Mechanistic Basis of Pannexin Channel Mechanosensitivity

By

Shubhamsingh Tanwar

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Department of Pharmacology and Therapeutics

University of Manitoba

Winnipeg, Canada

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ABSTRACT

Pannexin1 (Panx1) forms membrane bound mechanosensitive ion channels and forms an integral component of various physiological and patho-physiological processes. It is well established that Panx1 responds to a wide range of mechanical stimuli. However, the mechanisms governing the mechanosensitivity are not known. My thesis investigates the mechanistic basis of Panx1 mechanosensitivity by employing whole cell voltage-clamp electrophysiology, along with biochemical, molecular and imaging techniques. The results demonstrate that the filamentous actin network regulates the (i) basal channel activity of Panx1 under iso-osmotic condition, (ii) augmentation of channel activity under hypo-osmotic condition, and (iii) inhibition of channel activity under hyper-osmotic condition. Panx1 mechanosensitive responses are inhibited in cells expressing sequentially truncated Panx1 CT (356-414), suggesting that the Panx1 mechanosensitivity is conferred by the distal CT region. Data suggests that F-actin regulates Panx1 mechanosensitivity via interaction with distal Paxn1 CT using tethered mechanisms.

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium ion concentration
$[K^+]_o$	extracellular potassium ion concentration
µg	microgram
µL	microliter
µM	micromolar
^0C	degree Celsius
ANOVA	Analysis of Variance
Arp	actin related protein
ASIC	Acid Sensing Ion Channel
ATP	Adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BCA	bicinchoninic acid
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CBX	carbenoxolone
cc	cubic centimeter
$CD4^+$	cluster of differentiation 4

CNS	central nervous system
CO_2	carbon dioxide
co-IP	co-immunoprecipitation
CSD	cortical spreading depression
CsOH	cesium hydroxide
CT	Carboxyl terminus
Cxs	connexins
Cyt-D	cytochalasin-D
dH2O	distilled water
DMEM	Dubleco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
Doxy	doxycycline
EAE	experimental autoimmune encephalomyelitis
ECL	extracellular loop
ECS	extracellular solution
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ENaC	Epithelial sodium channel
ER	endoplasmic reticulum
F-actin	filamentous actin
FBS	fetal bovine serum
flag-mPanx1	flag-tagged mouse pannexin 1

FRAP	fluorescence recovery after photobleaching
GFP	green fluorescence protein
Gly0	core non-glycosylated form
Gly1	high mannose form
Gly2	complex glycosylated form
GΩ	giga Ohm
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1	high mobility group box 1
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cells
IB	immunoblotting
ICL	intracellular loop
ICS	intracellular solution
Inxs	innexins
IP	immunoprecipitation
I-V	current-voltage
JSK	jasplakinolide
K^+	potassium ion
KCl	potassium chloride
kDa	kilo Dalton
kg	kilogram

kHz	kilo Hertz
KO	knockout
$MgCl_2$	magnesium chloride
mM	millimolar
mm	millimeter
mmol	millimoles
mRNA	messenger ribonucleic acid
ms	millisecond
MscK	Mechanosensitive channel KefA
MscL	Mechanosensitive channel of Large conductance
MscS	Mechanosensitive channel of Small conductance
mV	millivolt
MΩ	mega Ohm
N	normal (of normality)
N/A	not applicable
NaCl	sodium chloride
NaOH	sodium hydroxide
NDZL	nocodazole
NMDA	N-Methyl-D-aspartic acid
NO	nitric oxide
NT	Amino-terminus
OPUS	l(1)ogre, Pas, unc-7, and shakB

$P2X_2$	ATP Purino receptor X2
$P2X_3$	ATP Purino receptor X3
$P2X_7$	ATP Purino receptor X7
P2Y2	ATP Purino rceptor Y2
pA	pico-ampere
PAGE	poly-acrylamide gel electrophoresis
Panx1	Pannexin 1
Panx2	Pannexin 2
Panx3	Pannexin 3
Panxs	Pannexins
PBS	phosphate buffered saline
PCTL	paclitaxel
PKD	polycystic kidney disease
PLP	Periodate-Lysine-Paraformaldehyde
PPK	pickpockets
pS	pico-Siemen
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
rpm	rotations per minute
RT-qPCR	qRT-PCR
sClC3	short Chloride Channel 3
SDS	sodium dodecyl sulphate

SEM	standard error measure
SFKs	Src Family Kinase
shRNA	short-hairpin RNA
TM	transmembrane domain
TREK-2	TWIK-related (2-pore domain) K+ channel - 2
TRPM3	Transient Receptor Potential Melastatin 3
TRPV1	Transient Receptor Vanilloid 1
WT	wild type

SECTION 1: INTRODUCTION

1.1 Mechanobiology

A German anatomist Dr. Julius Wolff, in 1892, postulated that bone tissues undergo re-modelling in accordance with the physical pressure exerted in the vicinity. In other words, bone deposition is higher in the regions subjected to demanding daily physical loading, compared to the lesser utilized regions where reabsorption is higher. This was the first time that the idea of mechanical forces driving tissue remodeling was voiced. A quarter of a century later Dr. D'Arcy Wentworth Thompson, a Scottish biologist and a pioneer of mathematical biology, published a controversial book in the year 1917 called "On Growth and Forms" in which he described how physical and mechanical forces influence cells and tissues during and after embryonic development. Given the modest level of technical advancement within biology at that time, the concepts of mechanobiology remained unarticulated until more than half a century later. A significant body of literature came into existence in the 1990s pertaining to mechanobiological work both *in vitro* and *in vivo*, evidently supporting the phenomenon of mechanical forces affecting and altering cellular and physiological processes. The research spanned from cell proliferation [1] and osteoblastogenesis [2] in response to mechanical loading on bone tissues, to cell proliferation and signaling alterations in vascular endothelial and smooth muscle cells in response to mechanical stretch similar to the pulsatile flow of blood in vasculature [3]–[5].

Today, mechanobiology is well established as a science that investigates the interaction of biological systems with their immediate physical environment, focusing particularly on how mechanical stimuli exerted on cells and tissues generate the corresponding biochemical responses. Mechanical sensation is transduced into a response which brings about molecular alterations at the cellular level [6], [7]. Some of the crucial questions in the field pertain to

understanding the ability of biomolecules to sense mechanical changes. The importance and depth of this question has facilitated the emergence of an area within mechanobiology now referred to as "mechanosensing".

1.2 Mechanosensing

Mechanosensing is a prevalent phenomenon in living cells which assist in translating environmental stimuli into biological responses. All cells are subjected to mechanical forces, either directly or indirectly through changes in the extracellular matrix. Cells need to be constantly on guard against even a slight change in any component in their vicinity. Their ability to sense, analyze, and react to these alterations is crucial for facilitating vital survival mechanics such as self-defence, self-renewal, and self-repair [8]–[10]. Mechanical stress may appear in the form of shear force, membrane stretch, physical stimuli, or osmotic swelling or shrinking. Cells are equipped with the machinery to sense and deal with these stimuli to prevent or mitigate the possible damage. The components of this machinery include, but are not limited to, stretch sensitive ion channels (Panx1, TRPV1, TRPM3, TREK-2, ASIC, etc.), force sensing proteins (titin, talin, vinculin, etc.), and cytoskeletal elements (actin, tubulin, actinin, intermediate filaments, actin crosslinkers, myosin-II, anchoring proteins, etc.).

1.3 Mechanically sensitive ion channels

Mechanosensitivity is one of the general properties of certain channels, much like voltage-sensitivity. Some of the channels well known as "voltage-sensitive" are also mechanosensitive [11]–[13]. The only requirement for a channel to be categorized as mechanosensitive is that the

mechanical stress reaches the channel leading to change in channel pore conformation. Mechanosensitive membrane channels, together with cytoskeleton, makes up an essential component of biological mechano-sensory machinery [14]. They are majorly involved in the transduction of mechanical stimuli that are exerted on a living cell, into intracellular electrical or chemical signals. The existence of mechanically sensitive channels had been postulated for a long time but their discovery and validation by patch-clamp electrophysiology took place only in 1984, by Guharay and Sachs [15]. They demonstrated the increased channel activity in response to the suction applied during patch clamping in cultured chick skeletal muscle cells. Since their first description in chick skeletal muscle cells, they have been identified in many cell types and organisms. In prokaryotic cells, mechanosensitive channels MscS, MscK, and MscL drives osmoregulation and prevents the cell from getting hypo-osmotic shock [16]–[18]. Lateral and posterior touch sensation is mediated by the channels Mec-4 and Mec-10 in *C. elegans* [19]. Epithelial Na-Channels (ENaC) helps in the regulation of salt-water concentration in vertebrate epithelia [20]. In Drosophila, external sensory organs utilize NompC and nanchung, whereas internal dendritic neurons utilize pickpocket (PPK) channels [21]–[23]. Mammalian TRPV4 channels are linked with osmosensory regulation in various tissues [24], and PKD1/PKD2 channels in mammalian kidney epithelium detects fluid flow [25]. TREK channels, a type of two-pore K^+ channels, are implicated in neuroprotective functions in mammalian nervous system [26].

1.4 Mode of activation of mechanosensitive channels

Ion channels are broadly classified as voltage gated, ligand gated, or mechanically gated. In each case, the channels derive energy from the external stimulus (change in voltage; ligand binding;

mechanical stress), which changes channel conformation through non-conducting, low-conducting, or high-conducting states [11], [27]–[30]. In the case of mechanosensitivity, the gating is either cytoskeleton-dependent or -independent [31]. Prokaryotic cells do not have cortical cytoskeleton, but they possess a rigid cell wall made up of lipid bilayer. All bacterial ion channels, such as MscL, follow a cytoskeleton-independent gating phenomenon. When exposed to mechanical strain or osmotic stress, force transmission occurs exclusively via the lipid bilayer without the involvement of any other cellular elements, leading to activation of these membrane channels. This type of gating is also called the "bilayer mechanism" [31], [32]. Unlike bacterial cells, animal cells lack a rigid cell wall and exhibit membrane ruffles & microvilli instead. These cortical regions in animal cells are supported by cytoskeletal elements, such as filamentous actin (F-actin) and microtubules. Contraction of cytoskeleton when subjected to mechanical stress transmits the driving force towards mechanically sensitive membrane channels, which brings about channel activation, augmentation, or inactivation based on the intrinsic property and interaction of the channel [33]. This type of cytoskeleton-dependent gating is also called the "tethered mechanism" or "gating spring model", reflecting the interaction of membrane channel with cytoskeleton [31], [34], [35]. However, it is not necessary that the mechanosensitive response of the channel results from direct cytoskeletal interaction. It mainly highlights that the cytoskeleton is essential for stress-sensing and force transmission for the activation/inactivation of these channels. Integrins are transmembrane receptor proteins that mediate adhesion of a cell to extracellular matrix. Integrins distribute external mechanical stimuli through the cells by forming focal adhesions on the cell membrane, which allow the mechanical stress to be focused on cytoskeletal elements, which directly or indirectly mediates transmission of forces to the membrane bound proteins (eg. ion channels) [33], [36], [37].

Hayakawa *et al.* used phalloidin-conjugated beads attached to actin stress fibers, in human umbilical vein endothelial cells (HUVECs), to demonstrate the involvement of actin cytoskeleton in the regulation of mechanosensitive channels [38]. Corresponding large currents were recorded each time actin fibers were pulled, using optical tweezers, during patch clamp recordings. Moreover, a significant inhibition of the mechanosensitive current response was seen in the cells exhibiting dissembled actin fibers (using cytochalasin-D), suggesting that mechanical stimulus was being transmitted via actin stress fibers. Short Chloride Channel – 3 (sClC3), which functions as a volume sensitive outwardly rectifying anion channel, responds to hypotonic and hypertonic stress by channel activation and inactivation, respectively. By using a co-sedimentation assay, McCloskey *et al.*., demonstrated that sClC3 directly interacts with actin filaments [39]. By diffusing a synthetic interfering peptide targeting sClC3-actin interaction via the patch pipette during patch clamp recordings, they showed successful inhibition of the mechanosensitive response of sClC3 channels during osmotic cell swelling. A similar approach was employed by Prager-Khoutorsky *et al.* to investigate the mechanosensitive response of transient receptor potential vanilloid type-1 (TRPV1) channels in osmosensory neurons [40]. These authors showed that hypotonicity-induced cell volume decrease leads to a corresponding depolarization of the cells due to TRPV1 channel activation. Using structured illumination microscopy, they showed that the density of microtubules is remarkably higher in osmosensory neurons compared to other types of neurons. The interweaved scaffold of microtubules within somata of osmosensory neurons occupies the full cell volume. Moreover, osmosensory neurons pretreated with the microtubule stabilizing drug, taxol, showed enhanced mechanosensitive responses mediated by increased current activity during hypotonic cell shrinking. Nocodazole

(microtubule disrupting drug), on the other hand, treated osmosensory neurons exhibited inhibition of the mechanosensitive response. Additionally, the same group showed that interference peptides targeting the microtubule-TRPV1 interaction inhibited the mechanosensitive currents mediated by the channels during voltage-clamp recordings. Treating the cells with these peptides, before immunoprecipitation assay, showed a significant decrease in the TRPV1-microtubule interaction complex. These experiments strongly support the tethered mechanism of ion channel mechanosensitivity, emphasizing the importance of cytoskeleton as a mediator for channel activation and regulation in animals. The focus of this thesis is to elucidate the mechanisms regulating the mechanosensitivity of Pannexin channel. In particular, my objective was to determine whether cytoskeletal interactions underlie pannexin channel mechanosensitivity. In the following sections I will provide a detailed introduction to the Pannexins (focusing on Pannexin-1), and review the literature investigating the mechanosensitive attributes of the channels.

1.5 Introduction to Pannexin

1.5.1 Discovery of Pannexins

Post decennium to the discovery of vertebrate gap junction protein Connexin in chordates, a family of membrane protein with distinct sequence [41], but similar topology, was proposed to function as gap junction proteins by the name OPUS [42], which is an acronym incorporating the names of genes with similar sequence – *l(1)ogre, Pas, unc-7, and shakB*. This new family of gap junction proteins was subsequently renamed Innexins, deciphering their function as "invertebrate analogs of the connexins" [43]. Although, there is no manifestation of

connexins in any other phylum except that of Chordata, the innexins were believed to not be restricted to the invertebrates and to be expressed in various other taxonomic groups as well (including chordates). Yuri Panchin was the first one to report a similar group of proteins in vertebrates, and because of their diverse phylogenetic expression, they were proposed to be termed "Pannexins" (Latin; pan = throughout, nexus = connection) [44]. The new nomenclature was applied only to the vertebrate proteins while invertebrate homologs continued to be innexins. Interestingly, the mRNA sequence of Panx1 was submitted to GenBank a couple of years before the discovery of pannexins. The sequence was deposited under the name MRS1 (GenBank Accession no. AF093239.1), and the first evidence of their functional role as ATP release channels was established 4 years after the discovery of Panx1 [45].

1.5.2 Pannexin isotypes and their distribution

Members of pannexin family consists of Pannexin 1 (Panx1), Pannexin 2 (Panx2), and Pannexin 3 (Panx3). According to a phylogenetic analysis using the maximum likelihood method of Molphy, before radiating through the vertebrata sub-phyla, the pannexin gene underwent two rounds of duplication, leading to the origination of Panx2 first followed by the origination of Panx1 and Panx3 [46]. Also, Panx1 and Panx3 are more closely related to each other in terms of sequence similarity, molecular weight, structure, and cellular localization, as compared with Panx2 [47], [48]. These duplication events not only created the pannexin paralogs but also maintained their orthologous attributes throughout vertebrate taxonomic groups [49].

Earlier Northern blot analysis by Baranova *et al.* showed ubiquitous expression of Panx1, with varying expression levels, in tissues derived from human heart, brain, lung, liver, skeletal

muscle kidney, spleen, thymus, prostate, testis, ovary, small intestine, and colon [47]. Later, a significant body of literature confirmed the presence of Panx1 in almost all the human tissues. Unlike Panx1, Baranova's work suggested that Panx2 is strongly expressed in the brain, and was believed to be restricted to the compartments of central nervous system. However, a handful of publications not only revealed low levels of Panx2 transcripts in other tissues but also co-expression with Panx1 [50]–[52]. Using a combination of RT-qPCR, Western blotting, and immunofluorescence, recently Le Vasseur *et al.* demonstrated substantial levels of Panx2 expression outside the CNS, for example in tissues of kidney, heart, skin and colon, [53]. Panx3 is expressed predominantly in the chondrocytes, osteoblasts, fibroblasts, and cartilage, and is reported to be co-expressed with Panx1 in mouse osteoblast, cartilage, and skin [47], [54], [55]. The ability of Panx2 and Panx3 to form functional ion channels is somewhat controversial. Likewise, identifying their clear function has been challenging. Panx1 is considered to be the major functional form amongst all three members of the pannexin family. It is expressed ubiquitously and is linked with a wide range of physiological processes and pathological conditions. Thus, it represents the focus of a majority of studies including the work presented in this thesis.

1.6 Pannexin1 structure and membrane trafficking

1.6.1 Pannexin1 Structure

Although, Panx1 was discovered due to their limited sequence homology to innexins, and were assumed to function as gap junction channels because of their topological similarity with

connexins, there is no sequence homology between Panxs and connexins. In case of Panx1, 6 subunits oligomerize

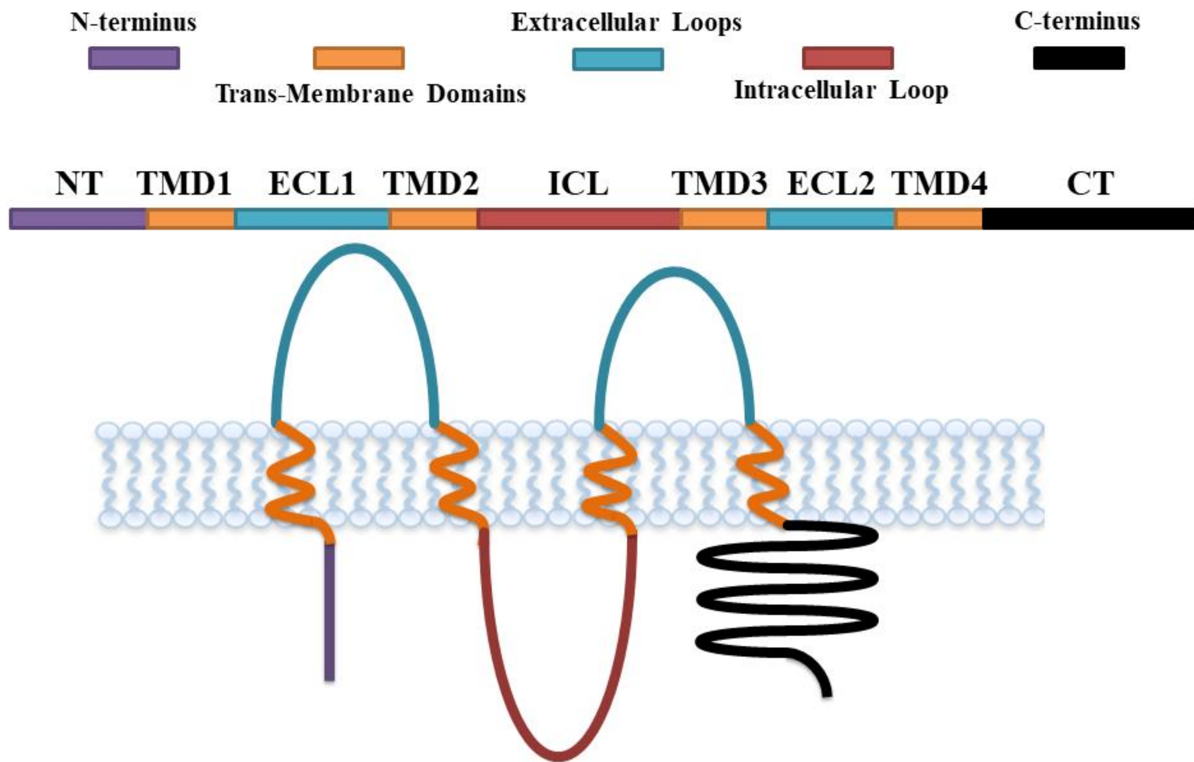


Figure 1.1 Illustration of Panx1 topology

together to form a single channel on the plasma membrane wherein each subunit consists of four α-helical transmembrane (TM) domains, two extra-cellular loops (ECL), one intra-cellular loop (ICL), along with cytoplasmic amino (NT) and carboxyl (CT) termini (fig. 1.1) [56], [57]. Interestingly, based on four different algorithm analyses, a unique "membrane" domain of 10 (mostly hydrophobic) amino acid within CT is predicted to form a membrane re-entrant loop within plasma membrane (not a transmembrane loop) [58]. Six subunits are combined to form a

single hexameric membrane channel. The lining of the channel pore is contributed by the transmembrane domain – 1 and extracellular loop of each subunit [59]. Most voltage-gated channels possess a channel pore size of 4.3 Å to 10 Å diameter, and are selectively permeable to either cations or anions [60], [61]. However, one of the unique attributes of Panx1 is that the channel pore diameter is estimated to be ~17-20 Å [57], and the channels are not only non-selectively permeable to ions but also to small molecules of up to 1 kDa in size [45], [62].

