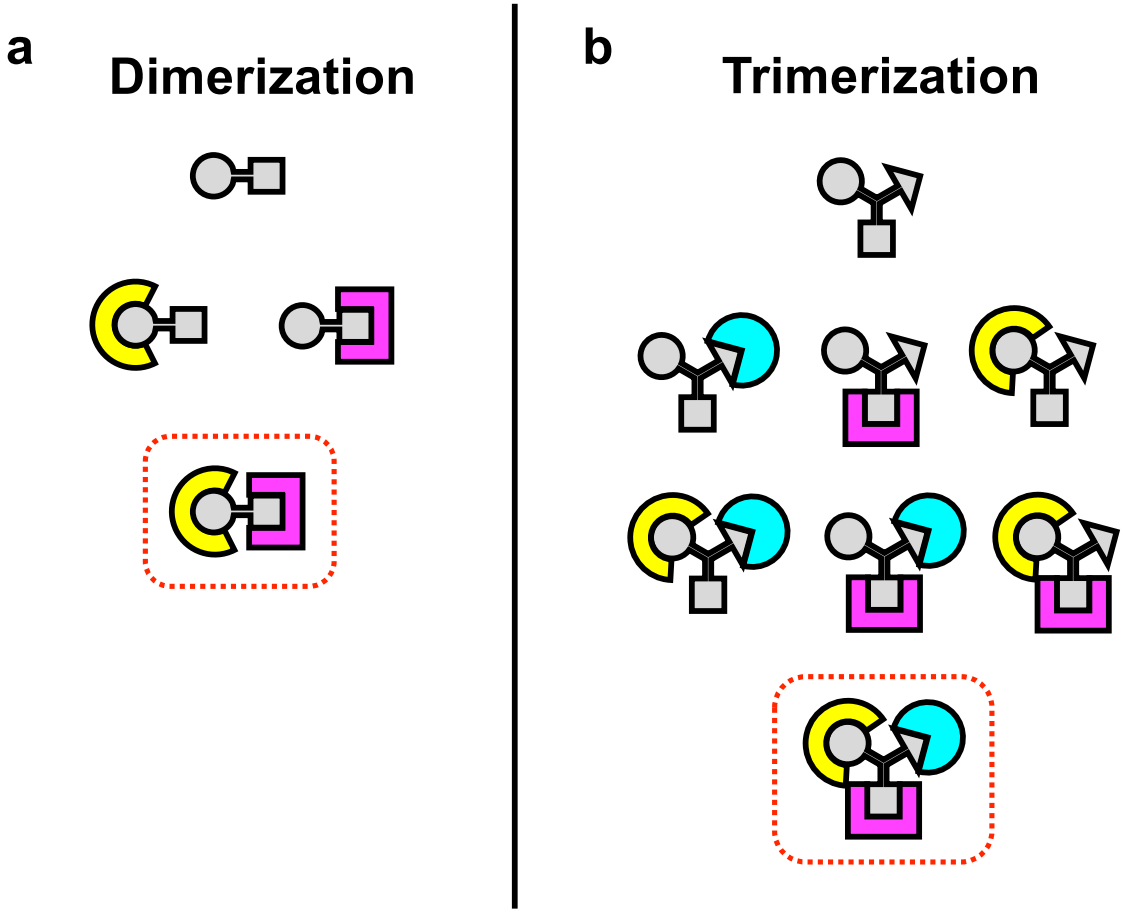
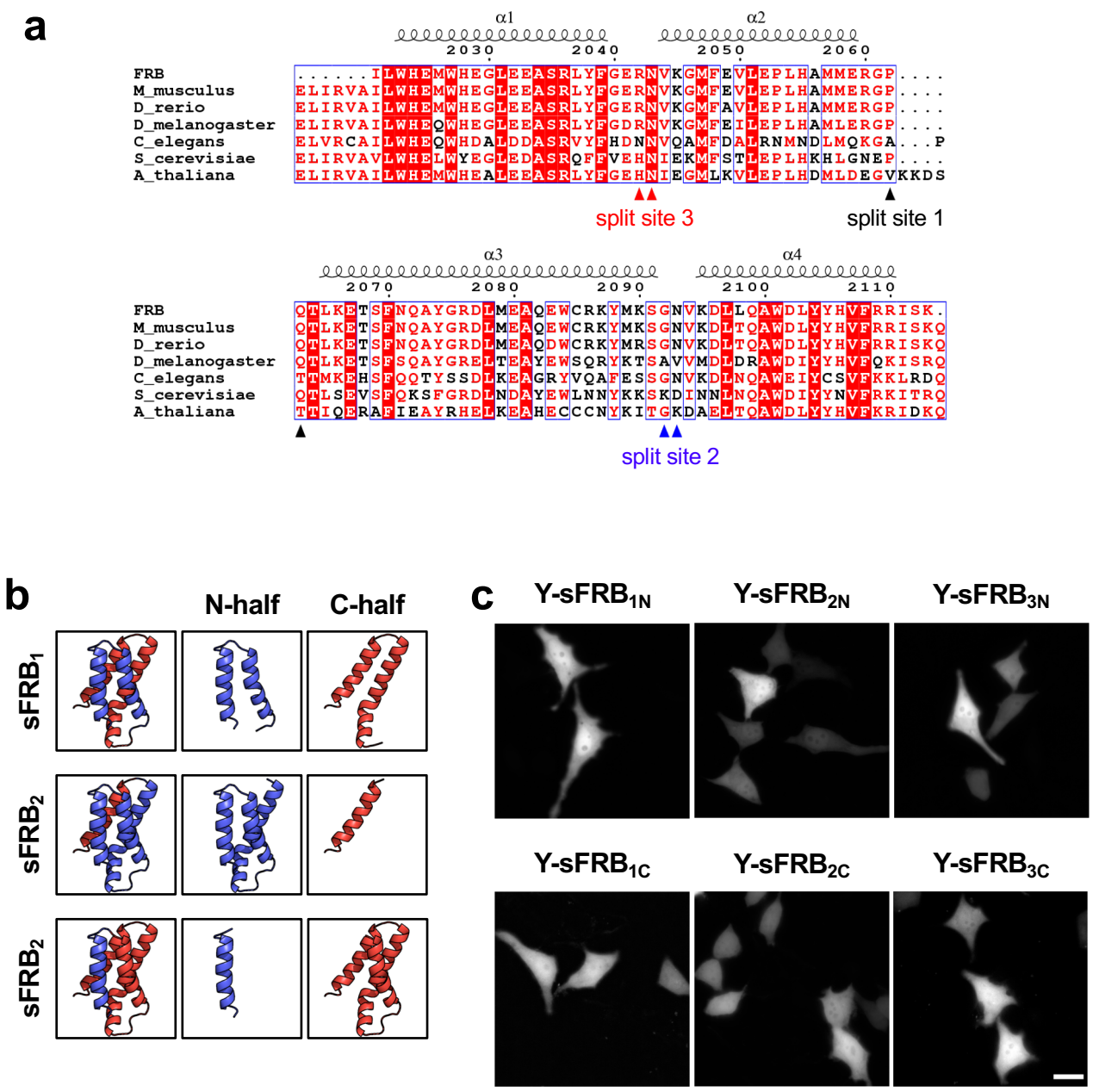
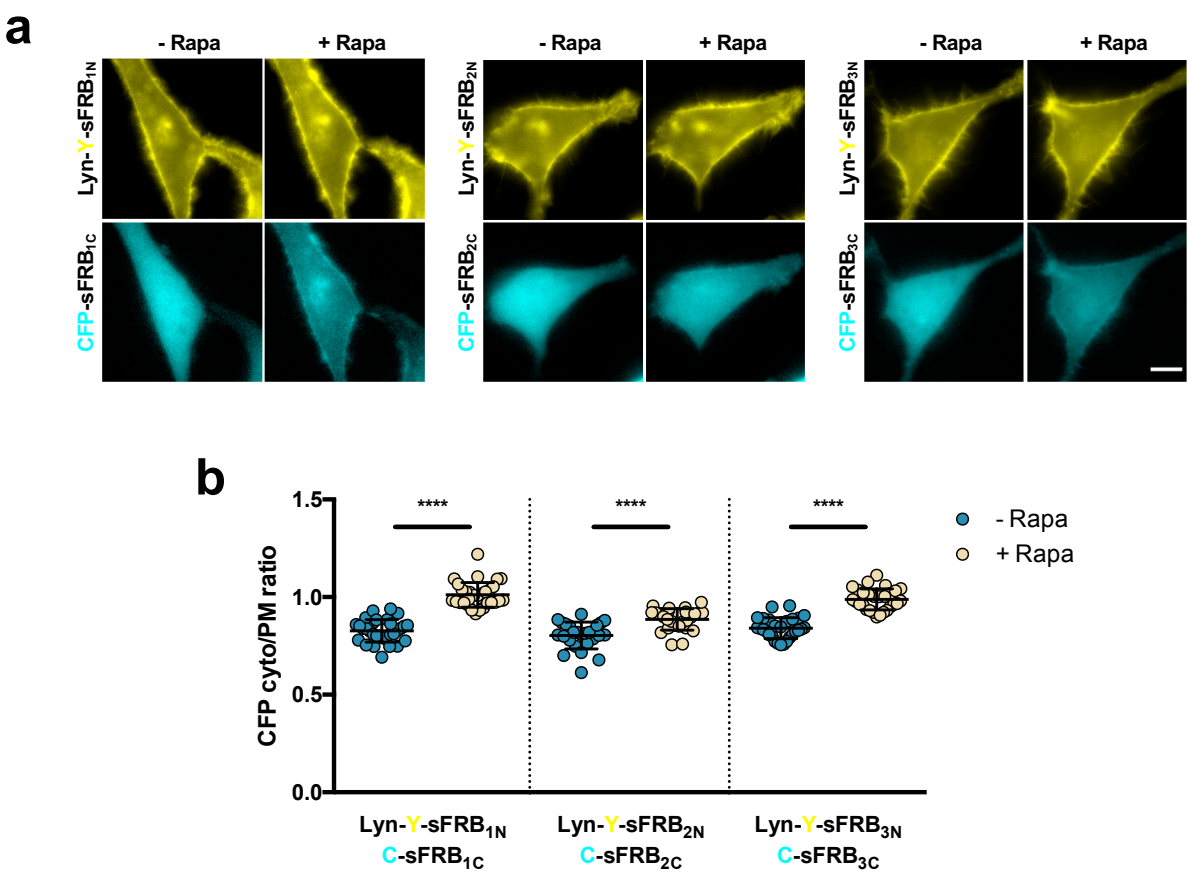


