Quantitative characterization of Tetraspanin 8 homointeractions in the plasma membrane

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Abstract

The spatial distribution of proteins in cell membranes is crucial for signal transduction, cell communication and membrane trafficking. Members of the Tetraspanin family organize functional protein clusters within the plasma membrane into so-called Tetraspanin-enriched microdomains (TEMs). Direct interactions between Tetraspanins are believed to be important for this organization. However, studies thus far have utilized mainly co-immunoprecipitation methods that cannot distinguish between direct and indirect, through common partners, interactions. Here we study Tetraspanin 8 homointeractions in living cells via quantitative fluorescence microscopy. We demonstrate that Tetraspanin 8 exists in a monomer-dimer equilibrium in the plasma membrane. Tetraspanin 8 dimerization is described by a high dissociation constant (K_d = 14700 ± 1100 Tspan/μm²), one of the highest dissociation constants measured for membrane proteins in live cells. We propose that this high dissociation constant, and thus the short lifetime of the Tetraspanin 8 dimer, is critical for Tetraspanin 8 functioning as a master regulator of cell signaling.

Introduction

Tetraspanins (Tspans) are widely expressed membrane proteins of 200 – 350 amino acids that cross the cell membrane four times. They are involved in diverse cellular processes, such as cell proliferation, trafficking, signaling, adhesion, spreading, cell-cell fusion, pathogen entry, and diseases such as cancer. This variety of biological processes is a direct result of the molecular interactions of Tspans with many different partner proteins, such as transmembrane receptors, signaling proteins, adhesion molecules, enzymes, and other Tspans. Thereby, Tspans organize the cell membrane and regulate the activity of other membrane proteins.
The functional units that Tspans form with their partner proteins in the membrane are called “Tetraspanin-enriched microdomains” (TEMs). TEMs were formerly described as large assemblies of multiple members of the Tspan family and their partner proteins. Within these TEMs, different members of the Tspan family are proposed to be associating with each other, thereby bringing their partner proteins in close proximity to form large islands of functional signaling clusters. Furthermore, Tspans are known to engage in a variety of Tspan-lipid interactions, e.g. with cholesterol or gangliosides, which are believed to be important for some biological processes, such as viral entry. Recent super-resolution and electron-microscopy studies have refined this picture, by revealing Tspan nanoclusters with only ~ 120nm diameters that contain a few copies of a single Tspan species.

The unique ability of TEMs to control a broad range of biological functions makes them an interesting target to study. A classification of primary and secondary interactions has been proposed for Tspans. Primary interactions involve a Tspan molecule and partner proteins and are believed to be mediated by contacts in the extracellular or intracellular domain of the Tspan protein. Common partner proteins include members of the integrin or immunoglobulin superfamilies. Secondary interactions are weaker and occur between individual members of the Tspan family, and include Tspan homointeractions. Specific interactions between Tspans have also been suggested, but they are not well understood.

Here we sought to quantitatively characterize Tspan homointeractions, the ‘molecular glue’ of the Tspan web. Most of our current knowledge about Tspan interactions is based on non-quantitative studies using techniques such as co-immunoprecipitation, that do not directly report on interactions within the native membrane environment. We use quantitative fluorescent microscopy and live cells to investigate homooligomerization of Tetraspanin 8 (Tspan8), the first Tspan molecule to be ever cloned. We determine both its oligomeric state, and the thermodynamics of association, which allows us to refine the current TEM model.

Materials and Methods

Plasmid constructs

The genes encoding for the Tspan8 constructs, Tspan8-mTurq and Tspan8-eYFP, were subcloned into the pcDNA3.1(+) vector. All Tspan8 constructs had either eYFP or mTurquoise attached to their C-
terminus via a flexible 15 amino acid (GGS)₅ linker. The point mutations C7A, C74A, C75A, C83A, and C232A were introduced into Tspan8-mTurq and Tspan8-eYFP plasmids to create the Tspan8-Δpalmitoylation versions.

The plasmids were amplified in competent DH5α E. coli cells and purified using Qiagen’s HiSpeed Plasmid Midi Kit (# 12643). All plasmids used in this work were verified by sequencing (Genewiz, Frederick, MD).

