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A study of the C-terminus in prestin by designing cleavable constructs

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ABSTRACT

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The membrane protein prestin plays a central role in the mammalian auditory system by enabling Outer Hair Cells (OHCs) in the cochlea to actively respond to electrical signals. Prestin belongs to the SLC26A family of membrane proteins, which all have a large cytosolic terminus containing a conserved region known as the STAS domain (Sulfate Transporters and Anti-Sigma factor antagonist), whose function remains unknown. In this work, we engineered prestin by inserting three types of short peptides, flexible, rigid and cleavable linkers, into different positions in the C-terminus and tested prestin’s functionality, self-interactions, and lateral mobility in the membrane. First, we inserted short peptides between the last trans-membrane domain and the STAS domain. We found that the prestin’s functionality is inhibited, and the self-interaction is significantly decreased. The FRAP data revealed that the mobility of prestin is altered by inserting different types of linkers before the STAS domain. Second, we inserted cleavable linkers in the disordered region within the STAS domain. The data revealed that prestin inserted with linkers between 596aa and 597aa, and between 620aa and 621aa, can successfully locate to the membrane and retain NLC function. Next, by using advanced fluorescence microscopy and immunoprecipitation, we discovered that prestin is confined by a binding site located between the 597aa and the 620aa. Together, these data suggest that the interface
between the C-terminus of prestin, STAS domain, and the last TMD is crucial to prestin’s function, self-interactions, and mobility, and that a binding site locates in the disordered region of STAS domain that can influence prestin’s lateral mobility.
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Chapter 1

Introduction

The mammalian auditory system has an amazing complexity of components, featured by its exquisitely high sensitivity and frequency selectivity, to detect and amplify sounds[1]. Briefly, collected by the external ears, sounds travel down the auditory canal to the eardrum, where the sound waves get converted into mechanical vibrations. The mechanical vibrations then transmit through the middle ear bones and enter the cochlea in the inner ear. In the cochlea, the organ of Corti actively amplifies the mechanical vibrations and analyzes the vibration frequency. Next, the mechanical vibrations are modulated and transduced into electrical signals which are further sent to the brain through auditory nerves, where specific sounds are recognized [2].

1.1 Cochlea physiology

The mammalian cochlea is a duct filled with fluid spiraling a bony axis, modiulus, and coiled into a compartment within the temporal bone on either side of head [1, 2], shown in Figure 1.1a. The typical length of an uncoiled human cochlea is \( \sim 34 \) mm. The cochlea has two openings to the middle ear, an oval and a round window. Sound is transmitted through the outer and middle ear and enter the cochlea from the oval window. The round window, covered by a flexible membrane, serves as an outlet to allow the vibration of cochlea fluid, which finally stimulates the sensory hair cells.
of the cochlea. The cochlea duct is partitioned into three compartments, the scala vestibuli, scala media and scala tympani, see Figure 1.1b.

Figure 1.1: The structure of the mammalian auditory system. a) The cochlea in the inner ear. Sound waves are collected by the outer ear, and travel through the middle and enter the cochlea. b) The cross-section of the cochlea. It contains three fluid-filled compartments, Scala vestibuli, Scala media and Scala tympani. c) The organ of Corti. It contains the gel-like tectorial membrane, hair cells sitting on top of basilar membrane, and other cells supporting the structure. Figure taken from reference [3].

The scala vestibuli, scala media and scala tympani are separated by Reissners membrane and Basilar Membrane (BM). On Basilar Membrane sits the sensory or-
gan of Corti (OC). The scala vestibuli and scala tympani are filled with perilymph, which contains a high concentration of Na\(^+\) and low concentration of K\(^+\). However, the scala media provides a specialized ionic environment, endolymph, containing a high concentration of K\(^+\) and low concentration of Na\(^+\), for the mechanotransducting membranes of the sensory hair cells [1, 2].

In the mammalian cochlea, the organ of Corti sits on the basilar membrane and is the essential sensory receptor of hearing. It contains a gel-like tectorial membrane and different types of cells, including the essential sensory hair cells and other cell types supporting the structure. The OC is bathed in the characteristic fluid environment of the cochlea with the apex region of hair cells bathed in endolymph and the cell bodies bathed in perilymph. Due to the specialized ionic environment of endolymph, this certain placement can effectively save ATP for K\(^+\) ions going in and out of hair cells. Two classes of sensory hair cells, inner hair cells and outer hair cells, are arranged in rows along the organ of Corti. As the name indicates, hair cells are neuroepithelial cells with hair bundles, stereocilia, in the apical region specialized for mechanotransduction, and the basal region is specialized for releasing neurotransmitters. Inner hair cells (IHCs) have a goblet-shaped morphology, and there are \(\sim 3,500\) lied in a single row in each human cochlea. IHCs are dominant sensory cells which stimulate \(\sim 90\%\) of the afferent nerve fibers and with no efferent nerve coming in. Distinct from IHCs, outer hair cells (OHCs) are mainly connected with efferent nerve fibers and responsible for the active amplification of the mechanical signal [1, 2, 4].

The organ of Corti functions as a frequency analyzer in the cochlea. The mechanical vibration of the cochlea fluid caused by sound waves travels along the cochlea
duct and causes the upward and downward movements of the basilar membrane. The upward movement of BM bends the stereocilia of both hair cells towards the modiolus, and the deflection of stereocilia opens mechanosensitive cationic channels for the influx of $K^+$ ions to depolarize the cells. Conversely, the downward motion of BM drives the hair cells outward from the modiolus resulting in hyperpolarization. The mechano-electrical signal is amplified by OHCs during the process. The depolarization of the cell potential leads to the release of neurotransmitters at the basal region into the afferent synapse. The BM is a microscopic structure consisting of collagen fibers. The fibers become longer and smaller in diameter towards the apex, which reduces the stiffness of BM as it goes to the apex. This structural change of collagen fibers within the BM from the base to the apex makes BM function as a frequency analyzer [5]. Basically, the stiffness of the fibers corresponds to the frequencies, and that is, the stiffer the fibers are, the higher frequencies they vibrate best at. As the mechanical vibration consisting of different frequencies travels along the cochlea, the vibration of the BM becomes strongest at the point where it has a resonant frequency equal to that of the traveling sound. The resonance would dissipate the traveling vibration and stop it at this point. As a result, high frequency sounds vibrate the BM in the base and low frequency sounds vibrate the BM in the apex [1, 2, 4].

1.2 Outer Hair Cell and electromotility

OHCs are cylindrical, neuroepithelial cells with an average radius of 4-5µm. There are roughly ~11,000 OHCs in each human cochlea. OHCs lie in three rows along the organ of Corti [2]. The positive internal pressure inside OHCs and OHCs’ unique cytoskeleton structure result in the characteristic cylindrical shape[6, 7, 8]. The apex
of OHCs is bathed in endolymph and the cell bodies are freely bathed in perilymph for its electromotile properties. Though with a constant diameter, OHCs axial length is a function of position and it becomes longer from the base to the apex. Morphologically, OHCs can be divided into three distinct regions, the apical top with the mechano-transduction receptor stereocilia, the lateral wall and the basal region where the nucleus is located. There is a great deal of evidence showing OHCs are necessary for normal function of cochlea. In the absence of OHCs, though IHCs are intact, the hearing sensitivity is severely comprised [9] and so for the frequency selectivity[10].

![Diagram of OHC membrane response](image)

Figure 1.2 : The response of the OHC membrane under changing transmembrane potentials. The OHC would contract under depolarization and elongate under hyperpolarization. The generated force feeds back to the cochlea to actively amplify the signal. Figure taken from reference [2].

The role of OHCs (Figure 1.2) in the cochlea is for the active amplification of
the mechanical vibration. When the sound vibrates the BM to deflect the stereocilia of OHCs, OHCs get depolarized and hyperpolarized, which results from that the mechano-sensitivity cationic channels are being turned on and off. Unlike other cell types, OHCs can axially contract under depolarization and elongate under hyperpolarization. This electrical potential-driven motion is termed as electromotility [11]. The force and energy generated by OHCs positively feed back to the cochlea partition which strengthens the vibration. The salient features of OHCs electromotility include the following [4]: first, electromotility could take place in the absence of ATP and Ca^{2+} [12, 13]; Second, the electromotile response occurs in a cycle-by-cycle mode at a microsecond rate [14]; Third, the electromotility is non-linear [15].

In electromotile OHCs, a measurable charge movement across the membrane accompanies the change of the transmembrane potential [16, 17]. This gating charge leads to a transient gating current. Usually, in ion channels, the gating current is generated by a conformational change of a S4 α-helix, the voltage sensor, that moves positively charged residues. The movement of the voltage sensor is manifested by a bell-shaped non-linear capacitance (NLC), which is voltage-dependent[16, 17]. As electromotility and NLC are highly correlated, it is generally accepted that NLC serves as the electrical signature of the electromotility of OHCs.

