Incorporation of sensing modalities into de novo designed fluorescence-activating proteins

Jason C. Klima, Lindsey A. Doyle, Justin Daho Lee, Michael Rappleye, Lauren A. Gagnon, Min Yen Lee, Emilia P. Barros, Anastassia A. Vorobieva, Jiayi Dou, Samantha Bremner, Jacob S. Quon, Cameron M. Chow, Lauren Carter, David L. Mack, Rommie E. Amaro, Joshua C. Vaughan, Andre Berndt, Barry L. Stoddard & David Baker

Through the efforts of many groups, a wide range of fluorescent protein reporters and sensors based on green fluorescent protein and its relatives have been engineered in recent years. Here we explore the incorporation of sensing modalities into de novo designed fluorescence-activating proteins, called mini-fluorescence-activating proteins (mFAPs), that bind and stabilize the fluorescent cis-planar state of the fluorogenic compound DFHBI. We show through further design that the fluorescence intensity and specificity of mFAPs for different chromophores can be tuned, and the fluorescence made sensitive to pH and Ca\(^{2+}\) for real-time fluorescence reporting. Bipartite split mFAPs enable real-time monitoring of protein–protein association and (unlike widely used split GFP reporter systems) are fully reversible, allowing direct readout of association and dissociation events. The relative ease with which sensing modalities can be incorporated and advantages in smaller size and photostability make de novo designed fluorescence-activating proteins attractive candidates for optical sensor engineering.
De novo designed mini-fluorescence-activating proteins (mFAPs) (Fig. 1a) bind and activate the fluorescence of the fluorogenic compound DFHBI (3,5-difluoro-4-hydroxybenzylidine imidazoline) (1, Fig. 1b) in vitro and in bacteria, yeast, and mammalian cells1. DFHBI does not fluoresce when free in solution2, but becomes brightly fluorescent upon stabilization of the cis-planar conformation (planar Z conformation) through macromolecular binding3. RNA aptamers have been evolved to bind similar fluorogenic DFHBI-derived compounds4,5 (e.g., DFHBI, DFHBI-1T [(Z)-4-(3,5-difluoro-4-hydroxybenzylidine)-2-methyl-1-(2,2,3-trifluoroethyl)-1H-imidazol-5(4 H)-one] (2, Fig. 1c) and DFHO [3,5-difluoro-4-hydroxybenzylidine imidazoline-2-oxime]), with up to 0.72 fluorescence quantum yield6, but to our knowledge so far no protein-based systems other than the mFAPs have been reported to bind and fluorescently activate DFHBI-1T or DFHO chromatophores.

Circularly permuted fluorescent proteins such as cpGFP and cpFAST enable the real-time detection of analytes of interest using fluorescence microscopy7-9. Likewise, self-complementing split fluorescent protein reporter systems based on green fluorescent protein (GFP) variants10-12 and FAST13 have been engineered to monitor protein-protein interactions in vitro, in cyto, and in vivo14 using fluorescence microscopy. The β-barrel structure of mFAPs suggests that mFAPs could be re-designed to monitor analyte fluxes and protein-protein interactions; such sensors could have complementary biophysical properties to existing fluorescent proteins (such as intrinsically fluorescent GFP and extrinsically fluorescent DiB9,15, Y-FAST16, Ca2+-responsive cpFAST17, and splitFAST13 reporters and sensors). mFAPs have several biophysical properties that make them attractive candidates for further development. First, they are less than half the size of GFP, so their genetic footprint is smaller, and fusions to proteins of interest are less perturbative. Second, the bound chromophore can readily exchange with free chromophore in solution, and hence mFAPs can be more photostable17 than GFP. Third, chemical derivatives of DFHBI with different fluorochromophores. Fourth, de novo mFAPs can be engineered to remain folded at low pH, facilitating the engineering of pH-responsive fluorogenic optical sensors. Finally, the chromophore-binding pocket is close to the protein surface, potentially enabling the design of allosteric coupling between chromophore binding and linked analyte binding domains for analyte-responsive fluorogenic optical sensors7,18,19.

Here, we explore the incorporation of sensing modalities into the mFAPs. We develop and apply methodologies for engineering chromophore-selective mFAP variants: mFAP2a, mFAP2b, and mFAP10 that incorporate 12, 10, and 11 mutations, respectively (Supplementary Fig. 1).

Titrations of mFAP2, mFAP2a, mFAP2b, and mFAP10 with either DFHBI (Fig. 1d) or DFHBI-1T (Fig. 1e) and quantum yield measurements (Table 1 and Supplementary Fig. 2) showed that: mFAP2, mFAP2a, and mFAP10 have ~2.7-fold, ~2.5-fold, and ~12-fold brighter fluorescence with DFHBI-1T than DFHBI, but bind DFHBI with ~30-fold, ~39-fold, and ~2.6-fold higher affinity than DFHBI-1T, respectively; and that mFAP2b has ~30-fold brighter fluorescence with DFHBI than DFHBI-1T and binds DFHBI with ~6.1-fold higher affinity than DFHBI-1T. The mFAP10-DFHBI-1T complex is the brightest, with 23.7% absolute quantum yield (under conditions with 99.9% of chromophore bound) and a 17.5-fold increased brightness over the previously reported mFAP2-DFHBI complex1, resulting in a 242-fold fluorescence activation over free DFHBI-1T (Table 1). Relative fluorescence intensities and thermodynamic dissociation constants (Kd) for the deprotonated (phenolate) states of DFHBI, DFHBI-1T, and DFHO for the Ca2+-independent mFAP variants presented in this study are given in Supplementary Table 1 (for example, mFAP3 binds the yellow colored DFHO chromophore with ~10-fold lower fluorescence intensity than mFAP2b with DFHBI). Using a laser scanning confocal fluorescence microscope to image E. coli expressing either mFAP2a or mFAP2b labeled with either DFHBI or DFHBI-1T, we observed pronounced chromophore selectivity of mFAP2b for DFHBI over DFHBI-1T, and chromophore promiscuity of mFAP2a for both DFHBI or DFHBI-1T (Fig. 1f-i). E. coli cultures expressing either mFAP2a or mFAP2b mixed in a 1:1 cellular ratio labeled with DFHBI-1T have ~49% of the total fluorescence signal of cultures labeled with DFHBI (Supplementary Fig. 3).

We next targeted mFAP2a or mFAP2b to the endoplasmic reticulum (ER) of mammalian COS-7 cells using a C-terminal sec16β localization sequence, and observed bright fluorescence of ER under fixed cell (Supplementary Fig. 4) and live cell (Supplementary Movie 1 and Supplementary Movie 2) epifluorescence microscopy after labeling with DFHBI. Under fixed cell imaging, following washing and re-labeling with DFHBI-1T (Supplementary Fig. 4) the fluorescence was altered as expected, demonstrating external spatiotemporal control over fluorescence. To compare the photostability of mFAP2a and mFAP2b to a monomeric enhanced GFP (EGFP) variant, we also targeted AcGFP1 to the ER of COS-7 cells. Upon continuous wave illumination imaging at ~0.885 Hz (1.13 s frame−1) of fixed COS-7 cells using laser scanning confocal fluorescence microscopy, we found at 50.0 µM chromophore (saturating conditions) a 6.2-fold, 3.5-fold, and 6.1-fold improved photostability of mFAP2a-DFHBI, mFAP2b-DFHBI-1T, and mFAP2b-DFHO complexes over AcGFP1, respectively. At 500 nM chromophore (sub-saturating conditions), the improvements in photostability of the three complexes over AcGFP1 were 5.0-fold, 3.8-fold, and 4.7-fold, respectively (Fig. 2).

Optimization of brightness and chromophore selectivity. We began by seeking to improve the stability of mFAPs at low pH, the binding affinity to the phenolic and phenolate forms of DFHBI, as well as the fluorescence intensity of both complexes. mFAP2 was chosen for optimization because it has the highest absolute fluorescence quantum yield (φ, of 2.1%) and highest affinity (Kd of ~180 nM) for the phenolate form of DFHBI compared to mFAP11. Through a series of library selections (see "Methods") targeting aliphatic and aromatic residues directly interacting with DFHBI or in the hydrophobic core of the β-barrel, as well as residues in the loop connecting the seventh and eighth β-strands (loop7) of the β-barrel, we obtained three brighter and incorporation of pH responsiveness. A prerequisite for designing a robust pH-responsive mFAP is the ability to bind both protonated (phenolic) DFHBI tautomers and both deprotonated (phenolate) DFHBI resonance structures (Fig. 3a). When stabilized in the cis-planar conformation, the phenolic and phenolate forms of DFHBI exhibit blueshifted and redshifted peak excitation wavelengths, respectively6 (Fig. 3d). mFAP2b binds both forms of DFHBI (Fig. 3g); to increase pH sensitivity we screened design variants based on the change in fluorescence between pH 3.61 and pH 7.34 (Supplementary Fig. 5), and identified a particularly pH-responsive variant we call mFAP_pH.
**Fig. 1** Characterization of brighter and chromophore-specific mFAPs. 

**a** Computational model of de novo designed β-barrel variant mFAP2b showing protein backbone (cartoon) and bound DFHBI chromophore (sticks).

**b, c** Chemical structures of DFHBI and DFHBI-1T, respectively.

**d, e** In vitro titration of DFHBI or DFHBI-1T with mFAP2 (gray), mFAP2b (lime), mFAP2a (violet), and mFAP10 (pink) proteins. Error bars represent s.d. of the mean of eight technical replicates. Normalized means were fit to a single-binding site isotherm function using non-linear least squares fitting to obtain $K_d$ values (Table 1), and the fits scaled to the maximum mean relative fluorescence unit (RFU) values (lines).

**f**–**i** Each panel shows a representative image of the fluorescence signal emitted by E. coli cells expressing the indicated mFAP variant labeled with 10.0 μM concentration of the indicated chromophore (left) and a zoom-in of the modeled-binding pocket of that mFAP variant bound to the chromophore (right). The images (left) are the pseudocolored normalized fluorescence intensity per pixel. Scale bars represent 10 microns. Imaging was independently repeated twice with similar results. The computational models (right) show the residues unique to mFAP2b (V13, M15) (lime sticks) or mFAP2a (A13, F15) (violet sticks). Intermolecular hydrogen bonds to the chromophore are shown as black dotted lines. Vacuum electrostatic contact potential around the chromophore is shown in a transparent gray surface.

**f** mFAP2b with DFHBI, **g** mFAP2b with DFHBI-1T does not emit a detectable fluorescence signal because binding is precluded by steric clashes of DFHBI-1T with V13 (red cylinders), **h** mFAP2a with DFHBI, and **i** mFAP2a with DFHBI-1T. Source data is available for Fig. 1f–i.
Table 1 Photophysical properties of mFAPs with DFHBI and DFHBI-1T compared with controls.

|                | Ex (nm) | Em (nm) | K<sub>abs</sub> (M<sup>–1</sup>)<sup>a</sup> | K<sub>em</sub> (M<sup>–1</sup>)<sup>a</sup> | Extinction coefficients | Bound % | Quantum yield % | Relative quantum yield | Reported quantum yield |
|----------------|---------|---------|------------------------------------------|------------------------------------------|------------------------|---------|----------------|------------------------|------------------------|
| EGFP           | 488     | 507     | 56,000                                   | 33,600                                   | EGFP                   | 99.9    | 0.15 ± 0.01   | 0.001                   | 0.0007                  |
| mFAP2a         | 430     | 494     | 505                                      | 37,800                                   | mFAP2a                 | 99.1    | 1.8 ± 0.25    | 0.005                   | 0.003                   |
| mFAP2b         | 470     | 475     | 497                                      | 48,900                                   | mFAP2b                 | 100.0   | 0.017 ± 0.009 | 0.026                   | 0.029                   |
| mFAP10         | 484     | 485     | 485                                      | 67,200                                   | mFAP10                 | 99.9    | 0.045 ± 0.005 | 0.045 ± 0.005            | 0.045 ± 0.005            |
| EGFP           | 418     | 423     | 489                                      | 30,100                                   | EGFP                   | 95.1    | 11 ± 3.1      | 0.001                   | 0.0007                  |
| mFAP2a         | 418     | 423     | 489                                      | 30,100                                   | mFAP2a                 | 100.0   | 0.017 ± 0.007 | 0.026                   | 0.029                   |
| mFAP2a         | 470     | 475     | 497                                      | 48,900                                   | mFAP2b                 | 100.0   | 0.017 ± 0.009 | 0.026                   | 0.029                   |

Note: Extinction coefficients are measured from absorption spectra, and quantum yields are obtained by non-linear least squares fits to the mean fluorescence intensity of the eight technical replicates per chromophore titration (Fig. 1d, e).

