Functional Studies of the Domains of Piezo1 Ion Channels

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School of Duke University

2018
ABSTRACT

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Abstract

Mechanosensation, or the ability to sense mechanical forces, is critical for the survival of all organisms. For vertebrates, the ability to sense and respond to environmental stimuli, or somatosensation, is not well understood at the neural circuit level, and the molecular underpinnings for somatosensation, especially in regards to mechanosensation, remain elusive and unresolved. In recent years, Piezo ion channels were discovered as the first mammalian excitatory bona fide mechanosensitive ion channel to be the initial molecules in sensing gentle touch in the somatosensory neural circuitry. Although physiological roles of Piezo ion channels for both somatosensation and other non-neuronal processes have been identified, the mechanisms for how these ion channels directly sense mechanical forces and transduce electrical signals remains unknown.

With the use of biochemical, molecular, and electrophysiological methods, I first developed a novel technique in which electrophysiology could be performed on microsomes, or vesicles formed from ER fractions containing proteins that are not trafficked to the plasma membrane. This experiment revealed that wild-type Piezo1 ion channels retain stretch activation in microsomes, and therefore this technique could be utilized to characterize mutants channels that lack trafficking to the plasma membrane in order to identify which domains are involved in activation and inactivation of Piezo
ion channels. I next generated two separate constructs that removed domains suggested to be involved in activation and inactivation of Piezo1. Removal of the proposed inactivation domain rendered a non-functional channel that could still trimerize, suggesting that this domain not only plays a role in inactivation but also is critical for activation of Piezo1 ion channels. Partial removal of the proposed membrane-spanning mechanosensor domains produced a channel that lacked the ability to conduct large macroscopic currents but formed a conducting pore with single channel openings. This finding suggests that membrane tension is not sensed and transduced to the pore by a single domain, but rather multiple domains in concert. Together the findings provide evidence that the mechanisms for both activation and inactivation require multiple domains moving in collaboration together, and will broadly be informative for continued studies of the molecular mechanisms of mechanosensitive ion channels.
Dedication

To all the people who have kept me sane.
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Acknowledgements

I would like to thank my advisor Jörg Grandl for all the patience and support and guidance over the years. I would like to thank previous and current lab members, Sairam Jabba, Raman Goyal, Jason Wu, Jason Sosa, Amanda Lewis, Ashley Henderson, and Michael Young for the immense amount of questions answered and exceptional support (through words and food).
1. Introduction

1.1 Overview

Mechanosensation is the physiological process through which mechanical stimuli are experienced, such as the rigid texture of a surface or a painful poke to the skin. Touch is one of the five main senses and is not only critical for us to respond to noxious stimuli and pain, but is also important for us to build perceptions of the world around us (Wu, Lewis, & Grandl, 2016). The photoreceptors for vision, olfactory receptors for smell, and the receptors for taste have been identified and characterized for many years, however, the mechanoreceptors for touch have remained elusive until recently and are still contested for hearing (Lamb, Collin, & Pugh, 2007; Fleischer, 2009; Chaudhuri & Roper, 2010; Wu, Lewis & Grandl, 2016; Pan & Holt, 2015). Although the first receptors for excitatory mammalian mechanosensation have been identified, little to none is known about the molecular underpinnings behind this physiological process and even less is understood about its role in pain. Mechanosensation is not only a molecular process that is important in understanding neurobiology, it also is critical for other vital physiological systems, such as vasculature, and is present in all organisms, including plants, protozoa, and bacteria (Prole et al, 2013). Interestingly, one family of proteins has been identified in having involvement in these processes across all organisms, the ion channels recently identified as Piezos (Coste et al., 2010; Wu, Lewis, & Grandl, 2016).
Piezo ion channels were identified as the first excitatory mammalian bona fide cation non-selective mechanically activated ion channels via a siRNA knockdown screen of 70+ potential candidates in Neuro2A cells in the lab of Ardem Patapoutian (Coste et al., 2010). Two mammalian isoforms of Piezo – Piezo1 and Piezo2 – have been identified, and invertebrates and protozoan contain one isoform and 3 or more, respectively (Coste et al., 2010; Prole et al., 2013). In mammals, both Piezo1 and Piezo2 are pore-forming subunits that are non-selective cationic channels activated via mechanical forces. In primary sensory neurons, Piezo ion channels sense mechanical touch and elicit an excitatory signal via membrane depolarization that leads to an action potential and signaling to the central nervous system; consistent with this ability, Piezo channels are important for gentle touch sensation, nociception, and proprioception (Ikeda et al, 2014; Woo et al., 2014; Maksimovic et al., 2014, Ranade et al., 2014). Although Piezo channels play significant roles in these sensory processes that are vital for our survival, these proteins lack homology to any other family of proteins, and therefore our current understanding of the mechanism for how these proteins function in sensing mechanical forces is in the early stages.

The aim of this dissertation is to investigate the mechanisms for gating of Piezo ion channels. I set out to determine which domains are necessary for channel activation and inactivation, similarly to how other families of ion channels contain modular
structures, such as ion channels containing voltage- or chemical-sensing domains. By combining a microsomal preparation with negative pressure patch clamp electrophysiology, I was able to not only develop a novel method to perform electrophysiology on vesicles formed from endoplasmic reticulum membrane fractions, but I also was able to begin to determine that specific domains are necessary for normal function of the channel. For most modular ion channels, such as voltage-gated ion channels, the mechanisms for activation and inactivation can be coupled but specific domains are involved in voltage-sensing and pore opening and closing; the findings in this dissertation provide the insight that the crossover of certain domains for the mechanisms of activation and inactivation of Piezo ion channels do specifically not fit the current models for other sensory ion channels.

1.2 *Mechanotransduction in cell biology*

Mechanotransduction is the conversion of mechanical forces into a downstream signal via either an electrical signal or a biochemical signaling process. Mechanical forces typically experienced by cells are membrane stretch or indentation, osmotic pressure, shear stress, or matrix stiffness. Force transduction can lead to a variety of cellular processes, such as migration, differentiation, apoptosis, transcription/translation, cytoskeletal reorganization, and many others (Vogel & Sheetz, 2006; Guillot & Lecuit,
Most of these processes are mediated by either cytoskeletal adhesion proteins or ion channels.

1.2.1 Force transduction via cytoskeleton adhesion

The cytoskeleton within a cell allows for force exertion and sensing between cells and substrates and the transmission of force between extracellular matrix and the cytoskeleton is mediated via focal adhesion molecules. Focal adhesions are comprised of molecular complexes that contain actomyosin cytoskeleton, integrins, and adaptor proteins, such as talin and vinculin, that allow for the sensing of friction between and cell and substrate to induce YAP and TAZ translocation to continue adhesion growth and cytoskeletal remodeling (Elosegui-Artola et al., 2016; Goldmann, 2012, Dupont et al., 2011).

The timescale for changes in focal adhesion complexes to translate to downstream signaling and adhesion growth or cytoskeletal remodeling is typically on the order of minutes to hours. For cellular processes that require immediate feedback, focal adhesion complexes would not be ideal, however for longer, i.e. hours to days, continuous changes necessary for cell migration and or differentiation, a slower timescale is advantageous so that excessive and changes do not lead to uncontrolled growth.
1.2.2 Force transduction via modulatory ion channels

Mechanosensitive ion channels have previously been identified in organisms from bacteria to fruit flies. Mechanosensitive ion channels conduct ion flow across the cellular plasma membrane in response to mechanical force application. The timescale between force application and ion flux through the mechanosensitive ion channels is within milliseconds, allowing for immediate responses, such as opening in response to osmotic stress or for membrane depolarization for an action potential.

In the mechanotransduction field, it has been established that in order for an ion channel to be considered a bona fide mechanically activated ion channel, the protein must fulfill all of the four following criteria: 1) the channel must be expressed in the mechanosensory tissue, 2) response to mechanical stimuli requires its expression and deletion of the channel ablates any mechanical response, 3) any alterations to channel biophysical properties directly affects the mechanical response, and 4) mechanical gating can be observed in heterologous expression systems (Arnadottir and Chalfie, 2010).

1.2.2.1 Bacterial mechanosensitive ion channels

Of all the mechanosensitive ion channels, possibly the most characterized are the bacterial Mechanosensitive channel of Small conductance (MscS) and Mechanosensitive channel of Large conductance (MscL). Both proteins are pore-forming pentameric channels that act as an osmotic release valve by opening via lateral membrane tension.
from hypoosmotic shock (Booth & Blount, 2012; Arnadottir & Chalfie, 2010). When open, MscL channels have a pore opening of 25Å or greater and a conductance of ~3nS, allowing ions and small molecules to pass through (Perozo et al., 2002). The channels are gated by hydrophobic mismatch, a mechanism by which the cellular membrane is stretched and thinned, exposing membrane-buried hydrophobic residues and shifting the channel into a different conformation; mutating lower pore residues based on hydrophobicity alters the channel function, demonstrating its mechanism for activation (Birkner, Poolman, & Kocer, 2012). MscS and MscL are functional in heterologous systems and considered to be mechanically activated ion channels.

1.2.2.2 Eukaryotic mechanosensitive ion channels

One of the largest families of sensory ion channels is the transient receptor potential (TRP) channels, which contain a members that have been identified as candidate mechanosensitive ion channels. First, the mammalian TRPV4 channel is ubiquitously expressed amongst neuronal and non-neuronal tissues, with highest expression levels in osmo-regulatory tissues, skin, and neurons, and ablation of TRPV4 affects nociceptive behaviors (Suzuki et al., 2003). TRPV4 in heterologous systems has been shown to be activated by osmotic pressure, however it has not been demonstrated to be activated by membrane stretch and has not been confirmed to be a bona fide mechanically activated ion channel (Liedtke et al., 2000). Another TRP family member is
the TRPN channel ortholog NompC in *Drosophila melanogaster*, which was discovered in a mutant screen for mechanical insensitivity (Kernan, Cowan, & Zuker, 1994). NompC mutations in the hair bristles of flies ablate mechanosensitive currents. NompC can produce mechanically induced currents in insect S2 cells, and the structure of NompC has been resolved, revealing a canonical TRP membrane-bound channel with long ankyrin repeats that are specific in length to transmit force to open the channel (Arnadottir & Chalfie, 2010; Zhang et al., 2015; Jin et al., 2017).

Another family of ion channels that contains members that are bona fide mechanically activated ion channels is the two-pore domain K+ channels (K2Ps). TREK-1, TREK-2, and TRAAK isoforms can be directly activated by mechanical stretch and permeate K+ ions for inhibition in mammalian nociceptive neurons (Maingret et al., 1999; Brohawn et al., 2014). Stretch-activated K2P channels can be activated in pressure patch clamp electrophysiology, both from negative and positive pressure stimulus, and can also be activated in a resting patch, which has a membrane tension of ~0.5-4.0mN/m (Brohawn et al., 2014). Interestingly, hyperpolarization of the membrane in result of TRAAK activation can inhibit Piezo1 mechanical response (Brohawn et al., 2014).

### 1.3 The function of Piezo ion channels

Mechanosensation is vital for the survival of organisms, both in neuronal and non-neuronal tissues. Piezo1 is predominantly expressed in non-neuronal tissues that
sense continuous forces, including repetitive pulsatile mechanical forces, i.e. vasculature and pulmonary tissues (Figure 1, right), whereas Piezo2 is predominantly expressed in neuronal tissue and the skin, and more specifically in proprioceptive and gentle touch sensory dorsal root ganglia (DRG) neurons (Figure 1, left) (Wu, Lewis, & Grandl, 2016).

Genetic ablation of Piezo1 in mice leads to embryonic lethality as a result of the lack of development of vasculature (Li et al., 2014), highlighting the significance of Piezo1 expression and activity as early as the embryonic stage. Piezo1 is expressed in endothelial tissue lining the blood vessels in vasculature and is believed to be the shear stress sensor in these cells, as well as in the renal and gastrointestinal tract of mammals (Lang, Breer, & Frick, 2017; Alcaino, Farrugia, & Beyder, 2017).

Figure 1: Examples of the physiological role of Piezo ion channels in the neuronal and non-neuronal organ systems (adapted from Wu, Lewis & Grandl, 2016).
(A) Piezo2 has been identified as being one of the primary mechanosensitive ion channels expressed in Merkel cells and DRG neuron afferents for mediating touch sensation. Merkel cells sense pressure and touch via indentations in the skin, or surrounding keratinocytes, and release neurotransmitters that can activate receptors in DRG neurons that leads to firing of action potentials. Piezo2 in the DRG neuron afferents can also sense mechanical stimuli and transduce an action potential. (B) Piezo1 has been shown to be vital for the development of vasculature in mice and for arterial remodeling. Piezo1 is expressed in endothelial cells that line the inner layer of blood vessels and is responsible for sensing shear stress of blood flow through the vessels. Piezo1 is also expressed in red blood cells and is required for regulation of blood cell volume.

Piezo channels can be transiently expressed in heterologous systems for direct biophysical characterization and manipulation via electrophysiological techniques. Ion channels such as Piezos, conduct ions across a resisting membrane at exceptionally fast rates. Depending on the electrophysiological patch configuration, the membrane potential can be ‘voltage-clamped’ in which a specific amount of current is injected to maintain a holding potential; in this dissertation the holding potential is typically -80mV or +80mV. When a stimulus is applied that activates the Piezo channels within the patch, the pore domain of the channel opens allowing millions of ions to flow through. This ion flux across the membrane will cause a change in the voltage of the membrane, which is then compensated by adjusting the amount of current that must be injected to maintain the holding potential. This current is equivalent to the amount of current going induced by the ion flux.
Activation of the channel is when a stimulus is applied and the pore domain opens and allows for the permeation of ions through the pore. Ion permeation can occur in or out of the cell depending upon the membrane potential and the specific ion channel’s ion selectivity. Deactivation of the channel is when the stimulus is released and the pore of the channel closes and no more ions can permeate across the membrane. Adaptation of the channel is when a stimulus is being applied and during that stimulus transitions to a non-conducting state, but when the stimulus intensity is increased a population of channels to transition back to a conducting state and unblocked ion permeation can occur. Inactivation of a channel is when a stimulus remains present yet the channels transition into a conformational state in which the channel’s pore cannot move into the open state and therefore ion permeation is blocked until the channel can transition into the closed state.