**Cell culture and transfection**

HEK293T cells, purchased from ATCC, were maintained at 37 °C, 5% CO₂, in Dulbecco’s modified eagle medium (Gibco, #31600034) supplemented with 10% fetal bovine serum (HyClone, #SH30070.03), 20 mM D-Glucose and 18 mM sodium bicarbonate. For imaging experiments, cells were seeded in 35 mm glass coverslip collagen coated Petri dishes (MatTek, P35GCOL-1.5-14-C) at a density of 2.5x10⁵ cells per dish. Transient transfection was performed 24 hours after seeding using Lipofectamine 3000 (Invitrogen, #L3000008) according to the manufacturer’s protocol. For FIF experiments, the cells were transfected with 1 – 2 μg of DNA encoding Tspan8-eYFP. For FRET experiments, the cells were co-transfected with 5 – 6 μg of mixtures of DNA encoding Tspan8-mTurquoise and Tspan8-eYFP or the mutants in varying ratios (1:3 – 1:1). For the spectral unmixing of co-transfected cell images in FRET experiments, single-transfections were performed using 3 μg of plasmid DNA. 12 hours after transfection, the cells were rinsed twice with phenol-red free, serum free starvation media and were then serum starved for at least 12 hours.

**Microscope imaging**

Fürster Resonance Energy Transfer (FRET) experiments were performed according to the published Fully Quantified Spectral Imaging-FRET (FSI-FRET) protocol.(26) Prior to imaging, the cells were subjected to osmotic stress with a pre-warmed (37 °C) 1:9 serum-free media:diH₂O, 25 mM HEPES solution. This reversible osmotic swelling was necessary because the cell membrane is normally highly ruffled and its topology in microscope images is virtually unknown (26). The reversible osmotic stress eliminates these wrinkles and allows to convert effective 3D protein concentrations into 2D receptor concentrations in the plasma membrane (26). After swelling, the cells were allowed to stabilize for 10 min at room temperature. FSI-FRET imaging was performed using a two-photon microscope equipped with the OptiMis True Line Spectral Imaging system (Aurora Spectral Technologies, WI). The details of the
microscope have been described previously (27, 28). For each cell, two microscope scans were acquired – a direct acceptor emission scan using an excitation wavelength of \( \lambda = 960 \text{ nm} \) and a donor plus sensitized acceptor emission scan using an excitation wavelength of \( \lambda = 840 \text{ nm} \). Each scan produced an image of 300x440 pixels, where every pixel contains a full fluorescence spectrum in the range of 420 – 620 nm. Each imaging session did not exceed 2 hours.

Fluorescence Intensity Fluctuations Spectroscopy (FIF) experiments were performed with a TCS SP8 confocal microscope (Leica Biosystems, Wetzlar, Germany) equipped with a HyD hybrid detector. Images (1024x1024, 12bit) were acquired in photon counting mode with a scanning speed of 20 Hz and a 488 nm diode laser excitation. The emission spectra of eYFP were collected from 520-580 nm.

**Fluorescent protein standards**

To convert measured intensities to concentration, the FSI method relies on calibrations with purified fluorescent protein solutions of known concentration.(26) Soluble monomeric eYFP and mTurquoise fluorescent proteins with an N-terminal 6x His tag were expressed and purified as described in (29). The purified fluorescent protein solutions were buffer exchanged into PBS buffer with 10 kDa molecular weight cutoff concentrators (Pierce, #88527). The stock solutions were filtered with a 0.2 \( \mu \text{m} \) syringe filter and diluted to 50% and 75% fluorescent protein solution standards in the micromolar range. The absorption of the eYFP (\( \lambda_{\text{abs}} = 514 \text{ nm} \)) and mTurquoise (\( \lambda_{\text{abs}} = 434 \text{ nm} \)) diluted and undiluted solution standards were measured in a NanoDrop 2000C (Thermo Scientific) with a 1 cm path length quartz cuvette. The concentrations of the solution standards were calculated using Lambert-Beer's law. Molar absorption coefficients of 83400 mol/cm and 30000 mol/cm were used for the calculation of eYFP and mTurquoise concentrations, respectively.