The electromotility is generally considered to be produced by a voltage-sensing motor residing in the lateral wall [4]. The speed and sensitivity of the electromotile response suggest a direct biophysical mechanism, coupling of the sensor and motor elements, which means changing transmembrane voltage is directly converted to displacement. Moreover, the motor is considered to reside within the plasma membrane,
since the changing voltage is restricted to the bilayer and OHCs retain their electromotile function after the digestion of internal structures [18]. It is also considered that motor molecules interact with OHC cytoskeletons directly or indirectly, so that the electromotile responses of the motor molecules can cumulate and feed the energy back to the cytoskeleton resulting in the whole cell axial length changes. In 2000, a transmembrane protein, prestin, was identified to be the motor molecules responsible for the electromotility in OHCs [19].

Conceptionally, prestin should comprise at least two functional elements: the voltage sensor that detects the changing transmembrane potential, and the actuator that undergoes a conformational change to generate the electromotile response [4]. Currently, there are two proposed model mechanisms describing OHC electromotility. The first is the area motor model, shown in Figure 1.3. Area motor model describes prestin has two states, a short state and a long state, each of which has a different surface area [20, 21] and prestin can sense the changing transmembrane voltage and change between the short and long states to generate electromotile response. Prestin uses monovalent anions in the cytoplasm as the extrinsic voltage sensor [4]. Without binding with monovalent anions, prestin stays in the short state. Monovalent anions bind to specific sites in prestin, and are translocated across the membrane. Monovalent anions are moved towards extracellular side during hyperpolarization and towards cytoplasmic side during depolarization. The translocation of monovalent anions triggers conformational changes in prestin which results in prestin switching between the short state in depolarization and long state in hyperpolarization.

The second model is the membrane bending model, shown in Figure 1.4. The
Figure 1.3: The area motor model for electromotility. Prestin uses monovalent anions as an extrinsic voltage sensor for transmembrane potentials. Prestin molecules can change between the short state and long state under depolarization and hyperpolarization to contract and elongate the cell. Figure taken from reference [4].

The membrane bending model describes that changing transmembrane potentials affects dipoles in the membrane causing changes in the membrane curvature and out-of-plane deformations [22]. The bending model does not specify whether prestin directly senses the transmembrane potential and undergoes a conformational change, but describes changes in the membrane curvature that results in the unfolding of plasma membrane when the OHC elongates. Studies have shown that the OHC plasma membrane does has an excess of membrane [23, 24].

Despite the difference between area motor model and membrane bending model, both models emphasize the interaction between prestin and cytoskeleton and prestin’s self-interactions that sense the transmembrane voltage and generate forces to contract and elongate cell bodies. For both models, the connection between prestin and cytoskeleton is important, since it’s hard to imagine that sole molecular conformational
Figure 1.4: A membrane bending model for electromotility. The alternations of transmembrane voltage cause the change of membrane curvature and deformation, which leads to axial length changes. Figure taken from reference [25].

changes could be significant enough to lead to cellular length changes. The study of the interaction between prestin and cytoskeleton and prestin’s self-interactions would provide supporting information and further improve both models.

1.3 Prestin and the STAS domain

The search for the molecular basis for OHCs electromotility has been for over decades. Due to several facts, an area motor model [20, 21] is proposed that the electromotility of OHCs is conferred by a membrane motor protein which could sense the changing potential across the membrane and accordingly change its state between contracted and elongated. In 2000, on the basis of subtractive cloning strategy between OHCs and IHCs, Dallos and co-workers managed to find a cDNA that is specifically expressed in OHCs and termed Prestin [19]. Prestin belongs to a family of anion transporters,
the solute carrier protein 26A (SLC26A) [26]. Expression of Prestin in a heterologous system enables the cells with all the hallmarks of the OHC [4]. First, it confers the transfected cells with NLC, which has similar characteristics to those observed in OHCs [19]. Second, Prestin-expressed cells are electromotile [19] with the motility magnitude approaching 0.2 µm. Third, force measurement experiments showed that prestin could generate mechanical forces [27]. Finally, the prestin-mediated electromotility is reciprocal [27].

Prestin is a transmembrane protein consisting of 744 amino acids with a molecular weight of 81.4 kDa, and is predicted to have 12 or 14 transmembrane domains (Figure 1.5) [28], with both N- (∼100aa) and C-terminals (∼240aa) in the cytosol. Prestin is thought to possess two functions as a voltage-driven motor protein. First, it contains a voltage sensor that can sense the changing voltage field across the membrane. Second, it also contains an actuator that undergoes a conformational change.

Prestin is a member of the mammalian SLC26A anion-transport family [29] and SLC26A further belongs to a larger SulP family, including anion transporters in eukaryotes, prokaryotes and plants [30, 31]. Ten mammalian members in the SLC26A family have been identified, and all of the others, except for prestin, transport monovalent or divalent anions across the membrane. Prestin serves as an incomplete anion transporter that utilizes Cl ions as extrinsic voltage sensors for translocating anions partially across the membrane [32, 33]. Binding of chloride enables prestin to undergo a conformational change to change the membrane surface area, which further causes the cell to contract or elongate. Among the SLC26A family, prestin is the only member that manifests unique characteristics of electromotility and NLC, and not
Figure 1.5: Proposed topology and structural motifs of rat-Prestin. Cylinders mean helices, arrows mean the β-strands. Prestin is predicted to have 14 transmembrane domains. Both the C- and N-terminus is in the cytosol. Figure taken from reference [28].

actually transporting anion across the membrane. As the cytoplasmic C-terminal is the least conserved region across the family, the C-terminus of prestin attracts much focus that it might confer prestin with unique features. In the C-terminus of prestin, a STAS (Sulphate Transporter and Anti-Sigma antagonist) motif is located [34], and thought to be involved in interactions between prestins and between prestin and other proteins [35, 36].

Recently, the crystal structure of STAS domain has been reported [37]. With the disordered regions (from 564aa to 636aa) replaced by a dipeptides GS, a variant of STAS [505aa-563aa]GS[637aa-718aa] was purified and crystallized. The crystal structure revealed that the prestin STAS has an overall ovoid shape. The core structure
The core structure of STAS domain is composed of a β-sheet by six β-strands. Figure taken from reference [37].

of STAS is composed of a β-sheet comprised by six β-strands (Figure 1.6). Notably, two rigid loops are formed during the first three β-strands, and this part is shown to interact with the C-terminal tail of STAS, mainly the last α-helix, through a hydrogen bond between the side chains of Y520 and H707, and a hydrophobic core formed by L511, Y520, I532 from the N-terminus and I706 and V710 from the C-terminus (Figure 1.7).

These findings indicate the STAS is a compact domain from Ser505 to Ala718. Besides, a long hollow binding site in STAS, near the C-terminus, between α2 and α4 helix is discovered. This site indicates that potential interactions with other molecules could modulate prestin function.
Figure 1.7: Interaction between N- and C-terminus of STAS domain. The N-terminus of prestin is in blue, and the C-terminus is in red. The highlighted residues are predicted to be involved in the interactions between N- and C-terminus. The remaining part of the structure is shown in light orange. Figure taken from reference [37].

Importantly, a proposed orientation of prestin STAS with respect to the plasma membrane is modelled by PPM algorithm. The simulation revealed the existence of the interaction between the plasma membrane and a non-polar region, comprised of Tyr545, Pro543, Tyr546, Phe651 and Val655 (Figure 1.8). With this relative position, the N-terminus of STAS is facing towards the lipid membrane while C-terminus is facing towards the cytoplasm. These residues that are predicted to be involved in the interaction with lipid membrane could be essential, and perturbations to the indicated residues may disrupt prestin function by compromising the STAS-membrane
Figure 1.8: One proposed orientation of prestin STAS with respect to the membrane (small green spheres) by PPM algorithm. The magenta residues are studied that have influence in STAS’s function. The bold residues are predicted by the PPM algorithm to be involved in the interaction between the STAS domain and the membrane. Figure taken from reference [37].

Up to date, the C-terminus of prestin has been studied by performing truncations, sites-directed mutagenesis and constructing chimera protein [38, 39, 40, 41]. Altogether, these works identified critical residues in the C-terminus/STAS domain that are essential for membrane targeting and the function of prestin. Systematic serial truncations have been conducted [39, 40] revealing that the sequence before 718aa in the C-terminus is necessary for prestin’s function. Truncated constructs that stopped 516, 525, 590, 630, 709 and 719aa were made and tested for expres-
sion and NLC, the electrical signature of electromotility. None of the above, except the construct stopped at 719, showed an NLC and delineated membrane localization (Figure 1.9).

![Figure 1.9](image)

Figure 1.9 : A systematic studies of prestin truncations. The residues after the 719aa are showed that don’t influence prestin’s NLC function. Figure taken from reference [40].