1.4 Electrophysiology and Piezo ion channels

Piezo ion channels are activated by a variety of stimuli, shear stress, membrane stretch and indentation. To study the activation and inactivation of Piezo ion channels, negative pressure patch clamp and mechanical stimulation electrophysiology have been the most commonly used techniques. Negative pressure patch clamp is performed in the cell-attached configuration, in which a patch of membrane is suctioned into a glass pipette and any ion flow through the channels within that membrane patch can be
measured as current. This allows for real-time measurement of dynamics of the protein and characterization of the channels’ biophysical properties, such as inactivation or adaptation. To induce a mechanical stimulus, negative suction is applied to the pipette to stretch the membrane of the patch, changing the overall lateral tension within the membrane (Figure 2, left). For Piezo channels, an increase in the amount of suction correlates to an increase in the mechanically evoked currents, and from this it can be measured the current as a function of pressure to determine the mechanical sensitivity, which can be quantified as the pressure for half-maximal activation, or the $P_{50}$ value. Mechanical indentation is performed in the whole-cell configuration by inducing mechanical currents with a blunt probe pushing onto the cell at increasing depths (Figure 2, right).

Figure 2: Mechanical force application and responsive currents. Left) cell-attached patch configuration records only currents from ion channels that are within the patch pipette. For negative pressure patch clamp recordings, a negative suction, controlled by a high-speed pressure clamp, is applied within the pipette to stretch the patch membrane and induce mechanical currents. Cell-attached recordings can be
used for single channel recordings. Right) Whole-cell patch configuration records currents from all channels that are activated within the cellular membrane. Mechanical indentation is induced by using a second blunt probe which pushes down on to the cell membrane to stretch the membrane.

1.5 The architecture of Piezo ion channels

Piezo ion channels contain 2500+ residues per subunit, making them one of the largest identified proteins, with little to no sequence homology to any other known proteins (Coste et al., 2010). Secondary topology prediction tools predict the channel to have 30-42 transmembrane domains per subunit, however the partial resolved cryo-EM structures have 24 transmembrane helices and two pore helices at the center of the channel, plus an unresolved N-terminal region, that form a trimeric propeller-like structure (Coste et al., 2010, Ge et al., 2015; Saotome et al., 2018; Guo et al., 2018). The channels have a width of approximately 200Å and a height of 140Å including the extracellular CED domain (Figure 3, left). Based on the structure, five prominent domains have been labeled (Figure 3, right): 1) a large, globular cap-like structure at the C-terminal is proposed to be positioned to the extracellular side and is referred to as the C-terminal extracellular domain (CED); 2) directly below the CED lies the pore domain, which contains the inner and outer pore helices that are directly connected to the CED; 3) the blades are comprised of the transmembrane helices that extend away from the pore helices and also include the unresolved N-terminal domain; 4) anchor domain is oriented directly at the cytoplasmic side of the pore helices and contains the proteins
most conserved sequence across isoforms and species; and 5) the beam domain consists of long amphipathic helices that lie perpendicular to the blade transmembrane helices.

Figure 3: The architecture and domains of Piezo1 in channel. (A) The top view shows the Piezo1 trimeric structure and the side view highlights the vertical alpha helices that are proposed to be the membrane-spanning domain and the CED that is directly above the pore. The side view demonstrates the membrane-spanning alpha helices and a globular domain that is above the center of the protein. (B) Five prominent domains make up the architecture of Piezo channels: the CED, the blade, the pore, the beam, and the anchor. The CED has been demonstrated to have a role in inactivation. Many identified disease mutations lie within the anchor domain, just below the pore domain.

Interestingly, protozoan Piezo genes appeared to have varying lengths of the N-terminal sequence that had repeating domains, however genetic analysis revealed that all species contained a conserved motif towards the C-terminal that was PF(X2)E(X6)W, which later was identified as being in the anchor domain (Prole & Taylor, 2013). When
the structure of Piezo1 was resolved these N-terminal repeats that had been predicted were present in the structure as repeating alpha helical bundles containing 4 helices per bundle and a membrane parallel helix at the proposed cytoplasmic-membrane interface (Prole & Taylor, 2013; Saotome et al., 2018; Guo et al., 2017). Six repeat bundles extend out from the pore helices forming the blades, with the beam connecting perpendicular to the helices, ending at the most distal resolved bundle (Figure 4) (Saotome et al., 2018; Guo et al., 2018). Although the N-terminal remains unresolved, it is suggested from topology prediction models of the sequence that the N-terminal possesses three more bundles (Saotome et al., 2018; Guo et al., 2018).

Figure 4: The structural topology of a Piezo1 ion channel subunit from the cryo-EM structures. The resolved structures are colored; the white domains at the N-terminal are the unresolved region that is predicted to be form alpha helical bundles. Each resolved helical bundle is comprised of four transmembrane helices that extend away from the center axis of the channel and pore domain. The helical bundles form a propeller-like structure that pivots and forms the blade domain. The beam lies perpendicular to the blade helices and interfaces with the anchor domain below the pore. The CED domain is connected between the IH and OH of the pore and the CED of one subunit is positioned over the IH of the adjacent subunit.
The pore is formed by the last two transmembrane helices, referred to as the inner pore helix (IH) and outer pore helix (OH), and a small helix-fold-helix motif that forms the anchor domain (Saotome et al., 2018; Guo et al., 2018). The anchor domain is suggested to be the selectivity filter, as residue E2133 is important for ion selectivity and unitary conductance; the resolved structure also reveals that residue R2482 lies adjacent to E2133, and has been identified as a disease mutation linked to dehydrated hereditary stomatocytosis (Coste et al., 2015; Saotome et al., 2018; Albuisson et al., 2013; Bae et al., 2013).

The CED is connected via the IH and OH and the resolved structure reveals possible fenestrations between the CED and the IH in which ions could enter at the top of the pore opening (Saotome et al., 2018). The structure of the CED had previously been resolved via crystallization, and revealed a unique β-sandwich fold, and in the channel structure, it appears that the interface of the CED from each subunit forms a cavity that is directly above the pore opening formed by the IHs (Kamajaya et al., 2014; Ge et al., 2015; Saotome et al., 2018; Guo et al., 2017). The CED has been suggested as being the inactivation gate, yet the lack of structural homology has left its function elusive. Although the structures of the Piezo1 ion channel offer insight into distinct domains of the channel, how these domains are involved in gating the channel remains to be identified.
1.6 Gating mechanisms and modulation of Piezo ion channels

1.6.1 Potential gating mechanisms of Piezo ion channels

In regards to mechanistic models proposed for how mechanically activated ion channels are gated, the two most prevalent models are the ‘bilayer model’ and the ‘tether model’ (Nilius & Honore, 2012). The bilayer model encompasses the process by which membrane tension from lipid is directly transduced to gating the ion channel, whereas the tether model has been described as an intracellular or extracellular auxiliary component is coupled or attached to the ion channel and pull directly on the channel to gate and open the pore (Figure 5, top left). Two families of ion channels have been characterized as tethered mechanically gated ion channels. The DEG/ENaC channels directly tether to the extracellular matrix, and in auditory hair cells, multiple components have been identified to be a part of the TMC-tip-link complex that gate the ion channel unidirectionally (O'Hagan, Chalfie, & Goodman, 2005; Pan & Holt, 2015).

For the bilayer model, force from lipids onto the channel can be transduced in a few different mechanisms. The mechanism of hydrophobic mismatch is the exposure of hydrophobic residues typically buried within the membrane by either membrane thinning or locally induced membrane curvature; this exposure is highly energetically unfavorable and therefore channels transition into different conformations, such as channel opening (Nilius & Honore, 2012). One of the best examples of the hydrophobic
mismatch mechanism is the exposure of hydrophobic pore residues in the MscL channel, which leads to the pore of the channel opening to >25Å (Booth & Blount, 2012; Perozo et al., 2002). Another mechanism for gating channels is via changes in lipid composition, either by incorporation or depletion of specific lipids. An example of channel gating via lipid composition is the activation of the K2P channel, TRAAK, via application of arachidonic acid in proteoliposomes (Brohawn, Su, & MacKinnon, 2014).

Piezo1 ion channels can be activated in preparations lacking cytoskeletal or ECM components, such artificial bilayers or blebs, suggesting that the channels do not require a tether for opening of the channel and are gated by the bilayer model (Syeda et al., 2015; Cox et al., 2016). Multiple findings have established that a membrane tension in the range of ~1.7-4mN/m is able to gate Piezo channels both at the plasma membrane and in artificial bilayers (Lewis & Grandl, 2015; Syeda et al, 2015; Cox et al., 2016). Piezo channels can be activated by both convex and concave membrane curvature within a pipette for patch clamp electrophysiology, suggesting the channel may sense changes in the lipid membrane as opposed to sensing the curvature of either the inner or outer leaflet of the membrane (Lewis & Grandl, 2015). It has been estimated that the in-plane area change of Piezo channels upon activation is ~6-20nm², similar to that of the bacterial MscL (~20nm²), suggesting that Piezo channels may have a similar mechanism for sensing tension as the MscL channels, i.e. hydrophobic mismatch (Bae et al., 2013;
Sukharev & Sachs; 2012). It is evident that Piezo channels can be gated via membrane tension, and it remains to be answered exactly how the lipid membrane interacts with Piezo channels for force transduction.

### 1.6.2 Modulation of Piezo ion channels

Although Piezo ion channels are functional in the absence of any tethers, Piezos may be indirectly modulated by tethers. Break down of the cytoskeleton via cyto-D treatment increases the open probability of the Piezo channels and shifts the pressure for half-maximal activation to lower pressures, increasing in the overall sensitivity of the channel (Gottlieb, Bae, & Sachs, 2012; Gnanasambandam et al., 2015). Additionally, Piezo channels in blebs, which lack cytoskeletal attachments, have increased sensitivity to a pressure stimulus, suggesting that the cytoskeleton or ECM components that tether to the plasma membrane may act in a mechanoprotective manner for Piezo channel function (Cox et al., 2016). STOML3 sensitizes Piezo1 and Piezo2 by recruiting cholesterol to the plasma membrane and increasing stiffness (Poole et al., 2014; Qi et al., 2014). Another lipid modulator of Piezo channels is PIP$_2$, which when depleted from the membrane by activation of phospholipase Cδ activation leads to the desensitization of Piezo channels (Borbiro, Badheka, & Rohacs, 2015).

Piezo ion channels can also be modulated by chemical targets. Ruthenium red, a polycationic pore blocker is able to directly interact with Piezo channels to block ion
permeation (Coste et al., 2012; Coste et al., 2015). The spider toxin GsMTx4 and

gadolinium are both known to be blockers of most mechanosensitive channels,
suggesting that these blockers may not directly interact or bind to Piezo channels, but
rather have a mechanism for channel inhibition by modulating local membrane
properties that affect any lipid interactions with channels (Coste et al., 2010; Coste et al.,
2012; Gottlieb et al., 2012). Two agonists have been identified that modulate Piezo1
function, Yoda1 and Jedi (Calahan et al., 2015; Syeda et al., 2015; Wang et al., 2018).

Yoda1 has been shown to activate Piezo1 in calcium assays and slow inactivation in
electrophysiology, and its proposed binding site is residues 1961-2063 (Calahan et al.,
2015; Syeda et al., 2015; Lacroix, Botello-Smith, & Luo, 2018). Jedi was determined to
interact extracellularly with Piezo1 at the distal end of the blades, and the end of the
beam, suggesting a possible ‘lever-like’ mechanism for activation of Piezo channels, in
which force is transmitted from the blades to move the beam and open the channels
(Wang et al; 2018). Piezo channels can also be modulated for inactivation by pH,
membrane voltage, and divalent ions (Coste et al., 2010; Gottlieb et al., 2012; Bae, Sachs,
& Gottlieb, 2015, Wu et al., 2017). Overall, Piezo channels are modulated by both tethers
and chemicals, both directly and indirectly. The use of modulators will be pivotal in
understanding the mechanisms of activation and inactivation.
1.7 Open questions and aims

This dissertation aims to answer the questions of which domains are necessary for the mechanisms of activation and inactivation of Piezo1 ion channels and how these domains contribute to both mechanisms. Piezo ion channels lack homology to any other ion channels and the gating domains of Piezo channels remain unknown. My overall goal is to elucidate the mechanisms for activation and inactivation of Piezo ion channels. My aims to achieve this are: 1) identify the domains necessary for mechanical activation in Peizo1 ion channels and 2) determine the necessity of the Piezo1 CED in inactivation. Channelopathies in Piezo ion channels, such as mutations in Piezo1 that slow inactivation leads to dehydration of red blood cells, directly connect these channels to diseases and affecting human health. The long term is to resolve the mechanisms of activation and inactivation so as to find future treatments for diseases.
2. Piezo1 ion channels retain mechanical activation in microsomes

This chapter contains unpublished data.

2.1 Introduction

In order for an ion channel to be considered a bona fide mechanically activated channel, 4 criteria must be met: 1) the channel must be expressed in the mechanosensory tissue, 2) response to mechanical stimuli requires its expression and deletion of the channel ablates any mechanical response, 3) any alterations to channel biophysical properties directly affects the mechanical response, and 4) mechanical gating can be observed in heterologous expression (Arnadottir and Chalfie, 2010). Piezo ion channels fulfill all of the four criteria and are the first excitatory mammalian non-selective cationic channel (Arnadottir and Chalfie, 2010; Coste et al., 2010, Coste et al., 2012). Mechanically activated ion channels can be gated by either one of two proposed mechanisms, the tether model or the bilayer model. The tether model posits that the ion channel is mechanically coupled to an auxiliary component that creates tension by pulling on the channel to open it, whereas the bilayer model suggests the direct activation of the ion channel occurs via lateral membrane tension from either local membrane curvature, hydrophobic mismatch, or lipid composition (Nilius & Honore, 2012; Wu, Lewis, & Grandl, 2016). The first step in understanding the mechanisms for activation of Piezo ion...
channels is to identify whether these channels are gated by a tether or membrane tension.

In heterologous cells, Piezo ion channels can be activated by membrane tension with a measured half maximal tension of 1.4-4.5 mN/m for Piezo1 in cell-attached patches, demonstrating membrane tension can directly gate Piezo1 ion channels (Coste et al., 2010; Coste et al., 2012; Lewis & Grandl, 2015; Cox et al., 2016). Additionally, Piezo ion channels retain stretch activation in cell-detached patches and blebs, suggesting that any tether or cytoskeletal components are not directly required for opening of Piezo ion channels (Lewis & Grandl, 2015; Cox et al., 2016). Purification and reconstitution of Piezo1 into droplet interface bilayers (DIBs) demonstrated that bilayer asymmetry, osmotic stretch, and lipid monolayer expansion are all sufficient enough to activate Piezo1, providing stronger evidence that Piezo channels are gated by the bilayer model (Syeda, et al., 2015).

