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Parts-Based Assembly of Synthetic Transmembrane Proteins in Mammalian Cells

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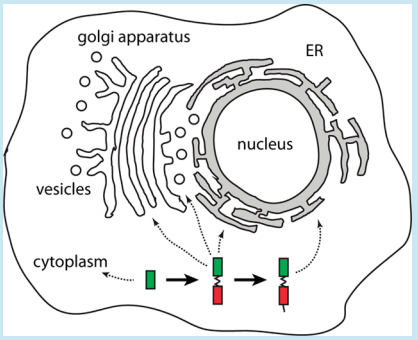
Supporting Information

ABSTRACT: Transmembrane proteins span cellular membranes such as the plasma membrane and endoplasmic reticulum (ER) membrane to mediate inter- and intracellular interactions. An N-terminal signal peptide and transmembrane helices facilitate recruitment to the ER and integration into the membrane, respectively. Using a parts-based assembly approach in this study, we confirm that the minimum requirement to create a transmembrane protein is indeed only a transmembrane helix (TM). When transfected in mammalian cells, our fusion proteins in the schematic form X-TM-Y were localized to vesicles, the golgi apparatus, the nuclear envelope, or the endoplasmic reticulum, consistent with ER targeting. Further studies to determine orientation showed that X was facing the cytoplasm, and Y the lumen. Lastly, in our fusion proteins with an N-terminal TM, the TM effectively reversed the orientation of X and Y. This knowledge can be applied to the parts-based engineering of synthetic transmembrane proteins with varied functions and biological applications.



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One of the goals of synthetic biology is to systematically engineer biological systems that reproduce natural functions or create novel ones.[1] Transmembrane proteins are important components in biological systems because they facilitate inter- and intracellular interactions such as cell adhesion and signal transduction. These transmembrane proteins span cellular membranes composed of phospholipid bilayers that separate the different cellular compartments from each other and the intra- from the extralumenal environment.[2] To eventually engineer transmembrane proteins with desired functions, it is important to have knowledge on how to precisely target their subcellular location (*e.g.*, plasma membrane or endoplasmic reticulum (ER) membrane) and the orientation of the protein domains (*i.e.*, which side of the membrane). To aid this effort, decades of research have revealed some key ideas on how transmembrane proteins are sorted to subcellular locations with specific orientations.[3−8]

Transmembrane proteins commonly have an N-terminal signal peptide to recruit the protein for ER targeting and have at least one transmembrane helix that spans the cellular membrane composed of approximately 22 mostly hydrophobic amino acids.[3] The N-terminal signal sequence is recognized by the signal recognition particle (SRP), recruited to the ribosomes on the ER for translation and later cleaved off by the signal peptidase.[3] While the signal peptide sequences vary widely between transmembrane proteins, they are typically 20−60 amino acids consisting of a tandem hydrophilic segment, a hydrophobic segment, and a signal peptidase cleavage site. Signal sequences are typically at the N-terminus but can also be found internally in the protein sequence where they are referred to as signal-anchor sequences.[4,5] As transmembrane proteins are translated in the ER, they are retained on the membrane by a hydrophobic transmembrane segment that folds into a helix and is embedded in the cellular membrane by hydrophobic interactions.[5] Subsequently, transmembrane proteins are sorted to different cell compartments (such as the plasma membrane, lysosome, or nuclear envelope) by vesicle trafficking through the golgi apparatus.[6] Lastly, transmembrane proteins can be retained in the ER by a C-terminal KDEL retention signal by binding to the KDEL receptor.[7]

While most of our current knowledge of the subcellular localization and orientation of transmembrane proteins arise from gene mutagenesis and truncation studies of individual proteins,[3−8] our group has applied a synthetic biology approach to assemble transmembrane proteins by parts. Here, we confirm that transmembrane proteins without a signal peptide can be recruited in the ER and that the only requirement is indeed a transmembrane helix. When the transmembrane helix is placed in the N-terminus of the protein, it alters the orientation of the transmembrane protein. The orientation of the protein domains for the synthetic transmembrane protein was further confirmed using protein domains that dynamically dimerize in the presence of rapamycin[9] or protein domains that

