), followed by solvation and energy minimisation by YASARA (Kelley, et al., 2015). To generate a model of the protein with a calcium ion bound at the second EF-hand the initial model was aligned using PyMol (www.pymol.org) to the highest ranked template (as judged by Phyre2) with a bound calcium ion (the mouse Reps1 EH domain; PDB: 1FI6 (Kim, et al., 2001)). This calcium-bound structure was then minimised using YASARA. As an independent check, 3DLigandSite was used to predict likely binding ligands in the apo-FhCaBP1 model (Wass, et al., 2010). The apo- and calcium bound FhCaBP1 structures are available as supplementary data to this paper.

Expression and purification of FhCaBP1

The sequence encoding FhCaBP1 was amplified from *F. hepatica* cDNA by PCR using primers designed to facilitate insertion into pET43 Ek/LIC (Merck, Nottingham, UK) using ligation independent cloning and following the manufacturer's protocol. This vector inserts sequence encoding the amino acids MAHHHHHHVDDDDK at the 5' end of the coding sequence, thus facilitating purification by metal affinity resins. The insert sequence was verified (GATC Biotech, London, UK).

The expression vector was transformed into *Escherichia coli* HMS174(DE3) and a single colony resulting from this transformation used to inoculate a culture (5ml, Luria-Bertani medium supplemented with 100 $\mu g ml^{-1}$ ampicillin) which was grown, shaking overnight, at 37 °C. This culture was diluted into 1 l of the same medium and grown until mid-log phase (i.e. when A_{600}=0.6-0.8, typically 3-4 h). The culture was then induced with IPTG (1.3 mM), grown for a further 3 h and harvested by centrifugation at 4200 *g* for 15 min. The supernatant was discarded, the pellets resuspended in approximately 20 ml of buffer R (50 mM Hepes-OH, pH 7.5, 150 mM NaCl, 10% (v/v)

glycerol) and the suspensions frozen at -80 °C until required.

The cell suspensions were thawed and disrupted by sonication on ice (three pulses of 100 W each lasting 30 s with 30-60 s gaps in between for cooling). Debris was removed by centrifugation (22,000 *g* for 20 min). The supernatant was immediately applied to a cobalt agarose column (His-Select, Sigma, 1 ml) which had been pre-equilibrated in buffer A (50 mM Hepes-OH, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol). The supernatant was allowed to pass through under gravity and the column was then washed with 20 ml of buffer A. FhCaBP1 was then eluted using buffer B (three time 2 ml aliquots of buffer A supplemented with 250 mM imidazole). SDS-PAGE was used to identify the protein-containing fractions and monitor purity. FhCaBP1 was dialysed overnight at 4 °C against buffer R supplemented with 1 mM DTT, then divided into aliquots (50-100 µl) and frozen at -80 °C.

Native gel electrophoresis

FhCaBP1 (10 µM) was resolved by discontinuous electrophoresis (Ornstein and Davis, 1964). Protein samples (total reaction volume was 10 µl) were incubated for 30 min at 20 °C in the presence of either EGTA (1 mM) or EGTA (1 mM)/metal salt (2 mM). An equal volume of non-denaturing gel loading buffer (120 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 1% (w/v) DTT) was then added and the samples resolved on 15% polyacrylamide gels (running buffer: 25 mM Tris-HCl, pH 8.3, 250 mM glycine) at 20 mA (constant current) until the bromophenol blue ran off the end of the gel (typically 45-60 min). Gels were then stained in 0.25%(w/v)Coomassie blue/10% (v/v) acetic acid/45% (v/v) ethanol overnight and destained in 0.75%(v/v) acetic acid/0.5%(v/v) ethanol.

Analytical methods

Protein-protein cross-linking was carried out using glutaraldehyde in a total reaction volume of 9 µl with 20 mM Hepes-OH, pH 7.4 as the buffer. FhCaBP1 (20 µM) was incubated at 37 °C for 60 min in

the presence of either 2 mM EGTA or 2 mM EGTA/4 mM calcium chloride. Glutaraldehyde (0.1-0.3%(v/v)) was then added and the reaction was allowed to proceed for 5 min before being terminated by the addition of Tris-HCl, pH 8.0 (1µl of 1 M solution) followed by 9 µl of SDS-PAGE loading buffer (as native gel loading buffer plus 4%(v/v) SDS) and heating at 95 °C for 3 min. Reactions were analysed by 15% SDS-PAGE.

Fluorescence spectra were collected using a Spectra Max Gemini XS fluorescence plate reader and SOFTmax PRO software. Samples (total volume 120 µl) were loaded in triplicate into black 96-well plates and contained 15 µM FhCaBP1 and either 1 mM EGTA or 1 mM EGTA/2 mM calcium chloride. These samples were incubated at 37 °C for 75 min before reading; excitation was at 280 nm and emission monitored over the range 310-410 nm.

Differential scanning fluorimetry (DSF) was carried out using a Rotor-Gene Q quantitative PCR machine and software as previously described (Thomas, et al., 2015, Thomas and Timson, 2015). Samples (total volume 20 µl with 10 mM Hepes-OH, pH 7.4) contained FhCaBP1 (15 µM) and SYPRO Orange (10×; manufacturer's concentration definition) and drugs or divalent ions as required.

Protein concentrations were estimated using the method of Bradford with BSA as a standard (Bradford, 1976).

Results

Expression and purification of FhCaBP1

Sequencing of the insert generated by PCR revealed three coding differences to the FH22 sequence deposited in the databases (**CAA06036**). These are at positions 85 and 86 where the residues are Pro-Pro in CAA06036, but Arg-Ala in our sequence and at position 180 (Tyr in CAA06036 and His in our sequence). Interestingly these changes are identical to the residues seen at the same positions

in the orthologous protein from *F. gigantica* (FgCaBP1; UniProt: **Q45TR6**; GenBank: **AAZ20312**). However, this protein differs from FhCaBP1 at four other positions, suggesting that the differences we observed are not the result of contamination. The new sequence has been submitted to GenBank with the accession code **KU647725**. FhCaBP1 can be expressed in, and purified from, *E. coli* (Fig. 1). The protein was produced in good yield (typically 4 mg per litre of bacterial culture).