Incorporation of Ca<sup>2+</sup>-responsiveness. To enable the engineering of additional environmental responsiveness in mFAPs, we used Rosetta<sup>22,23</sup> to de novo design 59 extensions of β-barrel loops 1, 3 and 7, and screened them for fluorescence after labeling with DFHBI. We identified five extended loop7 variants that maintain the β-barrel fold and are compatible with DFHBI binding (Supplementary Fig. 6). Using these extended loop sequences as linkers (see "Methods"), we grafted one EF-hand motif<sup>24</sup>, one EF-hand domain<sup>25</sup> (i.e., two EF-hand motifs), or calmodulin<sup>26</sup> (i.e., four EF-hand motifs) into loop7 of mFAP2b (Supplementary Fig. 7). Through DFHBI and Ca<sup>2+</sup> titrations, we found that Ca<sup>2+</sup> binding was allosterically coupled to DFHBI binding, with either positive<sup>8</sup> or negative<sup>8</sup> allosteric modulation of fluorescence (Fig. 4) depending only on the amino acid sequence of the linkers. As expected based on the cooperativity of Ca<sup>2+</sup> binding to calmodulin<sup>27,28</sup>, we found that as the number of grafted EF-hand motifs increases, so does the affinity for Ca<sup>2+</sup> ions (Fig. 4). While some existing fluorescent Ca<sup>2+</sup> sensors harboring calmodulin such as GCaMP6<sup>29</sup> are characterized by Hill coefficients of ~2–3, Ca<sup>2+</sup>-responsive mFAPs are characterized by Hill coefficients of ~1 (similar to previously reported ratiometric-pericam<sup>8</sup>, CatchER<sup>18</sup>, and XCaMPs<sup>30</sup>), suggesting that one Ca<sup>2+</sup>-binding site is allosterically coupled to chromophore affinity (Supplementary Table 2). Circular dichroism experiments showed that Ca<sup>2+</sup> binding increases the α-helical secondary structure, presumably in the calmodulin domain, and enhances thermostability for EF4n_mFAP2b harboring calmodulin (Supplementary Fig. 8).

Incorporation of the mFAP2a hydrophobic core amino acid substitutions (A13, F15) (Supplementary Fig. 9) increased Ca<sup>2+</sup> affinity by up to 11.7-fold for positively allosteric proteins and decreased Ca<sup>2+</sup> affinity by up to 11.6-fold for negatively allosteric proteins (Supplementary Table 2 and Supplementary Figs. 10, 11, 12, 13). The substitutions increase the affinity of DFHBI binding (Fig. 1d), and DFHBI and Ca<sup>2+</sup> titration data indicate thermodynamic coupling between DFHBI and Ca<sup>2+</sup> binding (Supplementary Fig. 14 and Supplementary Note 1). Overall, the computational models (Fig. 3b, c) suggest that the amino acid substitutions in mFAP<sub>pH</sub> compared to mFAP2b (i.e., W27M and W93F) improve pH-responsiveness of the protein–DFHBI complex by increasing shape complementarity toward the protonated (phenolic) form of DFHBI by removing a hydrogen bond between the W27 indole ring and the DFHBI imidazolinone moiety, and by reducing net positive charge in the β-barrel core via removing a buried unsatisfied hydrogen bond donor in the indole ring of W93, resulting in a higher result of protein-bound phenolic DFHBI to phenolate DFHBI at low pH (Fig. 3f, g). The phenolic and phenolate forms of DFHBI had nearly equivalent affinities (K<sub>d</sub> values) for mFAP<sub>pH</sub> of ~190 nM and ~160 nM, respectively (Supplementary Fig. 5). The pK<sub>a</sub> of free, unbound DFHBI in solution is ~5.4, and we observe the same pK<sub>a</sub> for the mFAP<sub>pH</sub>–DFHBI complex (Fig. 3h).

At peak excitation and emission wavelengths (Fig. 3d, e), mFAP<sub>pH</sub> showed a marked ~250-fold-change in ratiometric fluorescence (F<sub>ratio</sub>) from pH 8.38 to pH 3.63, compared with only a ~34-fold-change in F<sub>ratio</sub> from pH 8.38 to pH 4.79 for pHRed<sup>20</sup> and a ~3.5-fold-change in F<sub>ratio</sub> from pH 8.83 to pH 5.14 for pHluorin<sup>21</sup> (Fig. 3i). At low pH, the β-barrel fold of mFAP<sub>pH</sub> is more resistant to denaturation than those of pHRed and pHluorin2, and thus the mFAP<sub>pH</sub>–DFHBI complex has a higher dynamic range for ratiometric fluorescence across the physiologically relevant pH range. Ratiometric fluorescence imaging of the mFAP<sub>pH</sub>–DFHBI complex hence should enable real-time in situ quantification of pH.
Ca^{2+}-responsive mFAPs exhibit over 500-fold differences in affinity for Ca^{2+} (Supplementary Table 2), enabling the choice of a sensor with optimal fluorescence dynamic range in the anticipated Ca^{2+} concentration range under study.

To investigate the origin of the allosteric coupling between Ca^{2+} and DFHBI binding, we solved an X-ray crystal structure (Supplementary Table 3) of one of the positively allosteric Ca^{2+}-responsive mFAPs harboring one EF-hand motif, EF1p_mFAP2b, in complex with DFHBI and Ca^{2+} (Fig. 5a, b and Supplementary Fig. 15). The EF1p_mFAP2b–DFHBI–Ca^{2+} crystal structure revealed the residue K101 from the Ca^{2+}-bound EF-hand motif forms a hydrogen bond to the hydroxybenzylidene moiety of DFHBI, providing structural insight into the allosteric coupling mechanism between DFHBI and Ca^{2+} binding (Fig. 5c). Indeed, the K101A lysine-to-alanine substitution in EF1p_mFAP2b reduces DFHBI affinity ~21-fold in the presence of excess Ca^{2+} (K_d), and Ca^{2+} affinity ~29-fold in the presence of excess DFHBI, compared with EF1p_mFAP2b (Supplementary Fig. 16). This lysine residue is the second amino acid of the first EF-hand motif in all of the Ca^{2+}-responsive mFAPs, suggesting it influences the allosteroy in each case. Molecular dynamics (MD) simulations starting from the X-ray crystal structure coordinates of EF1p_mFAP2b in four conditions (apo, Ca^{2+}-bound, DFHBI-bound and with both Ca^{2+} and DFHBI) suggest Ca^{2+} binding to loop7 shifts the free energy landscape towards the holo (fluorescently active) conformation even in the absence of DFHBI (Supplementary Fig. 17), suggesting a conformational selection mechanism consistent with the experimentally observed allosteric coupling of DFHBI and Ca^{2+} binding (Supplementary Fig. 10).

To explore whether the Ca^{2+}-responsive mFAPs could detect Ca^{2+} fluxes in mammalian cells, we first expressed a positively allosteric Ca^{2+}-responsive mFAP harboring one EF-hand motif, EF1p_mFAP2b, in the extracellular matrix of HEK293 cells by fusion to an N-terminal immunoglobulin κ-chain leader sequence secretion signal and a C-terminal transmembrane anchoring domain from platelet-derived growth factor receptor (PDGFR). To optimize detection sensitivity and photostability while compromising on fluorescence dynamic range (see Supplementary Note 1), we chose to label HEK293 cells with DFHBI concentrations at approximately the (K_d · K_d)\(^{-1/2}\) for EF1p_mFAP2b, where K_d and K_d are the DFHBI K_d for the Ca^{2+}-bound or Ca^{2+}-free sensor, respectively (Supplementary Table 2). Titration of Ca^{2+} from 0 to 10 mM final concentration under constant DFHBI concentration resulted in a fluorescence fold-change (ΔF) of ~0.5 (Fig. 5d). The fluorescence response was similar after photobleaching the cells, presumably due to the high-chromophore concentrations improving the photostability of mFAPs (Fig. 2).

Next, we expressed negatively allosteric Ca^{2+}-responsive mFAPs containing two or four EF-hand motifs (EF2n_mFAP2a, EF4n_mFAP2b, and EF4n_mFAP2a) in the cytosol of HEK293 cells and stimulated Ca^{2+} release into the cytosol via endogenous muscarinic receptors\(^{19}\) by treatment with 100 μM acetylcholine (ACh). As expected, Ca^{2+} release into the cytosol resulted in a decrease in fluorescence upon ACh stimulation (Fig. 5e and Table 2) with DFHBI concentrations at approximately (K_d · K_d)\(^{-1/2}\), which balances detection sensitivity and photostability against fluorescence dynamic range (Supplementary Note 1). Compared to the positive control fluorescent Ca^{2+} sensor\(^{19}\) GCaMP6f (Supplementary Fig. 18), the Ca^{2+}-responsive mFAPs have lower fluorescence dynamic range at the DFHBI concentrations used, but are highly photostable (Fig. 5e, first 20 s).

As the fluorescence of negatively allosteric Ca^{2+}-responsive mFAPs increases when the Ca^{2+} concentration decreases, negatively allosteric Ca^{2+}-responsive mFAPs enable reporting
Chemical basis of pH-responsiveness in mFAP_pH. a-c Chemical structures of protonated tautomers and deprotonated resonance structures of DFHBI. b, c Computational models showing the residues unique to mFAP_pH (M27, F93) (magenta) and mFAP2b (W27, W93) (lime). The arrangement of intermolecular hydrogen bonds (black dotted lines) in b the mFAP_pH–DFHBI complex permits binding to both the phenolate and phenolic (shown) forms of DFHBI whereas c the mFAP2b–DFHBI complex only permits binding to the phenolic (shown) form of DFHBI. d Normalized fluorescence excitation spectra of the mFAP_pH–DFHBI complex at pH 3.6 and pH 8.4. e Normalized fluorescence emission spectra of the mFAP_pH–DFHBI complex at pH 3.6 and pH 8.4. f, g Fluorescence excitation spectra normalized to pH 3.8 and pH 7.5 of f pH-responsive mFAP_pH–DFHBI complex and g pH-unresponsive mFAP2b–DFHBI complex. h Normalized mean (n = 3) fluorescence intensity from the pH titration of the mFAP_pH–DFHBI complex (blue and red) and previously reported pHRed (dark gray and light gray), showing fluorescence emission by exciting the blueshifted fluorescence excitation peak (blue and dark gray) and fluorescence emission by exciting the redshifted fluorescence excitation peak (red and light gray). i Ratiometric fluorescence (F_{ratio}) from the pH titration of the mFAP_pH–DFHBI complex (magenta), pHRed (gray), and pHluorin2 (green). h, i The means are fit to h a sigmoid or inverse sigmoid function or i a logistic function using non-linear least squares fittings (lines). The dotted lines indicate pH values at which the measured $F_{ratio}$ coincides with two different pH values, and therefore are not used in the fittings. Error bars represent the s.d. of the mean of three technical replicates.
fluorescence in the SR consistent with inhibition of SERCA pumps34,36 (Supplementary Fig. 19).