For construct Del516, Del525, Del590, prestin was trapped in the Gorgi, while for Del630 and Del709, prestin was generally expressed in the cytosol, losing the membrane targeting. The truncated prestin that stopped at 719 displays normal membrane localization and NLC as the wild-type prestin. Although chimera constructs by adding the C-terminus of pendrin (SLC26A4) or PAT1 (SLC26A6) to the truncated prestin at 515aa and 644aa successfully rescue being trapped by Gorgi, the expression still didn’t show plasma membrane delineation, and none of these chimera proteins showed the desired NLC function. A Y520A/Y526A double mutation was made to abolish two tyrosine-containing motifs, and it resulted in the intracellular ac-
cumulation of prestin without membrane delineation. All these data together showed that the C-terminus of prestin plays an essential role in prestin’s normal expression and function, and some specific sequences are contained in the C-terminus that enables prestin to insert into the membrane and function [39].

Several studies have shown the evidence of molecular interactions between prestin and prestin. Freeze-fracture electron microscopy measured particles expressed on the OHC lateral wall which are considered to be prestin molecules but with a much larger size than what a 744 amino acids protein is expected to be [42]. The homooligomeric interactions of prestin have been measured by using fluorescence resonance energy transfer technique [36]. Studies using single-molecule sequential bleaching method indicated that prestin is obligate tetramers [43]. All together, these works suggest the existence of prestin-prestin homogeneous interactions.

Previous studies on prestin’s lateral mobility proposed a model of transient confinement of prestin in the HEK cells by the interaction with intracellular structures [44]. In the study, prestin-EGFP showed a much smaller diffusion coefficient, D, and a quite notable immobile fraction, which suggests that prestin-EGFP molecules may be transiently constrained within the HEK cells plasma membrane. Since C-terminus of membrane protein is usually involved in interactions with intracellular components, we hypothesize that the limited mobility of prestin is due to properties or interactions specific to prestin, which we think is between prestin and cytoskeleton. There’s another work [35] supporting the idea that prestin is interacting with cytoskeleton through intermediate molecules, such as MAP1S. In summary, STAS domain located in the C-terminus of prestin is one of the candidate regions that considered to be di-
rectly involved in prestin-prestin or prestin-cytoskeleton interactions, and this project used a novel approach to study the role of STAS domain in the interactions, which cannot be achieved by traditional genetic ways.

In this study, we engineered prestin by adding short linkers to different locations in the C-terminus of prestin to study the function of C-terminus/STAS domain. We used a flexible linker (GSSSS), a rigid linker (EAAAK) and a cleavable linker (ENLYFQG) which can be cleaved by the Tobacco Etch Virus (TEV) protease. We inserted these short linkers into four different locations in the C-terminus: 1) between the last transmembrane domain (TMD) and the STAS domain, 2) the loops connecting the secondary structures in the STAS domain, 3) the disordered region in the STAS domain and 4) after the STAS domain. These modified prestin constructs were tested for expression, NLC function, fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET). Our results showed that the interface between the last TMD and STAS domain is critical to function, mobility, and self-interactions, and that the disordered region in the STAS domain can tolerate the insertion of a short peptide with prestin’s membrane targeting ability and NLC function retained. Furthermore, the different types of short linkers can influence prestin’s lateral mobility. We further investigate the two functional prestin constructs with TEV recognition site inserted into the disordered region by testing the expression, distribution and lateral mobility before and after the cleavage. We showed that there is a binding site located in the disordered region, which can confine prestin’s mobility due to certain molecular interactions.
Chapter 2

Methodology

2.1 Cell Culture and Expression Plasmids

Human Embryonic Kidney (HEK) 293 cells were cultured in DMEM with 10% FBS and 1% Pen-Strep under standard conditions of 37°C, 95% humidity, and 5% CO₂. Cells were fed on alternative days and passaged when 100% confluency, usually five to seven days.

For whole-cell voltage patch clamping and FLIM-FRET experiments, cells are plated on 22 x 22 mm coverslip in 1.5cm² surface area tissue culture dish. For FRAP experiments, poly-D-lysine coated 22 x 22mm coverslips were utilized. Prior to plating, we coated the coverslips by placing 500µL of 50µg/mL poly-D-lysine in diH₂O in the coverslip at 37°C for one hour.

Cells were plated 1-48h prior to transfection at a density of roughly 600,000 ~ 840,000 cells/well in tissue culture treated 6-well plate. Cells were transfected by using Xtreme Gene HP transfection reagent. Briefly, 2µg plasmid together with 6µL Xtreme Gene HP transfection reagent were mixed well in 200µL opti-MEM in a 1.5 mL polypropylene tube. The transfection complex was incubated at room temperature for 15min, and then added dropwise to cell cultures. 8 - 18 hours post-transfection, cells were trypsinized and re-plated in coverslips in tissue culture dishes at a density
of ~67,200 cells/cm² for FRAP and FLIM-FRET, and 33,600 cells/cm² for patch clamping. All patch clamping, FRAP, FLIM experiments were performed at room temperature (22 °C) with cells bathed in Extra-cellular Buffer (ECB) or PBS buffer 48-96 hours post-translation for an optimal trafficking to the plasma membrane [45].

Presitin-EYFP and Prestin-mTFP constructs, gifts from Dr. Fred Pereira, Baylor College of Medicine, were created using vector pEGFP-N1 as previously described [36]. Briefly, gerbil prestin cDNA (AF230376) were cloned using PCR with high-fidelity pfu polymerase (Stratagene, La Jolla, CA) with forward primer 5 CG-GAATTCCACCATGGATCATGCCCAA3 and reverse primer 5 GGGATCCCGTCGCCTGGGGTGTTGGTGGT3. Amplified products were then subcloned in-frame to pEGFP-N1 vector and then EGFP was replaced by EYFP and mTFP from the vector pmTFP1-NT (Allele biotechnology). Prestin-mCitrine was made by the point mutation Q69M. The entire region encompassing prestin, EYFP, mTFP were verified by sequencing (Long Star Labs, Inc).

2.2 Western blot

HEK 293 cells were transfected 48h prior lysis. Cells were washed by pre-warmed PBS twice and treated with TrypLE for 1min and collected into 15ml tubes. Cells were centrifuged at 400rpm for 10min and were lysed by M-PER mammalian protein extract reagent (Thermo Fisher Scientific) for 15min with vortex. Lysed cells were centrifuged at 16,000rpm for 5min at 4°C and supernatant was collected for western blot analysis. The protein concentration was determined by Bradford Assay. Protein was separated by 12% SDS-PAGE. Western blots were conducted using anti-
nPres (Santa Cruz Biotechnology, Inc. 1:500), anti-GFP (Abcam, 1:750) and donkey anti-goat (Promega, 1:7500), GAPDH (V-18) (Santa Cruz Biotechnology, Inc. 1:500). Blots were visualized by LAS 4000 Imager.

2.3 Fluorescence Recovery After Photobleach

For double bleaching protocol [46], imaging was obtained by a Zeiss LSM 510 META laser scanning confocal microscopy at room temperature using a 63X Plan-Apochromat, NA1.4 objective. All fluorophores were excited with 514nm light from a 30mW argon/2 laser set at 50% output, corresponding to a tube current of 7.7-8.1 Amps. 12-bit 512x512 images, corresponding to 73.1 µm², were obtained at a digital zoom of 2, and with a pixel dwell time of 1.6 µs. Scanning and bleaching conditions were empirically determined to optimize saturated and zero-valued pixels and to maintain constancy in FRAP parameters across data pools. Each FRAP experiment consisted of a time series of 120 scans of the large ROI, each taking 192 ms, with a 1.5s delay between scans from 1 to 30 and from 61 to 90, and a 10s delay between scans from 31 to 60 and from 91 to 120. Bleaching is under 100% 514nm transmission for 120 iterations, which gives a 50-80% bleaching efficiency. A double bleaching protocol [46] was utilized where the first bleach occurred after 10 scans and was repeated after another 60 scans. Cell membranes were only used for FRAP if they were either completely free from other cells or the abutting membrane was non-fluorescent. Experiments were carried on different cells.

Fluorescence intensity was quantified using Zeiss AIM software package. The raw data output is a single time point corresponding to the beginning of each scan and the
average intensity of a selected ROI during that scan. To produce a recovery curve, the fluorescence intensity was normalized to the average intensity of 10 pre-bleaching scans, that is, scans 1–10 for the normalization of first bleach, and scans 61–70 for the second bleach. The normalized curve was used to fit the model and to calculate several parameters: the bleaching efficiency $B$, the undesired bleaching due to photobleaching $PB$, the immobile fraction $IF$, and the effective diffusion coefficient $D$.

Among the parameters, $B$ and $PB$ are to quantify the FRAP experiment, while $IF$ and $D$ are characteristic features for the mobility [46, 47, 48, 49, 50, 51, 52].