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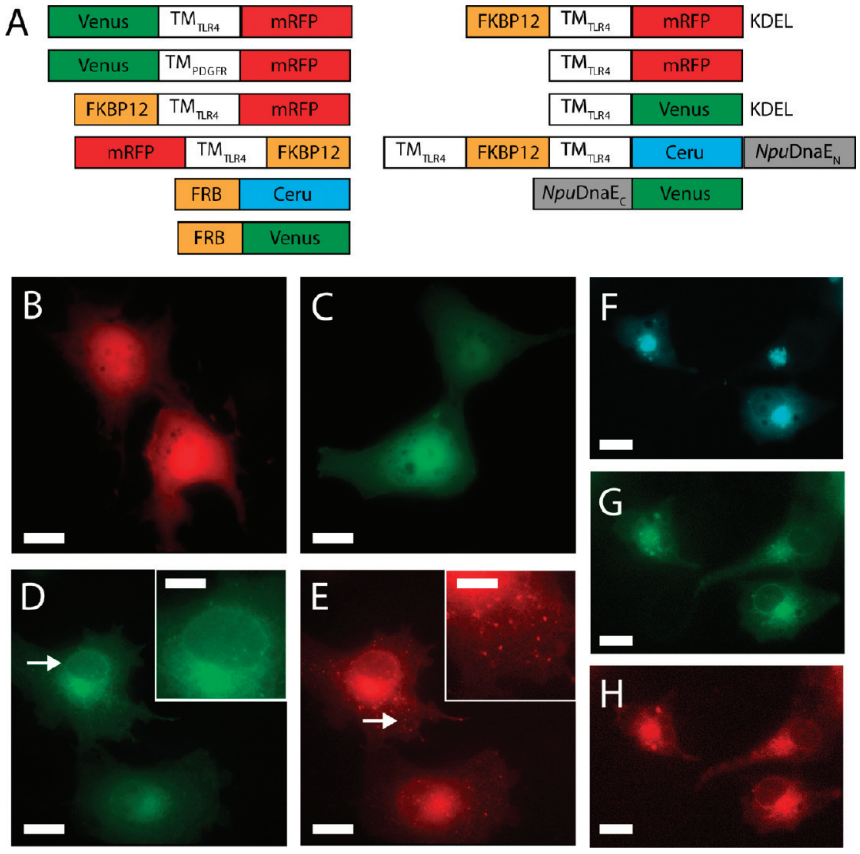

Figure 1. A transmembrane helix was sufficient for ER processing. (A) Schematic layout of fusion proteins created in the study. (B) mRFP or (C) Venus expressing alone in Cos-7 cells remained nuclear and cytoplasmic. (D) The green channel of Venus-TM_{TLR4}-mRFP expressing Cos-7 cells localized to regions resembling ER processing. The inset focused on the nuclear envelope also confirmed by brightfield microscopy. (E) The red channel of Venus-TM_{TLR4}-mRFP expressing Cos-7 cells mostly co-localized with the green channel but with more vesicles. The inset focused on the vesicles. (F–H) Arf1-CFP (cyan) and Venus-TM_{TLR4}-mRFP (green and red) co-expressing in Cos-7 cells showed that Venus-TM_{TLR4}-mRFP had strong fluorescence to regions of the golgi apparatus. Scale bar is 10 μm, inset scale bar is 5 μm. Images are false color: CFP, cyan; Venus, green; mRFP, red. See also Supplementary Figure S1 and Video S1.

bind and undergo protein splicing.[10] This knowledge serves as a basis for rational engineering of synthetic transmembrane proteins by parts with varied functions and biological applications.

While there are many examples in the literature of transmembrane proteins that have an N-terminal signal peptide, a survey of the transmembrane proteins in the UniProt database[11] (Release 2011_03) suggests that the majority of transmembrane proteins do not have an N-terminal signal peptide. To avoid discovering orthologous proteins, only human proteins were used. In total, there were 20,234 human proteins in the database, of which 5,153 were transmembrane proteins (*i.e.*, containing a confirmed/potential transmembrane helix). Of the transmembrane proteins, only 1,540 had an N-terminal signal peptide. There were 1,218 single pass transmembrane proteins, where 1,192 of these proteins had N-terminal signal peptides, making them the majority of proteins that have an N-terminal signal peptide. It also suggests that multiple pass transmembrane proteins do not typically require N-terminal signal peptides. The 26 single pass transmembrane proteins that did not have an N-terminal signal peptide had subcellular localizations that were, if further specified, golgi apparatus (*e.g.*, Golgin subfamily B member 1), nuclear envelope (*e.g.*, Serine/threonine-protein kinase LMTK1), or endoplasmic reticulum (*e.g.*, Neuropathy target esterase).

