

Supplementary Information

Structural implications of Ca²⁺-dependent actin-bundling function of human EFhd2/Swiprosin-1

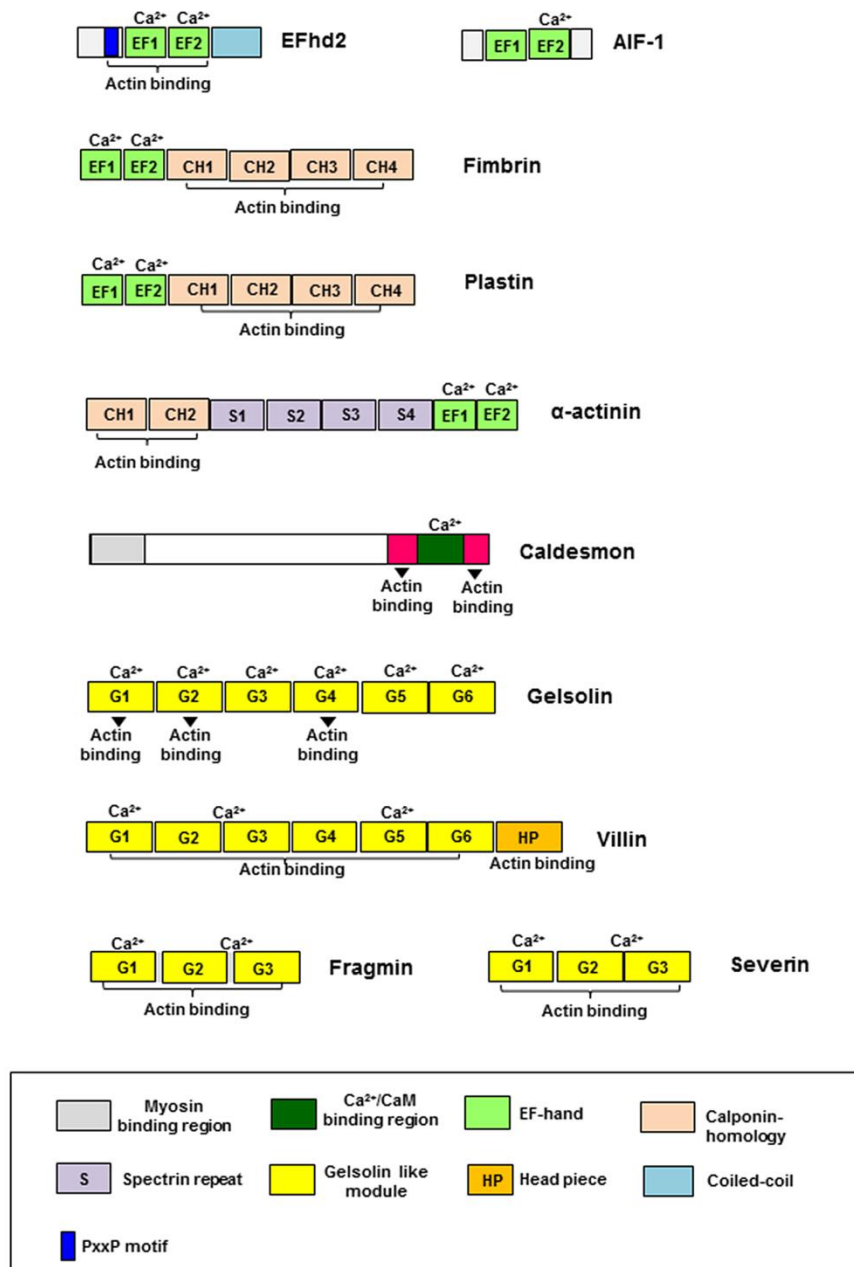
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Supplementary Figure S1. Domain structures of Ca²⁺-related actin-binding proteins.

Schematic showing the domain organisation of Ca²⁺-related actin-binding proteins. CH domains and gelsolin repeat modules function as actin-binding regions. EF-hands and the CaM-binding region function as Ca²⁺-binding regions; however, EF1 of AIF-1 does not bind to Ca²⁺ and the actin-binding site of AIF-1 is unclear.