For the single bleaching protocol, the FRAP experiment was performed on a Nikon A1-Rsi laser scanning confocal microscopy (LSM) using a 60X Plan-Apochromat Oil NA 1.4 objective. Prestin-mCitrine was excited with 514nm light from a 30-mW argon/2 laser. 12-bit 256 X 256 images were obtained with 10% laser transmission with a digital zoom of 4. The laser passed a 400-457/514 first dichroic mirror and excited the specimen and the emission signal was passed through a 585/65 filter cube. The detector gain is set at 100 with an offset of 0. During the FRAP experiments, cells were bathed in sterile PBS solution at room temperature. Three circular regions of interest (ROIs) were selected and monitored. The three circular ROI are all 3.0µm in diameter, representing bleaching region, a background region and a control region to monitor undesired photobleaching and drifting that may happen during the FRAP experiments. Each experiment consists of a time series of 50 scans. The first 10 scans were scanned with 1s delay between scans to establish the pre-bleaching intensity. Then the 514nm laser power was set at 100% to bleach the bleaching region for 1 scan. After the bleaching, another 10 scans with 2s delay between scans were monitored to capture the fast rising intensity right after the bleaching in a FRAP curve.
Next, the 30 scans with 10s delay in between were performed to capture the slow development of intensity. These settings were chosen to minimize the undesired photobleaching due to repeated scanning. Each experiment was performed on different individual cells.

Fluorescent intensity is quantified using the Nikon FRAP analysis module designed for A1-Rsi LSM. The raw data output is a series of mean fluorescence intensity in every ROI at different time points. The fluorescence intensity was normalized to the average intensity of the first 10 pre-bleaching scans. Then we averaged the intensity at each time point for each group. The normalized curve was used to fit in the 1-D diffusion model by running Matlab script.

Historically, both experimental and analytical methods for determining D have varied greatly, posing a hurdle for comparison of results between laboratories and sometimes between experiments within a laboratory. In this work, we fit our experiment data into a 1-D diffusion equation, which has been used in previous works [44].

$$I_{av}(t) = 1 - \left( \frac{1}{L} \left[ \frac{\sqrt{4Dt}}{\sqrt{\pi}} \left\{ \exp\left(-\frac{L^2}{4DT}\right) - 1 \right\} + L \times erf\left(\frac{L}{\sqrt{4Dt}}\right) \right] \right)$$

The analytical solution to the diffusion equation in 1-D required two main assumptions. First, the bleaching efficiency should be 100%, instantaneously, and the bleaching occurs solely along the line of membrane enclosed in the bleach ROI. Second, the line with length corresponding to the diameter of the bleach ROI, $\sim 2.9 \mu m$, has a negligible width, which we believe to be true based on the slow rate of flip-flop in cell membranes.
2.4 Fluorescence Lifetime Imaging Microscopy (FLIM) - FRET

Fluorescence resonance energy transfer or Förster resonance energy transfer (FRET) occurs whenever the emission spectrum of a fluorophore, termed donor, overlaps with the absorption spectrum of another fluorophore at proximity, termed acceptor. A donor fluorophore, initially in its electronically excited state, may non-radiatively transfer energy to an acceptor. It’s important to note that FRET does not involve emission of light by the donor nor absorption of light by the acceptor. The energy transfer is through the dipole-dipole coupling, and there is no intermediate photon in FRET [53]. The extent of energy transfer is determined by the distance between the donor and acceptor and the extent of spectral overlap [53]. The rate of energy transfer $k_T(r)$ is defined by

$$k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6$$

, where $r$ is the distance between the donor and acceptor fluorophore and $\tau_D$ is the lifetime of the donor in the absence of energy transfer, and $R_0$ is Förster distance which is characteristic for each FRET pair. The efficiency of energy transfer ($E$) is defined as the quantum yield of the energy transfer transition,

$$E = \frac{k_T(r)}{k_T(r) + k_f + \sum k_i}$$

, where $k_f$ is radiative decay rate and $\sum k_i$ is the sum of other de-excitation pathways. Thus, the efficiency ($E$) between a FRET pair at a fixed distance is

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

. Note that the efficiency varies inversely with the $6^{th}$ power of the distance between donor and acceptor ($r$) over the range of 1-10nm [54]. Such distances (10-100 Å) are relevant for most biomolecules or their constituent domains engaged in complex
formation and conformational transition. Thus, FRET provides a powerful tool to investigate protein-protein interaction in a nanoscale.

The average amount of time that a fluorophore stays in the excited state after excitation is referred as the fluorescence lifetime. The meaning of fluorescence lifetime is represented in Figure 2.1, where we only focus on those processes responsible for returning to the ground state. Electrons absorb photon energy of $\hbar \nu_A$ and transit from the ground state $S_0$ to the excited state $S_1$. After quickly ($\sim 10^{-12}$s) relaxing to the thermal equilibrant excited state, that is, the lowest energy vibrational state of $S_1$, the excited electrons then return to the ground state $S_0$ by the emission of photons or non-radiative decay. The lifetime of the excited state is defined by the average time the molecule stays in the excited state prior to returning to the ground state[53], and can be calculated by

$$\tau = \frac{1}{\Gamma + k_{nr}}$$

, where $\Gamma$ is the emissive rate of the fluorophore and $k_{nr}$ is the rate of non-radiative decay.

Suppose the initial population of fluorophores in the excited state is $n_0$, the number of excited molecules at time $t$ following excitation is

$$\frac{dn(t)}{dt} = \frac{1}{\tau} n(t)$$

. Emission is a random process and each excited fluorophore has the same probability of emitting photons. This results in an exponential decay of population of molecules in the excited state,

$$n(t) = n_0 e^{-\frac{t}{\tau}}$$
Figure 2.1: A simplified Jablonski diagram to illustrate the meaning of fluorophore lifetime. A fluorophore gets excited from ground state to excited state by absorption of photons, and the fluorescence lifetime is the average time that the fluorophore stays in the excited state before dropping to the ground state by photon-emitting or non-radiative decay. Figure is taken from reference [53].

In a typical fluorescence experiment, we expect

\[ I(t) = I_0 e^{-\frac{t}{\tau}} \]

where \( I \) is the fluorescence intensity.

Since the lifetime of fluorophores can be significantly altered by the FRET event, fluorescence lifetime imaging microscopy (FLIM) becomes a powerful tool to calculate the FRET efficiency by measuring the changes in the fluorescence decay curve. FLIM-FERT has the obvious benefits over other traditional FRET measurement [55]. The problem of steady-state FRET technique (sensitized emission FRET, after photobleach FRET) is that the concentrations of donor and acceptor are unknown and varies throughout the sample. Thus, the result depends on not only the FRET molec-
Figure 2.2: Fluorescence decay components in FRET systems. In a FLIM-FRET decay curve, two components in the fluorescence lifetime of donor fluorophores can be resolved between interacting and non-interacting population. Figure taken from reference [55].

The use of FLIM can intrinsically solve these problems. Donor fluorescence lifetime is affected by FRET event with acceptor, thus donor bleedthrough and cross-talk between donor and acceptor spectrum have no influence on FLIM-FRET. Also, by fitting the fluorescence decay curves, multiple components within the decay curves can be resolved, in Figure 2.2. This solves the donor and acceptor ratio problem existing in steady-state FRET techniques.
2.4.1 Configuration

FLIM-FRET experiments were performed using a Zeiss LSM 510 MEGA confocal microscopy equipped with Coherent Chameleon Ti:Sapphire femtosecond pulsed laser with tunable excitation wavelength from 700 to 1000nm. The laser was tuned to provide 867nm wavelength beam at a 90MHz repetition rate and aligned to go into the confocal microscopy. The architecture of the whole system is illustrated in Figure 2.3.

Figure 2.3: Architecture of the TCSPC system. Pulsed laser generated by Ti:Sapphire laser at a specific repetition rate is aligned to go into confocal microscopy and hit the sample, the emission photons are collected by PMT detector. The emission signal and the reference signal directly from the laser are together analyzed in a TCSPC module. Each pulse of laser may or may not excite photon emission. The collective data is analyzed and gives the fluorescence decay curve. Figure is modified from [55].

The pulsed laser hits the primary dichroic mirror (HFT KP 650) and then excites the specimen. A non-descanned detector (Hamamatsu R3809U-50; Hamamatsu Pho-
tonics UK Ltd, Herts., U.K) is used to capture the emission photons. The pulsed laser reference signal and fluorescence decay signal were integrated by using an SPC-150 TCSPC board (Becker & Hickl BmbH, Germany).