When transfected in mammalian cells, our fusion proteins in the schematic form X-TM-Y (such that TM was a transmembrane helix flanked by fluorescent proteins X and Y) appeared localized to the vesicles, golgi apparatus, nuclear envelope, or endoplasmic reticulum, consistent with ER targeting (Figure 1 and Supplementary Figure S1). Previous studies have shown that highly hydrophobic regions (*e.g.*, transmembrane domains) are recognized by the cellular machinery in eukaryotes called the translocon to directly insert the transmembrane domain into the ER membrane.[5] Thus, the protein Venus-TM_{TLR4}-mRFP was created as a tandem fusion of the yellow fluorescent protein mutant Venus,[12] the transmembrane domain from human toll-like receptor 4[13] (^{632}TIIGVSVLSVLVVSVVAVLVY^{652}) and monomeric red fluorescent protein (mRFP),[14] while the protein Venus-TM_{PDGFR}-mRFP used the transmembrane domain from human platelet-derived growth factor receptor[15] (^{532}VVVISAILALVVLTIISLIILIMLW^{556}) (Figure 1A). When Venus and mRFP were transfected in mammalian cells (Figure 1B,C and Supplementary Figure 1C–F), the fluorescence distribution was cytoplasmic and nuclear as expected from a small ~27 kDa protein without any signaling peptides that can diffuse across the nuclear envelope (n = 6/6 experiments). In contrast, when the Venus-TM_{TLR4}-mRFP or Venus-TM_{PDGFR}-mRFP were transfected, both had similar fluorescence distribution that appeared localized to vesicles, golgi apparatus,

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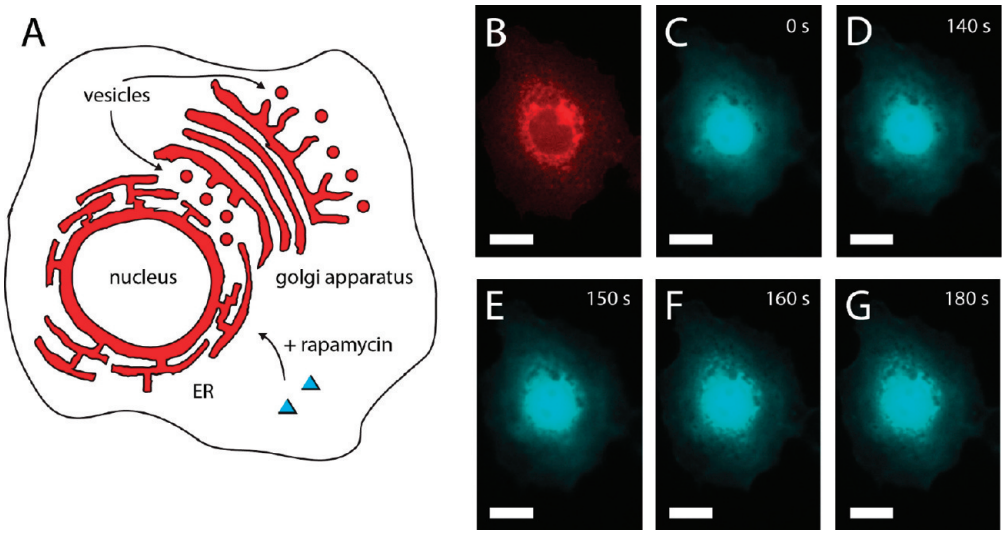

Figure 2. In fusion proteins of the form X-TM-Y, X faced the cytoplasm. (A) A cartoon depicting the recruitment of FRB-Ceru to FKBP12-TM_{TLR4}-mRFP after the addition of rapamycin. (B) FKBP12-TM_{TLR4}-mRFP and (C) FRB-Ceru co-expressing in Cos-7 cells at time 0 s. (D–G) When rapamycin was added at 80 s, FRB-Ceru translocated to FKBP12-TM_{TLR4}-mRFP within minutes, suggesting that FKBP12 was facing the cytoplasm. Scale bars are 10 μm. Images are false color: FRB-Ceru, cyan; FKBP12-TM_{TLR4}-mRFP, red. See also Supplementary Figures S2 and S3 and Video S2.