Although membrane tension may be the primary source for channel activation of Piezos, the cytoskeleton may play an indirect role in modulating activation, as breaking cytoskeletal connections either with CytoD treatment or in inside-out patches appears to alter the channel open probability and tension to open the channel (Gottlieb, Bae, & Sachs, 2012; Gnanasambandam et al., 2015; Lewis & Grandl, 2015). Therefore, the cytoskeleton may affect the overall membrane tension but it does not need to be tethered.
to Piezo ion channels in order for them to open. Although all of the above findings demonstrate Piezo channels are gated via membrane tension, each of the experimental set-ups have caveats: cell-attached patches may still retain some cytoskeletal connections and are not an isolated bilayer membrane, but with reconstitution of purified channels into proteoliposomes, only a few channels are present, hindering the measurement of macroscopic currents.

In order to achieve our goal of deepening the understanding of the mechanisms of activation and inactivation of Piezo ion channels, the domains involved in each mechanism need to be identified. Piezo ion channels are one of the largest ion channels and contain 38 transmembrane domains per subunit, providing a large number of possible domains that could be a part of the activation or inactivation gates (Ge et al., 2015; Saotome et al, 2018; Guo et al., 2017). Our lab has previously shown that pulling on individual domains with magnetic nanoparticles is not sufficient to directly open Piezo ion channels, suggesting that multiple domains may be a part of the activation gate (Wu et al., 2016). Removal of domains to determine which ones are necessary or involved in gating would be one method to assess their requirement for gating, however removal of the CED of Piezo1 prevented surface expression but not internal localization to the endoplasmic reticulum (ER) (Wu et al., 2017). Therefore, I set out to design an experimental set-up that would allow for the characterization of activation and
inactivation of any mutant Piezo channels that do no express at the plasma membrane surface.

In order to study how the removal of any domains affects the function of Piezo channels, I needed to be able to measure currents for biophysical characterization from any mutant Piezo channels that did not express at the cellular membrane. Previous work demonstrated that Piezo1 channels localized to the ER are part of an integrin activation pathway that controls cell adhesion, therefore suggesting that Piezo ion channels may not only function at the plasma membrane, but may also be stretch activated in ER membranes (McHugh et al., 2010; McHugh et al., 2012). I hypothesized that if I could successfully prepare vesicles from ER membrane fractions, that Piezo1 ion channels would retain mechanical sensitivity. My rationale is that Piezo1 ion channels retain mechanical sensitivity in a multitude of experiment set-ups – cell-attached, cell-detached, blebs, and DIBs – and we know that channels localize and function in signaling pathways initiated within the ER, as previously stated above (Coste et al., 2010; Coste et al., 2012; Lewis & Grandl, 2015; Cox et al., 2016; Syeda, et al., 2015; Gottlieb, Bae, & Sachs, 2012; Gnanasambandam et al., 2015). My aim was to design a novel experimental technique in which we could measure macroscopic currents of Piezo ion channels, and any mutated constructs that lacked surface expression in order to identify which domains are required for activation and inactivation.
To test the hypothesis that Piezo1 ion channels retain mechanical activation in ER membranes, I developed a novel technique which combined the preparation of microsomes, or vesicles formed from ER membranes, and patch clamp electrophysiology (Figure 5). Liver microsomes are commonly used in the study of drug and xenobiotic metabolism and drug delivery, however insect cell microsomes have been used for inserting membrane proteins into lipid planar bilayers for electrophysiological characterization (Zhang et al., 2015; Simon & Blobel, 1991; Dondapati et al., 2014). Microsomes containing transiently transfected mouse Piezo1 exhibited large macroscopic currents. The activation threshold was shifted towards higher pressures in microsomes as compared to HEK cell recordings, as well as almost a complete loss of inactivation, suggesting that membrane tension or other components can modulate activation and inactivation.
Figure 5: Microsome preparation procedure. Briefly, heterologous cells were harvested by centrifugation, homogenized, and fractionated. The fraction containing the endoplasmic reticulum membranes was spun by ultracentrifugation and resuspended to form microsomes. Mircosomes prepared from HEK-P1KO cells that had been transfected with Piezo ion channels should also contain Piezo protein.

2.2 Experimental Methods

2.2.1 Cell culture and transfection

HEK293T-P1KO cells (HEK-P1KO; Piezo1 knockout human embryonic kidney cells) were obtained from Ardem Patapoutian (Dubin et al., 2017) and used for all
experiments. Cells were cultured in DMEM-HG (Life Technologies) supplemented with 10% HyClone defined fetal bovine serum (GE Healthcare Life Technologies), 50U/mL penicillin and 50mg/mL streptomycin (Life Technologies), and incubated at 37°C and 5% CO₂. Cells were typically passaged every 2-3 days and used for 7-8 passages.

For microsome experiments, cells were seeded at 1 million cells per 10cm dish with supplemented DMEM. Three 10cm dishes were transiently transfected 24 hours post-seeding with 90µg specific construct DNA as described via the manufacturer protocol for FuGene6 or were left untransfected (unTF) as a negative control. Microsomes were prepared 36-48 hours post-transfection.

2.2.2 Microsome preparation

Microsomes were prepared using the microsome isolation kit (Abcam). Three 10cm dishes with transfected HEK-P1KO cells at 60-70% confluency were washed and resuspended with 3mL of ice cold DPBS (Sigma Aldrich). Cells were spun down at 700xg for 5 mins and then the supernatant removed. The cell pellet was resuspended in 500µL of chilled homogenization buffer with PIC, homogenized in a chilled 2mL Dounce homogenizer 10-15 strokes, and 750µL of homogenization buffer added and pipetted up and down to fully mix cell suspension. Cell homogenate was transferred to microcentrifuge tube and vortexed for 30s and chilled on ice for 60s. Homogenate was spun down at 4°C at 10,000xg for 15min. A thin lipid layer was aspirated of the top of
the supernatant, which contains dilute microsomes and cytosolic components, and the supernatant transferred to a new pre-chilled microcentrifuge tube. Microsomes were spun down at 25,000rpm (Beckman Coulter OptimaMAX, TLA100.3 rotor) at 4°C for 20-30min. The supernatant was removed and the microsome pellet resuspended in liposome buffer (200mM KCl, 40mM MgCl, pH=7.2). Microsomes were kept on ice for 2-3hrs while used for imaging or electrophysiology experiments.

### 2.2.3 Electrophysiology

All negative pressure cell-attached recording experiments were performed at room temperature in a balanced bath and internal solution. The solution contained (in mM) 130 NaCl, 5 TEA-Cl, 5 EGTA, 10 HEPES, pH= 7.2 with NaOH. For Yoda1 experiments, the recording solution was used with 40µM Yoda1 (dissolved in DMSO). Patch clamp recordings were performed with an EPC10 amplifier and Patchmaster software (HEKA Electronik), with a 5kHz sample rate and 2.9kHz filter rate. A high-speed pressure-clamp system (ALA Scientific Instruments) connected to Patchmaster software was used to control pressure during experiments. Thick-walled borosilicate glass (OD-1.5mm, ID-0.75mm) pipettes with resistances of 3.5-6.5MΩ were used for experiments. For negative potential experiments, patches were held at -80mV for the recordings. For sweeps, cells were held at 0mmHg for a 500ms pre-step, followed by
500ms of a pressure step, and ending with a 500ms 0mmHg post-step. Pressure steps increased from 0 to -150mmHg in 10mmHg increments.

For cell-attached recordings in HEK-P1KO cells, a bath solution was used containing (in mM) 140 KCl, 10 HEPES, 1 MgCl$_2$ and 10 glucose, pH=7.3 with KOH, and a pipette solution was used containing (in mM) 130 NaCl, 5 KCl, 10 HEPES, 1 CaCl$_2$, 1 MgCl$_2$, and 10 TEA-Cl, pH=7.3 with NaOH. The same pressure step protocol as the microsomes was used for recording from HEK-P1KO cells.

2.2.4 Imaging

Microsomes were prepared from HEK-P1KO cells as described and added to the sodium-based recording solution for imaging. A Nikon CoolSNAP ES camera and 4x relay lens (Nikon Instruments Inc, Melville, NY) was used with a 20X objective and 2sec exposure to take brightfield images.

2.2.5 Electrophysiology analysis

Electrophysiology data was analyzed using Igor Pro 8.02 (WaveMetrics). Pre-pressure step baseline currents were subtracted off-line and the peak current during the pressure stimulation was measured for each pressure step. All patches had a seal resistance >1GΩ, and any patches with maximum currents less than 50 pA or did not make it past the -70mmHg pressure step were excluded. The raw peak current at each pressure step was measured for WT mPiezo1 and untransfected microsome patch
recordings and the statistical significance for each pressure step was determined via unpaired t-test. The pressure of half-maximal activation (P50) was calculate by first normalizing the peak current of each individual cell or microsome to its maximum peak current and then fit with a Boltzmann function:

$$I = I_{\text{min}} + \frac{I_{\text{max}}}{1 + \exp \left( \frac{P_{50} - P}{k} \right)}$$

where the minimum and maximum current amplitude are $I_{\text{min}}$ and $I_{\text{max}}$, P is the pressure and $k$ is the slope. The mean P50 was calculated for each microsome and cell recording and statistical significance determined by unpaired t-test.

2.2.6 Statistical analysis

Unpaired t-test was used for all electrophysiological analysis to determine whether the function of Piezo1 was altered in microsomes by comparing the Boltzmann P50 and slope for a statistical significance with a $p<0.05$. All experiments were performed with at least 3 separate transfections.

2.3 Piezo1 retains mechanical sensitivity in microsomes

In order to test the hypothesis that Piezo1 would retain mechanical sensitivity in microsomes, the first step was to optimize a protocol for preparing microsomes from ER membrane fractions that could be used for electrophysiology, as negative pressure patch clamp electrophysiology on microsomes has previously never been performed. Since the
ER contains over half of the proteins within the cell, as well as calcium release ryanodine receptors and potassium channels, all microsomes were prepared or incubated in sodium-based solutions so as to ensure that any mechanically activated currents observed from microsomes would be from mechanosensitive non-selective cationic channels that conduct sodium ions, and not from any ryanodine receptors or stretch-sensitive K2P channels (Alberts, et al., 2002; van Petegem, 2012). Microsomes were visible at 20x magnification and were large enough for a patch pipette to patch onto the membrane (Figure 6A).

To test whether Piezo ion channels retain mechanical sensitivity in ER membranes, we transiently transfected HEK-P1KO cells with mouse Piezo1-IRES-EGFP (WT mPiezo1) and prepared cells and microsomes for electrophysiological experiments. For cellular recordings, WT mPiezo1-transfected HEK-P1KO cells were recorded in previously published cell-attached solutions (Coste et al., 2010). Microsomes were added to a bath containing a sodium-based solution and allowed 15-20min to settle onto a glass slide, and negative pressure patch clamp recordings were performed with a balanced bath and pipette solution. Mechanically induced currents were observed in the microsomes from cells that had been transfected with WT mPiezo1 (58.9±7.4pA at -100mmHg, n=16microsomes), as well as cellular recordings. Although WT mPiezo1 retained mechanical sensitivity in microsomes, there were apparent alterations in the
biophysical properties, i.e. activation and inactivation kinetics. whereas untransfected microsomes did not have any observable mechanical currents (8.9±0.9pA at -100mmHg, n=14microsomes). Untransfected microsomes were used as a negative control to confirm no presence of any other mechanosensitive ion channels.

Figure 6: WT mPiezo1 is mechanically activated in microsomes. (A) Top are microsomes at 20x magnification, scale bar 10µm. Bottom is microsome at 40x magnification with 4x coupler, scale bar 3µm. (B) Representative traces of WT mPiezo1 currents in microsomes (top) and HEK-P1KO cells (bottom). Cells and microsomes had a 500ms no stimulus, 500ms negative suction, and 500ms no stimulus protocol applied. Negative suction was applied 0 to -150mmHg or until patch burst.
2.4 Gating kinetics and pressure for half-maximal activation of Piezo are altered in microsomes

It was apparent that although Piezo1 retained mechanical function in microsomes, there were changes in activation and the half-maximal activation threshold (P50) compared to that of channels in the plasma membrane. Negative pressure cell-attached patch recordings were performed on HEK-P1KO cells transfected with WT mPiezo1 with regular glass pipettes and a previously published cell-attached solutions previously described (Coste et al., 2010; Lewis & Grandl, 2015). Patches were treated with the same negative suction protocol as the microsomes. As what is typically observed for Piezo1 cell-attached patches, the currents were rapidly-inactivating (Figure 7A, bottom). WT mPiezo1 currents in microsomes lacked any inactivation (Figure 7A, top) and mechanically activated currents were absent in untransfected microsomes as compared to WT mPiezo1-transfected microsome raw peak currents (Figure 7A, middle; 7B). Peak currents for both HEK cell and microsome WT mPiezo currents were normalized (Figure 7C). When fit with a Boltzmann equation, there was a significant difference in the slope constants (Figure 7D, right: WT mPiezo1 HEK-P1KO 12.5±2.4; WT mPiezo1 microsomes 23.8±2.8; unpaired t-test, p= 0.17, n=8cells; 20 microsomes) as well as a significant shift in the P50 (Figure 7D, left: WT mPiezo1 HEK 29.3mmHg ±4.9; WT mPiezo1 microsomes 90.5mmHg ±9.3; unpaired t-test, * p<0.05, ***, p= 0.00005, n=8cells; 20microsomes). WT mPiezo1 currents in microsomes do not have a significant difference
in activation (Figure 7E: microsomes 55.8ms ±24.4; HEK-P1KO cells 7.1ms ±1.5; unpaired t-test, n=8cells, 15microsomes) or deactivation (Figure 7E: microsomes 263.5ms ±49.7; HEK-P1KO cells 20.7ms ±7.8; unpaired t-test, n=8cells, 15microsomes) kinetics compared to HEK-P1KO cells (Figure 7E: microsomes 55.8ms ±24.4; HEK-P1KO cells 7.1ms ±1.5; unpaired t-test, n=8cells, 15microsomes). Due to a loss of inactivation in microsomes, inactivation taus could not be fit to the data. These results demonstrate that WT mPiezo1 is functional in microsomes, however there is a shift in the biophysical properties.
Figure 7: The biophysical properties of WT mPiezo1 are significantly shifted in microsomes. (A) Representative traces of WT mPiezo1 in microsomes (top) and HEK cells (bottom). WT mPiezo1 lacked inactivation in microsomes, whereas 90% of WT mPiezo1 in HEK cells contained rapidly-adapting currents. (B) WT mPiezo1 dose response curves of raw peak current from microsomes transfected with WT mPiezo1 vs. untransfected microsomes. Significant differences in current at each pressure step * p<0.05, ** p<0.005, *** p<0.0005. (C) WT mPiezo1 dose response curves in HEK cells and microsomes. Significant differences in current at each pressure step * p<0.05, ** p<0.005, *** p<0.0005. (D) WT mPiezo1 currents in microsomes had a significantly shifted P50 value (left) of 90.5mmHg ± 9.3 n=18 microsomes compared to HEK cells with 29.3mmHg ± 4.9 n=8 cells; unpaired t-test, p= 0.00005. The slope value (right) for microsomes 23.8±2.8 was significantly different from HEK cells 12.5±2.4. unpaired t-
test* p<0.05, ** p<0.005, *** p<0.0005 (E). * p<0.05, ** p<0.005, unpaired t-test, n=9 cells; n=15 microsomes. (E) WT mPiezo1 activation and inactivation taus at -90 mmHg were statistically insignificant for WT mPiezo1 between HEK-P1KO cells and microsomes. The activation taus for WT mPiezo1 in HEK-P1KO cells (7.1 ms±1.5) compared to in microsomes (55.8 ms±24.4) were not statistically significant; the deactivation taus for for WT mPiezo1 in HEK-P1KO cells (20.7 ms±7.8) compared to in microsomes (263.5 ms±49.7), unpaired t-test.