1.6.2 Membrane Trafficking

Panx1 is synthesized in the endoplasmic reticulum (ER) and traffics to the Golgi via Sar1-dependent Coat Protein - II vesicles [56], [63]. Protein glycosylation is crucial to the function and trafficking of Panx1 within intracellular organelles and ultimately towards the plasma membrane. This is highlighted by the fact that inhibition of glycosylation by drugs or site specific mutagenesis of key asparagine residues, disrupts membrane localization [54], [56]. Panx1 exists in three glycosylation species – Gly0 (core non-glycosylated form), Gly1 (high mannose form), and Gly2 (complex glycosylated form). Panx1 is first glycosylated to the Gly1 form within the ER before trafficking to the Golgi, where glycosylation at residue N254 occurs and leads to formation of the Gly2 species, which is then translocated to the plasma membrane [54], [56], [64], [65]. Non-glycosylated Panx1 is retained within the ER by calnexin for pre-mature degradation [66].

1.7 Physiological and pathophysiological roles of Panx1 channels

1.7.1 Panx1 channel in physiological processes

Pannexin channels are widely accepted to form large pore, non-selective membrane channels permeable to solutes of up to 1 kDa in size. The ubiquitous expression and non-selective permeability of Panx1 defines the unique attributes of these channels. Consistent with its broad expression profile, Panx1 plays a significant role in wide array of physiological processes, especially in paracrine signaling as an ATP-release channel. For example, Panx1 is implicated in phenylephrine-induced vascular smooth muscle cell contraction in resistance arteries by mediating the release of the vasoconstrictor ATP [67]. Additionally, ATP released via Panx1 from endothelial cells in large arteries facilitates vasodilation using endothelium-derived hyperpolarization like mechanisms [68]. Purinergic receptor stimulation through ATP release via stress-activated Panx1 channels initiates Ca^{2+} wave propagation leading to vasodilation by nitric oxide release on smooth muscle cells [62]. Ca^{2+} waves refer to a phenomenon of a rise in Ca^{2+} levels within a stimulated cell which leads to concentric propagation of rising Ca^{2+} levels in the neighbouring cells. The initiation of Ca^{2+} waves is mediated by the activation of ATP-sensitive purinergic receptors which enhances inositol 1,4,5-triphosphate levels releasing Ca^{2+} from the ER stores. Purinergic receptor stimulation by Panx1-mediated ATP release is also implicated in erythrocyte lysis post exposure to α-toxin from *E. coli* or *S. aureus*, which could be reduced by Panx1 channel blockers carbenoxolone, mefloquine, and probenecid [69], [70]. In addition to bacterial infection, ATP released through Panx1 channels, on interaction of $CD4^+$ cell receptors with HIV1 viral envelope, activates P2Y2 receptors facilitating viral entry within the cell by membrane depolarization and membrane-to-membrane fusion [71]. Panx1 is a part of purinergic $P2X_7$ receptor complex that mediates ATP release [72], [73]. P2Y-mediated activation of Panx1 is implicated in ATP-induced ATP- release [74]. Paracrine action of ATP released via Panx1

channels under hypotonic stress is implicated in mucociliary clearance, which is a crucial airway host defense mechanism controlled by human airway epithelium. This is achieved by regulating airway surface liquid volume and ciliary movement [75], [76]. In the taste buds, Panx1 channels are believed to mediate ATP release from tastant activated cells, which in return activates the $P2X_2$ and $P2X_3$ receptors on the presynaptic cell to induce the information relay towards the CNS via afferent nerves [77]–[79]. Also, Panx1 forms the pore unit as a part of $P2X_7$ death complex, which stimulates cellular apoptosis by release of ATP [73].

1.7.2 Panx1 channel in pathophysiology of diseases

The first characterization of Panx1 channel as a contributor to ischemic stroke neuronal injury was reported by Thompson *et al.* [80]. The group demonstrated that Panx1 channels conduct massive currents during ischemic conditions like oxygen/glucose-deprivation. This leads to ionic dysregulation and necrotic cellular death in acute brain slices. During ischemia, NMDA receptor activation leads to the potentiation of Panx1 channel activity [81]. This results in anoxic depolarization of pyramidal neurons due to ischemic conditions. NMDA receptor-induced Panx1 channel activation results in the stimulation of Src family kinases (SFKs), which leads to further activation of Panx1 channels by post-translational modifications. A custom developed interfering peptide targets the SFKs-Panx1 interaction and successfully attenuated anoxic depolarization of neurons without affecting SFK activation [81].

A stroke-model study using pannexin ablated mice shows that Panx1/Panx2 double KO mice exhibited a dramatic decrease in total infarct size and significant functional-behaviour recovery, compared to Panx1 or Panx2 single gene KO mice [82]. Likewise, pharmacological inhibition of

Panx1 using probenecid yielded similar results. Administration of probenecid in mice subjected to transient focal ischemia significantly (i) reduced infarct size and cerebral water volume; (ii) inhibited cell death; (iii) reduced inflammation 48 hours after stroke [83].

Another study conducted using Panx1-ablated mice investigated the role of Panx1 in encephalomyelitis [84]. Panx1 was knocked out in a mouse model for experimental autoimmune encephalomyelitis (EAE). Panx1 KO mice exhibited delayed onset of EAE and reduced mortality as compared to Panx1 WT. Spinal cord lesion size was significantly reduced in Panx1 KO mice. Thus, the study indicates Panx1-mediated progression of encephalomyelitis.

In addition to cerebral ischemia, Panx1, expressed by retinal ganglion cells, may also mediate the detrimental effects of retinal ischemia [85]. In an interesting work by Jiang *et al.*, transcranial direct current stimulation is shown to decrease the upregulation of Panx1 mRNA expression after inducing stroke in Sprague-Dawley rats, thereby preserving dendritic spine density and motor function scores [86].

As stated earlier, Panx1 is ubiquitously expressed in almost all the human tissues. Panx1 is expressed abundantly in myenteric ganglia which regulates the motility of colon. However, tissues derived from patients suffering from colitis exhibit a significant reduction in Panx1 expression [87]. It is anticipated that a feedback mechanism downregulates Panx1 channels upon induction of enteric dysfunction during colitis. Another study reported that Panx1 inhibition by probenecid prevented the death of enteric neurons during colitis in mice [88]. The study suggests

that inflammation during colitis causes neuronal death by the activation of Panx1 channels in conjunction with P2X7 receptors, Asc adaptor proteins, and caspases.

1.8 Mode of activation of Panx1 channels

Earlier studies suggested that Panx1 forms large-pore membrane channels which are non-selective. This means that the channel is permeable to cations, anions, and small molecules of up to 1 kDa in size (e.g. ATP). However, more recent studies provide evidence that Panx1 channel opens to form either a large pore or a small pore based on the mode of activation [28]. In the small pore configuration, channels have a unitary conductance of 40-70 pS. Such openings occur for example in response to voltage-dependent stimuli. Of note, the small pore configuration does not exhibit ATP permeability [89], and currents are largely based on the flux of chloride ions [90]. The majority of other stimuli facilitates large-pore opening of the channel with a unitary conductance of 400-500 pS. Large-pore openings exhibit one of four sub-conductance states (90%, 30%, 25%, or 5% of the full conductance) and are associated with ATP release from the channel [28]. Large-conductance channel opening is facilitated by high extracellular K^+ concentration, high intracellular Ca^{2+} concentration, hypoxic conditions, and mechanical stress. However, in excitable cells like neurons and myocytes, voltage-dependent gating does exhibit larger pore opening at positive voltages [91]. The specific gating mechanisms can be broadly categorized as:

i) Extracellular potassium: extracellular $[K^+]_0$ at the level of $\geq$ 10 mM facilitates pannexin channel opening. $[K^+]_0$ in the rage of 100 mM leads to Panx1 channel activation over a wide voltage range [62], [92]–[94].

ii) Intracellular calcium: although Panx1 channels are not sensitive to extracellular Ca^{2+}, they do respond to intracellular calcium ($[Ca^{2+}]_i$) elevations to millimolar levels, at normal resting potentials [95], [96]. Many physiological processes can provoke $[Ca^{2+}]_i$ accumulation at millimolar levels, including activation of various purinergic receptors which facilitates Ca^{2+} influx from the extracellular space further increasing $[Ca^{2+}]_i$ levels.

iii) Caspase cleavage: The Panx1 C-terminus is proposed to have an autoinhibitory region which interacts non-covalently with the inside of the channel pore and prevent channel activation at rest. Upstream of this C-terminal autoinhibitory region lies a caspase-cleavage site. During apoptosis, the C-terminal tail is cleaved by caspase 3 or caspase 7, at residues 376-379 (caspase-cleavage site) rendering the channel constitutively activated and able to release ATP. Even without the induction of apoptosis, C-term cleavage at residues 376-379 leads to constitutively open channel suggesting that the cleavage directly regulates channel opening without the additional apoptotic mediators. Interestingly, cleaved C-terminal tail blocks the channel pore by itself if covalently tethered to the channel pore [97]–[99].

iv) Mechanical stimulation: Panx1 channels respond to a wide range of mechanical stimuli and mechanical stimulation of channels is linked to various physiological and pathological process, details of which are reviewed in the following sections (1.9 and 1.10).

1.9 Panx1 mechanosensitivity in cellular processes

Although investigations pertaining to mechanosensitive attributes of Panx1 channels are still in their infancy, there is a considerable number of studies that explore the mechanosensitivity of these channels. Bao *et al.* demonstrated mechanosensitive activation of Panx1 channels in response to negative pressure (suction through patch pipette) applied to single channel patch [92]. Increased channel activity was seen during mechanical stimulation from (i) closed channel configuration to an open channel, and (ii) low sub-conductance state to a higher conductance state. This mechanical stimulation was exhibited over a wide range of membrane potentials. Using *Xenopus* oocytes, the same study for the first time showed that Panx1 also mediates the release of ATP. In response to shear stress, Panx1-mediated ATP release from erythrocytes results in activation of NO-dependent relaxation of smooth muscle cells and vasodilation [100].

In addition to blood-flow regulation, mechanosensitivity of Panx1 is also implicated in human airway defense mechanisms. ATP is released extensively in human bronchial airways due to mechanical pressure exerted during coughing, tidal breathing, or hypotonic cell swelling [101]–[103]. Seminario-Vidal *et al.* has shown that hypotonic-stress promotes Panx1-mediated ATP release in human bronchial epithelial cells [104]. Similarly, Ransford *et al.* found 60% inhibition in ATP released by airway epithelial cells in response to hypotonic stress after Panx1 channel blockade and shRNA-mediated Panx1 knockdown [75]. Furthermore, in a pre-clinical study, nasal mucosa (inferior turbinate samples) was retrieved from patients with chronic hypertrophic rhinitis and was confirmed for robust Panx1 expression by using qRT-PCR and immunohistochemistry [76]. The authors also showed that hypotonic stress-induced ATP release from the nasal mucosa was significantly inhibited by carbenoxolone (a non-specific Panx1 channel blocker).

Mechanical stress-induced Panx1-mediated ATP release has also been shown in neuronal cells. Retinal ganglion neurons release ATP when subjected to hypotonic cell swelling in a CBX-sensitive manner [105]. Swelling-activated currents were inhibited by the Panx1 channel blockers CBX and probenecid. In the same study, retinal ganglion cells stretched using a special pressure chamber confirmed the release of ATP via Panx1 channels. Mechanical stress-induced ATP release mediated by Panx1 activates neurotoxic signaling pathway by ATP-dependent activation of $P2X_7$ receptors [105].

ATP-released by mechanosensitive stimulation of Panx1 is also implicated in initiation and propagation of Ca^{2+} wave signaling. A study by D'hondt *et al.* investigated Ca^{2+} wave signaling elicited by a mechanical stimulus in a single layer of primary corneal endothelial cells [106]. Mechanical stimulus was provoked by an acute short-lasting deformation induced by touching the cell membrane with a glass pipette tip, to just one cell within a group of endothelial cells. An instantaneous rise in Ca^{2+} was seen at the point of stimulation and spreads throughout the mechanically stimulated cell. Upon reaching the cell boundary, the level of Ca^{2+} drops to the baseline within the cell and the wave is propagated to the surrounding cells in a concentric manner. Panx1 channels are implicated in the mechanically stimulated initiation and propagation of Ca^{2+} waves [107].

These studies highlight the increasing recognition of the importance of Panx1 mechanosensitive functions in various cell and tissue types. Similarly, mechanosensitive functioning of Panx1 is implicated in the patho-physiological conditions, as discussed in the following section.

1.10 Panx1 mechanosensitivity and diseases

1.10.1 Glaucoma

Glaucoma is associated with increased intraocular hydrostatic pressure and has been linked to the death of retinal ganglion cells [108], [109]. In patients with acute glaucoma, the magnitude of pressure increase is directly correlated with elevated levels of ATP in the extracellular space of the anterior chamber [110]. In a study published by Reigada *et al.*, application of pannexin channel blocker CBX to bovine retinal eye cups subjected to hydrostatic pressure of 20 mm Hg decreased pressure-dependent release of extracellular ATP by 87%, compared to control eye cups [111]. Release of ATP was mediated by Panx1 channels in response to the mechanical stimulus (hydrostatic pressure). Thus, Panx1 channels activation during swelling or stretching in the eye has been implicated to retinal ganglion cell death due to pathological Ca^{2+} influx.

In an extensive study on rat optic nerve head astrocytes, Beckel *et al.* confirmed that astrocytes release ATP, when subjected to either 5% of equibiaxial mechanical strain or hypotonic cell swelling, through Panx1 channels [112]. Astrocytes from $Panx1^{-/-}$ rats showed reduced baseline as well as stretch/swelling induced ATP release. Also, application of CBX and probenecid blocked the release of ATP in the cells subjected to stretch or swelling. Isolated astrocytes subjected to extended stretch in vitro exhibited upregulated levels of Panx1 and Panx2 mRNA. A mouse model of chronic glaucoma showed a similar increase in Panx1, Panx2, and Panx3 gene levels, in vivo in optic nerve head tissue. However, immunohistochemistry analysis revealed that only Panx1 protein expression was increased. This study implicated Panx1 as the mediator of

ATP release from astrocytes due to the elevated hydrostatic pressure and chronic strain induced by glaucoma.

1.10.2 Cortical spreading depression

Cortical spreading depression (CSD) causes migraine aura and initiates headache by activating perivascular trigeminal nerves, which is a part of trigeminocervical complex that mediates migraine headache [113]–[115]. Acute depolarization and over-activation of NMDA receptors is proposed to cause Panx1 channel activation during CSD [116], [117]. This stress-induced Panx1 activation in neurons is linked with the release of proinflammatory mediators called 'high-mobility group box 1' (HMGB1), initiating the inflammatory response from glia limitans prolonging the trigeminal stimulation [83], [93]. *In vivo* studies by Karatas *et al.* in mouse brain, illustrates that CBX completely blocks the CSD-induced late middle meningeal artery dilation, which is the result of trigeminal nerve activation [118]. Single or multiple CSDs was induced by pinprick, in lab settings, and the activation/inhibition of Panx1 channels was confirmed by monitoring propidium iodide uptake. Indeed, Panx1 channels are permeable to small molecules of up to 1 kDa in size, and propidium iodide (668 Da) is widely used as a reporter dye to confirm large-pore activation of Panx1 channels [104]. Pinprick induced rapid uptake of propidium iodide, which was inhibited by the application of CBX, implicating the activation of Panx1 channels in CSD [118].

1.10.3 Cancer cell survival

Primary tumour cells are rapidly transported away from their site of origin and dispersed throughout the body. 90% of these cells end up getting deformed due to mechanical stress acting upon them in capillaries of microvasculature of the secondary end organs [119]–[121]. Regardless of this hindrance, a small set of these cancer cells survive the mechanical stress and carry on the metastatic progression. By running whole-transcriptome RNA-sequencing on highly metastatic breast cancer cells, Furlow *et al.* found the enriched expression of a truncated form of Panx1 channel, called Panx1$^{1\text{-}89}$ [122]. This truncated form of Panx1, when expressed with WT full length Panx1 channels, significantly increases the release of ATP in the extracellular space (which is inhibited by the application of CBX). The ATP released is essential for the survival of metastatic cancer cells while under mechanical stress. Inhibition of Panx1 also decreased the cell survival after centrifugal stretch. Interestingly, cells with inhibited Panx1 channels were shown to be viable even when subjected to the hypotonic stress by the addition of extracellular ATP. Thus, ATP released from the metastatic cancer cells is mediated via Panx1$^{1\text{-}89}$-dependent Panx1 channel activation, and is essential for the survival of these cancer cells during their mechanical deformation in the vasculature. Moreover, pharmacological inhibition of Panx1 activation (by CBX) in highly metastatic cells in vivo, suppresses the metastatic progression and colonization of the cells. Therefore, Panx1 channels may prove to be a therapeutic target in blunting the spreading of cancer.

The mechanosensitive attribute of these channels surpasses other gating modalities when linked with various physiological processes, and has garnered much attention since the channels are proven to act as mechanosensitive conduits for ATP release in various cell types. Even though a

considerable amount of work in the literature directly links the mechanosensitive nature of Panx1 as key factor driving several physiological and pathophysiological processes, it is still unknown exactly how Panx1 channels respond to the mechanical stimuli. As stated previously, a major focus of my studies is to elucidate the mechanisms behind Panx1 mechanosensitivity. Interestingly, there is a precedence in the literature to suggest that pannexin channels interact with the cytoskeletal elements, as described in the following section.

1.11 Pannexin 1 and the cytoskeleton

During a FRAP (fluorescence recovery after photobleaching) analysis, Bhalla-Gehi *et al.* [63] noticed the movement of membrane-bound GFP-tagged Panx1 within the photobleached area from the periphery. To confirm if the membrane trafficking and movement of Panx1 within photobleached regions is regulated by cytoskeleton, they disrupted the cytoskeletal networks. This study first demonstrated that cytochalasin-B induced actin filament disruption severely affected the cell surface expression of Panx1 channels, whereas disruption of microtubules by nocodazole did not affect the cell surface dynamics of Panx1 channels. Moreover, by using co-immunoprecipitation and co-sedimentation assays, they also found that ectopically expressed Panx1 interacts with actin through the C-terminus. This was further validated by Wicki-Stordeur and Swayne [123]. By running liquid chromatography and tandem mass spectrometry, along with immunoprecipitation targeting Panx1, they found actin and actin-related protein 3 (Arp3) as Panx1 interacting proteins. Arp3 is a part of actin-modifying complex and serves as a nucleation site for new actin filaments, which is essential for actin-mediated mechanical force generation via Y-branched networking [124]. Despite the implication of cytoskeletal interaction with Panx1, the functional consequences of this interaction have not been determined. As mentioned earlier,

cytoskeletal elements regulate the stimulation of mechanosensitive channels by the tethered mechanism. Since past work has demonstrated potential direct interaction of Panx1 with filamentous actin it is intuitive to propose that the actin (or microtubules) may be involved in the mechanosensitive regulation of the Panx1 channels.

1.12 Rationale and Hypothesis

The ubiquitous expression of Panx1 channels in mammalian tissues, and their implications in numerous physiological processes and pathological conditions makes these channels worthy of extensive attention. We know that channels are potentiated by multiple components ranging from purely physiological to environmental, but we do not yet understand how exactly the channel responds to the stimulus. Undoubtedly, it is widely accepted that Panx1 channels respond to myriad of mechanical stimuli. Nonetheless, the mechanisms by which Panx1 channels exhibit mechanosensitive response are still practically unknown.

The principal objective of this work is to elucidate the mechanisms behind mechanosensitivity of Panx1 channels. Major past works in the field has shown that 1) Panx1 respond to the mechanical stimuli such as hypotonic cell swelling, liquid shear force, membrane stretch, etc; 2) Panx1 interacts with F-actin by C-terminal region; 3) cytoskeletal attachment is shown to be a major regulator of mechanotransduction by ion channels. Thus, my hypothesis is that the cytoskeletal elements regulate the mechanosensitive response of pannexin-1 channels during osmotic stress. To elaborate on this, I propose three potential direct interaction models for actin-tethered mechanosensitivity of Panx1 (Fig. 1.2).

i) **Association model:** Under regular isotonic conditions, cortical actin filaments do not interact with the C-terminus of membrane bound Panx1 channels (exhibiting basal channel activity). On subjecting the cell to hypo-osmotic stress-induced cell swelling, the actin filaments bind with the Panx1 C-terminus rendering the channel hyper-activated.

ii) **Dissociation model:** Panx1 interacts with F-actin under regular isotonic conditions, via its C-terminus, regulating the basal channel response. Hypo-osmotic stress induced cell swelling leads to the dissociation of the interaction resulting in augmented channel activity.

iii) **Alteration model:** F-actin regulates the basal channel activity by interacting with C-terminus of the Panx1 channels under iso-osmotic conditions. On exposure to hypo-osmotic stress, the interaction changes (still interacting) which brings about the mechanosensitive response of the channels.

The experiments described in the following sections investigates (i) the effects of osmotic stress on Panx1 channel activity, (ii) the role of cytoskeleton in the regulation of Panx1 channel mechanosensitivity, (iii) minimal domain responsible for Panx1 mechanosensitivity.