2.4.2 Data analysis

FLIM data is analyzed using SPCImage software provided by Becker & Hickl BmbH. After FLIM data being imported, IRF is loaded for a deconvolution of recorded fluorescence decay. We fit the “Single-Mode” FLIM data into a two-component decay curve by fixing the \( t_2 \), which is the lifetime of the non-interacting donor, with the value of the lifetime from a donor-only sample. The parameters of interest by fitting the data into a two-component decay curve are listed in Table 2.1. The energy transfer efficiency (E) can be calculated using the following equation.

\[
E = 1 - \frac{\tau_{DA}}{\tau_D}
\]

\( \tau_{DA} \) is the quenched lifetime of the donor by energy transfer from the specimens containing both donor and acceptor, and \( \tau_D \) is the unquenched lifetime of the donor in a donor-only specimen. Here, we use \( t_m \) as the \( \tau_{DA} \) and \( t_2 \) as \( \tau_D \) to calculate the \( E_{fret} \) according to established protocols [56].

2.5 Electrophysiological Recordings

In a whole-cell voltage-clamping setup, the capacitance is determined by applying a sinusoidal command voltage and recording the current response. The phase shift monitored between the output and the input signals allows for the determination of the membrane capacitance. Cells were recorded 48h post-transfection to allow for
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
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<tbody>
<tr>
<td>a1</td>
<td>percentage of the slow component</td>
</tr>
<tr>
<td>t1</td>
<td>fluorescence lifetime of the slow component</td>
</tr>
<tr>
<td>a2</td>
<td>percentage of the fast component</td>
</tr>
<tr>
<td>t2</td>
<td>fluorescence lifetime of the fast component</td>
</tr>
<tr>
<td>tm</td>
<td>combined fluorescence lifetime</td>
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Table 2.1: Parameters in analysis of a two-component fluorescence decay curve

stable measurement of nonlinear capacitance. Ionic blocking solutions were used to isolate capacitive currents. The bath solution (extra-cellular buffer) contains 99 mM NaCl, 20 mM TEA-Cl, 2 mM CoCl₂, 1.47 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPE (pH 7.2, 320 mosM), and the pipette solution (intra-cellular buffer) contains 130 mM CsCl, 2 mM MgCl₂, 10 mM EGTA and 10 mM HEPES. Osmolarity was adjusted to 320 mosM with dextrose. To determine the membrane capacitance ($C_m$), we used a phase-sensitive detector implemented in PatchMaster software (HEKA, Mahone Bay, Canada). We applied an 800-Hz, 10-mV sine wave and measured the current response as DC holding potential was stepped from -160 to +160 mV in 2 mV increments. At each DC potential, four complete sinusoidal voltage cycles occurred, and discrete capacitance values were calculated from the last three.

When prestin is expressed and functional, a bell-shaped curve of the nonlinear capacitance is measured, and the resulting capacitance versus voltage curve can be fitted to a two-states Boltzmann function.

$$C_m = C_{lin} + Q_{max} \frac{ze}{kT} \frac{b}{(1 + b)^2}$$
where,

\[ b = \exp\left(-\frac{ze(V_m - V_h)}{kT}\right) \]

where \( Q_{\text{max}} \) is the maximum nonlinear charge movement; \( V_h \) is the voltage at which half-maximal charge transfer occurs; \( z \) is the valence of charge movement. The valence may represent a fractional elementary charge moved across the membrane or the fraction of bilayer traversed by a whole elementary charge; \( C_{\text{lin}} \) is the linear capacitance, derived from the dielectric properties of the cell membrane; \( Q_{sp} \), the specific charge density, \( i.e. \) total charge moved normalized to linear capacitance, is reported as on a cell-by-cell basis. In this work, \( NLC \) is reported as \( NLC_{sp} \) which is normalized to linear capacitance by calculating \((C_m - C_{\text{lin}})/C_{\text{lin}}\).

### 2.6 Molecular dynamics simulation

The structure of STAS variant [505-563]GS[637-718] from \( R. \) norvegicus prestin has been crystallized [37]. In this STAS domain structure, the disordered region was replaced by a GS linker due to the instability the disordered region caused during the crystallization. We used Modeller to add the original 73 amino acids in the disordered region back to the structure as a random loop structure[57]. Then in an npt all-atom simulation, at temperature 300K and under 1 bar pressure, the STAS domain, neutralized by 0.15M potassium and chloride to mimic physiological condition, relax in a water solvent environment for 10ns using Gromacs 4.5 and Amber99sb-ildn forcefield. Then we use VMD to visualize the simulated structure of full-length prestin’s STAS domain.
Chapter 3

Experimental Result

3.1 Insertion of linkers at different locations reveals crucial interface in the C-terminus

To rationally engineer the C-terminus of prestin, we choose three locations to insert the linkers, and they are before, within and after the STAS domain. The predicted ending residue of the last trans-membrane domain is 500aa [39] and the starting residue of the STAS is 505aa [37]. Firstly, we respectively inserted three types of linkers, a flexible linker made of GSSSS, a rigid linker made of AEAAAKA which forms an α-helix and a TEV protease cleavable linker of ENLYFQG, between the last TMD and STAS domain. Secondly, we inserted the TEV protease cleavable linker into the loops connecting second structures within the STAS domain. Thirdly, we inserted the cleavable linker between 596aa/597aa and 620aa/621aa, which are within the disordered region in the STAS domain. Finally, we inserted the cleavable linker after the STAS domain at 718aa. These modified prestin constructs and an unmodified prestin construct which serves as the positive control were fused with mCitrine to the C-terminus. We transfected HEK 293 cells with these prestin constructs, and tested expression by confocal microscopy and functionality by patch clamping to measure NLC feature. The results, summarized in Figure. 3.1, showed that all the prestin constructs with the insertion between the last TMD and the STAS domain successfully trafficked to the membrane but none of the constructs conferred the cells with
NLC feature. These data suggest that the interface between the last TMD and the STAS domain is critical and such disruptions in this region inhibit prestin’s function.

In the second group, where prestin is inserted with linkers into the loops connecting the secondary structures in the STAS domain, the functionality of prestin is inhibited in all the conditions. Insertions into this region also cause trafficking issues that prestin is largely trapped in the cytosol. These data suggests that the STAS domain is a compact core and such disruptions would completely inhibit prestin’s function and membrane targeting.
Figure 3.1: Four types of position in the C-terminus of prestin has been tested inserting with short peptides. The insertions between the last TMD and the STAS domain and the insertions into the loops connecting the secondary structures deprived the NLC function of prestin. Prestin inserted with linker into the disordered region and after the STAS domain retained the wild-type NLC function.

In the third group, where prestin inserted with TEV protease cleavable linker at 596aa/597aa and 620a/621aa respectively in the disordered region, the modified prestin successfully trafficked to the membrane and the prestin-expressed cells had NLC function, shown in Figure 3.2. In the fourth group, where prestin inserted with TEV protease cleavable linker after the STAS domain at 718aa, the prestin-expressed cell also showed membrane delineation and NLC feature. These results indicate that the disordered region and the region after the STAS domain can tolerate the insertion
of short linkers and retain the functionality.

Figure 3.2: NLC measurement of prestin variants by whole-cell voltage patch clamp. “prestin596TEV” is the modified prestin construct that is inserted with TEV protease recognition site between 596aa/597aa. “prestin620TEV” is the modified prestin construct inserted with TEV protease recognition site between 620aa/621aa. “prestin718TEV” is the modified prestin construct inserted with TEV protease recognition site between 718aa/719aa. “prestin503trun” is the modified prestin construct that has been truncated at 503aa and is the example of the NLC measurement of a non-functional prestin construct. “prestin” is the wild-type unmodified prestin with mCitrine fused to the C-terminus and is the positive control of functional prestins. NLCsp is evaluated using the calculation discussed in Methods.

To further investigate how the prestin protein sequence conserves between different species and neighbour proteins in the SLC26A family, we performed a protein
sequence alignment for prestin (see Figure 3.3). As the alignment result indicates, the regions between 500aa and 505aa, and STAS domain, except for disordered region, of SLC26A5 is highly conserved across species. These regions are conserved even compared to neighbour gene in the SLC26A family. However, we can see the disordered region is highly conserved between gerbil and human, but has more variations when being aligned with chicken prestin and SLC26A4 and SLC26A6 of mouse. These suggested that the interface between membrane (the last TMD) and STAS is sensitive and critical, it could be involved in orientation or has some certain functions, and the loops in STAS domain is highly conserved and changes of them could completely disrupt the prestin function.