FhCaBP1 is similar in sequence and structure to FhCaBP2

The most similar known protein to FhCaBP1 is FgCaBP1 (**AAZ20312**; 186/190 identical residues) and the most similar *F. hepatica* protein is FhCaBP2 (**AJF23779**; 147/187 identical residues). FhCaBP1 has a predicted molecular mass of 22.0 kDa and a computationally estimated pI of 5.55.

Prediction of the three-dimensional structure of FhCaBP1 revealed a two domain protein joined by a linker (Fig. 2a). This is similar to the predicted structures of the other FhCaBP and SmTAL family members (Banford, et al., 2013, Orr, et al., 2012, Thomas, et al., 2015, Thomas and Timson, 2015). In common with the other family members, the region linking the two domains is expected to be flexible, allowing the two domains to alter their relative orientation. Two of the three residues (Arg-85, Ala-86) which differ between our sequence and the previously published one lie at the beginning of this linker. If the linker is flexible, it may be more tolerant of amino acid changes than other parts of the protein.

Alignment of the apo-FhCaBP1 structure to the highest ranked template with calcium bound, suggested that the ion binds to the second EF-hand in the N-terminus. This is consistent with what was observed in FhCaBP2 where site-directed mutagenesis of a key glutamate residue in this EF-hand abolished calcium binding activity (Thomas and Timson, 2015). That a calcium ion binds at this site was also supported by predictions from 3DLigandSite (data not shown). This predicted calcium ion binding to the second EF-hand based on data from 21 different crystal structures. Therefore, a

calcium-bound structure was generated with the ion bound at the second EF-hand (Fig. 2a). Calcium ion binding was not predicted to result in major structural changes; the apo and calcium-bound structures aligned with a root mean square deviation (rmsd) of 0.433 Å over 2642 equivalent atoms. Comparison of energy-minimised models based on the primary sequence reported here and the sequence previously submitted to the database also showed no major predicted structural differences (rmsd 0.400 Å over 2466 equivalent atoms).

EF-hands provide coordination for the bound ions using both main chain and backbone functional groups. These are arranged approximately at right angles and are known at the X, Y, Z, -X, -Y and –Z positions. Bioinformatics studies have established the consensus residues at these positions which are consistent with calcium ion binding (Gifford, et al., 2007). In the first EF-hand in FhCaBP1, the sequence deviates from the consensus only at the –Y position (Ala-27) (Fig. 2b). The preferred residue here is threonine (Gifford, et al., 2007). However, since it is the backbone carbonyl group which coordinates the ion at this position, considerable deviation from the consensus is possible. Despite this, it appears that this site is non-functional. The second EF-hand conforms to the consensus at all positions except -Y (Lys-61, Fig. 2c). Despite the introduction of a positive charge in the side chain, this has been shown to be consistent with calcium and manganese ion binding at this site (Sanchez-Barrena, et al., 2005, Thomas and Timson, 2015). Interestingly all members of the FhCaBP family characterised to date have a lysine at this position in the second EF-hand. The functional significance of this, if any, is not yet clear.

FhCaBP1 binds calcium and manganese ions

Native gel electrophoresis showed that addition of EGTA (a calcium-specific chelating agent) to FhCaBP1 caused the protein to have increased mobility in native PAGE. Addition of a molar excess of calcium ions to the FhCaBP1/EGTA mixture resulted in a similar electrophoretic mobility to the wild-type protein (Figure 3a). This suggests that the purified, recombinant protein is largely isolated in a

calcium-bound form. EGTA removes these calcium ions, but adding exogenous calcium ions in molar excess over the EGTA results in a calcium-bound form of the protein. The ability of the protein to bind a range of other ions was tested (each in the presence of EGTA to remove any calcium ions bound to the purified protein). Of these ions, only manganese caused a reduction in mobility similar to calcium (Figure 3a). Thus, like other CaBP family members, FhCaBP1 can interact with this ion in addition to calcium. FhCaBP3 and FhCaBP4 both bind other ions in addition to calcium and manganese; however, like FhCaBP1, FhCaBP2 only binds these two ions (Banford, et al., 2013, Orr, et al., 2012, Thomas and Timson, 2015). FhCaBP1's ability to bind calcium ions was confirmed by intrinsic fluorescence measurements and differential scanning fluorimetry. The intrinsic fluorescence of FhCaBP1 was quenched in the presence of 2 mM calcium chloride/1 mM EGTA compared to treatment with 1 mM EGTA (Figure 3b). This suggests that calcium ions bind to the protein resulting in conformational changes which affect the environment of tryptophan residues. In the presence of 1 mM calcium chloride/0.5 mM EGTA the melting temperature (T_m) of FhCaBP1 was 69.2±0.6 °C compared to 67.5±0.0 °C in the presence of 0.5 mM EGTA (Figure 3c). This suggests that calcium ions bind to FhCaBP1, stabilising the protein and make it more resistant to thermal denaturation.

FhCaBP1 dimerises in a calcium-independent manner

Addition of glutaraldehyde to FhCaBP1 resulted in the appearance of an additional band when the reaction was analysed by SDS-PAGE (Fig. 4). This band corresponds to a molecular mass of 45 kDa, i.e. approximately twice the mass of the monomeric protein. In contrast to FhCaBP3 and FhCaBP4, but similar to FhCaBP2, the presence of calcium ions did not markedly affect the crosslinking. Similar results were obtained when bissulfosuccinimidyl suberate (BS^3) was used as a crosslinker (Supplementary Figure S1). Therefore, FhCaBP1 forms dimers which are not affected by the calcium ion concentration.

FhCaBP1 binds some calmodulin antagonists

DSF revealed that the calmodulin antagonists trifluoperazine (TFP) and chlorpromazine (CPZ) cause a reduction in the overall thermal stability of FhCaBP1 (Table 1; Supplementary Figure S2). In contrast, praziquantel (PZQ), *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) and thiamylal had no significant effect on the T_m of FhCaBP1. The most likely explanation is that TFP and CPZ bind to a partially folded form of the protein thus shifting the equilibrium between folded and partially folded protein away from the folded form, resulting in an overall destabilisation and lower T_m value. These results are similar to those seen in FhCaBP2, which is also destabilised by TFP and CPZ (Thomas and Timson, 2015).