**Split fluorescence-activating proteins.** We next sought to design bipartite split fluorogenic sensors13 from mFAPs by creating split points in the β-hairpins and loop7 of the mFAP2a scaffold. With eight β-strands1 per β-barrel, there are seven possible bipartite split mFAPs (Supplementary Fig. 20). As the split mFAP fragments would have solvent-exposed hydrophobic patches that could hamper solubility, we initially tagged split mFAP fragments to maltose-binding protein (MBP) to improve soluble expression levels. β-barrel complementation assays in excess DHFBI-1T showed that split mFAP fragments m12 and m38 displayed the highest fluorescence activation above background, with 7.34-fold higher mean fluorescence intensity over mean background fluorescence intensity. After background subtraction, the brightest fragment combination, m12 and m38, had 184-fold higher mean fluorescence intensity than the dimmest fragment combination, m1 and m28. Differences in the fluorescence excitation spectra of the fluorescently active β-barrel complexes in excess DHFBI-1T suggest that bipartite split mFAPs stabilize the fluorescently active cis-planar conformation of DHFBI-1T in slightly different chromophore environments (Supplementary Fig. 20).

Titrations of MBP-tagged split mFAP fragments into their complementary MBP-tagged split mFAP fragments in excess DHFBI-1T resulted in reconstitution of fluorescence at high-protein concentrations, but the signal did not plateau even at the highest concentrations tested. The estimated split mFAP fragment dissociation constants (K_d values) are ≥281 µM for m12 and m38, ≥22.0 µM for m14 and m58, ≥323 µM for m16 and m78, and ≥354 µM for m17 and m8 (Supplementary Fig. 20). In contrast, when we fused complementary split mFAP fragments to BCL2 family member proteins and high affinity (K_d ∼ 1 nM) designed binding partners37 (Fig. 6a), the fluorescence increased linearly until reaching a plateau at equimolar concentrations of complementary split mFAP fragments (Fig. 6b).

To assess whether split mFAPs could be used for real-time monitoring of protein–protein association, we pre-incubated equimolar BCLXL_m58 with unfused aBCLXL in excess DHFBI-1T to pre-assemble non-fluorescent BCLXL_m58–aBCLXL complex. Upon addition of equimolar m14–aBCLXL (or buffer as a negative control), the fluorescence increased as m14–aBCLXL competed with unfused aBCLXL for the BCLXL-binding cleft of BCLXL_m58, resulting in assembly of the m14–m58 complex,

**Fig. 4 In vitro characterization of Ca^2+–responsive mFAPs.** a–c DHFBI titration in the absence of Ca^2+ (squares) and presence of Ca^2+ (circles). a For mFAP2b, Ca^2+ does not affect DHFBI binding. b For EF1p2_mFAP2b, binding of Ca^2+ and DHFBI exhibit positive allosterism. c For EF1n_mFAP2b, binding of Ca^2+ and DHFBI exhibit negative allosterism. a–c Normalized fluorescence intensities (n = 1) fit to a sigmoid function using non-linear least squares fitting (lines). d–f Ca^2+ titrations with excess DHFBI concentration compared to protein concentration. d Unnormalized mean fluorescence intensities of mFAP2b demonstrating a lack of Ca^2+-responsiveness. e Normalized mean fluorescence intensities of EF1p2_mFAP2b (with one EF-hand motif, K_d = 2300 µM) demonstrating positive allosterism between DHFBI and Ca^2+ binding. f Ca^2+-responsiveness is dependent on the number of EF-hand motifs inserted into loop7, as exemplified by the normalized mean fluorescence intensities of EF1n_mFAP2b (with one EF-hand motif, K_d = 260 µM), EF2n_mFAP2b (with two EF-hand motifs, K_d = 60 µM), and EF4n_mFAP2b (with four EF-hand motifs, K_d = 7.0 µM), demonstrating negative allosterism between DHFBI and Ca^2+ binding. d–f Error bars represent the s.d. of the mean of three technical replicates. The means (n = 3) are fit to a d constant function, or e sigmoid or f inverse sigmoid function with Hill coefficients of 1, using non-linear least squares fitting (lines) to obtain K_d values (Supplementary Table 2).
which activates the fluorescence of DHFBI-IT (Fig. 6c, d). The reaction evolved analogously for BFL1–aBFL1 and BCL2–aBCL2 cognate-binding partners. Different peak fluorescence fold-changes observed amongst split mFAP fusions to BCLXL–aBCLXL, BCL2–aBCL2, and BFL1–aBFL1 complexes suggest that the molecular geometry of the heterodimer interaction affects the brightness of the assembled β-barrel complex. Fluorescence excitation spectra revealed a prominent peak in fluorescence excitation wavelength at 488 nm upon combining split mFAP fragments compared to buffer negative controls (Supplementary Fig. 21).

To assess whether split mFAPs could be used for real-time monitoring of protein–protein dissociation, we pre-incubated BCL2_m58 with equimolar m14_aBFL1 in excess DHFBI-IT to pre-assemble fluorescent complexes. As the non-cognate BCL2–aBFL1 complex has a dissociation constant ($K_d$) of 320 ± 40 nM, the cognate BCL2–aBCL2 complex has a $K_d$ of 0.8 ± 0.5 nM, and aBFL1 and aBCL2 interact with the same binding clef of BCL2, aBCL2 should outcompete aBFL1 for binding to BCL2 (Fig. 6e). Indeed, titration of aBCL2 into pre-assembled BCL2_m58–m14_aBFL1 complex in excess DHFBI-IT resulted in an aBCL2 concentration-dependent decrease in fluorescence (Fig. 6f). Fluorescence excitation spectra showed the disappearance of the fluorescence excitation peak at 488 nm wavelength consistent with chromophore unbinding and deactivation of fluorescence upon split mFAP fragment disassembly (Supplementary Fig. 21).
Table 2 Fluorescence response to acetylcholine stimulation of HEK293 cells expressing cytosolic Ca^{2+}-responsive mFAPs or GCaMP6f.

| Sensor         | Peak $\Delta F/F_0$ | [DFHBI] (µM) | Regions of interest | Fluorescence response to increased $[Ca^{2+}]$ |
|----------------|---------------------|--------------|---------------------|-----------------------------------------|
| EF2n_mFAP2a    | 0.12 ± 0.092        | 20.0         | 15                  | Negative                                |
| EF4n_mFAP2b    | 0.42 ± 0.14         | 43.3         | 10                  | Negative                                |
| GCaMP6f       | 0.46 ± 0.12         | 43.3         | 15                  | Negative                                |
| EF4n_mFAP2a    | 11 ± 1.8            | 0.00         | 12                  | Positive                                |

The mean and s.d. of the mean of peak absolute values of the fluorescence fold-change (peak $\Delta F/F_0$) upon acetylcholine (ACh) stimulation over the indicated number of regions of interest surrounding single cells (3 technical replicates per sensor; Fig. 1c and Supplementary Fig. 18).

Circular permutation. To further explore the range of possibilities for mFAP-based sensors, we circularly permuted mFAPs (cpmFAPs) using Rosetta and the split points from the four brightest bipartite split mFAPs. The brightest cpmFAP tested, c35-34_mFAP2a_12, has a de novo designed α-helical linker and displayed ~93% of the fluorescence intensity of mFAP2a at equimolar concentration and excess DFHBI-IT (Supplementary Fig. 22). Size-exclusion chromatography with multi-angle light scattering showed c35-34_mFAP2a_12 to be monomeric (Supplementary Fig. 23).

Discussion
We have demonstrated that the functionality and brightness of mFAPs can be readily extended by structure-based design and engineering. It should be emphasized, however, that the engineering of useful fluorogenic sensors based on mFAPs is still in its early days—the mFAPs were first described in September 2018. Currently, existing fluorescent protein-based sensors still have numerous advantages over mFAPs in brightness (e.g., EGFP is ~2.1-fold brighter than the mFAP10–DFHBI-IT complex) for applications involving single molecule localization microscopy, fluorescence spectral diversity, higher Ca^{2+} affinity, and higher fluorescence dynamic range, as in self-labeling chemigenetic indicators. These sophisticated reporters and sensors reflect decades of work by many groups; we hope this report will stimulate exploration of de novo designed fluorescence-activating proteins. With further optimization using both selection and computational design methodologies, there is likely considerable room for improvement of brightness, photo-stability, pH-responsiveness, and Ca^{2+}-responsiveness.

At this stage, the possible advantages of de novo designed mFAP sensors over existing fluorescent protein-based reporters are listed in Table 3. Two notable advantages of split mFAPs over existing split GFP-based approaches for monitoring transient protein–protein interactions are the rapid activation of fluorescence upon assembly of split mFAP fragments that enables tracking of protein–protein association, and the rapid deactivation of fluorescence upon disassembly of split mFAP fragments that enables tracking of protein–protein dissociation (similar to splitFAS with ~2 orders of magnitude lower fragment affinities). mFAPs can activate the fluorescence of DFHBI-derived chromophores with emission spectra in different color ranges, as illustrated by the activation of DFHOB fluorescence by mFAP3. mFAPs can be used as modular fluorogenic optical sensors for detection and quantification of other small-molecules, ions, or proteins by insertion of their respective binding peptides into the loops of mFAPs without circularly permuting the mFAPs, as in construction of Ca^{2+}-responsive mFAPs. The cpmFAPs enable design of modular fluorogenic sensors by fusing analyte binding peptides (e.g., calmodulin and M13) directly to the juxtaposed termini or within β-sheets, as in construction of GCaMP19,20. More generally, as brighter and more photostable fluorogenic compounds are developed, the methodologies described herein should be readily applicable to creating protein-based fluorogenic optical sensors from binders to these compounds.

Methods
Design of brighter and pH-responsive mFAPs. A previously described mFAP2 computational design model was used as a template for manual selection and design of mutable residues using Rosetta33,43 macromolecular modeling software. Guided by the previously generated deep mutational scanning maps of stability and fluorescence of b1IL5, we constructed three mFAP2 mutational variants mFAP2(P50T,S52V), mFAP2(S52T), and mFAP2(P50T,S52V,G100D) (Supplementary Fig. 21), and two mFAP2 variants (V13A and M15F) known as loop7 that were expected to improve the stability of the protein while also aiding crystallization. Circular dichroism in the absence of DFHBI revealed that mFAP2(P50T,S52V), hereafter called mFAP2.1, demonstrated improved stability at pH 2.93 (Supplementary Fig. 1b, c) and higher fluorescence in the presence of DFHBI at pH 3.66 (Supplementary Fig. 1d, e) compared to mFAP2, consistent with improved binding of the phenolic form of DFHBI to the stabilized protein. A minimal site-directed mutagenesis (SDM) library (Supplementary Data 3) was generated at 15 residue positions on mFAP2.1 encoding mutations hypothesized to improve the fluorescence ratio fold-change from low to high pH, increase DFHBI affinity, and reduce conformational flexibility of the loop connecting the seventh and eighth β-strands (known as loop7) juxtaposing the DFHBI-binding pocket.

