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Localization and function of Pannexin2 isoform 202

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Abstract

Pannexins (PANX) are a family of membrane glycoproteins comprised of 3 members: pannexin 1 (PANX1), pannexin 2 (PANX2), and pannexin 3 (PANX3). Oligomerized pannexin proteins form channels that allow for the passage of ions and small molecules. While PANX1 and PANX3 mostly localize to the cell surface, PANX2 remains intracellular within the endoplasmic reticulum (ER). Recently, a splice variant of PANX2 termed Panx2-202, was detected during development of murine skin. However, the localization and function of this shorter Panx2-202 isoform remains unknown. We found that Panx2-202 localizes to the ER in immortalized rat epidermal keratinocytes. We also found that expression of Panx2-202 in keratinocytes regulates mitochondria morphology. Collectively, we then hypothesized that Panx2-202 may regulate mitochondria morphology at the ER-mitochondria interface. We found that Panx2-202 colocalizes with split-GFP-based contact site sensor for the ER-mitochondria membrane. Lastly, Panx2 is a known substrate of caspase-3/7 cleavage at two residues in the C-terminal tail. These residues are present in Panx2-202, we presumed that the isoform also undergoes caspase-3/7 cleavage. Thus, we developed a novel tool to study C-terminal cleavage in living cells. Upon induction of apoptosis, the C-terminal tail of the Panx2-202 fused to a GFP tag containing a nuclear localization sequence was released from the ER membrane then trafficked to the nucleus. Overall, we defined the localization of Panx2-202 to the ER and identified a potential role in the regulation of mitochondria.

Keywords

Pannexins, Pannexin 2, Pannexin 2-202, Pannexin 2-isoform 202, endoplasmic reticulum, mitochondria, endoplasmic reticulum-mitochondria contact sites, keratinocytes, caspase cleavage

Summary for Lay Audience

Pannexins are a family of membrane proteins consisting of three members: pannexin 1 (PANX1), pannexin 2 (PANX2), and pannexin 3 (PANX3). Pannexins proteins form channels responsible for intracellular communication through the passage of small ions and molecules. These unique channels are characterized to play important roles in skin development as well as in various pathological diseases including a variety of cancers and osteoarthritis. The Panx2 gene encodes two versions of the Panx2 protein or what are called splice variants (PANX2 isoform 1 and PANX2 isoform 202). To date, no literature is available on the function of the isoform 202, or where it localizes within a cell. However, a recent study found expression of the Panx2 isoform 202 in the skin of developing mice. Therefore, we sought to define the localization and function of Panx2 isoform 202 in skin cells (keratinocytes). Using fluorescent fusion proteins, we were able to determine that Panx2 isoform 202 localizes to the endoplasmic reticulum (ER), an organelle responsible for protein synthesis and calcium regulation. In addition, when Panx2 isoform 202 is expressed in keratinocytes, we see a change in the shape of mitochondria. Lastly, we have preliminary data that suggests Panx2 isoform 202 may play a role in programmed cell death of keratinocytes. Collectively, we have defined a localization of Panx2 isoform 202 to the ER where it regulates mitochondria shape and have new data that suggests a potential role of this isoform in programmed cell death in the skin.

Co-authorship Statement

The work and writing were performed and carried out by the author, under the supervision of Dr. Patrick Lajoie. Julie Genereaux assisted with the cloning of the various plasmids.

Dedication

In memory of my brilliant, courageous and inspiring Teta Louise.

And to my family, thank you for the constant love and support to help me reach my goals.

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List of Abbreviations

3D, three dimensional

aa, amino acids

ATP, adenosine triphosphate

BSA, bovine serum albumin

CBX, carbenoxolone

CCCP, cyanide m-chlorophenyl hydrazine

CNS, central nervous system

COX4, cytochrome oxidase 4

CPT, camptothecin

CT, C-terminus

dKO, double knockout

DLP1, dynamin-like protein 1

EGFP, enhanced green fluorescent protein

ER, endoplasmic reticulum

FBS, fetal bovine serum

FLIP, fluorescence loss in photobleaching

Gly1, high mannose glycosylation species

Gly2, complex glycosylation species

HA, hemagglutinin

HEK293T, human embryonic kidney cells

IL, intracellular loop

IP3R, 1,4,5-triphosphate receptor

kDa, kilodalton

MAMs, mitochondria associated membranes

Mff, mitochondrial fusion factor

Mfn1, mitofusin1

Mfn2, mitofusin2

NLS, nuclear localization signal

NPCs, neural stem and progenitor cells

NRK, normal rat kidney cells

NT, N-terminus

OMM, outer mitochondrial membrane

OPA1, optic atrophy1

Panx2-202, pannexin 2 isoform 202

PDI, protein disulfide isomerase

PTMs, post-translational modifications

REKwt, rat epidermal keratinocytes wild-type

REK-Panx2-KO, rat epidermal keratinocytes CRISPR-Cas9 knockout of Panx2

ROS, reactive oxygen species

RT, room temperature

SERCA, sarco/endoplasmic reticulum calcium ATPase

SC, stratum corneum

SRP, signal recognition peptide

STS, staurosporine

TM, transmembrane

UPR, unfolded protein response

UVA, ultraviolet A

UVB, ultraviolet

Chapter 1

1.0 Introduction

1.1 Overview of Pannexins

Pannexins are a family of tetra-spanning membrane glycoproteins: Pannexin 1 (PANX1), Pannexin 2 (protein name designated PANX2 in humans and Panx2 in mice) and Pannexin 3 (PANX3), discovered in 2000 due to their sequence similarity to the invertebrate gap junction proteins; innexins [1,2]. Pannexin proteins possess a membrane topology of four transmembrane domains (TM), two extracellular loops (EL), one intracellular loop (IL) and oriented in the membrane where the N- and C-termini (NT and CT respectively) are facing the cytosol (**Figure 1-1**) [3,4]. This membrane topology resembles that of connexins, a family of gap junction forming proteins responsible for intercellular communication [4]. However, unlike connexins, pannexins are unable to form gap junctions with adjacent cells to participate in intercellular communication [5,6]. Oligomerized pannexin proteins form large-pore channels that allow for the passage of ions and small molecules up to 1 kilodalton (kDa) in size [5,7–9]. PANX1 is the most characterized pannexin family member due to its ubiquitous expression across various cell types and tissues. PANX1 is known to play a role in a variety of functions including, but not limited to keratinocyte differentiation, immune response, tumorigenesis and metastasis [10–17]. During these processes, PANX1 channels are involved in regulating the transport of ions, metabolites or nucleotides dependent on cell type [7,9,18,19]. The functional formation of a PANX1 channel consists of seven PANX1 monomers to form a heptameric channel [20]. Through the secretory pathway, the PANX1 channel translocates to either the plasma membrane or the ER where it participates in cellular homeostasis. Pannexin trafficking will be explained later in this chapter. In contrast to PANX1, PANX3 expression is more restricted to the bone, skin, skeletal muscle and cartilage [21,22]. A few functions of PANX3 are described to be involved in both cell proliferation and differentiation [10]. PANX3 function is implicated in a more definite list of diseases including obesity, osteoarthritis, osteosarcoma, and non-melanoma skin cancers [23]. Participation in these processes, like PANX1, involve activation of the PANX3 channel through membrane depolarization and small molecule stimulation [21,22]. Although proposed functions of PANX3 channels exist, the stoichiometry of the PANX3 channel remains to be elucidated. Unlike PANX1 and PANX3, there is limited data available on PANX2's functions and relation to human diseases.

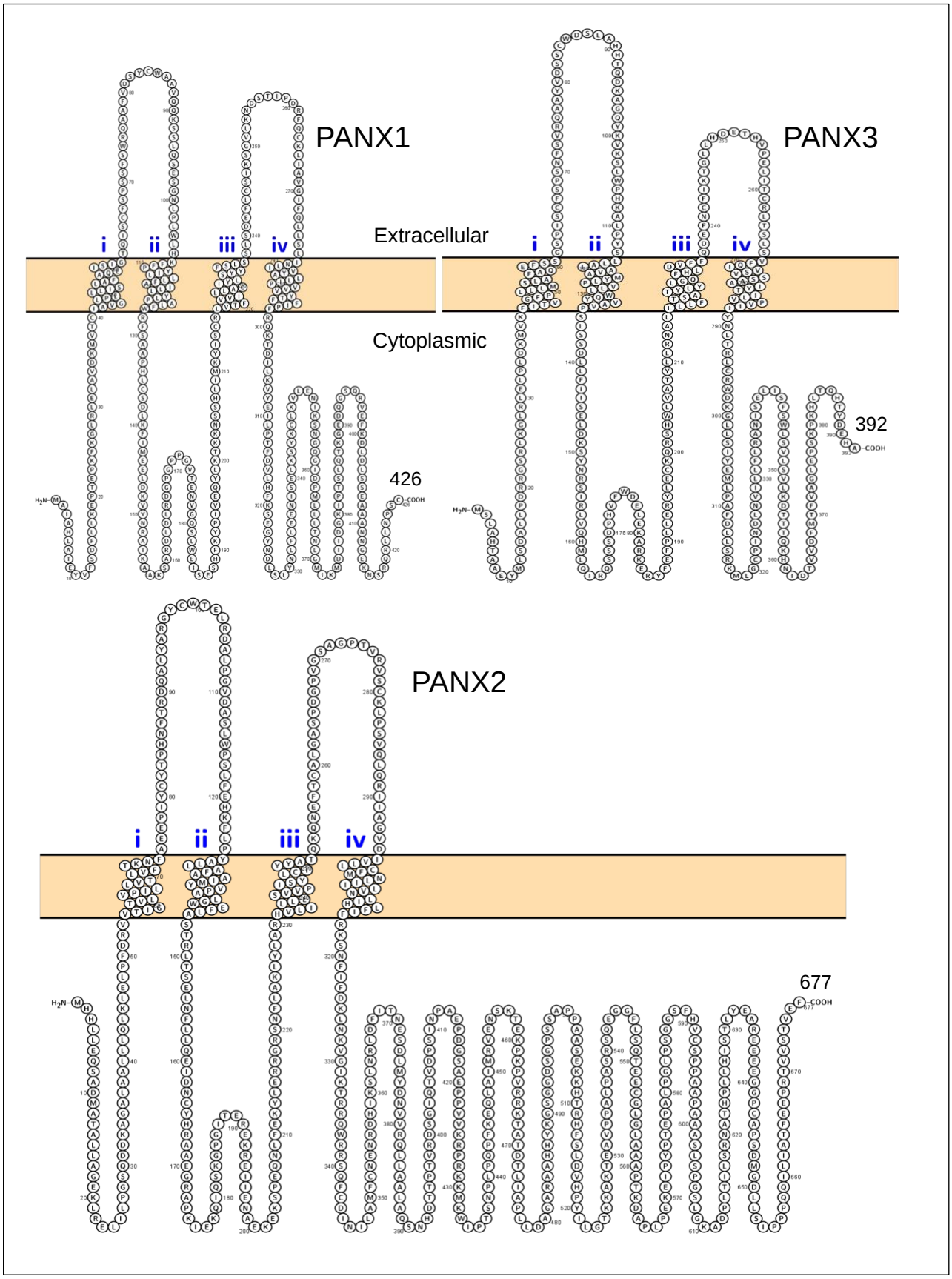

PANX1
PANX3
PANX2
Extracellular
Cytoplasmic
i
ii
iii
iv
426
392
677
COOH

Figure 1-1 Pannexin structure overview.

Pannexins are a family of three tetra-spanning membrane glycoproteins. They possess two extracellular loops (EL), one intracellular (IL), and N- and C-termini facing the cytosol. Panx2 is the largest of the three pannexins with a length of 677aa. Figure created with Protter[24] using the *Mus muculus* pannexin protein sequences.

1.2 Pannexin 2

Panx2 is the largest pannexin made of 677 amino acids (aa) with a total protein weight of ~75kDa (**Figure 1-1**) [2]. Notably, the C-terminal tail of PANX2 is longer than both PANX1 and PANX3 C-termini (**Figure 1-1)**. PANX2 is encoded by the *Panx2* gene located on chromosome 22 in humans and chromosome 15 in mouse, whereas both *Panx1* and *Panx3* are located on the same chromosome, chromosome 11 in humans and chromosome 9 in mouse. The amino acid sequence of the PANX2 protein diverges from PANX1 and PANX3 which are much more similar [2,25,26]. Early work in pannexin biology revealed that Panx2 transcripts were detectable at a greater level in the brain and spinal cord [26]. This expression was identified from *in situ* hybridization of both *Panx1* and *Panx2* in mice determining an overlap in mRNA expression of both pannexin transcripts in the cortex, olfactory bulb, hippocampus and cerebellum [26]. Initial studies investigating Panx2 gene expression, revealed a more restricted expression to the central nervous system [2]. Recent studies have identified a more ubiquitous expression of Panx2 to other tissues and cell types including the liver, skin, digestive tract and testis [27–33]. To date, the literature available on PANX2, examines the localization and function of the mRNA *PANX2alt1* (encoding a protein of 633aa in humans and 677aa in mice) although, there is a second isoform: *PANX2alt2* (encoding a protein of 509aa in humans and 651aa in mice) [2]. Panx2alt1 and Panx2alt2 will be referred to as Panx2 or the canonical sequence and Panx2-202 or Panx2 isoform 202 respectively in regard to the murine protein. Computational analysis of mouse Panx2 isoforms (Uniprot identifier: Q6IMP4-2) indicates the difference between mouse Panx2-202 to the canonical protein is an insertion of eight amino acids at position 75 (insertion sequence: "AARVSSLPS") and a shorter C-terminus with a change in sequence from position 632-677 ("SSSPPSRSREQL"). A significant finding by Baranova et al 2004 indicated that transcription of *PANX2alt2* may lead to a decrease in expression of the isoform *PANX2alt1*, which leaves the question of whether examining the function and localization of *PANX2alt2* is more physiologically and functionally relevant than investigation conducted with canonical *PANX2alt1*. The data available regarding PANX2 focuses on the canonical or isoform 1, although the splice variant: isoform 202 exists in humans and mice, there is no information on the function or localization of isoform 202. Thus, my graduate work focused on the localization and function of the uncharacterized mouse Panx2-202 isoform.

1.3 Panx2 interactions with Panx1

Gene expression of *Panx1* and *Panx2* are inversely regulated throughout rodent brain development with *Panx1* more present in neonatal tissues and *Panx2* more abundant in the adult tissues [34,35]. Although different at the sequence level from each other, one study co-expressed rat *Panx1* and *Panx2* RNA in *Xenopus oocytes* where functional membrane channels were formed that varied in voltage compared to channels composed of only Panx2 [36]. Biochemical evidence from co-transfections of Panx2-eGFP and Panx1-myc into human embryonic kidney 293 (HEK293T) cells provided further evidence of an interaction between Panx1 and Panx2 [36]. The heteromeric Panx1/Panx2 channels were found to not be gated by extracellular calcium, but were sensitive to pannexin channel blocker carbenoxolone (CBX)[36] . Penuela et al (2009) investigated the potential of Panx1/Panx2 heteromeric channel formation by co-expressing Panx1 and Panx2 in HEK293T cells and observed a reduction in dye uptake, a physical interaction between the two pannexins, and an increased localization of Panx2 at the cell surface [25]. It remains unclear whether PANX2 is able to form a functional homomeric channel. It was first observed that rat Panx2 forms octameric channels in *Xenopus oocytes*, but the channels were non-responsive to extracellular potassium or PANX1 channel blockers CBX or probenecid [37]. Unlike PANX1[20], the exact structure of PANX2 channels is suggested to form octameric or heptameric channels. Until the 3D structure of PANX2 channels can be generated, the composition of a single PANX2 channel remains unknown.