Discussion

Overall, FhCaBP1 shows greatest similarity in its biochemical properties to FhCaBP2. Notably both bind both calcium and manganese ions (but failed to interact with any of the other ions tested) and both dimerise in a calcium independent manner. Given the overall sequence and predicted structural similarity, it is likely that other experimental findings about FhCaBP2 will also hold for FhCaBP2. Therefore, we predict that the second EF-hand in the N-terminal domain is most likely they key calcium ion binding site in FhCaBP1; this prediction is also consistent with the molecular modelling presented in this paper. The close sequence, structural and functional similarities between these two proteins suggests that they may have arisen from a relatively recent gene duplication event and it seems likely that there will be some functional overlap between the two proteins *in vivo*. It remains to be determined how extensive this overlap is. Indeed the physiological roles of this protein family remain elusive. However, it is reasonable to speculate that one function may be to link calcium signalling and dynein-mediated processes.

In order to function as a signalling protein, calcium binding must result in some change to the protein, which can be sensed by other cellular systems. Here we detected changes to the electrophoretic mobility, tryptophan fluorescence and thermal stability as a consequence of calcium

ion binding. All these indicate some form of conformational change in the protein resulting from ion binding. However, molecular modelling did not predict substantial changes to the overall fold and so it is likely that these changes largely affect the arrangement of loops and the conformation amino acid side chains on the surface of the protein. The linker between the domains is likely to be highly flexible, but the modelling techniques employed here are unlikely to predict accurately changes in this region. Ideally, experimental structures of the full-length protein in the presence and absence of bound calcium ions would be available to facilitate more detailed molecular dynamics studies. To date, only two experimental structures of proteins from this family have been determined and both are only of the C-terminal DLC-like domain (Costa, et al., 2014, Nguyen, et al., 2016). Interestingly, in both cases, the initial protein in the crystallisation mixes was the full-length one which degraded to the DLC-like domain. It has been suggested that this susceptibility to degradation may be an important feature of the *in vivo* function of the protein (Nguyen, et al., 2016). The crystal structure of the DLC-like domain of FhCaBP2 confirmed biochemical studies that this domain is a dimer (Nguyen, et al., 2016, Thomas and Timson, 2015). The EF-hand domain of this protein also dimerises (Thomas and Timson, 2015). It is likely that the same is true for FhCaBP1. The importance of dimerization to the functioning of these proteins is currently unknown. It is also interesting to speculate that heterodimers of FhCaBP family members may also be found *in vivo*. If these do occur they would add another potential layer of complexity to FhCaBP-mediated signalling.

By analogy with calmodulin, it is likely that changes to the surface characteristics of FhCaBP1, alter its affinity for other proteins thus facilitating its signalling role. Identification of these binding partners is a priority for future research. The availability of recombinant FhCaBP1 should facilitate experiments such as pull-downs, affinity chromatography and co-immunoprecipitation. The recent publication of the draft *F. hepatica* genome (Cwiklinski, et al., 2015) will facilitate the interpretation of molecular genetic approaches (for example two-hybrid screening) to the discovery of binding partners. Selective knock-down of the mRNA coding for FhCaBP1 (and consequent reduction in the

amount of protein produced) may enable studies of the effects of reduced concentrations of the protein *in vivo*. Such experiments would need to be designed carefully to ensure that only FhCaBP1 (and not other family members) were knocked-down and it would also be necessary to consider what phenotypic outputs would be measured. A recent study on RNAi knock-down of calmodulin-like proteins in *F. hepatica* demonstrates that it is possible to target highly similar sequences and observe consequent phenotypes (McCammick, et al., 2016). Once binding partners have been identified, it will be necessary to study the downstream signalling processes which are mediated by FhCaBP1. To do this it should be possible to use many of the methods used to study signalling pathways in higher eukaryotes. However, the current lack of reliable cell culture systems for *F. hepatica* will be a significant barrier in doing so.

The likely role of FhCaBP1 (and other members of the family) in calcium-mediated signalling makes this protein an interesting potential drug target. Calmodulin is a target of a number of drugs used in the treatment of schizophrenia and other psychiatric conditions (Levin and Weiss, 1977, Marques, et al., 2004, Marshak, et al., 1985). It has also been investigated as a possible anti-cancer drug target (Hait, 1987). This suggests that targeting calcium-binding proteins is pharmacologically viable. The *S. mansoni* protein SmTAL1 (which has a similar domain structure to FhCaBP1) interacts with the highly effective antischistosomal drug praziquantel providing tantalising evidence that the drug's mechanism may involve antagonism of this protein's signalling actions (Thomas, et al., 2015). However, praziquantel is not considered to be active against *F. hepatica* and there was no evidence for interaction between this drug and FhCaBP1 in our studies. Trifluoperazine and chlorpromazine do interact with FhCaBP1 and TFP has been shown to disrupt calmodulin signalling and reduce viability in *S. mansoni* (Thompson, et al., 1986). However, they are unlikely to be effective drugs for the treatment of liver fluke infections: their actions on brain are likely to result in severe side effects. Therefore, if FhCaBP1 was to be targeted, a drug discovery programme would be required. It is possible that TFP and CPZ may be useful lead molecules for medicinal chemistry optimisation aimed

at increasing the selectivity for FhCaBP1 over host calmodulin (and other calcium binding proteins). The similar structural and biochemical properties of FhCaBP1 and FhCaBP2 suggest that any drug designed to antagonise FhCaBP1 would, most likely, also affect the action of FhCaBP2. This is unlikely to result in problems: indeed hitting two targets rather than one may be beneficial in providing effective therapy.

Conclusions

Structurally and biochemically, FhCaBP1 is most similar to FhCaBP2. It interacts with calcium and manganese ions and forms homodimers. Dimerisation appears to be unaffected by the presence or absence of divalent cations. The protein interacts with some calmodulin antagonists suggesting that it may be possible to identify other small molecules which bind to it with higher selectivity and affinity. Further work is required to identify FhCaBP1's *in vivo* roles and the extent of overlap between these roles and those of other members of the same protein family in *F. hepatica*.

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Conflict of interest statement

The authors have no conflicts of interest to report.