2.5 Discussion

Piezo ion channels are able to retain mechanical sensitivity in a multitude of experimental set-ups that break or remove cytoskeletal components, including cell-attached patches, inside-out patches, blebs and DIBs, giving strong support that these channels follow the bilayer model for activation (Coste et al., 2010; Lewis & Grandl, 2015; Cox et al., 2016; Syeda et al., 2015). Piezo ion channels not only lack structural and sequence homology to any other ion channel, but also contain 38 transmembrane domains per subunit, which has made it challenging in piecing together the domains and mechanisms for activation and inactivation of these channels. Our lab set out to identify the domains involved in activation of Piezo ion channels by pulling on individual transmembrane domains to directly open the channel, however our findings were that pulling on individual domains was not sufficient to activate the channels but rather slowed inactivation kinetics when pulling on the CED and end tip of the blades (Wu et al., 2016). These findings suggested that perhaps it is not single transmembrane domains acting as the activation gate for Piezo ion channels, but instead larger domains
may work in cooperatively to open and close the channels. To identify the domains that
are necessary for activation and inactivation, the next step would be to remove domains
and test for functionality, however it has been shown that removal of the CED abolished
surface expression of the protein, providing a challenge to characterizing any deletion
mutants (Wu et al., 2017). Therefore, we needed to design a technique that would allow
for the study of deletion mutants and for the measurement of macroscopic currents for
biophysical characterization.

It was not surprising that Piezo1 channels retained mechanical sensitivity in
microsomes, as Piezo1 has been identified in having a functional role in ER integrin
pathways that affect cell adhesion (McHugh et al., 2010; McHugh et al., 2012). Piezo ion
channels localized to the surface of the plasma membrane have been the population of
focus for characterizing Piezo channel function, as it is most accessible for performing
electrophysiological and immunohistochemistry experiments and has significant
physiological relevance for disease research; however, Piezo1 may have more functions
than being the primary excitatory mechanosensor in cells. Functionality in the
microsomes further supports that gating of Piezo1 is in accordance with the bilayer
model, and more strikingly, there appears to be changes in kinetics that could be a result
of the bilayer. WT Piezo1 currents in microsomes displayed a slowing activation rate, as
well as a loss of rapid inactivation, which is observed infrequently in cell-attached HEK
cell recordings. Membrane tension has been shown to directly gate Piezo1 ion channels, therefore the changes in the biophysical properties for Piezo1 in the microsomes could be a result of changes in membrane tension due to the lipid composition within the ER microsome membranes (Lewis & Grandl, 2015; Cox et al, 2016; Syeda et al., 2015).

Sphingolipids and sterols are rigid lipids that contribute to membrane stiffness, and these lipid levels vary among cellular organelles; plasma membranes have much higher levels of sterols and sphingolipids, giving it more stability and stiffness, whereas the ER membrane contains low levels of these lipids and is much more fluid (van Meer, Voelker, & Feigenson, 2008). Piezo channels are sensitized by increasing the membrane stiffness through recruitment of cholesterol to the plasma membrane via STOML3, suggesting that lipid composition indirectly modulates Piezo activity via membrane stiffness (Qi et al., 2014). Reduced stiffness in the microsomes would lower the resting membrane tension and would therefore require a much larger force or tension to activate Piezo channels, as the results demonstrate with the significant increase in the half-maximal activation threshold and time to peak current for Piezo1 in the microsomes compared to cell-attached HEK cells.

It has repeatedly been demonstrated that membrane tension gates activation of Piezo ion channels, yet less has been studied on the effects of membrane tension on the inactivation mechanism of Piezos. Our lab has previously demonstrated that
inactivation can modulate the sensitivity of Piezo channels, as the offset of a stimulus that reduces the resting membrane tension within a patch pipette recovers any channels that have populated an inactivated state and increasing the overall mechanical response (Lewis & Grandl, 2015). It is possible that if inactivation is indirectly modulated by membrane tension, then the membrane tension within the microsomal membranes may alter inactivation kinetics, i.e. remove inactivation. One way to assess using our microsome preparation would be to record from WT Piezo1 in microsomes upon the application of cholesterol or other molecular compounds that could alter membrane stiffness, to see if there is a leftward shift in the pressure for half-maximal activation or if inactivation can be rescued by membrane stiffness alone. The advantage of the microsome preparation over cholesterol depletion experiments performed on cells is that any lipid could be incorporated into the microsomes during preparation, ensuring that the lipids are in fact inserted into the microsome membranes, as opposed to typical perfusion of cholesterol depleting compounds during electrophysiological recordings. Although the microsome preparation does not directly replicate the membrane tension of the plasma membrane for any experimental testing, it however does offer an opportunity for more fine-tuned manipulation of experimental design, which will be necessary in order to determine the mechanistic underpinnings of activation and inactivation of Piezo ion channels.
3. The C-terminal extracellular domain is necessary for Piezo1 function

This chapter contains unpublished data.

3.1 Introduction

After verification of the Piezo ion channels as bona fide mechanosensitive ion channels that contained pore-forming subunits, it became evident that Piezo channels were unique in structure as well as their potential mechanisms for mechanical gating (Coste, 2010, Coste, 2012, Syeda, 2016). Piezo channels lack any sequence homology to any other protein, and cryo-EM structures of Piezo1 reveal a large globular extracellular domain that is positioned directly over the membrane-spanning pore region, later termed the C-terminal extracellular domain (CED) (Ge et al., 2015; Saotome et al., 2018; Guo et al., 2017). Pulling with extracellular magnetic nanoparticles directly on the CED domain does not activate Piezo ion channels, however it is sufficient to significantly slow the inactivation kinetics of mouse Piezo1 ion channels (Wu et al., 2016). Piezo1 and Piezo2 channels have markedly different inactivation kinetics, and a chimeric approach of swapping the CED domains between Piezo1 and Piezo1 is sufficient in conferring the inactivation kinetic of its corresponding CED (Wu et al., 2017). Manipulating the CED of Piezo channels directly affects inactivation of the channel, however this does not mean that the CED is the inactivation gate. It is possible that the CED is critical for the structural changes as the channel transitions from conducting to non-conducting states,
but does not directly block ion permeation during inactivation. It is known that lateral membrane tension activates Piezo1 in membranes; however the mechanism of inactivation, and the specific function of the CED in inactivation, remains elusive.

Although the CED lacks homology and structural motifs to any known protein, its direct positioning over the pore, along with its apparent role in Piezo channel inactivation, suggests the possibility that the CED could serve as being the inactivation gate for Piezo in channels. However, it still remains a possibility that the CED is necessary for both gating mechanisms of activation and inactivation. My goal was to characterize the mechanism for inactivation of Piezo ion channels by determining the function of the CED for Piezo channel inactivation.

I hypothesized that deletion of Piezo1 CED will slow or remove inactivation. My rationale is that certain families of ion channels, such as Shaker K+ channels, contain inactivation domains that act as a ball-and-chain and block the pore during stimulation (Hoshi, Zagotta, & Aldrich, 1991; Long, Campbell, & MacKinnon, 2005). The CED of Piezo in channels has also been demonstrated in being directly involved in inactivation, as swapping the CED of Piezo1 and Piezo2 confers its respective inactivation kinetics (Wu et al., 2016; Wu et al., 2017).

To test this hypothesis, the entirety of CED domain of Piezo1 ion channel was removed and tested the CED deletion channel for mechanical sensitivity via negative
pressure patch clamp electrophysiology. Deletion of the CED of Piezo1 prevented trafficking of the mutant protein to the cellular plasma membrane and rendered a channel unable to be activated via mechanical or chemical activation in microsomes. Although the mutant channel was non-functional in a stretch activation assay, NativePAGE gel analysis demonstrated that the removal of the CED did not prevent trimerization of the ion channel.

3.2 Experimental Methods

3.2.1 Cloning

The CED deletion constructs, mP1-ΔCED and mP1_TNXL-ΔCED, were generated by first inserting AgeI restriction cut sites at amino acid positions P2223 and S2450 in mouse Piezo1-pIRES-EGFP in pcDNA3.1(+) (Ardem Patapoutian lab) and mouse Piezo1-TNXL (site) (previously described), respectively, using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). AgeI cut site primers were synthesized and desalted (Millipore Sigma). Constructs were restriction enzyme digested with AgeI-HF (NEB) and the large DNA fragment lacking the CED fragment was purified via gel electrophoresis. Purified digested DNA was ligated together with T4 DNA ligase (NEB). The restriction enzyme site remained as a ‘TG’ residue expression in the deletion site. For both constructs, successful fragment removal and ligation was verified by Sanger sequencing (Genewiz).
An α-bungarotoxin binding site was inserted to at residue 86 of WT mPiezo1 and mP1-ΔCED to be used for immunostaining experiments, as this residue has been previously demonstrated to be extracellular exposed (Wu et al., 2016). The 13 amino acid α-bungarotoxin binding site (BBS) sequence (WRYYESSLEPYPD) was cloned into mP1-ΔCED using a Q5® Site-Directed Mutagenesis Kit (NEB). Successful insertion of the BBS was verified via Sanger sequencing (Genewiz).

3.2.2 Cell culture and transfection

HEK293T-P1KO cells (HEK-P1KO; Piezo1 knockout human embryonic kidney cells) were obtained from Ardem Patapoutian (Dubin et al., 2017) and used for all experiments. Cells were cultured in DMEM-HG (Life Technologies) supplemented with 10% HyClone defined fetal bovine serum (GE Healthcare Life Technologies), 50U/mL penicillin and 50mg/mL streptomycin (Life Technologies), and incubated at 37°C and 5% CO₂. Cells were typically passaged every 2-3 days and used for 7-8 passages.

3.2.2.1 Seeding and transfection for HEK-P1KO electrophysiology and immunostaining

For cell-attached electrophysiology and confocal fluorescence imaging experiments, HEK-P1KO cells were seeded onto 12mm coverslips coated with poly-L-lysine and laminin (Millipore Sigma) at a seeding density of 20,000 cells per well in a 24-well plate. Cells were transiently transfected 24 hours later with WT mPiezo1-pIRES-GFP, mP1-ΔCED, or GFP for electrophysiology experiments and with mPiezo1-pIRES-
GFP BTX86, mP1-ΔCED BTX86, or GFP for confocal imaging experiments. For transfection, specified DNA constructs (1.5 µg) were mixed with OPTI-MEM (Gibco) and FuGene6 (Promega) as described by the manufacturer protocol. 10µM ruthenium red was added to media. Cells were recorded or immunostained 48 hours post-transfection.

3.2.2.2 Seeding and transfection for HEK-P1KO microsome preparation

For microsome experiments, cells were seeded at 1 million cells per 10cm dish with supplemented DMEM. Three 10cm dishes were transiently transfected 24 hours post-seeding with 90µg specific construct DNA as described via the manufacturer protocol for FuGene6 or were left untransfected (unTF) as a negative control. Microsomes were prepared 36-48 hours post-transfection.

3.2.3 Microsome preparation

Microsomes were prepared using the microsome isolation kit (Abcam). Three 10cm dishes with transfected HEK-P1KO cells at 60-70% confluency were washed and resuspended with 3mL of ice cold DPBS (Sigma Aldrich). Cells were spun down at 700xg for 5 mins and then the supernatant removed. The cell pellet was resuspended in 500µL of chilled homogenization buffer with PIC, homogenized in a chilled 2mL Dounce homogenizer 10-15 strokes, and 750µL of homogenization buffer added and pipetted up and down to fully mix cell suspension. Cell homogenate was transferred to
microcentrifuge tube and vortexed for 30s and chilled on ice for 60s. Homogenate was spun down at 4°C at 10,000xg for 15min. A thin lipid layer was aspirated of the top of the supernatant, which contains dilute microsomes and cytosolic components, and the supernatant transferred to a new pre-chilled microcentrifuge tube. Microsomes were spun down at 25,000rpm (Beckman Coulter OptimaMAX, TLA100.3 rotor) at 4°C for 20-30min. The supernatant was removed and the microsome pellet resuspended in liposome buffer (200mM KCl, 40mM MgCl pH=7.2). Microsomes were kept on ice for 2-3hrs while used for imaging or electrophysiology experiments.

### 3.2.4 Electrophysiology

All negative pressure cell-attached recording experiments were performed at room temperature in a balanced bath and internal solution. The solution contained (in mM) 130 NaCl, 5 TEA-Cl, 5 EGTA, 10 HEPES, pH= 7.2 with NaOH. For Yoda1 experiments, the recording solution was used with 40µM Yoda1 (dissolved in DMSO). Patch clamp recordings were performed with an EPC10 amplifier and Patchmaster software (HEKA Electronik), with a 5kHz sample rate and 2.9kHz filter rate. A high-speed pressure-clamp system (ALA Scientific Instruments) connected to Patchmaster software was used to control pressure during experiments. Thick-walled borosilicate glass (OD-1.5mm, ID-0.75mm) pipettes with resistances of 3.5-6.5MΩ were used for experiments. For negative potential experiments, patches were held at -80mV for the
recordings and for positive potential experiments, cells were held at 0mV between sweeps and at +80mV during the sweeps. For sweeps, cells were held at 0mmHg for a 500ms pre-step, followed by 500ms of a pressure step, and ending with a 500ms 0mmHg post-step. Pressure steps increased from 0 to -150mmHg in 10mmHg increments.