Fluorescence screening of the SDM library at pH 3.66 and pH 7.36 revealed that the most pH-responsive mutant mFAP2.1(T30P), also known as mFAP2.2, demonstrated ~1.3-fold higher fluorescence ratio fold-change from pH 3.66–7.36 than mFAP2.1 (Supplementary Fig. 1f). Subsequently, two independent combinatorial libraries were generated from mFAP2.2: one at five positions aimed at increasing loop7 rigidity (Supplementary Data 4), and another at eight positions aimed at optimizing hydrophobic packing of residues in the hydrophobic β-barrel core (Supplementary Data 5). The brightest variant from the first library mFAP2.2 (A100E,G101N,N102D,T104H), hereafter known as mFAP2.3, and the brightest variant of the second library mFAP2.2(M27T,W39L,V57A,F93W), hereafter known as mFAP2.4, showed an increase in fluorescence intensity from the phenolate form of DFHBI of ~1.1-fold and ~3.4-fold from mFAP2 at pH 7.36, respectively (Supplementary Fig. 1g, h). The mutations producing mFAP2.3 and mFAP2.4 were combined on one scaffold generating mFAP2.5. A single mutation (V57I) was identified by screening a combinatorial library (Supplementary Data 6) of mutations at 7 positions aimed at packing more methyl groups into the hydrophobic β-barrel core of mFAP2.5. The protein (Fig. 1a, referred to as mFAP2b (“b” for bright)), is ~1.2-fold brighter than mFAP2.1 and ~1.3-fold brighter than mFAP2.4 at neutral pH (Supplementary Fig. 1i). It was demonstrated that mFAP2b had ~10-fold weaker affinity for DFHBI than the initial mFAP2 design. Another combinatorial library (Supplementary Data 7) was generated at five positions of mFAP2b aimed at increasing affinity for the deprotonated state of DFHBI without compromising fluorescence intensity by packing both aromatic and aliphatic residues in the hydrophobic β-barrel core of mFAP2b. Screening for fluorescence intensity of the phenolate state of DFHBI using a relatively low-DFHBI concentration (555 nM) resulted in mFAP2b(V13A,M15F), known as mFAP2a (“a” for affinity). mFAP2a displayed ~1.3-fold brighter fluorescence than mFAP2b at low-DFHBI concentration (Supplementary Fig. 1i). Aiming to further improve packing of methyl groups in the hydrophobic β-barrel core to increase the fluorescence intensity of the phenolate state of DFHBI at neutral pH while accommodating the interesting geometry of the Y71W mutation, a combinatorial library (Supplementary Data 8) was constructed at three positions of mFAP2a, resulting in designs mFAP3 through mFAP5 (Supplementary Table 1). However, these mutants were dimmer and demonstrated lower expression levels than mFAP2b or mFAP2a (Supplementary Table 1), although mFAP3 showed to be the brightest DFHBO-binding variant (mFAP3 with 10.0 µM DFHBO is ~10-fold dimmer than mFAP2b with 10.0 µM DFHBO). In order to increase the fluorescence brightness of mFAP2a with DFHBI-IT, a final combinatorial library was constructed at six positions of mFAP2a (Supplementary Data 9) by mutating aliphatic and aromatic residues in the chromophore-binding pocket of mFAP2a.
Fig. 6 Assembly and disassembly of bipartite split mFAP fragments m14 and m58. a–d Assembly of split mFAP fragments. a Association model in which BCLXL is fused to m58 (BCLXL_m58) (violet cartoon), aBCLXL is fused to m14 (m14_aBCLXL) (yellow cartoon), and fluorescence of DFHBI-1T (green spheres) is activated upon association (arrow) of BCLXL_m58 and m14_aBCLXL. b Normalized fluorescence intensity (points) of BCLXL_m58 titration into a constant m14_aBCLXL concentration in excess DFHBI-1T after reaching equilibrium, showing the fit to a bimolecular association model (line) using non-linear least squares fitting. c Split mFAP competitor pre-incubation model in which fluorescence of DFHBI-1T (green spheres) is activated upon competition (arrow) of m14_aBCLXL with unfused aBCLXL (yellow cartoons) for the BCLXL-binding cleft of BCLXL_m58 (violet cartoon) (the reaction evolves analogously for BFL1-aBFL1 and BCL2-aBCL2 cognate-binding partners). d Temporal evolution of fluorescence fold-change in excess DFHBI-1T upon (n = 1) addition of equimolar m14_aBCLXL (orange points) or buffer (black points) to pre-incubated equimolar BFL1_m58 and aBFL1, addition of equimolar m14_aBCLXL (violet points) or buffer (black points) to pre-incubated equimolar BCL2_m58 and aBCL2, and addition of equimolar m14_aBCLXL (blue points) or buffer (black points) to pre-incubated equimolar BCLXL_m58 and aBCLXL, showing the fits to a monophasic exponential function (lines) using non-linear least squares fitting. e, f Disassembly of split mFAP fragments. e Pre-assembled split mFAP competition model in which BCL2 is fused to m58 (BCL2_m58) (violet cartoon), aBFL1 is fused to m14 (m14_aBFL1) (orange cartoon), and fluorescence of DFHBI-1T (green spheres) is activated before unfused aBCL2 (yellow cartoon) competes with m14_aBFL1 for the BCL2-binding cleft of BCL2_m58 (arrow), resulting in fluorescence deactivation. f Temporal evolution of fluorescence fold-change in excess DFHBI-1T of pre-incubated equimolar BCL2_m58 and m14_aBFL1 at 2.00 μM final concentrations with unfused aBCL2 titrated in at (n = 1) 0 μM (black points), 4.00 μM (green points), and 10.0 μM (red points) final concentrations, showing the fits to a monophasic exponential function (lines) using non-linear least squares fitting. Source data is available for Fig. 6b, d, f.

**Design of chromophore-selective mFAPs.** Computational modeling of DFHBI (Fig. 1b) into the binding pocket of mFAP2b (Fig. 1f) and mFAP2a (Fig. 1h) using Rosetta34 macromolecular modeling software showed that the mutations V13A and M15F resulted in a void in the binding pocket of mFAP2a. It was hypothesized that a commercially available derivative of the DFHBI chromophore with a trifluoromethyl group, DFHBI-1T4, could pack into the void without causing steric clashes. Computational modeling of DFHBI-1T in the pocket of mFAP2a (Fig. 1i) demonstrated good protein–chromophore shape complementarity, whereas DFHBI-1T modeled into the mFAP2b pocket (Fig. 1g) resulted in steric clashes. Studying the fluorescence of mFAP2a and mFAP2b in the presence of DFHBI-1T experimentally validated this (Fig. 1d, e). Relative fluorescence intensities and binding affinities of DFHBI, DFHBI-1T, and DFHO for selected mFAP variants were then measured at neutral pH (Supplementary Table 1). The fluorescence intensity of mFAP2a with DFHBI-1T was improved upon screening the “IN2” combinatorial library, resulting in mFAP10.

**Design of extended loop library.** β-hairpin loop fragments from the RCSB Protein Data Bank [www.rcsb.org] were used to manually curate custom β-barrel loop fragment databases. RosettaRemodel36 was used to fix the β-hairpin loop termini to loops 1, 3, 5, and 7 of the de novo β-barrel scaffolds b11 and b321, picking 3-mer and 9-mer fragments from the custom β-barrel loop fragment databases, from which 2226 designs with successfully closed loops were generated.
**Table 3: Potential advantages of de novo designed mFAP sensors over existing fluorescent protein-based reporters and sensors.**

| Biophysical property | Advantage |
|----------------------|-----------|
| Size                 | Smaller size than GFP-based, DiBPs and FAST-based reporters (Supplementary Data 1). |
| Photostability       | Higher photostability over AgCfp1 in fixed mammalian cells (Fig. 2). |
| Reversibility        | Spatiotemporal control over fluorescence via on-demand labeling protocols (Supplementary Fig. 4), unlike intrinsically fluorescent proteins and like DiBPs and FAST-based reporters. |
| Ratiometric fluorescence | Higher ratiometric fluorescence dynamic range across the physiologically relevant pH range than pHRed and pHluorin for imaging Ca²⁺ transients in high-Ca²⁺ concentration environments (Fig. 3f and Supplementary Table 2). |
| Dynamic range of Low-Ca²⁺ affinity | Lower Ca²⁺ affinity than existing fluorescent protein-based Ca²⁺ sensors such as GCaMP3 and CatchER for imaging Ca²⁺ transients in high-Ca²⁺ concentration environments (Fig. 5f and Supplementary Table 2). |
| Rapid response of split mFAPs | Rapid change in fluorescence intensity upon split mFAP fragment assembly (Fig. 6d), similar to splitFASP in not requiring chrophore maturation like split GFP. |
| Reversibility of split mFAPs | Reversible fluorescence of split mFAPs, unlike split GFP, and similar to splitFASP but with lower fragment affinities (Fig. 6f and Supplementary Fig. 20). |

Loop coordinates were extracted as .pdb files and used as templates to generate Rosetta blueprint files specifying amino acid type, secondary structure, and ABEGO type of loops to be rebuilt onto loops 1, 3, 5, and 7 of a computational mFAP model of mFAP2b resulting in 8904 Rosetta blueprint files. For each blueprint file, a RosettaScript XML script (Supplementary Note 2) was used to graft the loop onto mFAP2p with a centroid energy function followed by Monte Carlo sampling of protein side-chain repacking and protein side-chain and backbone minimization steps in a fully Cartesian coordinate energy function. Seventeen thousand and four hundred forty-eight resulting designs were filtered for the following computational protein design metrics (as scored from the XML script described in Supplementary Note 2): geometry = 1; total scored score ≤ −3.7271; residues ≤ −1.2729; psia ≥ 0.755044; bnn_sc heavy ≤ 2; bnn_bb heavy ≤ 2; interface ≤ −38.375; SC ≥ 0.734076; paa_pp ≤ −40.8947; and omega ≤ 2.8757. Only 959 designs with extended loops grafted onto loops 1, 3, 5, and 7 of mFAP2b passed the filter criteria for experimental testing (Supplementary Data 1).

**Design of Ca²⁺-responsive mFAPs.** The mFAP2p loop7 sequence and the five extended loop7 sequences shown to confer fluorescence (Supplementary Fig. 1c) were sampled as linkers for grafting the sequence of one EF-hand motif from Protein Data Bank (PDB) accession code 1NKP onto loop7 of mFAP2b. An in-house script (Supplementary Note 3) was written to prune the experimentally validated extended loop7 sequences one residue at a time keeping up to four residues on the N-terminal and C-terminal linkers relative to the grafted EF-hand motif, optionally adding an additional glycine residue on the N-terminal linker and optionally adding an additional glycine or proline residue on the C-terminal linker. This combinatorial library (Supplementary Data 12) had a theoretical diversity of 385 linkers. The linkers resulting in negatively allosteric Ca²⁺-responsive mFAPs containing one EF-hand motif were combinatorially sampled to act as linkers for grafting two EF-hand motifs from PDB accession code 1FW4 onto loop7 of mFAP2b, where the N-terminal helix of PDB accession code 1FW4 was truncated up to homologous residues on successfully grafted single EF-hand motif designs. This combinatorial library (Supplementary Data 13) had a theoretical diversity of 1140 linkers. The linkers resulting in positively and negatively allosteric Ca²⁺-responsive mFAPs containing two EF-hand motifs were combinatorially sampled to act as linkers for grafting four EF-hand motifs from PDB accession code 1PRW onto loop7 of mFAP2b, where the N-terminal helix of PDB accession code 1FW4 was truncated up to homologous residues on successfully grafted single EF-hand motif designs. This combinatorial library (Supplementary Data 14) had a theoretical diversity of 25 linkers.

**Synthetic DNA construction.** For combinatorial libraries, oligonucleotides with degenerate codons encoding desired mFAP sequences were designed using SwiftLab. To reverse engineering forward a combinatorial library of oligonucleotides with degenerate and non-degenerate codons spanning the mFAP gene of interest were synthesized (IDT DNA). Oligonucleotides spanning identical gene regions were pooled at equimolar ratios relative to the theoretical amino acid diversity encoded by each gene region. Full-length genes were constructed using assembly polymerase chain reaction (PCR) with Phusion polymerase (NEB). For the extended loop library, the loop1, loop3, and loop7 libraries were assembled separately, and the concentrations of assembly PCR products consisting of full-length genes were quantified on a NanoDrop 8000 (Thermo Scientific) and the three libraries pooled in quantities proportional to the theoretical library diversities of assembly PCR products. Successfully assembled full-length genes, as well as synthetic gBlock (IDT DNA) oligonucleotides encoding full-length protein sequences, with 5’ and 3’ flanking vector backbone sequences were sub-cloned into the pet15b vector (Novagen) or the pcDNA5/FRT/TO vector (ThermoFisher Scientific) using Gibson assembly. The mFAP2.2 loop library was constructed via Gibson assembly of polyacrylamide gel electrophoresis (PAGE)–purified duplex oligonucleotides into pet15b–mFAP2.2 linear vector DNA. The full-length genes encoding pHRed and pHluorin were synthesized and cloned into the pet29b (+) vector (IDT DNA). Cloned DNA constructs were transformed into Lemo21(DES) competent E. coli (NEB) and plated onto lysogeny broth (LB) agar plates supplemented with 50 μg.mL⁻¹ carbenicillin or 0.100–10.0 μg.mL⁻¹ kanamycin.