Figure legends

Figure 1: **Expression and purification of FhCaBP1.** SDS-PAGE (15%) showing the expression and purification of FhCaBP1 (indicated with an arrow to the right of the gel). M, molecular mass markers with masses shown to the left of the gel in kDa; U, total bacterial cell protein extract prior to induction with IPTG; I, total bacterial cell protein 3 h after induction; S, proteins released on sonication; W1, proteins flowing through the column on the first wash; W2, proteins flowing through the column on the second wash; E1, proteins released from the column on the first elution with imidazole; E2, proteins released from the column on the second elution with imidazole.

Figure 2: **Predicted structure of FhCaBP1**. (a) The overall predicted fold of the protein with a calcium ion bound at the second EF-hand in the N-terminal domain. The N- and C-termini are indicated. (b) The first EF-hand showing potential ion coordinating residues. (c) The first EF-hand showing predicted ion coordinating residues.

Figure 3: **FhCaBP1 binds calcium and manganese ions.** (a) Native gel electrophoresis of FhCaBP1 (10 μM) in the presence of various metal ions (for electrophoresis conditions, see Materials and Methods). All samples except U (untreated) contained 1 mM EGTA. Where present, ions were 2 mM. (b) Intrinsic fluorescence spectra of FhCaBP1 (15 μM) in the presence of 1 mM EGTA (dotted line) or 1 mM EGTA/2 mM calcium chloride (solid line). (c) First derivative curves of the Sypro orange fluorescence as a function of temperature for FhCaBP1 (15 μM) in the presence of 0.5 mM EGTA (dotted line) or 0.5 mM EGTA/1 mM calcium chloride (solid line).

Figure 4: **FhCaBP1 is a dimeric protein**. FhCaBP1 (20 μM) forms dimers (and some higher order oligomers), as detected by cross-linking with glutaraldehyde (concentrations shown above the gel). Reaction products were resolved on 15% SDS-PAGE and dimers were detected in the presence of 2 mM EGTA (EGTA) and 2 mM EGTA/4 mM calcium chloride ($CaCl_2$). M, molecular mass markers (sizes

shown to the left of the gel in kDa); U, untreated protein.

References

Banford, S., Drysdale, O., Hoey, E. M., Trudgett, A., and Timson, D. J., 2013. FhCaBP3: A *Fasciola hepatica* calcium binding protein with EF-hand and dynein light chain domains. Biochimie 95**,** 751-758.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72**,** 248-254.

Brennan, G. P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G., and Brophy, P. M., 2007. Understanding triclabendazole resistance. Experimental and molecular pathology 82**,** 104-109.

Cabada, M. M., Lopez, M., Cruz, M., Delgado, J. R., Hill, V., and White, A. C., Jr., 2016. Treatment Failure after Multiple Courses of Triclabendazole among Patients with Fascioliasis in Cusco, Peru: A Case Series. PLoS Negl Trop Dis 10**,** e0004361.

Costa, M. A., Rodrigues, F. T., Chagas, B. C., Rezende, C. M., Goes, A. M., and Nagem, R. A., 2014. Preliminary crystallographic studies of a *Schistosoma mansoni* antigen (Sm21.7) dynein light-chain (DLC) domain. Acta crystallographica.Section F, Structural biology communications 70**,** 803-807.

Cwiklinski, K., Dalton, J. P., Dufresne, P. J., La Course, J., Williams, D. J., Hodgkinson, J., and Paterson, S., 2015. The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome Biol 16**,** 71.

Fitzsimmons, C. M., Jones, F. M., Stearn, A., Chalmers, I. W., Hoffmann, K. F., Wawrzyniak, J., Wilson, S., Kabatereine, N. B., and Dunne, D. W., 2012. The *Schistosoma mansoni* tegumental-allergen-like (TAL) protein family: influence of developmental expression on human IgE responses. PLoS neglected tropical diseases 6**,** e1593.

Fitzsimmons, C. M., McBeath, R., Joseph, S., Jones, F. M., Walter, K., Hoffmann, K. F., Kariuki, H. C., Mwatha, J. K., Kimani, G., Kabatereine, N. B., Vennervald, B. J., Ouma, J. H., and Dunne, D. W., 2007. Factors affecting human IgE and IgG responses to allergen-like *Schistosoma mansoni* antigens: Molecular structure and patterns of in vivo exposure. International archives of allergy and immunology 142**,** 40-50.

Fitzsimmons, C. M., Stewart, T. J., Hoffmann, K. F., Grogan, J. L., Yazdanbakhsh, M., and Dunne, D. W., 2004. Human IgE response to the *Schistosoma haematobium* 22.6 kDa antigen. Parasite immunology 26**,** 371-376.

Francis, P. and Bickle, Q., 1992. Cloning of a 21.7-kDa vaccine-dominant antigen gene of *Schistosoma mansoni* reveals an EF hand-like motif. Molecular and biochemical parasitology 50**,** 215-224.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., and Bairoch, A., 2005. Protein identification and analysis tools on the ExPASy server. In: Walker, J. M., (Ed., The Proteomics Protocols Handbook. Humana Press, New York, USA, pp. 571-607.

Gifford, J. L., Walsh, M. P., and Vogel, H. J., 2007. Structures and metal-ion-binding properties of the Ca2+-binding helix-loop-helix EF-hand motifs. The Biochemical journal 405**,** 199-221.

Gil, L. C., Diaz, A., Rueda, C., Martinez, C., Castillo, D., and Apt, W., 2014. Resistant human fasciolasis:

report of four patients. Rev Med Chil 142**,** 1330-1333.

Hait, W. N., 1987. Targeting calmodulin for the development of novel cancer chemotherapeutic agents. Anti-Cancer Drug Design 2**,** 139-149.

Havercroft, J. C., Huggins, M. C., Dunne, D. W., and Taylor, D. W., 1990. Characterisation of Sm20, a 20-kilodalton calcium-binding protein of *Schistosoma mansoni*. Molecular and biochemical parasitology 38**,** 211-219.

Hoffmann, K. F. and Strand, M., 1997. Molecular characterization of a 20.8-kDa *Schistosoma mansoni* antigen. Sequence similarity to tegumental associated antigens and dynein light chains. The Journal of biological chemistry 272**,** 14509-14515.