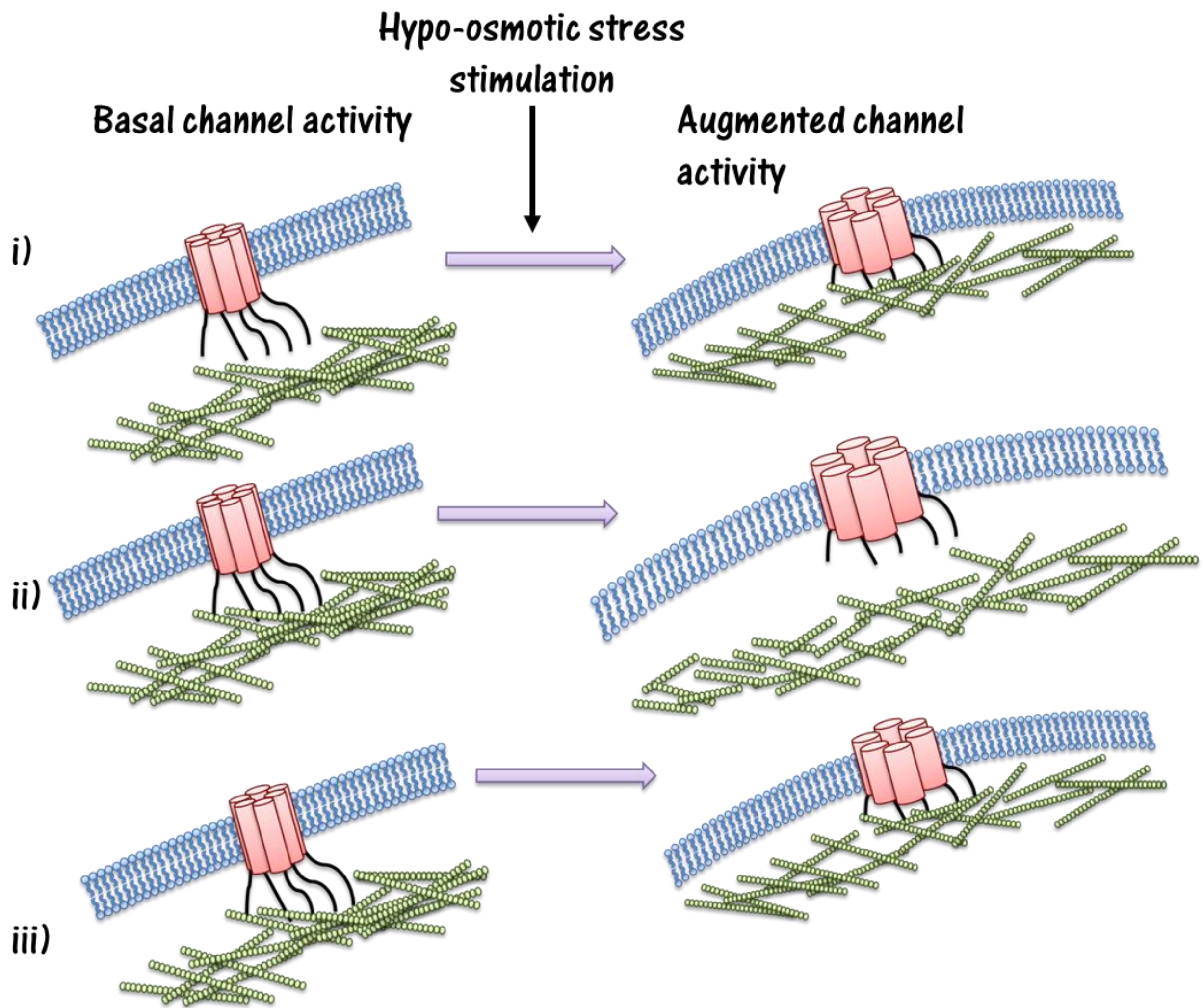


Figure 1.2 Illustration of three proposed tethered interaction models of Panx1-actin.

SECTION 2: MATERIALS AND METHODS

2.1 Cell culture

Two different cell lines were used for the experiments: 1) HEK 293T cells, and 2) flag-mPanx1 Flp-In T_REx 293 cells. The latter are derived from HEK 293T but engineered to express the Tet-repressor system and Flp-recombinase. Flp-recombinase and associated Flp-In integration sites were exploited to insert the flag-mPanx1 sequence downstream of the Tet repressor site. In this scheme, transcription of flag-Panx1 is enabled in the presence of doxycycline (doxy), which binds to the Tet repressor. Hence, flag-mPanx1 T-Rex 293 cells express Panx1 in doxy-inducible manner (Fig. 2.1).

Cells were cultured at 37 ^{0}C with 5% CO_2 using Dubleco's Modified Eagle Medium (DMEM; Sigma-Aldrich) enriched with 10% fetal bovine serum (FBS), in 100 mm culture dish. Cells were passaged every 3-4 days with 80% dish confluency till the maximum of 40 passages.

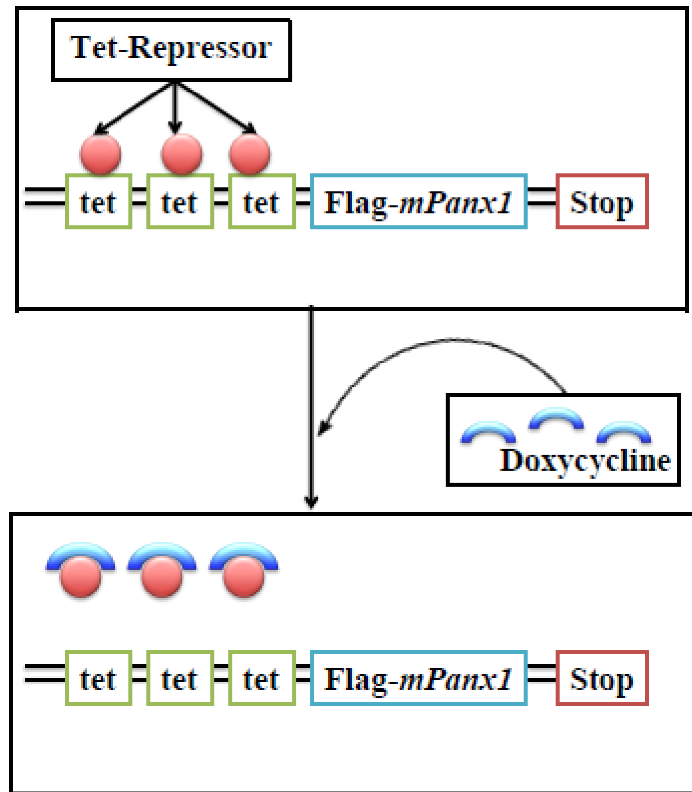


Figure 2.1 Illustration of Panx1 expression induced by doxy

In the modified Flp-In T-REx 293 cells, flag-mPanx1 gene undergoes transcription only in the presence of doxy. Doxy binds to the Tet-repressor complex allowing transcription factor binding to Tet regulated gene, initiating the transcription of flag-mPanx1 gene.

2.2 Transient Transfection

For transient expression of selected plasmid described below, HEK 293T cells were transfected using jetPRIME^{TM} (Polypus Transfection, NY), according to the protocol provided by the manufacturer. Briefly, 9.2 µg of DNA (2 µg of flag-mPanx1 wild type or deletion constructs + 0.2 µg of green fluorescent protein/pLB-GFP + 7 µg of pCDNA 3.1, the latter used as carrier DNA) was added to the jetPRIME^{TM} buffer (Polypus Transfection, NY) mL microtubes and

vortexed thoroughly followed by addition of 12.5 µL of jetPRIME^{TM} reagent and further vortex for 15 sec. The transfection mix was then incubated for 10 min at RT, and then added drop-wise to the dish with 60% confluency. Dishes were gently rocked back and forth before incubating for 4 h at 37 ^{0}C with 5% CO_2. After 4 hours, the transfection-mix was replaced by fresh warm (37 ^{0}C) DMEM with 10% FBS, and the dishes were incubated overnight at 37 ^{0}C with 5% CO_2. Transfection was confirmed the following day by observing the cells for GFP since the HEK 293T cells were co-transfected with flag-mPanx1 (WT or truncated) and GFP plasmids pLB-GFP plasmid. Only cells positive for GFP were selected for electrophysiological recording.

2.3 Whole-cell voltage-clamp electrophysiology

Flp-In T-Rex 293 cells stably expressing flag-mPanx1 gene were seeded at 5% confluency in 35 mm dishes 24 hours before the beginning of recordings. Panx1 expression was induced by adding 1 µg/mL of doxy to the dishes, and the recordings were completed within 24-48 hours of the Panx1 expression. After taking the petri dish out of the incubator the media was immediately replaced with the basic extracellular solution (ECS) containing (in mM): 110 NaCl, 5.4 KCl, 25 HEPES, 33 Glucose, 2 $CaCl_2$, 1 $MgCl_2$, (pH = 7.4, adjusted using 10 N NaOH). By default, the osmolarity value of ~250 mmol/kg (hypo-osmotic) was confirmed using a vapour pressure based osmometer. Other desired osmolarity values of ~310 (iso-osmotic) and ~360 mmol/kg (hyper-osmotic) were achieved by adding Mannitol (an inert membrane impermeable solute) to the basic ECS. In some experiments, ECS contained 100 µM carbenoxolone (CBX) – a Panx1 channel blocker.

Borosilicate glass capillaries (World Precision Instruments, FL) were used to make recording electrodes, having resistance of 3-5 MΩ, using a two-stage pipette puller (PP 83; Narishige, NY). Patch pipettes were freshly pulled before recordings and were filled with intracellular solution (ICS) containing (in mM): 150 Cs-gluconic acid, 10 HEPES, 2 $MgCl_2$, pH adjusted to 7.2 using 100 CsOH, and osmolarity set to ~300 mmol/kg.

For voltage-clamp recordings, the selected cell was approached with an ICS filled patch electrode while maintaining a positive pressure of ~0.05 cc. Once the tip of the electrode made contact with the cell membrane, negative pressure was applied to suction a small fraction of the cell membrane within the patch electrode. A holding potential of -60 mV, near the resting membrane potential, was applied to ultimately achieve the resistance of at least 1 GΩ (Giga-seal). Whole-cell access was achieved by applying a "zap", which is a pulse of 2 kHz for 0.6 ms. On attaining the whole-cell configuration, Panx1 channel mediated currents were recorded using 500 ms voltage ramps every 10 seconds (Clampex 10.2 software; Molecular devices), wherein the voltage is stepped down from -60 mV to -100 mV and ramped up to +100 mV.

2.4 Protocol to record osmotic response

On attaining the whole-cell configuration with a selected cell voltage-clamped at -60 mV, the barrel for iso-osmotic ECS perfusion was turned on from the gravity-driven multi-barreled perfusion system, with the flow rate of 1 ml/min, to record a stable baseline activity of Panx1 channels for ~5 mins. Barrel position was then switched to the next barrel containing hypo/hyper

ECS and the current response was recorded for ~5 mins, before turning on the CBX perfusion or a washout perfusion.

2.5 Cytoskeletal drug treatment

To test the involvement of cytoskeletal elements in the mechanosensitive response of Panx1 channels, HEK 293 cells were pre-treated with cytoskeleton stabilizing (Jasplakinolide[125], paclitaxel[126]) or de-stabilizing agents (cytochalasin-D[127], nocodazole[128]). Stock solutions of the drugs were made in DMSO. Table 2.1 illustrates the drugs added to the iso-osmotic ECS and the cells were incubated for 30-40 mins at RT before beginning the standard recording procedure.

Table 2.1 Drugs used to stabilize or de-polymerize cytoskeleton

	De-stabilizing agent	**Stabilizing agent**
F-actin	Cytochalasin-D (100 µM)	Jasplakinolide (2.5 µM)
Microtubules	Nocodazole (5 µM)	Paclitaxel (2.5 µM)

2.6 Recordings with the transiently transfected HEK 293T cells

As mentioned in the section “Transfection”, the 100 mm dish with transiently transfected cells is used to seed 35 mm dishes, which were then incubated overnight before recording. All recordings were performed within 24 to 36 hours of the transfection. Only GFP positive cells were selected for recording as the Panx1 channel expression was well correlated with this selection transfection marker.

2.7 Immunofluorescence staining

For this, cells were plated on imaging-grade plastic-bottom dishes (ibidi, WI) of 35 mm in size, coated with poly-D-lysine. The dishes were incubated overnight and cells were fixed using a Periodate-Lysine-Paraformaldehyde (PLP) fixative. Briefly, dishes were washed with iso-osmotic ECS thrice, and incubated with PLP-fixative for 10 mins at RT before washing with PBS for 5 mins each. The fixed cells were then permeabilized in 0.2% Triton-X/PBS for 10 mins and blocked in 5% normal donkey serum for 30 mins. The permeabilized cells were then incubated with primary antibodies in 0.5% BSA/PBS for 2 hours, before washing with PBS thrice (5 mins each wash). Cells were then incubated with fluorophore conjugated secondary antibodies in 0.5% BSA/PBS for 1 hour, before washing thrice with PBS (5 mins each). To stain F-actin, selected dishes were treated with ActinGreen™ 488 (2 drops/ml) in PBS, for 30 mins before washing with PBS thrice. ActinGreen™ 488 is Alexa Fluor® 488 conjugated phalloidin probe that binds to F-actin with high selectivity. All the dishes were then incubated for 5 mins with DAPI to stain the nuclei. After a final three washes with PBS, dishes were preserved in PBS at 4 ^{0}C for imaging within two weeks.

Table 2.2 Antibodies and stains used for immunofluorescence experiments

	Primary antibody	**Secondary antibody**	**Stain**
Flag-mPanx1	Anti-flag M2 (1:200) Host: Mouse	Anti-mouse Alexa Fluor® 555 Host: Donkey	N/A
F-Actin	N/A	N/A	ActinGreen™ 488 (2 drops/mL in PBS)
Microtubules	Anti-α-tubulin (1:100) Host: Rabbit	Anti-rabbit Alexa Fluor® 488 Host: Donkey	N/A
Nucleus	N/A	N/A	DAPI (5µg/ml) (1:2000)

2.8 Immunoprecipitation

Doxy-induced Flp-In T-Rex 293 cells, expressing flag-mPanx1, were washed 2 times with cold PBS and collected in microtubes with 1 mL PBS, on ice. Microtubes containing cells suspended in PBS were centrifuged for 5 min at 500x g, at 4 ^{0}C. Supernatant was discarded and 250 µL of Complexiolyte-48 buffer containing protease inhibitor was added to the pellet. A 25 G needle and 10 mL syringe were used to lyse the cells by aspiration. Microtubes were then placed on end-over rotator for 1 h at 4 ^{0}C. This was followed by centrifugation at 14,000 rpm for 10 min, at 4 ^{0}C. 20 ul of cell lysates were used as inputs. For Panx1 pull down, the supernatant (cell lysate) was mixed with 50 µL flag-tagged beads, and incubated on end-over rotator overnight, at 4 ^{0}C. For actin pull down, protein G beads were washed with cold PBS for 3 times, and incubated with anti-actin antibody (1:50; Abcam) for 4 h, at 4 ^{0}C, to coat them with the antibody before mixing with cell lysate. The microtubes containing beads were then centrifuged at 500x g for 5 min, followed by three washes of 500 µL, each, of Complexiolyte-48 buffer. This was followed by another centrifugation at 500X G for 5 mins, and dilution in 2X sample (gel loading) buffer. The samples were then subjected to SDS-PAGE, followed by western blotting. Antibodies used for Western blotting are (i) anti-flag-HRP (1:1000; Sigma) – to probe flag-mPanx1 in actin-pulldown blot; (ii) anti-actin (1:7000; Abcam) – to probe β-actin in flag-mPanx1 pull-down blot.

2.9 Western blotting

Cells were washed twice with PBS at RT, and re-suspended in microtubes with 500 µL chilled lysis buffer (1% Triton-X-100, 0.1% SDS, 0.5% Sodium deoxycholate, 150 mM NaCl, 50 mM Tris, 10 mM EDTA) supplemented with protease inhibitor cocktail (Mini cOmplete Tablets,

Roche Applied Science), on ice. The tubes were then kept on shaker for 30 min at 4 ^{0}C for lysing, followed by centrifugation at 14000 g for 30 min at 4 ^{0}C. The supernatant was collected in separate microtubes and protein concentration was estimated using Pierce® BCA protein assay kit (Thermo Scientific). Samples were mixed with 2X loading buffer (3.5mL 1M Tris-Cl pH6.8, 4.5mL Glycerol, 1g SDS, 1mL 0.5% Bromophenol Blue, 1mL dH_2O, 70 µl 2-β-mercaptoethanol) and boiled at 95 ^{0}C for 5 min. Tubes with boiled samples were spun down for 60 sec. 15 µg of whole cell lysates from each sample were loaded on 12% SDS gel, and the setup was run at 120 mV for 40-50 mins. Resolved samples were transferred to nitrocellulose membranes (blots) and blocked in 5% skimmed milk solution for 1 hr. Blots were then probed for flag-mPanx1 or actin using HRP-conjugated mouse anti-Flag M2 antibody (1:1000; Sigma) or anti-β-actin (1:7000; Abcam), respectively, and treated for 60 secs with SuperSignal® West Pico enhanced chemiluminescent substrate (Thermo Scientific) for visualization using Molecular Imager® Gel Doc™ imaging system (Bio-Rad Laboratories, Inc.).

2.10 Generation of Panx1 constructs for transient transfection

All deletion constructs were generated using polymerase chain reaction (PCR) by insertion of a stop codon at the desired site where the truncation was intended to be introduced. Primers (Sigma) were designed using Vector NTI software. A touchdown PCR was done using KOD Hot-Start DNA Polymerase (Novagen) to generate a single PCR product, which was then sub-cloned into pCDNA3.1-Flag plasmid. Thermal cycling conditions were as follows: 1) 95 ^{0}C for 2 minutes, 2) 10 cycles of 94 ^{0}C for 15 seconds, 60 ^{0}C (-1 ^{0}C/cycle) for 15 seconds, and 72 ^{0}C for 30 seconds, 3) 25 cycles at 94 ^{0}C for 15 seconds, 50 ^{0}C for 15 seconds, and 72 ^{0}C for 30 seconds, and 4) 72 ^{0}C for 10 minutes. PCR products were resolved on a 1.2% agarose gel containing 1X

SYBR Safe DNA gel stain (Invitrogen). Gel fragments were extracted from the agarose using GenElute Gel Extraction Kit (Sigma) according to the manufacturer's protocol. The eluted PCR product was digested overnight at 37 ^{0}C with AscI and NotI (New England Biolabs) and subsequently purified through the GenElute Gel Extraction Kit (Sigma). Eluted DNA was ligated into AscI/NotI-digested pCDNA3.1-Flag plasmid at a 1:3 (vector:insert) molar ratio and then transformed into chemically-competent JM109 bacteria (Promega) and grown overnight at 37 ^{0}C on LB-agar plates containing 100 µg/mL ampicillin. Four to six individual colonies were grown in 5 mL LB containing 100 µg/mL ampicillin and grown overnight in a shaker incubator at 37 ^{0}C at 250 rpm. Overnight cultures were centrifuged at 3,200x g for 5 minutes and the bacterial pellets were subjected to GenElute Plasmid Miniprep Kit (Sigma) according to the manufacturer's protocol. Purified plasmid DNA was digested with AscI and NotI to confirm the presence of an insert. Positive clones were DNA sequenced (MICB DNA Sequencing Service, University of Manitoba) to confirm sequence accuracy.

Table 2.3 Primers used in the generation of Panx1 constructs

Construct	Forward Primer (5' - 3')	Reverse Primer (3' - 5')
Panx1 (1-372)	ATTATTAGGCGCGCCAACCATGGCCATCGCCCACTT	ATAGCGGCCGCAATTCAAATCATGCCCAGGTTTGTCAGGAGT
Panx1 (1-359)	ATTATTAGGCGCGCCAACCATGGCCATCGCCCACTT	ATAGCGGCCGCAATTCAGCCCTGCCCATTGCTTTTAATGTTC
Panx1 (1-299)	ATTATTAGGCGCGCCAACCATGGCCATCGCCCACTT	ATAGCGGCCGCAATTCACCGGAATGGGATGAAGAACGTGTAG
Panx1 (36-426)	ATTAAGGCGCGCCAACCATGAAGATGGTCACATGTATTGCCGTGG	ATGCGGCCGCATTTAGCAGGACGGATTCAGAAGCCT
Panx1 (1-378)	ATTATTAGGCGCGCCAACCATGGCCATCGCCCACTT	TCCGGCGGCCGCATTTAATCAATGATGTCCATCTTAA
Panx1 (1-390)	ATTATTAGGCGCGCCAACCATGGCCATCGCCCACTT	TATGGCGGCCGCATTTACTCTCCCTTGGTCTGTAGGG
Panx1 (1-402)	ATTATTAGGCGCGCCAACCATGGCCATCGCCCACTT	TATGGCGGCCGCATTTACAAATCTTTGAACTCCACTC
Panx1 (1-414)	ATTATTAGGCGCGCCAACCATGGCCATCGCCCACTT	TATGGCGGCCGCATTTACTCCCCATTGTTTGCTGCAG

2.11 Statistical Analysis

Raw data from voltage-clamp recordings were acquired & extracted using pCLAMP Clampex 10.2 software (Molecular Devices), and processed & analyzed

using pCLAMP Clampfit 10.6 software (Molecular Devices). GraphPad Prism 5 (GraphPad Software, CA) was used for statistical analysis. All the data values reported are expressed as mean ± SEM. Means values are compared using Student's t-test for single group comparison, and one-way ANOVA with Dunnett's post-hoc test for multiple group comparisons. $P<0.05$ was considered significant for all the tests.

SECTION 3: RESULTS

3.1 Characterization of Panx1 in doxy-induced cells

Pannexin channels and connexin hemichannels share similar characteristic properties, i.e. they are both sensitive to similar pharmacological blockers, and exhibit similar solute permeability. Therefore, it is challenging to tease apart responses of Panx1 from those of connexins (or other mechanosensitive) channels expressed endogenously, for example in neurons. Thus, the Jackson lab created a model system, based on Flp-In T-REx 293 cells, to stably express flag-tagged mouse pannexin1 (flag-mPanx1) in doxycycline (doxy) inducible manner. The cells only express Panx1 in the presence of doxy (Fig. 2.1). These cells do not exhibit endogenous expression of Panx1 (or connexin), making it easier to evaluate the mechanistic basis of mechanosensitivity of Panx1.