**Screening libraries.** The number of E. coli colonies picked for functional screening was approximately 4-fold the theoretical diversity of each library. E. coli colonies were inoculated into 1.00 mL of LB media supplemented with 50.0 μg.mL⁻¹ carbenicillin in Nunc 2.0 mL DeepWell 96-well plates (Thermo Scientific), and were grown at 37 °C shaking at 1200 rpm overnight. Twenty-five microliters of these clones were inoculated into 1.00 mL of fresh LB media supplemented with 50.0 μg.mL⁻¹ carbenicillin, grown at 37 °C and 1200 rpm for 3–4 h, then 0.5 μM isopropl β-D-thiogalactopyranoside (IPTG) final concentration was added to each well, and protein expression induced for 4 h at 37 °C. Cells were pelleted at 2272 × g for 2–5 min and pellets were resuspended in 50.0 μL of lysis buffer #1 (25.0 μM Tris, 300 μM NaCl, 2.00 μM imidazole, pH 8.0) supplemented with 1.00 mg.mL⁻¹ PMSE, a small amount of deoxyriboonucleic acid (DNase I) from bovine pancreas (Sigma Aldrich), and 2.00 mg.mL⁻¹ lysozyme from chicken egg white (Sigma) for lysis. Crude lysates were vigorously shaken at 25–37 °C for 12–48 h, then clarified by centrifugation. Clarified lysates were assayed on a Synergy Neo2 hybrid multi-mode reader (BioTek) in 96-well non-binding surface microplates (Corning 3650) with Gen5 (version 3.03.14) software.

For each clone encoding a β-barrel core variant, loop7 variant, or extended loop variant, 15.0 μL of clarified lysate was combined with 185.0 μL of NaHPO₄-citrate (pH 7.36 or pH 3.66) buffer supplemented with 150 μM NaCl and either 1.08 μM DFBHI (Lucerna), 555 nM DFBHI (Lucerna), or 250 μM DFBHI-1T (Lucerna). NaHPO₄-citrate buffer was made from 200 mM NaHPO₄ and 100 mM citrate stock solutions, and final pH was adjusted using hydrochloric acid (HCl) or sodium hydroxide (NaOH). DFBHI and DFBHI-1T stock solutions were 2.00 μM in 22.8 μM Tris (pH 8.0), 95.0 mM NaCl and 5% dimethyl sulfoxide (DMSO). Clones that demonstrated fluorescence were Sanger sequenced via colony PCR of overnight cultures, and the brightest designs or designs with highest fluorescence.
fold-change across pH 7.36–3.66 were further characterized with large-scale protein purification. For each clone, recoding one or more EF-hand motifs grafted onto β-barrel loop 7, 15.0 µL of lysate was combined with 185.0 µL of either 2.0 mM CaCl₂ (Sigma Aldrich), 25.0 mM Tris (pH 8.00), 100 mM NaCl, 1.08 µM DFHBI or 2.00 mM EGTA (Sigma Aldrich), 25.0 mM Tris (pH 8.00), 100 mM NaCl, 1.08 µM DFHBI. Clones that demonstrated greater than an approximately twofold change in fluorescence intensity between CaCl₂ and EGTA conditions were sequenced, and the designs demonstrating the highest fold-change in fluorescence intensity between CaCl₂ and EGTA conditions were further characterized with large-scale protein purification.

Fluorescence intensity assays. To measure fluorescence intensities of the phenol-nal type of chromophores, fluorescence was measured on a Synergy Neo2 hybrid multi-mode reader (BioTek) in black polystyrene, non-reflective 96-well microplates (Corning 3650). Fluorescence intensity was measured in triplicate by exciting at λ_{ex} = 484 nm and measuring fluorescence emission at λ_{em} = 505 nm (or λ_{em} = 511 nm for clones harboring the W27 indole ring) (Supplementary Fig. 22b). The W27 mutation redshifts the peak emission wavelength from λ_{em} = 505 nm to λ_{em} = 511 nm, presumably due to the W27 indole ring donating a hydro bond to the imidazolone moiety of the deprotonated state of DFHBI, which stabilizes the chromophore conjugated π-electron system in the excited state causing the redshift in emission. Fifty microliters of small-scale purified protein was combined with 185.0 µL of Na₂HPO₄-citrate (pH 7.36) buffer supplemented with 100 mM NaCl and 108.0 mM DFHBI for a final concentration of 100 nM DFHBI. In measuring the excitation spectra from λ_{em} = 525 nm of each clone in triplicate (Supplementary Fig. 1), 30.0 µL of large-scale purified protein was combined with 170.0 µL of 25.0 mM Tris (pH 8.00) supplemented with 100 mM NaCl and 914.0 nM DFHBI for a final concentration of 7.77 µM protein and 777 nM DFHBI. Fluorescence intensity at λ_{ex} = 484 nm and λ_{em} = 505 nm of each clone in triplicate (Supplementary Fig. 1), 30.0 µL of large-scale purified protein was combined with 170.0 µL of 25.0 mM Tris (pH 8.00) supplemented with 100 mM NaCl and 653.0 nM DFHBI for a final concentration of 5.55 µM protein and 555 nM DFHBI. In measuring fluorescence intensity at λ_{em} = 468 nm and λ_{em} = 530 nm of each clone in technical triplicate (Supplementary Fig. 1k), 24.0 µL of 35.4 µM large-scale purified protein was combined with 1.00 µL of 1.25 µM DFHBI-1T (Lucerna) (from 2.5% DMSO and 97.5% high-salt Tev cleavage buffer) was added to wells containing pHRed or pHluorin2. Blank wells for background subtraction were prepared identically except using 20.0 µL of high-salt Tev cleavage buffer instead of purified protein. Wells were excited at λ_{ex} = 387 nm or λ_{ex} = 484 nm and fluorescence emission measured at λ_{em} = 501 nm or λ_{em} = 505 nm, respectively. Following background subtraction from the mean endpoint fluorescence of buffer controls, the fluorescence ratio fold-change from pH 3.61–7.36 was calculated as:

\[ \text{Fluorescence Ratio Fold-Change (pH 3.61-7.34) =} \]

\[ \frac{I(\text{pH} 3.61 - \text{pH} 7.34)}{I(\text{pH} 3.61)} \]

where \( I(\text{pH} 3.61) \) is the endpoint fluorescence measurement at the subscripted pH value and superscripted fluorescence excitation (λ_{ex}) and fluorescence emission (λ_{em}) wavelengths.

In measuring pH-dependent fluorescence signals (Fig. 3d–i), Na₂HPO₄-citrate buffer supplemented with 150 mM NaCl at each unique pH value were prepared via mixing various volumes of 100 mM citric acid (Sigma Aldrich), 200 mM Na₂HPO₄ (Sigma Aldrich), Na₂HPO₄ buffer and final pHs quantified with a Basic pH meter (Fisher Scientific). pHRed and pHluorin2 were produced via large-scale protein purification, 6xHis-tag removal and size-exclusion chromatography (SEC) purification, and mFAP_P and mFAP2b were produced via large-scale protein purification and SEC purification. pH-dependent fluorescence was measured at 562 nm for protein concentration in mFAP2b, mFAP_P, pHRed, and 170.0 mM final concentration for pHluorin2, at each pH in technical triplicate in 200.0 µL final volumes per well. To prevent pH fluctuations upon addition of protein and DFHBI, 193.0 µL of Na₂HPO₄-citrate buffers supplemented with 150 mM NaCl at each pH was aliquoted per well, 4.00 µL of purified protein was aliquoted per well and 1.00 µL of 1.00 mM DFHBI (in 2.5% DMSO and 97.5% high-salt Tev cleavage buffer) was added to wells containing mFAP_P, pHRed, whereas 1.00 µL of chromophore buffer (2.5% DMSO and 97.5% high-salt Tev cleavage buffer) was added to wells containing pHRed or pHluorin2. Blank wells for background subtraction for mFAP2b, mFAP_P, pHRed and pHluorin2 were prepared identically, respectively, except adding 4.00 µL of high-salt Tev cleavage buffer instead of purified protein. In measuring fluorescence excitation spectra at each pH (Fig. 3d), excitation wavelengths were set to the range λ_{ex} = 300–530 nm and fluorescence emission measured at λ_{em} = 562 nm. In measuring pH-dependent fluorescence excitation spectra at pH 3.76 and pH 7.34 (Fig. 3f, g), excitation spectra were measured using excitation wavelengths in the range λ_{ex} = 300–540 nm and emission wavelength λ_{em} = 592 nm. In measuring fluorescence excitation spectra at two pH values (Fig. 3e), emission spectra for the blueshifted excitation peak was measured at pH 3.63 using excitation wavelength λ_{ex} = 379 nm and emission wavelengths in the range λ_{em} = 460–700 nm, and the emission spectra for the redshifted excitation peak was measured at pH 8.38 using excitation wavelength λ_{ex} = 430 nm and emission wavelengths in the range λ_{em} = 460–700 nm. In measuring fluorescence from both the blueshifted and redshifted excitation peaks (Fig. 3h, i), for mFAP_P, pH the fluorescence excitation wavelengths were λ_{ex} = 379 nm and λ_{ex} = 463 nm and fluorescence emission wavelengths were λ_{em} = 409 nm, respectively, whereas for pHRed the fluorescence excitation wavelengths were λ_{ex} = 440 nm and λ_{em} = 575 nm and fluorescence emission wavelengths were both λ_{em} = 635 nm, and for
pHluorin2 the fluorescence excitation wavelengths were $\lambda_{\text{ex}} = 405$ nm and $\lambda_{\text{em}} = 483$ nm and fluorescence emission wavelengths were both $\lambda_{\text{em}} = 535$ nm. In Fig. 3i, for the mFAP pH-DFFHI complex, pHRed, and pHluorin2 the ratiometric fluorescence ($F_{\text{ratio}}$) is calculated from the background-subtracted, unnormalized fluorescence measurements using fluorescence emission from the redshifted excitation peak ($F_{\text{em},\text{ratio}}$) as the numerator and fluorescence emission from the blueshifted excitation peak ($F_{\text{em},\text{ratio}}$) as the denominator:

$$F_{\text{ratio}} = \frac{F_{\text{em},\text{ratio}}}{F_{\text{em},\text{ratio}}}$$

(3)

In Fig. 3i, mean ratiometric fluorescence values are fit to continuous logistic functions with the formula $F_{\text{ratio}} = \frac{1}{1 + e^{-\left(\frac{\lambda_{\text{em}}}{K_{d}}\right)}}$ for the mFAP pH-DFFHI complex, $F_{\text{ratio}}$ was measured for pHRed, and $F_{\text{ratio}}$ was measured for pHluorin2 using non-linear least squares fitting, which serve as continuous calibration curves for real-time quantification of pH.[49]

In measuring the fluorescence (phenolic and deprotonated (phenolate) DFFHI affinities with mFAP pH (Supplementary Fig. 5c), fluorescence endpoints were measured on a Synergy Neo2 hybrid multi-mode reader (BioTek) in flat bottom, black polystyrene, non-binding surface 96-well microplates (Corning 3650). mFAP pH was produced by large-scale protein purification[49] and aliquoted to 500 nM final volumes at 500 nM final concentration in seven serial dilutions ($\lambda_{\text{dilution factor}}$ of DFFHI starting from 10.0 μM DFFHI final concentration, including an eighth condition without chromophore, in Na$_2$HPO$_4$-citrate buffer supplemented with 143 mM NaCl final concentration at either pH 3.61 or pH 7.34. For pH 3.61 fluorescence was excited at $\lambda_{\text{em}} = 397$ nm and fluorescence emission measured at $\lambda_{\text{em}} = 483$ nm and fluorescence emission was measured at $\lambda_{\text{em}} = 536$ nm. At each pH, background fluorescence endpoints of wells with identical chromophore concentrations but purified protein replaced with an identical volume of buffer were measured, and fluorescence endpoints subtracted from those measured with protein. Background-subtracted data were normalized from 0 to 1 and fit to a single-binding site isotherm function using non-linear least squares fitting to obtain the reported $K_d$ values, which were less than the protein concentrations tested.