3.2 The interface between the last TMD and STAS domain is crucial to prestin’s self-interactions

Although the modified prestin constructs that were inserted with linkers between the last TMD and the STAS domain didn’t show a NLC feature, the prestin was successfully located in the membrane for insertions of all three types of linkers. To evaluate the influence of the insertions on prestin’s self-association, we co-transfected HEK 293 cells wild-type prestin tagged with mTFP to the C-terminus and four types of prestins tagged with EYFP to the C-terminus and conducted FLIM-FRET experiments to measure the prestin-prestin interactions. The four types of prestin constructs co-transfected with prestin-mTFP were: prestin construct inserted with a 5 amino acids flexible linker GSSSS between the 500aa and 501aa, prestin construct inserted with a 7 amino acids rigid linker AEAAA KA between the 500aa and 501aa, prestin
Figure 3.3 : Protein sequence alignment of prestin from different species and with neighbour proteins in the SLC26A family

| slc26a5_prestin_gerbil_ | AVIALLTVIYRTGSPSYKVLQGLPDTVYIDTAYBVEKREIPGIFIQ | 540 |
| slc26a5_prestin_human_ | AVIALLTVIYRTGSPSYKVLQGLPDTVYIDTAYBVEKREIPGIFIQ | 540 |
| slc26a5_prestin_chicken_ | AVAFAMITVIYRTQPQYRLQIPDTIYCDVELVEKREIPGIFIQ | 546 |
| slc26a6_mouse_ | SVFSSLLTVVSWQNLPHSSSLQGVPTDDTIDYRADVYESGAEKAVGVTFRSS | 523 |
| slc26a4_pendrin | GLLFALLTVLAVGFPWNGLGVSPDTIYKSGTMLPEEPGKLRF | 550 |
| slc26a5_prestin_gerbil_ | NAPIYANSILSNALKRKTGVNPAILMGARRKAMRY---AKVGNAN- | 586 |
| slc26a5_prestin_human_ | NAPIYANSILSNALKRKTGVNPAILMGARRKAMRY---AKVGNAN- | 586 |
| slc26a5_prestin_chicken_ | NTSLYFANSEYTSIALKKTGVPDCAILARARRQKH---AREIKKANK | 593 |
| slc26a6_mouse_ | SATLYFANSEYSLKKEKCGVDDRRTQKXXXIKQEMKLMKAK | 573 |
| slc26a4_pendrin | SSPIFYGWGFKCNSSTVGFDARIRYVNKLFAALRI---QKLQKQGL | 597 |
| slc26a5_prestin_gerbil_ | ------IANAVKVDVGEVGDENATKPEEEDDEVYPPIVIKTTPPEE | 628 |
| slc26a5_prestin_human_ | ------MANAVKADAEDVEDGDEATKPEEEDDEVYPPIVIKTTPPEE | 628 |
| slc26a5_prestin_chicken_ | VKKAVSLKKNSTNDWEAHSVHEIANDGLPANFQFADYDAGQDSDPE | 643 |
| slc26a6_mouse_ | SQQDASSKISSSVNNTLGEDVSNVDGAEKQVQELQGDIVSSQO | 623 |
| slc26a4_pendrin | RATKN---GIISDGSNNAFEDPDVEPEELPNIPKKEIEIQWENSEP | 645 |
| slc26a5_prestin_gerbil_ | ------LQRFMPQETVHTVHTLDFTQVFNSDSVGKTVLAEMKEYEG | 668 |
| slc26a5_prestin_human_ | ------MQRFMPQEDVHTVHTLDFTQVFNSDSVGKVLAGVEYEG | 668 |
| slc26a5_prestin_chicken_ | ------LHREFVPTNVHSLILDFAPFVNFSDSGAKLRSVIEYN | 683 |
| slc26a6_mouse_ | EDAKAPTNTSKLSSLQPGFHSLLLDSTSFVTTCVKSLKINFRDF | 673 |
| slc26a4_pendrin | ------VKNVPVKPIHSLVLDCCAVSDLVGVRSLRAWKEFQ | 684 |

We conducted FLIM-FRET experiment on the transfected cells, and the data showed that the FRET efficiency of unmodified prestin was 11%. For all other three prestin constructs that have been inserted with different types of linkers between the last TMD and the STAS domain, the FRET efficiencies were significantly decreased.
to $\sim 5\%$, as shown in Figure 3.4. Together these data showed that the insertion of short peptides between the last TMD and the STAS domain disrupted the functionality of the prestin, and significantly weakened prestin’s self-association.
Figure 3.4: The insertion of either flexible, rigid or cleavable linker between the last TMD and the STAS domain significantly decreased the self-interaction of prestin. The normal level of FRET efficiency of unmodified prestin construct is around 11%, while the modified prestins have FRET efficiencies of around 5%. Loading with water-soluble cholesterol can increase the prestin-prestin interaction and the FRET efficiency of prestin inserted with flexible linker was elevated to the normal level. A student t-test is used to evaluate the confidence. In the figure, control is co-transfection of prestin-mTFP and prestin-EYFP (N=24 without cholesterol loading, N=11 with cholesterol loading), 503/504TEV is co-transfection of prestin-mTFP and prestin-EYFP inserted with TEV recognition site between 503 and 504 amino acid (N=10), 500/501GS is co-transfection of prestin-mTFP and prestin-EYFP inserted with flexible linker between 500/501aa (N=9 without cholesterol loading, N=7 with cholesterol loading), 500/501EA is co-transfection of prestin-mTFP and prestin-EYFP inserted with rigid linker between 500/501aa (N=6 with cholesterol loading).

To further investigate the relationship between the decreased self-association of prestin in membrane and the inhibition of NLC, a question was raised based on this
observation: whether the inhibition of NLC in these constructs is due to the decreased self-association of prestin or because of the inhibition of specific molecular interaction? To answer the question whether increasing self-association of prestin in the membrane can recover the NLC feature, we loaded the transfected HEK 293 cells with 1mM M\(\beta\)CD-containing (water-soluble) cholesterol, which has previously been shown to increase the prestin-prestin interaction by increasing the membrane mobility [58]. The data showed that for the unmodified prestin, the FRET efficiency increased to 15\%, and for the prestin construct inserted with flexible GSSSS linker between the 500aa and 501aa, the FRET efficiency had been increased to around 10\%, which is the level of previous unmodified untreated prestin. However, even loading the HEK 293 cells with excess water-soluble cholesterol brought up the FRET efficiency to the normal level, it still didn’t rescue prestin’s NLC functionality. Together, these data suggested that the elevation of the prestin’s self-interaction level did not rescue prestin’s functionality and that specific molecular interactions are necessary for prestin’s function.

3.3 The flexibility of linkers between the last TMD and the STAS domain can influence prestin’s mobility

Besides FLIM-FRET measurement of prestin’s self-interaction, we also conducted FRAP experiment to measure the diffusion coefficient of the prestin constructs inserted with either flexible or rigid linker between the last TMD and the STAS domain. We transfected the HEK 293 cells with modified prestin constructs fused with EYFP to the C-terminus and unmodified prestin tagged with EYFP as the positive con-
trol. We used a double bleach protocol to study the transient confinement of prestin as reported by the previous study [44]. For the unmodified prestin construct, the averaged diffusion coefficient of the first bleach is 0.033 µm²/s and 0.013 µm²/s for the second bleach. The data showed that the diffusion coefficients of prestin inserted with 5 amino acids of GSSSS flexible linker had been increased to 0.061 µm²/s for the first bleach and 0.03 µm²/s for the second bleach. However, the diffusion coefficient of prestin inserted with 7 amino acids of AEAAAKA rigid linker at the same position remained the same with the control group. The diffusion coefficient of the first bleach is 0.034 µm²/s and the second bleach is 0.016 µm²/s. Together, these data suggested that different types of linkers influence the diffusion behavior of prestin, and the interface between the last TMD and the STAS domain is not only important to prestin’s function and self-interaction, but also important to prestin’s lateral mobility.
Figure 3.5: The FRAP measurement of prestin’s mobility. The insertion of a flexible or rigid linker can alter the mobility of prestin. Prestin inserted with a flexible linker between the last TMD and the STAS domain showed a significant increase in the diffusion coefficient, however, the prestin construct inserted with a rigid linker at the same position remained the same level of diffusion coefficient with unmodified prestin. prestin-(GSSSS)5-STAS-mCIT donates the prestin inserted with the flexible linker between the last TMD and the STAS domain (N=8). prestin-(EAAAK)5-STAS-mCIT donates the prestin inserted with rigid linker between the last TMD and the STAS domain (N=9).
3.4 The disordered region can tolerate peptides insertion and could be potentially used for further study of STAS domain

We inserted a 7 amino acids TEV protease cleavable linker into the disordered region in STAS domain and the engineered prestin retained the function of trafficking to the membrane and the prestin-expressed cells had NLC feature. The disordered region in STAS domain is between 564aa and 636aa and had been replaced by a GS linker during the crystallization to increase the stability in the previous study [37]. We used the resolved structure of STAS domain and add the 73aa back to the position and ran an all-atom molecular dynamics to evaluate the behavior of this region. As shown in Figure 3.6, the core of the STAS domain is comprised of two halves, where one half consists of 59 residues from 505aa to 563aa and the other half consists of 82 residues from 637aa to 718aa. The two halves are connected by the disordered region that consists of 73 residues from 564aa to 636aa and does not form any noticeable secondary structures. The positions that we inserted the cleavable linker locate in the disordered region. The cleavage at the designed positions in the disordered region would split the STAS domain into two halves with roughly equal size. Together, these data showed that the disordered region forms variable loops without having any secondary structures, and it serves as a place to insert linkers and make modification without interfering prestin’s function.
Figure 3.6: MD of STAS domain from *R. norvegicus* prestin with disordered region. The red box showed the 596aa and 597aa. The green box showed the 620aa and 621aa. The orange line indicates where the core of the STAS domain would be split into two halves if the STAS domain gets cleaved in the disordered region.