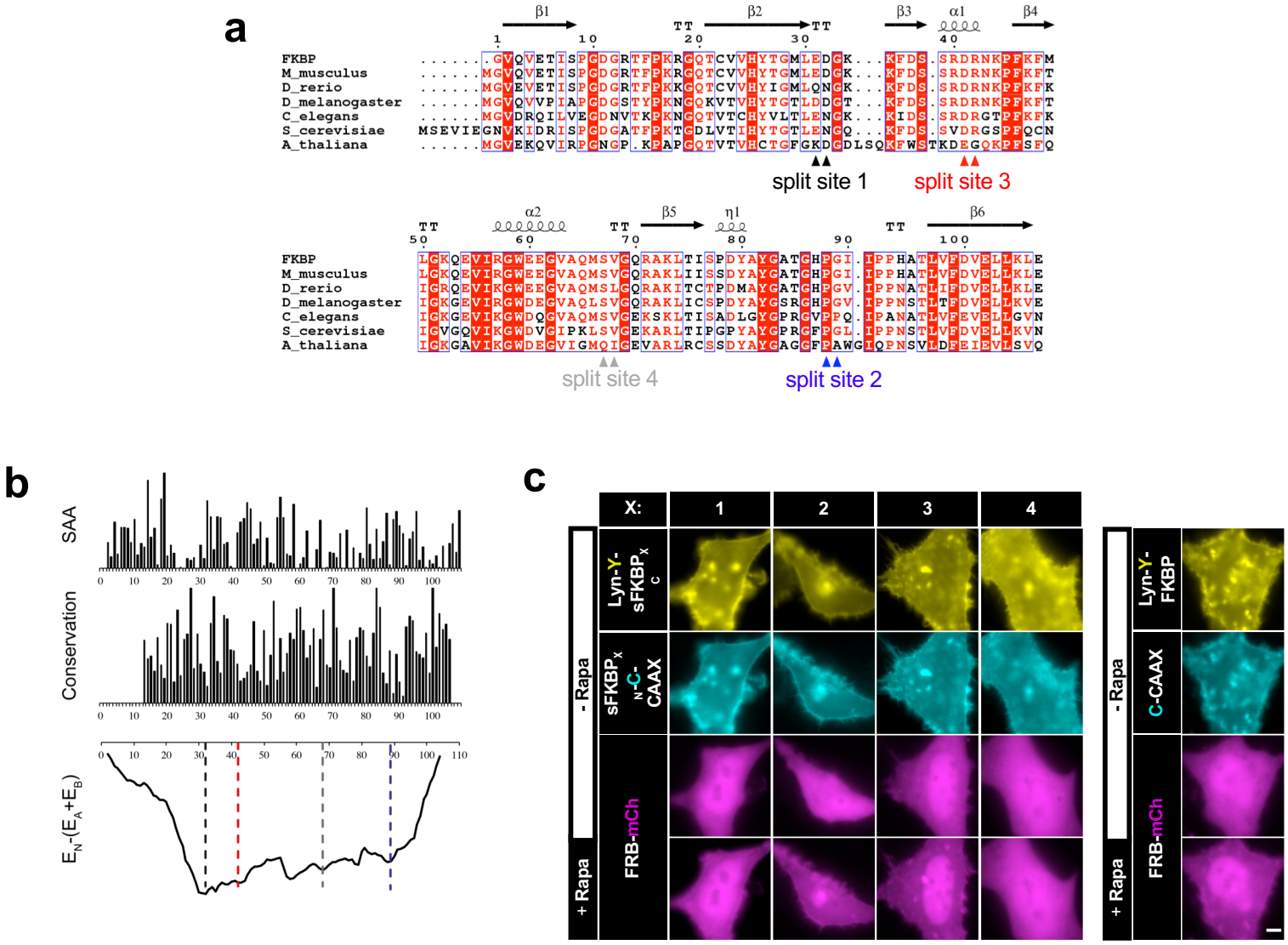
Supplementary Figure 1: Possible binding configurations of CID and CIT. (a) A small molecule dimerizer binds both proteins (yellow and magenta) in one of four configurations (red dotted box). (b) A small trimerizer binds all three proteins (yellow, magenta, and blue) in one of eight configurations (red dotted box).