1.4 Pannexin post-translational modifications

Following protein translation some proteins may undergo post-translational modifications (PTMs) which alter the properties of the protein through the chemical addition of a methyl, glycosyl or phosphoryl groups or by proteolytic cleavage [38] onto one or more amino acid(s). PTMs are essential in biological processes which aid in protein structure, protein lifespan, cellular trafficking, protein folding and protein-protein interactions. Pannexins are substrates for several PTMs, including but not limited to N-linked glycosylation, phosphorylation, ubiquitination and caspase cleavage [25,39–43]. As my project focuses on PANX2, I will discuss predicted and identified PTMs relevant to PANX2 including S-palmitoylation, caspase cleavage and N-glycosylation [40,44].

1.4.1 S-palmitoylation

One of the first identified PTMs on Panx2 via in silico analysis was S-palmitoylation at predicted site C246 [41]. Palmitoylation is the covalent attachment of lipids to proteins. Swayne et al 2010 utilized biochemical assays in neural stem and progenitor cells (NPCs) to identify a reduced intensity in the palmitoylated species of Panx2 (~85kDa) when treated with hydroxylamine there was a greater intensity in un-palmitoylated Panx2 species (~60kDa) [41]. Interestingly, the same study suggested that two Panx2 species found in NPCs have a different subcellular localization compared to terminally differentiated neurons. This suggests a role for the different PANX2 species throughout neuron differentiation.

1.4.2 Caspase cleavage

Proteolytic cleavage is another method of PTMs associated with the initiation of apoptosis or pyroptosis. Caspases are a family of a dozen cysteine proteases that cleave the C-terminal end of an aspartate residue (Asp; D) [45]. Specifically, caspase-3 and -7 are classified as executioner caspases as activation of these caspases initiate apoptosis [46,47]. Caspase-3/7 recognize the motif Asp-Glu-Val-Asp (D-E-V-D) where proteolytic cleavage occurs following the C-terminal end of the first Asp residue[48]. Hundreds of proteins are substrates to proteolytic cleavage by caspases, including PANX1 and PANX2 but not PANX3 [40]. Using Jurkat immune cells as a model, through the induction of apoptosis, PANX1 channel was activated via caspase cleavage in the C-terminal tail which initiated the release of ATP and UTP, two substrates classified as “find-me” signals for phagocytes during apoptosis [9]. As PANX1 is found in this plasma membrane, this was a new implication of how pannexins participate in apoptosis through communication with the extracellular environment. This finding led to the investigation into how exactly caspase cleavage mediates PANX1 channel activation. In addition, this study revealed that the C-terminal tail of PANX1 acted as a channel blocker, upon proteolytic cleavage of the C-terminal tail at the caspase recognition site, it was sufficient to activate the channel without the presence of apoptotic conditions [39]. This form of channel inhibition is referred to as a “ball-and-chain” mechanism, commonly associated with voltage-gated channels [39]. Furthermore, Panx2 was first identified as a possible substrate for caspase -3/7 cleavage, due to a shift in band migration to a detectable band the size of the cleaved C-terminal tail, although the exact location of the cleavage site had yet to

be confirmed [25]. Recently, the caspase -3/7 cleavage sites on Panx2 isoform 1 were validated using mutagenesis and *in vitro* caspase 3 assays at residues D400 and D416 within the C-terminal tail [40,49]. Sanchez-Pupo et al 2022 also identified that upon induction of apoptosis via UVB irradiation, Panx2 was cleaved primarily at D416 when overexpressed in rat epidermal keratinocytes (REK)-Panx2-KO cells (REK-Panx2-KO) [49]. In addition, it was prevalent that with both genomic ablation of Panx2 in REK cells and overexpressing Panx2 in REK-Panx2-KO cells promotes induction of apoptosis, independent of caspase cleavage.

1.4.3 N-linked glycosylation

N-linked glycosylation is a reversible PTM occurring on all three pannexins that is catalyzed by the addition of one or more glycosyl group(s) on an asparagine (Asn; N) residue [5,43,50]. Glycosylation can occur on a variety of amino acids, but specifically N-linked glycosylation occurs on pannexin proteins. N-linked glycosylation reactions occur within the endoplasmic reticulum (ER) lumen with potential modifications continuing in the Golgi apparatus. Panx2 is found at a high mannose N-glycosylation (termed Gly1) where Panx1 and Panx3 exist as un-glycosylated (Gly0), high mannose, and complex glycosylated (Gly2) species [25,36]. The glycosylation process of Panx1 and Panx3 begin within the ER lumen and continues into the Golgi apparatus then transported to the plasma membrane via Sar1-dependent COPII vesicles [51,52]. Although there is no evidence whether Panx2 Gly 1 does the same. The variation in glycosylated states between Panx2 versus Panx1 and 3 support the narrative where PANX2 behaves in a different manner than its family members. Recently, the predicted asparagine (Asn; N) residue at position 86 (N86) was confirmed to be the only glycosylated site within Panx2's protein sequence [43]. Using mutagenesis, the N residue was substituted with a glutamine (Q) residue to develop the construct Panx2-N86Q to confirm if this residue was the location of N-glycosylation. Not only was the N-glycosylation site confirmed in Panx2, in that study the glycosylation site was found to be essential for subcellular localization as the N86Q mutant was unable to correctly target to the ER membrane whereas the wildtype Panx2 trafficked to the ER and Golgi apparatus but was not required for interactions with Panx1. As N-glycosylation occurs in the ER lumen that modulates protein trafficking, some authors believe that PANX2 existing in a Gly1 state explains the reason for its predominantly intracellular localization [27,37].

1.5 Subcellular localization of pannexins

1.5.1 Structure and function of the ER

The ER is the largest membrane-bound organelle that spans the cytoplasm in eukaryotic cells. The ER is a dynamic organelle that plays a pivotal role in a variety of cellular functions including lipid metabolism, calcium storage, protein synthesis and protein quality control of secretory proteins[53]. The first step in secretory protein biogenesis is initiated by cytosolic ribosomes, once a hydrophobic peptide sequence emerges it can then be recognized by a signal recognition particle (SRP) that redirects the protein to the ER. The cytosolic ribosome then binds to the ER membrane, translation continues, and the newly synthesized protein enters the ER lumen co-translationally. Once the polypeptide enters the ER lumen it can be subjected to PTMs, one example is N-linked glycosylation that occurs on all pannexins. N-glycosylation is the addition of an oligosaccharide group to an Asn residue at the prerequisite recognition motif Asn-X-Ser/Thr. This reaction occurs in tandem with protein translocation into the ER lumen and is catalyzed by oligosaccharide transferase enzyme [54]. The exact role(s) of N-linked glycosylation is not well understood, but it is suggested that the N-linked glycan provides thermodynamic stability and is an integral part of folding and sorting of secretory proteins [55]. In respect to pannexins, the degree of N-glycosylation represented by a shift in molecular weight, dictates whether the pannexin will continue sorting to the Golgi apparatus through Sar1-COPII dependent pathway [52,56]. COPII vesicles are transport vesicles which bud off from the ER, translocate through the cytoplasm to the Golgi apparatus. The Golgi apparatus serves as a second destination for modifications of proteins bound for the plasma membrane. Most common reactions within the Golgi are the addition of carbohydrates or oligosaccharides. As mentioned previously, Panx1 and Panx3 exist in complex glycosylated states where Panx2 is present in a high-mannose state. This differing N-glycosylation states of pannexins dictates whether their localization remains intracellular or if the protein can traffic to the cell surface.

In addition to protein folding and sorting, the ER plays a vital role in cellular homeostasis related to calcium storage and signaling. The ER must maintain a high concentration of calcium as chaperones responsible for efficient protein folding and maturation require calcium as a cofactor to operate[57]. To actively maintain this optimal level of calcium, the ER membrane contains

sarco/endoplasmic-reticulum Ca2+ ATPase (SERCA) proteins responsible for the transport of calcium from the cytosol to the ER lumen which metabolize ATP in the process. Calcium is also released from the ER in a passive manner, although this mechanism is poorly understood, many proteins have been characterized to mediate this activity [58]. Through inhibition of known calcium channels in the ER, PANX1 was identified as a calcium leak channel in overexpressing LNCaP cells [59]. Overexpression of PANX3 in C2C12 cells revealed localization to the plasma membrane and the ER, where it too functioned as an ER calcium leak channel that promoted osteoblast differentiation [21]. Recently, Panx2 has been characterized to localize to the ER yet its function within the ER remains to be elucidated [43,60].

1.5.2 Overview of Pannexin localizations

The function of a protein is reliant on its subcellular localization, therefore discovering the localizations of proteins is key to uncover their role in cellular homeostasis. PANX1 and PANX3 are both characterized to localize to the plasma membrane and the ER. The function of PANX1 at the plasma membrane involves mediating ATP release, whereas at the ER it functions as a calcium leak channel [9,25,59]. Similarly to PANX1, PANX3 also functions as a calcium leak channel at the ER, and mediates ATP release into the extracellular environment [21]. On the contrary, PANX2 has conflicting evidence on intracellular localizations dependent on the study and cell type (**Figure 1-2; Table 1-1**).

1.5.3 Intracellular localizations of Panx2

Most studies available which assess the localization of PANX2, suggest a more intracellular localization, although in some overexpression systems PANX2 can be found at the cell surface. Initial studies illustrated Panx2 ectopically expressed in normal rat kidney (NRK) epithelial cells possessed a more intracellular localization compared to paralogs Panx1 and Panx3 localizing to the cell surface [25]. Although, when Panx1 and Panx2 were co-expressed in NRK, untagged Panx2 was detectable at the plasma membrane whereas there was no change in localization during co-expression of Panx2 and Panx3 [25]. Another group also confirmed that Panx2 tagged with enhanced green fluorescent protein (eGFP) localizes to an intracellular compartment when overexpressed in rat C6 glioma cells, although the exact intracellular location was not defined [61]. In order to investigate where Panx2 was localizing intracellularly, another group generated stable-cell lines

with HeLa cells containing untagged or human influenza hemagglutinin (HA)-tagged rat Panx2 constructs [62]. The data suggested Panx2 colocalizes with early or recycling endosomes *in vitro* and that protein trafficking of Panx2 differs from its paralogs [62]. In addition to this study, another group reported that in a neuroblastoma cell line, Panx2-eGFP construct colocalized with mannose-6-phosphate receptor found in endosomal compartments [63].

1.5.4 Pannexin 2 at the ER and ER-mitochondria contact sites

Two groups separately have localized Panx2 to the ER. Sanchez-Pupo et al (2018) have shown that ectopically expressing an untagged Panx2 localizes primarily to markers of the ER and cis-Golgi matrix, protein disulfide isomerase (PDI) and GM-130 respectively [43]. In addition to their findings, they found no significant colocalization with late endosomes or mitochondria. This study found that the localization of Panx2 to the ER was reliant on the glycosylation site N86. When this site was mutated to a glutamine (Q), targeting was disrupted, and aggregation occurred with the N86Q mutant which was not seen with the wildtype Panx2. Although aggregation of the Panx2-N86Q mutant occurred, there was still partial colocalization with the ER and cis-Golgi matrix. It is important to note that these experiments were conducted in normal rat kidney (NRK) cells that have low expression of Panx1. Even though Panx2 has a more predominant intracellular localization, when ectopically expressed in a cell line with high Panx1 expression, there is a small pool of Panx2 capable of trafficking to the cell surface which was identified by cell surface biotinylation assays and immunofluorescence microscopy. Therefore, Panx2 is shown to primarily localize to the ER [43]. More recently, Le Vasseur et al (2019) confirmed previous findings where Panx2 colocalized to the ER but their findings further claimed that Panx2 localizes at ER-mitochondria membrane contact sites. This study developed a stably expressing glioma cell line overexpressing Panx2 tagged with eGFP as well as mouse brain sections for endogenous Panx2 expression to conduct their colocalization studies [60]. The conflicting evidence assessing the localization of PANX2 may reflect the different DNA constructs, antibodies and cell lines employed in those studies (Table 1-1).

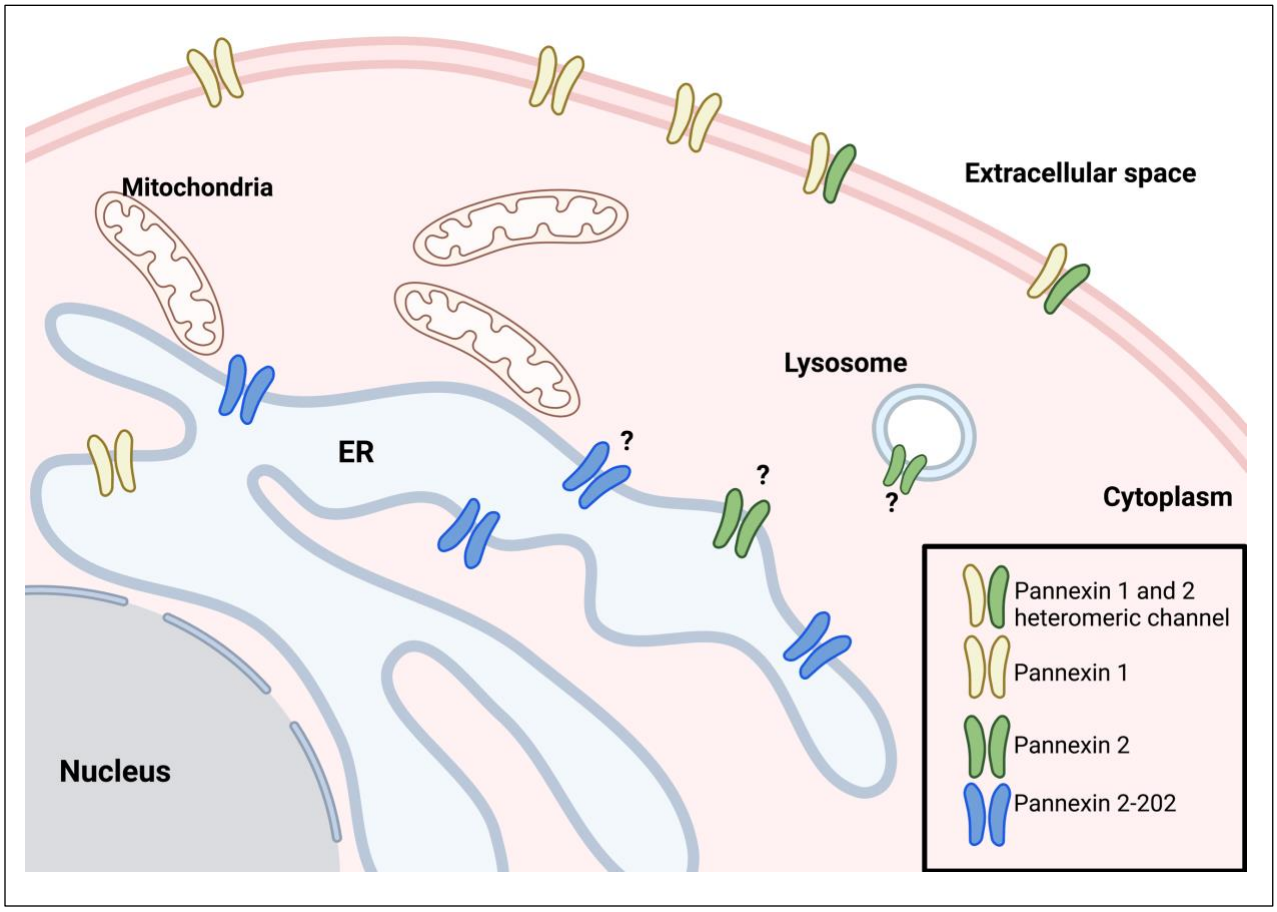

Mitochondria
Extracellular space
Lysosome
ER
?
?
?
Cytoplasm
Nucleus
Pannexin 1 and 2 heteromeric channel
Pannexin 1
Pannexin 2
Pannexin 2-202

Figure 1-2 Localizations of pannexin 2.