Table 1. Co-localization Coefficients for Fusion Proteins in Cos-7 Cells[a]

	PC	PC with Costes' thresholding	Van Steensel's peak CCF[b]	PC (% change)	PC with Costes' (% change)	Van Steensel's peak CCF (% change)
Venus-TM_{TLR4}-mRFP (yellow fluorescence) and Venus-TM_{TLR4}-mRFP (red)	0.975	0.975	0.975			
Venus-TM_{PDGFR}-mRFP (yellow fluorescence) and Venus-TM_{PDGFR}-mRFP (red)	0.965	0.964	0.965			
FKBP12-TM_{TLR4}-mRFP and FRB-Ceru (resting)	0.805	0.804	0.806			
FKBP12-TM_{TLR4}-mRFP and FRB-Ceru (rapamycin)	0.859	0.863	0.860	6.7	7.3	6.7
mRFP-TM_{TLR4}-FKBP12 and FRB-Ceru (resting)	0.814	0.814	0.816			
mRFP-TM_{TLR4}-FKBP12 and FRB-Ceru (rapamycin)	0.818	0.818	0.819	0.5	0.5	0.4
FKBP12-TM_{TLR4}-Venus-KDEL and STIM1-mRFP	0.973	0.974	0.973			
FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru (resting)	0.760	0.760	0.762			
FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru (rapamycin)	0.920	0.920	0.920	21.0	21.0	21.1
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-NpuDnaE$_N$ and NpuDnaE$_C$-Venus	0.902	0.917	0.902			
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-NpuDnaE$_N$ and FRB-Venus (resting)	0.784	0.804	0.806			
TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-NpuDnaE$_N$ and FRB-Venus (rapamycin)	0.791	0.802	0.813	0.9	−0.2	0.8

[a]PCs, PCs with Costes' automatic thresholding, and Van Steensel's peak CCF values for the co-localization of fusion proteins are reported for the given conditions. Percentage change is reported relative to resting conditions. [b]All CCF values were statistically significant with Van Steensel's R^2 values greater than 0.95.[28]

nuclear envelope, or endoplasmic reticulum, consistent with ER targeting (Figure 1D,E and Supplementary Figure S1A,B) (n = 6/6 experiments). Although not readily apparent in the images, there was a faint web-like fluorescence distribution that appeared like an ER localization with fluorescent vesicles that moved a few micrometers between frames acquired once every 5 s (Supplementary Video S1). These vesicles occasionally appeared only red but not yellow fluorescent, suggesting that in some instances Venus is cleaved or unfolded. Furthermore, co-transfection with golgi apparatus localized Arf1-CFP[16] showed co-localization with the most dense region of Venus-TM_{TLR4}-mRFP fluorescence (Figure 1F–H and Supplementary Figure S1G–L) (n = 6/6 experiments). Lastly, in many cells fluorescence distribution clearly outlined the nuclear envelope as determined by brightfield microscopy. Since the two transmembrane helices did not have differences in fluorescence distribution, we arbitrarily chose TM_{TLR4} for further studies.

When transfected in mammalian cells, our fusion proteins in the schematic form X-TM-Y had an orientation where X was facing the cytoplasm (Figure 2, Supplementary Figures S2A–C and S3A–C). The precise orientation of transmembrane proteins is determined by a complex interplay of factors including the length and hydrophobicity of the TM sequence as well as the charge and folding state of the flanking protein components.[8] Thus, to determine the orientation of our fusion proteins, the protein FKBP12-TM_{TLR4}-mRFP was created as a tandem fusion of a 12-kDa FK506-binding protein (FKBP12),[9] TM_{TLR4}, and mRFP, while the protein FRB-Ceru was a tandem fusion of the FKBP-rapamcyin binding (FRB) domain of mTOR[9] (mammalian targets of rapamycin) and the cyan