a

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EFhd2_Human   MATDELATKLSRRRLQMEGEGGGTPEQPLNG...AAAAAAGA...PDEAAEALG.SADCELSAKLLRRAD.LNQGIGF
EFhd2_Monkey  MATDELATKLSRRRLQMEGEGGGTPEQPLNGAA...AAAAAAGA...PDEAAEALG.SADCELSAKLLRRAD.LNQGIGF
EFhd2_Rat     MATDELASKLSRRRLQMEDEGG.EATEQPLNG...AAAAAAGA...PDETAQALG.SADDELSAKLLRRAD.LNQGIGF
EFhd2_Mouse   MATDELASKLSRRRLQMEDEGG.EATEQPLNGA...AAAAAAGA...PDETAQALG.SADDELSAKLLRRAD.LNQGIGF
EFhd2_Zebrafish MATDELSKLSRRRLKIE.DGSHEPAA...ALDEAHEN.GAHDKPTTS...SADSELSAKLLRRAD.LNQGIGF
EFhd1_Human   MASEELACKLERRLR...REEAEESGPQL...APLGAPAPPEPKP.EPEPPARAPTASADAELSAQLSRRLD.INEGAARP
EFhd1_Monkey  MASEELACKLERRLR...REEAEESGPQL...APLGAPAPPEPKP.EPEPPARAPTASADAELSAQLSRRLD.INEGAARP
EFhd1_Rat     MSSEELACKLQRRRLRELETRAETHQDQPQA...APCTAPAGHR...EPEPPAPAPTASADSELNKLSRRLD.IHQGAVRS
EFhd1_Mouse   MSSEELACKLQRRRLRELETRAETHQDQPQA...APCTAPAGHR...EPEPPAPAPTASADSELNKLSRRLD.IHQGTARP
EFhd1_Zebrafish MSSEELARKLQRRRLD...ADTNPG...TQDEET...PVKPAITMSDDTSELTSLNRRLD.LDGTAKP
AIF-1_Human   .....ADTNPG...TQDEET...MSQTRDLQGGKAFGLLKAQQEERLDEINQKFLDD
AIF-1_Monkey  .....ADTNPG...TQDEET...MSQTRDLQGGKAFGLLKAQQEERLDEINQKFLDD
AIF-1_Rat     .....ADTNPG...TQDEET...MSQSKDLQGGKAFGLLKAQQEERLDGINKFLDD
AIF-1_Mouse   .....ADTNPG...TQDEET...MSQSKDLQGGKAFGLLKAQQEERLDGINKFLDD
AIF-1_Zebrafish .....ADTNPG...TQDEET...MPSNMDLQGGKAFGLLKAQQERLDEINQKFMED
  
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..... PxxP EF-hand 1 EF-hand 2

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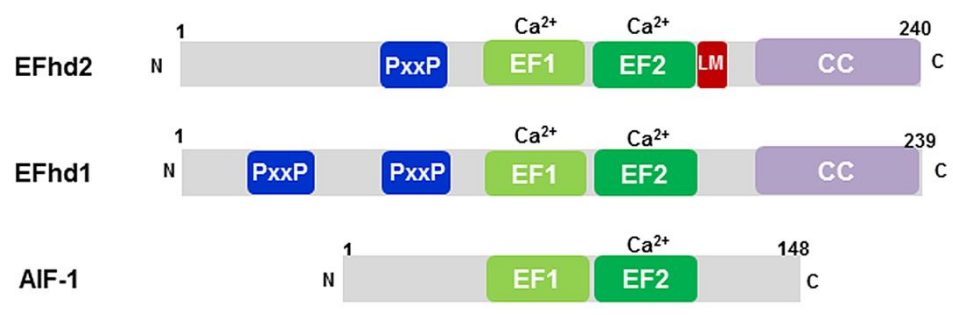
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EFhd2_Rat     QSPSRRVFNPTTFKQFSRQIKDMEKMFQYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
EFhd2_Mouse   QSPSRRVFNPTTFKQFSRQIKDMEKMFQYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
EFhd2_Zebrafish QSPSRRVFNPTTFKQFSRQIKDMEKMFQYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
EFhd1_Human   RR...SRVFNPTTFKQFSRRLIKDLESMPKLYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
EFhd1_Monkey  RR...SRVFNPTTFKQFSRRLIKDLESMPKLYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
EFhd1_Rat     GR...SKVFNPTTFKQFSRRLIKDLESMPKLYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
EFhd1_Mouse   GR...SKVFNPTTFKQFSRRLIKDLESMPKLYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
EFhd1_Zebrafish KQ...MKVFNPTTFKQFSRRLIKDLESMPKLYDAGRGGFIDLMELKLMMEKLGAPCPTHLGLKSMIKEVDEDFDGLKSFREIL
AIF-1_Human   PK.....YSDEDLPSK.LEGFKEKYMEDLNGNGDIDMSLKRMLEKLGVPKPTHLGLKSMIKEVDEDFDGLKSFREIL
AIF-1_Monkey  PK.....YSDEDLPSK.LEGFKEKYMEDLNGNGDIDMSLKRMLEKLGVPKPTHLGLKSMIKEVDEDFDGLKSFREIL
AIF-1_Rat     PK.....YSDEDLPSK.LEAFKVKYMERDLNGNGDIDMSLKRMLEKLGVPKPTHLGLKSMIKEVDEDFDGLKSFREIL
AIF-1_Mouse   PK.....YSDEDLPSK.LEAFKVKYMERDLNGNGDIDMSLKRMLEKLGVPKPTHLGLKSMIKEVDEDFDGLKSFREIL
AIF-1_Zebrafish QK.....YRDEEDLPEK.LDSFNKMYAEDLNDQGEIDMSLKRMLEKLGVPKPTHLGLKSMIKEVDEDFDGLKSFREIL
  
