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Skin Diseases and Non-Syndromic Hearing Loss Linked to Cx30 Mutations Arise Through Several Distinct Mechanisms

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology

SKIN DISEASES AND NON-SYNDROMIC HEARING LOSS LINKED TO Cx30 MUTATIONS ARISE THROUGH SEVERAL DISTINCT MECHANISMS

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by

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Abstract

Connexin 30 (Cx30), a member of the large gap junction protein family, plays a role in the homeostasis of the epidermis and inner ear through gap junctional intercellular communication (GJIC). Here, we investigated four autosomal dominant Cx30 gene mutations linked to hearing loss and/or various skin diseases. First, the T5M mutant linked to non-syndromic hearing loss formed functional gap junction channels and hemichannels, similar to wild-type Cx30. The V37E mutant associated with keratitis-ichthyosis-deafness (KID) syndrome was retained in the endoplasmic reticulum (ER) and significantly induced unfolded protein response (UPR)-mediated apoptosis. The loss-of-function G59R mutant linked to Vohwinkel and Bart-Pumphrey syndromes was retained primarily in the Golgi apparatus. Lastly, the Clouston syndrome-linked A88V mutant significantly induced apoptosis, primarily through an UPR-independent mechanism. Collectively, we discovered that four unique Cx30 mutants cause disease through different mechanisms, highlighting the overall complexity of connexin-linked diseases and the importance of GJIC in disease prevention.

Keywords

Connexin 30 (Cx30), connexin mutation, rat epidermal keratinocytes (REKs), Clouston syndrome, Vohwinkel syndrome, Bart-Pumphrey syndrome, keratitis-ichthyosis-deafness (KID) syndrome, non-syndromic hearing loss, apoptosis, unfolded protein response (UPR), dominant-negative, hemichannel, gap junctional intercellular communication (GJIC)

Co-Authorship Statement

All experiments were performed by Amy Berger, however Dr. Qing Shao designed the primers used to generate wild-type Cx30 constructs used in this study.

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List of Abbreviations and Nomenclature

3D	3 dimensional
A2	alanine at amino acid position 2
A40V	alanine to valine substitution at amino acid position 40
A88	alanine at amino acid position 88
A88V	alanine to valine substitution at amino acid position 88
A88V-GFP	A88V Cx30 mutant linked to green fluorescent protein
ANOVA	analysis of variance statistical test
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
C86S	cysteine to serine substitution at amino acid position 86
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CHOP	C/EBP homologous protein
CMTX	Charcot-Marie-Tooth disease
CO_2	carbon dioxide

Cx	connexin
Cx26-RFP	connexin 26 linked to red fluorescent protein
Cx30-GFP	connexin 30 linked to green fluorescent protein
Cx30-RFP	connexin 30 linked to red fluorescent protein
D50N	aspartic acid to asparagine substitution at amino acid position 50
D66H	aspartic acid to histidine substitution at amino acid position 66
Da	dalton
DCF-ECS	divalent cation free extracellular solution
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECS	extracellular solution
EDTA	ethylene-diamine-tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
eIF2α	alpha subunit of eukaryotic translation initiation factor 2
EKV	erythrokeratoderma variabilis
EP	endocochlear potential
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
F137L	phenylalanine to leucine substitution at amino acid position 137
G11R	glycine to arginine substitution at amino acid position 11

G12D	glycine to aspartic acid substitution at amino acid position 12
G12R	glycine to arginine substitution at amino acid position 12
G22R	glycine to arginine substitution at amino acid position 22
G45E	glycine to glutamic acid substitution at amino acid position 45
G57	glycine at amino acid position 57
G59	glycine at amino acid position 59
G59A	glycine to alanine substitution at amino acid position 59
G59R	glycine to arginine substitution at amino acid position 59
G59R-GFP	G59R Cx30 mutant linked to green fluorescent protein
G59S	glycine to serine substitution at amino acid position 59
GFP	green fluorescent protein
GJA1	gap junction alpha 1
GJB6	gap junction beta 6
Gjb6$^{-/-}$	connexin 30 knock out mice
GJIC	gap junctional intercellular communication
GM130	Golgi matrix protein 130
GRP78	glucose regulated protein 78
GRP94	glucose regulated protein 94
HED	hidrotic ectodermal dysplasia
HeLa	Henrietta Lacks cervical cancer cells