It was crucial to functionally characterize the doxy-induced flag-mPanx1 T-Rex 293 cell line to build a strong foundation for further experiments. My first set of experiments validated the cell line for its expression of Panx1 channels, both biochemically and functionally. Panx1 exists in three distinct glycosylation forms – Gly0 (un-glycosylated core form), Gly1 (high mannose form), and Gly2 (complex glycosylated form). Post-translation within the ER, Panx1 is N-glycosylated at the residue N254 in second extracellular loop to form the high mannose (Gly1) form. The Gly1 form is trafficked to the Golgi where further glycosylation results in the formation of the complex glycosylated Gly2 form. The complex glycosylated species of Panx1 are trafficked to the plasma membrane. Indeed, inhibition of Panx1 glycosylation severely disrupt the translocation of Panx1 to the cell surface [54], [56]. Of note, the expression of distinct Panx1 glycosylation species is reflected on Western blot by band shift. This shift results in the exhibition of three separate bands, representing three distinct glycosylation species, within the

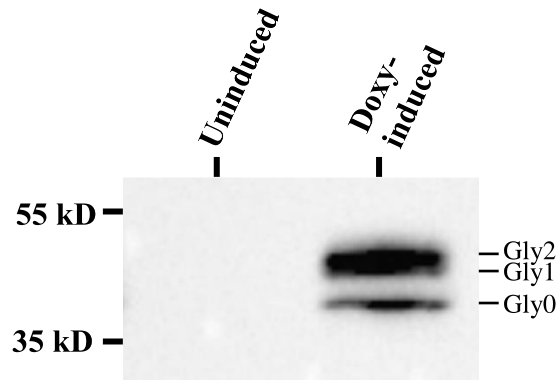
range of 38 kDa to 48 kDa. The presence of Panx1 bands representing the fully glycosylated species on Western blots reflects is consistent with the presence of membrane localized Panx1 in cells [129]. In the present study, I performed Western blotting using lysates derived from doxy-induced cells and uninduced cells, used as a negative control. The blot probed with HRP conjugated anti-flag confirms the existence of distinct bands representing the expression of glycosylated Panx1 (fig. 3.1 A) in doxy-induced cell. Lysates from the uninduced control cells did not exhibit any bands for flag-mPanx1. This confirms that (1) the flag-mPanx1 expression is not constitutive i.e. flag-mPanx1 protein expression is suppressed in the absence of doxy; and (2) anti-flag-HRP antibody is highly specific and reliable for further use in the project. To further confirm the expression of doxy-induced Panx1 in these cells, I used immunofluorescence microscopy. Doxy-induced and uninduced cells were fixed and immunolabelled for flag-mPanx1 (red) and counterstained with DAPI (blue) to identify nuclei. Images in figure 3.1 (B) supports the Western blotting data suggesting that Panx1 is expressed at the surface of doxy-induced cells. In contrast, uninduced cells do not exhibit Panx1 immunolabelling.

Whole-cell voltage-clamp electrophysiology was employed to provide an evidence of functional Panx1 expression in doxy-induced cells. Cells were voltage-clamped at -60 mV, and currents were monitored using voltage ramps by stepping the voltage to -100 mV and ramping to +100 mV over 500 ms. Recordings revealed that the currents recorded from the doxy-induced cells were inhibited by the Panx1 channel blocker carbenoxolone (CBX; 100 μM) (fig. 3.1 C). Similar CBX sensitive currents could not be elicited from uninduced cells. IV (current-voltage) traces derived from the doxy-induced cells showed that the currents are outwardly rectified with reversal potential value close to -50 mV (figure 3.1 D). Collectively, the outward rectification, -

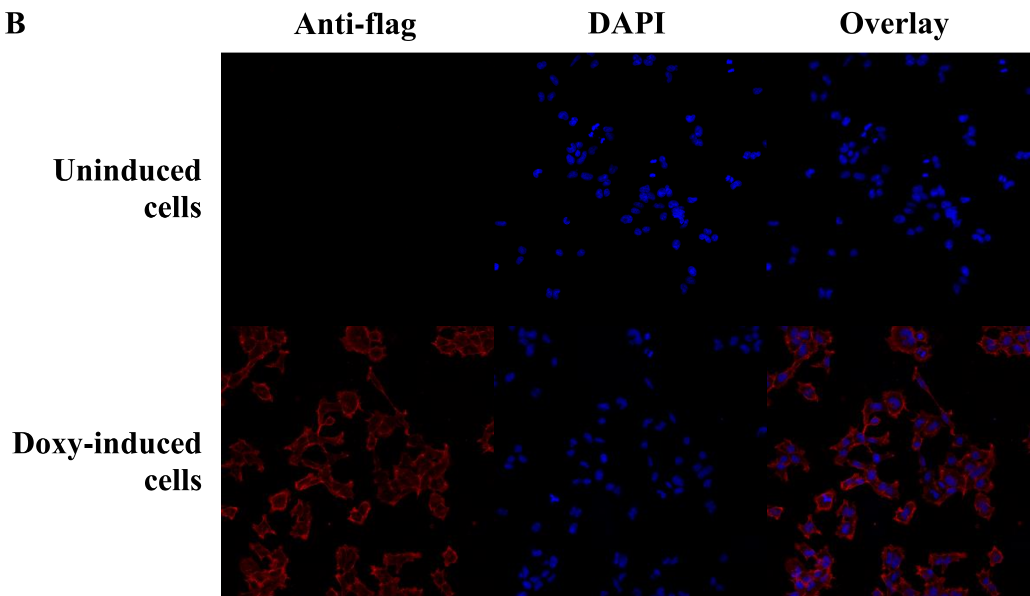
50 reversal potential value, and CBX sensitive nature of the currents serve as hallmark for Panx1 channel mediated currents. On the contrary, currents recorded from uninduced cells (i) displayed modest rectification (ii) did not exhibit the required reversal potential, (iii) and were not blocked by CBX.

Thus, my results confirm the functional expression of Panx1 in doxy-induced cells, and the absence of any leaked expression of Panx1 in uninduced cells. The model system is, hence, successfully validated for the functional expression of Panx1 channels and was deemed qualified for further experimentation.

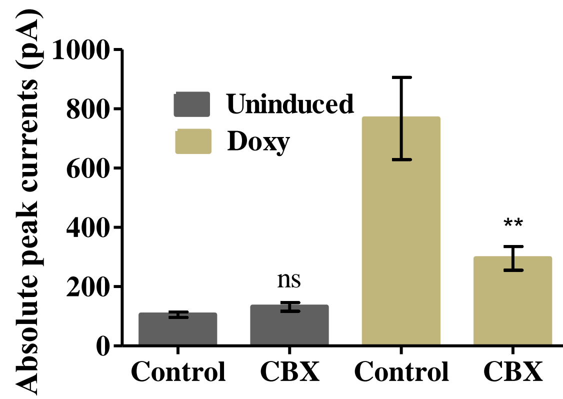
A



B



C



D

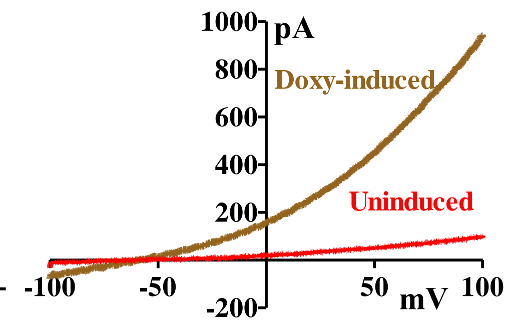


Figure 3.1. Validation of doxy-induced Panx1 expression in flag-mPanx1 T-Rex 293 cells

(A) Western blot depicting the expression of flag-mPanx1 in the cells treated with doxy, in Gly0, Gly1, Gly2 glycosylated forms. (B) Confocal images of cells stained for flag-mPanx1 (red) and DAPI (blue) illustrating the expression of Panx1 in the doxy-induced cells and the absence of Panx1 expression in uninduced cells. (C) In the cells treated with doxy, large amplitude currents were observed which were sensitive to the application of CBX (Panx1 channel inhibitor). Currents recorded from uninduced cells were not sensitive to the application of CBX. (D) I-V curve recorded from the doxy-induced cells depicting that the currents are outwardly rectified with reversal potential of -50 mV. Statistical analysis: values compared to respective control group using Student's t-test (unpaired); $^{**}p<0.005$; all $n > 6$.

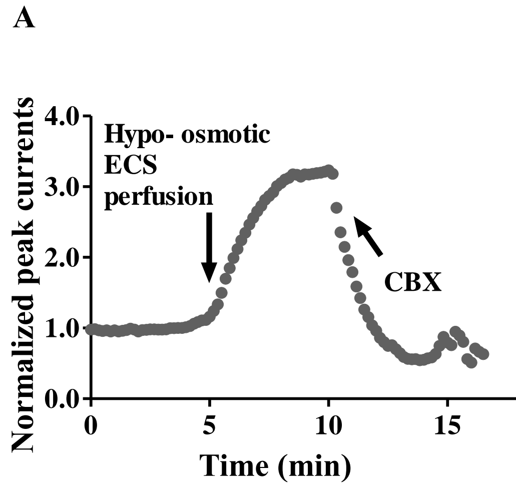
3.2 Hypo-osmotic stress elevates Panx1 channel-mediated current response

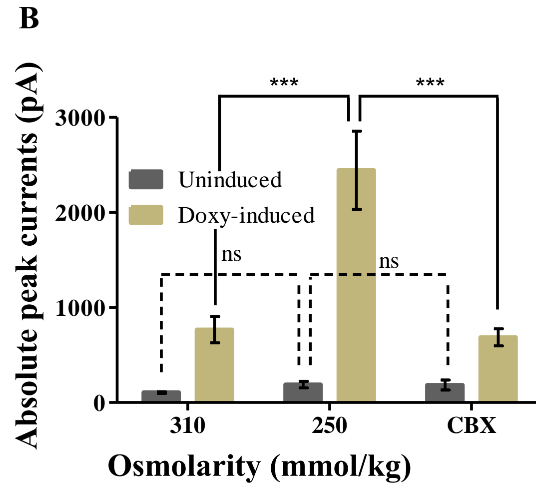
Since the project is focused on investigating the mechanosensitive properties of Panx1, it was important to establish the osmolarity-dependent response of Panx1 channels in our model system. To confirm that the mechanosensitive response was apparent in flag-mPanx1 T-Rex 293 model system, I performed whole-cell voltage-clamp recordings while subjecting the cells to hypo-osmotic stress-induced cell swelling. Figure 3.2 (A) illustrates the recording protocol that was followed. After establishing a stable baseline of 5 mins with iso-osmotic perfusion, cells were perfused with ECS of desired hypo-osmotic value. After 5 mins of hypo-osmotic stress, cells were exposed to CBX (added to hypo-osmotic ECS to make certain that the activity recorded is mediated solely by Panx1 channels). In cells expressing Panx1 (i.e. doxy induced) Panx1 channel-mediated currents were augmented when exposed to hypo-osmotic (250 mmol/kg) stress (figure 3.2 B). Moreover, IV traces recorded during hypo-osmotic ECS application exhibited the characteristic properties of Panx1 (figure 3.2 C). Lastly, the augmented current was completely blocked by application of CBX. Notably, the inhibition by CBX reduced current amplitude below baseline. This reflects the fact that Panx1 channels are active at rest during baseline recordings in iso-osmotic conditions.

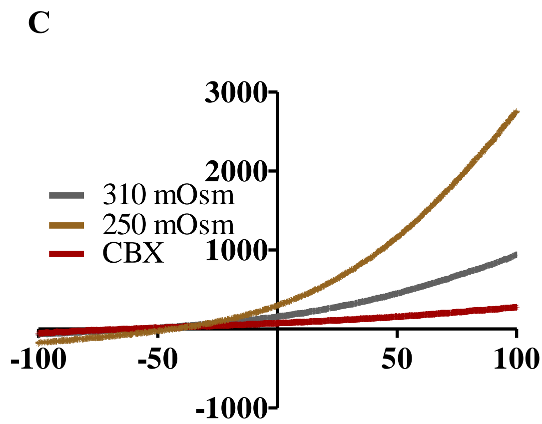
It was necessary to confirm the absence of any endogenous mechanosensitive channels which may contaminate the current responses in my recordings. Therefore, I performed the voltage-clamp recordings using uninduced control cells and repeated the protocol illustrated in fig. 3.2 A. Results from uninduced cells clearly show that the currents were not sensitive to the change in osmotic condition suggesting that the cells do not express any endogenous mechanosensitive

channels (fig. 3.2 B). Also, the currents were insensitive to the application of CBX, further confirming the absence of flag-mPanx1 channels in uninduced cell. This validates 2 major points: i) Panx1 channel respond to the mechano-osmotic stimuli in flag-mPanx1 T-Rex 293 model system; and ii) the cells do not express any endogenous mechanosensitive channels.

The results I have shown so far validate and confirm the mechanosensitive functionality of Panx1 expressed in our model flag-mPanx1 T-Rex cell system. Next, I wanted to establish the range over which channels are responsive to osmotic stress. This would establish if the osmotic response of Panx1 is bi-directional i.e. augmentation of channel activity with hypo-osmotic condition and inhibition of channel activity with hyper-osmotic condition. To address this, I recorded Panx1-mediated responses from cells subjected to varying osmolarity of 340, 310, 280, 250, 210 mmol/kg. Figure 3.2 (D) demonstrates the progressive potentiation of Panx1-mediated currents in response to corresponding decrease in osmolarity. Indeed, lowering the osmolarity exhibits corresponding progressive increase in the channel activity, which seems to plateau beyond the osmolarity of 250 mmol/kg. An important finding from this experiment is that the hyper-osmotic bath solution triggered reduction in current amplitude, which has never been reported before, suggesting that the hyper-osmotic stress-induced cell shrinking inhibits Panx1 channel activity. Collectively, these results indicate that the response of Panx1 channels to osmotic stress is bi-directional from the iso-osmotic setpoint.


A
Normalized peak currents
4.0
3.0
2.0
1.0
0.0
Hypo- osmotic
ECS
perfusion
CBX
0
5
10
15
Time (min)


B
Absolute peak currents (pA)
3000
2000
1000
0
***
***
Uninduced
Doxy-induced
ns
ns
310
250
CBX
Osmolarity (mmol/kg)


C
3000
2000
1000
-1000
310 mOsm
250 mOsm
CBX
-100
-50
50
100

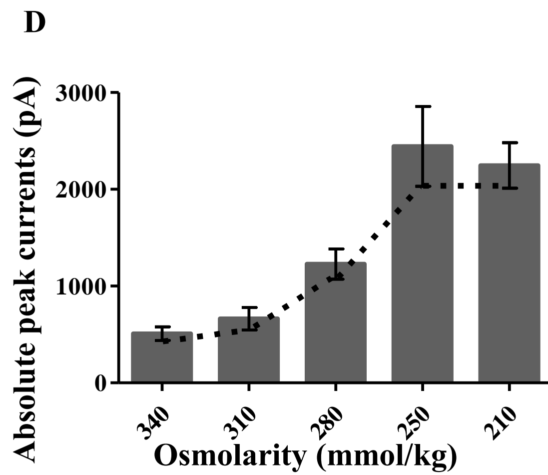

D
Absolute peak currents (pA)
3000
2000
1000
0
340
310
280
250
210
Osmolarity (mmol/kg)

Figure 3.2. Effect of varying osmolarity on Panx1-mediated currents in doxy-induced flag-mPanx1 T-Rex 293 cells.

(A) Illustration of the protocol followed during whole-cell voltage-clamp recordings with 5 min of baseline activity under iso-osmotic condition followed by 5 min of osmotic-stress induced response culminating in the CBX treatment to confirm Panx1-mediation. (B) Doxy-induced cells exhibit augmented current response which is abolished by CBX, suggesting mediation by Panx1 channels. Uninduced cells remained nonresponsive to the osmotic stress and CBX application. (C) I-V curves representing the current responses from doxy-induced cells to different osmotic conditions. (D) Absolute current amplitudes reflecting the varying current responses with respect to the varying osmotic conditions. Progressive increase in the current amplitude is observed with the corresponding sequential decrease in the ECS osmolarity. Statistical analysis: (B): One-way ANOVA with Dunnett's multiple comparison post-test; ***$p<0.0005$. (D) One-way ANOVA with post-test for linear trend; $p<0.0001$. All $n>6$.

3.3 Cyt-D treatment inhibits Panx1 mechanosensitivity

It was established that Panx1 channels are mechanosensitive and respond to osmotic stress. In past reports from studies of other mechanosensitive and stress activated channels, channel interaction with cytoskeletal elements, either direct or indirect, has been shown to play an important role in imparting mechanosensitivity. I proposed to investigate the role of cytoskeletal elements in the regulation of Panx1 channel mechanosensitivity. Cytoskeletal components that I focused on are filamentous actin (F-actin) and microtubules. The first step to narrow down to just one cytoskeletal elements was to confirm which of these play a major role in contributing to the mechanosensitive attributes of Panx1 channels. To address that, I pre-treated the cells with well-established cytoskeleton stabilizing or de-stabilizing drugs. Cytochalasin-D (Cyt-D; 100 µM) was used as an F-actin depolymerizing agent and jasplakinolide (JSK; 2.5 µM) was used as F-actin stabilizing agent. In case of microtubules, nocodazole (NDZL; 5 µM) was used for depolymerizing while paclitaxal (PCTL; 2.5 µM) was used for stabilizing the tubulin network.

I pretreated the cells with respective drugs for 30-40 mins before performing whole-cell voltage-clamp recordings. In contrast to the control set (767 pA; n=7), cells pre-treated with Cyt-D exhibited reduced current amplitude of 319 pA (n=8) during baseline recordings under iso-osmotic (310 mmol/kg) condition (p=0.02) (fig. 3.3 A). This suggests that the intact F-actin network is crucial for basal channel activity of Panx1, under iso-osmotic conditions. Interestingly, stabilizing F-actin by JSK pre-treatment also lead to the decreased basal channel activity of 309 pA (n=8; p=0.02) reflecting the importance of actin dynamics in the basal channel regulation. Stabilizing or depolymerizing the network of tubulin did not affect the basal channel activity.

Likewise, recordings from the cells pretreated with Cyt-D exhibited significantly reduced mechanosensitive response (1033 pA; n=8) as compared to the control cells (2443 pA; n=7) during hypo-osmotic stress condition (fig. 3.3 A). This suggests that F-actin plays a role in regulation of Panx1 channel mechanosensitivity during hypo-osmotic stress. However, stabilizing the F-actin network did not affect the mechanosensitivity significantly. Reduced mechanosensitivity due to F-actin disruption hints towards the tethered interaction of Panx1 with cortical actin. Unlike Cyt-D, JSK pre-treatment did not deprive Panx1 of F-actin, rather it stabilizes cortical F-actin. The actin network is still intact and Panx1 continues to receive force transmitted by the network. As anticipated, stabilizing or depolymerizing tubulin network had no significant effect on the hypo-osmotic stress response of Panx1 channels, suggesting that microtubules do not play an important role in governing the mechanosensitive attributes of Panx1.

So far, I have established that F-actin plays a role in mechanosensitive as well as basal channel regulation of Panx1. To further testify the importance of intact F-actin network in the Panx1 channel regulation, I pretreated the cells with Cyt-D and performed whole-cell voltage-clamp recordings with bathing solution having three different osmolarity (310, 280, 250 mmol/kg). A steep increase in channel activity was observed with depreciating osmolarity in recordings from control cells (fig. 3.3 B). On the contrary, the growth in Panx1 current amplitude with increasing hypo-osmotic stress was blunted in cells treated with Cyt-D. This supports the fact that F-actin plays a major role in regulating Panx1 channel mechanosensitivity.

As suggested previously in section 3.2, I demonstrated for the first time that hyper-osmotic stress inhibits channel activity. I therefore wanted to confirm if F-actin similarly regulates the hyper-osmotic stress response of Panx1 channels. To address this, I followed a similar approach as in the hypo-osmotic stress response tests. I pretreated the cells with Cyt-D and performed whole-cell voltage-clamp recordings following the protocol illustrated in figure 3.3 (C). Five min of baseline recording was followed by hyper-osmotic ECS perfusion and finally by a washout period. If F-actin regulates hyper-osmotic stress response of Panx1, then disruption of the actin network must reduce the inhibition of Panx1 channel activity seen with hyper-osmotic stress. In other words, channel activity of Cyt-D pre-treated cells must not change in response to the hyper-osmotic stress and should remain similar to the baseline. In the untreated control cells, hyper-osmotic stress resulted in the inhibition of Panx1 channel activity (265 pA; n=6), as compared to the basal channel activity of 768 pA (n=7) (p=0.004) under iso-osmotic condition (fig. 3.3 D). Note that the inhibition in channel activity is a mechanosensitive response of the channel. Interestingly, cells with disrupted actin network did not exhibit inhibition of the channel activity in response to the hyper-osmotic stress. In other words, Panx1 mediated current response in Cyt-D treated cells was not changed with the change in osmotic condition. This highlights the fact that Panx1 channels in the Cyt-D pre-treated cells lost their mechanosensitivity. Thus, the data suggests that actin regulates hyper-osmotic stress response of Panx1 channels. Therefore, collectively the data suggests that the intact and dynamic F-actin network regulates not only the mechanosensitive responses of Panx1 channels but also the basal channel activity.