Subcellular localizations of PANX1 and PANX2. PANX1 primarily localizes to the plasma membrane but is also found to function as a calcium leak channel at the endoplasmic reticulum (ER). PANX2 is characterized to form heteromeric channels with PANX1 that localize to the plasma membrane. PANX2 channels are characterized to localize to lysosomal compartments, the ER, and ER-mitochondria contact sites. Yet, its function at these subcellular localizations remains to be elucidated. Figure created with BioRender.com.

Table 1-1 List of characterized localizations of pannexin 2.

Paper	Cell line	Fluorescent protein or epitope tag?	Localization
Penuela et al 2009	NRK and HEK293T	untagged mouse Panx2	Intracellular but cell surface when co-expressed with Panx1
Lai et al 2009	Rat C6 glioma cells	Stable Panx2-HA and transient Panx2-EGFP expression	Suggests endosomal intracellular localization
Wicki-Stordeur et al 2013	N2a	mouse Panx2-EGFP and endogenous detection with Aviva antibody	mannose-6-phosphate receptor (endosomal)
Boassa et al 2014	HeLa, HEK293T and MDCK	Rat Panx2 with HA or 4Cys epitope tag or untagged Panx2	Early or recycling endosomes
Sanchez-Pupo et al 2018	AD293 and NRK	Ectopic expression of Panx2	ER and cis-Golgi apparatus
Le Vasseur et al 2019	Rat C6 glioma cells	Stable cell line over expressing Panx2-eGFP	ER-mitochondria contact sites

1.5.5 ER-mitochondria contact sites

Direct communication between the ER and mitochondria is facilitated through the physical contact of their membranes forming structural domains or mitochondria-associated membranes (MAMs) [64]. Crosstalk between the ER and mitochondria regulate lipid homeostasis, transport of calcium and reactive oxygen species (ROS) and contribute to apoptotic signaling [65–68]. One of the first characterized ER-mitochondria contact sites was via the transfer of phospholipids between the two organelles [69]. Calcium in the mitochondria is crucial for metabolism and adenosine triphosphate (ATP) production, as well as critical for proper protein folding in the ER [70]. Calcium diffusion in the mitochondria occurs across the outer mitochondrial membrane (OMM) by the voltage-dependent anion channel (VDAC) driven by an electrochemical gradient of the inner mitochondrial membrane (IMM)[71]. This calcium uptake at the mitochondria happens to occur juxtaposed to the site of calcium release from the ER. The multi-protein complex involved in calcium transfer from the mitochondria to the ER includes the inositol-1,4,5-trisphosphate receptor (IP3R) at the ER membrane and the VDAC in the OMM [72]. As mentioned earlier, there are other characterized proteins that function as a calcium leak channel at the ER, leaving the possibility that calcium uptake can occur from other membrane protein complexes. For example, both PANX1 and PANX3 act as calcium leak channels at the ER, and the localization of Panx2 to the ER suggests the possible function to be a calcium leak channel [21,59]. Note that PANX1 and PANX3 are more similar in amino acid sequence than PANX2 is to either pannexin, so whether it possesses similar functions remains unclear.

ER-mitochondria contact sites are integral in calcium and lipid homeostasis, in addition these membrane contact sites are also fundamental players in initiation of apoptosis signaling [68,73]. An example where these two organelles would communicate to induce apoptosis would be upon ER stress via overload of calcium in the ER [73]. This would cause a signaling cascade where the mitochondria would then uptake more calcium than normal and would then release caspase cofactors to induce cell death. Another instance of contact sites and their role in apoptosis is through autophagosome formation [74]. Autophagy is a regulated cellular recycling system that removes unnecessary components from the cell. ER-mitochondria contact sites are characterized to facilitate calcium exchange that initiates autophagosome formation [74,75]. In addition to autophagy, ER-mitochondria contact sites are suggested to play a role in mitophagy- which is the

removal of dysfunctional mitochondria through the autophagy pathway[74]. ER-mitochondria contact sites have been characterized as hubs for recruitment of mitochondrial fission and fusion machinery [76,77]. Therefore, the possibility of PANX2 to be present at these membrane contact sites could suggest a potential role for either lipid or calcium homeostasis as well as a role in autophagy.

1.6 Pannexin 2 in disease

Early work investigating the function and expression of PANX2, revealed that Panx2 transcripts were detectable at a greater level in the brain and spinal cord [26]. This led to further assessment of endogenous PANX2 expression and its role in neurogenesis using primary NPCs, where endogenous PANX2 expression in NPCs exists as a S-palmitoylated species, the first evidence of a cell-specific post translational modification [41]. With promising findings of Panx2 transcripts in the brain and PANX2 levels *in vitro*, the functional role of PANX2 channels *in vivo* was not clear. The development of a Panx2-knock out (KO) mouse model was used to assess the contribution of Panx2 in ischemic brain damage[18]. In this stroke model, the Panx2-KO ($Panx2^{-/-}$) mice had a reduction in infarct size, which was further reduced in Panx1 and Panx2 ($Panx1^{-/-}$ and $Panx2^{-/-}$) double knock-out mice suggesting a compensatory role of the channels during neurodegeneration [78,79]. Early pannexin literature focused on PANX2 being solely localized to the central nervous system (CNS), which was contradicted in Le Vasseur et al (2014) was the first group to detect some Panx2 protein levels in murine skin, skeletal muscle, kidney, eye and colon relative to levels in the spinal cord [27]. The presence of PANX2 in regions outside of the CNS, suggests a more global role in a variety of cellular functions.

1.6.1 Cancer and apoptosis

Unlike PANX1 and PANX3, there is little evidence available to define the role of PANX2 in disease. PANX1 is characterized to have a critical role in a variety of cancers acting as both a tumor suppressor[12,80,81] and promoter[82]. In available literature, PANX2 has been characterized to be both pro-apoptotic and anti-apoptotic. Using C6 glioma cells stably expressing Panx2 reduced tumour growth and oncogenicity *in vitro* and *in vivo* [61]. In addition to the anti-apoptotic role of PANX2, another group revealed that pancreatic β-cells lacking PANX2 were sensitized to cytokine-induced apoptosis *in vitro* [29]. The novel localization of Panx2 at the ER-mitochondria contact sites, supports the pro-apoptotic role as ER-mitochondria contact sites are well

characterized signaling centers that can modulate cell survival, metabolism and response to cellular stressors [60]. This current study also identified that ectopic over-expression of Panx2 in C6 glioma cells sensitized the cells to staurosporine (STS)-induced apoptosis [60]. In a more pro-apoptotic role of PANX2, another group identified a link between upregulated PANX2 and a malignant phenotype in prostate cancer cell lines, PC3 DU145 and LNCaP [32]. The authors found that by silencing the expression of PANX2 in prostate cancer cell lines, there was a decrease in migration and invasion abilities yet an accumulation of intracellular ferrous iron (Fe^{2+}), an indicator of ferroptosis initiation [32]. More recently, Sanchez-Pupo et al 2022 sought to investigate the role of canonical Panx2 during UVB-induced apoptosis of keratinocytes. To study the role of Panx2 during cell death in keratinocytes, this study utilized CRISPR/Cas9 to remove endogenous Panx2 in rat epidermal keratinocytes (REK) to yield REK-Panx2-KO cell line [49]. The genetic deletion of Panx2 delayed but did not prevent apoptosis in UVB-damaged cells [49]. Overall, the studies combined suggest a role of PANX2 in apoptosis, or types of apoptosis that is dependent on cell type.

1.7 Pannexins in the skin

1.7.1 General overview of the skin

The skin is the largest organ and comprises ~15% of total adult body weight and with various functions [83]. The skin acts as a barrier from biological pathogens and harmful chemical and UVB radiation [84]. In addition to protective functions, the skin prevents water loss, aids in the body in thermoregulation and participates in vital metabolic processes including vitamin D production [85]. The skin is composed of three layers: (1) epidermis, (2) dermis and (3) subcutaneous layer [86]. The outermost layer, the epidermis, consists of several layers or strata starting with the basal layer (the stratum basale) to the spinous and granular layers, to the outermost layer the stratum corneum (Figure 1-3) [87]. The predominant cell type of the epidermis are keratinocytes [88], that are functionally different depending on the layer of the epidermis [89]. The outermost layer of the epidermis, the stratum corneum (SC) serves as the physical barrier to microbes and chemical irritants and is composed of terminally differentiated, protein-enriched keratinocytes: corneocytes [88]. In order to maintain a healthy epidermis, there is a balance between proliferation and desquamation resulting in complete regeneration every 28 days [90]. Calcium is a key regulator in

the proliferation and differentiation of keratinocytes that contributes to a healthy epidermis [89]. There is a steep calcium gradient in a healthy epidermis with the highest concentration in the stratum granulosum [89]. Keratinocyte differentiation begins as cells move from the stratum basale to the stratum spinosum where keratins K1 and K10, and involucrin are expressed that help redevelop the epidermis [89]. Dysregulation in proliferation and desquamation processes are common characteristics of skin disorders.

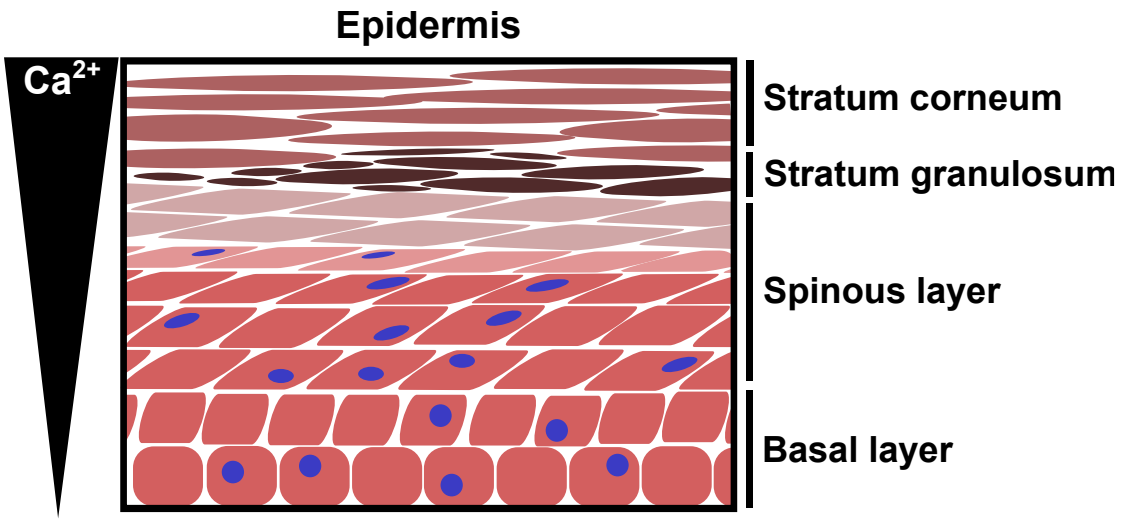

Epidermis
Ca2+
Stratum corneum
Stratum granulosum
Spinous layer
Basal layer

Figure 1-3 Overview of epidermis structure.

The epidermis consists of three main layers: basal layer, spinous layer and stratum corneum all composed of keratinocytes at different phases of differentiation. The stratum granulosum is a thin layer in the epidermis that lies above the stratum spinous and below the stratum corneum. Keratinocytes within the stratum granulosum are known as granular cells. Mammalian epidermis possesses a characteristic calcium gradient, the lowest levels in the stratum basale and the highest in the stratum granulosum and stratum corneum layers. Illustration created with Inkscape adapted from [Visscher & Narendran 2014][91].

1.7.2 Mitochondria in skin function

The skin is a continuously regenerating organ. To maintain healthy cell turnover, skin progenitor cells require a sufficient amount of energy in the form of ATP sourced primarily from mitochondria. Mitochondria produce ATP through a process known as oxidative phosphorylation, a by-product of this process are ROS that are known players in pathologies such as aging and cancers [92]. ROS levels can have pathogenic consequences but, in the skin, they also play a role in differentiation of epithelial stem cells through activation of Notch and β-catenin signaling cascades [93]. One group identified mitochondrial transcription factor A-KO mice had higher neonatal mortality rate due to impaired epidermal barrier, supporting the notion that proper mitochondrial function is a critical component of a healthy epidermis [94]. Aside from regeneration of the epidermis, mitochondria also play a key role in skin aging. The skin undergoes expected natural aging and is highly susceptible to photo-aging from ultraviolet-(UV)A and UVB radiation [95]. With natural aging, there is a decline in mitochondrial function, increase in ROS production, altered mitochondrial membrane potential [96] in turn, an increase in mitophagy[97] and apoptosis[98]. Chronic exposure to UV radiation can result in nuclear and mitochondrial DNA damage, oxidative stress and lead to skin cancer. UVA rays can penetrate deep into the dermis while UVB primarily affects epidermal keratinocytes [95,99]. A key marker in chronological and photo-aging in the mitochondria is present in deletions in mitochondrial DNA [100,101]. These deletions in mitochondrial DNA can eliminate genes encoding key complexes apart of the electron transport chain, that are required for ATP production and in turn function and differentiation of keratinocytes [102–104]. Persistent exposure to UV radiation can accelerate the natural aging process and increases the risk of developing into skin cancer [105]. Dysfunction of mitochondria can be characterized in all cancers, including those that affect the skin primarily due to deletions in mitochondrial DNA or their role in ROS production and apoptosis [106,107]. Not only is the proper function of mitochondria implicated in skin cancer but also other rare clinical disorders causing an imbalance in mitochondrial proteins, enzymes, ROS generation or balance of mitophagy and mitochondrial biogenesis. Taken together, the mitochondria are a critical player in epidermis regeneration and general function of the skin. It is important to understand how the mitochondria is functioning in skin disorders and diseases in order to develop treatments with more specific targets. Therefore, as Panx2 has a predominant

intracellular localization and recent localization to ER-mitochondria contact sites, it is possible Panx2 could play a role in mitochondria function and regulation in the skin.