### 3.5 Discover a potential binding site

We successfully constructed three functional prestin constructs with TEV protease cleavable site inserted into the 596aa/597aa, 620aa/621aa and 718aa/719aa, respectively. First, we examined the prestin expression. The results showed that all functional prestin constructs expressed well and had the same expression profile like unmodified prestin construct which successfully trafficked to the membrane.

Next, we wanted to cleave the cleavable linker inserted at certain positions to study the behavior and role of the STAS domain in prestin after the cleavage. We co-transfected HEK 293 cells with the plasmid coded with wild-type TEV protease fused with mTFP to the C-terminus, and the functional prestin constructs fused with mCitrine to the C-terminus. Then, we examined the prestin-expressed cells
under confocal microscopy to look at the distribution of the mCitrine signal, shown in Figure 3.7. The data showed that the unmodified prestin still remained in the membrane, which indicates that the wild-type prestin does not have internal TEV protease recognition site and could not be cleaved by TEV protease. However, the prestin with cleavable linker inserted at 620/621aa and 718/719aa showed a totally different expression profile that the mCitrine was evenly distributed in the cytosol. These data indicates that the TEV protease cleaved the prestin constructs inserted with TEV protease recognition site at 620a/621aa and 718aa/719aa. The cleavage split prestin into two fragments, and the cleaved fragment diffused into the cytosol.

However, for prestin inserted with the same cleavable linker at 596/597aa, the mCitrine still stayed near the membrane. Based on the confocal image, this could possibly mean that 1) the TEV protease could not access the inserted TEV recognition site and the prestin was not cleaved; or 2) the TEV protease did cleave the inserted recognition site, however, rather than diffused into the cytosol like what happened for the other two constructs, the cleaved fragment stayed near the membrane.
Figure 3.7: Confocal images of prestin constructs. Both prestin inserted with cleavable linker at 596/597aa and 620/621aa and prestin inserted with cleavable linker at 718/719aa after the STAS domain can successfully traffic to the membrane. When co-transfected with TEV protease, the unmodified prestin remained on the membrane. For prestin inserted with cleavable linker at 620/621aa and at 718/719aa, the cleaved fragments diffused into the cytosol while for prestin inserted with cleavable linker at 596/597aa, the cleaved fragment retained near the membrane.
To further answer the question whether the prestin with cleavable linker inserted at 596/597aa was cleaved and investigate the disparity between insertions at 596/597aa and 620/621aa, both in the STAS domain’s disordered region, we conducted an immunoprecipitation experiment to evaluate the cleavages. The antibody which targets the N-terminus of prestin was used in the Western Blot experiment. As shown in Figure 3.8, the unmodified prestin is confirmed that it did not get cleaved. The missing bands at the position corresponding the size of prestin fusion protein indicate all three constructs did get cleaved by the TEV protease. These findings suggested that prestin constructs inserted with cleavable linker at 596/597aa, 620/621aa and 718/719aa can be accessed and successfully cleaved by the wild-type TEV protease.

Together with the confocal images data, these data showed that the cleaved fragments of prestin inserted with cleavable linker at 620/621aa and 718/719aa were not confined near the membrane, and after the cleavable, they diffused into the cytosol. For prestin inserted with cleavable linker at 596/597aa, the cleaved fragment was confined by some specific interactions. Thus, the cleaved fragment was not allowed to diffuse freely, and stayed near the membrane or on the membrane after the cleavage.
Figure 3.8: Western blot of the cleavage of cleavable prestin constructs. Cells were co-transfected with unmodified and cleavable prestin constructs with TEV recognition sites at 596/597aa, 620/621aa, and 718/719aa together with wild-type TEV protease. The size of the prestin fusion protein is around 130kDa. The first two columns were unmodified prestin constructs without insertion of TEV recognition site co-transfected with and without TEV protease, and the prestin constructs were not cleaved by the protease. The third and fourth columns were prestins inserted with TEV recognition site between 596aa and 597aa, and TEV protease can successfully access and cleave this modified prestin and split it into two fragments. The fifth and sixth columns were prestin inserted with cleavable linker at 718aa/719aa after STAS domain and co-transfected with and without TEV protease. This construct could be cleaved by TEV protease.
3.6 The binding site in disordered region confines prestin

To further investigate the biophysical property of two functional cleavable prestin constructs, prestin-tetS-596, which is prestin inserted with the cleavable linker between 596aa/597aa and prestin-tetS-620, which is prestin inserted with cleavable linker between 620aa/621aa, we conducted FRAP experiments to measure the mobility of prestin molecules after the cleavage. We co-transfected HEK 293 cells with cleavable prestin constructs labeled with mCitrine to the C-terminus and TEV protease labeled with mTFP to the C-terminus. Then we measured the diffusion coefficient of the cleaved fragment based on the mCitrine signal. We measured the diffusion coefficient of the unmodified prestin molecules labeled with mCitrine to the C-terminus as the positive control. The recovery curves in FRAP measurement are shown in Figure 3.9 and the diffusion coefficients from fitting the curve are summarized in Table. 3.1.

The results showed that the unmodified prestin molecules, corresponding to a molecular weight of 109.7 kDa, had a diffusion coefficient of $0.097 \mu m^2/s$. In prestin-tetS-596, the cleaved fragment showed a decreased diffusion coefficient of $0.045 \mu m^2/s$. In prestin-tetS-620, the cleaved fragment showed a significantly increased diffusion coefficient of $0.50 \mu m^2/s$, which is over 10 times higher than the diffusion coefficient of the cleaved fragment in prestin-tetS-596. Together, these data provides evidence that there is a binding site located in the disordered region in STAS domain between 597aa and 620aa, and the binding site may interact with some other components to confine prestin molecules and prevent prestin’s free diffusion.
Table 3.1: Diffusion coefficients of prestin constructs in FRAP experiments. The diffusion coefficient is calculated by fitting the averaged FRAP curve into 1-D diffusion equation discussed in the methods section. (N = 5 for unmodified prestin, N = 8 for prestin-tetS-596 and N = 6 for prestin-tetS-620)

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Diffusion Coefficient (µm²/s)</th>
<th>R-squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmodified prestin</td>
<td>0.097</td>
<td>96%</td>
</tr>
<tr>
<td>prestin-tetS-596</td>
<td>0.045</td>
<td>98%</td>
</tr>
<tr>
<td>prestin-tetS-620</td>
<td>0.50</td>
<td>74%</td>
</tr>
</tbody>
</table>

Figure 3.9: FRAP measurement of cleaved and uncleaved prestin. (a) The averaged, normalized fluorescence recovery curve of unmodified prestin, prestin-tetS-596 and prestin-tetS-620 in the FRAP experiments. (b) Fitting to the averaged and normalized recovery curve. The prestin-tetS-620 construct showed an increased mobility and prestin-tetS-596 construct showed a decreased mobility compared to the unmodified prestin construct.
Chapter 4

Discussion

The C-terminus of prestin, where the STAS domain is located, drew a lot of focus regarding its potential function in prestin’s function, self-interaction and mobility. Previous studies have shown that specific residues in the C-terminus are necessary for prestin’s expression and function [39], and the C-terminus is considered to be the candidate mediating interactions with the cytoskeleton and prestin-prestin interactions [35, 36]. In our work, we tried to engineer prestin by inserting different type of short linkers to the C-terminus at different locations. By rationally designing the prestin construct, we tested modified prestin constructs expression, function, self-interaction and mobility to study the role the C-terminus in prestin’s biophysical behaviors. Our data showed that the interface between the last TMD and STAS domain is critical to prestin’s function, self-interaction and mobility. Tuning the types of linker connecting prestin’s hydrophobic membrane core the long C-terminus could influence prestin’s self-interaction, and mobility. We also found a specific region in the STAS domain could be used for modifying the C-terminus without interfering prestin’s function. Furthermore, by designing functional modified prestin constructs that are inserted with a cleavable linker, we can leverage the power of synthetic biology to dynamically study the role of the C-terminus in prestin’s biophysical properties by conditionally activating protease to cleave the protein.