Huang, Y., Zhou, Z., Hu, X., Wei, Q., Xu, J., Wu, Z., and Yu, X., 2007. A novel tegumental protein 31.8 kDa of *Clonorchis sinensis*: sequence analysis, expression, and immunolocalization. Parasitology research 102**,** 77-81.

Jeffs, S. A., Hagan, P., Allen, R., Correa-Oliveira, R., Smithers, S. R., and Simpson, A. J., 1991. Molecular cloning and characterisation of the 22-kilodalton adult *Schistosoma mansoni* antigen recognised by antibodies from mice protectively vaccinated with isolated tegumental surface membranes. Molecular and biochemical parasitology 46**,** 159-167.

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., and Sternberg, M. J. E., 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protocols 10**,** 845-858.

Kim, S., Cullis, D. N., Feig, L. A., and Baleja, J. D., 2001. Solution structure of the Reps1 EH domain and characterization of its binding to NPF target sequences. Biochemistry 40**,** 6776-6785.

Kim, Y. J., Yoo, W. G., Lee, M. R., Kim, D. W., Lee, W. J., Kang, J. M., Na, B. K., and Ju, J. W., 2012. Identification and characterization of a novel 21.6-kDa tegumental protein from *Clonorchis sinensis*. Parasitology research 110**,** 2061-2066.

Krieger, E., Joo, K., Lee, J., Lee, J., Raman, S., Thompson, J., Tyka, M., Baker, D., and Karplus, K., 2009. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. Proteins 77 Suppl 9**,** 114-122.

Levin, R. M. and Weiss, B., 1977. Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. Molecular pharmacology 13**,** 690-697.

Li, Y., Auliff, A., Jones, M. K., Yi, X., and McManus, D. P., 2000. Immunogenicity and immunolocalization of the 22.6 kDa antigen of *Schistosoma japonicum*. Parasite immunology 22**,** 415-424.

Lopes, D. O., Paiva, L. F., Martins, M. A., Cardoso, F. C., Rajao, M. A., Pinho, J. M., Caliari, M. V., Correa-Oliveira, R., Mello, S. M., Leite, L. C., and Oliveira, S. C., 2009. Sm21.6 a novel EF-hand family protein member located on the surface of *Schistosoma mansoni* adult worm that failed to induce protection against challenge infection but reduced liver pathology. Vaccine 27**,** 4127-4135.

Marques, L. O., Lima, M. S., and Soares, B. G., 2004. Trifluoperazine for schizophrenia. Cochrane database of systematic reviews (Online) (1)**,** CD003545.

Marshak, D. R., Lukas, T. J., and Watterson, D. M., 1985. Drug-protein interactions: binding of chlorpromazine to calmodulin, calmodulin fragments, and related calcium binding proteins.

Biochemistry 24**,** 144-150.

McCammick, E. M., McVeigh, P., McCusker, P., Timson, D. J., Morphew, R. M., Brophy, P. M., Marks, N. J., Mousley, A., and Maule, A. G., 2016. Calmodulin disruption impacts growth and motility in juvenile liver fluke. Parasit Vectors 9**,** 46.

Mohamed, M. M., Shalaby, K. A., LoVerde, P. T., and Karim, A. M., 1998. Characterization of Sm20.8, a member of a family of schistosome tegumental antigens. Molecular and biochemical parasitology 96**,** 15-25.

Nguyen, T. H., Thomas, C. M., Timson, D. J., and van Raaij, M. J., 2016. *Fasciola hepatica* calcium-binding protein FhCaBP2: structure of the dynein light chain-like domain. Parasitol Res 115**,** 2879-2886.

Ornstein, L. and Davis, B. J., 1964. Disc electrophoresis-I: Background and theory. Ann NY Acad Sci 121**,** 321-349.

Orr, R., Kinkead, R., Newman, R., Anderson, L., Hoey, E. M., Trudgett, A., and Timson, D. J., 2012. FhCaBP4: a *Fasciola hepatica* calcium-binding protein with EF-hand and dynein light chain domains. Parasitology research 111**,** 1707-1713.

Ruiz de Eguino, A. D., Machin, A., Casais, R., Castro, A. M., Boga, J. A., Martin-Alonso, J. M., and Parra, F., 1999. Cloning and expression in *Escherichia coli* of a *Fasciola hepatica* gene encoding a calcium-binding protein. Molecular and biochemical parasitology 101**,** 13-21.

Russell, S. L. and Timson, D. J., 2014. Calcium binding proteins in the liver fluke, *Fasciola hepatica*, New Developments in Calcium Signaling Research. Nova Science Publishers, Inc, pp. 89-104.

Sanchez-Barrena, M. J., Martinez-Ripoll, M., Zhu, J. K., and Albert, A., 2005. The structure of the *Arabidopsis thaliana* SOS3: molecular mechanism of sensing calcium for salt stress response. Journal of Molecular Biology 345**,** 1253-1264.

Santiago, M. L., Hafalla, J. C., Kurtis, J. D., Aligui, G. L., Wiest, P. M., Olveda, R. M., Olds, G. R., Dunne, D. W., and Ramirez, B. L., 1998. Identification of the *Schistosoma japonicum* 22.6-kDa antigen as a major target of the human IgE response: similarity of IgE-binding epitopes to allergen peptides. International archives of allergy and immunology 117**,** 94-104.

Senawong, G., Laha, T., Loukas, A., Brindley, P. J., and Sripa, B., 2012. Cloning, expression, and characterization of a novel *Opisthorchis viverrini* calcium-binding EF-hand protein. Parasitology international 61**,** 94-100.

Subpipattana, P., Grams, R., and Vichasri-Grams, S., 2012. Analysis of a calcium-binding EF-hand protein family in *Fasciola gigantica*. Experimental parasitology 130**,** 364-373.

Thomas, C. M., Fitzsimmons, C. M., Dunne, D. W., and Timson, D. J., 2015. Comparative biochemical analysis of three members of the *Schistosoma mansoni* TAL family: Differences in ion and drug binding properties. Biochimie 108**,** 40-47.

Thomas, C. M. and Timson, D. J., 2015. FhCaBP2: a *Fasciola hepatica* calcium-binding protein with EF-hand and dynein light chain domains. Parasitology**,** 1375-1386.

Thomas, C. M. and Timson, D. J., 2016. A mysterious family of calcium-binding proteins from parasitic worms. Biochem Soc Trans 44**,** 1005-1010.