**Fluorescence Intensity Fluctuations Spectroscopy (FIF) analysis**

The basolateral membrane of HEK 293T cells was imaged with a Leica TCS SP8 confocal microscope. The images were analyzed using the FIF software described in (33). The basolateral membrane outlined by the researcher was segmented into 15x15 pixel segments by a moving squares method. The resulting segments were analyzed using the brightness and concentration calculator in the FIF software (33). The molecular brightness \( \varepsilon \) is related to the variance of fluorescence \( \sigma^2 \) across segments and the average intensity \( <I> \) by

\[
\varepsilon = \frac{\sigma^2 - \sigma_0^2}{<I>}
\]  
(1)
where $\sigma_D^2$ is the variance of the noise of the detector. For a photon-counting detector, the brightness is (34):

$$\varepsilon = \frac{\sigma^2}{\langle I \rangle} - 1$$  \hspace{1cm} (2)

**Calculation of dissociation constants**

Tspan dimerization was described by a two-state thermodynamic model describing equilibrium between Tspans in the monomeric (m) and dimeric (d) states:

$$m + m \rightleftharpoons d$$  \hspace{1cm} (3)

Thus, the equilibrium dissociation constant, $K_d$, is given by

$$K_d = \frac{[m]^2}{[d]}$$  \hspace{1cm} (4)

Since the total Tspan concentration is given by

$$[T] = [m] + 2[d] = [m] + \frac{2[m]^2}{K_d}$$  \hspace{1cm} (5)

we can solve this quadratic equation for $[m]$ as a function of $K_d$ and $[T]$ and take the positive root as its solution:

$$[m] = \frac{\sqrt{1 + \frac{4[T]}{K_d}} - 1}{4}$$  \hspace{1cm} (6)

Using equations (5) and (6), one can calculate the fraction of dimeric membrane protein $f_d$ as a function of $K_d$ and $[T]$:

$$f_d = 1 - f_m = 1 - \frac{[m]}{[T]} = 1 - \frac{\sqrt{1 + \frac{4[T]}{K_d}} - 1}{\frac{4[T]}{K_d}}$$  \hspace{1cm} (7)
The fraction of dimeric proteins can be linked to the FRET efficiency due to specific dimerization, $E_D$, according to (26, 30, 31)

$$E_D = f_d x_A \bar{E}$$

$x_A$ in eq. 8 is the fraction of acceptors, which is measured for each cell (since both the donor and the acceptor concentration are measured in each cell). $x_A$ accounts for the fact that each cell has different expressions of donors and acceptors, and FRET depends on their relative expressions. It also accounts for the fact that only some of the dimers have both a donor and an acceptor and therefore contribute exclusively to the measured FRET efficiency.

$\bar{E}$ in eq. 8 is the “intrinsic FRET”, the FRET efficiency in a dimer containing a donor and an acceptor fluorophore. It is a structural parameter, which depends only on the separation, orientation, and dynamics of the two fluorescent proteins in the dimeric complex and is constant for each dimer. Under the assumption that the fluorescent proteins can rotate freely, the intrinsic FRET $\bar{E}$ can be related to the inverse sixth power of the distance between the donor and acceptor fluorescent proteins $d$.

$$\bar{E} = \frac{1}{1 + \left( \frac{d}{R_0} \right)^6}$$

where $R_0$ is the Förster radius of the eYFP-mTurquoise FRET pair, 54.5 Å.

The assumption of free rotation can be justified since the fluorescent proteins are attached to the Tspan molecule via a flexible 15 amino acid (GGS)$_5$ linker. However, this assumption might not hold in all cases.

The measured FRET efficiency has contributions due to 1) specific dimerization of Tspans, $E_D$, and 2) stochastic FRET. Stochastic FRET, which is significant within the two-dimensional plasma membrane, occurs when a donor and an acceptor approach each other by chance within close proximity (<100 Å). The stochastic FRET contribution depends on the unknown values of $K_d$, $\bar{E}$, and the measured acceptor concentrations, [A]. (32) A Stochastic FRET library for various $K_d$, $\bar{E}$ and [A] values has been tabulated and verified in previous published work. (32) This stochastic FRET library, along with the specific FRET contributions given by Eq (8), was used to calculate theoretical FRET efficiencies for different $K_d$ and $\bar{E}$.
values. The experimentally measured FRET efficiencies for the measured Tspan concentrations are then compared to the theoretical FRET efficiencies for different $K_d$ and $\hat{E}$ values. The best fit $K_d$ and $\hat{E}$ values are determined using a non-linear least square algorithm as described. (32)