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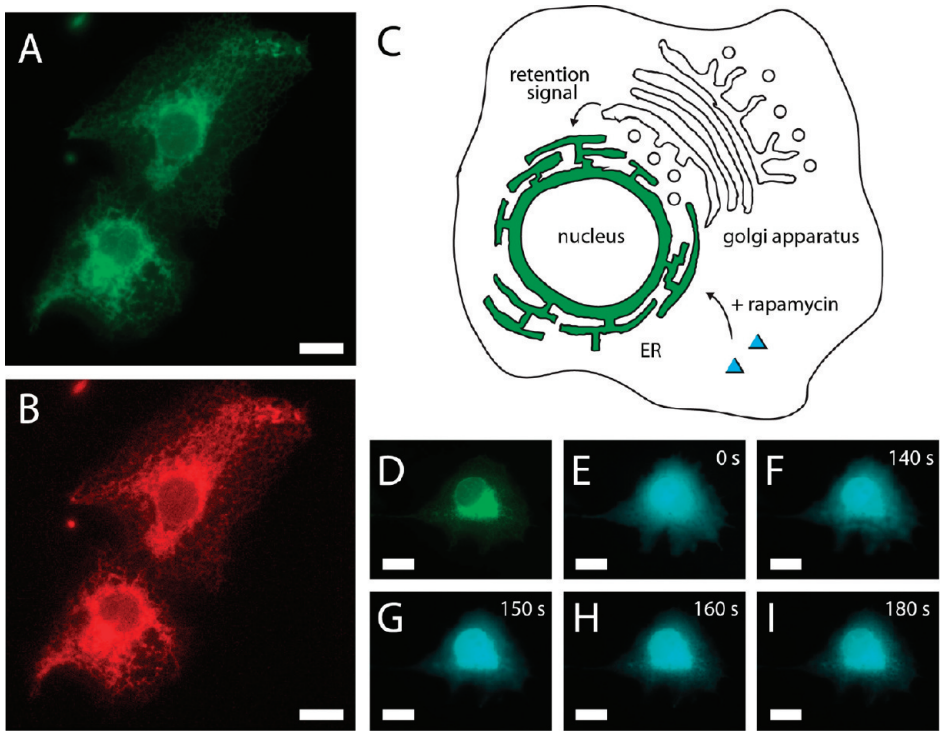

Figure 3. Fusion proteins of the form X-TM-Y-KDEL were retained in the ER. (A) A cartoon depicting the recruitment of FRB-Ceru to FKBP12-TM_{TLR4}-Venus-KDEL after the addition of rapamycin. (B) FKBP12-TM_{TLR4}-Venus-KDEL and (C) STIM1-mRFP co-expressing in Cos-7 cells showed co-localization at the ER, suggesting that Venus is in the lumen of the ER. (D) FKBP12-TM_{TLR4}-Venus-KDEL and (E) FRB-Ceru co-expressing in Cos-7 cells at time 0 s. (F–I) When rapamycin was added at 80 s, FRB-Ceru translocated to FKBP12-TM_{TLR4}-Venus-KDEL within minutes, suggesting that FKBP12 was still facing the cytoplasm. Scale bars are 10 μm. Images are false color: FRB-Ceru, cyan; FKBP12-TM_{TLR4}-Venus-KDEL, green; STIM1-mRFP, red. See also Supplementary Figures S2 and S3 and Video S3.

fluorescent protein mutant Cerulean[17] (Figure 1A). Upon addition of rapamycin, the FKBP12 and FRB dynamically heterodimerize with high affinity (k_d = 2.5 nM)[18] in a mechanism that has been exploited to engineer rapamycin-induced control over various cellular processes such as filopodia formation[9] and kinase activity[19] (Figure 2A). When both proteins were transfected in mammalian cells, FKBP12-TM_{TLR4}-mRFP had a fluorescence distribution similar to that of Venus-TM_{TLR4}-mRFP, while FRB-Ceru had a cytoplasmic and nuclear fluorescence distribution similar to that of Venus or mRFP (Figure 2B, Supplementary Figures S2A and S3A) (n = 12/12 experiments). When $[1\ \mu M]_f$ of rapamycin was added, FRB-Ceru translocated within minutes to some areas of the ER/golgi apparatus but not the vesicles (Figure 2B; Supplementary Figures S2B,C and S3B,C and Video S2) (n = 6/6 experiments). To quantify the change in the co-localization between FKBP12-TM_{TLR4}-mRFP and FRB-Ceru, we calculated co-localization coefficients of the resting and rapamycin-induced states, which showed an increase of about 7–9% (Table 1; Supplementary Tables S1 and S2). This suggests first that the FKBP12 must be facing the cytoplasm and second that not all areas of FKBP12-TM_{TLR4}-mRFP expression are in a form where FKBP12 is accessible (such as misfolded or unfolded proteins or cleaved FKBP12). This result does not preclude the possibility that some FKBP12 may be facing the lumenal side. Thus, the protein mRFP-TM_{TLR4}-FKBP12 was created and co-transfected with FRB-Ceru. When induced with $[1\ \mu M]_f$ of rapamycin, there was no observable translocation of FRB-Ceru to mRFP-TM_{TLR4}-FKBP12 (Supplementary Figure S1M–O) (Table 1).