Thompson, D. P., Chen, G. Z., Sample, A. K., Semeyn, D. R., and Bennett, J. L., 1986. Calmodulin: biochemical, physiological, and morphological effects on *Schistosoma mansoni*. The American Journal of Physiology 251**,** R1051-1058.

Vichasri-Grams, S., Subpipattana, P., Sobhon, P., Viyanant, V., and Grams, R., 2006. An analysis of the calcium-binding protein 1 of *Fasciola gigantica* with a comparison to its homologs in the phylum Platyhelminthes. Molecular and biochemical parasitology 146**,** 10-23.

Waine, G. J., Becker, M. M., Scott, J. C., Kalinna, B. H., Yang, W., and McManus, D. P., 1994. Purification of a recombinant *Schistosoma japonicum* antigen homologous to the 22-kDa membrane-associated antigen of *S. mansoni*, a putative vaccine candidate against schistosomiasis. Gene 142**,** 259-263.

Wass, M. N., Kelley, L. A., and Sternberg, M. J., 2010. 3DLigandSite: predicting ligand-binding sites using similar structures. Nucleic acids research 38**,** W469-473.

Webster, M., Correa-Oliveira, R., Gazzinelli, G., Viana, I. R., Fraga, L. A., Silveira, A. M., and Dunne, D. W., 1997. Factors affecting high and low human IgE responses to schistosome worm antigens in an area of Brazil endemic for *Schistosoma mansoni* and hookworm. The American Journal of Tropical Medicine and Hygiene 57**,** 487-494.

Winkelhagen, A. J., Mank, T., de Vries, P. J., and Soetekouw, R., 2012. Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. Emerging infectious diseases 18**,** 1028-1029.

Xu, J., Ren, Y., Xu, X., Chen, J., Li, Y., Gan, W., Zhang, Z., Zhan, H., and Hu, X., 2014. *Schistosoma japonicum* tegumental protein 20.8, role in reproduction through its calcium binding ability. Parasitology research 113**,** 491-497.

Zhang, Z., Xu, H., Gan, W., Zeng, S., and Hu, X., 2012. *Schistosoma japonicum* calcium-binding tegumental protein SjTP22.4 immunization confers praziquantel schistosomulumicide and antifecundity effect in mice. Vaccine 30**,** 5141-5150.

Figure 1

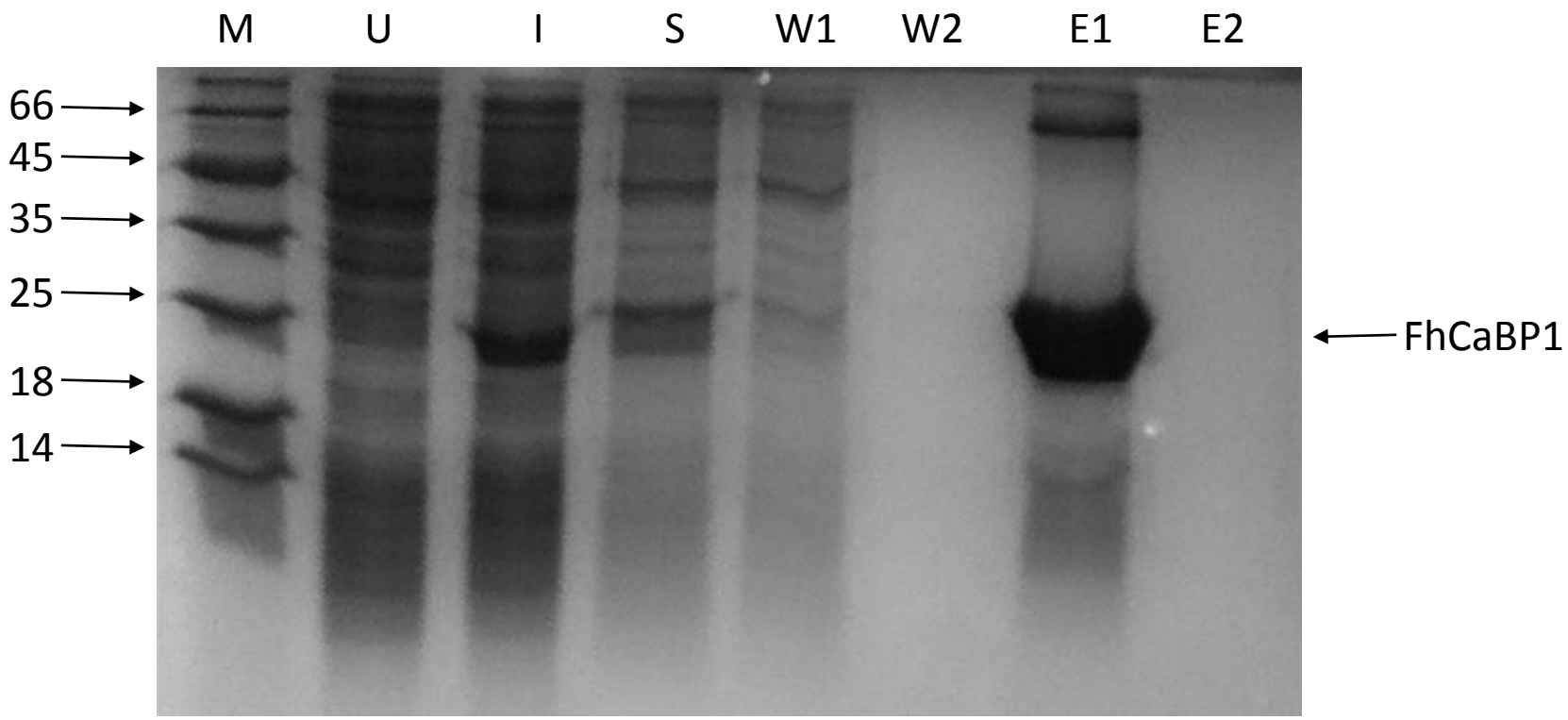
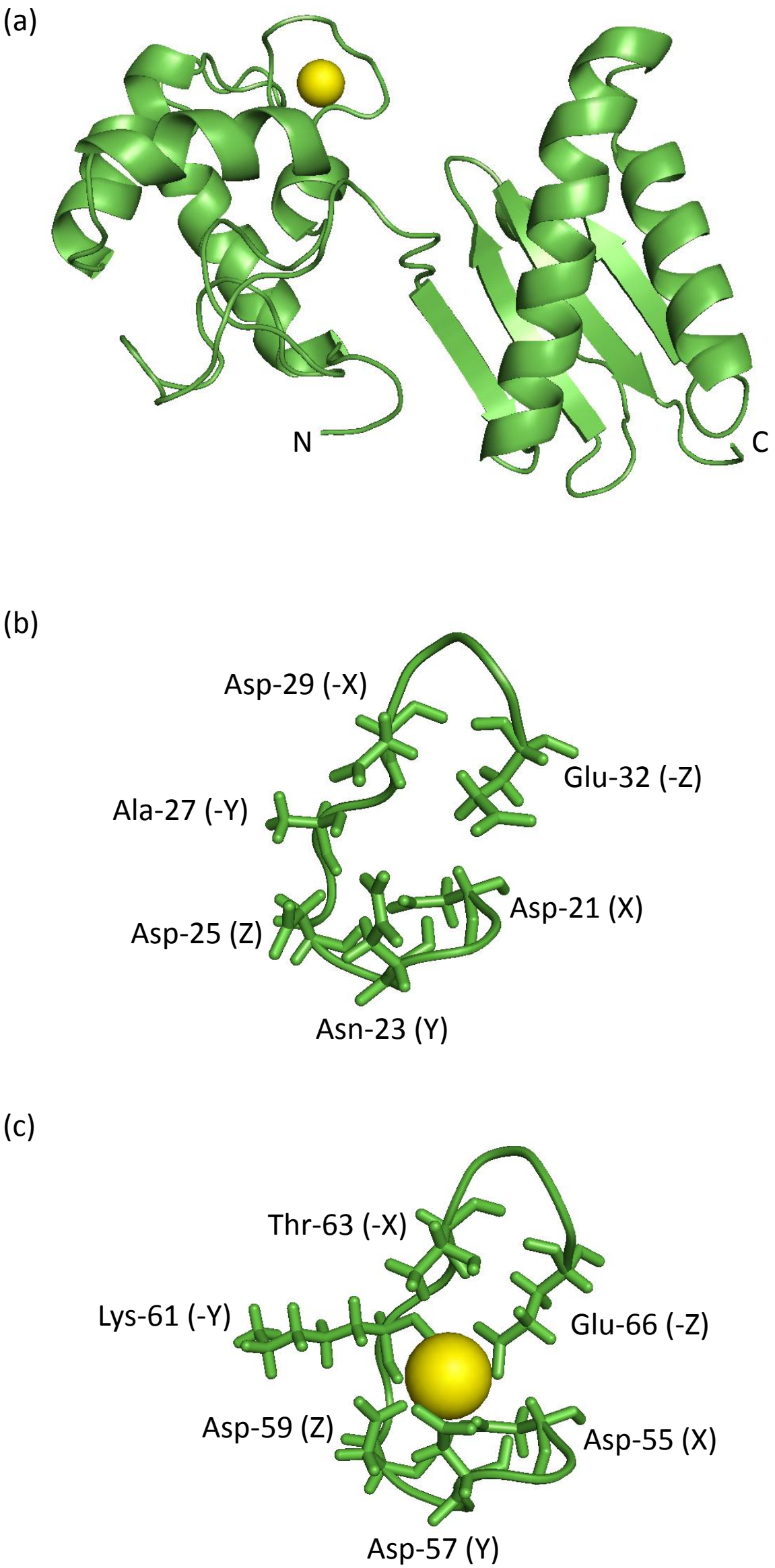