For cell-attached recordings in HEK-P1KO cells, a bath solution was used containing (in mM) 140 KCl, 10 HEPES, 1 MgCl₂ and 10 glucose, pH=7.3 with KOH, and a pipette solution was used containing (in mM) 130 NaCl, 5 KCl, 10 HEPES, 1 CaCl₂, 1 MgCl₂, and 10 TEA-Cl, pH=7.3 with NaOH. The same pressure step protocol as the microsomes was used for recording from HEK-P1KO cells.

3.2.5 Immunostaining

For surface labeling experiments, cells transfected with BBS-tagged constructs or GFP were washed 3 times for 5min with DPBS (Millipore Sigma) and then incubated with DPBS, 10mM HEPES, 1:100 (10µg/mL) of an α-bungarotoxin conjugated with AlexaFluor-555 (B35451; Molecular Probes) for 15min at 37°C in the dark. Cells were washed 3 times for 5min and were fixed with 4% paraformaldehyde for 30min at room temperature in the dark. Cells were again washed 3 times for 5min and then mounted with Fluoromount-G (SouthernBiotech) onto glass slides.

For cytoplasmic labeling experiments, cells transfected with BBS-tagged samples or GFP were fixed with 4% paraformaldehyde for 30min at room temperature and then
permeabilized for 15min with Triton X-100 (201614; ThermoFisher). Cells were then treated for 15min with 10% normal goat serum (NGS) before being incubated with α-bungarotoxin conjugated with AlexaFluor-555 in 1% NGS for 60min at room temperature in the dark. Cells were then washed 3 times with DPBS and mounted with Fluoromount-G (SouthernBiotech) onto glass slides.

3.2.6 Fluorescence imaging

All surface and cytoplasmic labeling immunostained cells were imaged on a Zeiss 780 inverted confocal microscope at 40x or 63x magnification with an aperture of 1,4. A custom-written script (Wu, Goyal, & Grandl, 2016) in Fiji image processing software measured the mean fluorescence intensity along the bounding cell membrane for surface labeling experiments and within the cytoplasm for cytoplasmic labeling experiments.

For fluorescence imaging of WT mPiezo1 and mP1-ΔCED at the surface of microsomes, the TNXL-tagged constructs were transfected into 10cm dishes and microsomes prepared as previously described. The microsomes were added to the microsome electrophysiology solution and imaged with a 488-filter using a Nikon CoolSNAP2 with 4x coupler at 20x magnification. Images were analyzed using a custom-written script for Matlab that measured the bounding mean intensity of the microsome.
3.2.7 Electrophysiology analysis

Electrophysiology data was analyzed using Igor Pro 8.02 (WaveMetrics). Pre-pressure step baseline currents were subtracted off-line and the peak current during the pressure stimulation was measured for each pressure step. All patches had a seal resistance >1GΩ, and any patches with maximum currents less than 50 pA or did not make it past the -70mmHg pressure step were excluded. The raw peak current at each pressure step was measured for WT mPiezo1, mP1-ΔCED, and untransfected microsome patch recordings and the statistical significance for each pressure step was determined via unpaired t-test.

3.2.8 Statistical analysis

Unpaired t-test was used for all electrophysiological analysis to determine whether the function of Piezo1 was altered in microsomes by comparing the raw peak current for a statistical significance with a p<0.05. All experiments were performed with at least 3 separate transfections.

3.3 The CED of Piezo1 ion channel is necessary for surface expression in HEK-P1KO cells

In order to determine whether removal of the CED of Piezo1 ion channels would remove inactivation, a construct was engineered in which the entire extracellular CED region (residue P2225-S2450) was removed from Piezo1-IRES-GFP (WT mPiezo1) and will be further referred to as mP1-ΔCED and can be visualized in Figure 8.
Figure 8: The structure of WT mPiezo1 (top left) and a predicted structure of mP1-ΔCED (top right, modified PDB 3JAC). The predicted full structure of mP1-ΔCED on the left with each subunit colored different. A schematic of the proposed mP1-ΔCED alpha helical structure (bottom).

mP1-ΔCED was first transfected into HEK-P1KO cells and negative pressure patch clamps electrophysiology was performed to measure mechanical currents to observe any changes in inactivation kinetics. WT mPiezo1 and pcDNA-GFP were both transfected as positive and negative controls, respectively. All recordings were
measured in the cell-attached patch configuration. To induce changes in membrane tension via stretch activation, negative suction was applied to the patch pipette and would stretch the membrane in a concave geometry. Cells transfected with WT mPiezo1 exhibited large macroscopic currents at both negative (-80mV, n=9cells, Fig 9A,B) and positive (+80mV, n=4cells, Fig9C, D) potentials, whereas both mP1-ΔCED (-80mV, n=9cells; +80mV, n=7cells) and pcDNA-GFP (-80mV, n=6cells; +80mV, n=5cells) transfected cells lacked any mechanically activated currents at either negative or positive potentials (Fig 9A-D).

Figure 9: mP1-ΔCED lacks mechanical currents in HEK-P1KO cells. (A) Negative pressure patch clamp recordings at -80mV 0 to -160mmHg on HEK-P1KO
cells transfected with WT mPiezo1 (top), mP1-ΔCED (middle), and untransfected (bottom). (B) Peak current averages for each construct at -80mV. WT mPiezo1 was statistically significant from untransfected with p<0.05. (C) Negative pressure patch clamp recordings at +80mV 0 to -160mmHg on HEK-P1KO cells transfected with WT mPiezo1 (top), mP1-ΔCED (middle), and untransfected (bottom). (D) Peak current averages for each construct at +80mV. WT mPiezo1 was statistically significant from untransfected with p<0.05.

To determine whether the lack of mechanical activity for mP1-ΔCED was due to loss of function or reduced membrane expression, surface labeling experiments were performed to verify protein localization and expression. An α-bungarotoxin binding site (BBS) was inserted at residue 86 of both WT mPiezo1 (WT mP1-BTX86) and mP1-ΔCED (mP1-ΔCED-BTX86), as this residue had previously been characterized as an extracellular exposed residue (Figure 10) (Wu et al., 2016).

Figure 10: Schematic of immunolabelling of Piezo1. An α-bungarotoxin binding site was inserted at residue 86, which has previously been demonstrated to be exposed to the extracellular side (Wu et al., 2015). Briefly, α-bungarotoxin is conjugated to fluorophore Alexa555, which can then be used for imaging on a confocal microscope.
For both surface and internal labeling experiments, α-bungarotoxin conjugated with the fluorescent tag AlexaFluor-555 was used to bind the BBS of each construct. Immunostaining revealed that while mP1-ΔCED-BTX86 was expressed internally, there was minimal to no expression of mP1-ΔCED-BTX86 at the plasma membrane surface compared to WT mP1-BTX86 (Figure 11).

Figure 11: mP1-ΔCED-BTX86 does not express at the plasma membrane surface of HEK-P1KO cells. Immunnostaining demonstrated that although mP1-ΔCED is
expressed in the cytoplasm (permeabilized; quantified in bottom right), there is a significant reduction in expression of mP1-ΔCED at the plasma membrane as normalized to WT mPiezo1 surface expression (bottom left), unpaired t-test.

Together these results demonstrate that the CED is necessary for normal trafficking of Piezo ion channels to the plasma membrane. No membrane trafficking signal sequence(s) have been identified and it is possible that the CED contains a recognition site for transport to the plasma membrane. Another possibility is that the removal of the CED causes structural misfolding and cannot be trafficked out of the endoplasmic reticulum.

3.4 The CED is necessary for stretch activation in microsomes

To test whether mP1-ΔCED retained mechanical function in the endoplasmic reticulum (ER), negative patch clamp electrophysiology was performed on vesicles, called microsomes, formed from ER membrane fractions. Briefly, HEK-P1KO cells that were transfected with either WT mPiezo1 (positive control), mP1-ΔCED, or untransfected (negative control) were homogenized and the ER membrane fractionated out and microsomes allowed to form. Microsomes were added to a sodium-based bath solution and allowed to settle for 15mins onto the glass slide. Since the ER contains many proteins, including Ca2+ -activated and -sensitive proteins, a balanced pipette solution to that of the bath was used for all microsome experiments. Microsomes containing WT mPiezo1 exhibited macroscopic currents at both negative (-80mV,
n=20 microsomes) and positive (+80mV, n=9 microsomes), while both mP1-ΔCED (-80mV, n=15 microsomes; +80mV, n=5 microsomes) and untransfected microsomes (-80mV, n=18; +80mV, n=10) elicited no mechanically activated currents (Figure 12).

Figure 12: mP1-ΔCED is not stretch activated in microsomes. (A) Negative pressure patch clamp recordings at -80mV 0 to -160mmHg on microsomes transfected with WT mPiezo1 (top), mP1-ΔCED (middle), and untransfected (bottom). (B) Peak current averages for each construct at -80mV. WT mPiezo1 was statistically significant from untransfected with p<0.05. (C) Negative pressure patch clamp recordings at +80mV 0 to -160mmHg on microsomes transfected with WT mPiezo1 (top), mP1-ΔCED (middle), and untransfected (bottom). (D) Peak current averages for each construct at +80mV. WT mPiezo1 was statistically significant from untransfected with p<0.05.

Since the CED domain lies directly over the pore domain and is part of the subunit-subunit interface, it is possible that the subunits cannot trimerize without the
CED domain. To first determine whether mP1-ΔCED could in fact trimerize and potentially still form a pore, NativePAGE gel analysis was used to verify trimerization. A cellular preparation of HEK-P1KO cells were prepared and transfected the same as previously described for Piezo1 purification (Syeda et al., 2016). Cells that had been transfected in 500cm² dishes with either WT mPiezo1 or mP1-ΔCED were harvested and homogenized, with the proper scaling up of buffers, as described for the microsome preparation. Any membrane bound protein in the ER microsomes was released by detergent (10%CHAPS) and run on a NativePAGE gel to determine whether any monomers were present or unfolding occurred. The gels had bands.

To test whether mP1-ΔCED was also present the membrane of microsomes, a fluorescent sensor TNXL was inserted into WT Piezo1 (WT mP1-TNXL) and mP1-ΔCED (mP1-ΔCED-TNXL) at position so that it could be determined whether the channels were localized at the microsome membrane. Microsomes were prepared from HEK-P1KO cells transfected with either WT mP1-TNXL or mP1-ΔCED-TNXL and added to the sodium-based bath solution for imaging with a Nikon CoolSnap2 (4x coupler) camera. Image analysis revealed that both WT mP1-TNXL and mP1-ΔCED-TNXL had no significant difference in expression levels of the microsomal membranes (Figure 13).
Figure 13: Piezo constructs localize to the microsomal membrane. (A) Microsomes with mPiezo1-TNXL or mP1-ΔCED-TNXL imaged for quantifying localization to the membrane of microsomes. (B) Normalized mean fluorescence intensity to mPiezo1-TNXL. mP1-ΔCED-TNXL did not have a significant difference in localization, unpaired t test.

Collectively, these findings suggest that the removal of the CED has no effect on the folding and trimerization of Piezo ion channels. The loss of function from the absence of the CED could possibly be a result of either of the following: 1) removing the CED creates a Piezo ion channel that is populating an inactivated state and requires a significantly large driving force to open the channel or 2) the CED is part of the ion conduction pathway in Piezo ion channels.
3.5 Total deletion of Piezo1 CED does not populate an inactivated state

Previous research has shown that Piezo ion channels are gated by membrane tension, and that the resting membrane tension within a patch pipette can transition populations of Piezo ion channels into the inactivated state, and can be relieved by application of a positive prepulse before negative suction (Lewis and Grandl, 2015). Since the ER membranes contain different lipid and protein compositions compared to the plasma membrane, it is possible the resting membrane tension of microsomal membranes shifts a larger population of Piezo ion channels into an inactivated state. The removal of the CED, along with a different resting membrane tension, could possibly have mP1-ΔCED channels populating an inactivated state. To address this, a precondition protocol was applied to cell-attached patches on microsomes with either WT mPiezo1, mP1-ΔCED, or untransfected. Briefly, a 3sec prepulse of increasing +10mmHg, 1mmHg increments) positive pressure was applied before a saturating negative pressure step. Contrary to what has been observed for WT mPiezo1 in HEK293 cells, WT mPiezo1 in microsomes exhibited a decrease in maximal peak current upon application of positive pressure prepulse that almost completely blocked channel activation at +10mmHg prepulse (Figure 14). Both mP1-ΔCED and untransfected microsomes did not elicit any currents at any prepulse, suggesting that mP1-ΔCED is not transitioned into an inactivated state. Therefore the results suggest that the CED is
either part of the ion conduction pathway, is one of the components that transduces force and opens the channel, or is integral for local folding that allows the pore to open and close.

Figure 14: Preconditioning does not recover any mP1-ΔCED channels from an inactivated state. (A) Microsomes were given a 3sec prepulse of 0 to +10mmHg in 1mmHg increments, before a saturating (-90mmHg) negative suction pulse was applied to remove any channels in an inactivated state to a closed state. Microsomes were transfected with WT mPiezo1 (top), mP1-ΔCED (middle), untransfected (bottom). (B) Maximal current averages during the negative suction stimulus were plotted at each prepulse pressure.
3.6 Removal of the CED renders Piezo1 insensitive to chemical modulation

Although removal of the CED of Piezo1 results in a channel that is not activated via mechanical stretch, Piezo1 ion channels can be activated via the agonist Yoda1 (Calahan et al., 2015; Syeda et al., 2015). To test whether mP1-ΔCED retained Yoda1 sensitivity, negative pressure patch clamp electrophysiology was performed in the presence of Yoda1. Yoda1 was added to the patch pipette solution for negative pressure step recordings at negative potentials on microsomes transfected with either WT mPiezo1, mP1-ΔCED, or untransfected. Although Yoda1 did not directly activate channels in the absence of the pressure stimulus, it severely slowed down the deactivation kinetics for WT Piezo1 (Figure 15). In the presence of Yoda1, neither mP1-ΔCED nor untransfected cells exhibited currents (Figure 15). A specific binding site for Yoda1 has not been identified yet, however, these results are highly suggestive that the CED is critical and an integral component in the structure of a conductive Piezo1 ion channel, either through maintaining local structural integrity at the pore or possibly being part of the ion conduction pathway.
Figure 15: Yoda1 does not recover mP1-ΔCED mechanical activation. (A) Negative pressure patch clamp recordings at -80mV in a balanced sodium-based solution and 40µM Yoda1 (completely dissolved)Microsomes were transfected with WT mPiezo1 (top), mP1-Δ1-573 (middle), untransfected (bottom). (B) Maximal current averages for each construct was plotted against the pressure.
3.7 Discussion

The CED of Piezo ion channels has been implicated in being a part of the inactivation mechanism(s), however the knowledge of the mechanism(s) is unresolved. The goal has been to identify which domains of Piezo ion channels are involved in activation and inactivation so that the understanding of the mechanisms for gating of these channels can become as in depth as is for most other ion channel families. As the first characterized mammalian mechanically gated channels, gaining understanding of how this unique ion channel family functions at the biophysical level will lead to the advancement of these channels being used as a potential biological tool for manipulating neurological processes. In order to resolve these mechanisms, it is critical that the specific domains involved in each mechanism is known so that more direct manipulations can be done to further deepen our understanding of these mechanisms.