When transfected in mammalian cells, our fusion proteins in the schematic form X-TM-Y-KDEL (such that KDEL is an ER retention signal on the lumenal side) had an ER fluorescence distribution, where X was facing the cytoplasm, and Y the ER lumen (Figure 3, Supplementary Figures S2D–H and S3D–H). The protein FKBP12-TM_{TLR4}-Venus-KDEL was created as a tandem fusion of FKBP12, TM_{TLR4}, Venus, and the KDEL ER retention signal (Figure 1A). When FKBP12-TM_{TLR4}-Venus-KDEL was transfected in mammalian cells, it had a web-like fluorescence distribution that appeared localized to the ER with no vesicles (Figure 3A, Supplementary Figures S2D and S3D) (n = 6/6 experiments). Furthermore, co-transfection with ER-localized STIM1-mRFP[20] (a transmembrane protein involved detection of ER Ca^{2+} depletion) showed co-localization with a Pearson's correlation coefficient of 0.973 (Figure 3B, Supplementary Figures S2H and S3H) (n = 6/6 experiments) (Table 1). Since the KDEL receptor retains transmembrane proteins in the ER by binding KDEL retention signal on the lumenal side,[7] the Venus must be in the lumen of the ER (Figure 3C). When FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru proteins were co-transfected in mammalian cells, FRB-Ceru translocated within minutes to the ER upon stimulation with $[1\ \mu M]_f$ of rapamycin (Figure 3D–I; Supplementary Figures S2E–G and S3E-G and Video S3) (n = 6/6 experiments). To quantify the change in the co-localization between FKBP12-TM_{TLR4}-Venus-KDEL and FRB-Ceru, we also calculated co-localization coefficients of the resting and rapamycin-induced states which showed an increase of about 7–21% (Table 1, Supplementary Tables S1 and S2). Thus, the FKBP12 must still be facing the cytoplasm. Given the efficiency