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linker LM Coiled-coil 240

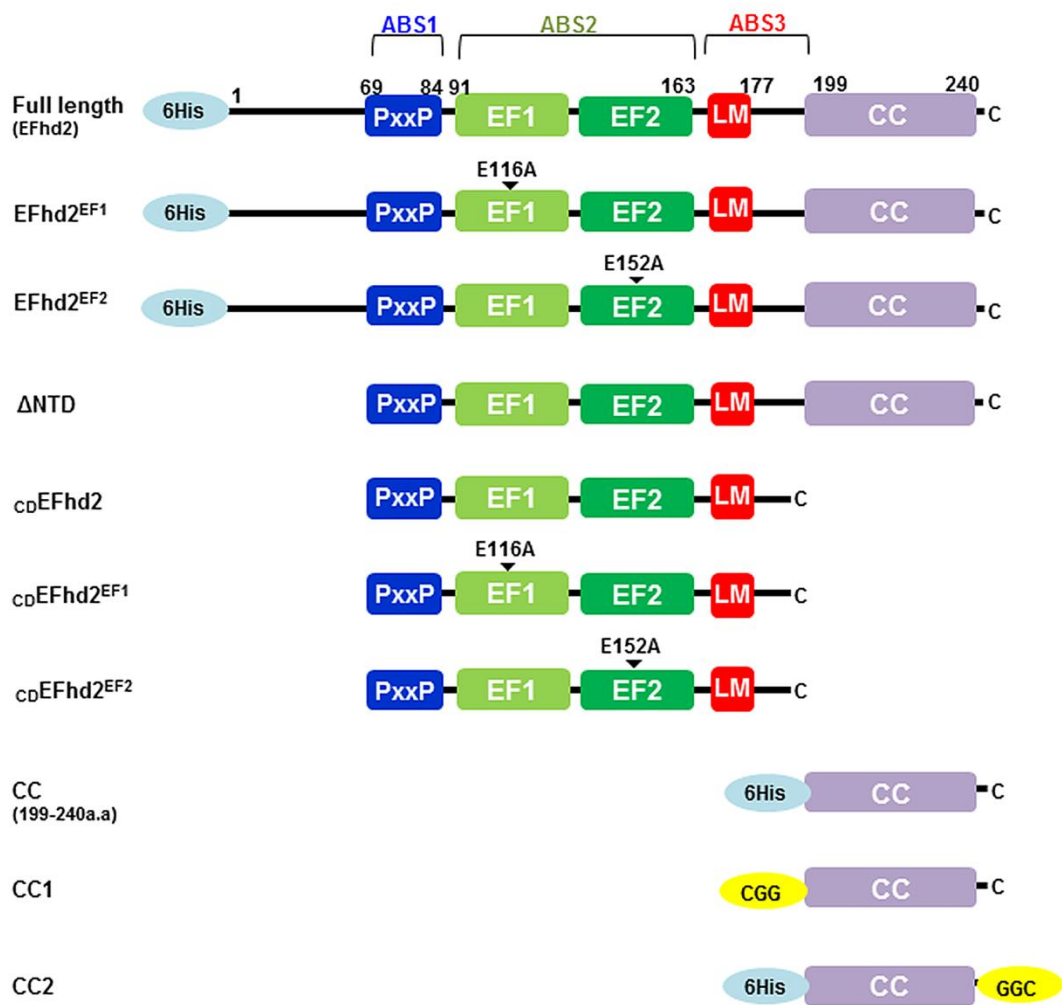
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EFhd2_Human   LIFRKAAGELQEDSGLCVLARLSEIDVSTEGVKGAKSFFFEAKVQAINVSSRFEIKAQERKQAEEMKQRKAFAFKELQSTFK.
EFhd2_Monkey  LIFRKAAGELQEDSGLCVLARLSEIDVSTEGVKGAKSFFFEAKVQAINVSSRFEIKAQERKQAEEMKQRKAFAFKELQSTFK.
EFhd2_Rat     LIFRKAAGELQEDSGLHVLARLSEIDVSTEGVKGAKNFFFEAKVQAINVSSRFEIKAQERKQAEEMKQRKAFAFKELQSTFK.
EFhd2_Mouse   LIFRKAAGELQEDSGLHVLARLSEIDVSTEGVKGAKNFFFEAKVQAINVSSRFEIKAQERKQAEEMKQRKAFAFKELQSTFK.
EFhd2_Zebrafish LIFRKAAGELQEDSGLHVLARLSEIDVSTEGVKGAKNFFFEAKVQAINVSSRFEIKAQERKQAEEMKQRKAFAFKELQSTFK.
EFhd1_Human   LIFRKAAGELQEDSGLMALAKLSEIDVLEGVKGAKNFFFEAKVQALSASKFAELKARQDERKREERLRQAFAFKELQANFNT.
EFhd1_Monkey  LIFRKAAGELQEDSGLMALAKLSEIDVLEGVKGAKNFFFEAKVQALSASKFAELKARQDERKREERLRQAFAFKELQANFNT.
EFhd1_Rat     LIFRKAAGELQEDSGLLALAKFSEIDVLEGVGAKNFFFEAKQAALACSSKFAELKARQDERKREERLRQAFAFKELQAFAF.
EFhd1_Mouse   LIFRKAAGELQEDSGLLALAKFSEIDVLEGVGAKNFFFEAKQAALACSSKFAELKARQDERKREERLRQAFAFKELQAFAF.
EFhd1_Zebrafish LIFRKAAGELQEDSGLMALARLSEIDVSTEGVLGARDFFEAKQAALSVRSKFAELREQDERKREERLRQAFAFKELQSTFCS.
AIF-1_Human   RMMLGKRS.....AILKMILMYEKAR.EKXKPTGPPAKKAISELP.....
AIF-1_Monkey  RMMLGKRS.....AILKMILMYEKAR.EKXKPTGPPAKKAISELP.....
AIF-1_Rat     RMMLGKRS.....AILRMILMYEKAR.EKXKPTGPPAKKAISELP.....
AIF-1_Mouse   RMMLGKRS.....AILRMILMYEKAR.EKXKPTGPPAKKAISELP.....
AIF-1_Zebrafish XMMLGKRS.....AVLKLVMYDKANGSKXKPDGPPPKRDIITSLP.....
  