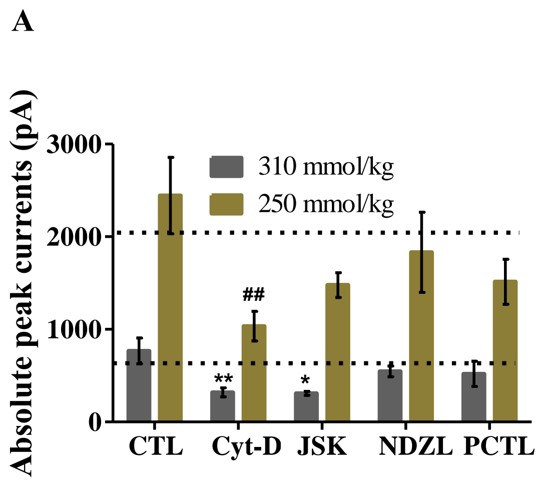
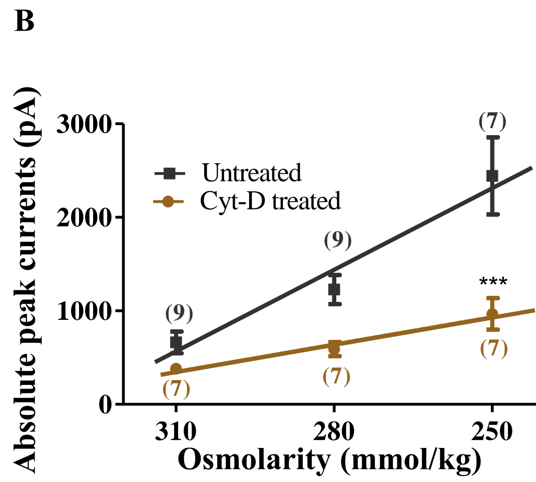
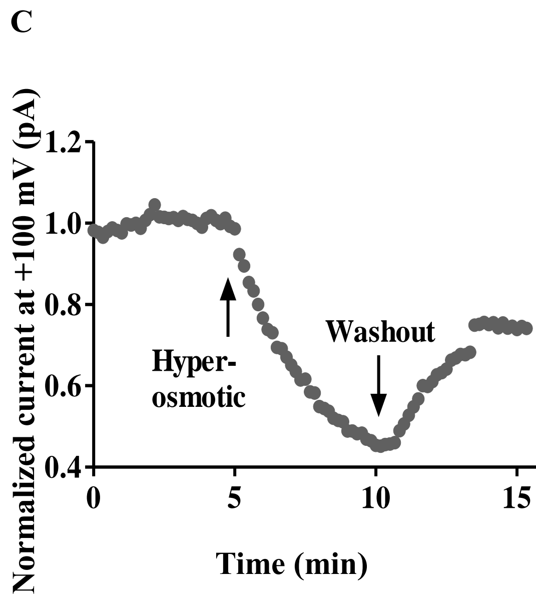
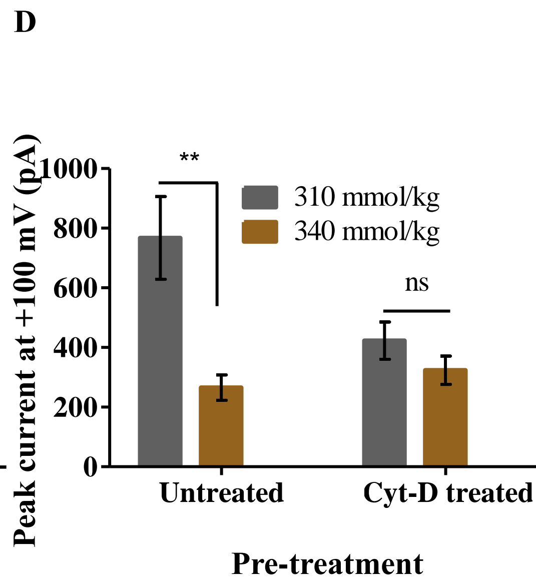

A
Absolute peak currents (pA)
3000
2000
1000
0
310 mmol/kg
250 mmol/kg
##
**
*
CTL
Cyt-D
JSK
NDZL
PCTL

B
Absolute peak currents (pA)
3000
2000
1000
0
Untreated
Cyt-D treated
(7)
(9)
(9)
***
(7)
(7)
(7)
310
280
250
Osmolarity (mmol/kg)

C
Normalized current at +100 mV (pA)
1.2
1.0
0.8
0.6
0.4
Hyper-
osmotic
Washout
0
5
10
15
Time (min)

D
Peak current at +100 mV (pA)
1000
800
600
400
200
0
**
310 mmol/kg
340 mmol/kg
ns
Untreated
Cyt-D treated
Pre-treatment

Figure 3.3 Effect of cytoskeleton stabilizing and de-stabilizing agents on the Panx1 channel activity during osmotic stress.

(A) Whole-cell voltage-clamp recordings in cells pre-treated with cytoskeleton depolymerizing and stabilizing drugs. Pre-treatment with F-actin depolymerizing drug (Cyt-D) reduces basal channel activity and the hypo-osmotic stress response of Panx1. Tubulin network disruption did not exhibit significant effect on Panx1 channel activity. (B) Panx1 mediated currents recorded from untreated control cells and Cyt-D treated cells exposed to ECS of three different osmolarity. Disruption of F-actin inhibited Panx1 mechanosensitivity. (C) Illustration of the protocol followed during whole-cell voltage-clamp recordings with 5 min of baseline activity under iso-osmotic condition followed by 5 min of hyper-osmotic stress-induced response culminating in washout with isotonic ECS. (D) Peak current values recorded during the hyper-osmotic stress conditions, normalized to the respective control (baseline) activity, suggesting the loss of Panx1 mechanosensitivity due to the disruption of F-actin by Cyt-D. Statistical analysis: (A) One-way ANOVA with Dunnett's post hoc analysis (values compared with CTL group); *$p<0.05$ **$p<0.005$; ##$p<0.005$. (B) One-way ANOVA with Bonferroni post-test; ***$p<0.001$. (D) Student's t-test (unpaired); $p=0.07$; all $n \geq 6$.

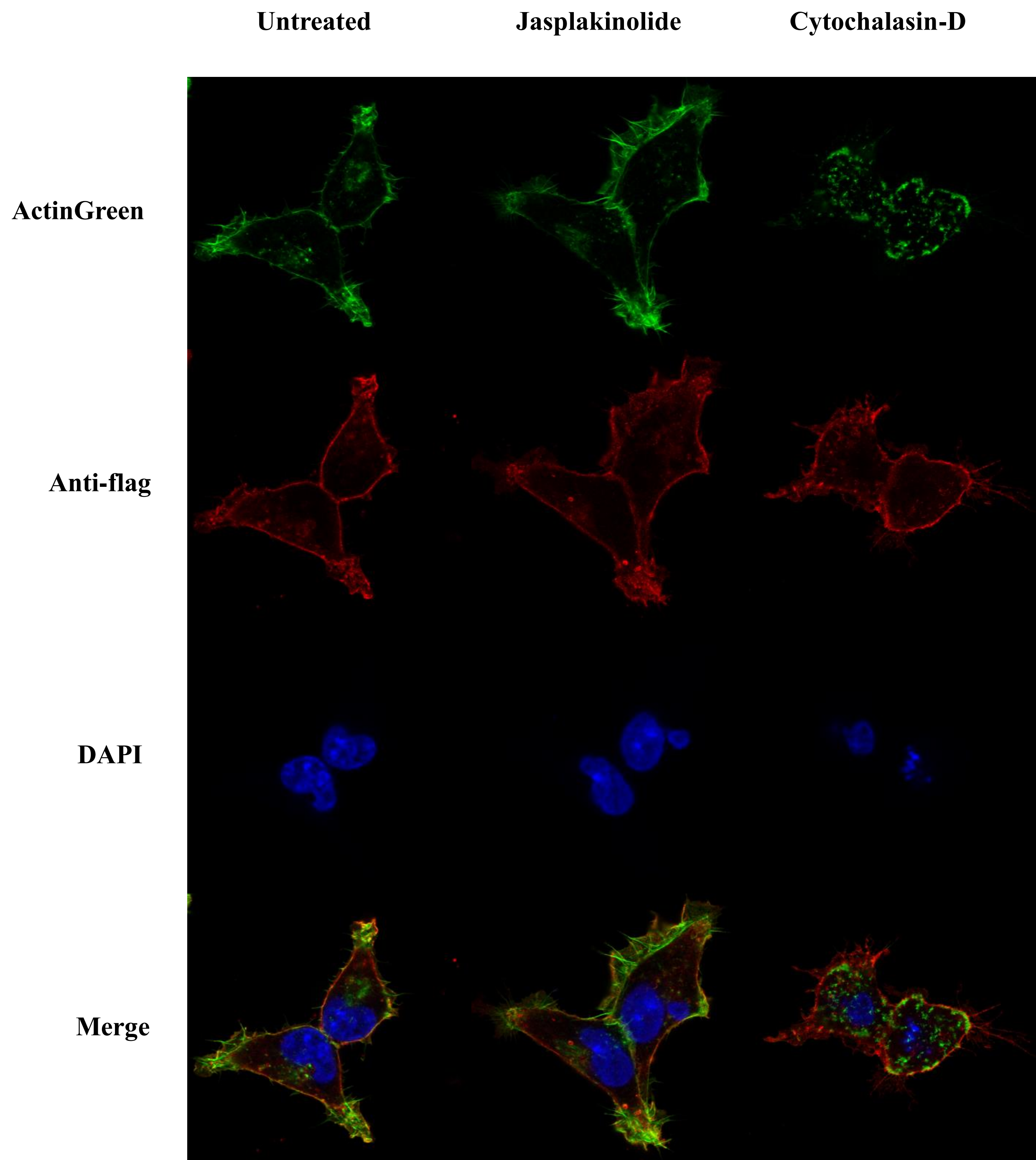
3.4 Panx1 colocalizes with cortical F-actin

So far, my experiments establish that F-actin regulates basal channel activity and is a major contributor to osmotic-stress induced Panx1 responses. With confocal imaging, I wanted to visualize the localization of actin and Panx1. Co-distribution or colocalization of actin and Panx1 would supplement the past evidence suggesting that Panx1 interacts with F-actin. Untreated control cells and the cells treated with cytoskeleton stabilizing and depolymerizing agents were fixed and immuno-staining for actin, tubulin, nucleus, and flag-mPanx1. Airyscan images of the cells were taken using a confocal microscope. Figure 3.4 (A) shows images of cells treated with Cyt-D and JSK along with untreated control cell with unmodified F-actin network [flag-mPanx1 (red), actin (green), and nucleus (blue)]. Images of the untreated cell illustrates the localization of Panx1 with actin at the cell membrane. Also, Panx1 is well expressed at filopodia, lamellipodia, and membrane ruffles, which are actin-rich regions in cells. Both the proteins seem to co-localize suggesting a possible interaction. Additionally, it is also evident from the images that the Cyt-D pre-treatment used for this study was effective in disruption of the cortical actin network depriving the Panx1 of F-actin. This strengthens the conclusion drawn from the previous experiments that the F-actin interacts with Panx1 to regulate channel functioning.

Similarly, I also tested the localization of Panx1 with respect to tubulin. Untreated cells and cells treated with NDZL (depolymerizing agent) and PCTL (stabilizing agent) were fixed and stained for tubulin (green), flag-mPanx1 (green), and nucleus (blue). Figure 3.4 (B) illustrates the expression of Panx1 in the regions not accessed by microtubules (eg. membrane ruffles). This suggests that tubulin is unlikely to interact with Panx1 and hence, is not essential for Panx1

channel functioning. Images also confirm the disruption of tubulin network by NDZL pre-treatment.

A



B

Untreated **Paclitaxel** **Nocodazole**

Anti-Tubulin

Anti-flag

DAPI

Merge

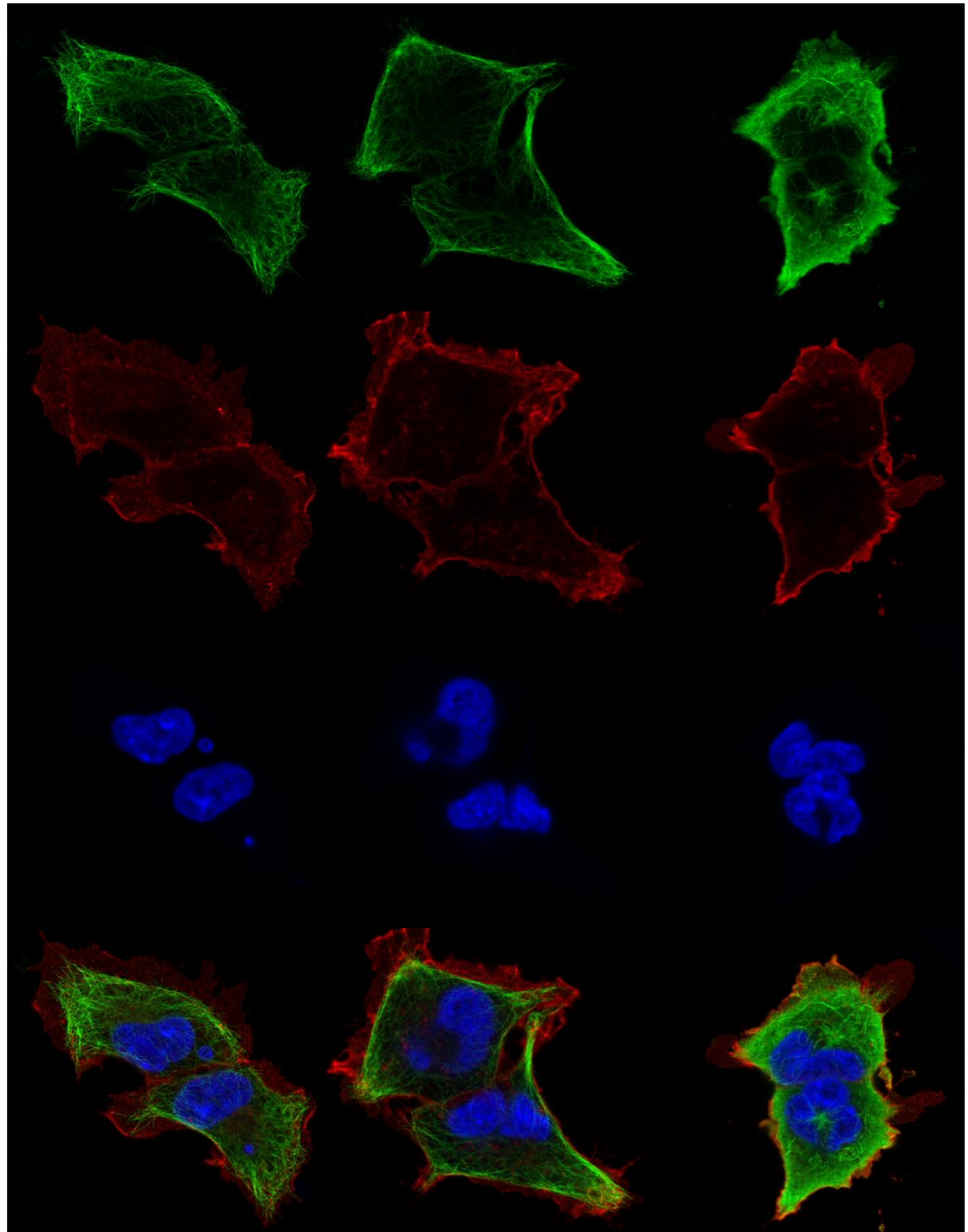


Figure 3.4 Immunofluorescence imaging depicting the localization of Panx1, actin, and tubulin networks.

(A) Panx1 (red) seems to localize at actin (green) rich regions such a filopodia, lamellipodia, and membrane ruffles suggesting the interaction of the two proteins. Actin network was visible depolymerized because of the Cyt-D pre-treatment. (B) Microtubules (green) do not approach the peripheral regions of lamellipodia, membrane ruffles, and filopodia where Panx1 (red) is well expressed. NDZL pre-treatment was effective in tubulin network disruption.

3.5 Panx1 interacts with actin in flag-mPanx1 T-Rex 293 cells

Bhalla-Gehi *et al.* [63] demonstrated the interaction of actin with Panx1 by using immunoprecipitation wherein lysates of Panx1 or Panx1-GFP expressing BICR-M1R_k cells were used. They pulled down Panx1 or Panx1-GFP and found β-actin to co-precipitate with Panx1. Furthermore, they also showed that polymerized actin filaments bind with the C-terminus region of Panx1 protein, using a co-sedimentation assay. This latter finding suggests that Panx1 and actin can directly associate. Nevertheless, *in situ* Panx1 and actin may interact via a larger protein complex with multiple adapter proteins facilitating their interaction. In either case, such physical interaction may underlie the Panx1 channel mechanosensitivity. Having said that, work published by Bhalla-Gehi *et al.* is the single publication which employed co-IP or co-sedimentation assay of actin and Panx1. It is important to confirm the interaction by co-IP in my model system. Co-IP was performed as described in the section 2.8. Briefly, Panx1 expression was induced by doxy treatment in flag-mPanx1 T-Rex 293 cells before lysis. Lysates were incubated with anti-β-actin coated beads to pulldown β-actin which was assessed using Western blotting. Panx1 was observed to co-immunoprecipitate with β-actin, as seen in the immunoblotting probed with HRP-conjugated anti-flag (fig. 3.5 A). The reciprocal pulldown of Flag-Panx1 was also performed and the resulting immune complexes subjected to immunoblotting to probe for the presence of β-actin. As shown in Fig. 3.5.B, the presence β-actin in the flag pulldown was confirmed. This two-way co-IP assay strongly suggests the physical interaction of Panx1 with actin in the model system used for this study. Regardless of whether the binding is direct or via multiple adapter proteins, the co-precipitation of actin with Panx1 strongly emphasized that the interaction is natural under our experimental conditions, and play a major role in channel regulation.

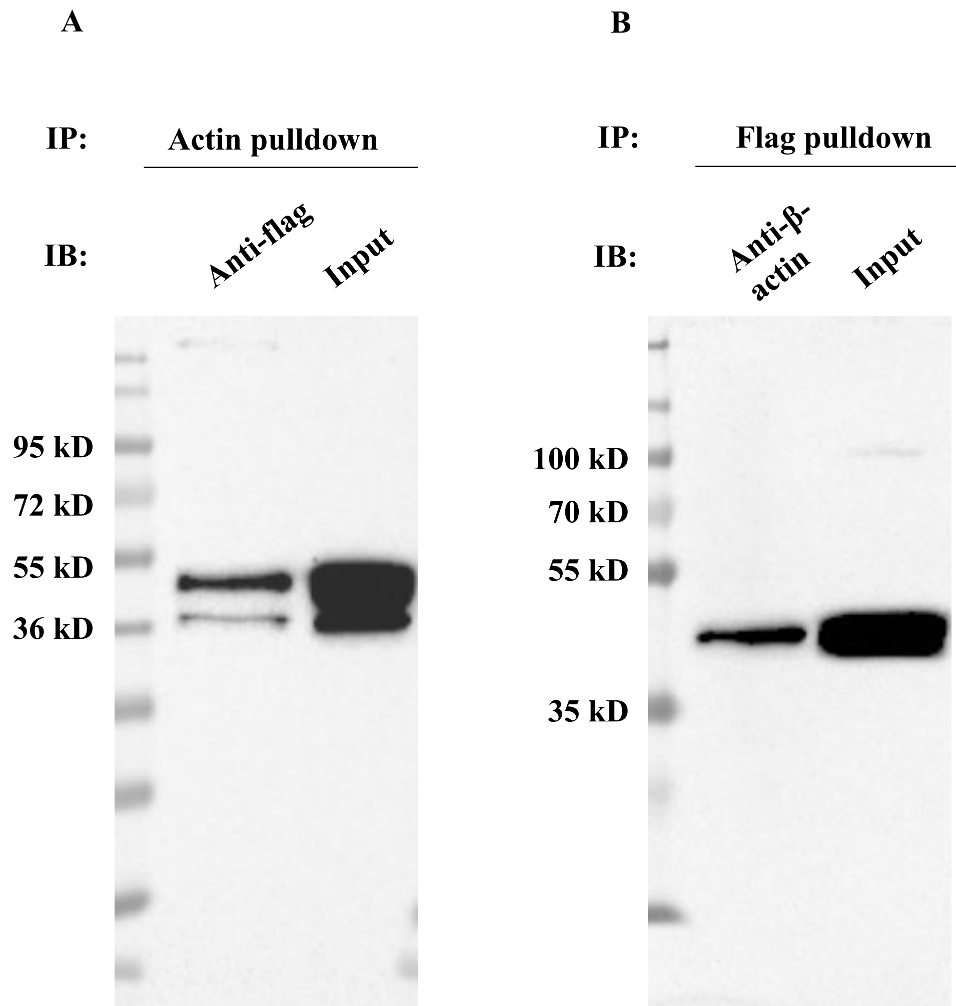


Figure 3.5 Panx1 interacts physically with actin

Immunoblotting performed with actin (A) and flag (B) pulldown using lysates derived from doxy-induced flag-mPanx1 T-Rex 293 cells shows the presence of flag-mPanx1 in the actin pulldown and actin in the flag pulldown, suggesting a physical interaction of the two proteins in this cell line.

3.6 The hypo-osmotic stress response of Panx1 channels is Ca^{2+} dependent

Hypo-osmotic stress can induce a rise in the levels of intracellular Ca^{2+} [96], [130] which may play a role in the stress-response of Panx1 channels because the channels are sensitive to the $[Ca^{2+}]_i$ increase [96]. To investigate this, I supplemented the ICS with well-established calcium chelators, EGTA (11 mM) or BAPTA (20 mM), before patching the cells. Voltage-clamp data revealed that chelation of $[Ca^{2+}]_i$ by EGTA reduced the current amplitude to 356 pA (n=7) as compared to the control recordings (767 pA; n=7) ($p<0.05$) under the iso-osmotic (310 mmol/kg) condition. Likewise, chelation of $[Ca^{2+}]_i$ with BAPTA also significantly reduced the basal channel currents to 343 pA (n=7) ($p<0.005$), under iso-osmotic condition. This suggests that $[Ca^{2+}]_i$ is important for basal channel function.