The loss of function due to removal of the CED begins to identify that the domains involved in either activation or inactivation are not modulatory, but suggest rather the domains may be complementary and interdependent for function.

To begin to tease apart the inactivation mechanism of Piezo channels, it is interesting to point out the orientation of the CED directly over the pore domain, almost reminiscent of other ion channel families that are characterized by N-type activation. N-type activation, commonly referred to as the “ball-and-chain” mechanism, of Kv and
Nav channels occurs when a domain blocks the pore during activation and blocks ion conduction (Hoshi, et al., 1991; Goldin, 2003; Armstrong and Hollingworth, 2017). As enticing of a fit this model has structurally with Piezo ion channels, N-type inactivation is characterized by removal of the blocking particle to remove inactivation, followed by restoration of the blocking particle to return inactivation; the removal of the CED led to a loss of function instead of a loss of inactivation suggesting that the canonical N-type mechanism is not at play for Piezo ion channels. On the other hand C-type inactivation is characterized by conformational changes of pore and selectivity filter of K+ channels and occupancy of ions within the pore and filter that lead to slower inactivation kinetics (Goldin, 2003; Armstrong and Hollingworth, 2017). Although the inactivation mechanism of Piezo ion channels may require conformation changes at the pore openings, the rapid inactivation of Piezo in channels is independent of ion occupancy do not make a strong case for typical C-type inactivation. It is possible that the CED is necessary for the conformational changes of inactivation to occur, however it is apparent that the inactivation mechanism does not exactly fit N-type or C-type inactivation mechanisms.

Pulling or mutating the CED of Piezo channels did not directly demonstrate any effects on channel activation, however the CED may still play an indirect role for this mechanism. Removal of the CED did not prevent trimerization or localization of the
channel to the microsomal membrane. Therefore the loss of mechanical and chemical activation may result from the pore collapsing, as the N-terminal of the CED is connected to the inner pore helix and the C-terminal connected to the outer pore helix. Should the pore collapse and be dependent upon the CED to allow for any transition of structural conformations, this could explain the loss of function. On the contrary, the CED possesses a cavity that structurally is similar to a pore opening and is oriented directly over the inner pore helices. One possible mechanism could be that the CED pulls down toward the pore and transmembrane helices during activation to become part of the ion conduction pathway, and then as begins to pull away from the pore as changes in membrane potential induces either conformation changes or lipid interactions.

Specific residues within the CED, as well as the entire domain itself, modulate inactivation kinetics, and our findings that that absence of the CED renders a non-functional channel suggests that the domain may not only be a component of the inactivation gate, but may also be a critical component for either the activation gate or maintaining the structure of the ion conduction pathway.
4. The N-terminus of Piezo1 is necessary for normal mechanical activation

This chapter contains unpublished data.

4.1 Introduction

Three labs have independently resolved cryo-EM structures of Piezo1, and all contain almost identical global structural features. Piezo1 forms a trimeric propeller-like channel, with five predominant features: 1) the “blades” consisting of tetrameric alpha helical bundle repeats that extend ~120Å from the pore helices and are the membrane-spanning domain, 2) a long “beam” that lies perpendicular to the bundles towards the intracellular side, 3) the “pore” region formed by two inner and outer membrane-spanning alpha helices per subunit, 4) the “CED” that lies in the extracellular space directly over the pore and contains a central cavity, and 4) the “anchor” domain that lies within the membrane towards the intracellular end of the pore helices (Ge et al, 2015; Ardem 2017, Mackinnon 2017). Interestingly, all the resolved structures of Piezo1 lack resolvable density of the first few hundred N-terminal residues proposed to be due to high flexibility and movement of these membrane-spanning helices. Lateral membrane tension has been demonstrated to be the primary driving force for activation of Piezo1 ion channels, however the specific domains that sense or transduce changes in tension to open the pore helices have yet to be identified (Lewis and Grandl, 2015; Cox et al., 2016; Syeda et al., 2016). Pulling directly onto specific membrane helices is not adequate
enough to directly open Piezo1 channels, suggesting that multiple helical bundles may sense membrane tension in concert and transduce the force to open the pore (Wu et al., 2016). Membrane tension could be sensed by the channels via a few potential mechanisms, such as local membrane curvature or hydrophobic mismatch, and the upward curved geometry of the blades of Piezo1 highlight them as the likely candidates for transducing force from the membrane to the pore (Nilius and Honore, 2012; Wu, Lewis, and Grandl, 2017). Although evidence suggests that the CED actively plays a role in inactivation of Piezo ion channels, much less evidence exists narrowing down the domains and mechanism for gating activation, and therefore it is my goal to understand the activation mechanism(s) and domains involved for Piezo ion channels.

Voltage-gated ion channels contain separate domains that sense changes in membrane potential and that act as an inactivation gate (source), and thus, the hypothesis is that Piezo ion channels may also have separate modulatory domains for gating activation and inactivation and that activation may be sensed and transduced via the blade and beam domains. The resolved cryo-EM structures lack resolution for the first 500+ residues, however, there remains stability in the alpha helical bundles that are intact with the beam domain. Therefore, the aim of this project was to identify the minimal domains necessary for stretch activation of Piezo1 ion channel.
To test this, a minimal construct was generated that was approximately the length of the resolved cryo-EM structures, and functionality tested via negative pressure patch clamp electrophysiology. Removal of the first 500+ N-terminal residues in the blade domain prevented trafficking of the minimal channel to the plasma membrane in mammalian cells. The minimal construct lacked normal stretch activation function compared to wild-type Piezo1 channels, however single channel openings during mechanical were observed, suggesting that the minimal construct still has retains the ability to conduct ions.

4.2 Experimental Methods

4.2.1 Cloning

The N-terminal deletion construct, mP1-Δ1-573, was generated by first inserting a NotI restriction cut sites at amino acid position 573 in mouse Piezo1-pIRES-EGFP in pcDNA3.1(+) (Ardem Patapoutian lab) and two residues prior to the start codon in the vector sequence using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). NotI cut site primers were synthesized and desalted (Millipore Sigma). Constructs were restriction enzyme digested with NotI-HF (NEB) and the large DNA fragment was purified via gel electrophoresis. Purified digested DNA was ligated together with T4 DNA ligase (NEB). The restriction enzyme site remained as a residue
expression in the deletion site. For both constructs, successful fragment removal and ligation was verified by Sanger sequencing (Genewiz).

An α-bungarotoxin binding site was inserted to at residue 2422 of the mP1-Δ1-573 to be used for immunostaining experiments, as this residue has been previously demonstrated to be extracellularly exposed (Wu et al., 2016). The 13 amino acid α-bungarotoxin binding site (BBS) sequence (WRYYESSLEPYPD) was cloned into mP1-Δ1-573 using a Q5® Site-Directed Mutagenesis Kit (NEB). Successful insertion of the BBS was verified via Sanger sequencing (Genewiz).

4.2.2 Cell culture and transfection

HEK293T-P1KO cells (HEK-P1KO; Piezo1 knockout human embryonic kidney celrdem ls) were obtained from Ardem Patapoutian (Dubin et al., 2017) and used for all experiments. Cells were cultured in DMEM-HG (Life Technologies) supplemented with 10% HyClone defined fetal bovine serum (GE Healthcare Life Technologies), 50U/mL penicillin and 50mg/mL streptomycin (Life Technologies), and incubated at 37°C and 5% CO₂. Cells were typically passaged every 2-3 days and used for 7-8 passages.

3.2.2.1 Seeding and transfection for HEK-P1KO electrophysiology and immunostaining

For cell-attached electrophysiology and confocal fluorescence imaging experiments, HEK-P1KO cells were seeded onto 12mm coverslips coated with poly-L-lysine and laminin (Millipore Sigma) at a seeding density of 20,000 cells per well in a 24-
well plate. Cells were transiently transfected 24 hours later with WT mPiezo1-pIRES-GFP, mP1-Δ1-573, or GFP for electrophysiology experiments and with mPiezo1-pIRES-GFP BTX2422, mP1-Δ1-573 BTX2422, or GFP for confocal imaging experiments. For transfection, specified DNA constructs (1.5 µg) were mixed with OPTI-MEM (Gibco) and FuGene6 (Promega) as described by the manufacturer protocol. 10µM ruthenium red was added to media. Cells were recorded or immunostained 48 hours post-transfection.

3.2.2.2 Seeding and transfection for HEK-P1KO microsome preparation

For microsome experiments, cells were seeded at 1 million cells per 10cm dish with supplemented DMEM. Three 10cm dishes were transiently transfected 24 hours post-seeding with 90µg specific construct DNA as described via the manufacturer protocol for FuGene6. Microsomes were prepared 36-48 hours post-transfection.

4.2.3 Microsome preparation

Microsomes were prepared using the microsome isolation kit (Abcam). Three 10cm dishes with HEK-P1KO cells at 60-70% confluence, transfected with either WT mPiezo1, mP1-Δ1-573, or untransfected, were washed and resuspended with 3mL of ice cold DPBS (Sigma Aldrich). Cells were spun down at 700xg for 5 mins and then the supernatant removed. The cell pellet was resuspended in 500µL of chilled homogenization buffer with PIC, homogenized in a chilled 2mL Dounce homogenizer.
10-15 strokes, and 750μL of homogenization buffer and pipette up and down to fully mix cell suspension. Cell homogenate was transferred to microcentrifuge tube and vortexed for 30s and chilled on ice for 60s. Homogenate was spun down at 4°C at 10,000xg for 15min. A thin lipid layer was aspirated of the top of the supernatant, which contains dilute microsomes and cytosolic components, and the supernatant transferred to a new pre-chilled microcentrifuge tube. Microsomes were spun down at 25,000rpm (Beckman Coulter OptimaMAX) at 4°C for 20-30min. The supernatant was removed and the microsome pellet resuspended in liposome buffer (200mM KCl, 40mM MgCl2, pH=7.2). Microsomes were kept on ice for 2-3hrs while used for imaging or electrophysiology experiments.

4.2.4 Electrophysiology

All negative pressure cell-attached recording experiments were performed at room temperature in a bath solution containing (in mM) 140 KCl, 10 HEPES, 1 MgCl2 and 10 glucose, pH=7.3 with KOH, and a pipette solution containing (in mM) 130 NaCl, 5 KCl, 10 HEPES, 1 CaCl2, 1 MgCl2, and 10 TEA-Cl, pH=7.3 with NaOH. Glass pipettes (OD 1.5mm, ID 0.86mm) with a resistance of 2-3.5MΩ were used for cell-attached recordings. For microsome cell-attached recording experiments, a balanced bath and pipette solution was used, containing (in mM) 130 NaCl, 5 TEA-Cl, 5 EGTA, 10 HEPES, pH=7.2 with NaOH. For microsome Yoda1 experiments, the microsome recording
solution was used with 40µM Yoda1. Thick-walled glass pipettes (OD 1.5mm, ID 0.75mm) with resistances of 3.5-6.5MΩ were used for all microsome experiments. All patch clamp recordings were performed with an EPC10 amplifier and Patchmaster software (HEKA Electronik), with a 5kHz sample rate and 2.9kHz filter rate. A high-speed pressure-clamp system (ALA Scientific Instruments) was used to control pressure during experiments connected to Patchmaster software. For negative potential experiments, patches were held at -80mV for the recordings and for positive potential experiments, cells were held at 0mV between sweeps and at +80mV during the sweeps. All patches formed a gigaseal before any pressure protocol was started. For sweeps, cells were held at 0mmHg for a 500ms pre-step, followed by 500ms of a pressure step, and ending with a 500ms 0mmHg post-step. Pressure steps increased from 0 to 150mmHg in 10mmHg increments, although some seals broke before all 16 sweeps were complete.

4.2.5 Immunostaining

For surface labeling experiments, cells transfected with BBS-tagged samples or GFP were washed 3 times for 5min with DPBS (Millipore Sigma) and then incubated with DPBS, 10mM HEPES, 1:100 (10µg/mL) of an α-bungarotoxin conjugated with AlexaFluor-555 (B35451; Molecular Probes) for 15min at 37°C in the dark. Cells were washed 3 times for 5min and were fixed with 4% paraformaldehyde for 30min at room
temperature in the dark. Cells were again washed 3 times for 5min and then mounted with Flouromount-G (SouthernBiotech) onto glass slides.

For cytoplasmic labeling experiments, cells transfected with BBS-tagged samples or GFP were fixed with 4% paraformaldehyde for 30min at room temperature and then permeabilized for 15min with Triton X-100 (201614; ThermoFisher). Cells were then treated for 15min with 10% normal goat serum (NGS) before being incubated with α-bungarotoxin conjugated with AlexaFluor-555 in 1% NGS for 60min at room temperature in the dark. Cells were then washed 3 times with DPBS and mounted with Fluoromount-G (SouthernBiotech) onto glass slides.

4.2.6 Fluorescence Imaging

All surface and cytoplasmic labeling immunostained cells were imaged on a Zeiss 780 inverted confocal microscope at 40x or 63x magnification with an aperture of 1.4. A custom-written script (Wu, Goyal, & Grandl, 2016) in Fiji image processing software measured the mean fluorescence intensity along the bounding cell membrane for surface labeling experiments and within the cytoplasm for cytoplasmic labeling experiments.

