Supporting Information

Supplemental Figure S1. A transmembrane helix was sufficient for ER processing in 3T3 and Hela cells. Related to Figure 1 but in different cell lines to show results are not cell line specific.

Supplemental Figure S2. Fusion proteins behaved similarly in 3T3 cells. Related to Figure 2-4 but in different cell lines to show results are not cell line specific.

Supplemental Figure S3. Fusion proteins behaved similarly in Hela cells. Related to Figure 2-4 but in different cell lines to show results are not cell line specific.

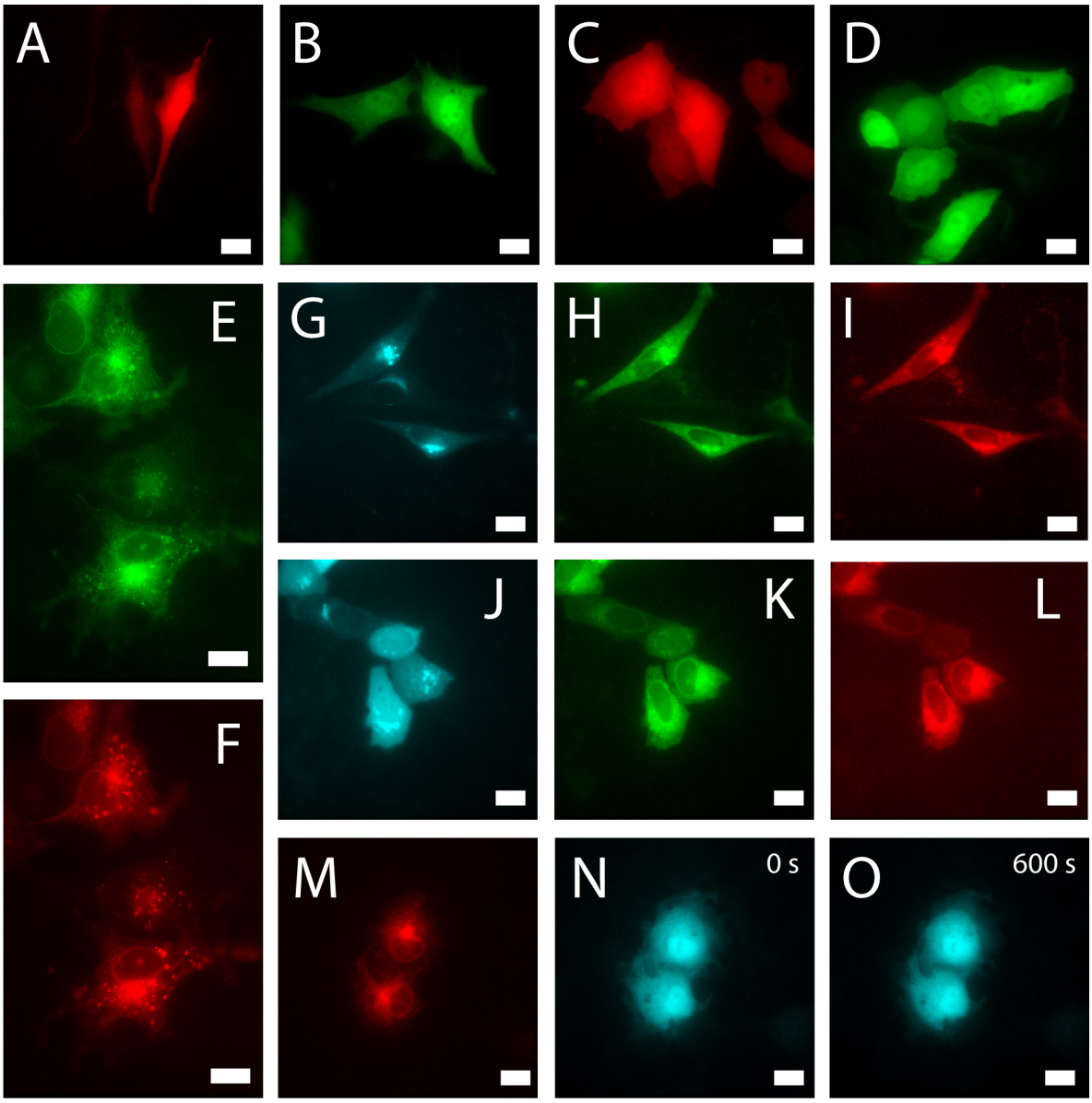
Supplemental Table 1. Co-localization coefficients for fusion proteins in 3T3 cells. Related to Figure S2.