**Chromophore titrations.** Fluorescence endpoints were measured on a Synergy Neo2 hybrid multi-mode reader (BioTek) in flat bottom, black polystyrene, non-binding surface 96-well microplates (Corning 3650). In measuring chromophore-binding affinities (Fig. 1d, e), mFAP2, mFAP2a, and mFAP10 were produced by large-scale protein purification[49] and aliquoted to 500 nM final volumes of Chelex 100 pre-treated high-salt Tev cleavage buffer) or high-salt Tev cleavage buffer, along with ten serial dilutions (Fig. 4a – 5, f, i, t, 11c, 1f, 13c, i, f, 1, i, t, c, i, t, i, f) of wells with identical chromophore concentrations lacking protein (substituted for equivalent volumes of high-salt Tev cleavage buffer) were measured and subtracted from protein measurements prior to normalization.

**Ca$^{2+}$-responsive mFAP Ca$^{2+}$ titrations.** In measuring Ca$^{2+}$ affinity of mFAPs on a Synergy Neo2 hybrid multi-mode reader (Fig. 4d, f and Supplementary Fig. 13c), large-scale purified proteins were substituted for equivalent volumes of high-salt Tev cleavage buffer) were measured and subtracted from protein measurements prior to normalization.

**Ca$^{2+}$-responsive mFAP fluorescence spectra.** Fluorescence spectra of Ca$^{2+}$-responsive mFAPs were measured on a Synergy Neo2 hybrid multi-mode reader (BioTek) in flat bottom, black polystyrene, non-binding surface 96-well microplates (Corning 3650). In measuring Ca$^{2+}$ affinity of mFAPs on a Synergy Neo2 hybrid multi-mode reader (Fig. 4d, f and Supplementary Fig. 13c), large-scale purified proteins were substituted for equivalent volumes of high-salt Tev cleavage buffer) were measured and subtracted from protein measurements prior to normalization.

**Ca$^{2+}$-responsive mFAP DHFII titrations.** In measuring Ca$^{2+}$-dependent DHFII affinity of Ca$^{2+}$-responsive mFAPs on a Synergy Neo2 hybrid multi-mode reader (Fig. 4d, f and Supplementary Figs. 10a, b, c, d, e, g, h, 12a, b, d, e, g, h, 16a), large-scale purified proteins[49] were aliquoted to a final concentration of 500 nM in either 450 mM CaCl$_2$ (Sigma Aldrich) (prepared in high-salt Tev cleavage buffer) or high-salt Tev cleavage buffer, along with ten serial dilutions ($\lambda_{\text{dilution factor}}$) of DHFII starting from 316 μM DHFII including an eleventh condition without chromophore. Final volumes were 25.0 μL in flat bottom, black polystyrene, non-binding surface 96-well microplates (Corning 3686) or 200 μL in flat bottom, black polystyrene, non-binding surface 96-well microplates (Corning 3650). Fluorescence endpoints were measured using excitation wavelength $\lambda_{\text{ex}} = 488$ nm and emission wavelength $\lambda_{\text{em}} = 510$ nm. Background fluorescence endpoints of wells with identical chromophore concentrations but purified protein replaced with a small amount of Chelex 100 was prepared and mixed at room temperature overnight, and a 2.00 mM DHFII stock solution (in 5% DMSO and 95% high-salt Tev cleavage buffer) with a small amount of Chelex 100 pre-treated DHFII stock (in high-salt Tev cleavage buffer) including a twelfth condition without DHFII (Fig. 4c, f, i, t, 11c, f, 13c, i, f, 1, i, t, c, i, t, i, f) were measured and subtracted from protein measurements prior to normalization.

**Ca$^{2+}$-responsive mFAP fluorescence spectra.** Fluorescence spectra of Ca$^{2+}$-responsive mFAPs were measured on a Synergy Neo2 hybrid multi-mode reader (BioTek) in flat bottom, black polystyrene, non-binding surface 96-well microplates (Corning 3650). In measuring Ca$^{2+}$ affinity of mFAPs on a Synergy Neo2 hybrid multi-mode reader (Fig. 4d, f and Supplementary Fig. 13c), large-scale purified proteins were substituted for equivalent volumes of high-salt Tev cleavage buffer) were measured and subtracted from protein measurements prior to normalization.

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EF1p_mFAP2b and 600 nM DFHBI (Supplementary Fig. 9b); 51.4 µM EF1p2_mFAP2b and 5.40 µM DFHBI (Supplementary Fig. 9c); 900 nM EF1p2_mFAP2b and 700 nM DFHBI (Supplementary Fig. 9d); 47.4 µM EF1p3_mFAP2b and 70.0 µM DFHBI (Supplementary Fig. 9e); 30.6 µM EF1n_mFAP2b and 1.30 µM DFHBI (Supplementary Fig. 9f); 47.7 µM EF1n2_mFAP2b and 13.8 µM DFHBI (Supplementary Fig. 9g); 30.6 µM EF1n3_mFAP2b and 2.40 µM DFHBI (Supplementary Fig. 9h); 125 µM EF3n1_mFAP2b and 1.60 µM DFHBI (Supplementary Fig. 9i); 15.3 µM EF3n3_mFAP2b and 700 nM DFHBI (Supplementary Fig. 9j); 83.5 µM EF2n_mFAP2b and 5.00 µM DFHBI (Supplementary Fig. 9m); 6.90 µM EF2n_mFAP2b and 1.20 µM DFHBI (Supplementary Fig. 9n); 7.10 µM EF2n2_mFAP2b and 7.30 µM DFHBI (Supplementary Fig. 9o); 1.60 µM EF2n2_mFAP2b and 1.30 µM DFHBI (Supplementary Fig. 9p); 21.1 µM EF2n3_mFAP2b and 13.7 µM DFHBI (Supplementary Fig. 9q); 1.50 µM EF3n3_mFAP2b and 4.60 µM DFHBI (Supplementary Fig. 9r); 14.7 µM EF4n_mFAP2b and 14.5 µM DFHBI (Supplementary Fig. 9s); or 5.60 µM EF4n_mFAP2b and 31.4 µM DFHBI (Supplementary Fig. 9t).

**EF2n_mFAP2b DFHBI titration versus Ca2+ titration heatmap.** EF2n_mFAP2b was produced by large-scale protein purification and aliquoted to a final concentration of 500 nM in eleven serial dilutions of CaCl2 starting from 4.50 mM CaCl2 along columns, and eight serial dilutions of CaCl2 starting from 0.10 mM in rows. Fluorescence endpoints were measured using excitation wavelength λexc = 484 nm and emission wavelength λem = 508 nm. Raw data (without background subtraction) were normalized from 0 to 1 and reported (Supplementary Fig. 14c, top row).

**Split mFAP titration assays.** To measure fluorescence intensities in a protein fragment complementation assay (Supplementary Fig. 20b), fluorescence was measured on a Synergy Neo2 hybrid multi-mode reader (BioTek) in flat bottom, black polystyrene, non-binding surface 96-well microplates (Corning 3686). In technical triplicate, 12.0 µL of each split mFAP fragment covalently fused to maltose-binding protein (MBP) was mixed to an equimolar concentration supplemented with 1.00 µL of 1.25 mM DFHBI-1T (Lucerna) at 25.0 µL final volume per well. Fluorescence endpoints were measured using excitation wavelength λexc = 478 nm and emission wavelength λem = 520 nm. In technical triplicate, background fluorescence endpoints of wells with identical chromophore concentrations lacking protein (substituted for equivalent volumes of high-salt Tev cleavage buffer) were measured, and the mean fluorescence endpoints were subtracted from the mean fluorescence endpoints of samples containing protein.

Split mFAP fragment affinities (Supplementary Fig. 20d–g) were estimated by preparing MBP-tagged split mFAP fragments by large-scale protein purification in high-salt Tev cleavage buffer, with 25.0 µM DFHBI-1T final concentration at 28.0 µL final volumes per well. Fluorescence endpoints were measured using excitation wavelength λexc = 486 nm and emission wavelength λem = 530 nm. For each titration, the fluorescence intensity of the condition without titrant was subtracted from the fluorescence intensities of samples containing titrant, then the background-subtracted data was normalized from 0 to 1. In collecting fluorescence excitation and emission spectra (Supplementary Fig. 20c), the conditions with the highest protein concentrations and 25.0 µM DFHBI-1T were used. Excitation spectra were measured using excitation wavelengths in the range λexc = 384-676 nm and emission wavelengths in the range λem = 500-660 nm. Fluorescence excitation and emission spectra of conditions without the addition of the complementary split mFAP fragment were measured and used for background subtraction at the corresponding wavelengths.

For titrating BCLXL_m58 into m14_aBCLXL (Fig. 6b), m14_aBCLXL and BCLXL_m58 were prepared by large-scale protein purification in high-salt Tev cleavage buffer. Fluorescence endpoints were measured on a Synergy Neo2 hybrid multi-mode reader (BioTek) in flat bottom, black polystyrene, non-binding surface 384-well microplates (Corning 4514) using fluorescence excitation wavelength λexc = 486 nm and fluorescence emission wavelength λem = 530 nm. Nine wells each with 3.90 µL of 19.6 µM m14_aBCLXL and 2.20 µL of 114.0 µM DFHBI-1T were prepared, and 3.90 µL of each high-salt Tev cleavage buffer or BCLXL_m58 was aliquoted per well to reach final concentrations of 0.µM, 251 nM, 501 nM, 1.00 µM, 2.01 µM, 8.02 µM, 16.0 µM, 32.1 µM, 64.2 µM, and 128 µM. Fluorescence endpoints were measured on a Synergy Neo2 hybrid multi-mode reader using excitation wavelength λexc = 480 nm and fluorescence emission wavelength λem = 530 nm. Background-subtracted fluorescence intensities were measured after 2847 s of double orbital shaking in the dark. Fluorescence from the 0 µM BCLXL_m58 condition was subtracted from each condition, and the background-subtracted fluorescence in relative fluorescence units (RFU), F, was normalized by the formula:

\[
\text{Norm Fluorescence} = \frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}},
\]

where \(F_{\text{min}}\) (RFU) was the minimum fluorescence intensity, and \(F_{\text{max}}\) (RFU) was the fit to a constant function using non-linear least squares fitting of the fluorescence intensities of the four highest BCLXL_m58 concentrations. Using a bilinear association model:

\[
\text{BCLXL}_m58 + m14_aBCLXL = \frac{k_1}{\text{BCLXL}_m58} \frac{\text{BCLXL}_m58}{m14_aBCLXL} = K_{\text{d}1}
\]

it can be shown that:

\[
\frac{\text{BCLXL}_m58 + m14_aBCLXL}{\text{BCLXL}_m58} = \frac{k_1}{K_{\text{d}1} + k_1} \left( \frac{\text{BCLXL}_m58}{m14_aBCLXL} + K_{\text{d}1} \right)
\]

\[
\text{BCLXL}_m58 + m14_aBCLXL + K_{\text{d}1}
\]

where \(k_1 = K_{\text{d}1} + k_1\).