IP_3	inositol 1,4,5-trisphosphate
IRE1	inositol requiring kinase 1
K^+	potassium
kDa	kilodalton
KID	keratitis-ichthyosis-deafness
L34P	leucine to proline substitution at amino acid position 34
L56	leucine at amino acid position 56
Mg^{2+}	magnesium
mRNA	messenger ribonucleic acid
N45K	asparagine to lysine substitution at amino acid position 45
N54	asparagine at amino acid position 54
NaCl	sodium chloride
NAD+	nicotinamide adenine dinucleotide
NaF	sodium fluoride
NBF	neutral buffered formalin
NP-40	nonyl phenoxypolyethoxylethanol
ODDD	oculodentodigital dysplasia
Opti-MEM	optimal minimal essential medium
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + Tween 20

PCR	polymerase chain reaction
PDI	protein disulfide isomerase
pEGFP-N1	enhanced green fluorescent protein vector
PERK	double stranded RNA-activated protein kinase-like ER kinase
PI	propidium iodide
PPK	palmoplantar hyperkeratosis
pTagRFP-N	mammalian red fluorescent protein vector
R42P	arginine to proline substitution at amino acid position 42
REKs	rat epidermal keratinocytes
RFP	red fluorescent protein
S17F	serine to phenylalanine substitution at amino acid position 17
S59P	serine to proline substitution at amino acid position 59
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Stauro	staurosporine
T5	threonine at amino acid position 5
T5M	threonine to methionine substitution at amino acid position 5
T5M-GFP	T5M Cx30 mutant linked to green fluorescent protein
Tdt	terminal deoxynucleotidyl transferase
Tm	tunicamycin

TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UPR	unfolded protein response
V37	valine at amino acid position 37
V37E	valine to glutamic acid substitution at amino acid position 37
V37E-GFP	V37E Cx30 mutant linked to green fluorescent protein
V37I	valine to isoleucine substitution at amino acid position 37
VVAA	valine-valine-alanine-alanine sequence motif
wt	wild-type

Chapter 1

1 Literature Review

1.1 Connexins and Gap Junctions

Connexins (Cx) are polytopic integral membrane proteins that make up the gap junction protein family, of which there are 21 members in humans (Sohl and Willecke, 2003; Sohl and Willecke, 2004). Connexins are named according to their molecular weights in kilodaltons (kDa) and are categorized into α, β, γ, δ and ε subgroups based on sequence homology (Cruciani and Mikalsen, 2006; Mese et al., 2007; Sonntag et al., 2009). The polypeptide backbone of a connexin protein spans the cell membrane four times, forming two extracellular loops and one cytoplasmic loop, with both the amino and carboxy termini extending into the cytoplasm of the cell (Falk et al., 1997; Kumar and Gilula, 1996). While all connexins share a similar structural topology and highly conserved transmembrane and extracellular domains (Maeda et al., 2009; Mese et al., 2007), the length of the cytoplasmic loop and carboxy terminal tail varies between connexin family members (Goodenough et al., 1996; Saez et al., 2003; White and Bruzzone, 1996).

Six connexins oligomerize to form a connexon, which can be termed homomeric or heteromeric according to whether it is composed of one or more connexin isoforms, respectively (Mese et al., 2007). At the plasma membrane, a connexon can act as a hemichannel to allow for communication between the cytoplasm of the cell and its extracellular environment, or dock with a connexon from an apposing cell to form a functional intercellular communication channel (Laird, 2006). These extensively characterized channels, known as gap junction channels, are termed homotypic or heterotypic depending on whether they are composed of identical or different connexons (Mese et al., 2007). The ability of connexins to co-oligomerize and form mixed channels ultimately depends on the connexin expression profile of each cell type as well as the compatibility of the connexins.

Connexins are temporally and spatially expressed in almost every cell type and tissue in the human body (Goodenough et al., 1996; Saez et al., 2003), and Cx43 (encoded by the