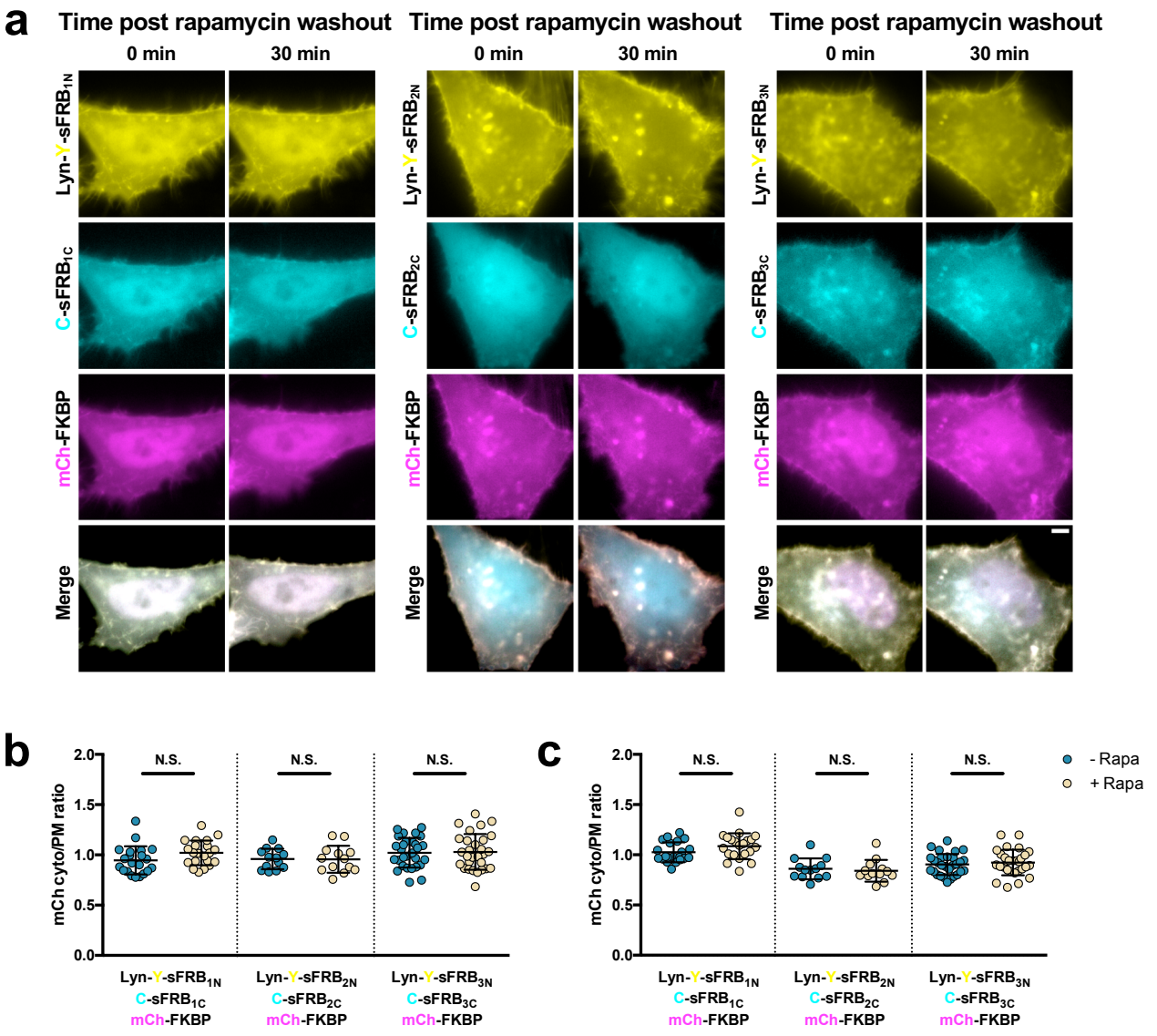
Supplementary Figure 2: FRB split sites and cytosolic sFRB expression check. (a) Sequence alignment of FRB used in this study with the FRB region of MTOR proteins across various species. Aligned though EMBL-EBI ClustalW and visualized with ESPript 3.0. Double arrowheads indicate split sites. (b) Visualization of sFRB N- and C-terminal halves (blue and red) across 3 split sites (PDB 1AUE). (c) Representative images of fluorophore-fused sFRB from 1 experiment per condition. Scale bar, 20 μm.