Supplemental Table 2. Co-localization coefficients for fusion proteins in Hela cells. Related to Figure S3.

Supplemental Video S1. Vesicles from Venus-TMTLR4-mRFP were moving. Related to Figure 1 to show the fluorescence specks are vesicles.

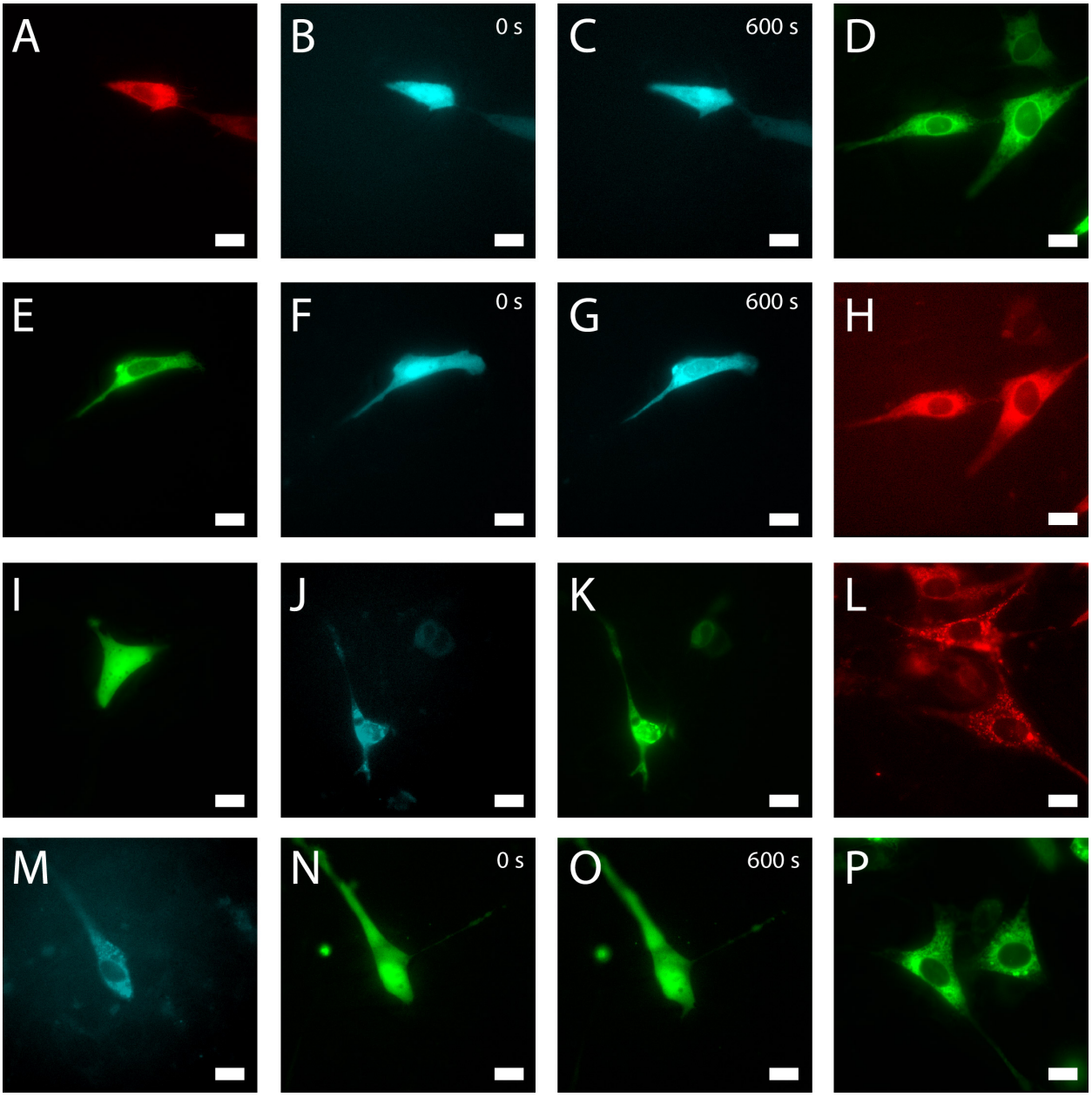
Supplemental Video S2. Rapamycin induced translocation of FRB-Ceru to FKBP12-TMTLR4-Venus-mRFP was observed in Cos-7 cells. Related to Figure 2 to show the dynamic recruitment of FRB-Ceru to establish protein orientation.