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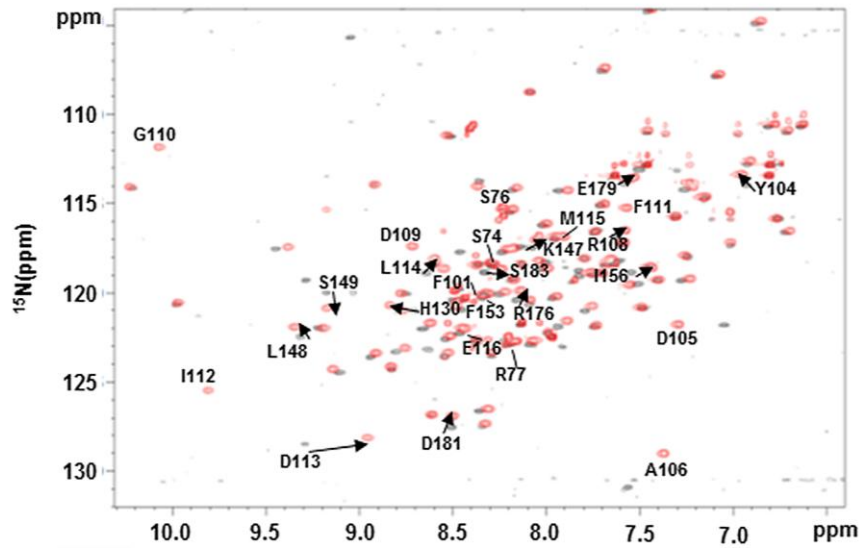
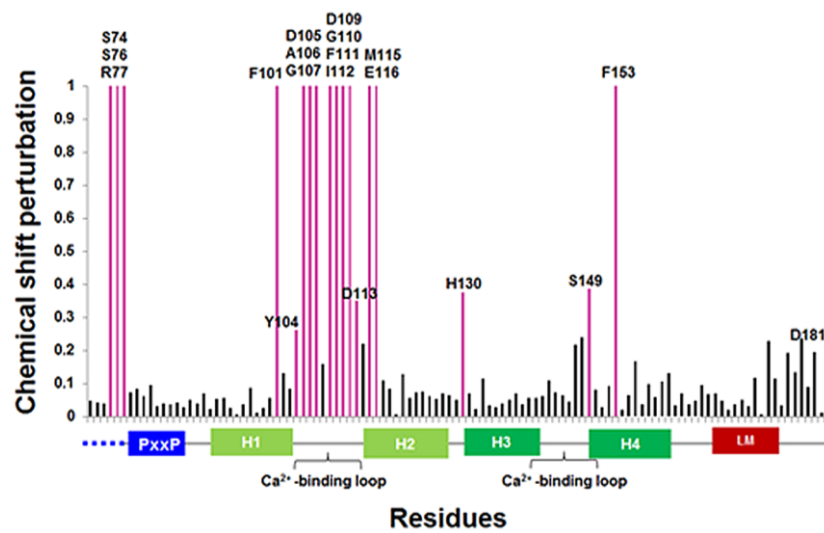
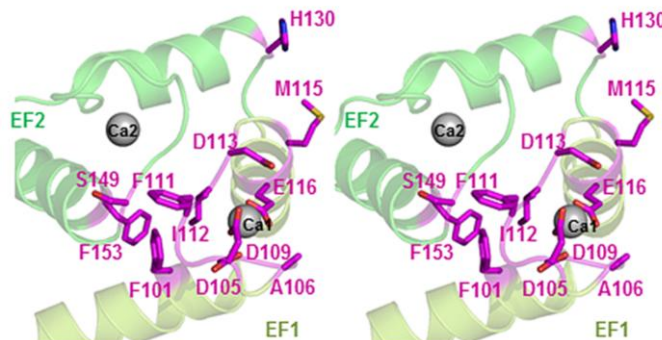
b



Supplementary Figure S2. Comparison of the domain organisation of EFhd2, EFhd1 and AIF-1. The multiple sequence alignment was carried out using the ENDscript server⁷¹. PxxP : proline-rich region; EF: EF-hands; LM: ligand mimic; CC: coiled-coil. The disordered region of the PX domain in the crystal structure of _{CD}EFhd2 is shown by the blue dashed line.