The goal of our work is to design functional modified prestin construct inserted
with different linkers to study the role of the STAS domain. We first considered inserting linkers to the gap between the membrane core and the STAS domain, since it would perfectly partition the prestin. The results showed that the unmodified prestin can be expressed in HEK 293 cells and prestin trafficked to the membrane and have a normal distribution like wild-type prestin. However, all the prestin constructs inserted with either flexible, rigid or cleavable linkers in this region did not have NLC functionality. The shortest linker we tested was the flexible GSSSSS linker with only five amino acids, whose length is estimated to be around 5nm. These data indicates that the region between the last TMD and the STAS domain is quite critical that even 5nm long insertion could not be tolerated. We hypothesize that the insertion of excess residues to the gap destroyed the original hydrophobic environment between the STAS domain and the membrane core by letting water molecules come in the gap. Previous work [37] proposed a model of the orientation of STAS domain and indicated the potential interaction between the N-terminus and C-terminus of the STAS domain. Our modifications were between 500aa and 505aa, and the loss of NLC function could be possibly due to the modification disrupting the orientation of STAS domain or the interactions between the N- and C- terminus of STAS domain.

The previous study reported the crystal structure of the STAS domain and indicated that the STAS is a single compact domain [37]. We further selected three possible locations to insert linkers to design functional prestin with insertion. Since the residues between secondary structures don’t form any structures, the function of these residues is often connecting two secondary structures, which provides potentials to make modification in these regions [59]. We selected five such loops to insert our linkers. Our data showed that the loops connecting the secondary structures in the
STAS domain still could not tolerate the insertion. These suggested that the STAS domain is very sensitive to modifications and could not even tolerate modifications to the non-functional loops connecting secondary structures. One of the functional modified prestin constructs was the prestin that is inserted with the cleavable linker right after the STAS domain between 718aa/719aa. Previous work has shown that a truncated prestin construct that stopped at 719aa still had NLC functionality [40]. This is consistent with our results that the last 26 residues are not necessary for prestin’s function.

To further search for a possible location to place the insertion, we examined the disordered region in the STAS domain and successfully designed two functional prestin constructs with TEV protease cleavable linker inserted. Previous work indicated that there is a disordered region in the roughly middle of the STAS domain that does not form structures and only consists of variable loops [37]. During the crystallization, this region has been replaced by a GS linker to increase the stability. We hypothesize that the disordered region may tolerate the insertion since it is a relatively large region, consisting of 73 amino acids that don’t form any structures. We first run an all-atom simulation on the full-size STAS domain by adding the 73 amino acids corresponding to the disordered region back to the resolved STAS domain structure. The result, as shown in Figure 3.6, indicates that the disordered region forms multiple variable loops that connect the two halves of STAS domain. We successfully designed two TEV protease cleavable prestin constructs where the recognition site was inserted between 596aa/597aa and 620aa/621aa. We evaluated the cleavable prestin constructs’ functionalities by testing the NLC function and the results showed that the cleavable prestin constructs have a bell-shaped NLC like the wild-type prestin, as
shown in Figure 3.2.

Although the prestin construct inserted with linkers between the last TMD and the STAS domain did not have NLC function, but they still drew much interest. Interestingly, these constructs showed a normal expression and distribution, where the prestin molecules trafficked to the membrane as wild-type prestin did. To further investigate the relationship between prestin’s function and prestin’s self-interaction, we conducted FLIM-FRET experiment to evaluate prestin’s self-interaction. We found that these modified constructs have a decreased FRET efficiency. The wild-type prestin had a FRET efficiency of around 11%, while the modified unfunctional prestin had a FRET efficiency of around 5%. This suggests that the insertion of linkers into the region between the last TMD and the STAS domain significantly decrease the prestin-prestin interaction. These data further provides evidence showing that STAS domain is actively involved in prestin’s self-interactions. Furthermore, we loaded the cells with cholesterol, which is shown to increase the prestin’s self-interaction [58], to increase prestin’s self-interaction. The results in Figure 3.4 showed that even though the FRET efficiency level had been brought up to the normal level, the cells still did not have NLC function. Our work, together with previous studies [36], indicates that the STAS domain is actively involved in the prestin-prestin interaction and the prestin’s self-interaction is not sufficient for its NLC function. The modification to the STAS domain not only disrupts the prestin’s self-association, but also inhibits some certain molecular interactions. This indicates that to ensure a certain level of prestin-prestin interactions is not sufficient for prestin’s function, some certain molecular interactions mediating through the STAS domain is necessary for prestin’s function.
Besides using FLIM-FRET to measure prestin’s self-interaction, we also conducted FRAP measurement to determine the mobility of these modified prestin constructs inserted with either flexible or rigid linker between the last TMD and STAS domain. The results showed that the prestin construct inserted with the rigid linker in this region has a similar diffusion coefficient with the wild-type prestin, while the prestin construct inserted with flexible linker has an increased diffusion coefficient. We hypothesize that one possible mechanism is that through STAS domain, prestin interacts with cytoskeleton directly or through other intermediating proteins [35], and this interaction with cytoskeleton confines prestin molecules from freely diffusing on the membrane. The insertion of a flexible linker to the region between the last TMD and the STAS domain weakens the direct or indirect molecular interactions between the STAS domain and the cytoskeleton component, which results in that prestin’s mobility increases.

To further study the effect of cleaving STAS domain on prestin’s function, we did experiments to co-express TEV protease and cleavable functional prestin constructs. Interestingly, the disparity of the distribution profile of the cleaved fragment leads us to take a closer look. For prestin-tetS-596 and prestin-tetS-620 construct, both cleavages took place in the disordered region, however, the cleaved fragment of the prestin-tetS-596 construct still stayed on the membrane or near the membrane, while the cleaved fragment of the prestin-tetS-620 construct diffused into the cytosol and showed a homogeneous distribution. Based on this observation, we hypothesize that there is a specific molecular interaction which confines the cleaved fragment of prestin-tetS-596. However, this specific molecular interaction does not exist in the
cleaved fragment of prestin-tetS-620. These results together lead to the hypothesis that there is a binding site located between the 597aa and the 620aa that is involved in specific molecular interactions, which could be direct or indirect interaction with the cytoskeleton, to confine prestin molecules. When the cleavage happens after the binding site, the cleaved fragment does not have the specific molecular interaction anymore, which results in the cleaved fragment freely diffuses into the cytosol. However, if the cleavage happens before the binding site, the cleaved fragment still has the molecular interaction so that it still stays on the membrane or near the membrane.

To provide further evidence for the hypothesis that there is a binding site located in the disordered region. We conducted FRAP experiment to study the mobility of the cleaved fragment. Figure 3.9b showed that the diffusion coefficient of the cleaved fragment from prestin-tetS-596 was decreased when compared to the unmodified prestin. The molecular weight of the unmodified prestin molecule is 109.7 kDa, while the cleaved fragment from prestin-tetS-596 has a molecular weight of 44.8 kDa. The cleaved fragment has a smaller size while has a smaller diffusion coefficient. This is consistent with our hypothesis that the cleaved fragment from prestin-tetS-596 still has the molecular interactions to confine diffusion. After the cleavage, there is less molecular weight to trap and thus it showed a slower diffusion behavior. For the cleaved fragment from prestin-tetS-620, it showed a roughly 5 times larger diffusion coefficient compared to the unmodified prestin molecule. The cleaved fragment from prestin-tetS-620 has a molecular weight of 42.1 kDa, but has roughly 10 times larger diffusion coefficient than the cleaved fragment from prestin-tetS-596. This provides evidence to support our hypothesis that the cleaved fragment from prestin-tetS-620 does not have interactions to confine diffusion so that it quickly diffuses into the
cytosol.
Chapter 5

Conclusion and future work

In this work, we studied the role of the C-terminus in prestin’s function, self-association and lateral mobility in the membrane using a novel approach of designing cleavable functional prestin constructs. Our result showed that the interface between the last transmembrane domain and the STAS domain in prestin is critical for prestin’s function. A small insertion into this region can inhibit prestin’s function. Our data also points out that the STAS domain is important for prestin’s self-association, and maintaining prestin’s self-association is not sufficient for prestin’s function. The disruption in the interface between the last TMD and the STAS domain weakens the prestin-prestin interaction and inhibits specific molecular interactions, which are necessary for prestin’s function. Next, our results indicate that the STAS domain is actively involved in molecular interactions which influence prestin’s lateral mobility in the membrane. The flexibility of the linker in the interface between the last TMD and the STAS domain could influence the interaction between the STAS domain and other components, which would further influence prestin’s mobility. Then, our FRAP experiments provide evidence that the STAS domain is involved in molecular interactions to influence prestin’s mobility through a binding site located in the disordered region. In summary, this works showed that the STAS domain is critical to prestin’s function, and involved in prestin’s self-association and molecular interactions with other components that influence prestin’s mobility.
For the future work, we would like to add tunability to the TEV protease activation which could enable us to conditionally activate the TEV protease and dynamically study the role of STAS domain in prestin's function, self-association and mobility. We can leverage the heterodimerization system and the split-TEV technique [60] to achieve this goal. Also, we could label prestin in its N-terminus to study the behavior of the membrane core after the cleavage, from which we could learn more about the role of the C-terminus and the role of the membrane core.
Bibliography