Supplemental Video S3. Rapamycin induced translocation of FRB-Ceru to FKBP12-TMTLR4-Venus-KDEL was observed in Cos-7 cells. Related to Figure 3 to show the dynamic recruitment of FRB-Ceru to establish protein orientation.



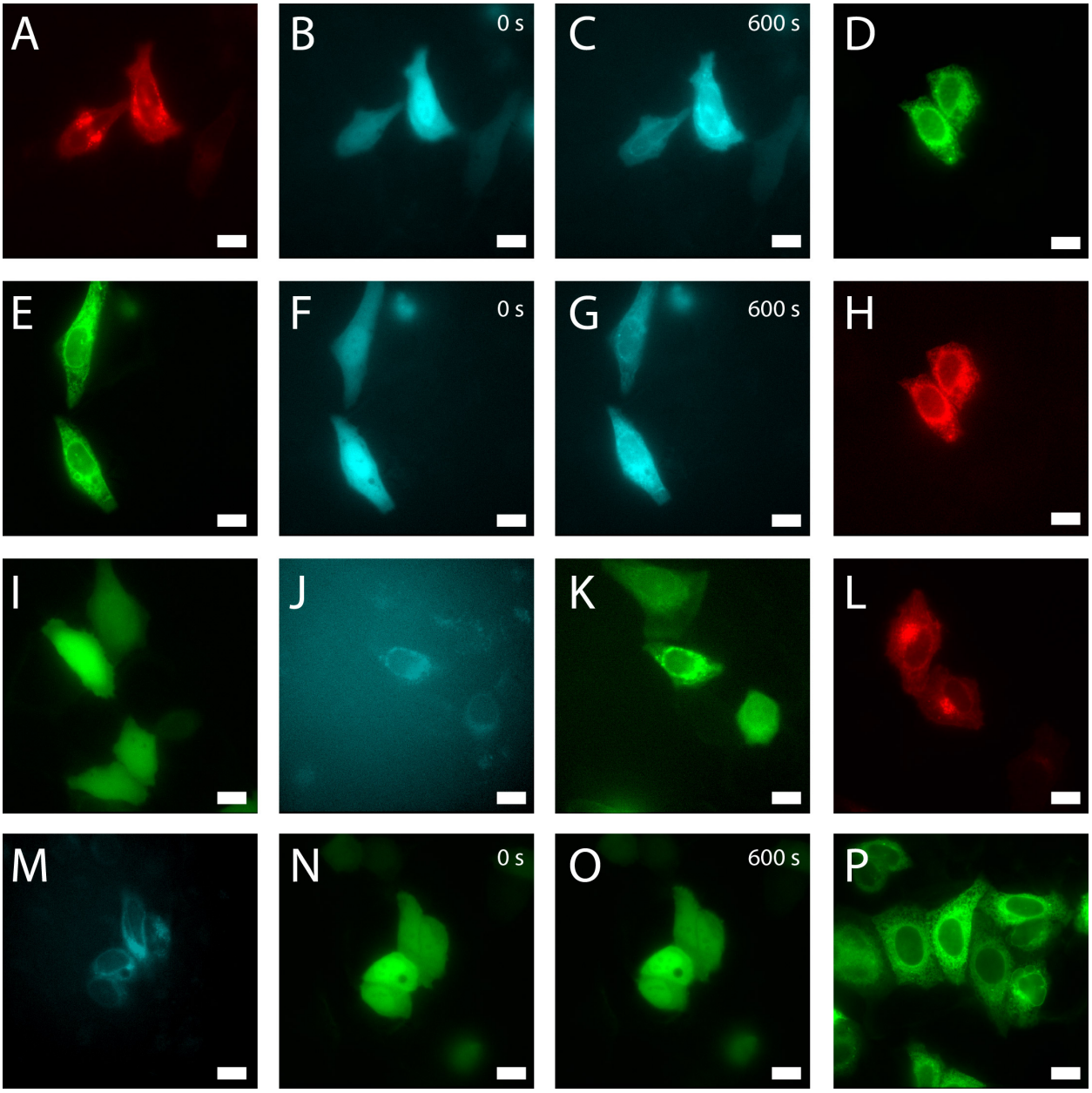
Supplemental Figure S1. A transmembrane helix was sufficient for ER processing in 3T3 and Hela cells