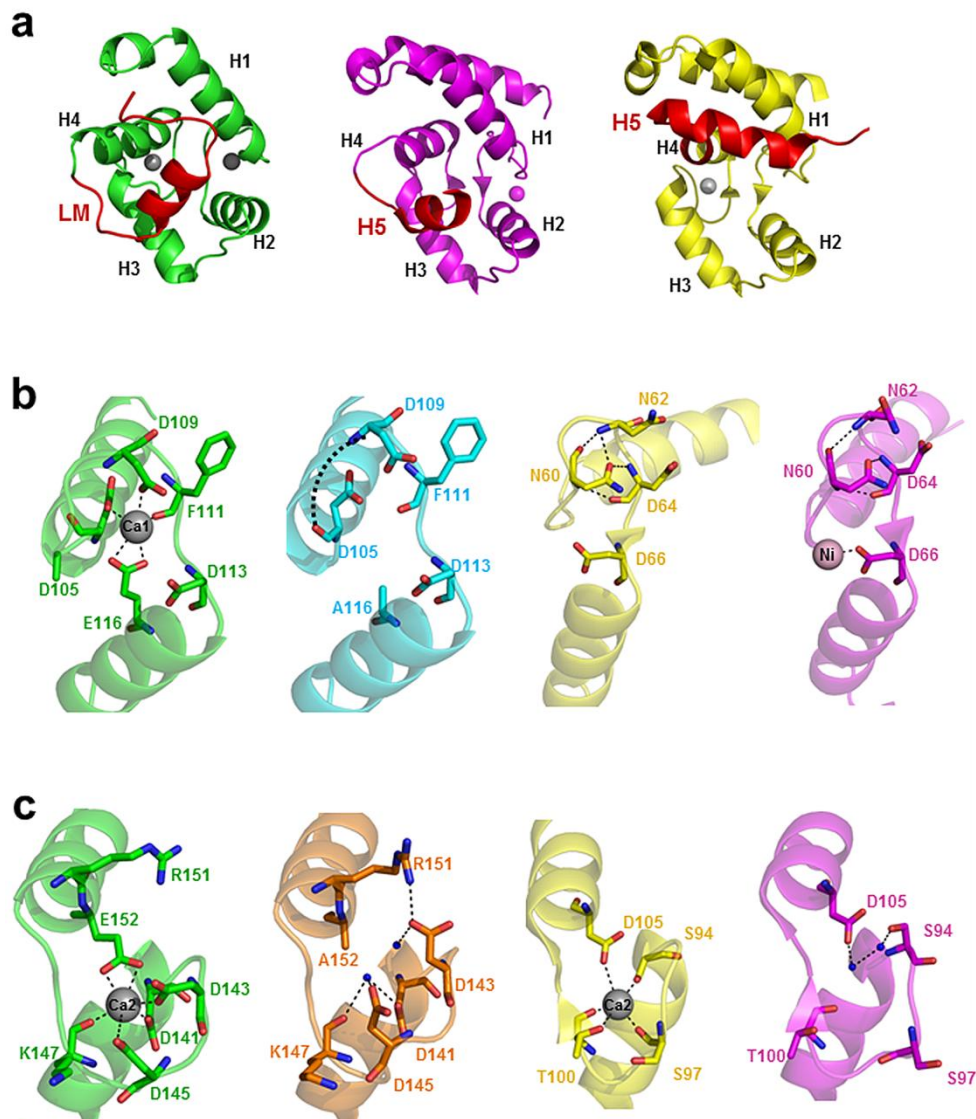
Supplementary Figure S3. Schematic of the EFhd2 constructs.

a**b****c**

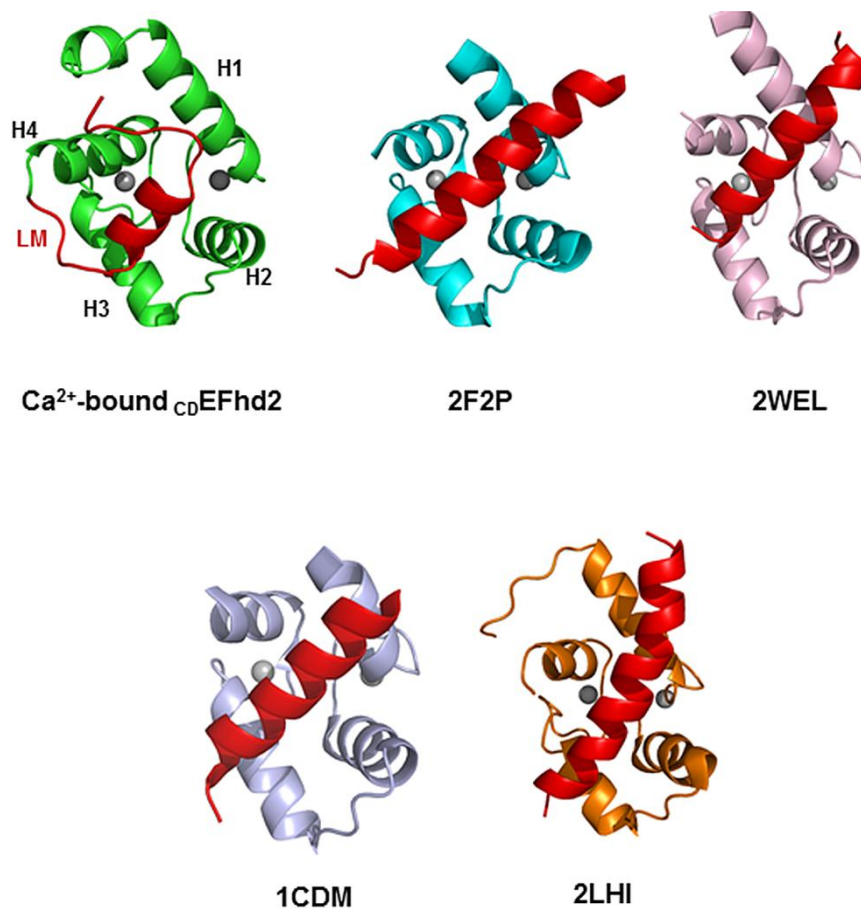
Supplementary Figure S4. Structural characteristics of $_{CD}EFhd2$ in the absence of Ca^{2+} .