Results

Tspan8 homooligomerization within the plasma membrane

We used Förster Resonance Energy Transfer (FRET) to characterize homointeractions between Tspan8 proteins, using the Fully Quantified Spectral Imaging-FRET (FSI-FRET) methodology that yields both thermodynamic and structural information about membrane protein dimers and oligomers. (26) The experiments are designed such that membrane protein concentrations are varied over a wide range, and are measured directly, along with the FRET efficiencies. Thus, the donor-labeled, acceptor-labeled and total membrane protein concentrations are known for each cell. The acceptor fraction, which is proportional to the measured FRET efficiency, is also known for each cell. FRET reports both on the propensities for interactions and on oligomer structure; the two contributions are decoupled in the FSI-FRET method for comprehensive quantitative interpretation of the FRET results.

The Tspan8 molecules were tagged with fluorescent proteins (either mTurquoise or eYFP, a FRET pair) at their C-termini and expressed in HEK293T cells (Figure 1 a). The cells were imaged with a 2-photon microscope to acquire the full fluorescence emission spectra for each pixel at $\lambda_1 = 840$ nm in which the donor, mTurquoise, is primarily excited (FRET scan) and at $\lambda_2 = 960$ nm in which the acceptor, eYFP, is primarily excited (acceptor scan). The fluorescent emission spectra of pixels belonging to the cell membrane are then spectrally unmixed and analyzed to obtain i) the donor concentrations, ii) the acceptor concentrations, and iii) the FRET efficiencies in hundreds of live cells. The analysis process and underlying equations are described in detail in (26). Figure 1 b shows an exemplary cell and the spectra acquired in the FRET and acceptor scans. Then, the experimental emission spectra are unmixed as a linear sum of three contributions: the fluorescence of the donor, the fluorescence of the acceptor, and a background contribution as described in (26).

The results of the analysis of 466 individual membrane regions are shown in Figure 2a-b. Figure 2a shows the FRET efficiencies as a function of acceptor concentrations. The FRET efficiencies increase as a function of acceptor concentrations, suggesting a concentration-dependent Tspan homoassociation in the cell membrane. Figure 2b shows the expression of Tspan8-eYFP versus the expression of Tspan8-mTurquoise; these data yield both the ratio of donors and acceptor and the total Tspan8 expression,
needed for quantification of the interactions. The high variability in receptor expression levels (Figure 2a and b), as well as in donor:acceptor ratios (Figure 2b), is due to transient transfection of HEK293T cells and is the strength of the FSI method to ultimately produce the best-fit binding curves. (26)

Next, we sought to interpret the measured FRET binding curves and determine association constants by fitting the FRET data to a protein association model. However, the oligomer size must be known in order to perform the fit. In the literature, oligomer sizes ranging from dimers (hypothesized to occur via specific interactions) to higher-ordered oligomers (involving promiscuous interactions) have been proposed. (14, 17, 35-38) A limitation of FRET is that the oligomer size is not measured directly but is inferred from fits to different association models. Therefore, we performed fluorescence intensity fluctuation (FIF) experiments to directly assess the oligomer size (Figure 1c). (33) In these experiments, the basolateral membrane of HEK293T cells expressing Tspan8 tagged with eYFP was imaged (Figure 1c,i). The molecular brightness, which scales with the oligomer size, was calculated in small sections of the plasma membrane by constructing histograms of fluorescence intensities in these sections (Figure 1c,ii). The brightness is calculated in each section as the ratio of the variance and average intensity. (33) Then, histograms of the calculated molecular brightness values are created as shown in Figure 1c,iii. In previous work, we have collected data for LAT (39, 40), a monomer control, and E-cadherin (41), a dimer control. Their histograms are shown in Figure 3. As expected, the maximum of the molecular brightness for the dimer control E-cadherin (2.0 ± 0.25) occurs at twice the maximum position of the monomer control LAT (1.0 ± 0.25). (41) The maximum value of Tspan8 (1.5 ± 0.25) falls between LAT and E-cadherin, suggesting that it exists in a monomer-dimer equilibrium. Higher-ordered oligomers (n>2) would be expected to 1) have a maximum position of n times the maximum position of LAT and 2) have the whole molecular brightness distribution shifted to values higher than E-cadherin. As we do not observe this, we conclude that Tspan8 does not form higher-order (n>2) oligomers in the plasma membrane but exists in either a monomeric or a dimeric form.