(A, C) mRFP or (B, D) Venus expressing alone in 3T3 and Hela cells, respectively, were nuclear and cytoplasmic. (E, F) The green and red channel, respectively, of Venus-TM_{PDGFR}-mRFP expressing Cos-7 cells localized to regions resembling ER processing. (G-L) Arf1-CFP (cyan) and Venus-TM_{TLR4}-mRFP (green and red) co-expressing in (G-I) 3T3 and (J-L) Hela cells showed strong Venus-TM_{TLR4}-mRFP fluorescence to regions of the golgi apparatus. Scale bar is 10 μm. Images are false color: CFP, cyan; Venus, green; mRFP, red. (M) FKBP12-TM_{TLR4}-mRFP and (N) FRB-Ceru were co-expressed in Cos-7 cells. (O) Rapamycin failed to induce the translocation of FRB-Ceru to FKBP12-TM_{TLR4}-mRFP.



Supplemental Figure S2. Fusion proteins behaved similarly in 3T3 cells

(A) FKBP12-TM_{TLR4}-mRFP and (B) FRB-Ceru were co-expressed in 3T3 cells. (C) Rapamycin induced translocation of FRB-Ceru to FKBP12-TM_{TLR4}-mRFP. (D) FKBP12-TM_{TLR4}-Venus-KDEL and (H) STIM1-mRFP co-expressing in 3T3 cells showed co-localization at the ER. Similarly, (E) FKBP12-TM_{TLR4}-Venus-KDEL and (F) FRB-Ceru were co-expressed in 3T3 cells. (G) Rapamycin induced translocation of FRB-Ceru to FKBP12-TM_{TLR4}-Venus-KDEL. (L) TM_{TLR4}-mRFP expressing in 3T3 cells labeled vesicles, while (P) TM_{TLR4}-Venus-KDEL labeled the ER. (I) *Npu*$DnaE_C$-Venus expressing in 3T3 cells was cytoplasmic and nuclear. (J) TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and (K) *Npu*$DnaE_C$-Venus co-expressing in 3T3 cells showed co-localization. (M) TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and (N) FRB-Venus were co-expressed in 3T3 cells. (O) Rapamycin failed to induce the translocation of FRB-Venus to FKBP12-TM_{TLR4}-Venus-KDEL. Scale bar is 10 μm. Images are false color: CFP, cyan; Venus, green; mRFP, red.



Supplemental Figure S3. Fusion proteins behaved similarly in Hela cells

(A) FKBP12-TM_{TLR4}-mRFP and (B) FRB-Ceru were co-expressed in Hela cells. (C) Rapamycin induced translocation of FRB-Ceru to FKBP12-TM_{TLR4}-mRFP. (D) FKBP12-TM_{TLR4}-Venus-KDEL and (H) STIM1-mRFP co-expressing in Hela cells showed co-localization at the ER. Similarly, (E) FKBP12-TM_{TLR4}-Venus-KDEL and (F) FRB-Ceru were co-expressed in Hela cells. (G) Rapamycin induced translocation of FRB-Ceru to FKBP12-TM_{TLR4}-Venus-KDEL. (L) TM_{TLR4}-mRFP expressing in Hela cells labeled vesicles, while (P) TM_{TLR4}-Venus-KDEL labeled the ER. (I) *Npu*$DnaE_C$-Venus expressing in Hela cells was cytoplasmic and nuclear. (J) TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and (K) *Npu*$DnaE_C$-Venus co-expressing in Hela cells showed co-localization. (M) TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and (N) FRB-Venus were co-expressed in Hela cells. (O) Rapamycin failed to induce the translocation of FRB-Venus to FKBP12-TM_{TLR4}-Venus-KDEL. Scale bar is 10 μm. Images are false color: CFP, cyan; Venus, green; mRFP, red.