(a) 2D 1H - ^{15}N HSQC spectra of $_{CD}EFhd2$ in the presence or absence of Ca^{2+} . The chemical

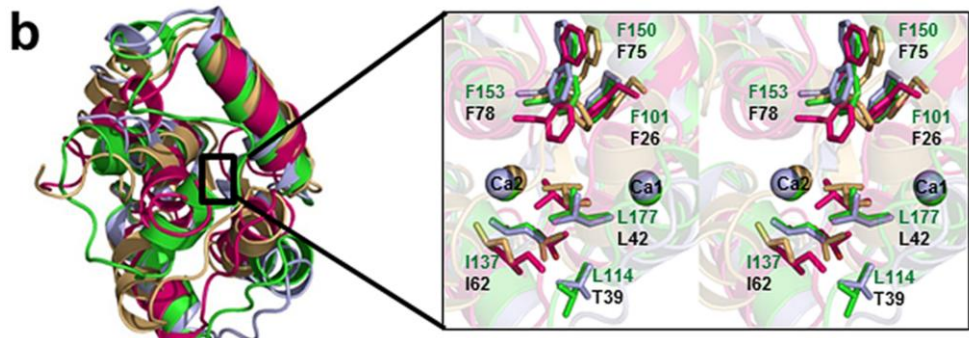
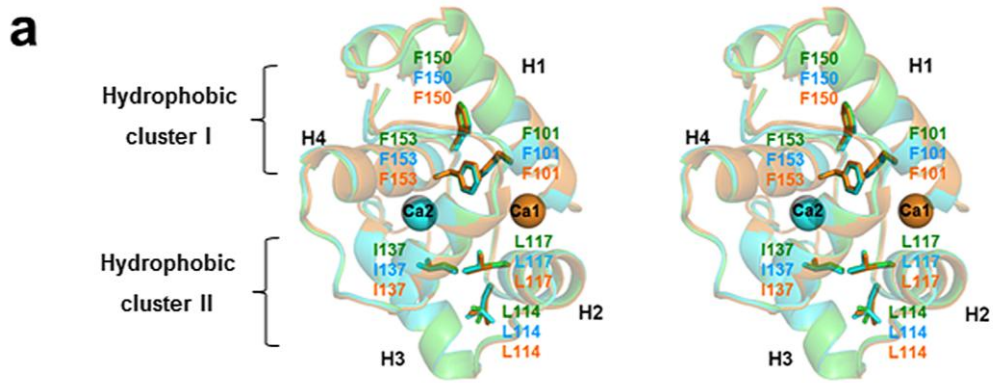
shifts are monitored in ^1H , ^{15}N correlation spectra. The resonances of $_{\text{CD}}\text{EFhd2}$ in the presence of Ca^{2+} are shown in red and resonances arising from $_{\text{CD}}\text{EFhd2}$ in the absence of Ca^{2+} are shown in black. Residues disappearing (set $\Delta\delta = 1$ ppm) or showing significant changes in chemical shifts ($\Delta\delta \geq 0.2$ ppm) upon addition of Ca^{2+} are labelled. **(b)** Chemical shift perturbation (CSP) analysis of $_{\text{CD}}\text{EFhd2}$ in the presence or absence of Ca^{2+} . The disordered region of the PxxP motif in the crystal structure of $_{\text{CD}}\text{EFhd2}$ is shown by the blue dashed line. **(c)** Representation of the perturbed residues (shown as stick in magenta) in the crystal structure of $_{\text{CD}}\text{EFhd2}$.