GJA1 gene) is the most ubiquitous, as it is endogenously expressed in over 35 distinct tissue types (Laird, 2006) including the heart, skin, brain and bone (Darrow et al., 1995; Dermietzel et al., 1989; Jones et al., 1993; Zhang et al., 1995). In addition, most cell types express multiple connexins and have overlapping connexin expression profiles (Laird, 2006; White and Bruzzone, 1996). For example, cardiomyocytes express Cx40, Cx43 and Cx45 (Coppen et al., 1999; Gourdie et al., 1993), and keratinocytes have been reported to express at least 10 different connexins including Cx26, Cx30, Cx31 and Cx43 (Di et al., 2005; Di et al., 2001). Mixed channels composed of Cx40 and Cx43 exist in the myocardium of the heart (Cottrell et al., 2001; Valiunas et al., 2000), while Cx26 and Cx30 mixed channels exist in the cochlea (Ahmad et al., 2003; Forge et al., 2003b; Sun et al., 2005; Yum et al., 2007). Mixed channels often exhibit distinct properties unique from their homomeric/homotypic counterparts (Beyer et al., 2001), or may be dominated by the properties of one of the contributing connexins (Ayad et al., 2006; Desplantez et al., 2004; Martinez et al., 2002). Importantly, not all co-expressed connexins are capable of co-oligomerizing to form mixed channels, as has been previously reported for Cx26 and Cx43 (Gemel et al., 2004).

1.2 Gap Junctional Intercellular Communication

Gap junction channels connect neighbouring cells to establish a direct cell-cell communication system through which electrical signals, ions and various hydrophilic cellular metabolites smaller than 1000 Da can pass (Alexander and Goldberg, 2003). This complex exchange process is known as gap junctional intercellular communication (GJIC), and gap junction channels aggregate into clusters on the plasma membrane, known as gap junction plaques, to allow for extensive GJIC between 2 apposing cells (Laird, 2006). Effective GJIC contributes to overall tissue maintenance and homeostasis, as it plays important roles in proliferation, differentiation and apoptosis, as well as growth and development (Choudhry et al., 1997; Kameritsch et al., 2013; Krysko et al., 2005; Langlois et al., 2007; McLachlan et al., 2007; White et al., 2007). For example, in wound healing, connexins are differentially expressed around the wound edge to regulate particular types of GJIC, altering the transjuctional molecules thought to be necessary to promote wound closure (Churko and Laird, 2013).

Two important secondary messengers that mediate intercellular signalling, cyclic adenosine monophsophate (cAMP) and inositol 1,4,5-trisphosphate (IP_3), permeate through most gap junctions, including those composed of Cx26, Cx32 and Cx43 (Bedner et al., 2006; Bedner et al., 2003; Niessen et al., 2000; Niessen and Willecke, 2000) as well as some heteromeric channels including Cx26/Cx30 and Cx43/Cx46 (Qu and Dahl, 2002; Zhang et al., 2005). However, the relative permeabilities of each channel to these molecules are variable. This is supported by Cx32 and Cx43 permeability studies, whereby Cx43 channels are 300-fold more efficient at mediating adenosine triphosphate (ATP) transfer than Cx32 channels, while adenosine permeates through Cx32 channels 10-fold better than through Cx43 channels (Goldberg et al., 1999; Goldberg et al., 2002). While the differential permeabilities of gap junction channels to certain molecules are dictated partly by limiting pore size, selectivity is also based on molecular size, flexibility, charge and charge distribution (Harris, 2007; Kwon et al., 2011; Maeda et al., 2009).

1.3 Hemichannels

In addition to forming gap junction channels, connexons have also been reported to act at the cell surface as undocked single membrane hemichannels, facilitating communication between the intra/extracellular environments. Hemichannels remain closed under physiological conditions, but open in response to changes in intra/extracellular ion concentrations, transmembane voltage, mechanical strain and post-translational modifications including phosphorylation (Wang et al., 2013). Hemichannel permeability is dictated by similar properties as for gap junction channels, as signalling molecules pass differentially through specific channels. For example, nicotinamide adenine dinucleotide (NAD^+) and prostaglandin E_2 have been reported to pass through Cx43 hemichannels (Bruzzone et al., 2001; Siller-Jackson et al., 2008), while ATP can permeate through Cx26, Cx30 or Cx43 homotypic (Essenfelder et al., 2004; Gomes et al., 2005; Tran Van Nhieu et al., 2003), as well as Cx26/Cx30 heterotypic hemichannels (Anselmi et al., 2008; Majumder et al., 2010). These molecules act on neighbouring cells in a paracrine fashion, triggering downstream cellular signalling pathways in many tissues (Wang et al., 2013). In the developing cochlea, ATP acts on purinergic receptors of inner hair cells to

elevate Ca^{2+} levels and maintain the survival of the spiral ganglion neurons (Tritsch et al., 2007). In addition, abnormal hemichannel activity has been implicated in a number of diseases caused by connexin gene mutations, including Charcot-Marie-Tooth disease (CMTX) (Liang et al., 2005), Clouston syndrome (Essenfelder et al., 2004) and keratitis-ichthyosis-deafness (KID) syndrome (Levit et al., 2012). It has also been reported that ATP release through leaky hemichannels may act in a paracrine fashion to facilitate cell death (Wang et al., 2013).