In contrast to control set (2443 pA; n=7), $[Ca^{2+}]_i$ chelation by EGTA significantly reduced the mechanosensitive response (1289 pA; n=7) ($p<0.005$) of Panx1 channels in response to hypo-osmotic stress. Similarly, recordings performed using BAPTA showed significantly reduced mechanosensitivity of Panx1-mediated currents (750 pA; n=7) ($p<0.005$). This suggests that $[Ca^{2+}]_i$ plays an important role in the hypo-osmotic stress response of Panx1 channels. Hypotonic cell swelling has been shown to result in a dramatic rise of $[Ca^{2+}]_i$ [130], which we speculate could contribute to Panx1 mechanosensitivity by two distinct processes: (1) Rise in $[Ca^{2+}]_i$ increases Src activity resulting in Panx1 channel potentiation [81], [131]. Indeed, Src activity has been shown to be augmented in response to hypotonic stress [132], [133]; (2) Rise in Ca^{2+} levels affect the functioning of a Ca^{2+}-dependent actin-binding protein α-actinin, which in return results in increased F-actin mediated Panx1 channel activity augmentation [134], [135].

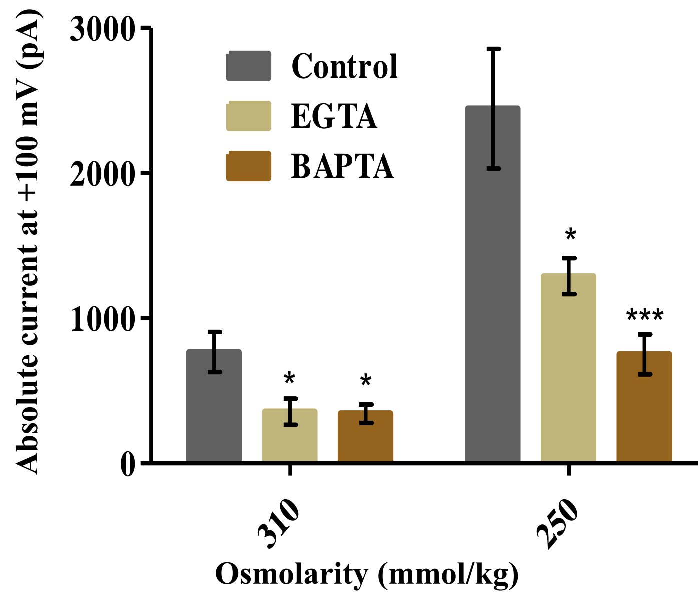


Figure 3.6 Effect of calcium chelators EGTA and BAPTA on the hypo-osmotic stress response of Panx1 channels.

Whole-cell voltage-clamp recordings performed with EGTA or BAPTA supplemented in the ICS filled in patch pipette revealed the Ca^{2+} dependent mechanosensitive response as well as basal channel regulation of Panx1. Statistical analysis: One-way ANOVA with Dunnett's multiple comparison post-test (Control vs BAPTA/EGTA); *$p<0.05$, **$p<0.005$; n=7.

3.7 Identification of the Panx1 domain responsible for mechanosensitivity

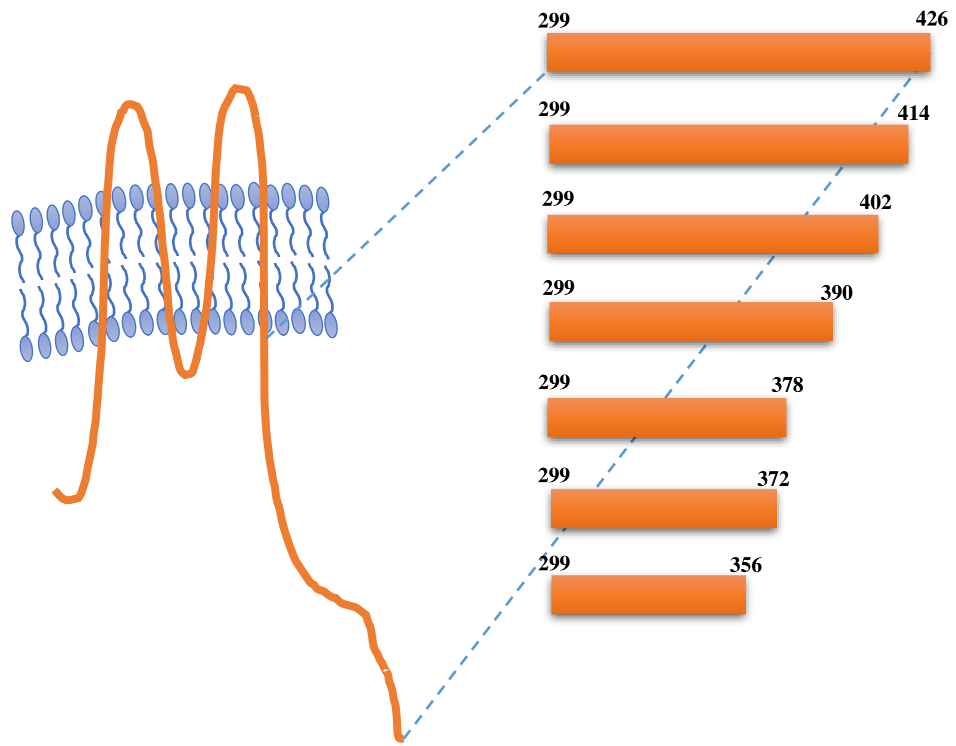
In the previous sections (section 3.1 to 3.6), experiments were conducted using flag-mPanx1 T-Rex 293 cells modified to stably express flag-tagged Panx1 in a doxy-inducible manner. From this section onwards, all the experiments described were performed using the parental cell line of flag-mPanx1 Flp-In T-Rex 293cells, namely HEK 293T, which do not express Panx1 endogenously. Data derived from the previous set of experiments established that (i) Panx1 interacts with F-actin; (ii) F-actin regulates basal channel activity; (iii) F-actin regulates mechanosensitive response of Panx1 during osmotic-stress conditions. The next phase of this project is to map the minimal domain responsible for Panx1 mechanosensitivity. To execute that, our lab created sequential deletion mutants of Panx1 which are progressively truncated at C-terminal region (fig. 3.7 A). Truncations were introduced after the amino acid residues- 1) 356, 2) 372, 3) 378, 4) 390, 5) 402, 6) 414. This was achieved by insertion of a stop codon following the base pairs coding for these amino acid residues. These truncation mutants are flag-tagged at N-terminus (flag-mPanx1). There are three predicted phosphorylation sites and one predicted glycosylation site present within the regions that are truncated [54]. This means that the truncations may lead to a change in expression profile of the channels. Therefore, to confirm the surface expression of the mutants, I used Western blotting and immunofluorescence imaging, before performing electrophysiological studies.

HEK 293T cells were transiently transfected with Panx1 truncation mutants or WT constructs of flag-mPanx1 using jetPRIME^{TM} (as described in sec. 2.3) and lysed. Western blotting was performed on the lysates using the anti-flag-HRP antibody to probe the blot. Image of a

representative blot in figure 3.7 (B) shows the protein bands for the truncation mutants and WT. Immunoblotting suggests that all the seven Panx1 constructs are expressed at varying levels in the transfected cells. Moreover, the blot exhibits protein bands representing glycosylation species of Panx1. This suggests that all the truncation mutants and WT Panx1 trafficked through the ER and Golgi and are expressed on the cell membrane in the transfected HEK 293T cells.

To further confirm the surface expression of the constructs, I performed immunofluorescence with the HEK 293T cells transfected with the constructs: WT, 1-378, 1-414, 1-299 (full C-term deletion). In preliminary work, I found that Panx1 with full C-term deletion is not translocated to the membrane, and is localized to the cytoplasm. So, construct 1-299 could serve as a negative control in this imaging experiment. Transfected cells were fixed and stained for Panx1, actin, and nucleus as described in the section 2.8. Figure 3.7 (C) shows the Airyscan images of the cells taken with a confocal microscope. It is apparent from the images that Panx1 (red) is expressed at the cell surface in the cells transfected with WT, 1-378, and 1-414 constructs (relative to actin; green). On the other hand, Panx1 is localized within the cytoplasm in case of full C-term deletion (construct 1-299). Images of the cells expressing WT, 1-378, and 1-414 constructs also suggests co-distribution of Panx1 with actin (merge column), which was already in the previous experiments. Collectively, the western blotting and immunofluorescence validates the surface expression of the truncation mutants, and lays the platform for next set of experiments.

A



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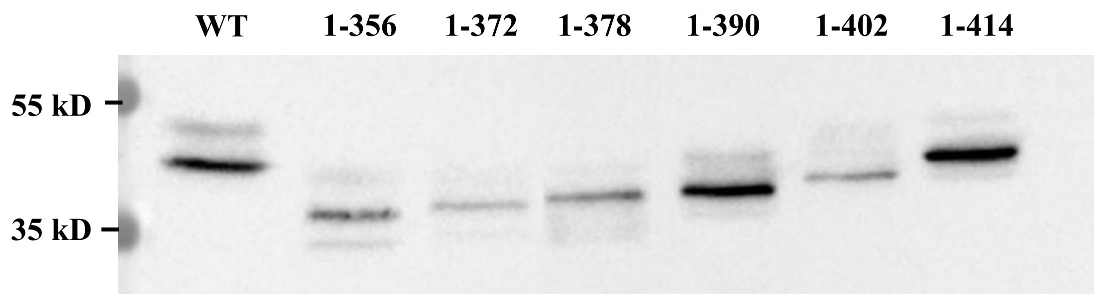


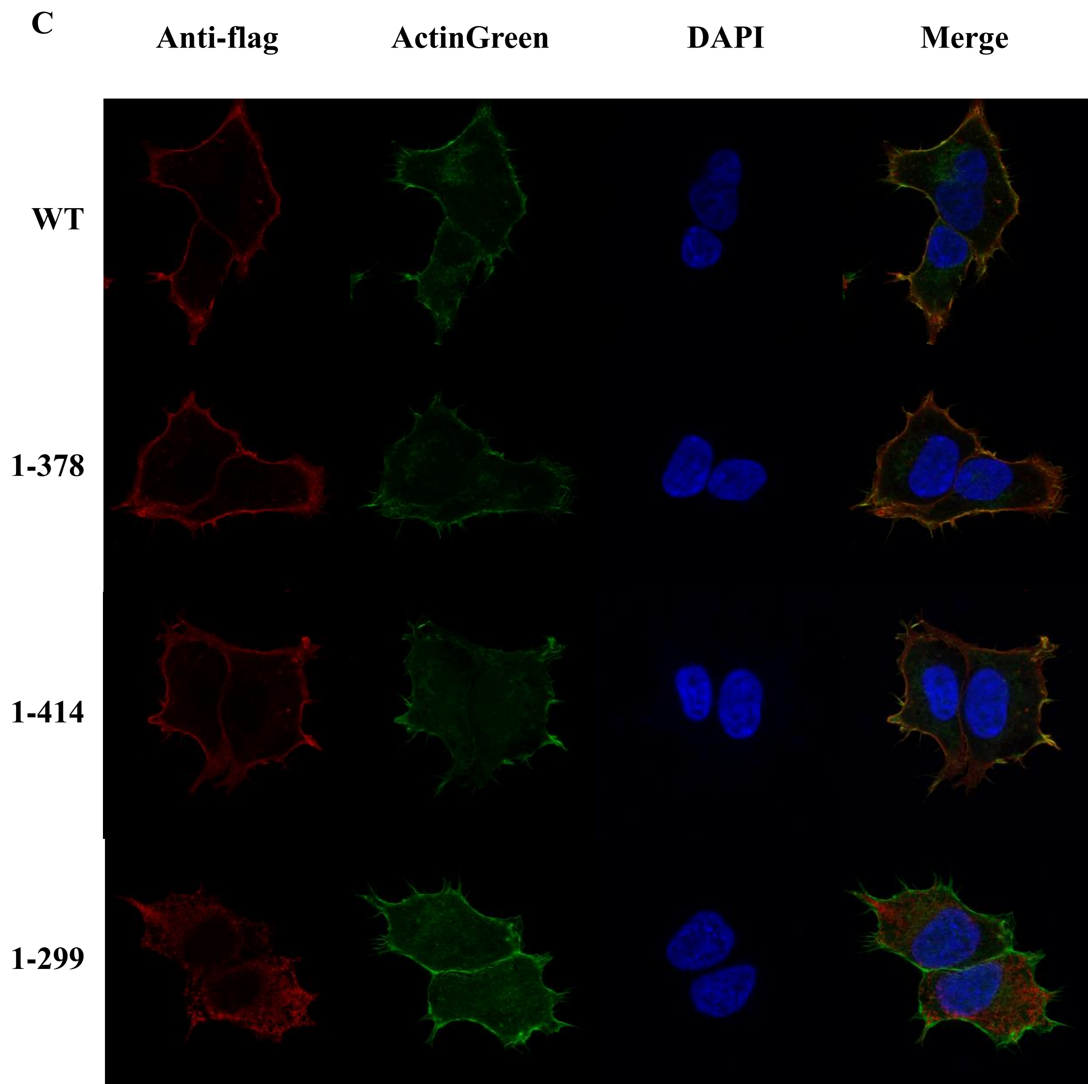

C
Anti-flag
ActinGreen
DAPI
Merge
WT
1-378
1-414
1-299

Figure 3.7 Validation of Panx1 truncation mutant expression

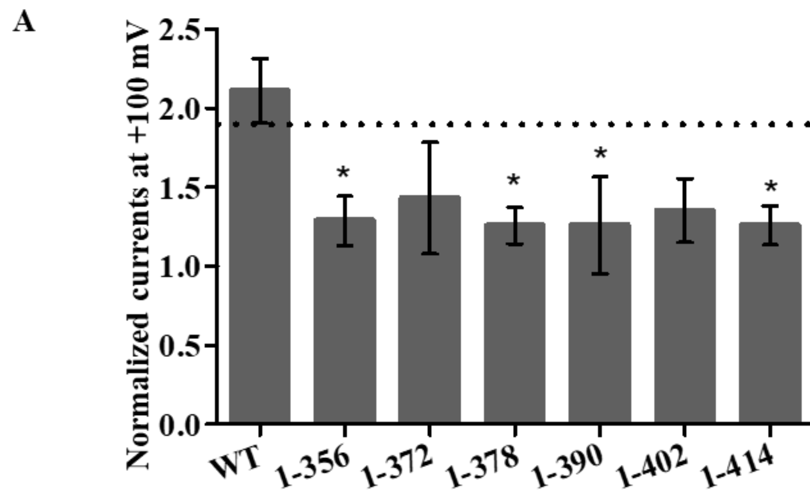
(A) Illustration of truncation positions at the C-terminal region of Panx1, expressed transiently in the HEK 293T cells. (B) Western blot reflecting the surface expression of the truncation mutants as signified by the band range representing Panx1 glycosylation. (C) 1-378 and 1-414 deletion constructs along with WT and full C-term deleted Panx1 constructs tested for surface expression. WT, 1-378, and 1-414 were well expressed at the surface whereas full C-term deleted Panx1 (1-299) was localized within the cytosol.

3.8 Panx1 CT regulates mechanosensitive behavior of the channel

The transfected cells expressing WT or truncated Panx1 were voltage-clamped at -60 mV. Cells were then perfused with iso-osmotic ECS to establish a stable baseline for 5 mins before subjecting the cells with hypo-osmotic stress. Data derived during hypo-osmotic recordings for each truncation construct were normalized with their respective baseline current amplitude (fig. 3.8 A). The cells transfected with deletion constructs 1-356, 1-378, 1-390, and 1-414 showed significant inhibition of mechanosensitive response ($n \geq 6$; $p=0.02$). Cells transfected with 1-372 and 1-402 did not exhibit statistically significant difference compared to the cells transfected with WT construct ($n \geq 6$). This may result from the fact that transient transfections are more variable in terms of expression. More experiments need to be undertaken to provide a more definitive answer. Nonetheless, the region 414 – 426 seems to play a role in mechanosensitive regulation of Panx1. This idea is articulated because that region was deleted in all the above truncation constructs affecting the mechanosensitive response of the channels. It is also possible for the region 372 to 414 to be the principal or supplementary regulator of the Panx1 channel along with the distal CT tail. To confirm that the current responses recorded from the transfected cells are Panx1 mediated, I recorded with middle (1-378) and distal truncation mutants (1-414) using CBX. Cells were exposed to CBX after establishing 5 min baseline activity under iso-osmotic bath solution. Figure 3.8 (B) shows the absolute current amplitudes recorded from the cells transfected with WT, 1-378, and 1-414 mutant constructs. As expected, current responses (1302 pA; $n=4$) recorded from the cells expressing WT Panx1 under iso-osmotic condition is inhibited by CBX (278 pA; $n=4$) ($p=0.01$). Similarly, baseline activity recorded from the cells transfected with mutants 1-378 (937 pA; $n=4$) and 1-414 (1531 pA; $n=6$) is blocked by CBX completely (1-378: 256 pA; $n=4$) (1-414: 266 pA; $n=6$) ($p<0.002$). This confirms that the

truncated channels are functional and the cells exhibit Panx1 mediated current responses. Figure 3.8 (C) depicts the I-V traces of the currents recorded during hypo-osmotic stress from the cells transfected with the respective Panx1 constructs. Traces for construct 1-356 depicts considerably low peak amplitude at +100 mV, as if the channel was not functional. Nonetheless, the constructs exhibit finger prints of Panx1 channel-mediated currents: CBX-sensitive, outwardly rectified with reversal potential close to -50 mV, suggesting the current recorded was mediated by Panx1 channels.

A



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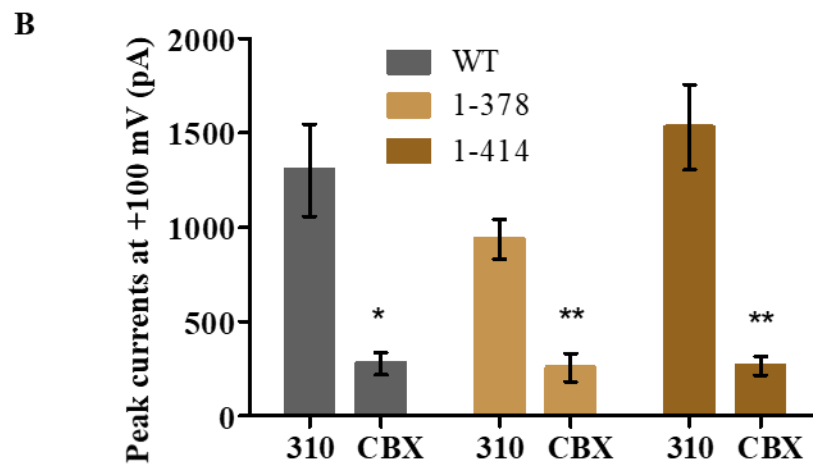

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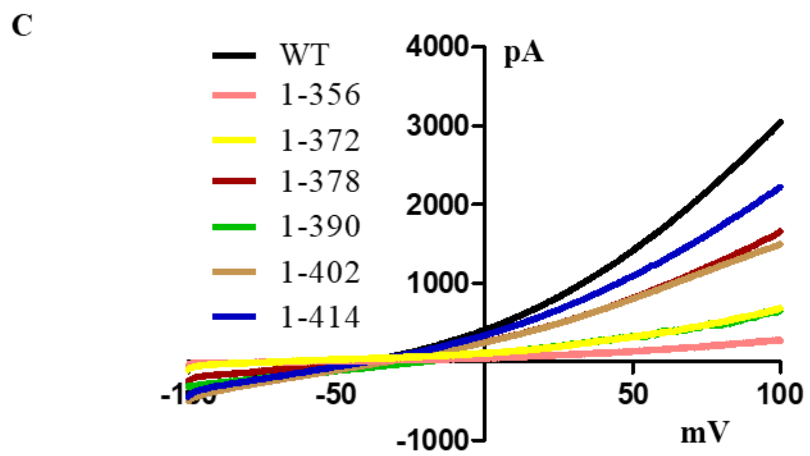


Figure 3.8 HEK 293T cells transfected with Panx1 sequential truncation mutants exhibit loss of mechanosensitivity.

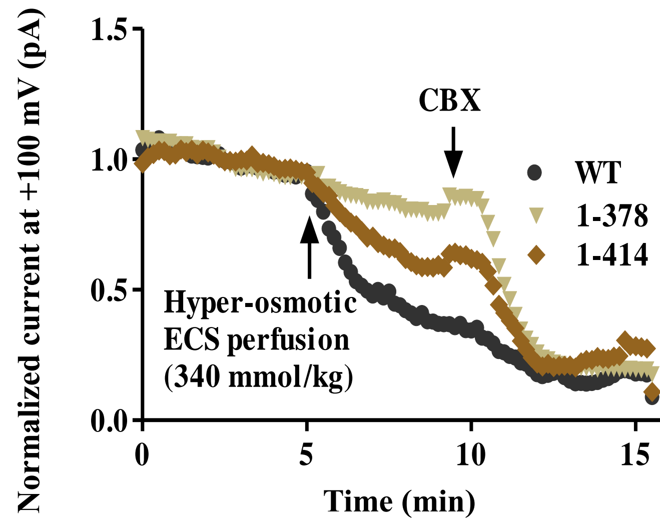
(A) Peak currents recorded at +100 mV during hypo-osmotic stress are normalized with respective baseline currents. Result shows the loss of mechanosensitive response in the cells transfected with the CT deletion mutants. (B) Absolute peak currents recorded from the cells transfected with WT, 1-378 and 1-414 constructs. The data confirms Panx1-mediated current activity and functional channel expression. (C) I-V curves representing the current activity mediated by Panx1 (WT or truncated) channels during hypo-osmotic stress. Statistical analysis: (A) one-way ANOVA with Dunnett's multiple comparison post hoc analysis; $^*p<0.05$; $n \geq 6$. (B) Student's t-test (unpaired); $^*p<0.05$, $^{**}p<0.005$; $n \geq 4$.