1.7.3 Pannexins in the skin

PANX1 and PANX3 are well characterized in the skin with their roles in keratinocyte differentiation and wound healing. PANX1 protein levels were first identified in the suprabasal epidermal layers, keratinocytes, fibroblasts and hair follicles in murine skin [5,10,11].While PANX3 has a more intracellular profile in the basal and sebaceous glands of thin and thick murine epidermal layers [10]. Similarly, to PANX1, upon analysis of Panx3 global knockout mouse model, there was a delay in wound healing, decreased inflammatory response and reduction in collagen remodeling [108]. Although PANX1 and PANX3 have well characterized roles in the skin, little is known about the function of PANX2 in the skin where expression has been identified yet no function was defined [27,28]. During the assessment of Panx1 and Panx3 double-KO (dKO) mice, Abitbol et al 2019 were not able to detect full length Panx2 or isoform 1 but did detect expression of isoform 202 in the wild-type and dKO skin samples [28]. This study was the first to identify expression of the isoform 202 in skin suggesting a more important role for PANX2 in the skin that is specific for this isoform. More recently, Sanchez-Pupo et al 2022 investigated expression of Panx2 and Panx2-202 throughout murine skin development and found elevated levels of Panx2-202 at the transcript and protein levels at P0 and P4 respectively [49]. This was the first confirmation of Panx2-202 protein expression in murine skin. Although an outstanding finding, the function or localization of Panx2-202 in mammalian cells were undefined. Therefore, studying Panx2 isoform 202 appears to be the more relevant form of Panx2 to study in respect to the skin. My thesis work sought to define the localization of Panx2-202 to begin characterizing its function in keratinocytes.

1.8 Rationale

Pannexins play an important role in intracellular communication to maintain skin homeostasis, structure, and function. Current pannexin 2 literature focuses primarily on the full length or isoform 1 as there is no literature that investigates the localization or function of isoform 202. The novel isoform 202 or Panx2-202 has recently been detected throughout murine skin development and differentiated immortalized keratinocytes. However, its localization and function remain to be determined. Therefore, we sought to characterize the Panx2 isoform 202 (Panx2-202) in epidermal keratinocytes.

1.9 Hypothesis

My hypothesis is that Panx2 isoform 202 localizes to the ER where it regulates ER-mitochondria interactions that alter mitochondria morphology in epidermal keratinocytes.

1.10 Objectives

The research objectives of this thesis are the following:

1. Characterize the intracellular localization of Panx2 isoform 202 in keratinocytes.
2. Investigate the impact of Panx2-202 expression on mitochondria in keratinocytes.
3. Determine if the C-terminal tail of Panx2-202 is cleaved during apoptosis.

Chapter 2

2.1 Materials and methods

2.1.1 Cell lines, transfections, and culture conditions

Rat epidermal keratinocytes (REK) and REK-Panx2-KO immortalized cell lines were a gift from Dr. Dale Laird and Dr. Silvia Penuela, respectively, and previously characterized in [11,49,109,110]. Cells were grown in DMEM (1X) (Corning, REF# 10-017-CV, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 1% penicillin-streptomycin (Pen Strep) (Corning, REF# 30-002-Cl, Manassas, VA, USA) and maintained at 37°C and 5% CO_2. For transient transfections, cells were transfected at 70% confluency using Lipofectamine 3000 (Invitrogen, REF# L3000-015, Carlsbad, CA, USA). Lab-Tek chamber transfections were conducted with the suggested amount of DNA (400ng total DNA) 6-well plates for lysate collection were transfected with 1µg of DNA and incubated for 48hours as per manufacturer instructions.

2.1.2 Plasmid source and construction

Mus musculus Panx2 isoform 202 (Panx2-202) fragment was synthesized by GENEWIZ Gene Synthesis using Uniprot ID: Q6IMP4-2. The Panx2-202 fragment was cloned into -moxGFP-N1 (Addgene plasmid #68070) using the EcoRI/AgeI sites. The moxGFP-NLS contains the moxGFP molecule followed by the nuclear localization sequence (PKKKRKV) of SV40 cloned into the AgeI/NotI sites of the parental plasmid. Panx2-Panx2-202-untagged were made by subcloning fragment into digested Panx2-moxGFP using EcoRI/NotI sites. SPLICS plasmid SPLICS Mt-ER Short P2A (SPLICS-Short; Addgene plasmid # 164108 ; http://n2t.net/addgene:164108 ; RRID:Addgene_164108) were obtained from Addgene and characterized in [100] [111].

2.1.3 Protein extractions and Western blots

Cell lysates were obtained using SDS and Triton X-100-based buffer (RIPA buffer; 0.1% SDS, 1% Triton X-100, 150mM NaCl, 50mM Tris-HCl, 0.5% sodium deoxycholate). At the time of cell lysate collection, lysis buffer was supplemented with Halt™ Protease & Phosphatase Inhibitor Cocktail (100X; Thermo Fisher Scientific) and 0.5 M EDTA solution (100X; Thermo Fisher Scientific) to a final dilution of 1:100 for each additive. 45µg of total protein was resolved by 10%

SDS-PAGE or Mini-PROTEAN® TGX Stain-Free™ 4-15% polyacrylamide Precast gels and transferred onto polyvinylidene difluoride (PVDF) membranes using a Trans-blot® Turbo™ Transfer System (BIO-RAD). Membranes were blocked with 5% bovine fraction V heat shock serum (BSA; Roche, REF# 10735086001, Mannheim, Germany) in 0.05% Tween20-Phosphate Buffer Saline (T-PBS) for 1hr at RT and probed overnight at 4°C with primary antibodies. Primary rabbit anti-PANX2-CT (1:200) and rabbit anti-GFP polyclonal (1:500, Novus Biologicals, Littleton, CO, USA #NPB2-37821) antibodies were used at ~100μg/mL final concentration: 0.1μg/mL. Secondary antibody goat anit-rabbit Alexa680 (Thermo Fisher Scientific, NY, USA, #A27042) was used at a 1:5000 dilution and the membranes were scanned using BIO-RAD ChemiDoc XRS+ System (Bio-RAD, CA, USA).

2.1.4 Immunofluorescence and imaging

REK-Panx2-KO cells were grown and maintained in Lab-Tek chambered coverglass (#155383 Thermo Fisher Scientific, NY, USA) for immunofluroescence experiments. Cell monolayers were fixed with 4% (w/v) paraformaldehyde (PFA; Fisher Scientific #BP531-500; 37% diluted with 1X PBS to 4%PFA) for 15 minutes at room temperature (RT). Cellular membranes were permeabilized with 0.05% Triton X-100 (#BP151-100) for 15 minutes at RT. Coverglass chambers were blocked for 1hr at RT with 10% Normal Goat Serum (NGS; Life Technologies Corp., REF# 50062Z, ORE, USA). Primary anti-PANX2-CT and anti-PANX2-NT antibodies were a gift from Dr. Silvia Penuela, anti-COX4 (#MA5-31470, Invitrogen), anti-PDI (Enzo Life Sciences Inc., CAT# ADI-SPA-891-D, NY, USA), were used in 1:200 dilutions in 1% NGS-2%BSA-PBS. Chambers were incubated with primary antibodies for 1hr at RT, washed with PBS and re-incubated with dye-conjugated secondary antibodies: goat-anti rabbit Alexa488 (#A-110088, Thermo Fisher Scientific, NY, USA), goat-anti rabbit Alexa680 (#A27042), goat-anti mouse Alexa647 (#A-21235) at a 1:500 dilution.

FLIP assays were performed by photobleaching a region of interest of a live cell at full power of the 488 nm laser and monitoring fluorescence loss over time [112]. Mean fluorescence intensity (MFI) of the photobleached live-cell was measured every 50 seconds of experiment and recorded to assess loss of overall fluorescence of the cell.

For colocalization assessment of Panx2-202-moxGFP with mitochondria (COX4), Z-stacks of transfected and fixed REK-Panx2-KO cells were collected. The Z-stack images were processed using the "3D projection" function in FIJI with a Y-axis of rotation. For colocalization of Panx2-202-moxGFP and mitochondria (mito-BFP) using live cells, a live-cell movie of a transfected REK-Panx2-KO cell was collected for ~45 seconds. The movie was then processed into a Kymograph using FIJI by selecting a region of interest to measure the movement of fluorescence over a measured period of time (Select line or rectangular region of interest > Analyze > Multi Kymograph).

Images were obtained on a Zeiss LSM 800 AiryScan confocal microscope on a Plan-Apochromat 63x/1.40 Oil DIC M27 objective -unless stated otherwise- located within the Anatomy and Cell Biology Departmental Imaging Core Facility.

2.1.5 Mitochondrial morphology analysis

Image analysis of mitochondria were conducted in FIJI (ImageJ)[113] as binary images as described in [114]. Once the images were processed to binary, measurements in FIJI were taken for the number of mitochondria, average area, circularity, and mean circularity. Measurements for mitochondria were selected to detect structures with the size between 0.20-infinity μm^2 and a circularity from 0-1.00. Circularity measurements in FIJI provide a score between 0 and 1, 1 being the most circular. Percentage of circular mitochondria (i.e., circularity) was calculated by using mitochondria that received a score of 1 from the FIJI measurements and divided by the total number of mitochondria measured in an individual cell, yielding the percent of circular mitochondria per cell. Mean circularity was calculated by taking the average (or mean) of all circularity scores from the mitochondria measured within an individual cell and divided by the total number of mitochondria.

2.1.6 Split-GFP contact site sensors quantification

Image analysis of ER-mitochondria contact site sensors (SPLICS) were conducted in FIJI (ImageJ)[113]. The threshold of 8-bit SPLICS images were automatically adjusted in FIJI, converted into binary, then measurements through FIJI were selected for number of contacts represented by structures with the size between 0.20-infinity μm^2 and a circularity from 0-1.00. Then, the area of the cell was measured by tracing the outline of the cell in FIJI using the draw tool and measurement

was taken for the area of the cell in μm^2. The number of contacts measured was then divided by the area of the cell to yield the number of contacts per square micron.

2.1.7 Camptothecin treatment for induction of apoptosis

REK-Panx2-KO cells were cultured in Labtek chambers and transfected with Panx2-202-moxGFP-NLS or Panx2-202-moxGFP. 48 hr post-transfection media of REK-Panx2KO cells were supplemented with 1μM of camptothecin and kept in the incubator for 8hrs and were fixed in 4% PFA for confocal microscopy. Mean fluorescence intensity (MFI) was measured by tracing the nucleus or cytosol and recording the value for mean gray value to use for statistical analysis.

2.1.8 Statistics

Statistical analysis stated in figure captions were performed using GraphPad Prism Software (version 9.4.0) (La Jolla, CA, USA). Mitochondria circularity, mean circularity, number of mitochondria and average area were assessed using Welch's unpaired two-tailed *t* tests. SPLICS contact site formation, nucleus MFI and cytosolic MFI were also analyzed with Welch's unpaired two-tailed *t* test. Probability of $p<0.05$ was considered statistically significant.

2.2 Results

2.2.1 Characterization of Panx2 isoform 202 in epidermal keratinocytes.

There is very limited literature available on Panx2 isoform 202 as only one article identifies the expression of Panx2 isoform 202 at mRNA and protein levels in developing murine skin, yet no group has uncovered the localization of the isoform [49]. As current Panx2 literature focuses primarily on isoform 1 or the canonical protein, we sought to develop a set of tools in order to investigate the localization of this isoform. We generated a construct tagged with fluorescent protein to yield Panx2-202-moxGFP (**Figure 2-1A**). moxGFP is an optimized GFP variant resistant to disulphide bond formation to maintain GFP monomers in the oxidative environment[115]. Thus, it represents the ideal fluorescent fusion partner for secretory proteins such as pannexins. We found that when Panx2-202-moxGFP was ectopically expressed in rat epidermal keratinocytes (REK) with genomic deletion of Panx2 (REK-Panx2-KO)[49] it colocalized with the ER marker: protein disulfide isomerase (PDI; **Figure 2-1C**). To ensure that the C-terminal moxGFP tag did not interfere with the intracellular sorting of Panx2-202 we also developed an untagged version of Panx2-202 (Panx2-202-untagged; **Figure 2-1A**) which could be detected using anti-Panx2-CT antibody (**Figure 2-1D**). This untagged Panx2-202 isoform also displayed an ER localization (**Figure 2-1C**). With these results we can conclude that both Panx2-202-moxGFP and Panx2-202-untagged colocalize with the ER. Of note, we found that unlike the isoform 202, tagging Panx2 isoform 1 with moxGFP resulted in its mis-localization into punctate structure that are not observed when the untagged version was expressed (**Supplemental Figure 2-1**). These results suggest that the C-terminal tagging of Panx2 isoform 1 with GFP can interfere with its localization and localization and trafficking studies generated with such fusion proteins should be interpreted with caution.

Previous groups have characterized Panx2-wildtype or isoform 1 to localize with endolysosomal compartments or recycling endosomes [62,63]. To assess whether a pool of Panx2-202 localizes outside of the ER, we conducted a photobleaching assay termed fluorescence loss in photobleaching (FLIP). FLIP is performed where a region of interest is continuously exposed to a

high intensity laser over a period of time [116]. The fluorescence of the cell is assessed to look for changes in fluorescence intensity in the regions of the cell that were not directly in the photobleaching area. Over the course of the photobleaching experiment, fluorescence of Panx2-202-moxGFP diminishes in the areas that were not in the direct region of photobleaching (**Figure 2-3A**). This occurs when a protein is associated to a continuous organelle like the ER. In our case, the fluorescence was homogenously depleted throughout the cells, consistent with the ER localization of Panx2-202 (**Figure 2-3**). The rate of depletion of ER associated Panx2-202moxGFP affected by photobleaching is much faster than the neighbouring cells (control) that undergo regular photobleaching as a result of microscopy (**Figure 2-3B**). Therefore, we can conclude that Panx2-202 remains associated to the interconnected ER network and does not appear to traffic out of the ER into the secretory pathway.