1.4 Connexin Life Cycle

Connexin proteins have relatively short half-lives of only a few hours, highlighting the importance of rapid gap junction turnover (Beardslee et al., 1998; Fallon and Goodenough, 1981; Laird et al., 1991). This continuous process is mediated by connexin synthesis and oligomerization, trafficking to the cell surface, channel assembly, internalization and degradation; processes that can all be regulated to allow cells to respond to many changes in physiological conditions (Laird, 2006). Connexins typically follow the traditional integral membrane protein secretory pathway to the cell surface, as they are co-translationally inserted in the endoplasmic reticulum (ER), pass through the Golgi apparatus and traffic to the cell surface (Laird, 2006; Musil and Goodenough, 1993). Protein folding occurs in the oxidizing environment of the ER with the assistance of a number of chaperone proteins and folding enzymes (Kleizen and Braakman, 2004), and it has been suggested that isomerases facilitate the formation of low energy disulfide bonds important for specific connexin conformation (Laird, 2006). Oligomerization has been reported to occur in the ER for a number of connexins including Cx32 (Das Sarma et al., 2002; Maza et al., 2005), but later in the *trans*-Golgi network for Cx43 and Cx46 (Koval et al., 1997; Musil and Goodenough, 1993). It has been previously suggested that following the oligomerization process, connexons are transported via microtubules to the cell surface in a closed state (Laird, 2006). An exception to this traditional secretory pathway may be Cx26, as it has also been reported to be post-translationally inserted into the ER (Ahmad and Evans, 2002) as well as directly inserted into the plasma membrane (Zhang et al., 1996), although these studies have not been widely accepted. A more recent study demonstrated that Cx26 follows the same secretory pathway as Cx43,

passing through the Golgi apparatus on the way to the cell surface, as gap junction assembly was sensitive to treatment with ER-Golgi protein transport blocker, brefeldin A (Thomas et al., 2005).

Once embedded in the plasma membrane, connexons can act as undocked hemichannels, or form gap junction channels that mediate GJIC (Laird, 2006). Gap junction plaque formation is a highly dynamic process by which newer gap junction channels form around the outer edges of an existing plaque, as demonstrated for Cx43 (Gaietta et al., 2002). Aged gap junction channels in the center of the plaque are destined for internalization by endosomal pathways or as double-membrane annular junctions termed connexosomes (Laird, 2006). Once internalized, gap junctions and connexins may be degraded by lysosomes or proteosomes, as there is evidence supporting both pathways (Laing et al., 1997; Qin et al., 2003). However, one study reported that Cx43 could be recycled back to the cell surface to participate once again in gap junction formation (VanSlyke and Musil, 2005).

1.5 Epidermal Differentiation

The epidermis is comprised mainly of keratinocytes, which continuously differentiate to form multiple stratified epidermal layers that maintain the integrity of the skin (Cartlidge, 2000). The skin acts as a physical barrier to protect against pathogen invasion, physical and chemical insults, as well as solute and water loss (Proksch et al., 2008). The outermost layer of the epidermis forms this specialized protective barrier, and is generated by the progressive differentiation of keratinocytes in the innermost layer, the stratum basale (Cartlidge, 2000). The stratum basale separates the epidermis from the underlying dermis and contains keratinocyte stem cells that replenish the basal keratinocyte population as they differentiate to form the suprabasal layers; the stratum spinosum and stratum granulosum (Kretz et al., 2004). The large keratinocytes in the stratum spinosum are involved in the synthesis and production of a number of large keratin molecules (Fuchs and Green, 1980), while granular keratinocytes produce lipids that contribute to the hydrophobic barrier of the stratum corneum (Proksch et al., 2008). Keratinocytes then undergo terminal differentiation and programmed cell death to form the outer epidermal layer, a process that shares some similarities with apoptosis,

including morphological changes and fragmented nuclei, but occurs through a distinct mechanism (Lippens et al., 2005). The dead cells residing in the stratum corneum maintain their enzymatic activity (Churko and Laird, 2013), differentiating them from apoptotic cells. Finally, as subcorneal keratinocytes continue to differentiate, cellular connections in the outermost corneal layers deteriorate and the outer layer of the epidermis is sloughed off (Cartlidge, 2000).