3.9 Panx1 CT regulates hyper-osmotic stress response

Previous experiments confirmed that the distal region of Panx1 CT regulates the hypo-osmotic response of the channel. I wanted to confirm if that region also regulates the channel response during hyper-osmotic stress. HEK 293T cells transfected with WT, 1-378, and 1-414 flag-Panx1 constructs were used for whole-cell voltage-clamp recordings. Voltage-clamped cells were perfused with hyper-osmotic ECS on achieving 5-min stable baseline, followed by CBX treatment to confirm the Panx1-mediation. Figure 3.9 (A) depicts the time-based plot of normalized currents (peak amplitude at +100 mV every 10 sec). 5-min of stable baseline activity can be seen in all the three groups, followed by a steep decrease in the current activity in case of WT Panx1. Panx1 with truncations at 378 and 414 did not exhibit significant reduction in the current activity during hyper-osmotic stress condition. Total inhibition of channel activity was seen in all the three cases because of the CBX treatment, confirming that the current response recorded was mediated by Panx1 channels. Figure 3.9 (B) shows the absolute current values recorded from the cells transfected with WT, 1-378, or 1-414 Panx1 constructs. In case of WT Panx1, a significant inhibition of channel activity was seen because of the hyper-osmotic stress from the basal channel activity of 1259 pA (n=5) to 468 pA (n=6) (p=0.001). Whereas the hyper-osmotic stress did not induce a significant inhibition of channel activity in the cells transfected with Panx1 1-378 (n=6). In case of the cells transfected with Panx1 1-414, hyper-osmotic stress seems to inhibit the channel activity, although statistically insignificant. This may reflect – (i) a considerable variability within transient transfections; OR (ii) the distal region of 12 amino acids may play a modest role in exhibiting hyper-osmotic response of Panx1. More experiments need to be performed to derive a definitive conclusion. The loss of mechano-sensation in the truncated

Panx1 channels further strengthen the main finding of the project that distal region of Panx1 CT regulates mechanosensitive response of the channel.

A



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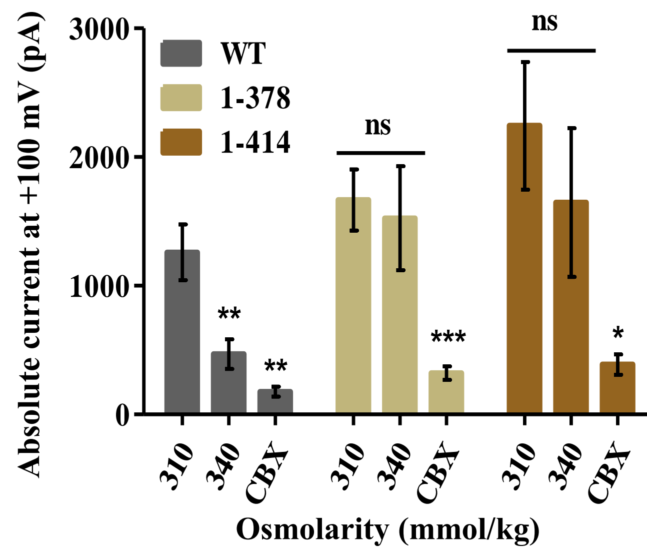

Figure 3.9 Hyper-osmotic stress response of Panx1 truncation mutants

(A) The normalized peak current amplitude recorded from cells transfected with WT, 1-378, 1-414 Panx1 constructs is plotted over time. (B) Contrary to the current responses recorded from the WT Panx1, Panx1 truncation mutants 1-378 exhibited inhibition of hyper-osmotic stress response. However, truncation 1-414 seems to have an inhibitory effect of hyper-osmotic stress (statistically insignificant). Statistical analysis: Student's t-test (unpaired); *$p<0.05$, **$p<0.005$, ***$p<0.0005$; all $n \geq 5$.

SECTION 4: DISCUSSION

4.1 Summary of the key findings

The principal objective of my research thesis was to elucidate the mechanistic basis of Panx1 channel mechanosensitivity. It is established that Panx1 interacts with F-actin, but the functional consequences of this interaction remained elusive. Evidently, the cytoskeletal network of actin and tubulin conduct and transmit mechanical forces to various cellular channels and organelles. More importantly, many membrane-bound mechanically sensitive channels are regulated by their interaction with the cytoskeletal elements [31], [32], [35]. Therefore, I investigated the involvement of the cytoskeleton in regulating Panx1 channel mechanosensitivity. Additionally, with the means of Panx1 truncation mutants, I also identified the region of the channel protein which confers the channel with the ability to sense and respond to the mechanical stress.

To investigate the mechanisms responsible for Panx1 mechanosensitivity, we developed a model system based on Flp-In T-Rex 293 cells, modified to express Flag-tagged mPanx1 in doxy-inducible manner. I validated the model system for functional expression of Panx1 channels responsive to mechanical stimuli. I employed whole-cell voltage-clamp recordings to characterize osmotic stress induced Panx1 channel activation. Hypotonic stress induced cell swelling leads to the augmentation of Panx1 channel activity, which has previously been shown in other cell types. However, there has been no data published to date demonstrating the effect of hypertonic stress on the Panx1 channels. I found that hypertonic stress inhibits the channel activity. I tested the involvement of actin and tubulin in the mechanosensitive response of Panx1. I found that actin contributes majorly in mediating the augmentation or inhibition of Panx1 activity in response to osmotic stress. Moreover, actin also plays a role in regulating basal activity of the channels. Co-IP experiments from our lab supports the earlier work which shows

interaction of Panx1 with actin. Additionally, the confocal imaging from my work demonstrates the co-distribution of Panx1 and actin. Also, Panx1 is well expressed within actin rich cellular structures (membrane ruffles, lamellipodia, and filopodia). Whole-cell voltage-clamp experiments conducted using cells transiently transfected with C-term truncation mutants of Panx1 shows a significantly reduced Panx1 response to osmotic stress. This suggests that the distal C-terminus region, comprising the final 12 amino acids, is responsible for imparting the mechanosensitive properties of the channel.

4.2 Influence of osmotic stress on pannexin channel activity

Even though it is well accepted that Panx1 channels are mechanosensitive, the gating mechanism responsible have remained elusive. Although, a considerable amount of pannexin literature explores the mechanical stress-response of the channels in various cell and tissue types, none investigated the mechanistic basis of channel activation during mechanical stress. Nonetheless, a substantial amount of literature within the area of mechanobiology highlights the importance of cytoskeletal interaction in mediating the mechanical stress response of numerous other mechanically sensitive proteins. Therefore, based on the previous work in the literature, I tested the involvement of cytoskeletal elements – actin and microtubules in the Panx1 mechanosensitivity.

The first step of this project was to characterize the flag-mPanx1 Flp-In T-Rex 293 model system developed in our lab which stably expresses the Panx1 in a doxy-inducible manner. I confirmed that the Panx1 channels are expressed only in doxy-induced, but not uninduced, cells. It was crucial to employ this recombinant expression system in my studies in order to unambiguously

identify the effect of mechanical stress on Panx1 channels. Indeed, it is difficult to tease apart Panx1-mediated responses in other cell types because of (1) overlapping expression profiles, and similar pharmacological and biophysical properties of Panx1 with Cxs; (2) the expression of many other endogenously expressed mechanosensitive channels, which hold the potential of contaminating current responses. So, I validated the Flp-In T-Rex 293 cells stably expressing flag-mPanx1 using electrophysiology, Western blotting, and immunofluorescence. I demonstrated that the currents recorded from these cells exhibit outward rectification with reversal potential close to -50, and they are inhibited by application of the Panx1 channel blocker CBX. Similar currents could not be recorded from uninduced cells. These three factors confirm that the currents recorded were mediated by Panx1 channels.

Since my project is focused on understanding the mechanisms pertaining to Panx1 mechanosensitivity, it was important to validate the mechanical response of Panx1 expressed in my model system. To elicit the mechanosensitive response, I used a basic method which is employed widely to study the mechanical stress i.e. to induce cell swelling or shrinking by exposing the cells to hypotonic or hypertonic conditions, respectively. Previous studies have already shown that hypotonic cell swelling or cellular membrane stretch leads to the augmentation of Panx1 channel activity [92], [96], [104], [105], and my results reflect the same. An important point to be noted here is that the uninduced control cells do not elicit any response to the application of hypotonic solution, confirming that these cells does not express any functional mechanosensitive channels endogenously. Also, in my experiments, I was careful not to alter the basic ionic (Na^+, Cl^-, and K^+) composition of the solutions. I achieved this by altering

the concentration of an inert osmolyte (mannitol). This approach avoids changes in driving force which itself would alter Panx1 current amplitude and thus confound interpretation of my results.

Although the hypotonic response of Panx1 has been described before, to my knowledge past studies have not examined the effects of hypertonic stress or cell shrinking on Panx1 channel activity. I found that exposing the cell to hyper-osmotic stress condition inhibits Panx1 channel-mediated currents. Thus, my results demonstrate that the response of Panx1 channels to osmotic stress is bi-directional. This means that the channel activity increases with decreasing osmolarity and the activity decreases with increasing osmolarity. This indicates that the channels exhibit a varying degree of activation based on the osmolarity of the immediate surrounding. This ability of Panx1 may highlight the importance of these channels as environmental, physical, or physiological mechanical stress sensors in cells. Furthermore, the fact that the Panx1 is responsive to relatively modest changes in osmolarity from a physiological setpoint of ~290 mmol/kg implies that Panx1 channels function as mechano-transducers of physiological and pathophysiological changes in osmolarity. Thus, Panx1 is an integral unit of the mechanotransduction machinery at cellular level, assisting in the sensing and transducing mechanical stress from the extracellular to the intracellular region.

4.3 Actin cytoskeleton regulates pannexin channel activity and mechanosensitivity

As stated earlier, I investigated the involvement of cytoskeletal elements in the mechanosensitive regulation of Panx1 channels. I used well established cytoskeleton stabilizing and depolymerizing drugs to strengthen or disrupt the network of actin or microtubules. The

objective here was to deprive the membrane-bound Panx1 of actin or tubulin. We know that actin interacts with Panx1, and if that interaction is essential for mechanosensitive responses of the channels, then it must be affected, at least, by depolymerization of actin filaments. It is evident from the voltage-clamp recordings obtained from cells pre-treated with actin/tubulin stabilizing and depolymerizing agents that actin is the major contributor to the Panx1 mechanosensitivity. Depolymerization of the actin filaments severely inhibited the augmentation of Panx1 channel activity during hypo-osmotic stress-induced cell swelling. On the contrary, microtubule disruption had minimal effect on Panx1 mechanosensitivity, suggesting that tubulin cytoskeleton may not play a role in the channel regulation. Furthermore, disruption of actin filaments also reduced basal channel function, suggesting that the actin network is not only essential for the mechanosensitivity of the Panx1 channels but also for the basal channel regulation. This is one of the major findings in this project and is consistent with the idea that Panx1 is tethered to the actin cytoskeleton.

In addition to the cytoskeleton depolymerizing agents, I also pre-treated the cells with cytoskeleton stabilizing agents. Stabilization of actin filaments by JSK pre-treatment resulted in the inhibition of basal channel activity, which was unexpected. It is possible that actin stabilization may have shifted the osmotic setpoint at which Panx1 conducts basal channel activity. Because of this shift in the osmolarity-dependent baseline activity, there may have been a shift of hypo-osmotic (potentiation) and hyper-osmotic (inhibition) setpoints and the corresponding current responses. Further experiments would be needed to confirm whether the setpoint for inhibition vs potentiation was altered by actin stabilizing agent. To address this, JSK pre-treated cells could be used to perform recordings over a range of varying osmolarity (340 to

250 mmol/kg). Actin stabilization reduced the basal channel activity observed at 310 mmol/kg. Therefore, it is possible to see a further inhibition with the osmolarity above 310 mmol/kg in the cells treated with JSK. Interestingly, the cells pretreated with tubulin stabilizer did not show a significant change in either the basal channel activity or the mechanosensitive response of the channels. This was consistent with the finding that Panx1 channel function was not affected by disruption of the tubulin network.

Additionally, voltage-clamp recordings performed using cells pre-treated with $[Ca^{2+}]_i$ chelators (EGTA, BAPTA) also reduced the mechanosensitive response of the channels when subjected to hypotonic cell swelling. This implicates that the mechanosensitive regulation of the Panx1 channels is Ca^{2+} dependent. One possibility is that $[Ca^{2+}]_i$ rises in response to hypo-osmotic stress induced cell swelling and thus triggers a post-translational modification of Panx1. For example, the activity of Src kinase, known to positively regulate Panx1 channel function, has previously been shown be augmented in response to osmotic stress [81], [130], [131], [136]. Another possibility is that chelating $[Ca^{2+}]_i$ alters actin dynamics by regulating the activity of a Ca^{2+}-dependent actin-binding protein, for example α-actinin. Collectively, my findings from experiments using actin stabilizer and $[Ca^{2+}]_i$ chelators indicate that the actin dynamics is an essential process required for the proper functioning of Panx1 channels and their response to hypo-osmotic stress.

Having established that F-actin plays a role in basal channel regulation and channel response to the hypo-osmotic stress, I next performed voltage-clamp recordings to determine the contribution of actin to the hyper-osmotic stress response of Panx1. I recorded from the cells pre-treated with

actin depolymerizing agent and then exposed to the hyper-osmotic stress. If F-actin contributes to the response of Panx1 to hyper-osmotic stress, then F-actin disruption should prevent Panx1 current inhibition during hyper-osmotic stress. This is precisely what I observed; the disruption of actin filaments by Cyt-D prevented the hyper-osmotic stress response of Panx1. Cells pre-treated with Cyt-D did not exhibit reduction in Panx1 currents in response to hyper-osmotic stress, unlike untreated cells. This demonstrates the loss of Panx1 mechanosensing due to actin depolymerization. This suggests that actin disruption prevents the inhibition of channel activity in response to hyper-osmotic stress, confirming that actin is essential for hypertonic response regulation of Panx1 channels.

My results strongly suggest that the interaction between F-actin and Panx1 is important for regulating Panx1 channel mechanosensitivity. Change in the osmolarity induces a corresponding change in the cortical actin structure, which in turn affects the Panx1 channel opening (activity) by tethering mechanisms as discussed in greater detail in the section to follow. Depolymerization of actin filaments disrupts the cortical F-actin network, which is now incapable of conducting and transmitting the mechanical forces exerted on the cell during osmotic stress. Although, depolymerization certainly does not deprive the Panx1 of actin availability. Perhaps actin is still tethered to the Panx1 channels in the monomeric form, but is unable to respond to the mechanical stress due to the lack of networking. Hence, Panx1 channels do not function normally in the absence of actin filaments.

4.4 Models of Actin-Pannexin interaction

In section 1.12, I proposed three models of actin-Panx1 interaction focused on different tethered mechanistic models (Fig. 1.2).

A) Model (i) or the Association Model assumes that under resting conditions, Panx1 does not interact with F-actin and therefore channel function is independent of F-actin. Once the cell is subjected to osmotic stress, actin associates with Panx1 to augment channel activity. However, this model is difficult to reconcile with (a) the inhibition of channel function seen during hyper-osmotic stress; and (b) the reduction in basal activity caused by the disruption of actin network.

B) Model (ii) or the Dissociation Model, conversely, assumes that the membrane-bound Panx1 channel is associated with actin cytoskeleton under resting condition, and actin regulates the channel activity at baseline. Once the cell is subjected to osmotic stress, the association is displaced resulting in channel activity augmentation. Much like Association Model, this model does not explain the channel activity inhibition seen during hyper-osmotic stress. Also, according to this model, dissociation of Panx1 from actin network stimulates augmentation of channel activity. However, disruption of actin network by Cyt-D treatment did not induce augmentation in channel activity. On the contrary, actin network disruption resulted in inhibition of basal channel activity and mechanosensitive response.

C) Model (iii) or the Alteration Model describes that Panx1 is tethered with F-actin network under resting condition and this interaction regulates basal channel activity. Once the cell is subjected to osmotic stress, the actin-Panx1 interaction pattern changes (actin still tethered with Panx1). Depending on the degree of osmotic stress the interaction pattern

changes. Hypo-osmotic stress results in the augmentation of channel activity and hyper-osmotic stress results in the inhibition of channel activity.

Based on my findings, model (iii) best suits the results among all the three proposed models. It explains the following observations satisfactorily-

(a) Co-precipitation of Panx1 with actin. According to the model, Panx1 is tethered to actin cytoskeleton under normal condition. This interaction regulates the basal activity of Panx1 channel. In the co-IP experiments, actin was co-precipitated with Panx1, and Panx1 was co-precipitated with actin. This shows that the two proteins interact under normal (isotonic) conditions.

(b) Bi-directional response to osmotic stress. The model explains that under regular condition actin-Panx1 interaction generates basal response of Panx1 channel activity. Osmotic stress (hypo- or hyper-osmotic) leads to the change in interaction pattern which results in the alteration in channel activity. Hence, hypo-osmotic stress results in the augmentation of Panx1 channel activity, whereas hyper-osmotic stress results in the inhibition of channel activity.

(c) Reduction in the basal channel activity due to disruption of actin network using Cyt-D. Since the actin-Panx1 interaction under normal condition regulates basal channel activity, disruption of actin filaments would certainly affect the resting state activity.

(d) Loss of mechanosensitivity due to F-actin disruption. Cyt-D treated cells did not exhibit an alteration in Panx1 channel activity in response to hypo-osmotic stress. According to the model, changes in the basal interaction of Panx1 with actin result in the hypo-osmotic or hyper-osmotic stress response. In Cyt-D treated cells there were no actin filaments in

the first place to interact with the channel. Hence, channel did not respond to the osmotic stress.

By default, Panx1 is tethered with actin filaments and constantly interacts with the network. Physical or mechanical changes exerted on the cell is sensed by the F-actin network and communicated to the Panx1 via that network. Any possible cell deformation, stretching, or shrinking modifies the cortical actin filament network. This change in the filament structure brings about a corresponding change of Panx1 channel activity. Figure 4.1 describes the interaction pattern of actin-Panx1 under normal and osmotic stress conditions. As stated earlier, under normal conditions, Panx1 is tethered to actin filaments at a certain position by its C-terminus. The interaction at this position confers the "basal" channel activity of Panx1. When the cell is exposed to the higher osmotic (hyper-osmotic) conditions, the actin filaments are re-structured at the cortex, which changes the actin-Panx1 CT interaction positions of each of the six subunits. This change in the interaction position leads to the lesser opening of the pore (a lower sub-conductance state or open probability), which results in the overall reduction of the collective channel activity in the cell. On the contrary, when the osmotic condition changes to that of a lower osmotic measure (hypo-osmotic stress) compared to the osmotic value of "normal/basal" condition, it leads to cell swelling. Cortical actin filaments are re-structured and are perhaps more stretched, leading to the change in actin-Panx1 CT interaction position. Because of the stretching of the filaments, actin-tethered Panx1 subunits are pulled along with the actin network. This leads to a larger opening of the pore (higher sub-conductance state), resulting in the collective augmentation of the Panx1 channel activity in the stress- subjected cell.

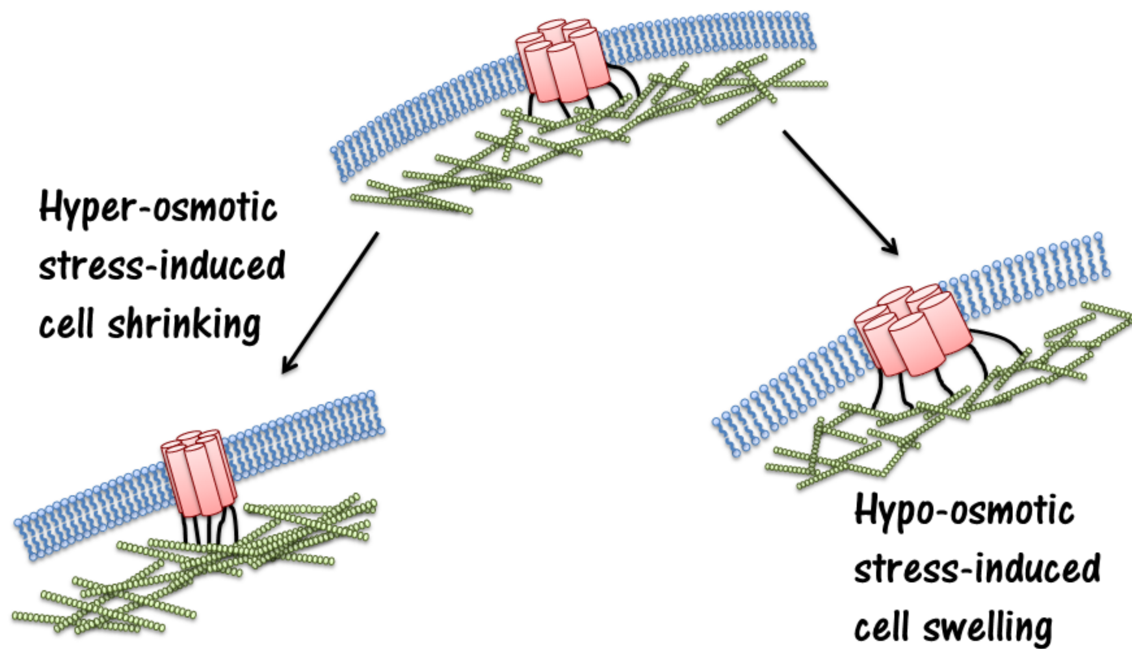

Figure 4.1 Illustration of the actin-pannexin interaction pattern with respect to the change in osmotic conditions

In other words, actin acts like a lever attached to Panx1 CT, which induces submaximal opening of the channels at rest according to the cortical tension exerted by the cell. This point can be articulated as the point no. 1 on a three-point lever scale (0, 1, 2) and the lever (actin) is at this point at rest (or under iso-osmotic condition). Under the hypo-osmotic stress, cortical tension is increased (lever is positioned at point no. 2) leading to the maximal opening of the channels. Whereas, under the hyper-osmotic stress the cortical tension is minimal (lever is positioned at point no. 0) resulting in lesser opening of the channels.