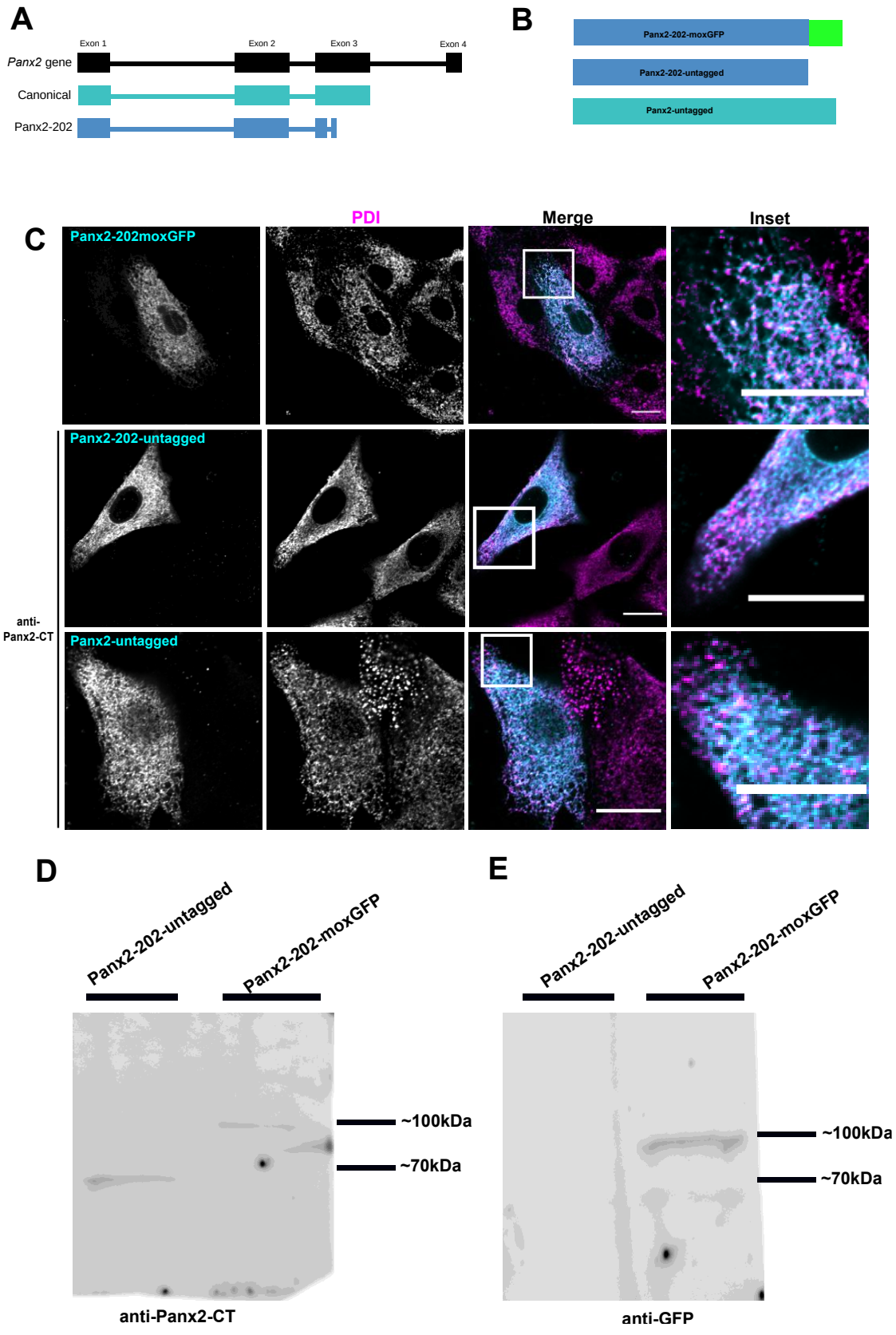
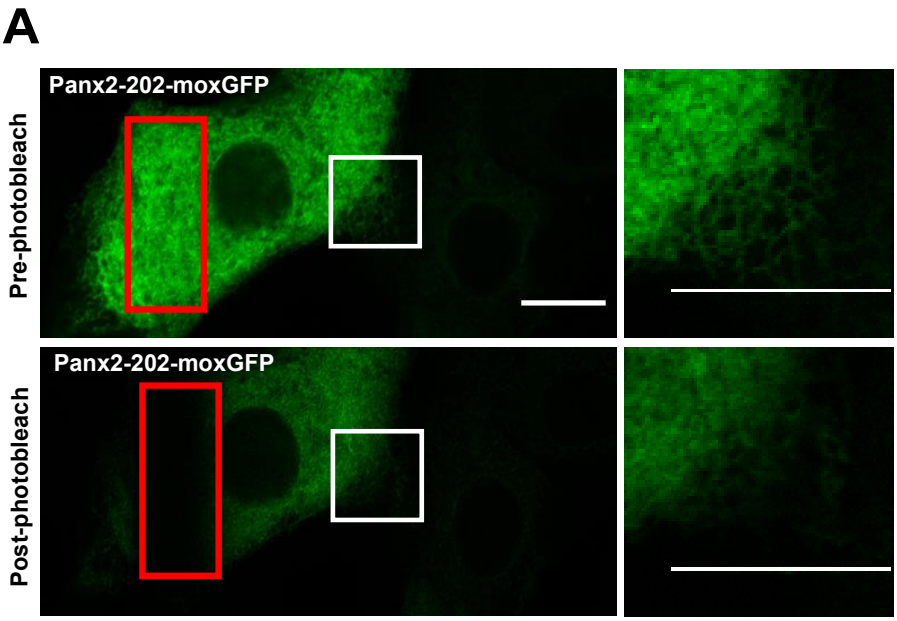

A
Exon 1
Exon 2
Exon 3
Exon 4
Panx2 gene
Canonical
Panx2-202
B
Panx2-202-moxGFP
Panx2-202-untagged
Panx2-untagged
C
PDI
Merge
Inset
Panx2-202moxGFP
Panx2-202-untagged
Panx2-untagged
anti-
Panx2-CT
D
Panx2-202-untagged
Panx2-202-moxGFP
~100kDa
~70kDa
anti-Panx2-CT
E
Panx2-202-untagged
Panx2-202-moxGFP
~100kDa
~70kDa
anti-GFP

Figure 2-1. Ectopic expression of Panx2-202 in REK-Panx2-KO cells has an ER distribution.

(A) Schematic representing the *Panx2* gene (black), teal and blue rectangle diagrams beneath represent coding sequence of Panx2 splice variant for canonical and isoform 202 respectively. (B) Cartoon schematic of tools to study Panx2 and Panx2-202 localization: Panx2-202-moxGFP), Panx2-202-untagged, and Panx2-untagged. (C) Representative immunofluorescence confocal images of REK-Panx2-KO cells taken 48hr post transfection with Panx2-202-moxGFP, Panx2-202-untagged (Panx2-202-UT) or Panx2-untagged (Panx2-UT; cyan) then immunostained for protein disulfide isomerase (PDI; shown in magenta). REK-Panx2-KO cells transfected with Panx2-202-untagged or Panx2-202moxGFP then collected and lysed for Western blot analysis with anti-Panx2 (D) and anti-GFP antibodies (E). Panx2-UT and Panx2-202-UT were detected with anti-Panx2CT antibody. Scale bar = 15μm.


A
Panx2-202-moxGFP
Pre-photobleach
Panx2-202-moxGFP
Post-photobleach

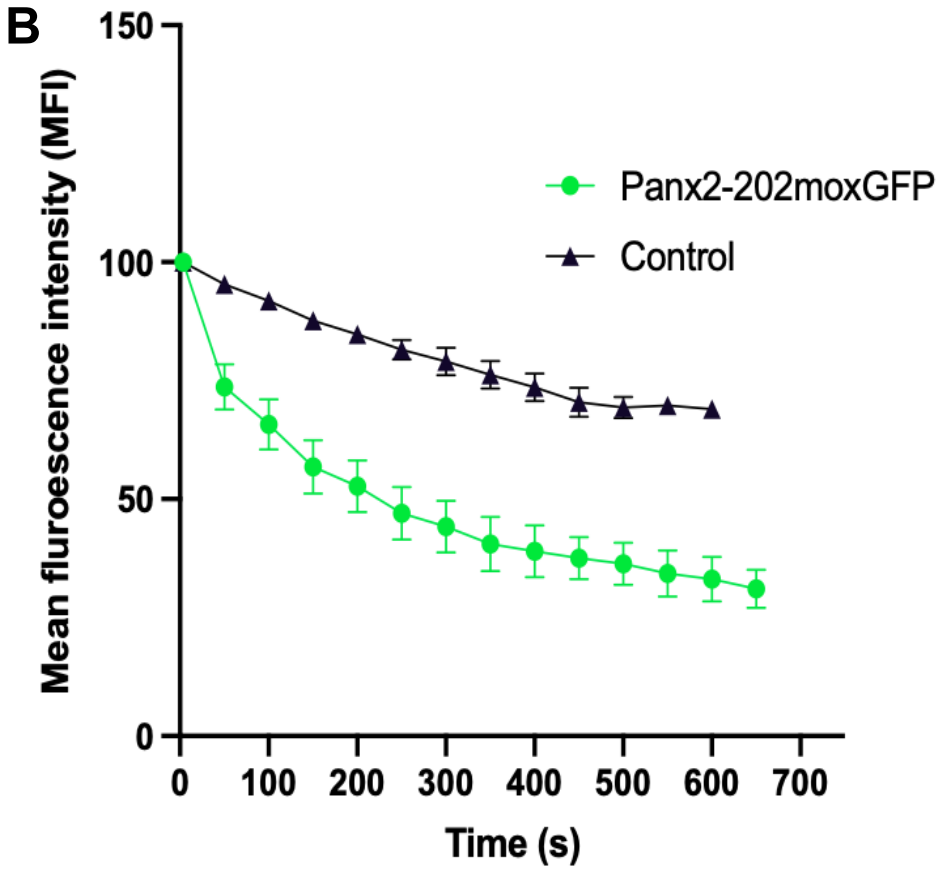

B
Mean fluroescence intensity (MFI)
150
100
50
0
0
100
200
300
400
500
600
700
Time (s)
Panx2-202moxGFP
Control

Figure 2-2. FLIP assay performed on live REK-Panx2-KO cells transfected with Panx2-202moxGFP.

(A) FLIP assay performed on live REK-Panx2-KO cell transfected with Panx2-202moxGFP in the red rectangular region of interest. Top set of images show the cell pre-photobleaching and bottom set of images show the same cell after photobleaching was complete (t= ~10min). The white boxes indicate the magnified area shown to the right of each panel. (B) FLIP curves. Normalized fluorescence intensity of the cell over the course of the experiment, the control is a Panx2-202-moxGFP expressing cell that was not undergoing photobleaching. Representative cell out of 6 cells analyzed. Scale bar = 15μm.

2.2.2 Expression of Panx2-202 regulates mitochondria morphology.

Prior to validating the localization of Panx2-202 to the ER, we conducted colocalization assessments with the mitochondria since canonical Panx2 was previously characterized at ER-mitochondria contact sites [27]. We co-transfected REK-Panx2-KO cells[49] with Panx2-202-moxGFP and stained for mitochondrial marker: cytochrome oxidase 4 (COX4; **Figure 2-3A**). Upon colocalization assessments, we did not see colocalization of Panx2-202 with the mitochondria but there was a distinct difference between the mitochondria in the cells transfected with Panx2-202-moxGFP versus the untransfected cells stained with COX4 in the same frame (**Figure 2-3A**). We observed more elongated, clustered mitochondria in the transfected cells while the untransfected cells maintained more punctate/circular mitochondria. We then performed quantitative analysis to assess the change seen in mitochondria morphology. There was a significant difference in the circularity and mean circularity between the mitochondria in transfected and untransfected cells (**Figure 2-3B**). Transfected cells meaning they are overexpressing Panx2-202-moxGFP. Although there was a significant difference in the circularity of the mitochondria, there was no significant difference in the number of mitochondria per cell or the average size of mitochondria between transfected and untransfected cells (**Figure 2-3C**). Next, using 3D surface projection of transfected REK-Panx2-KO cells with Panx2-202-moxGFP and stained with COX4, we wanted to see whether there was colocalization of Panx2-202 with mitochondria. 3D reconstruction did not show significant colocalization with mitochondria (**Figure 2-3D**). Moreover, timelapse imaging in living cells show that Panx2-202 does not colocalize with mitochondria but is rather juxtaposed to mitochondrial marker mito-BFP (**Figure 2-3E**). Therefore, ER protein Panx2-202 appears to regulate mitochondria morphology without colocalizing with mitochondria.

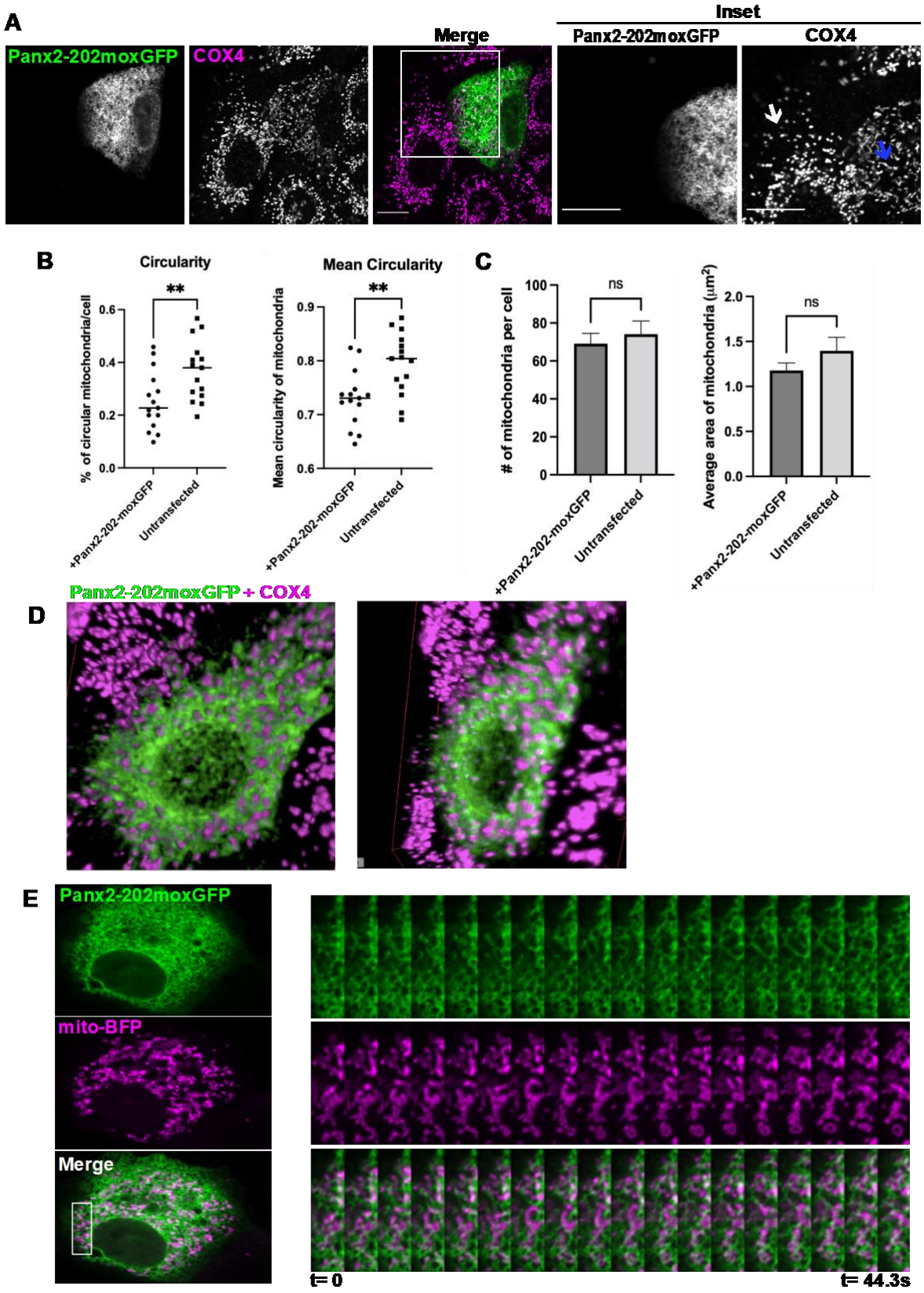

A
Panx2-202moxGFP
COX4
Merge
Inset
Panx2-202moxGFP
COX4
B
Circularity
**
% of circular mitochondria/cell
0.6
0.4
0.2
0.0
+Panx2-202-moxGFP
Untransfected
Mean Circularity
**
Mean circularity of mitochondria
0.9
0.8
0.7
0.6
+Panx2-202-moxGFP
Untransfected
C
ns
# of mitochondria per cell
100
80
60
40
20
0
+Panx2-202-moxGFP
Untransfected
ns
Average area of mitochondria (μm²)
2.0
1.5
1.0
0.5
0.0
+Panx2-202-moxGFP
Untransfected
D
Panx2-202moxGFP + COX4
E
Panx2-202moxGFP
mito-BFP
Merge
t= 0
t= 44.3s

Figure 2-3. Ectopic expression of Panx2-202 in REK-Panx2-KO cells alters mitochondria morphology but does not colocalize with mitochondria.