The theoretical maximum fluorescent complex concentration, \(\text{BCLXL}_m58 + m14_aBCLXL\), is reached at excess \(\text{BCLXL}_m58\), taken at \(\text{BCLXL}_m58_{\text{extr}} = 10.0\). Similarly, it can also be shown that:

\[
\frac{\text{BCLXL}_m58 + m14_aBCLXL}{\text{BCLXL}_m58} = \frac{k_1}{K_{\text{d}1} + k_1} \left( \frac{\text{BCLXL}_m58}{m14_aBCLXL} + K_{\text{d}1} \right)
\]

As fluorescent complexes only form with the folded fraction of m14_aBCLXL, \(P_{\text{folded}}\), under the condition that \(\text{m14_aBCLXL}_{\text{folded}} = P_{\text{folded}} \times 7.64 \mu M\) in the \(\text{m14_aBCLXL}_{\text{local}}\) fold it was fit to a free parameter to the normalized fluorescence intensity with the formula:

\[
\frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} = \frac{[\text{BCLXL}_m58 + m14_aBCLXL]}{[\text{BCLXL}_m58 + m14_aBCLXL]_{\text{max}}}
\]

where:

\[
K_{\text{d}1} = K_{\text{d}1_{\text{local}}} = \frac{\text{m14_aBCLXL}_{\text{local}}}{[\text{BCLXL}_m58 + m14_aBCLXL]_{\text{local}}} = 1.23 \times 10^{-13} M
\]

because the aBCLXL domain of m14_aBCLXL associates with the binding cleft of the BCLXL domain of BCLXL_m58 with the previously reported BCLXL–aBCLXL thermodynamic dissociation constant of \(K_{\text{d}1} = 22.0 \times 10^{-9} M_{\text{ex}}\) at 25.0 µM DFHBI-1T (Supplementary Fig. 20e), under an approximation that the BCLXL_m58–m14_aBCLXL interaction energy comprises only the BCLXL–aBCLXL and m14–m58 interaction energies:

\[
\Delta G_{\text{BCLXL-m58-m14_aBCLXL}} = \Delta G_{\text{BCLXL-aBCLXL}} + \Delta G_{\text{m14-m58}}
\]

where \(\Delta G\) is the change in Gibbs free energy upon the superscripted protein–protein interaction in 25.0 µM DFHBI-1T. Non-linear least squares fitting yielded \(P_{\text{folded}} = 0.532 \pm 0.0160\), and therefore the reported

\[\text{m14_aBCLXL}_{\text{folded}} = 4.06 \mu M\]
Chelex 100 was added to the protein sample and nutated overnight at 4 °C. A stock was monitored at 220 nm wavelength from 25 °C to 95 °C at 2 °C evenly spaced denaturations were performed with 0.500 mg mL\(^{-1}\) of Chelex 100 overnight. Far-ultraviolet CD wavelength scans and thermal emission spectra of the protein without calcium or magnesium (DPBS) (Thermo Scientific) were measured in DPBS (pH 7.40) at 4.00 mL. Final sample conditions were 25.0 µM DFHBI-1T, 2.00 μM EF4n_mFAP2b (Supplementary Fig. 8) was purified by large-scale protein purification and SEC purification in phosphate-buffered saline (PBS) (25.0 mM phosphate, 150 mM NaCl, pH 7.40), and far-ultraviolet CD wavelength scans recorded from 195 nm to 260 nm. mFAP2b was measured at 0.441 mg mL\(^{-1}\) and mFAP2.1 at 0.500 mg mL\(^{-1}\) in NaHPO\(_4\)-citrate buffer with pH adjusted to 7.75, 3.96, or 2.93 using NaOH and HCl. The reported data was background-subtracted using buffer only controls (Supplementary Fig. 1b, c).

**Circular dichroism.** Circular dichroism (CD) measurements were recorded at 25 °C in a 1 mm cuvette on an AVIV model 420 CD spectrometer (Biomedical, Inc.). mFAP2 and mFAP2.1 were purified by large-scale protein purification and SEC purification in phosphate-buffered saline (PBS) (25.0 mM phosphate, 150 mM NaCl, pH 7.40), and diazylated overnight into DPBS that was adjusted to pH 7.40 using NaOH. Chromophore purifications of DHBI (Lucerna) and DHFBI-1T (Lucerne) were dissolved to 20.0 mM in 100% DMSO, and diluted in DPBS (pH 7.40) to measure absorbances on a Jasco V-750 spectrophotometer at peak absorbance wavelengths (417 nm for DHFBI and 422 nm for DHFBI-1T). Following background subtraction of identical buffer without chromophore, Beer’s Law was used to calculate the molar chromophore concentrations of the stock solutions using previously reported extinction coefficients\(^5\).

**Preparation of protein–chromophore complexes.** For quantum yield measurements, 1.00 µM, 836 nm, or 919 nM chromophore solutions in DPBS (pH 7.40) at 4.00 mL final volumes were prepared for the following eight conditions: DHFBI only, DHFBI-1T only; 43.5 µM 6xHis-mFAP20 with DHFBI 43.5 µM 6xHis-mFAP20 with DHFBI-1T, 134 µM 6xHis-mFAP20 with DHFBI-1T, 206 µM 6xHis-mFAP20 with DHFBI and 206 µM 6xHis-mFAP20 with DHFBI-1T. Extinction coefficients: Absorbance spectra of protein–chromophore complexes were fit to measured values using a Perkin-Elmer Luminescence Spectrophotometer (1 nm interval, 800 nm min\(^{-1}\)). The extinction coefficients were then calculated using Beer’s Law:

\[ A = e \cdot c \cdot b \cdot c, \]

where \(A\) is absorbance, \(e\) is extinction coefficient, \(b\) is path length (1 cm), and \(c\) is concentration (1.00 µM, 836 nm, or 919 nM).

Excitation and emission wavelengths in the range \(430 \text{ nm} \leq \lambda_{\text{ex}} \leq 478 \text{ nm}\) and emission wavelengths in the range \(438 \text{ nm} \leq \lambda_{\text{em}} \leq 530 \text{ nm}\) were measured by Eq. (11) where

\[ F(\lambda_{\text{ex}}) = \frac{1 - 10^{-\varepsilon_0(\lambda_{\text{ex}})} 
\times F(\lambda_{\text{em}}) \cdot \lambda_{\text{ex}}^\varepsilon_0(\lambda_{\text{ex}}) \cdot \lambda_{\text{em}}^\varepsilon(\lambda_{\text{em}})}{1 - 10^{-\varepsilon_0(\lambda_{\text{ex}})} 
\times F(\lambda_{\text{em}}) \cdot \lambda_{\text{ex}}^\varepsilon_0(\lambda_{\text{ex}}) \cdot \lambda_{\text{em}}^\varepsilon(\lambda_{\text{em}}) \times n^2} \]

(13)

where \(F\) is the observed fluorescence intensity after excitation wavelength \(\lambda_{\text{ex}}\) and absorbance at the excitation wavelength \(\lambda_{\text{ex}}\) is absorbed at 440 nm, \(F\) is fluorescence emission, \(n\) is refractive index of the solution (1.3350 for DPBS at pH 7.40 and 1.3284 for methanol), and the subscripts “c” and “r” refer to the protein–chromophore complex measured and the reference dye, respectively. The reference dye Acidine Yellow G (in methanol) has a quantum yield value of 0.57 that was used\(^4\). Absolute quantum yield: An integrating sphere instrument (Hamamatsu C9920-12) (6 nm excitation bandwidth, 1 nm interval) and a high-sensitivity photonic multi-channel analyzer (Hamamatsu C10227-01) were used to measure a light emission spectrum. Absolute quantum yields were measured for solutions of protein–chromophore complexes in DPBS (pH 7.40) in which 95% of the total chromophore was occupying the protein-binding pocket (Table 1).

Protein–chromophore complex samples and control samples were excited at \(\lambda_{\text{ex}} = 440 \text{ nm}\) and absolute quantum yields were calculated according to the equation:

\[ \phi = \frac{f_m}{f_m} \]

(14)

where \(f_m\) is the emitted photon flux and \(f_m\) is the absorbed photon flux. The absolute quantum yields of the two control samples (Acridine Yellow G and fluorescein) agreed well with literature values\(^34,55\). Absolute quantum yield data was analyzed with U6039-05 PLQY measurement software.

**Size-exclusion chromatography with multi-angle light scattering.** Protein samples were prepared at 2.0 mg mL\(^{-1}\) and applied to a Superdex 75 10/300 GL column (GE Healthcare) on a LC 120 Series HPLC machine (Agilent Technologies) for size-based separation, a Heleos detector (Wyatt Technologies) for light scattering signals, and a t-Rex detector for differential refractive index detection. Results were analyzed using ASTRA 7.2 software for weighted average molecular weight.

**COS-7 cell culture and transfection.** COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1x NEAA, 100 units mL\(^{-1}\), penicillin, 100 µg mL\(^{-1}\) streptomycin, and 10% fetal bovine serum (FBS). For transfection, cells were collected using 0.25% trypsin EDTA, and approximately 1 million cells transfected by nucleofection using 2 µg of plasmid DNA (Supplementary Data 10), 18.0 µL of Lonza s.e. cell supplement, 82.0 µL of Lonza s.e. nucleofection solution, and pulse code DS-120 on a Lonza 4X Nucleofector system. Cells were seeded into ibidi µ-Slide 8-well glass bottom chambers at a density of approximately 30,000 cells well\(^{-1}\) and recovered overnight at 37 °C.

**COS-7 cell fixation.** COS-7 cells were treated at 37 °C with PFA/GA fixation solution (containing 100 mM aqueousPIPES buffer at pH 7.0, 1 mM MgCl\(_2\), 3.2% paraformaldehyde, and 0.1% gluteraldehyde) for 10 min, reduced with 10 mM sodium borohydride for 10 min, then rinsed with 1x PBS (11.9 mM phosphates, 137 mM NaCl, 2.70 mM KCl, pH 7.40) (Fisher Scientific #BP3991-5) for 5 min (Supplementary Fig. 4).

**Epifluorescence microscopy of COS-7 cells.** Conventional widefield epifluorescence imaging of cultured live COS-7 cells (Supplementary Movie 1 and Supplementary Movie 2) and fixed COS-7 cells (Supplementary Fig. 4) was performed on an inverted Nikon Ti-S microscope configured with a 60x/1.2 NA water-immersion objective lens (Nikon), a multiband filter set (LF405/488/532/635-A-000, Semrock), and a Zyla 5.5 sCMOS camera (Andor). Micro-Manager software with MM Studio and MMCore were used for acquisition. For widefield epifluorescence microscopy of fixed COS-7 cells expressing mFAP2a or mFAP2b targeted to the endoplasmic reticulum (ER) (Supplementary Fig. 4), samples were labeled with either 4.00 µM DHFBI or 4.00 µM DHFBI-1T in 1x PBS for at least 10 min before imaging. Cells were rinsed three times with 1.00 mL of 1x PBS. Samples were illuminated with 470 nm light at an intensity of ~2 W cm\(^{-2}\). Exposure times were 500 ms and current was 500 mA. For time-lapse widefield epifluorescence microscopy of live COS-7 cells expressing mFAP2a or mFAP2b targeted to the ER (Supplementary Movie 1, Supplementary Movie 2), cells were labeled with 4.00 µM DHFBI in 1x PBS. The time-lapse movies were acquired using 200 ms exposure times every 5 s for 25 total frames, with 100 mA excitation current. The total acquisition time per movie was just over 2 min, and movie playback speeds adjusted to 5 frames s\(^{-1}\).