Based on this result, we analyzed the FRET data in Figure 2a and b with a dimerization model (equation 3). Dimer formation is characterized by i) the dissociation constant $K_d$ and consequently the dimeric fraction as a function of Tspan8 concentration and ii) the structural parameter $\tilde{E}$ (or “intrinsic FRET”), which depends on the positioning of the fluorescent proteins in the Tspan8 dimers. However, the measured FRET data have two contributions: i) FRET due to specific Tspan8 dimerization and ii) FRET as a result of random approach (within 100 Å) of two Tspan8 molecules in the confined two-dimensional plasma membrane. The latter is also known as “stochastic” or “proximity” FRET and is well understood.
and can be corrected for. Its contribution to the measured FRET efficiency is shown in Figure 2a with the solid line. The measured FRET efficiencies lie above the proximity FRET line, indicating that Tspan8 is self-associating in the plasma membrane, confirming the FIF results. Following a verified two-step fitting procedure, as outlined briefly in Materials and Methods, we fit the data to different dimerization models, varying \( K_d \) and \( E^\theta \). The model with the smallest mean squared error best represents the experimental data. For Tspan8 homodimerization we find a dissociation constant of \( 14700 \pm 1100 \) Tspan/\( \mu m^2 \) and an \( E^\theta \)-value of 0.60 ± 0.03. Another way to characterize dimer formation is through its dimerization curve (dimeric fraction as a function of Tspan8 concentration). The Tspan8 dimerization curve depends on \( K_d \) and the Tspan8 concentration, and is calculated using equation 7. The best fit dimerization curve (solid blue line) to the data (blue circles) is shown in Figure 4a. The dashed lines indicate the 68% confidence intervals of the fit.

Tspans are posttranslationally modified via glycosylation of residues in the extracellular region and palmitoylation of intracellular juxtamembrane cysteine residues (13, 44, 45). It has been suggested that Tspan homointeractions are affected by the palmitoylation (12, 46). To explore the significance of palmitoylation in the interactions that we measured, we mutated all juxtamembrane cysteine residues (C7, C74, C75, C83, and C232) to alanine (Tspan8-Δpalmitoylation) to completely block palmitoylation. The FRET data as a function of acceptor concentration and the FIF data are shown in Figure 2c-d and Figure 3, respectively. The peak of the molecular brightness histogram is at 1.5 (± 0.25), suggesting that the Tspan8-Δpalmitoylation construct also exists in a monomer-dimer equilibrium just as the WT. The FRET data were thus fitted to the same monomer-dimer model as the WT and the fitted dissociation constant and the \( E^\theta \)-value for the palmitoylation deficient protein are \( 14500 \pm 600 \) Tspan/\( \mu m^2 \) and 0.86 ± 0.02, respectively. We thus observed that the dissociation constant was not affected by the removal of cysteine residues that are palmitoylated (p-value = 0.83; 2-tailed t-test). This suggests that hydrophobic interactions mediated by palmitoyl chains are neither necessary, nor the driving force for Tspan8 homointeractions. The best fit dimerization curve for the mutant is shown in Figure 4b. The \( E^\theta \) value is larger for the palmitoylation-deficient mutant (p-value < 0.0001; 2-tailed t-test). Most likely, this is due to repositioning of the attached fluorescent proteins in the Tspan8 dimer, as a result of decreased steric hindrance caused by the palmitoyl membrane anchors in the wild-type protein or structural rearrangement in the dimer interface.