(A) Representative REK-Panx2-KO cell fixed following a 48hour transient expression of Panx2-202moxGFP (green) and immunostained for cytochrome oxidase 4 (COX4; magenta). The white boxes indicate the magnified area to the right of the image (inset). White and blue arrows in the COX4 Inset panel indicate untransfected and transfected mitochondria respectively. (B) The REK-Panx2-KO cells transfected with Panx2-202moxGFP had significantly fewer circular mitochondria per cell (***p=0.0089)* and the average circularity of mitochondria was lower in the transfected cells (***p=0.0029)*. N=3, n=15. (C) There was no difference in the number of mitochondria per cell and average area of mitochondria between the two groups. (N=4, $n_{transfected}$= 43 cells, $n_{untransfected}$= 38). (D) 3D surface projection of cell transfected with Panx2-202-moxGFP (green) and stained with COX4 (magenta). (E) Kymograph of live cell transfected with Panx2-202-moxGFP (green) and mito-BFP (magenta). White rectangle is the magnified area to the right of the representative image. Left image is t=0 and right image is t=44.3seconds. Statistical significance considered when $p = <0.05$ using Welch's unpaired two-tailed *t* test. Scale bar = 15μm.

2.2.3 Overexpression of Panx2-202 does not interfere with ER-mitochondria contact site formation in epidermal keratinocytes.

Previous work has identified PANX2-EGFP to colocalize at ER-mitochondria contact sites in rat glioma cells [60]. In connection to our current findings of Panx2-202 colocalizing with the ER and influencing mitochondria morphology, we sought to investigate if Panx2-202 also colocalizes at ER-mitochondria contact sites in keratinocytes. In addition, we wanted to assess whether the presence of Panx2-202 influences the formation of ER-mitochondria contact sites. For this objective we utilized an *in vitro* detection with a split-GFP-based contact site sensors (SPLICS) that can measure multiple contact sites with a scalable range (**Figure 2-4A**) [111]. Our co-transfection experiments with Panx2-202-untagged and SPLICS-short, showed that there was colocalization with Panx2-202 with the short distance ER-mitochondria contact site reporter, as shown on the linescan analysis of a region of interest within the cell image (**Figure 2-4A, B**). These data suggest that Panx2-202 may be regulating mitochondria function through membrane contact sites. Next, we wanted to investigate whether the presence of Panx2-202 influences the number of times the ER and mitochondria come into contact with one another. The SPLICS (green) images were processed, and the number of contacts represented by a GFP signal were counted and measured. The data indicates that the presence of Panx2-202 did not influence contact site formation in rat epidermal keratinocytes (**Figure 2-4C**). We also conducted control transfections with moxBFP and SPLICS-Short to serve as a comparison to ER-mitochondria contact site formation to when Panx2-202 was present (**Figure 2-4A, C**). Therefore, Panx2-202 may be a regulator of mitochondria morphology through the ER but does not affect how many times the ER and mitochondria come into contact.

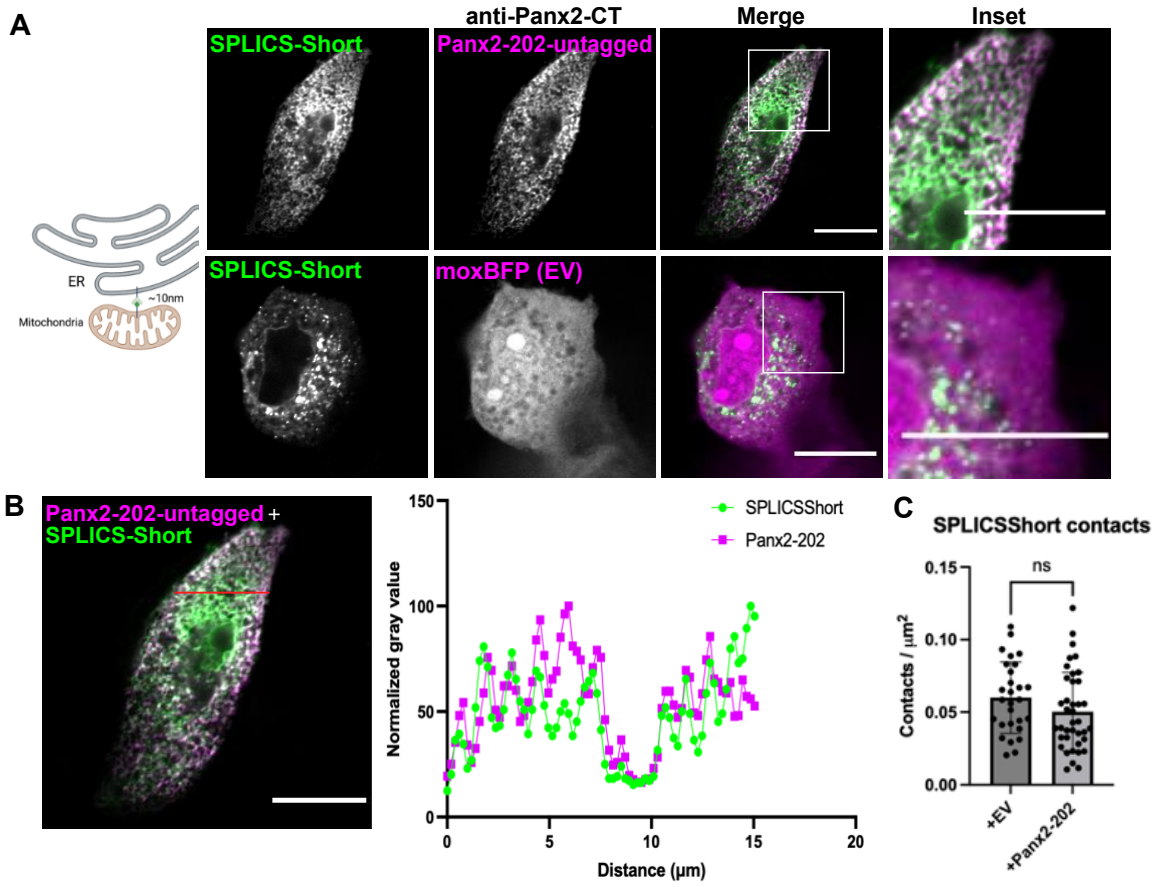

A
anti-Panx2-CT
Merge
Inset
SPLICS-Short
Panx2-202-untagged
SPLICS-Short
moxBFP (EV)
ER
~10nm
Mitochondria
B
Panx2-202-untagged +
SPLICS-Short
SPLICSShort
Panx2-202
Normalized gray value
Distance (µm)
C
SPLICSShort contacts
ns
Contacts / µm²
+EV
+Panx2-202

Figure 2-4. Overexpression of Panx2-202 does not interfere with ER-mitochondria contact site formation in epidermal keratinocytes.

(A) Schematic of SPLICS-Short plasmid (Created with BioRender.com adapted from Vallesse et al 2020. Representative images of REK-Panx2-KO cells co-transfected with SPLICS-short (green) and Panx2-202-untagged (magenta; top row) or an empty vector: moxBFP (EV; magenta; bottom row). White boxes indicate magnified region to the right of the image. (B) Red line indicated in image used for linescan analysis represented by the graph to the right. Panx2-202, an ER protein appears to colocalize with both SPLICS-Short. (C) The presence of Panx2-202 has no significant difference on the formation of ER-mitochondria contact sites. Each dot represents an individual cell. ($n_{SPLICSshort+EV}$=27, $n_{SPLICSshort+202UT}$=39, N=3 independent experiments). Statistical significance was considered when p= <0.05 using Welch's unpaired two-tailed *t* test. Scale bar= 15μm.

2.2.4 Panx2-202-moxGFP-NLS as a novel tool to study C-terminal caspase cleavage.

PANX2 is a known substrate of caspase 3/7 cleavage but until recently the exact localization of this cleavage was unknown [40]. The caspase 3/7 cleavage site of Panx2 was recently identified at sites D400 and D416, which are conserved in the isoform 202 [49]. Within the Panx2-202 amino acid sequence the conserved cleavage sites are found at position D408 and D424 since there is an addition of 8 amino acids in the N-terminal region in the isoform 202 that is not present in the canonical protein (**Supplemental Figure 2-2**). Therefore, the conservation suggests that Panx2-202 also undergoes caspase 3/7 cleavage during apoptosis in keratinocytes. We sought to develop a novel tool in order to study C-terminal caspase cleavage of Panx2-202 in living cells. We cloned the Panx2-202 sequence into plasmid containing a moxGFP variant followed by a nuclear localization signal (NLS) to yield Panx2-202-moxGFP-NLS (**Figure 2-5A**). Upon induction of apoptosis, the relocalization of the GFP signal to the nucleus would indicate that cleavage has occurred within the C-terminal tail of Panx2-202 to release the fragment from the ER membrane. In these experiments REK-Panx2-KO cells were transfected with Panx2-202-moxGFP-NLS, 48hr post transfection cells were either treated with 1μM camptothecin (CPT) or left untreated for an additional 8hours (**Figure 2-5B**). Notably, in the treatment group there was an increase in GFP signal based on measured mean fluorescence intensity (MFI) in the nucleus indicative of C-terminal cleavage allowing for translocation of the GFP to the nucleus because of the NLS (**Figure 2-5C-D**). It should be recognized that free GFP is found to localize to the nucleus in the absence of an NLS sequence [117]. As a control, we do not observe any change in the localization of the GFP signal upon induction of apoptosis with the Panx2-202-moxGFP in the absence of an NLS sequence (**Figure 2-5B**).

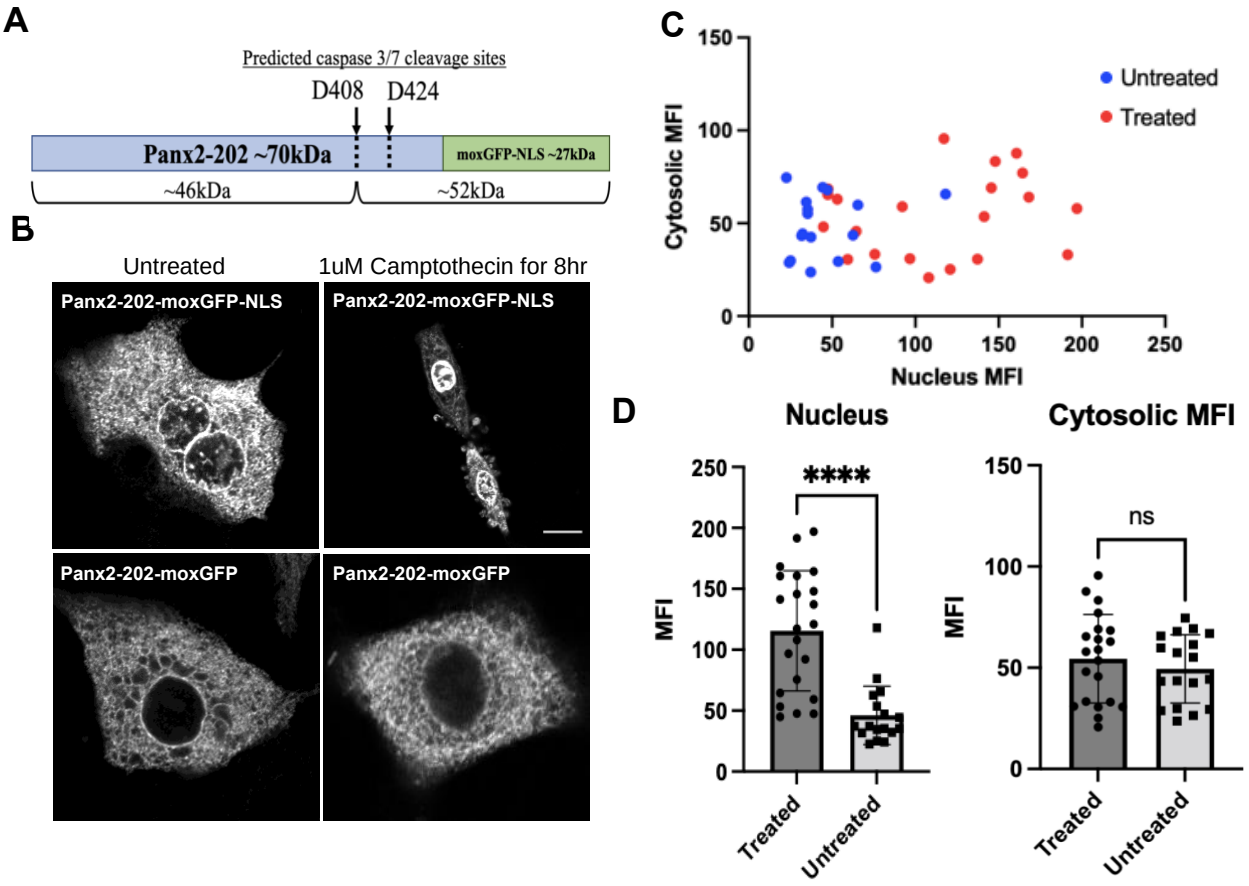
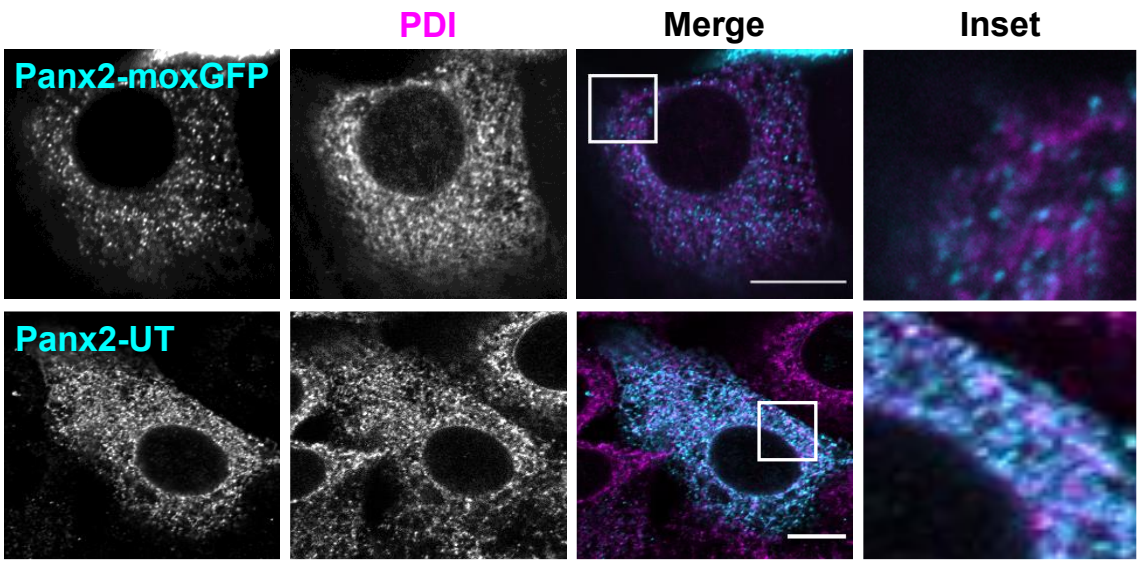
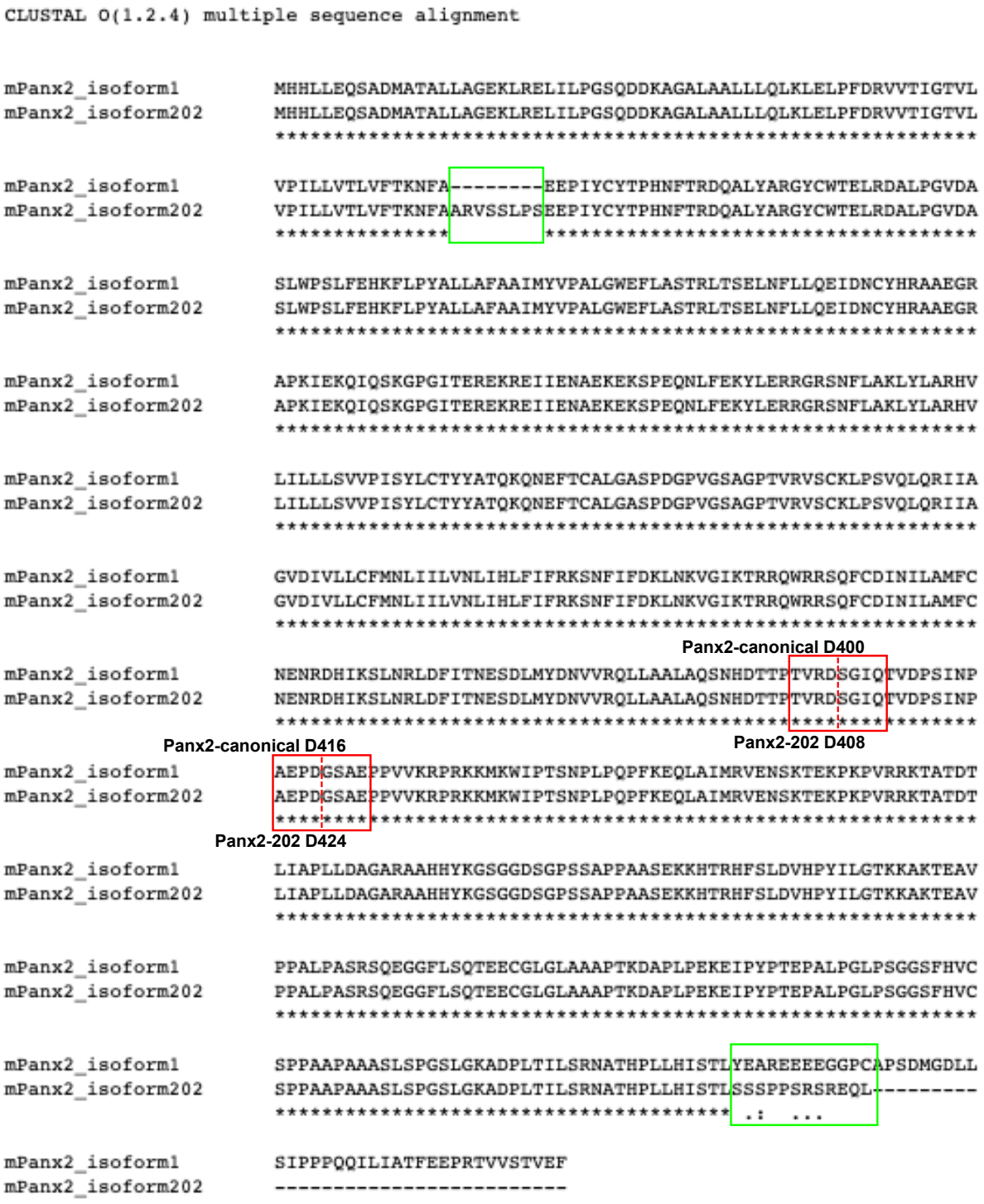