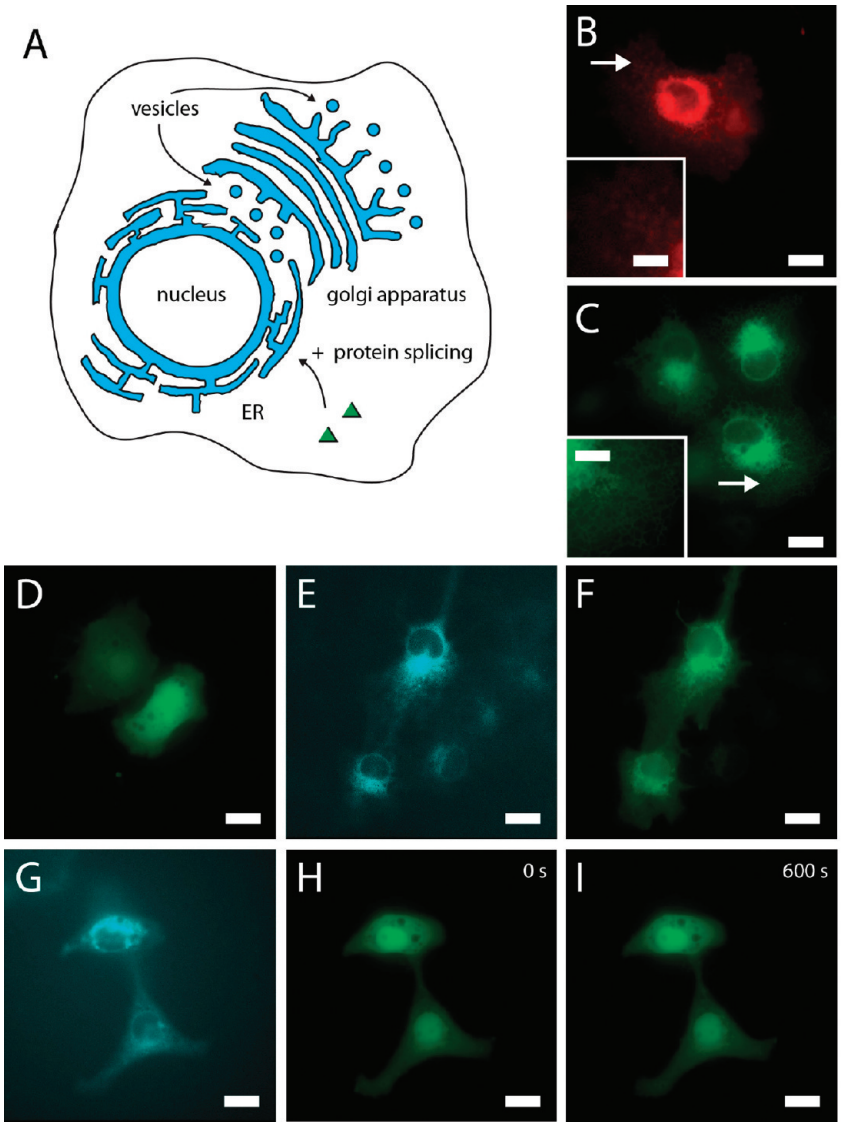

Figure 4. A transmembrane helix can act as a signal peptide and reverse the orientation of the transmembrane protein. (A) A cartoon depicting the recruitment of *Npu*DnaEC-Venus to TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*DnaEN by split intein-mediated protein splicing. (B) TM_{TLR4}-mRFP expressing alone in Cos-7 cells labeled vesicles. The inset focused on the vesicles. (C) TM_{TLR4}-Venus-KDEL expressing alone in Cos-7 cells labeled the ER. The inset focused on the web-like fluorescence distribution characteristic of the ER. (D) *Npu*DnaEC-Venus expressing alone in Cos-7 cells was cytoplasmic and nuclear. (E) TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*DnaEN and (F) *Npu*DnaEC-Venus co-expressing in Cos-7 cells showed co-localization, suggesting that *Npu*DnaEN is facing in the cytoplasm. (G) TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*DnaEN and (H) FRB-Venus co-expressing in Cos-7 cells at time 0 s. (I) When rapamycin was added at 80 s, FRB-Venus did not translocate to FKBP12-TM_{TLR4}-Venus-KDEL over 10 min, suggesting that FKBP12 was inaccessible. Scale bar is 10 μm, inset scale bar is 5 μm. Images are false color: TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*DnaEN, cyan; *Npu*DnaEC-Venus and FRB-Venus, green. See also Supplementary Figures S2 and S3.

of ER retention, it is unlikely that there is any large protein population where FKBP12 is facing the lumenal side.

In fusion proteins with an N-terminal TM, the TM targeted the protein to the ER and reversed the orientation of synthetic transmembrane proteins (Figure 4, Supplementary Figures S2I–P and S3I–P). When the fusion protein TM_{TLR4}-mRFP and TM_{TLR4}-Venus-KDEL were transfected into mammalian cells, they were localized to the vesicles and the ER, respectively, suggesting that the transmembrane helix targeted the protein to the ER (Figure 4B,C, Supplementary Figures S2L,P and S3L,P) (n = 6/6 experiments). Next, the fusion proteins TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and *Npu*$DnaE_C$-Venus were created, where *Npu*$DnaE_N$ and *Npu*$DnaE_C$ are the N-terminal and C-terminal fragments of the split DnaE intein from *Nostoc punctiforme* cyanobacterium,[10] respectively (Figure 1A). When co-expressed, *Npu*$DnaE_N$ and *Npu*$DnaE_C$ rapidly bind, self-splice, and create a peptide bond between the fragment N-terminal to *Npu*$DnaE_N$ and C-terminal to *Npu*$DnaE_C$ in a process that requires no exogenous cofactors or energy[10] (Figure 4A). When these proteins were transfected alone in mammalian cells, TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ had an ER fluorescence distribution, while *Npu*$DnaE_C$-Venus had a cytoplasmic and nuclear fluorescence distribution (Figure 4D,E,G, Supplementary Figures S2I,J,M and S3I,J,M) (n = 6/6 experiments). When TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and FRB-Venus were co-transfected in mammalian cells, rapamycin failed to induce translocation to the ER (Figure 4G-I, Supplementary Figures S2M–O and S3M–O) (n = 6/6 experiments). As expected, the co-localization coefficients generally changed less

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than 1% (Table 1, Supplementary Tables S1, S2). In contrast, when TM_{TLR4}-FKBP12-TM_{TLR4}-Ceru-*Npu*$DnaE_N$ and *Npu*$DnaE_C$-Venus were co-transfected, the cyan and yellow fluorescence were co-localized with a pearson's correlation coefficient between 0.844 and 0.902 (Table 1, Supplementary Tables S1, S2), suggesting that the *Npu*$DnaE_N$ is facing the cytoplasm (Figure 4E,F, Supplementary Figures S2J,K and S3J,K) (n = 6/6 experiments). Thus, adding an N-terminal TM can effectively reverse the orientation of the transmembrane protein. The TM is not expected to be cleaved like a signal peptide because it does not have a signal peptidase cleavage site.