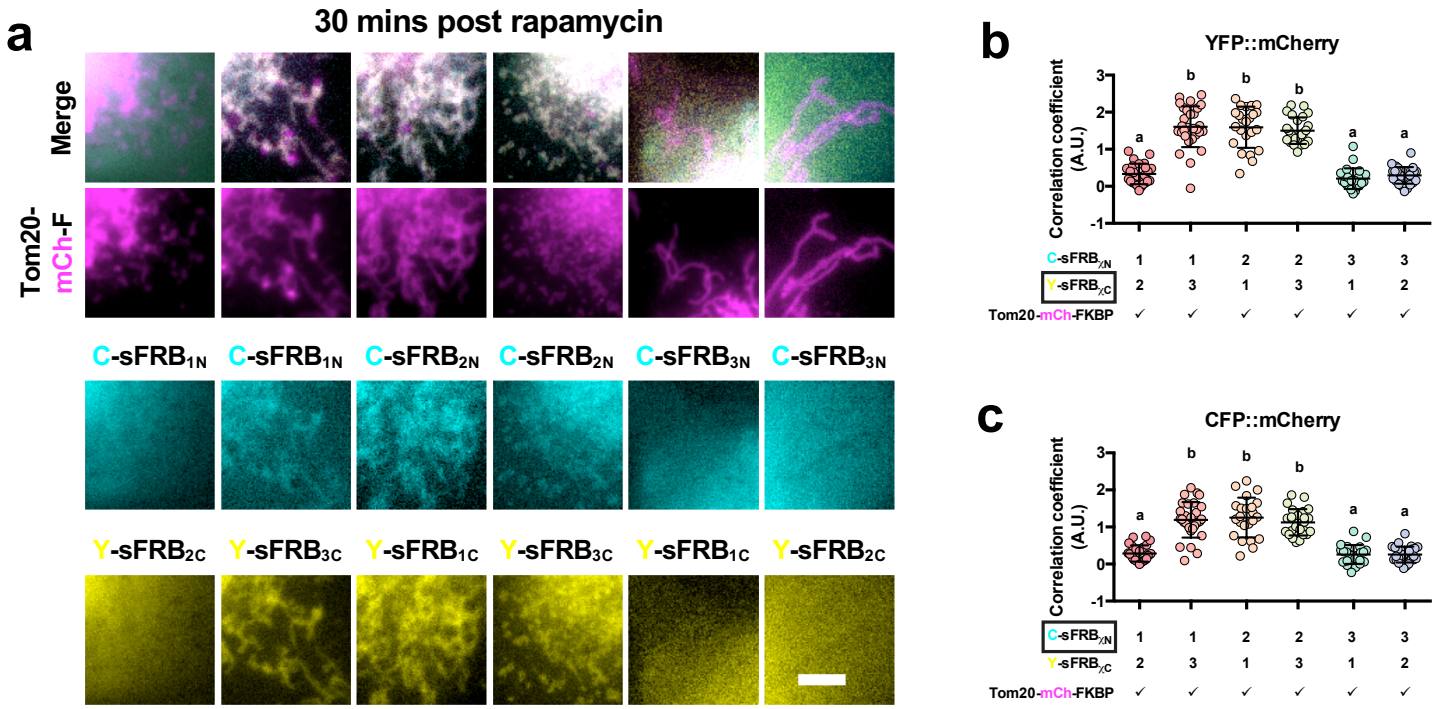
Supplementary Figure 3: sFRB dimerization in the absence of FKBP overexpression. (a) Epifluorescence snapshots of HeLa cells overexpressing N-terminal sFRB on the PM and its corresponding C-terminal sFRB in the cytosol, but not cytosolic FKBP, pre- and 12 mins post- 100 nM rapamycin addition. All three split sFRB pairs are tested. (b) PM/cytosolic ratios of C-terminal sFRB signal in cells pre- and 12 min post-rapamycin addition were determined through user-defined line-scan analysis. Data are presented as mean values +/- SD. From left to right: *n* = 31, 26, and 28 cells; 3 independent experiments each. Two-tailed Student's t test assuming equal variance was used, p-values indicated in graph. Scalebar, 10 µm. (****; $p < 0.0001$)

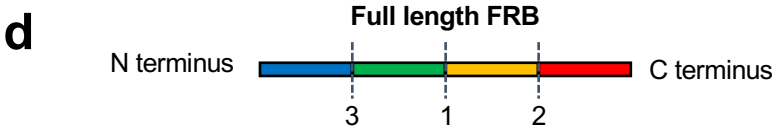


Supplementary Figure 4: FKBP split site generation and trimerization characterization. (a) Sequence alignment of FKBP12 used in this study with FKBP12 across various species. Aligned though EMBL-EBI ClustalW and visualized with ESPript 3.0. Double arrowheads indicate split sites. (b) Computational analysis to determine split sites for FKBP domain only. Loop areas with high solvent accessibility area (SAA), low conservation and favorable split energy profiles were chosen. (c) Representative images of mCh-FRB translocation from cytosol to PM in HeLa cells co-expressing PM-targeted $sFKBP_1$ – $sFKBP_4$ pairs and full length FKBP. Pre-rapamycin; top 3 rows. 9 mins post-rapamycin; bottom row. Corresponds to quantification of cells in experiments conducted in Fig. 3f. Scalebar, 5 μm.