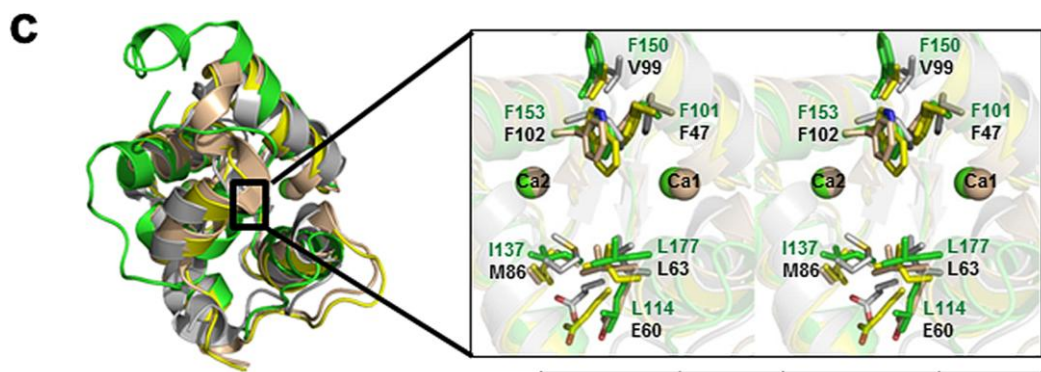
Supplementary Figure S5. Structural comparison between $\text{CD}\epsilon\text{Fhd2}$ with homologous protein, AIF-1. (a) Structures of Ca^{2+} -bound $\text{CD}\epsilon\text{Fhd2}$ (green), Ca^{2+} -free AIF-1 (PDB ID: 2D58, magenta) and Ca^{2+} -bound AIF-1 (PDB ID: 1WY9, yellow)³⁵. Grey and magenta spheres indicate calcium and nickel ions, respectively. (b) Ca^{2+} -binding loop of EF1 of the Ca^{2+} -bound $\text{CD}\epsilon\text{Fhd2}$ (green), $\text{CD}\epsilon\text{Fhd2}^{\text{EF1}}$ (cyan), Ca^{2+} -bound AIF-1 (yellow) and Ca^{2+} -free AIF-1 (magenta). (c) Ca^{2+} -binding loop of EF2 of the Ca^{2+} -bound $\text{CD}\epsilon\text{Fhd2}$ (green), $\text{CD}\epsilon\text{Fhd2}^{\text{EF2}}$ (orange), Ca^{2+} -bound AIF-1 (yellow) and Ca^{2+} -free AIF-1 (magenta). Blue spheres indicate water molecules.



Supplementary Figure S6. Structural comparison between _{CD}EFhd2 and EF-hand-containing proteins. Structure of Ca²⁺-bound _{CD}EFhd2 (green) and CaM with the target peptide complex (PDB ID: 2F2P, 2WEL, 1CDM and 2LHI)³⁸⁻⁴¹. Red ribbon diagram shows the LM of the _{CD}EFhd2 and target peptides of CaM. Silver spheres indicate Ca²⁺ ions. A search for structures similar to _{CD}EFhd2 using the Dali program³⁷ gave nearly 100 Ca²⁺-bound EF-hands (>10 for Z-scores and < 3.0 Å in RMSD), in which most of the EF-hand matches were calmodulin (CaM) and troponin C (TnC). In addition, the structure of _{CD}EFhd2 fits well with those of Ca²⁺-peptide-CaM such as PDB IDs 2F2P (Z-score = 11.5, RMSD = 1.6 Å for 79 Cα atoms), 2WEL (Z-score = 11.2, RMSD = 1.74 Å for 79 Cα atoms), 1CDM (Z-score = 10.6, RMSD = 1.7 Å for 73 Cα atoms) and 2LHI (Z-score = 10.5, RMSD = 1.87 Å for 81 Cα atoms).



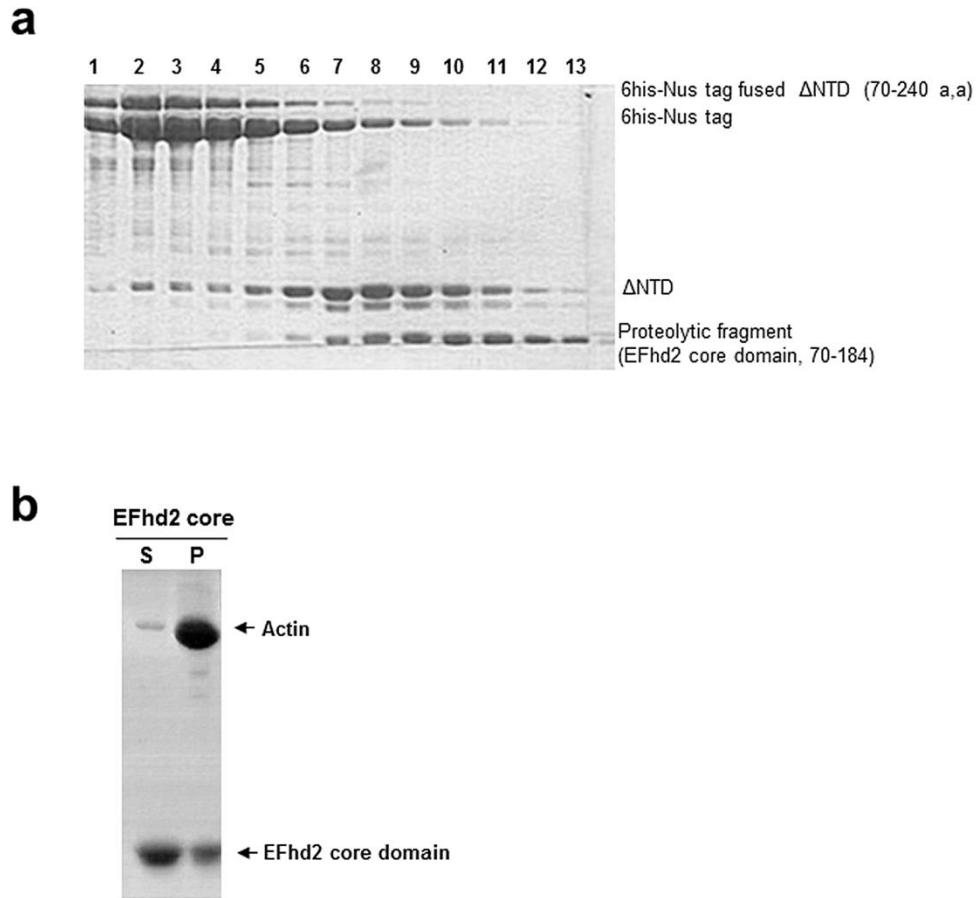
cdEFhd2	PDB	Ca ²⁺		RMSD
		EF1	EF2	
TnC (Class IV)	1AVS	o	o	1.45 Å
	1SMG	o	x	2.48 Å
	1SKT	x	x	3.13 Å