**Photostability assay.** COS-7 cells transfected with either pcDNAs/FRT/To-AsGFP1-sec61β, pcDNAs/FRT/To-mFAP2a-sec61β, or pcDNAs/FRT/To-mFAP2b-sec61β (Supplementary Data 10) were fixed and imaged (Fig. 2) at 25 °C using a Zeiss LSM-510 laser scanning confocal fluorescence microscope equipped with a 10x/0.4 Imm Infinity objective lens and SP1 software. The Argor/2 488 nm excitation laser was set to 50% output power (4.0 A tube current) and at 10% transmission resulting in 10.3 µW laser power. The pinhole size was set...
to 98 µm (1.02 Airy units). The excitation laser source passed through a HFT488 dichroic beam splitter to the specimen, and fluorescence captured through the HFT488 dichroic beam splitter to a 505 nm long-pass emission filter and detected. Acquiring 740 × 740 pixel (71.43 µm²) images with a single laser scan dichroic beam splitter to the specimen, and 16 µm buffer, solution pelleted again, and the pellet resuspended in either 10.0 mM HEPES pH 7.50 and 25% PEG 3350 (Index, Hampton Research). The drop was incubated for 10 min at 25 °C. Five microliters of each cellular mixture in different cultures (Fig.1f) were analyzed. For all samples, amplification calculations were each molded using six stacked crystallographic waters were kept. Protonation states at pH 7 were assigned using Schrödinger’s Maestro (version 10.4, Schrödinger, LLC, New York, NY) and all crystallographic waters were solvated in TIP3P water boxes with a buffer distance of 16 Å to the box edges and NaCl ions were added to provide charge neutrality at a total concentration of 150 mM. The Amber14SB force field, used for the protein and NaCl. The DFHBI ligand was parametrized using Antechamber and the generalized Amber Force Field (GaFF)69, with geometry optimization performed with Gaussian09. Cα2+ parameters were obtained from Bradbury et al.80.

The systems were minimized in five stages with increasing number of unconstrained atoms (proton only, solvent, ligand, side-chains, and the full system) totaling 13,000 steps of steepest descent and conjugate gradient methods. This was followed by equilibration involving an initial heating to 100 K at constant volume for 50 ps, followed by heating to 298 K at a constant pressure of 1 bar for 200 ps. The systems were further equilibrated at 298 K and 1 bar for 2.25 ns. Production runs were performed using GPU accelerated Amber1457, at 1 bar and 298 K with periodic boundary conditions and a 2 fs timestep, with non-bonded short-range interactions evaluated within a cutoff of 10 Å. Each of the four system conditions for all crystallography covering the full range of data (Fig.1f). Structure was determined using PROCHECK71 and aligned to the co-crystal protein structure. The protein backbone atoms coordinates were used to probe the conformational free energy landscape using in-house scripts. The DFHBI residue coordinates of all simulations were jointly clustered into 25 clusters, resulting in whole protein cluster centroids with an average backbone heavy atom root mean square deviation (RMSD) of 2.49 Å to each other. Clustering was performed using the loop7 backbone coordinates using PyEMMA’s k_means algorithm, and each cluster centroid structure was defined as the structure, which minimized the sum of the RMSD values to all other clusters. CPPTRAJ73 and MDTRAJ74 were used for RMSF and RMSD analysis.

HEK293 cell transfections. Plasmid DNAs (Supplementary Data 15 and Supplementary Data 17) were purified in deionized water for transfections. pGP-CMV-GCaMP6f was a gift from Douglas Kim & GENIE Project (Addgene plasmid #40735; http://www.addgene.org/40735/). RRID: Addgene_40735. Wild-type HEK293 cells (ATCC CRL-1573) were cultured on 24-well plates in DMEM media (4.5 g/L D-glucose, L-glutamine; ThermoFisher #11965-092) supplemented with 10% FBS and 1% Penicillin/Streptomycin in a 5% CO₂ atmosphere at 37 °C. Cells were seeded into 24-well plates (ThermoFisher #FBS102929) at 100,000 cells well−1. Twenty-four hours after seeding, cell media was aspirated and replaced with 200 µL of DMEM media. Lipofectamine 3000 (ThermoFisher Scientific) reagents were prepared according to the manufacturer’s instructions by resuspending the Lipofectamine 3000 reagent diluted in 25.0 µL of OPTI-MEM and 1 µg of plasmid DNA diluted in 25.0 µL OPTI-MEM and 2.00 µL of P3000 reagent, allowing for formation of DNA–lipid complexes for ~10 min after combination of the two mixtures. Reagent volumes were increased for the number of wells to be transected. Fifty microliters of complexed Lipofectamine reagents were split into each well, cells were incubated for 30 min, and the volume in each well was raised to 750 µL with DMEM media. Approximately 6 h after transfection, media was replaced with 1.00 µL of DMEM media. Cells were incubated for 48 h to allow for protein expression.
and 10 mM glucose, pH between 7.3–7.4 with NaOH) with the identical DFHBI concentration was added at a rate of 2 mL min−1 to the wells. In technical triplicate, the final conditions of each well were 400 µL volume and 10 nM Ca2+ with constant 7.0 µM DFHBI concentration throughout the experiment. “After Photobleaching” (see Fig. 5d) cells in a different region of interest (ROI) were illuminated for the same duration and imaging conditions used for each imaging experiment, prior to the titration experiment.

Data analysis: Regions of interest (ROIs) surrounding single cells from three technical replicates were hand-drawn in ImageJ software41. For each cellular ROI in each frame the average fluorescence was calculated in ImageJ. In order to perform background subtraction, cellular ROIs were then moved proximal to the origin of eachroi (where there was no fluorescence). Each cellular ROI average fluorescence had the average background intensity subtracted for each frame. Following background subtraction, fluorescence fold-change was calculated by Eq. (11) where F is the background-subtracted average fluorescence per ROI per frame, and F0 is the background-subtracted average baseline fluorescence as measured 1 s prior to the Ca2+ titration42 (Fig. 5d).

HEK293 cell acetylcholine stimulations. Stimulation protocol: A stock solution of 20.0 mM DFHBI was prepared in 100% anhydrous DMSO. Media was aspirated from the wells and the cells were rinsed once with 200 µL of Tyrode’s solution (120 mM NaCl, 2 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM HepES, 10 mM glucose, pH adjusted to 7.3–7.4 using NaOH). Cells were placed in 750 µL of Tyrode’s solution supplemented with 20.0 µM DFHBI for EF2n_mFAP2b-expressing cells, 43.3 µM DFHBI for EF4n_mFAP2b-expressing cells, and 93.3 µM DFHBI for EF4n_mFAP2b-expressing cells. CaGMP6-expressing cells were placed in 750 µL Tyrode’s solution. Cells were placed in the dark at 37 °C for 30 min prior to stimulation. Imaging was performed on a Leica DM18 microscope controlled by MetaMorph software. Cells were imaged at 5 frames s−1 with a 20x magnification lens (Leica HCX PL FLUOTAR L 20x/0.4 NA CORR). Ca2+-responsive mFAP-expressing cells were continually illuminated at 7.65 µW cm−2, and CaGMP6-expressing cells were continually illuminated at 1.24 µW cm−2, using a LumenCor Light Engine (Semrock filters: Excitation 474/27 nm; Emission 520/35 nm). Twenty seconds into the imaging, 100 µL of Tyrode’s solution spiked with 1.0 M CaCl2 was added by a syringe pump (Harvard Apparatus P10) and triggered by a TTL pulse. One-hundred microliters of Tyrode’s solution spiked with the identical DFHBI concentration and percent DMSO, and 850 mM acetylcholine (ACh) was added at a rate of 2 mL min−1 to the wells. In technical triplicate per sensor, the final conditions of each well were 850 µL volume of 100 µM DFHBI and Tyrode’s concentration throughout the addition of the ACh solution.

Data analysis: Regions of interest (ROIs) surrounding single cells from three technical replicates were hand-drawn in ImageJ software41. For each cellular ROI in each frame the average fluorescence was calculated in ImageJ. In order to perform background subtraction, cellular ROIs were then moved proximal to the original ROI per frame. Following background subtraction, fluorescence fold-change was calculated by Eq. (11) where F is the background-subtracted average fluorescence per ROI per frame, and F0 is the background-subtracted average baseline fluorescence as measured 1 s prior to the ACh stimulation. A maximal fluorescence response was determined as the maximum absolute value of the fluorescence fold-change after the stimulation frame (the peak fluorescence fold-change over the whole field of view was calculated for each frame in which fluorescence in each frame was calculated by Eq. (11) where F is the fluorescence per frame, and F0 is the fluorescence of the frame with the minimum fluorescence (Fig. 5f and Supplementary Fig. 19b). Temporal analysis was performed over 20 cardiac contraction cycles by averaging for each contraction the temporal difference between the peak whole field of view normalized fluorescence fold-change and the peak normalized average fluorescence from three ROI traces in the fluorescence channel (Fig. 5f).

Pharmacological inhibition of SERCA Ca2+ pumps: Cardiomyocytes in 2.0 mL of Tyrode’s solution loaded with 3.00 µM DFHBI were imaged at 2 Hz for 30 min using a 20x magnification lens (Leica HCX PL FLUOTAR L 20x/0.4 NA CORR). A stock solution of 20.0 mM cyclosporin-A (Cyclosporine A, ThermoFisher) was administrated at ~10 min from initial image acquisition to inhibit SERCA pumps and disrupt Ca2+ recovery into the SR after contraction. Fluorescence fold-change over the whole field of view in each frame was calculated by Eq. (11), where F is the fluorescence per frame, and F0 is the fluorescence of the frame just before contraction (Supplementary Fig. 19c).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The atomic coordinates and experimental data of the EFLp2_mFAP2b-DFHBI–Ca2+–crystal structure have been deposited in the RCSB Protein Data Bank with the accession code 6OHJ. Time-lapse widefield epifluorescence microscopy movies of live COS-7 cells are available in Supplementary Movie 1 and Supplementary Movie 2. The normalized time-lapse widefield epifluorescence microscopy movie of live hiPSC-derived CMs expressing SR-targeted EF2n_mFAP2b labeled at 3.00 µM DFHBI is available in Supplementary Movie 3. Amino acid and sequence alignment data used in the construction of the combinatorial library are available in Supplementary Data 1 and Supplementary Data 2. Other amino acid, DNA and oligonucleotide sequences used throughout this research are reported in Supplementary Data 3 through Supplementary Data 17. Plasmid DNA that support the findings of this study are available at Addgene (www.addgene.org/David_Baker). Source data are provided with this paper and online [https://doi.org/10.5281/zenodo.3960574]. Other DNA plasmid and data that support the findings of this study are available from the corresponding author upon reasonable request. Computational models for mFAP2a, mFAP2b, mFAP10, the 59 extended loop decoys, 5 refined extended loop decoys, 8 circularly permuted mFAP2a or mFAP2b decoys, and 12 circularly permuted mFAP2a or mFAP2b decoys with designed linkers are available to download; the β-barrel loop frameworks used in this study are available in Supplementary Table 1, and Supplementary Table 2 are available to download [https://github.com/klimaj/mFAPs].

Code availability. The Rosetta macromolecular modeling suite [https://www.rosetta-commons.org] is freely available to academic/non-commercial users and commercial licenses are available via the University of Washington Technology Transfer Office. The Rosetta design and refinement protocols for the extended loop library are available in Supplementary Note 2. The sequence design protocol to generate the combinatorial library of linkers to graft one EF1n_mFAP2b decoy onto loop7 of mFAP2b is available in Supplementary Note 3. The PyRosetta design protocol to generate circularly permuted mFAP2a and mFAP2b is available in Supplementary Note 4. The RosettaScripts script to design linkers onto cpmFAPs incorporates code in development and is available from the corresponding author upon request. Python scripts, RosettaScripts scripts, and parameterization files are available for download at [https://github.com/klimaj/mFAPs].
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