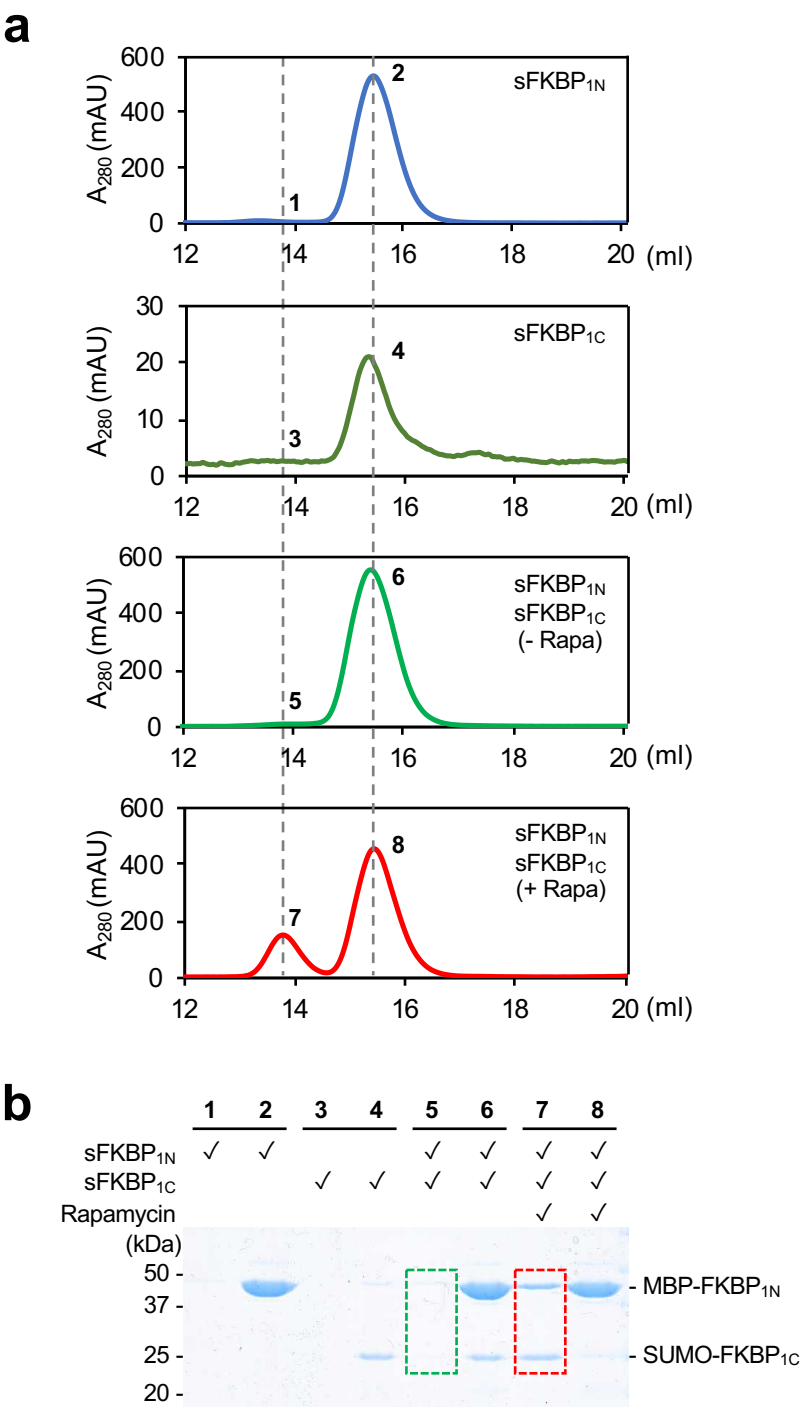
Supplementary Figure 5: Effects of rapamycin washout on sFRB-FKBP trimer stability in cells. (a) HeLa cells overexpressing PM localized Lyn-Y-sFRB$_{\chi N}$, cytosolic C-sFRB$_{\chi C}$, and cytolic mCh-FKBP, χ being split sites 1, 2, and 3, treated with 100 nM rapamycin for 30 mins and washed 10x prior to image capture at time 0 mins. Cells were imaged over 30 mins. PM/cytosolic ratios of mCh-FKBP (b) C-sFRB$_{\chi C}$ in cells pre- and 12 min post- rapamycin addition were determined through user-defined line-scan analysis. Data are presented as mean values +/- SD. For both graphs from left to right: *n* = 21, 13, and 29 cells; 3 independent experiments each. Two-tailed Student's t test assuming equal variance was used, p-values indicated in graph. Scalebar, 5 μm.





Part 1	Part 2	Illustration	Overlap	Functional?
$sFRB_{1N}$	$sFRB_{2C}$		No	No
$sFRB_{1N}$	$sFRB_{3C}$		Yes	Yes
$sFRB_{2N}$	$sFRB_{1C}$		Yes	Yes
$sFRB_{2N}$	$sFRB_{3C}$		Yes	Yes
$sFRB_{3N}$	$sFRB_{1C}$		No	No
$sFRB_{3N}$	$sFRB_{2C}$		No	No

Supplementary Figure 6: Assessing cross-reactivity of sFRB pairs. (a) 30 mins post- 100 nM rapamycin treatment, Cos-7 cells expressing Tom20-mCh-FKBP at mitochondria and co-expressing all combinations of CFP-$sFRB_{\chi N}$ and YFP- $sFRB_{\chi C}$, where χ are different. (b,c) For each combination of sFRB, trimerization quantified