Table S1. Co-localization coefficients for fusion proteins in 3T3 cells.
PCs, PCs with Costes' automatic thresholding and Van Steensel's peak CCF values for the co-localization of fusion proteins are reported for the below conditions. Percentage change is reported relative to resting conditions.

	PC	*PC with Costes' Thresholding*	*Van Steensel's Peak CCF*[1]	*PC (% Change)*	*PC with Costes' (% Change)*	*Van Steensel's Peak CCF (% Change)*
mRFP-TM_{TLR4}-FKBP12 and FRB-Ceru (resting)	0.804	0.820	0.835			
mRFP-TM_{TLR4}-FKBP12 and FRB-Ceru (rapamycin)	0.877	0.893	0.889	9.1	8.9	6.5
FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru (resting)	0.824	0.824	0.830			
FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru (rapamycin)	0.906	0.908	0.909	10.0	10.1	9.5
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and *Npu*$DnaE_C$-Venus	0.873	0.889	0.875			
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and FRB-Venus (resting)	0.703	0.725	0.704			
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and FRB-Venus (rapamycin)	0.710	0.724	0.710	1.0	-0.1	0.9

[1] All CCF values were statistically significant with Van Steensel's R^2 values greater than 0.95. (*28*)

Table S2. Co-localization coefficients for fusion proteins in Hela cells.
PCs, PCs with Costes' automatic thresholding and Van Steensel's peak CCF values for the co-localization of fusion proteins are reported for the below conditions. Percentage change is reported relative to resting conditions.

	PC	*PC with Costes' Thresholding*	*Van Steensel's Peak CCF*[1]	*PC (% Change)*	*PC with Costes' (% Change)*	*Van Steensel's Peak CCF (% Change)*
mRFP-TM_{TLR4}-FKBP12 and FRB-Ceru (resting)	0.795	0.795	0.801			
mRFP-TM_{TLR4}-FKBP12 and FRB-Ceru (rapamycin)	0.855	0.855	0.855	7.5	7.5	6.7
FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru (resting)	0.789	0.791	0.791			
FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru (rapamycin)	0.845	0.845	0.845	7.1	6.8	6.8
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and *Npu*$DnaE_C$-Venus	0.844	0.855	0.845			
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and FRB-Venus (resting)	0.671	0.707	0.675			
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and FRB-Venus (rapamycin)	0.680	0.712	0.683	1.3	0.7	1.1

[1] All CCF values were statistically significant with Van Steensel's R^2 values greater than 0.95. (*28*)

Supplemental Video S1. Vesicles from Venus-TM_{TLR4}-mRFP were moving

Venus-TM_{TLR4}-mRFP expressing in Cos-7 cells was observed in the red channel. Each 1 s of video represents approximately 15 s of elapsed time. Images are false color.

Supplemental Video S2. Rapamycin induced translocation of FRB-Ceru to FKBP12-TM_{TLR4}-Venus-mRFP was observed in Cos-7 cells

FRB-Ceru was localized in the nucleus and cytoplasm of Cos-7 cells at the start of the experiment. When rapamycin was added near the start of the video, FRB-Ceru translocated to FKBP12-TM_{TLR4}-mRFP. Each 1 s of video represents approximately 75 s of elapsed time. Images are false color.

Supplemental Video S3. Rapamycin induced translocation of FRB-Ceru to FKBP12-TM_{TLR4}-Venus-KDEL was observed in Cos-7 cells

FRB-Ceru was localized in the nucleus and cytoplasm of Cos-7 cells at the start of the experiment. When rapamycin was added near the start of the video, FRB-Ceru translocated to FKBP12-TM_{TLR4}-Venus-KDEL. Each 1 s of video represents approximately 75 s of elapsed time. Images are false color.