A
Predicted caspase 3/7 cleavage sites
D408
D424
Panx2-202 ~70kDa
moxGFP-NLS ~27kDa
~46kDa
~52kDa
B
Untreated
1uM Camptothecin for 8hr
Panx2-202-moxGFP-NLS
Panx2-202-moxGFP-NLS
Panx2-202-moxGFP
Panx2-202-moxGFP
C
Untreated
Treated
Cytosolic MFI
Nucleus MFI
0
50
100
150
200
250
D
Nucleus
Cytosolic MFI
MFI
****
ns
Treated
Untreated

Figure 2-5. Panx2-202-moxGFP-NLS is a novel tool to study caspase cleavage.

(A) Cartoon schematic of plasmid Panx2-202moxGFP-NLS with putative caspase cleavage sites: D408 and D424. (B) REK-Panx2-KO cells transfected with Panx2-202moxGFP-NLS (top) or Panx2-202moxGFP (bottom; control) and were either untreated (left) or treated (right) with 1μM camptothecin (CPT) for 8hours to induce apoptosis 48hours post-transfection then fixed for confocal imaging. Untreated Panx2-202moxGFP-NLS remains as an ER distribution whereas the treated cells had an increase in nucleus fluorescence. Panx2-202moxGFP served as a control for this experiment. (C) Scatter plot illustrating the mean fluorescence intensity (MFI) for the nucleus and cytosol of each cell. The treated group has a higher nucleus fluorescence. (D) Nucleus MFI is significantly higher in the CPT treated group than untreated. Each point represents one cell. Statistical significance Statistical significance considered when $p = <0.05$ using Welch's unpaired two-tailed *t* test (****$p = <0.0001$, p_{ns}=0.4426, $n_{treated}$= 22, $n_{untreated}$=17, N=2 independent experiments).


PDI
Merge
Inset
Panx2-moxGFP
Panx2-UT

Supplemental Figure 2-1. **Tagging PANX2 with moxGFP impairs its localization.** Representative immunofluorescence confocal images of REK-WT (top) and REK-Panx2-KO (bottom) cells taken 48hr post transfection with Panx2-moxGFP (top) or Panx2-untagged (Panx2-UT; bottom; shown in cyan) and immunostained for protein disulfide isomerase (PDI; shown in magenta). The white boxes indicate the magnified area shown to the right of each panel. Scale bar = 15μm.


CLUSTAL O(1.2.4) multiple sequence alignment
mPanx2_isoform1 MHHLLEQSADMATALLAGEKLRELILPGSQDDKAGALAALLLQLKLELPFDRVVTIGTVL
mPanx2_isoform202 MHHLLEQSADMATALLAGEKLRELILPGSQDDKAGALAALLLQLKLELPFDRVVTIGTVL
mPanx2_isoform1 VPILLVTLVFTKNFA--------EEPIYCYTPHNFTRDQALYARGYCWTELRDALPGVDA
mPanx2_isoform202 VPILLVTLVFTKNFAARVSSLPSEEPIYCYTPHNFTRDQALYARGYCWTELRDALPGVDA
mPanx2_isoform1 SLWPSLFEHKFLPYALLAFAAIMYVPALGWEFLASTRLTSELNFLLQEIDNCYHRAAEGR
mPanx2_isoform202 SLWPSLFEHKFLPYALLAFAAIMYVPALGWEFLASTRLTSELNFLLQEIDNCYHRAAEGR
mPanx2_isoform1 APKIEKQIQSKGPGITEREKREIIENAEKEKSPEQNLFEKYLERRGRSNFLAKLYLARHV
mPanx2_isoform202 APKIEKQIQSKGPGITEREKREIIENAEKEKSPEQNLFEKYLERRGRSNFLAKLYLARHV
mPanx2_isoform1 LILLLSVVPISYLCTYYATQKQNEFTCALGASPDGPVGSAGPTVRVSCKLPSVQLQRIIA
mPanx2_isoform202 LILLLSVVPISYLCTYYATQKQNEFTCALGASPDGPVGSAGPTVRVSCKLPSVQLQRIIA
mPanx2_isoform1 GVDIVLLCFMNLIILVNLIHLFIFRKSNFIFDKLNKVGIKTRRQWRRSQFCDINILAMFC
mPanx2_isoform202 GVDIVLLCFMNLIILVNLIHLFIFRKSNFIFDKLNKVGIKTRRQWRRSQFCDINILAMFC
Panx2-canonical D400
mPanx2_isoform1 NENRDHIKSLNRLDFITNESDLMYDNVVRQLLAALAQSNHDTTPTVRDSGIQTVDPSINP
mPanx2_isoform202 NENRDHIKSLNRLDFITNESDLMYDNVVRQLLAALAQSNHDTTPTVRDSGIQTVDPSINP
Panx2-canonical D416
Panx2-202 D408
mPanx2_isoform1 AEPDGSAEPPVVKRPRKKMKWIPTSNPLPQPFKEQLAIMRVENSKTEKPKPVRRKTATDT
mPanx2_isoform202 AEPDGSAEPPVVKRPRKKMKWIPTSNPLPQPFKEQLAIMRVENSKTEKPKPVRRKTATDT
Panx2-202 D424
mPanx2_isoform1 LIAPLLDAGARAAHHYKGSGGDSGPSSAPPAASEKKHTRHFSLDVHPYILGTKKAKTEAV
mPanx2_isoform202 LIAPLLDAGARAAHHYKGSGGDSGPSSAPPAASEKKHTRHFSLDVHPYILGTKKAKTEAV
mPanx2_isoform1 PPALPASRSQEGGFLSQTEECGLGLAAAPTKDAPLPEKEIPYPTEPALPGLPSGGSFHVC
mPanx2_isoform202 PPALPASRSQEGGFLSQTEECGLGLAAAPTKDAPLPEKEIPYPTEPALPGLPSGGSFHVC
mPanx2_isoform1 SPPAAPAAASLSPGSLGKADPLTILSRNATHPLLHISTLYEAREEEEGGPCAPSDMGDLL
mPanx2_isoform202 SPPAAPAAASLSPGSLGKADPLTILSRNATHPLLHISTLSSSPPSRSREQL---------
mPanx2_isoform1 SIPPPQQILIATFEEPRTVVSTVEF
mPanx2_isoform202 -------------------------

Supplemental Figure 2-2: CLUSTAL protein sequence alignment of mouse Panx2-isoform 1 and Panx2 isoform 202. Protein sequence alignment of Panx2 isoform 1 (or canonical Panx2) and Panx2 isoform 202. Green boxes highlight the change in sequence. The difference in the canonical sequence to the isoform sequence: 75A (canonical) → AARVSSLPS (isoform 202) and 632-677aa: YEAREEEEGGPCAPSDMGDLLSIPPPQQILIATFEEPRTVVSTVEF (canonical) → SSSPPSRSREQL (isoform 202). The red box highlights characterized caspase 3/7 cleavage site in the Panx2 canonical (D400, D416) and the same sites present in isoform 202 (D408 and D424 respectively).

2.3 Discussion

Pannexins (PANX) are a family of tetra spanning membrane glycoproteins (PANX1, PANX2 and PANX3) that participate in intracellular communication through the passage of small ions and molecules[3,118]. There is a vast amount of research available on PANX1 and PANX3 while there is limited and conflicting evidence of the function and localization of PANX2. The studies that are available which assess PANX2 localization vary depending on cell type and rely on overexpression systems. Within these studies, Panx2 has been characterized to localize to early or recycling endosomes, endo-lysosomal compartments, the ER and ER-mitochondria contact sites [25,43,60–63]. One commonality of all PANX2 studies is that they investigate the localization or function of PANX2 isoform 1, or the canonical protein although a splice variant exists: PANX2 alt 2 or isoform 202 (PANX2-202) [28,49]. The isoform is slightly smaller in molecular weight and with a partial sequence difference than the canonical PANX2 that was first identified in the brain [26]. The isoform 202 can be found in both humans (509aa) and mice (651aa). Abitbol et al 2019 found Panx2-202 to be consistently expressed in the skin of Panx1/Panx3 double KO mice as well as wildtype group whereas there was no significant expression of wildtype Panx2. More recently, the isoform was found at higher mRNA and protein levels in murine skin throughout development compared to canonical PANX2 [49]. This difference in expression between the isoforms may suggest a possible tissue-specific role of the isoform 202 in keratinocytes. As Abitbol et al 2019 and Sanchez-Pupo et al 2022 were the first to identify expression of Panx2-202 at the mRNA and protein levels, its localization and function within epidermal keratinocytes, or any cell type remained unknown. As our work is the first to investigate localization and function of this isoform, there are many avenues to complement the work conducted in this thesis.

Panx2 isoform 202 localizes to the ER in epidermal keratinocytes

Here, we developed tools to study the localization and function of Panx2-202 to build onto these new findings in PANX2 literature. As Panx2 was detected in the suprabasal layer of the epidermis[49], we thought it would be appropriate to conduct our localization studies using immortalized keratinocytes as a model [49]. We found that Panx2-202 colocalizes with the ER when tagged with a fluorescent protein (Panx2-202-moxGFP) as well as untagged (Panx2-202-untagged) and detected using anti-Panx2CT antibody. As other groups have reported canonical Panx2 to smaller vesicular organelles like endosomes, we decided to utilize a photobleaching

technique FLIP to verify if Panx2-202 was associated to a smaller organelle, or a continuous one like the. FLIP continuously photo bleaches a region of interest where the fluorescence is representative of Panx2-202-moxGFP which slowly depleted without a smaller vesicle remaining, suggesting an association to a larger, continuous organelle. Combined with our immunofluorescence data together, we can propose that Panx2-202 localizes to the ER in epidermal keratinocytes. However, the use of an overexpression system represents a limitation of my studies. For a more accurate representation of localization of endogenous Panx2-202 there are methods available that utilize the Panx2 native promoter to assess endogenous localization. One method is to fuse fluorescent proteins genomic level using CRISPR/Cas9 which may provide/validate intracellular localization[119]. In addition, the development of antibody capable of specifically detecting endogenous Panx2-202 should be explored further. The antibodies that are available to detect Panx2 have limitations as they are not clean when it comes to protein detection. In addition, there is limited information on whether there is an immortalized cell line that has a high enough endogenous expression of the Panx2 at the protein level. Considering the studies available on Panx2 investigate isoform 1 or the canonical protein, it would be interesting to test whether isoform 202 is expressed in the other cell lines used, or if it behaves similarly to the canonical protein in respect to individual studies. As it is shown in literature, the localizations of canonical Panx2 seem to differ depending on cell type, suggesting a cell-type dependent subcellular localization. Therefore, it would be interesting to investigate if Panx2-202 remains ER-localized in different cell types.

Two separate groups have identified Panx2 at the ER, but the exact function(s) of Panx2 at the ER remains to be elucidated. In addition, previous work has suggested that ER-localized PANX1 and PANX3 function as calcium leak channels [59,120]. Therefore, it is possible that Panx2-202 functions as a calcium leak channel within the ER membranes. One method to test this notion includes selecting a cell line which lacks Panx1 and Panx3 to ensure results obtained are from the overexpression of Panx2-202 in selected cell line, followed by treatment with SERCA inhibitor: thapsigargin and labeling calcium with fura-2, as described in Vanden Abeele et al 2006. This experiment would be an indication of whether overexpression/presence of Panx2-202 still allows the passage of calcium ions from the ER while a known calcium pump is inhibited.