Using a synthetic biology approach to assemble transmembrane proteins by parts, we confirm that the only requirement for creating a transmembrane protein is a transmembrane helix. Furthermore, when the transmembrane helix is placed at the N-terminus, it targets the protein to the ER and also reverses the orientation of our synthetic transmembrane proteins. The modular approach used in this study can be applied to engineer other synthetic transmembrane proteins with varied functions and biological applications. For instance, using this knowledge we can engineer synthetic transmembrane proteins by parts to investigate the fundamental mechanisms for transducing a signal across a cellular membrane such as induced oligomerization changes or more subtle conformational changes as in the piston model.[21] Lastly, the precise orientation of protein components relative to the transmembrane helix will likely further depend on protein folding and charge as these have been shown to be important in the literature.[8] In particular, well-folded N-terminal domains are less likely to flip orientations, while positive charged sequences are more likely to face the cytoplasm.[8]

METHODS

Plasmids. Arf1-CFP, FKBP12, FRB, *Npu*$DnaE_N$, and *Npu*$DnaE_C$ were from Addgene (Cambridge, MA) plasmids 11381, 15285, 15289, 12172 and 15335, respectively. STIM1-mRFP was subcloned from STIM1-YFP (Addgene plasmid 19754). TM_{TLR4} (uniprot accession no.: O00206), TM_{PDGFR} (uniprot id: P09619), and KDEL were created by overlap PCR and inserted in the pCfVtx3.[22] FKBP12, FRB, *Npu*$DnaE_N$, and *Npu*$DnaE_C$ were amplified and inserted into pCfVtx3.[22] All subsequent fusion proteins were subcloned as previously described.[22−24]

Cell Culture and Transfection. COS7, HeLa, and NIH3T3 cells were maintained in Dulbecco's Modified Eagle's Medium containing 25 mM D-glucose, 1 mM sodium pyruvate, and 4 mM L-glutamine (Invitrogen, Carlsbad, CA) with 10% supplemented fetal bovine serum (FBS) (Sigma Aldrich, St. Lois, MO) in T5 flasks (37 °C and 5% CO_2). Cells were passaged at 95% confluency using 0.05% trypsin with EDTA (Sigma) and seeded onto 35 mm glass-bottom dishes (MatTek, Ashland, MA) at 1:15 dilution. Cells were transiently transfected using Lipofectamine 2000 according to manufacturer's protocols (Invitrogen).

Illumination and Imaging. Imaging was performed using an inverted IX81 microscope with a Lambda DG4 xenon lamp source and QuantEM 512SC CCD camera with a 60x oil immersion objective (Olympus). Filter excitation (EX) and emission (EM) bandpass specifications were as follows (in nm): CFP (EX: 438/24, EM: 482/32), YFP (EX: 500/24, EM: 542/27), RFP (EX: 580/20, EM: 630/60) (Semrock). Image acquisition was done with MetaMorph Advanced (Olympus).

Co-localization Analysis. To quantify the comparison of co-localization between different fusion proteins, commonly used co-localization coefficients have been calculated (Tables 1). A number of methods to measure co-localization have been reported.[25−28] However, not all available measures are suitable for assessing the co-localization of non-uniform, discrete protein localizations within a cell.[25,28] Here we report Pearson's coefficient (PC), which is commonly used to compare the occurrence of co-localization.[25] The accuracy of this co-localization analysis relies heavily on the signal-to-noise ratio, which can be improved post-acquisition using background subtraction and thresholding. In order to avoid user bias, Costes' automatic thresholding was additionally used to calculate thresholded PC values.[29] Van Steensel's cross correlation coefficients (CCF) were also reported to provide PC values with a measure of statistical significance.[28] The *Colocalization_Finder* plug-in from ImageJ was used to help visualize the regions of co-localization.

ASSOCIATED CONTENT

Supporting Information

Supplementary figures, tables, and videos. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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