Discussion
Tspan homooligomers are believed to be fundamental building blocks of the Tspan web. So far, Tspan interactions have only been characterized by the strength of the detergent that they can withstand in co-immunoprecipitation experiments. In this study, we have quantitatively investigated homoassociation of Tspan8 in the plasma membrane. The results are summarized in Table 1. For the first time, we directly measured the oligomer size of Tspan8 in living cells and found that Tspan8 exists as either monomers or dimers (brightness value $\varepsilon = 1.5 \pm 0.25$), which are likely in equilibrium with each other, in accordance with the law of mass action. This homodimerization is described by a dissociation constant of $14700 \pm 1100$ Tspan/\(\mu\)m\(^2\). Compared to other membrane proteins, such as receptor tyrosine kinases (RTKs) which have dimerization constants ranging from $12 \sim 3235$ RTK/\(\mu\)m\(^2\), Tspan8 dimerization is weak. Furthermore, we found that, contrary to the literature (12, 13, 22), dimerization is not significantly affected by palmitoylation of juxtamembrane cysteine residues.

As homodimers seem to play an important role in Tspan function, we calculated what fraction of Tspans would be present in a dimeric form as a function of Tspan concentrations (Figure 4). To estimate the dimeric fraction in a cell, we searched the literature for Tspan expression levels. Tspans expression varies considerably by cell type and by family members. In blood, Tspan29 expression is one of the highest, while Tspan9 expression is one of the lowest. Specifically, Tspan29 expression is $38000 \sim 65000$ copies per platelet, while Tspan9 expression is $2800$ copies per platelet. A human platelet is reported to have a surface area of $15 \sim 30$ \(\mu\)m\(^2\). Thus, the expression range of Tspans in human cells varies between $\sim 90 \sim 4500$ Tspan/\(\mu\)m\(^2\). This would correspond to a fraction of $\sim 0 \sim 40\%$ dimeric Tspan (Figure 4), which increases with concentration, as expected from the law of mass action.

Specific Tspan homodimerization could be the molecular mechanism that underlies Tspan function. Tspan8 has one of the highest dissociation constants quantitatively measured in living cells, and thus it exhibits one of the weakest self-interaction propensity among membrane proteins. Association rates are believed to be diffusion limited, and thus similar for all proteins, while dissociation rates reflect the strength of the interactions. Thus, the measured high dissociation constant implies high dissociation rates and therefore short lifetimes of Tspan dimers. A short dimer lifetime may be an indispensable property of a protein that regulates signaling. Indeed, master organizers and regulators of the cell membrane, like Tspans, likely need to constantly adjust to cellular signals by remodeling their microenvironment.

It is generally believed that the strength of heterointeractions between Tspans and other membrane proteins are stronger than Tspan homointeractions (5, 14). The weak homodimerization that we
measure here is entirely consistent with this view. Therefore, we envision that Tspans organize other molecules around them into patches which are longer-lived compared to the lifetime of a Tspan dimer. These Tspan nanoclusters have been experimentally observed and consist of only a few Tspan molecules of the same family and their partner proteins.(23) These patches, which are associating and fusing transiently via Tspan homodimerization, are likely the building blocks of TEMs.

One can imagine the assembly of a TEM starting by the association of a Tspan8 molecule with its partner protein, as this is the stronger, longer-lived interaction. These TEM nanoclusters then associate via Tspan8 homodimerization to form larger TEMs as shown in Figure 5. In the case of exclusive Tspan8 homodimerization and a 1:1 Tspan8:partner protein ratio (Figure 5 a), a TEM could maximally contain 2 Tspan8 molecules and 2 partner proteins. The fast dissociation rates would allow Tspan8 to organize its different partner proteins. However, such a scenario is unlikely, as larger TEMs have been reported.(62-64) The formation of larger TEMs can be achieved only if there are additional interactions that bring the patches together. These could be heterointeractions between Tspan8 and other Tspans, for instance (Figure 5c). The latter is consistent with reports that TEMs contain not just one, but multiple Tspan species (63, 64). It is also possible that the molecules in the patches bind more than one Tspan, while all Tspans are capable of homodimerization (Figure 5b). The weak interactions that stabilize the TEMs are likely sensitive to the lipid environment, and thus the spatio-temporal changes in membrane lipid composition can regulate TEM organization. Such lipid-induced effects have been reported in the literature, consistent with the model that we propose here. For instance, the presence or absence of the ganglioside GM3 in Tspan29 microdomains has been shown to greatly influence the integrin-FGFR cross talk by altering FGFR tyrosine phosphorylation. (65)

As Tspans regulate a variety of cellular processes, it is important to acquire a better understanding of the molecular mechanisms that underly their function. Here we focus on the direct interactions between Tspan molecules. Based on the results, we refine the current model of the TEM, and we argue that the high dissociation constant measured here is critical for TEM organization and signaling. Future studies to characterize the kinetics of Tspan interactions, as well as the interaction thermodynamics and stoichiometries of other members of the Tspan family will further enhance our knowledge of Tspan biology.