by calculating pairwise Fisher's transformation of Pearson's correlation coefficients between YFP-$sFRB_{\chi C}$ and Tom20-mCh-FKBP (b) or between CFP-$sFRB_{\chi N}$ and Tom20-mCh-FKBP (c). For both graphs from left to right: *n* = 26, 30, 23, 25, 23, and 27 cells; 3 independent experiments each. p <0.0001 by one-way ANOVA for both graphs. Tukey-Kramer test indicates two significantly different groups, labeled by a/b. Data are presented as mean values +/- SD. (d) Summary of combinations of sFRB based on findings in (c), describing their overlap and functionality. Scalebar, 5 μm.



Supplementary Figure 7: Gel filtration chromatography of sFKBP$_1$. (a) Elution profiles from a Superdex 200 Increase 10/300 GL chromatography column. Mixed samples (100 µM MBP-sFKBP$_{1N}$, 10 µM SUMO-sFKBP$_{1c}$, or 10 µM rapamycin) are shown inside the chromatograms. The x- and y-axis denotes elution volume (ml) and milli-absorbance unit (mAU) at 280 nm, respectively. MBP-sFKBP$_{1N}$, SUMO-sFKBP$_{1c}$, and the sFKBP1 complex was eluted at 15.4, 15.3, and 13.8 ml, respectively. (b) SDS-PAGE profile of the sFKBP1 complex. Eluted fractions were concentrated and subjected to 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. The sFKBP1 complex is formed saturatively in a rapamycin-dependent manner, as comparatively indicated by green and red dotted squares (*n* = 4 independent experiments).