Although my proposed and selected model is emphasized solely on the tethered mechanism of the mechanosensitive channel regulation, the possibility of miniscule bilayer mechanism being at play cannot be completely discounted. Panx1 is membrane bound, and mechanical stress induces membrane-tension. It is possible for the tension to be transmitted to the channels, which may contribute to the channel regulation in response to the mechanical stress. This mechanism was suggested in an earlier study done by Bao *et al.* [92], in which the group claimed that Panx1 may directly respond to stretch through effects transmitted via the tension built-up in the lipid bilayer. This conclusion was based on the fact that they examined the effects of stretch by negative pressure applied to excised patches of membrane containing Paxn1 channels, which they assumed was free of actin cytoskeleton. However, the cortical actin network is tightly adjoined with the membrane and continues to function in the excised membrane patches as well, unless a measure is taken to specifically delaminate the cortical actin network [137], [138]. Conclusively, it is evident from the experiments I performed that F-actin and Panx1 interacts with each other via tethered mechanisms. Additionally, the cytoskeletal network of cortical actin filaments constantly regulates the channel activity with regards to the extracellular osmotic conditions, and transmits the mechanical force exerted upon cell to the membrane-bound Panx1 channels mediating the generation of a resulting channel response to the stress condition. A question to be resolved in future studies is - *what are the cellular consequences of altering the Panx1 channel activity during mechanical stress?* Panx1 constitutes an integral part of mechanotransduction machinery in a cell. Thus, it is anticipated that the augmentation or inhibition of channel activity may contribute to the stress-induced modification in cellular morphology. Change in Panx1 activity reflects a corresponding change in the permeability state of the channel. This stress-induced alteration in permeability state may result in induction of a certain cascade by Panx1-

mediated release of signaling molecules (eg. ATP). Mechanosensing attributes of Panx1 may also play a role in guiding the cell motility with respect to the extracellular physical environment (such as extracellular matrix).

4.5 Distal C-terminal region confers mechanosensitivity to Pannexin

As stated earlier, this project was focused on two objectives: (i) to investigate the involvement of cytoskeletal elements in the mechanosensitivity of Panx1 channels, (ii) to identify the minimal Panx1 domain responsible for the mechanosensitivity. Experiments from the first section evidently illustrated the regulation of Panx1 channels by actin filaments. One of the important findings from the experiments pertaining to the first section is that the actin-Panx1 interaction is essential not only for the stress response of the channels but also for the basal channel functioning. Now that I have tested the two cytoskeletal elements, and restricted the focus of my further study to actin, the next step was to identify the minimal mechanosensitive domain of the Panx1 protein. Since we already know that Panx1 interacts with actin via intracellular C-terminus, the next series of the experiments were focused on the Panx1 CT region.

The C-terminal region of Panx1 is cytoplasmic and spans from amino acid sequence 299 to 426. The strategy I pursued was to locate a region, within the span of these 128 amino acids, that is responsible for the channel regulation during mechanical stress. To achieve the minimal region responsible for mechanosensitivity, I employed voltage-clamp recordings on cells expressing truncation mutants of Panx1. The Jackson lab developed the following truncation mutant constructs of the Panx1: (1) WT / un-truncated, (2) 1 – 356, (3) 1 – 372, (4) 1 – 378, (5) 1 – 390, (6) 1 – 402, (7) 1 – 414, (8) 1 – 299 / entire C-terminal deletion, (9) 36 – 426 / entire N-terminal

deletion. HEK 293T cells were transiently transfected with truncation mutant or WT Panx1 using jetPRIME™. Expression of Panx1 WT or truncated forms was confirmed before employing the cells for electrophysiology. Preliminary immunofluorescence imaging revealed that the entire C- and entire N – terminal deletion mutants were not expressed on the cellular membrane, but rather remained restricted within the cytoplasm. Hence, they were not considered for further experiments or analysis.

Whole-cell voltage-clamp recordings revealed that truncation mutants exhibited a reduced mechanosensitive response to osmotic stress when compared to WT Panx1. Reduced mechanosensitivity was observed in all six C-terminal truncation mutant-transfected cells. This indicates that the region responsible for the Panx1 mechanosensitivity was absent in those truncations. The common region which was deleted in all truncations, is the 12 amino acid segment spanning from amino acids 415 to 426. This region is located at the most distal section of the C-terminus. Since all the truncations lack this region and they all show inhibited response to the hypo-osmotic stress-induced cell swelling, it is most likely that this 12-amino acid region is essential for the mechanosensitivity of Panx1. Nevertheless, it is possible that the entire region of 12-amino acids may not take part in the interaction governing the mechanically sensitive response of the channels. A few amino acids within that region could form the domain responsible for the mechanosensitivity of Panx1.

Consider the following evidence:

(i) actin interacts with Panx1 C-terminus [63];

(ii) actin regulates membrane trafficking of the channel [63];

(iii) actin regulates Panx1 channel functioning (present work);

(iv) distal C-terminal region of Panx1 is essential for the mechanical response of the channels (present work).

All the above separate findings sum up the mechanosensitive regulation of Panx1 channels. Although, previous studies have shown that actin interacts with the Panx1 CT, it was not known where this interaction takes place. Regarding this, the above-mentioned findings point out that actin-Panx1 interaction may take place at the distal C-terminal region of the channel, specifically within the span of terminal 12 amino acid sequence. However, currently we do not have evidence supporting if this interaction occurs via direct protein binding. The possibility of a mediator protein(s) will always exist until it is investigated further. In any case, it is likely that the distal C-terminal region is interacting with actin (directly or indirectly) and this interaction is an essential factor in the regulation of Panx1 channels, during normal and osmotic stress conditions.

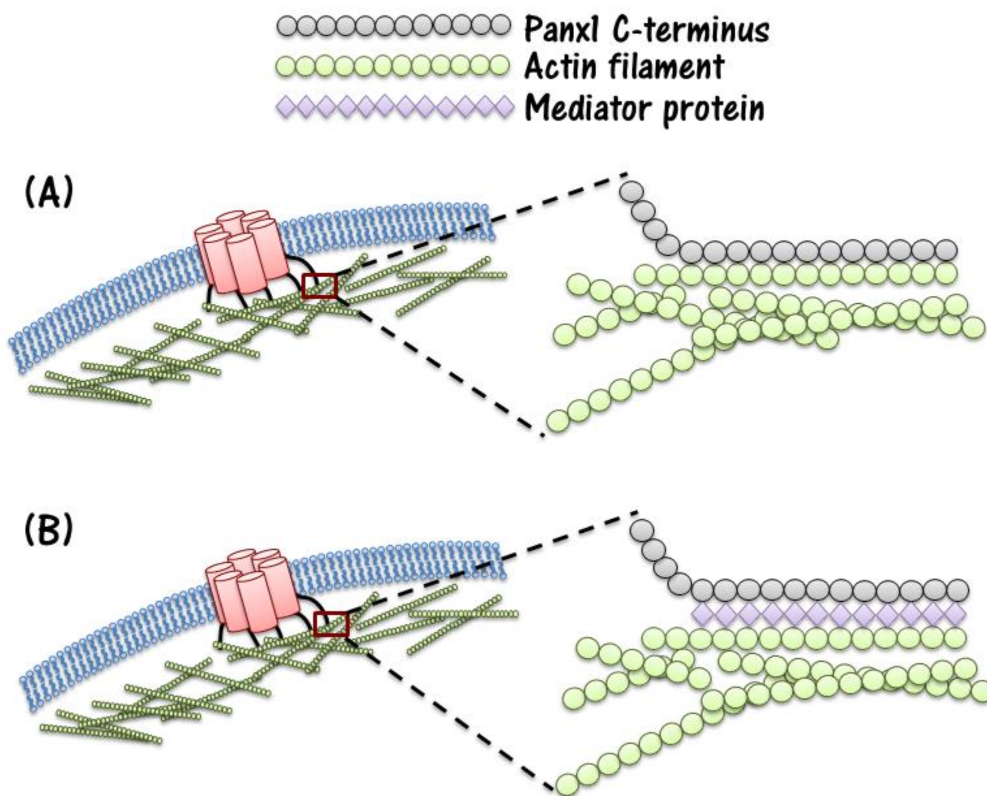

Figure 4.2 Depiction of proposed direct or indirect tethering of pannexin with actin

Figure 4.2 illustrates the potential direct and indirect interaction of Panx1 with actin. In fig. 4.2 (A) actin is tethered directly to the distal region of Panx1 CT. The interaction domain spans over the terminal 12 amino acids, or at least, is located within that region. Fig. 4.2 (B) showcases the involvement of a mediator protein for the interaction, illustrating the indirect tethering of Panx1 to actin. Nevertheless, the interaction region remains the same as in the direct tethering. However, the mediator protein(s) does not regulate the channel functioning by itself but merely facilitates the actin-Panx1 interaction. Actin related protein 3 (Arp3), a subunit of the actin-modifying Arp2/3 complex, is an essential component of the actin cytoskeleton [139]. Arp3 binds with actin and mediates the branching of actin filaments [124]. Recently, using immunoprecipitation along with liquid chromatography and mass spectrometry, Wicki-Stordeur

and Swayne found that Arp-3 interacts with Panx1 [123] . Additionally, they co-precipitated actin, Arp3, and Panx1 endogenously, suggesting that these interactions are default under regular conditions, and may serve as a factor in Panx1 regulation. This finding may imply that Arp3 could be a possible mediator protein mediating actin-Panx1 interaction, if the interaction is not direct.

4.6 Experiments for the future studies

From the experiments that I have performed in the present study, I have established that actin-Panx1 interaction follows a tethered model mechanism to regulate Panx1 mechanosensitivity. We now know that the membrane-bound Panx1 channels are tethered with actin cytoskeleton which regulates the functioning of the channel. However, we still do not know if the actin-Panx1 interaction is direct or indirect. To confirm the direct interaction, a follow-up experiment would be to perform a proximity ligation assay (PLA). In PLA, primary antibodies bind to the proteins of interest (actin and Panx1), and secondary antibodies binds to the respective primary antibodies. Oligonucleotides attached to secondary antibodies participate in rolling circle DNA synthesis only if the proteins are within 40 nm of each other. The newly synthesized DNA is detected by fluorescently labelled complimentary oligonucleotides, using confocal microscope. Fluorescence detection by microscope would confirm that actin and Panx1 are localized within 40 nm of each other, and most likely the interaction is direct. This would further clarify if they interact directly or through the employment of mediator protein(s). If the presence of a mediator protein is detected, then the protein should be scanned for the known actin binding proteins, and tested for its ability to directly interact with Panx1 using bioinformatics approach and co-

sedimentation assays. The idea is to narrow down the actin-Panx1 interaction to either direct binding or protein-mediated interaction.

Once it is established if the interaction is direct or indirect, then the next set of experiments would be to specifically identify the amino acid domain(s) responsible for actin or mediator protein interaction. The truncation experiments from the present study has ensured the presence of mechanosensitive domain within the distal C-terminal region of Panx1. Whether this distal region of 12-amino acid contains the actin-binding domain or the interaction sites for the mediator protein (which is linked with actin) is an area of further investigation. By looking carefully at the amino acid sequence of the distal 12 amino acids, we have noted the presence of a series of positively charged amino acids (lysine, arginine, histidine). These could form the basis of an interacting domain. To assess that, one strategy is to substitute the charged amino acids with neutral amino acids. Several point mutations within the distal region of Panx1 CT will be generated by replacing positively charged amino acids with neutral amino acids. Using these mutated Panx1, the minimal domain responsible for the mechanosensitive response and basal channel regulation would be identified. Co-sedimentation or co-precipitation experiments performed with cells expressing Panx1 having those specific point mutations will further shed light if the mutation leads to the disruption of actin-Panx1 interaction. If the results are negative (i.e. actin is not present in mutated-Panx1 pull down), it will undeniably establish that actin and Panx1 directly interacts, and the minimal binding domain would be known as well. This further study would not only characterize the mechanosensitivity of Panx1 channels thoroughly but also provide more information regarding the overall channel functioning.

The osmotic stress experiments mentioned earlier involved subjecting the cells to the stress condition for 5-10 min. It is rather uncertain how longer-term exposure to the osmotic stress would affect the channel functioning. The prolonged osmotic stress could (i) induce further potentiation of currents and increase surface expression of channels; or (ii) may lead to desensitization by internalization of the channels. To address this, mPanx1-GFP HEK 293 cells stably expressing GFP-tagged Panx1 will be voltage-clamped at -60 mV and exposed to the prolonged osmotic stress conditions (beyond 10 mins). Voltage-clamp recordings will be coupled with live cell imaging wherein the membrane-bound GFP-Panx1 will be tracked for (i) change in the surface expression level, and (ii) internalization of the channels because of longer-term exposure to the osmotic stress. The temporal analysis of the patch clamp recordings with the live cell imaging data will draw out a potential relation between the change in current activity and the surface expression of the channels due to the osmotic-stress. Also, the live cell imaging using confocal microscopy will be employed to monitor the changes in the actin cytoskeleton because of the augmented channel activity during hypo-osmotic stress. It is possible that stress-induced augmentation of Panx1 channel activity induces, mediates, or facilitates cortical actin cytoskeletal reorganization in time-dependent manner contributing to the cellular volume regulation. An extension of this study would be to expose the cells to hypo-osmotic or hyperosmotic stress for more than 60 mins. This would illustrate the long-term effects of osmotic stress on the Panx1 channel activity, surface distribution, and Panx1-mediated cellular responses. Panx1 is anticipated to take part in the apoptosis or necrosis induction. It is also possible that cells may downregulate surface expression of Panx1 to avoid detrimental effects.

The mechanistic information derived from the experiments performed and discussed above, is crucial in translating this study to investigate Panx1 mechanosensitivity in cells expressing Panx1 endogenously under conditions of mechanical stress. This includes for example neurons, astrocytes, airway epithelial cells, or retinal ganglionic cells. Once the minimal domain responsible for actin-mediated channel gating is known, then a peptide could be synthesized targeting that domain. This custom-synthesized interference peptide will be tested for its ability to inhibit the mechanical stress-induced response by supplementing it in a patch pipette for the patch clamp recordings. The peptide would diffuse from the pipette to the interior of the cell and displace the actin-Panx1 interaction, resulting in the inhibition of the osmotic stress-induced response of the Panx1 channels. If successful, the peptide could be used a potential pharmacological tool to specifically block the mechanical stress-induced channel functioning regulated by the actin-mediated interaction, while preserving other gating modalities. A similar approach has been successfully applied to disrupt the TRPV1-tubulin interaction, by supplementing the peptide within patch pipette during patch-clamp recordings [40].

4.7 Significance of the study

The significance of my study is accentuated by the scarce information currently available concerning mechanosensitivity of pannexin channels. Despite a substantial body of scientific literature implicating the role of Panx1 channels in numerous physiological functions, merely a handful of the investigations dealt with regulation of Panx1 channel functioning. Likewise, a considerable amount of work emphasized the mechanosensitive role of Panx1 in various diseases and physiological processes, but none scrutinized the mechanistic basis of Panx1 mechanosensitivity. My findings contribute to the Panx1 literature by providing mechanistic

information of channel regulation during mechanical stress. With this study, I have established that the cortical network of actin filaments contributes to the channel regulation with respect to the extracellular osmotic conditions. Also, for the first time, I demonstrated that the hyper-osmotic stress condition inhibits the Panx1 channel activity. Actin interacts with pannexin directly or indirectly, regulating the mechanosensitive response of the channels during osmotic-stress conditions. Additionally, my work confirms that the domain responsible for the mechanosensitivity in Panx1 is located within the distal region of the C-terminus, probably within the final 12-amino acid sequence.

The broader application of this work within scientific studies includes the development of targeted pharmacological inhibitory peptide quarrying the mechanosensitive responses of the pannexin channels, which could serve as a tool to further the advancement of our understanding in the regulation of the channel functioning in various detrimental pathophysiological processes. This could potentially lead to a therapeutic, specifically targeting the inhibition of the undesirable mechanosensitive augmentation of pannexin channels, which leads to the cellular death, without affecting the otherwise normally required functioning of the channels.

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APPENDIX: SUPPLEMENTARY FIGURES

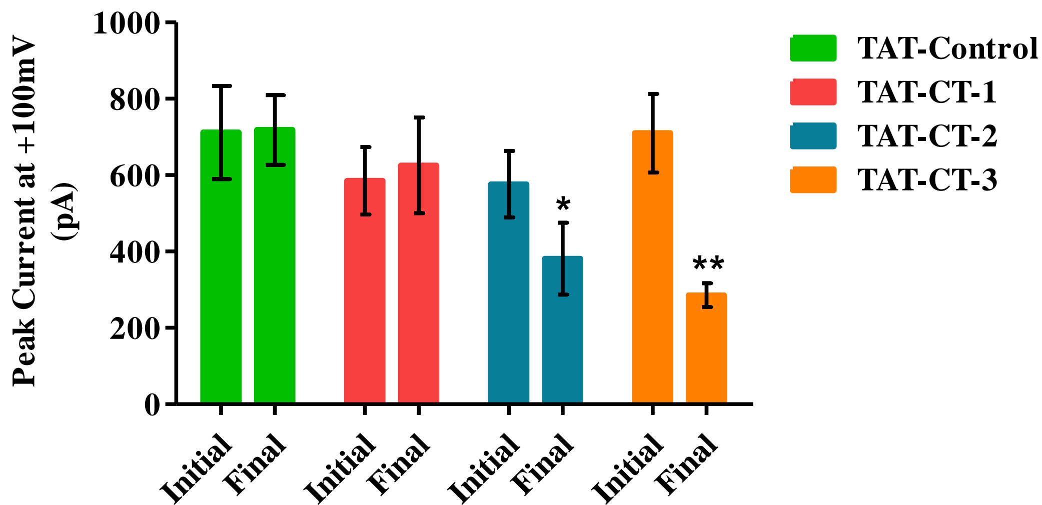

Figure S1. Unpublished lab data. TAT-conjugated synthetic peptide mimicking the regions of Panx1 CT were provided by Dr. Leigh-Anne Swayne (University of Victoria, BC): TAT-CT-1 (proximal CT; 53 aa), TAT-CT-2 (mid CT; 53 aa), TAT-CT-3 (distal CT; 61 aa). Whole-cell voltage-clamp recordings were performed with flag-mPanx1 T-Rex cells by supplementing the peptides in patch pipette, for 20 min duration. Figure shows the current amplitudes recorded for the initial 3 min and final 3 min of the recording duration. Data show that the cells diffused with TAT-CT-3 shows dramatic inhibition of Panx1-mediated current response. This suggests that the distal region of Panx1 CT is important for regulating channel activity.

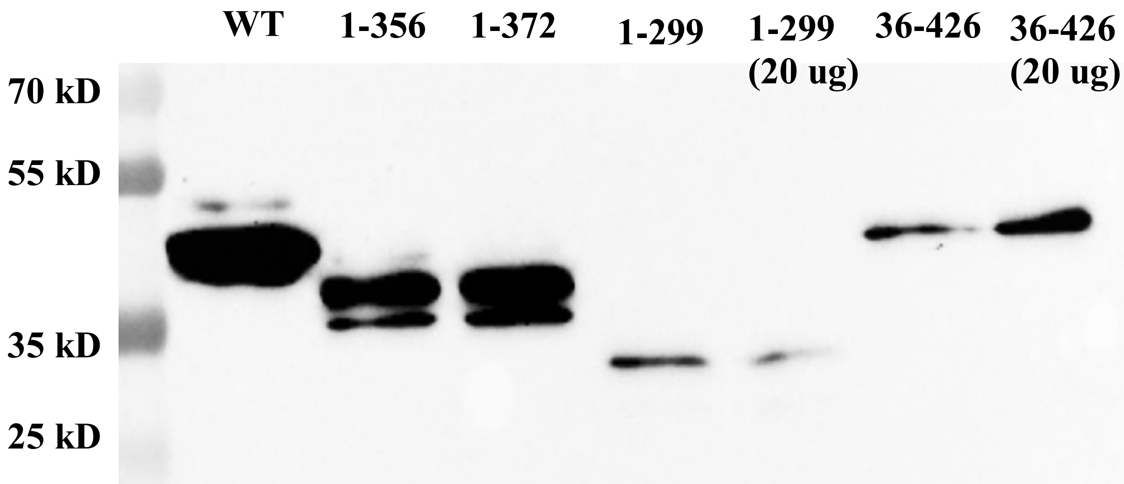

Figure S2. Unpublished data illustrating Western blotting results derived from the lysates collected from transfected HEK 293T cells. The blot image shows the absence of glycosylation presenting bands in case of 1-299 and 36-426 Panx1 truncations. This suggests that the full CT deletion (1-299) and full NT deletion (36-426) mutants were not expressed at the cell membrane.