The ER plays an essential role in cellular homeostasis in the skin. Various internal and external stressors such as overproduction of proteins and UV radiation respectively, activating ER stress response known as the unfolded protein response (UPR). ER stress occurs in both pathological and physiological conditions that can modulate pro or anti-apoptotic signaling cascades[121,122]. In the skin, pathological ER stress can be induced via UV irradiation that leads to oxidative stress and the upregulation of ER chaperones and transcription factors to return to cellular homeostasis[123,124]. Therefore, it would be interesting to investigate whether the presence of Panx2-202 has a protective role when the cell is exposed to ER stress.

The expression level of canonical and Panx2-202 varies during the development of murine skin at P0 and P4, at the mRNA and protein level respectively [49]. This difference in canonical versus Panx2-202 levels could be a regulatory mechanism/balance required of either protein during keratinocyte differentiation. It is not known whether Panx2 or Panx2-202 change localization during differentiation as our experiments were conducted using undifferentiated epidermal keratinocytes. REKs can differentiate in culture through the supplementation of $CaCl_2$ to growth media as well as forming an organotypic epidermis when cultured on a collagen substrate [49,109,125]. It would be insightful to investigate whether the localization of both Panx2 and Panx2-202 vary during the process of keratinocyte differentiation.

There is evidence of an interaction between Panx1 and Panx2 when the two paralogs are co-expressed [25,36,43]. It is suggested that Panx1 and Panx2 form functional heteromeric channels at the plasma membrane as well as co-expression of PANX1 with PANX2 drives PANX2's intracellular localization to the cell surface [25,43]. Therefore, this suggests that Panx2-202 may be able to interact with PANX1 as previously described for canonical PANX2. Currently, there is no data that suggests if Panx2-202 binds Panx1. As the C-terminal tail of Panx2 and Panx2-202 is the location of the major difference between the isoforms, it may play a role in the interaction with Panx1. Therefore, we should investigate whether Panx2-202 colocalizes with Panx1 at the plasma membrane when co-expressed as previously described in Sanchez-Pupo et al 2018. Understanding this interaction could provide insight into the importance of the C-terminal tail in both Panx2 and Panx2-202 and whether it is required for its interaction with Panx1. It still remains to be clearly shown that Panx2 and/or Panx2-202 form functional channels. In addition to the expression of

isoform 202, a route worth investigating related to pannexin interactions would be to determine whether Panx2 and Panx2-202 are able to interact with one another, as well as potentially form functional channels. Another avenue for interactions would be to investigate other protein interactors of Panx2 or Panx2-202. BioID is a proximity dependent-biotin identification screen which looks for protein interactions in living cells[126], where recently the overexpression of PANX1 in patient-derived rhabdomyosarcoma cell lines[127] was investigated. This BioID screen with the overexpression of PANX1 aided in the generation of transcriptomic and proteomic public searchable databases to identify new pathways and avenues modulating PANX1 functions[127]. Therefore, it would be intriguing to determine whether a similar data analysis can be conducted using overexpression of PANX2 or PANX2-202 to reveal novel protein interactions of both PANX2 isoforms.

Panx2-202, an ER resident protein regulates mitochondria morphology

During our colocalization assessments, we not only looked for localization with the ER but also with mitochondria. Considering one of the more recent papers published characterized canonical Panx2 to localize with ER-mitochondria contact sites [60], we thought this would be an appropriate organelle to assess colocalization with. Upon preliminary experiments there seemed to be no direct colocalization of Panx2-202 with mitochondria marker: COX4. It appeared Panx2-202 was juxtaposed to the mitochondria while maintaining a clear ER distribution. An interesting finding, we did observe was a change in mitochondria morphology in the cells that were ectopically expressing Panx2-202. Although there was no direct colocalization, it was apparent that from the ER, Panx2-202 was able to regulate mitochondria morphology, to yield more elongated and less punctate/circular mitochondria. The exact mechanism to which mitochondria morphology changes remains to be elucidated. The dynamic shape of mitochondria is controlled by cellular fission and fusion processes and is influenced by the bioenergetic state of the cell. Mitochondrial fission and fusion are controlled enzymatic reactions by proteins: soluble cytosolic dynamin-like protein 1 (DLP1), mitofusin (Mfn) 1 and 2, mitochondrial fission factor (Mff) and optic atrophy 1 (OPA1) [128]. Mfn and OPA1 are associated with the outer and inner mitochondrial membranes respectively where they control fusion of their affiliated membranes [32]. It would be interesting to see if Panx2-202 colocalizes with these known mitochondrial fission/fusion markers. In addition, it is possible that overexpression of Panx2-202 can be causing an upregulation of these mitochondrial fission

and fusion proteins, conducting mRNA analysis using qPCR and Western Blot analysis could indicate whether the presence of Panx2-202 leads to an increase in mitochondrial fission. One group recently identified Mfn proteins to colocalize directly with ER tubules, collectively showing that fusion events of outer and inner mitochondrial membranes occur at ER membrane contact sites [128]. Considering canonical Panx2 is present at ER-mitochondria contact sites in rat glioma cells[60], and our results show colocalization with an ER-mitochondria contact site marker, it is possible that Panx2 and/or Panx2-202 participate in mitochondrial fusion/fission events. Our results found that overexpression of Panx2-202 in REK cells, resulted in less punctate and more elongated mitochondria. Elongated mitochondria are prevalent during nutrient depletion induced autophagy [129]. Autophagy degrades mitochondria through a process known as mitophagy in response to mitochondrial damage [130]. Mitophagy can be classified into three groups: basal, stress-induced and programmed. Basal levels of mitophagy are homeostatic processes for cells to maintain energy production and maintain a healthy mitochondrial pool. Investigating whether Panx2-202 plays a role in mitophagy and mitochondrial biogenesis can be conducted through the treatment of mitochondria depolarization agent such as cyanide m-chlorophenyl hydrazine (CCCP) and mitochondrial morphology/fluorescence can be measured in transfected versus untransfected cells. This type of experiment would assist in identifying whether Panx2-202 has a role in promoting mitophagy or would play a protective role when overexpressed. This elongation of mitochondria is a form of maintenance of mitochondrial ATPase in order to maintain energy levels during starvation [129]. This suggests that presence of mouse Panx2-202 in a rat cell line could be inducing autophagic processes where we see the shift in mitochondrial morphology from punctate to elongated mitochondria. To test this, one could nutrient-deprive the same REK cells and assess mitochondrial morphology to see if we have a similar result as when Panx2-202 is present. Aside from autophagy, development of a cell line stably expressing Panx2-202 in order to investigate mitochondrial ATP levels, this would help determine whether ATP production/synthesis is affected by the presence of Panx2-202. Nevertheless, the best way to identify a role of Panx2/Panx2-202 in metabolic processes would be through a Panx2/Panx2-202-knockout (KO) mouse model and investigating whether Panx2-KO mouse models has defects in metabolism related to mitochondria function.

This left us with a remaining question of how can the presence of an ER protein alter mitochondria morphology? One answer to this question could be through ER-mitochondria contact sites which are communication hubs that facilitate calcium transport and lipid homeostasis between the two organelles [64]. Considering canonical Panx2 was characterized at ER-mitochondria contact sites in glioma cells, there was a possibility that Panx2-202 could also localize at these membrane contact sites. Utilizing the SPLICS plasmids [111], we assessed whether Panx2-202 colocalizes with ER-mitochondria contact sites, and if the presence of Panx2-202 influences how many contact sites that are formed. As these split-GFP reporters identify contact of the ER and mitochondria membranes at a measured distance, they are static and are not representative of the dynamic/fluid interaction that occurs at membrane contact points. Therefore, it would be interesting to investigate other known ER-mitochondria contact site markers such as glucose-related protein (Grp) 75 or Grp 78[131–133]. One interesting take away from this altered morphology is that in Sanchez-Pupo et al 2018, they found that expression of N-glycosylation mutant Panx2-N86Q resulted in an alteration in ER morphology [43]. With this mutant being resistant to N-glycosylation, it may have caused a disruption in regulatory machinery that controls protein folding within the ER. These data together suggest a possible regulatory role for Panx2 and isoform 202 in the maintenance of both ER and mitochondria.

Live cell reporter suggests that the C-terminal tail of Panx2-202 is cleaved upon induction of apoptosis

Previous studies have identified both pro- and anti- apoptotic roles of Panx2 in glioma cells, pancreatic β-cells, prostate cancer cells and keratinocytes [29,32,49]. The role of Panx2 seems to vary dependent on the cell type: in glioma cells Panx2 expression reduced oncogenicity, sensitized pancreatic beta-cells to cytokine-induced apoptosis, is a regulator of ferroptosis in prostate cancer cells and delayed UVB-induced apoptosis in keratinocytes. Upon induction of apoptosis, a multitude of proteolytic proteins are activated to disassemble cellular structures [45]. Panx2 was recently characterized to undergo caspase cleavage upon UVB-induced apoptosis at residues D400 and D416 in the cytosolic exposed C-terminal tail [49]. These residues are present in the C-terminal tail of Panx2-202 but located at residues D408 and D424, the presence of these sites suggest caspase cleavage is likely to occur in Panx2-202.

To study cleavage in the C-terminal tail, we utilized a fluorescent reporter that would change localization upon proteolytic cleavage following the induction of apoptosis. Here, we were able to quantify the shift in fluorescence from the ER in the untreated group to a more nuclear localization present in the treated group (1μM CPT for 8hrs) in REK-Panx2-KO cells transfected with Panx2-202-moxGFP-NLS. It is important to note there is no significant difference among the cytosolic MFI between the treated and untreated groups. This may be because each cell will be at its own point of apoptosis upon fixation following treatment, as well as that we cannot assume each Panx2-202 in each cell had undergone proteolytic cleavage. This should be accounted for in the cytosolic fluorescence compartment. In addition, in the nucleus there are small sections without fluorescence which we account for chromatin within the nucleus. It should also be considered that each cell measured may be at different points in the cell cycle which would influence positioning of chromatin upon prior to CPT-induced apoptosis. Furthermore, this is the first indication that cleavage is occurring in the C-terminal tail of Panx2-202 which we assume is at D408 and/or D424. Therefore, we have been the first to identify the potential role of Panx2-202 during apoptotic conditions, and that cleavage occurs in the C-terminal tail like canonical Panx2. As the shift in fluorescence to the nucleus identifies cleavage within the C-terminal tail, we did not confirm whether the exact residues of caspase cleavage in Panx2-202. When characterizing caspase cleavage sites at D400 and D416, Sanchez-Pupo et al 2022 only detected cleavage at one site D416 (D424 in Panx2-202) following exposure to UVB radiation. As the skin is subject to UV irradiation[134], it would be most relevant to expose Panx2-202 transfected keratinocytes to UVB irradiation and further assessed for protein fragmentation with Western Blot analysis, similar to the experiment conducted with canonical Panx2 in Sanchez-Pupo et al 2022. To validate the caspase cleavage sites, development of proteolytic resistant mutants: Panx2-202^{D408A}, Panx2-202^{D424A} and Panx2-202$^{D408A+D424A}$ like the mutagenesis conducted in Sanchez-Pupo et al 2022 with canonical Panx2. Following the same UVB-irradiation induced apoptosis[49], cells transfected with the Panx2-202 caspase resistant mutants should be lysed and assessed for fragmentation, or lack of fragmentation to determine whether caspase cleavage has occurred at these designated residues. Therefore, it is possible that either one or both predicted sites in Panx2-202 (D408 and D424) undergo proteolytic cleavage upon induction of apoptosis.

Panx2-202 in the skin

Genetic deletion of Panx1 and Panx3 resulted in impaired keratinocyte and fibroblast differentiation during would healing [11,108]. Although the presence of both canonical Panx2 and isoform 202 detected in this skin, their implication in wound healing and keratinocyte differentiation *in vivo* have yet to be determined. The Panx2-KO mouse model available has not been reported to possess an altered skin phenotype[78,79], although with recent work from Sanchez-Pupo et al 2022 it is possible that under stress conditions such as UVB irradiation there may be significant differences compared to wildtype counterpart. Further work investigating the role of Panx2 and Panx2 isoform 202 in skin healing, differentiation and structure remain to be addressed.

2.4 Conclusions

In conclusion, this work is the first to localize Panx2-202 to the ER in keratinocytes. In addition, we found ER resident protein Panx2-202 to regulate mitochondria morphology when overexpressed in epidermal keratinocytes, potentially through ER-mitochondria membrane contact sites. Previous literature characterizes Panx2 to various intracellular compartments dependent on cell type, future work should consider investigating the localization of isoform 202 in a variety of cell types. This study also identifies cleavage of Panx2-202 C-terminal tail upon induction of apoptosis using our novel fluorescent reporter Panx2-202moxGFP-NLS. Collectively, these findings provide insight into the localization and potential function of the previously uncharacterized Panx2-202.

2.5 References

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2.6 Curriculum Vitae

Marina Sertsis

Education

The University of Western Ontario, London, ON

Masters of Science candidate in Anatomy and Cell Biology- Research (Sept 2020- Present)

- Supervised by Dr. Patrick Lajoie
- Coursework
    - Advanced topics in cell and neurobiology 9555 - Completed
    - Research and seminars in cell and neurobiology 9520 - Completed

The University of Guelph, Guelph, ON

Honours Bachelor of Science in Molecular Biology and Genetics (Sept 2015- Feb 2020)

- Dean's Honour List Winter 2019

Life-science related experience

Student representative, Microscopical Society of Canada (June 2021-June 2022)

Research: Volunteer role in planning the Microscopical Society of Canada's student chapter in Ontario. Assisted in hosting career panel and developing guidelines / expectations for the Ontario chapter starting in 2022.

Mullen Lab, University of Guelph

Undergraduate Research Assistantship Recipient (May 2019-August 2019)

Undergraduate thesis student (September 2018- April 2019)

Research: Investigating the targeting signals of peroxisomal tail-anchored proteins in plant cells.

*Manuscript in progress for research contribution to this project.

Program Delta, Shoppers Drug Mart Inc.

Pharmacy Student: Roll-out Specialist (May 2018-August 2018)

Role: Leverage pharmacy workflow expertise to coach retail pharmacy teams on the new virtual workflow and changes as the result of the new system across Ontario. Assist in testing of new software and provide 2-3 days of in-store support on the modernized HealthWATCH system

Scholarships

- Western Graduate Research Scholarship: Awarded to Western graduate students

Conference oral presentations:

- Sertsis Marina (August 2022) ER-localized Pannexin 2 isoform 202 regulates mitochondria function in epidermal keratinocytes. Ontario Cell Biology Symposium. Toronto, Ontario. August 16-17, 2022. Three-minute lightning talk.

Conference Poster Presentations

- Sertsis Marina (August 2022) ER-localized Pannexin 2 isoform 202 regulates mitochondria function in epidermal keratinocytes. Ontario Cell Biology Symposium. Toronto, Ontario. August 16-17, 2022. (Poster)
- Sertsis Marina (May 2022) ER-localized Pannexin 2 isoform 202 regulates mitochondria function in epidermal keratinocytes. London Health Research Day. Online poster.