Figure 2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C
(b)
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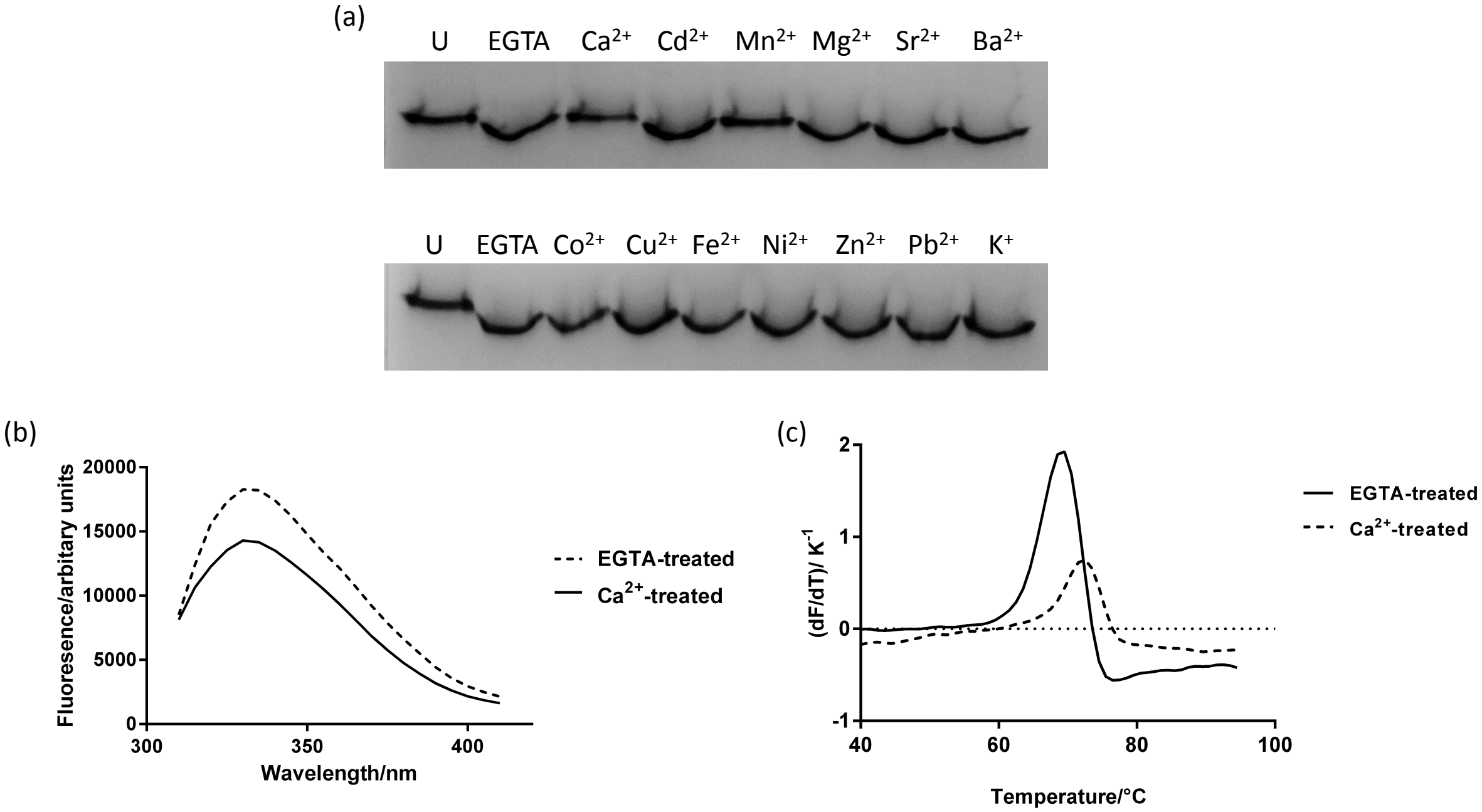
Asp-57 (Y)