Data Availability

All data are available upon request.
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Tables

Table 1: Characterization of Tspan8 and Tspan8-Δpalmitoylation homodimerization. \( K_d \) is the dissociation constant and \( \bar{E} \) a structural parameter that depends on the separation of donor and acceptor fluorophore.

| Oligomeric state       | \( K_d \)     | \( \bar{E} \)     |
|------------------------|---------------|------------------|
| Tspan8                 | 14700 ± 1100 Tspan/\( \mu m^2 \) | 0.60 ± 0.03 |
| Tspan8-Δpalmitoylation | 14500 ± 600 Tspan/\( \mu m^2 \) | 0.86 ± 0.02 |

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Figures

Figure 1: The fluorescence techniques Fully Quantified Spectral Imaging-FRET Forster Resonance Energy Transfer ((FSI-FRET) and Fluorescence Intensity Fluctuation (FIF) Spectrometry, as applied to Tetraspanin 8 (Tspan8). a i) The Tspan8 construct linked to a fluorescent protein (either mTurquoise or eYFP) used in the experiments. a ii) The fluorescence emission spectra of mTurquoise (blue) and eYFP (green) (a FRET pair). b) The FSI method: i) A HEK293T cell expressing Tspan8-mTurquoise and Tspan8-eYFP. Pixels within a selected membrane region (yellow polygon) are chosen for further analysis. ii), iii) A single pixel’s fluorescence emission signal (black circles) is decomposed as a linear sum (black line) of donor (blue line), acceptor (green line), and background (purple) contributions. c) The FIF method (33): i) The basolateral membrane of a HEK293T cell expressing Tspan8-eYFP is imaged and an area of interest (red polygon) is chosen to be analyzed. ii) The software (33) segments the chosen area by a moving-squares method. iii) The distribution of fluorescence intensity per pixel (circles) of a randomly chosen segment, together with its Gaussian fit (black line). The mean and width of the Gaussian are used to calculate the brightness for each segment. Histograms of these brightness values for thousands of segments derived from hundreds of cells are shown in Figure 3.

Figure 2: FRET data for WT-Tspan8 and its Δpalmitoylation mutant. a) and c) FRET as a function of acceptor concentration. Data for WT-Tspan8 is shown in blue and data for Tspan8-Δpalmitoylation is in orange. Every data point corresponds to one region in a cell, as shown in Figure 1. Up to four regions are typically chosen per cell. The solid black line represents proximity FRET which is due to random approach of donor and acceptor labeled Tspans (within 100 Å). b) and d) Expression of the donor Tspan8-mTurq and the acceptor Tspan8-eYFP.

Figure 3: FIF data for WT-Tspan8 (blue) and its Δpalmitoylation mutant (orange). The molecular brightness distributions for both fall between the monomer control LAT (black) and the dimer control E-cadherin (green). This data suggests that both Tspan constructs are present in either monomeric or dimeric form in the plasma membrane.

Figure 4: Dimeric fraction as a function of total Tspan8 concentration for WT-Tspan8 (a) and its Δpalmitoylation mutant (b). The solid line is the best fit and the circles are the binned experimental data, (error bars: standard error). The dashed lines show the 68% confidence interval to the fit.

Figure 5: Models for the assembly of TEMs. Circles represent Tspan proteins, stars depict partner proteins. a) Tspan8 partner proteins (red and teal) can only bind one Tspan molecule (blue). The fast dissociation rates allow Tspan8 to bring different partner proteins in proximity, but only small TEMs can form. b) Tspan8 partner protein (red and teal) can bind more than one Tspan molecule (blue). The formation of large TEMs is possible. c) Tspan 8 (blue) can associate with other Tspans (green), but only one Tspan can bind to its partner protein (red, grey). Large TEMs can form. Within TEMs Tspan8 explores a monomer-dimer equilibrium with fast dissociation rates enabling dynamic remodeling of the TEMs.