4.2.7 Electrophysiology analysis

Electrophysiology data was analyzed using Igor Pro 8.02 (WaveMetrics). Pre-pressure step baseline currents were subtracted off-line and the peak current during the
pressure stimulation was measured for each pressure step. The average peak current at each pressure step was plotted and the statistical significance for each construct was determined via unpaired t-test.

### 4.2.8 Fluorescence imaging analysis

Fluorescence imaging analysis was performed using a code previously published by a prior member of the lab (Wu et al., 2016). The custom Fiji script would first set a threshold limit on the GFP channel of each cell in images. This processed image would then create a binary image with each generated ROI and that was analyzed after delineation of cells. The script would generate a 1um band around the ROIs and take the mean fluorescence intensity within the band. Each transfection was normalized to the fluorescence intensity of WT mPiezo1 to account for any variability and the normalized data across transfections quantified together.

### 4.2.9 Statistical analysis

Unpaired t-test was used for all electrophysiological and fluorescence imaging analysis to determine whether the function and expression of the mutant constructs had a statistical significance with a $p<0.05$. All experiments were performed with at least 3 separate transfections.
4.3 The N-terminus is necessary for trafficking of Piezo ion channels to the plasma membrane of HEK-P1KO cells

In order to identify the minimal domains necessary for mechanical activation of Piezo1 ion channels, a minimal construct lacking the first 500+ residues of the N-terminus were engineered using mouse Piezo1-IRES-GFP; one construct mimicked the domains present in the resolved cryo-EM structures (mP1-Δ1-573) (Figure 16).

![Figure 16: A schematic of the proposed mP1-Δ1-573 alpha helical structure. The cryo-EM structure of Piezo1 revealed that the N-terminus of Piezo1 forms tetrameric alpha helical bundles, however the first 500+ residues, or proposed first 3 helical bundles, have not been resolved in any of the three structures.](image)

Construct mP1-Δ1-573 was first transfected into HEK-P1KO cells and negative pressure patch clamp electrophysiology was used performed to measure mechanical activation. Briefly, cell-attached patches were formed and negative suction applied to the membrane within the patch to stretch the membrane. WT mPiezo1-IRES-GFP (WT mPiezo1) was used for a positive control and pcDNA-GFP for a negative control. While stretch induced large macroscopic currents in WT mPiezo1-transfected cells at both -
80mV (n = 9 cells, ) and +80mV (n = 4 cells) recordings (Fig 17A,C), mP1-Δ1-573 transfected cells exhibited no currents (n = 10 cells at -80mV; n = 6 cells at +80mV) similar to pcDNA-GFP-transfected cells (n = 6 cells at -80mV; n = 5 cells at +80mV) (Fig 17A-D).

Figure 17: Removal of the N-terminus of Piezo1 abolishes stretch activation in HEK-P1KO cells. (A) Negative pressure patch clamp recordings at -80mV 0 to -160mmHg on HEK-P1KO cells transfected with WT mPiezo1 (top), mP1-Δ1-573 (middle), and untransfected (bottom). (B) Peak current averages for each construct at -80mV. WT mPiezo1 was statistically significant from unTF with p<0.05. (C) Negative pressure patch clamp recordings at +80mV 0 to -160mmHg on HEK-P1KO cells transfected with WT mPiezo1 (top), mP1-Δ1-573 (middle), and untransfected (bottom). (D) Peak current averages for each construct at +80mV. WT mPiezo1 was statistically significant from unTF with p<0.05.
To determine whether the loss of stretch activation with the mP1-Δ1-573 construct was a result of loss of function or reduced expression at the plasma membrane, we inserted an α-bungarotoxin binding site (BBS) at residue 2422 in both WT mPiezo1 (WT mP1-BTX2422) and mP1-Δ1-573 (mP1-Δ1-573-BTX2422), as this residue has previously been demonstrated to be exposed to the extracellular side, to be used for labeling for surface expression (Figure 18) (Wu et al., 2016). For both surface and internal labeling, an α-bungarotoxin conjugated with AlexaFluor-555 was used as a fluorescent tag for imaging on a confocal microscope. Surface staining revealed that mP1-Δ1-573-BTX2422 was not present at the plasma membrane, however, it had internal expression levels comparable to that of WT mP1-BTX2422 (Figure 19).

Figure 18: Schematic of immunolabelling of mP1-Δ1-573. An α-bungarotoxin binding site was inserted at residue 2422, which has previously been demonstrated to be exposed to the extracellular side (Wu et al., 2015). Briefly, α-bungarotoxin is conjugated to fluorophore Alexa555, which can then be used for imaging on a confocal microscope.
Figure 19: mP1-Δ1-573-BTX2422 does not localize to the plasma membrane of HEK-P1KO cells. (A) Hek-P1KO cells were transfected with mPiezo1-BTX2422, mP1-Δ1-573-BTX2422, or GFP and immunostained with an α-bungarotoxin conjugated to AlexaFluor555 (BTX-555). Surface expression was probed by staining cells before fixing (top row), while cytoplasmic expression was first fixed, permeabilized, and stained so as to allow BTX-555 into the cell. (B) Mean fluorescence intensities for non-permeabilized cells normalized to mPiezo1-BTX2422 for each transfection (n=2 transfections non-permeabilized, n=4transfections for permeabilized). Unpaired t-test for mP1-Δ1-573-BTX2422 and GFP to mPiezo1-BTX2422, ** p < 0.005. (C) Mean fluorescence intensities for permeabilized cells normalized to mPiezo1-BTX2422 for each transfection (n=2 transfections non-permeabilized, n=4transfections for permeabilized). Unpaired t-test for mP1-Δ1-573-BTX2422 and GFP to mPiezo1-BTX2422, ** p < 0.005. Scale bars 15µm.
Together these results demonstrate that removal of the first 573 residues of mouse Piezo1 abolishes trafficking of the ion channel to the plasma membrane. One possibility is that the membrane trafficking signal sequence lies within the N-terminus of Piezo1 and therefore removal of this region leaves the channels to remain in the endoplasmic reticulum (ER). The other possibility is that the removal of the N-terminus prevents the channel from structurally folding correctly, and thus the channel is misfolded and stuck in the ER.

4.4 The N-terminus is necessary for macroscopic stretch-activated currents, but not for structural integrity of the channel

To test whether mP1-Δ1-573 retained mechanical activity in the ER, negative pressure patch clamp electrophysiology was performed on microsomes prepared from ER fractions of cells that had been transfected with mP1-Δ1-573 or WT mPiezo1. A protocol has already been established (Chapter 2) for preparing microsomes and cell-attached negative pressure recordings, and thus if mP1-Δ1-573 were a functional channel comparative to that of WT mPiezo1, then the microsome preparation would be a much more efficient for characterizing functionality than purifying the protein and using proteoliposomes.

Microsomes were prepared from HEK-P1KO cells either untransfected or transfected with WT mPiezo1 or mP1-Δ1-573. A sodium-based balanced solution was used for microsome electrophysiological recordings, as there potentially could be other

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proteins and ion channels that are activated or sensitive to calcium or potassium, which is used for cell-attached recordings for Piezo ion channels in HEK cells. WT mPiezo1 retained stretch activation in the microsome preparation (Fig 20A,C), however mP1-Δ1-573 lacked large macroscopic currents compared to that of WT mPiezo1 (Figure 20A,C). Although mP1-Δ1-573 lacked large currents, single channel openings were observed during the pressure stimulus step of protocols (Fig 20B), which was not observed in untransfected microsomes.
Figure 20: mP1-Δ1-573 forms a channel but does not retain normal mechanical activation. (A) Negative pressure patch clamp recordings at -80mV 0 to -150mmHg on microsomes transfected with WT mPiezo1 (top), mP1-Δ1-573 (middle), and untransfected (bottom). (B) Single channel openings for WT mPiezo1 (top) and mP1-Δ1-573 (bottom). (C) Mean peak current averages for each construct at -80mV. WT mPiezo1 was statistically significant from untransfected with p<0.05. (D) Negative pressure patch clamp recordings at +80mV 0 to -160mmHg on microsomes transfected with WT mPiezo1 (top), mP1-Δ1-573 (middle), and untransfected (bottom). (E) Peak current averages for each construct at +80mV. WT mPiezo1 was statistically significant from untransfected with p<0.05.
Together all of these results suggest that the entire N-terminal membrane-spanning domain is required for normal stretch activation to produce macroscopic currents. Single channel openings of mP1-Δ1-573 suggest that the N-terminus is not required for the protein to form an ion conducting pore, however the entire N-terminal domain may work in concert to transduce changes in membrane tension to the pore. Although mP1-Δ1-573 does not retain normal macroscopic currents in microsomes, there is a possibility that the lipid composition of the microsomes affects any protein-lipid interaction that is necessary for activation or may be lacking from loss of the N-terminus.

4.5 Preconditioning and chemical modulation do not recover macroscopic stretch-activated currents in N-terminal Piezo1

It has been previously shown that the resting membrane tension within a patch pipette under no suction can prevent populations of Piezo1 ion channels within the patch from shifting out of an inactivated state into a closed state ready to open, yet application of a small positive pressure to the patch prior to the negative pressure step can relieve the tension and allow the inactivated population to close and more readily open up the stimulus (Lewis and Grandl, 2015). Since single channel, but not macroscopic, currents were observed for mP1-Δ1-573 and WT mPiezo1 exhibits a rightward shift in its P50 value in microsomes, it is possible that the resting tension within microsomal membranes is different than that of the cellular plasma membrane,
and therefore a large fraction of mP1-Δ1-573 channels within the microsomes are populating an inactivated state.

In order to address this, a preconditioning protocol was used in which a 3sec ‘prepulse’ of 0mmHg to +10mmHg was applied immediately before a saturating negative pressure pulse. Surprisingly, when the preconditioning protocol was applied to microsomes containing WT mPiezo1 it actually reduced the maximal peak current during the pressure step (Fig 21A,B). Application of a prepulse to mP1-Δ1-573 did not recover any currents (Figure 21A,B).
Figure 21: mP1-Δ1-573 cannot be recovered from a non-conducting state with preconditioning. (A) Microsomes were given a 3sec prepulse of 0 to +10mmHg in 1mmHg increments, before a saturating (-90mmHg) negative suction pulse was applied to remove any channels in an inactivated state to a closed state. Microsomes were transfected with WT mPiezo1 (top), mP1-Δ1-573 (middle), untransfected (bottom). (B) Maximal current averages during the negative suction stimulus were plotted at each prepulse pressure.

A chemical agonist, Yoda1, has been identified for Piezo1 channels that removes inactivation in electrophysiology experiments and activates in calcium imaging assays (Cahalan et al., 2015; Syeda et al. 2015). To test whether mP1-Δ1-573 retained Yoda1 sensitivity, Yoda1 was applied via the patch pipette solution and a negative pressure
protocol measured. WT mPiezo1 retained stretch-activated currents and had a significant shift in deactivation kinetics (Figure 22A-C), whereas mP1-Δ1-573 did not exhibit any macroscopic currents and was comparable to that of untransfected microsmes (Fig 22A,B). Together these results suggest that while mP1-Δ1-573 may trimerize and form a pore, partial removal of the N-terminus removes normal stretch activation.

![Image](image_url)

**Figure 22:** Yoda1 application does not rescue normal mechanical activation of mP1-Δ1-573. Yoda1 application does not rescue normal mechanical activation of mP1-Δ1-573.
Δ1-573. (A) Negative pressure patch clamp recordings at -80mV in a balanced sodium-based solution and 40µM Yoda1 (completely dissolved). Microsomes were transfected with WT mPiezo1 (top), mP1-Δ1-573 (middle), untransfected (bottom). (B) Maximal current averages for each construct was plotted against the pressure

4.5 Discussion

The mechanism(s) and domains involved in activation of Piezo ion channels have been difficult to identify as the channels lack any structural homology or motifs to any known or characterized on channels. In order to achieve the long term goal of gaining an understanding of the mechanisms of gating Piezo ion channels at level comparable to that of other well-characterized modulatory ion channels, the domains that are directly and indirectly gating these mechanisms need to be identified.

Previous work from our lab demonstrated that pulling with magnetic nanoparticles on residues 86 and 300 of Piezo1 caused a slowing of in activation, suggesting that although these residues are at the N-terminal and away from the pore and CED domain, the domains involved in the mechanisms of activation and inactivation may not be distinct and modulatory, as is the case for the voltage-sensing and inactivation domains of Kv and Nav channels (Wu, et al., 2017). In order to address which domains are involved, an N-terminal deletion mutant of Piezo1 was engineered and characterized for mechanical activation in microsomes, as removal of the N-terminal prevented trafficking. Removal of the N-terminal blade domain past the beam severely
hindered mechanical activation, however single channel openings were observed during the pressure stimulus occasionally.
5. Conclusions

5.1 Major results and interpretations

In this dissertation, I aimed to develop a new technique to identify the specific domains that are involved in the molecular mechanisms of activation and inactivation of Piezo1 ion channels. Piezo channels are a unique family of ion channels that lack homology to any other protein families. Piezo channels are the first mammalian bona fide excitatory mechanically activated ion channels that have been characterized and the molecular underpinnings for activation and inactivation have yet to be determined. These channels can be activated by various mechanical stimuli and modulated by different factors within the different tissues of the human body (Wu, Lewis, & Grandl, 2016). Although Piezo channels were only identified less than a decade ago, channelopathies in Piezo channels have already been identified as causing diseases in tissue specific manner, highlighting the importance in addressing the questions of what are the molecular mechanisms for function of these ion channels and how can these mechanisms be modulated to potentially treat diseases. In order to do this, my aim was to determine which domains of Piezo1 ion channels are involved in activation and inactivation. As certain mutation of Piezo channels result in the loss of trafficking of the channel to the plasma membrane, I developed a novel technique in which I would generate microsomes, or lipid vesicles comprised of endoplasmic reticulum membrane.
fractions and the proteins bound to it. I then performed patch clamp electrophysiology and fluorescence imaging experiments to determine whether removal of domains that have been implicated in activation or inactivation are required for function.