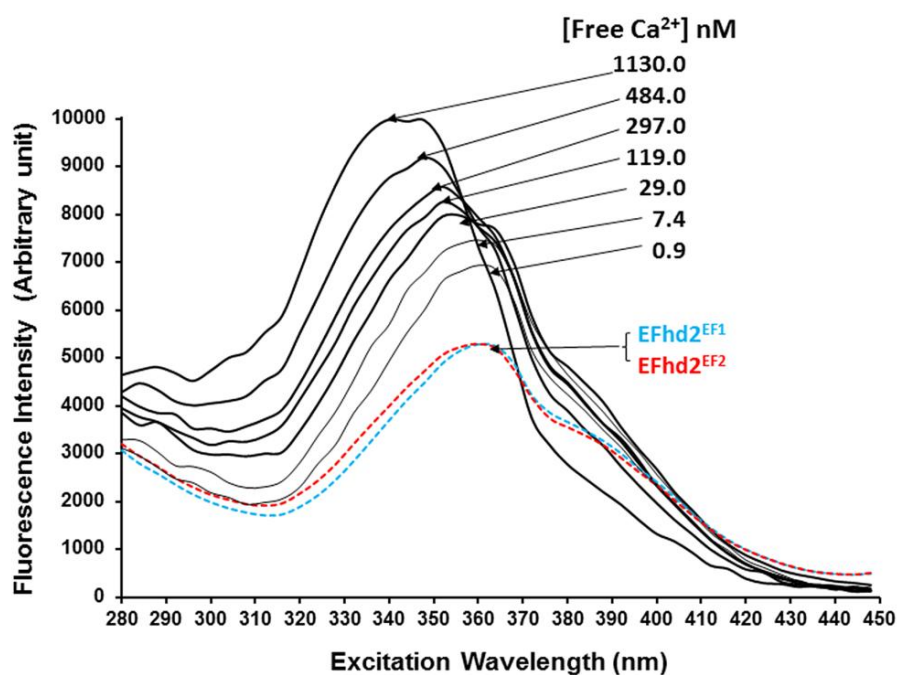
cdEFhd2	PDB	Ca ²⁺		RMSD
		EF1	EF2	
PVALB (Class I)	1RWY	o	o	1.49 Å
	1B8L	o	x	1.58 Å
	2JWW	x	x	2.28 Å

Supplementary Figure S7. Hydrophobic clusters of EFhd2. (a) Hydrophobic residues within hydrophobic cluster of the Ca²⁺-bound cdEFhd2 (green), cdEFhd2^{EF1} (cyan), and cdEFhd2^{EF2}

(orange) **(b)** Structural superposition of Ca²⁺-bound _{CD}EFhd2 (green) and TnC (1AVS, Ca²⁺-bound form, RMSD = 1.45 Å for 77 Cα atoms; 1smg, one Ca²⁺-bound form, RMSD = 2.48 Å for 72 Cα atoms; 1SKT, apo-form, RMSD = 3.13 Å for 64 Cα atoms)⁴⁹⁻⁵¹. **(b)** Structural superposition of Ca²⁺-bound _{CD}EFhd2 (green) and PVALB (1RWY, Ca²⁺-bound form, RMSD = 1.49 Å for 67 Cα atoms; 1B8L, one Ca²⁺-bound form, RMSD = 1.58 Å for 62 Cα atoms; 2JWW, apo-form, RMSD = 2.28 Å for 67 Cα atoms)⁵²⁻⁵⁴. Residues of Ca-bound and PVALB are labelled green and black, respectively.



Supplementary Figure S8. Identification of $_{CD}EFhd2$. (a) All samples collected during the size exclusion chromatography procedure. Purified ΔNTD (residues 70–240) was incubated with TEV protease for 10 h at 4 °C prior to buffer exchange through size exclusion chromatography. The resulting fragments were resolved by SDS-PAGE. (b) Co-sedimentation (actin-binding) assay of $_{CD}EFhd2$. Protein samples (5 μM) were added to pre-polymerised actin (2 μM) in the presence of 1 mM $CaCl_2$.



Supplementary Figure S9. Fluorescence spectra of fura-2 in the presence of various concentrations of Ca²⁺ are represented by black lines. Fluorescence spectra of fura-2 mixed with EFhd2^{EF1} and EFhd2^{EF2} is represented by cyan and orange dashed line, respectively. Standard solutions to determining Ca²⁺ concentration were prepared refer to the method of Kong *et al*⁶⁹.