1.6 Connexins in the Epidermis

Connexins play an important role in the proliferation and differentiation of the epidermis (Choudhry et al., 1997; Langlois et al., 2007; Risek et al., 1992). Human epidermal keratinocytes contain messenger RNA (mRNA) for at least 10 different connexins, and many are differentially expressed at the protein level throughout the epidermal strata, excluding the stratum corneum (Di et al., 2001; Kretz et al., 2004). Connexin expression overlaps in the layers of the epidermis, however, distinct subpopulations of keratinocytes express particular connexins. For example, Cx26 is expressed in the basal and granular layers, Cx30 and Cx45 are localized mainly to the granular layer with some expression in the stratum spinosum, and Cx40 is uniformly expressed across both suprabasal layers (Di et al., 2005; Di et al., 2001). Rat epidermal keratinocytes (REKs) express mRNA for a similar number of connexins (Maher et al., 2005), and adult rat epidermis exhibits the overlapping expression of Cx26, Cx31, Cx31.1, Cx37 and Cx43 (Goliger and Paul, 1994; Risek et al., 1992). In the epidermis of all mammalian species, complex connexin expression facilitates GJIC within an epidermal layer as well as across different epidermal layers (Richard, 2000), forming intricate communication systems to regulate keratinocyte proliferation and differentiation. Furthermore, connexin mutations that disregulate epidermal GJIC have been reported to result in a number of hyperproliferative and mutilating skin diseases (Bakirtzis et al., 2003; Bondeson et al., 2006; Jan et al., 2004; Smith et al., 2002; Tattersall et al., 2009).

1.7 Hearing and Sound Transduction

Hearing is a complex process involving the transmission of sounds from the outer ear to a compartmentalized structure in the inner ear known as the cochlea. Middle ear bones

convert sound waves into pressure waves that travel through the cochlea, along a basilar membrane where sensory hair cells and supporting cells of the Organ of Corti are located (Fettiplace and Hackney, 2006). The cochlea contains two fluid-filled compartments, which hold the perilymph or endolymph, the latter of which is high in K^+ (Kikuchi et al., 2000), establishing the endocochlear potential (EP) necessary for sensory transduction (Wangemann, 2006). When an acoustic stimulus activates hair cells, endolymphatic K^+ rushes into them, relaying important information about the sound to the brain (Fettiplace and Hackney, 2006). K^+ is then released into the extracellular space of the Organ of Corti, and two gap junction systems, known as the epithelial and connective tissue gap junction networks, play important roles in the recycling of K+ back to the endolymph (Hibino and Kurachi, 2006; Kikuchi et al., 2000) to maintain the EP (Hibino and Kurachi, 2006; Kikuchi et al., 2000; Wangemann, 2006).

1.8 Connexins in the Inner Ear

To date, five connexins, including Cx26, Cx29, Cx30, Cx31 and Cx43, have been identified in the epithelial and connective tissue gap junction networks of the mature mammalian cochlea, with evidence of overlapping expression (Nickel and Forge, 2008). For example, while Cx31 expression is restricted to the connective tissue network (Xia et al., 2000), Cx26, Cx30 and Cx43 are expressed in both (Lautermann et al., 1998). Importantly, Cx26 and Cx30 are the most predominantly expressed connexins in the inner ear (Hoang Dinh et al., 2009), and many studies support the notion that they are capable of forming functional mixed channels (Ahmad et al., 2003; Forge et al., 2003a; Forge et al., 2003b; Sun et al., 2005; Zhao and Santos-Sacchi, 2000). Mutations in these two connexins account for over half of all reported cases of inherited pre-lingual non-syndromic hearing loss (Chang et al., 2009; Schutz et al., 2010; Wang et al., 2011), which is classified as hearing loss in the absence of other disease manifestations. While it has been suggested that connexin mutations commonly affect K^+ recycling due to their expression in cochlear gap junction networks, evidence also supports that mutations cause defects in biochemical or metabolic coupling rather than ionic coupling (Beltramello et al., 2005; Schutz et al., 2010; Zhang et al., 2005). Furthermore, Cx30 knock out (*Gjb6*$^{-/-}$, referred to as Cx30$^{-/-}$ in this thesis) and epithelial network-Cx26

ablated mice were found to be deaf, as the loss of these connexins resulted in epithelium degeneration and loss of EP. However, the cochlea and vestibular organs were normally shaped, suggesting that connexins may not play essential structural roles in cochlear development (Cohen-Salmon et al., 2002; Teubner et al., 2003).