5.1.1 Piezo1 ion channels retain mechanical sensitivity in microsomes

In Chapter 2, I developed a technique for performing negative pressure patch clamp electrophysiology on microsomes containing Piezo1 ion channels. My technique allowed for the study of Piezo1 ion channels in a system that was free of any cytoskeletal network and did not require the tedious purification and insertion of channels into lipid bilayers. I had expected that if Piezo channels are gated by membrane tension and have been implicated in having a functional role in ER-initiated integrin-activating pathways, then Piezo1 would maintain its mechanical sensitivity in microsomal fractions. Indeed, Piezo1 ion channels were activated by negative pressure in microsomes, however activation kinetics and pressure for half-maximal activation were significantly slowed and rightward shifted, respectively. The plasma membrane and cellular organelles all possess different functions and are known to be characterized by different lipid compositions, and thus could be one possibility for the altered biophysical properties of Piezo1 in microsomes (van Meer, Voelker, & Feigenson, 2008). The plasma membrane must be more stiff and rigid in order to give the cell more stability, and its lipid composition is higher in sterols and sphingolipids that give more rigidity, as well as
direct tethers to the cytoskeleton and extracellular matrix. The ER membrane is low in sterols and sphingolipids, making it more fluid and with a different resting membrane tension than the plasma membrane and therefore could account for the changes we observed in Piezo1 function in microsomes.

Interestingly, it has been proposed that the cytoskeleton acts in a mechanoprotective manner with regards to Piezo ion channel function, in that removal or breakdown of the cytoskeleton increases Piezo1 channel activity at lower pressures and its open probability in a cell-attached configuration (Gottlieb, Bae, & Sachs, 2012; Gnanasambandam et al., 2015; Cox et al., 2016). This is partially contradictory to our findings in the microsomes, in that reduced mechanoprotection of the membrane from the absence of the cytoskeleton actually increased the amount of pressure required to open the channels. However, the lack of inactivation of Piezo1 in microsomes would suggest that the open probability is increased in the microsomal membranes, which is in agreement that mechanoprotection reduces channel open probability. The differences in results could suggest that the gating of Piezo ion channels is modulated by the lipid composition of the membrane, but that the open probability is dependent upon the membrane tension, regardless of the membrane lipid composition.

Our findings demonstrate a new method for studying mechanosensitive ion channels in a membrane that lacks any cytoskeletal mechanoprotection, but retains a
more native lipid composition than proteoliposomes. In chapter 3 and 4, this technique became especially useful for the study of the deletion of Piezo1 constructs that do not express at the plasma membrane surface.

5.1.2 The CED of Piezo1 is critical for activation

In Chapter 3, I engineered a Piezo1 construct that lacked the CED domain, which has been identified as potentially having a role in inactivation, as pulling and chimeric constructs of the CED of Piezo1 and Piezo 2 results in altered inactivation kinetics (Wu et al., 2016; Wu et al., 2017). I had expected that if the CED were part of the inactivation gate of Piezo ion channels, then removal of the domain would remove inactivation of the channel. However, removal of the CED prevented trafficking of the channel to the plasma membrane and rendered a non-functional channel that could still trimerize and localize to microsomal membranes. A few possibilities could account for the loss of function from the removal of the CED: 1) the CED is integral for orienting and positioning the pore helices to transition between the open, inactivated, and closed states; 2) the cavity within the CED is also a part of the ion conduction pathway and therefore ion permeation cannot occur in the absence of the CED; 3) the CED is indirectly connected to the activation gate and in its absence the activation gate cannot function correctly; or 4) removal of the CED shifts the activation threshold rightward,
and the membrane tension required to activate that channel is not within a physiological range (i.e. the patch bursts before reaching the threshold).

The chemical agonist, Yoda1, has been shown to be able to activate and slow inactivation of Piezo1 but not Piezo2, and recently a binding site for Yoda1 on Piezo1 was proposed to be in the region just upstream of the pore and CED (residues 1961-2063) (Calahan et al., 2015; Syeda et al., 2015; Lacroix, Botello-Smith, & Luo, 2018). In the microsomes, application of Yoda1 with negative suction had a small but significant shift in the pressure of half-maximal activation and a dramatic slowing of deactivation for WT mPiezo1, suggesting that Yoda1 one may stabilize a conducting state of the ion channel. Although the CED deletion construct contains the proposed Yoda1 binding site, application of Yoda1 and negative suction were not sufficient to activate the CED deletion channel. Three possible reasons for this could be that: 1) the Yoda1 binding site is in fact in the CED domain, preventing chemical activation in the CED deletion channel; 2) the absence of the CED prevents the conformational changes necessary for either binding or opening of the channel into a conducting state; or 3) the CED is part of the ion conduction pathway, and although Yoda1 is able to bind, no ion permeation can occur. One way to determine which of these possibilities is occurring would be to resolve a structure with either Yoda1 bound or with ions present in the permeation. This would determine whether the CED is a part of the Yoda1 binding pocket or if the cavity
within the center of the CED is able to permeate ions, and potentially could provide insight into structural identities of ligand-bound or conducting structures of Piezo1.

Ultimately our results demonstrate that the CED of Piezo1 ion channels is necessary for activation, suggesting that the CED plays a role, directly or indirectly, in both activation and inactivation. Whether this is due to the inability of the channel to transition between non-conducting and conduction states or that the CED is a part of the pore remains to be answered.

5.1.3 The N-terminal is required for normal activation of Piezo1

In Chapter 4, I engineered a Piezo1 construct that possessed only the domains resolved in the cryo-EM structures by partially removing the N-terminal. Whether Piezo channels sense membrane tension through individual helical bundles of the transmembrane region or the entire blade domain remains unknown. Since the first 500+ residues of Piezo1 have not been able to be structurally resolved, I expected that removing the highly flexible domain would render a channel that could still sense membrane tension and transduce changes in tension to the pore, as these structures are most stable in cell-free systems for structural experiments. Since the beam domain that lies perpendicular to the blade and ends at the tip of the blade of the resolved Piezo1 structure, it is plausible that the blade senses tension within the membrane and any movement of the blade helical bundles would pull on the beam and shift the anchor and
pore domains to either open or close. However, our results demonstrated that the N-terminal is important for trafficking to the plasma membrane, as well as producing macroscopic mechanical currents. Single channel openings were observed repeatedly only in microsomes prepared from cells containing the N-terminal deletion mutant and not in any negative control preparations, suggesting that the entire blade domain is necessary for sensing membrane tension but not for pore formation.

Our lab has previously demonstrated that pulling with magnetic nanoparticles on residues in the N-terminal (86 and 300) of Piezo1 is not sufficient to directly open the channel but rather results in a small but significant slowing of inactivation (Wu et al., 2016). Although these residues are located at the end of the blade tips and with no proximity to the pore domain, it is interesting that these residues would affect inactivation but not activation of Piezo1. One possibility is that pulling on these residues stabilized the open conformation of the pore, thus slowing the transition of the channel from a conducting state to a non-conducting state. If the N-terminal is necessary for stabilization of an open conformation of the channel, this could be the reason that the N-terminal deletion construct lacks the ability to open and produce macroscopic mechanical currents. This possibility could suggest that the N-terminal, either the tip or the entire blade, senses membrane tension and is required for the channel to transition into a stable open conformation. Until the N-terminal of Piezo1 or a conducting state
confirmation is resolved, it will be challenging to identify which domains orchestrate the opening and closing of Piezo ion channels.

Although these findings demonstrate that removal of the N-terminal abolishes normal activation of Piezo1 ion channels, the results suggest that not one specific domain is the mechanical gate for Piezo ion channels. One domain that could potentially give more insight into how the blades may sense tension or stabilize the open conformation of the channel would be the beam, which interfaces with both the blade and the anchor and pore domains. Further studies of how the blade and beam interact and function will push our understanding of the mechanisms for gating of Piezo ion channels.

5.2 Implications for the hypotheses of activation and inactivation of Piezo ion channels

Based on our minimal construct and the resolved Piezo1 structure, I propose that tension and force are sensed via the transmembrane domains directly linked to the perpendicular beam helices, from which the force is transmitted to the pore for gating. All of the upstream transmembrane helices may create a longer lever and surface for sensing force to be transmitted via the beam. Our findings demonstrate that both the N-terminal and CED are directly integral for activation of Piezo ion channels, and thus suggest that the domains of Piezo ion channels do not in fact act as distinct sensors or gates, but rather there is an interplay between the mechanisms of activation and
inactivation, which suggests that Piezo channels possess an entirely novel mechanism for mechanical gating that is yet identified.

Based on the resolved structure and loss of function from deletion of the CED, the CED either affects the overall architecture of the channel required for activation and gating or may be a part of the ion conduction pathway.

5.3 The significance of these findings

Narrowing down the specific residues and domains provides insight into how this unique family of mechanically activated channels are modulated by tension. Identification of the gating domains will contribute deeper insight to the bilayer model. Knowledge of the gating residues and domains will allow for more targeted design of potential agonists/antagonists.

5.4 Future directions and potential alternative approaches

The findings in the dissertation provide insight into the necessity of two distinct domains for normal function of Piezo ion channels: the N-terminal blade and the CED, yet how these domains specifically sense membrane tension and transition the channel into conducting and non-conducting states still are unanswered.

5.4.1 Identifying modulation of Piezo ion channels via tethers

Although Piezo ion channels can be activated in the absence of any cytoskeletal or ECM components, this does not rule out that the channels can be modulated by
tethers. In our microsome assay, Piezo1 channels had significant slowing of activation and a loss of inactivation. It is possible that an auxiliary protein or tether could be bound to the channel and modulate force transduction by changing the kinetics of activation and inactivation. Evidence that supports this possibility is that our lab has demonstrated already that pulling with magnetic nanoparticles, similarly to that of a tether, directly on the CED slows inactivation, suggesting that inactivation could be modulated by a tether (Wu et al., 2016). It has also been shown with atomic force microscopy (AFM) that the presence of collagen IV sensitizes the channel to mechanical pulling, but not other ECM proteins, suggesting that in vivo, this could be a tether mechanism for activating Piezo ion channels (Gaub & Müller, 2017). One way to assess whether a tether can modulate Piezo ion channels directly would be to combine our microsome assay with that of the AFM pulling assay. Since Piezo1 channels already exhibit slowed kinetics in the microsomes, it could be tested to see whether pulling with collagen IV could sensitize the channels in microsomes or rescue typical activation and inactivation rates observed in cellular recordings. It would also be intriguing to pull on either the N-terminal or the CED deletion constructs to observe whether modulation via a tether could rescue mechanical activation.
5.4.2 Understanding the effect of membrane stiffness and lipid composition on mechanical activation

Piezo1 ion channels retain mechanical sensitivity in various lipid membrane environments, such as inside-out patches, artificial bilayers, and now microsomes, yet these different preparations result in changes in the biophysical properties, such as shifted pressure of half-maximal activation or inactivation rates (Lewis & Grandl, 2015; Cox et al., 2016; Syeda et al., 2015). Depletion of PIP and PIP₂ from the membrane directly inhibits Piezo1 and Piezo2 channels, supporting that lipid composition can affect or alter Piezo channel function (Borbiro, Badheka, & Rohacs, 2015). Additionally, the plasma membrane provides stability and structure to cells and therefore has higher levels of rigid lipids, such as sterols and sphingolipids, than most other organelles within the cell (van Meer, Voelker, & Feigenson, 2008). In the microsome assay, WT Piezo1 exhibited slowing of activation and a rightward shift in the pressure for half-maximal activation, and since microsomes are prepared from ER membranes which contain little to no sterols or sphingolipids, these changes in the biophysical properties could be a result of a reduction in stiffness of the microsomal membrane. One method to address whether lipid composition and membrane stiffness directly modulate Piezo channel function would be to incorporate cholesterol or phosphoinositides into microsomes and measure any changes in the biophysical properties. The hypothesis would be that if lipid composition or membrane stiffness modulates activation of Piezo1...
channels, then incorporating specific lipids, such as PIP$_2$ or cholesterol, during sample preparation into the microsomal membranes would rescue activation rates comparable to that of Piezo1 at the plasma membrane of cells. Unlike cholesterol depletion experiments in which you cannot verify that all cholesterol has been removed from the plasma membrane, the advantages of this proposed preparation would be: 1) direct incorporation of lipids into the microsomes, as lipids can be adding during the microsome formation step, and 2) direct manipulation as to the concentration of lipids incorporated. This would allow for an in-depth analysis of how lipid composition can directly modulate the kinetics and activation of Piezo1 ion channels. If incorporation of sterols or sphingolipids into microsomes were to rescue activation rates and inactivation of Piezo1 to that of cellular Piezo1, it would also be worth testing the N-terminal and CED deletion constructs to determine whether lipid composition or membrane stiffness could also elicit mechanical sensitivity.

5.4.3 Rescue activation of the CED deletion constructs

Our results demonstrate that removal of the CED of Piezo1 renders a non-functional channel in our microsome assay. Since the function of the CED of Piezo channels has yet to be fully characterized, it is still unknown whether the domain is a part of the ion conduction pathway, is the inactivation gate, or provides structural stability for the conducting and non-conducting conformational states of Piezo channels.
Both mouse and *C. elegans* Piezo CED have been crystallized and their structures resolved, suggesting that the CED domain is soluble and structurally stable without any attachment to Piezo channels (Kamajaya et al., 2014; Ge et al., 2015). In order to determine the necessity of the CED for Piezo1 function, a rescue experiment would be ideal. To test this, the CED could be over-expressed and purified and applied to CED deletion Piezo channels in microsomes to test for mechanical sensitivity. Any rescued function would suggest that the CED is in fact required for activation, but also that the CED can function as an independent particle and direct linkage to the pore domain is not required for opening of the channel.

The aim of the studies in this dissertation were to gain a better understanding for the gating mechanisms of mechanosensitive ion channels, with a specific focus on Piezo ion channels. The development of a novel assay for studying ion channels in absence of cytoskeletal and ECM components allowed for the discovery that both the N-terminal and CED domains of Piezo ion channels are necessary for mechanical sensitivity. These findings contribute to our understanding that distinct domains within Piezo channels may function in accompaniment for the mechanisms of activation and inactivation. Our current understanding of the gating mechanisms of Piezo ion channels will assist in future studies of the role of Piezo channels in mechanosensation and somatosensation.
and the development of technologies to assess and more-directly treat Piezo-related diseases.
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Biography

Breanna Kalmeta attended and received her Bachelors of Science in Biochemistry from Rochester Institute of Technology in May 2012. In the fall of 2012, she joined the Department of Biochemistry at Duke University to pursue her doctoral degree in Biochemistry. In the fall of 2013, Breanna was accepted and transferred to the Department of Neurobiology at Duke University and joined the lab of Jörg Grandl in the Department of Neurobiology. She published the following articles as a secondary author, titled “Inactivation of mechanically activated Piezo1 ion channels is determined by the C-terminal extracellular domain and the inner pore helix” and “Directionality of temperature activation in mouse TRPA1 ion channel can be inverted by single-point mutations in ankyrin repeat six.”