Figure 3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Fe2+
Ni2+
Zn2+
Pb2+
K+
(b)
Fluoresence/arbitary units
20000
15000
10000
5000
0
300
350
400
Wavelength/nm
EGTA-treated
Ca2+-treated
(c)
(dF/dT)/ K-1
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-1
40
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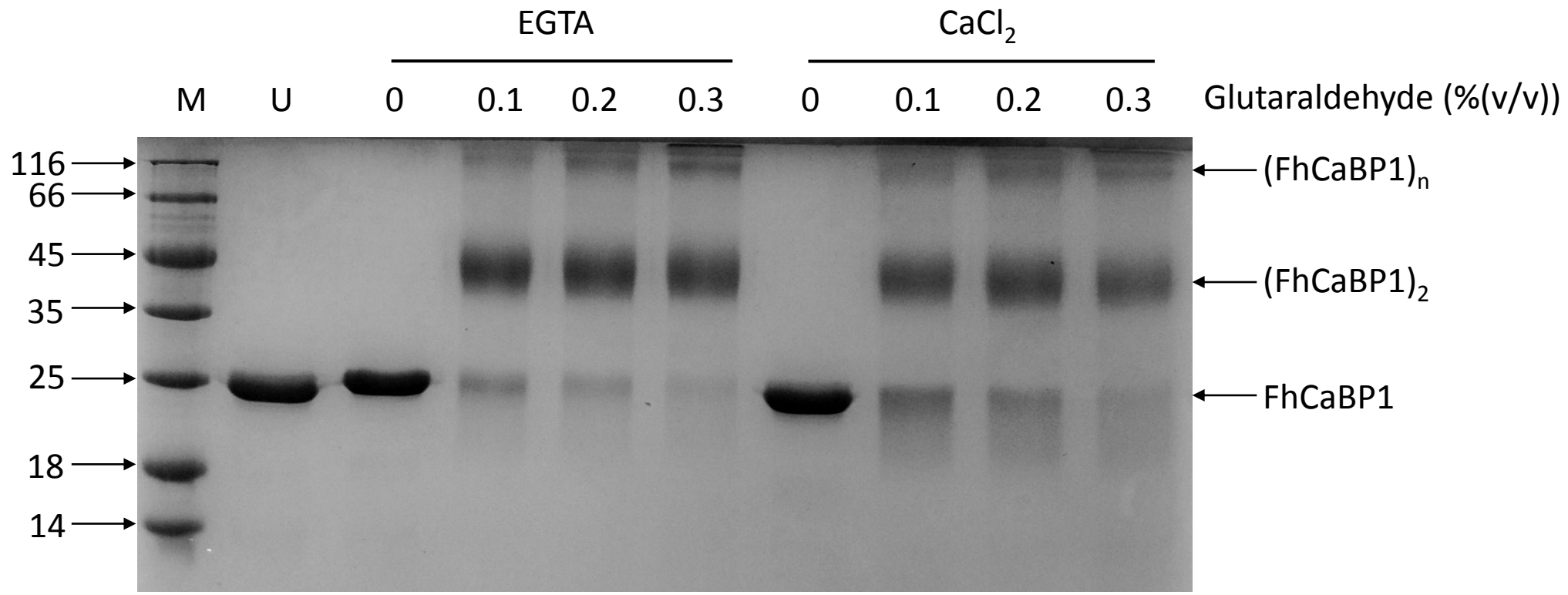
100
Temperature/°C
EGTA-treated
Ca2+-treated

Figure 4


EGTA
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Glutaraldehyde (%(v/v))
116
66
45
35
25
18
14
(FhCaBP1)n
(FhCaBP1)2
FhCaBP1

Table 1

Table 1: The effects of selected drugs on the thermal stability of FhCaBP1 (10 µM) in the presence of calcium chloride (1 mM) and drugs (250 µM) as indicated. Values shown are the mean T_m ± standard deviation (n=3).

	No drug	**DMSO**	**PZQ**	**CPZ**	**W7**	**TFP**	**Thiamylal**
Mean T_m (^oC)	71.9 ± 0.17	71.77± 0.25	71.57± 0.12	71.2± 0.17	71.6± 0.17	70.8± 0.0	71.9± 0.17
Mean ΔT_m (^oC)	$+0.13^{ns}$	n/a	-0.2^{ns}	-0.57*	-0.17^{ns}	-0.97****	$+0.13^{ns}$

Statistical significance determined by comparison to the 1% (v/v) DMSO-treated protein using one-way ANOVA with Tukey's post hoc test for multiple comparisons: ns, not significant ($p > 0.05$); * $p \leq 0.05$; **** $P \leq 0.0001$.

Molecular model

Click here to download Supplementary Material: FhCaBP1-FINAL-mini.pdb

Molecular model

Click here to download Supplementary Material: FhCaBP1-FINALplusCA-mini.pdb

Supplementary Figure S1

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Supplementary Figure S2

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