1.9 Connexins in Disease

Connexin mutations have been linked to a number of distinct diseases. Oculodentodigital dysplasia (ODDD) is a rare disease distinctly associated with Cx43 mutations, and to date, over sixty five mutations have been identified (Huang et al., 2013; Paznekas et al., 2009). Since Cx43 is ubiquitously expressed, this disease manifests in a number of ways and is characterized by craniofacial abnormalities, syndactyly of the digits, as well as ophthalmic and dental abnormalities (Paznekas et al., 2003). In addition, hearing loss and distinct hyperproliferative skin disorders have been associated with mutations in Cx26, Cx30, Cx30.3 and Cx31 (Laird, 2006). Mutations in both Cx30.3 and Cx31 result in erythrokeratoderma variabilis (EKV) (Richard et al., 2003; Tattersall et al., 2009), while hidrotic ectodermal dysplasia (HED), also known as Clouston syndrome, is distinctly caused by Cx30 mutations, of which there have been four identified to date (Baris et al., 2008; Common et al., 2002; Smith et al., 2002). Other diseases including KID, Vohwinkel and Bart-Pumphrey syndromes, previously linked to Cx26 mutations (Lee and White, 2009), have also been associated with mutations in the highly homologous Cx30 family member (Jan et al., 2004; Nemoto-Hasebe et al., 2009), highlighting the heterogeneity of these particular connexin-linked skin diseases. Furthermore, many of these hyperproliferative disorders are associated with hearing loss, and to date, approximately eight Cx30 mutations and over one hundred Cx26 mutations have been linked to various forms of hearing loss, including non-syndromic hearing loss (Baris et al., 2008; Nemoto-Hasebe et al., 2009; Wang et al., 2011; Zhang et al., 2005).

1.10 Cx30 Mutations Linked to Various Skin Diseases and Non-Syndromic Hearing Loss

Cx30, encoded by the *GJB6* gene, is an understudied member of the connexin protein family, and is highly expressed in the mammalian epidermis, inner ear and brain (Dahl et

al., 1996; Scott et al., 2012). To date, seven autosomal dominant missense mutations have been identified in the Cx30 gene that result in skin disease, hearing loss or a combination of both **(Fig. 1.1)**. Clouston syndrome linked distinctly to Cx30 mutations (Lamartine et al., 2000a), is a rare autosomal dominant disease first described by Clouston (1929). This disease is characterized mainly by nail deformities and increased susceptibility to nail infections, partial to complete hair loss and palmoplantar hyperkeratosis (PPK), which includes thickening of the skin on the palms of the hands and soles of the feet (Ando et al., 1988; Pierard et al., 1979; Rajagopalan and Tay, 1977). Clouston also reported that some patients had craniofacial abnormalities, ocular problems, as well as clubbed fingers and toes (Fraser and Der Kaloustian, 2001), while hearing loss and abnormal cardiac findings have also been reported in some cases (Christianson and Fourie, 1996; Lamartine et al., 2000b). Clouston syndrome affects approximately 1 in 100,000 people, and while the disease has affected families of different ethnic origins (Christianson and Fourie, 1996; Kibar et al., 2000; Lamartine et al., 2000b; Rajagopalan and Tay, 1977), it is most prevalent in the French-Canadian population due to a founder effect (Kibar et al., 2000). This loss of genetic variation occurs when a small number of individuals from a large population segregate to form a new population. Four particular Cx30 mutations have been identified, including G11R (11^{th} amino acid - glycine to arginine substitution) (Zhang et al., 2003), V37E (37^{th} amino acid - valine to glutamic acid) (Smith et al., 2002), D50N (50^{th} amino acid - aspartic acid to asparagine) (Baris et al., 2008) and A88V (88^{th} amino acid - alanine to valine) (Lamartine et al., 2000b). Interestingly, one patient harbouring the V37E mutant exhibited symptoms of KID syndrome, which is characterized by a similar skin phenotype as Clouston syndrome, but also includes erythrokeratoderma, hearing loss and vascularising keratitis (Jan et al., 2004). The V37E and D50N mutations have been identified in sporadic cases of Clouston syndrome (Baris et al., 2008; Smith et al., 2002), meaning these mutations appear in a more isolated and scattered fashion, while the G11R and A88V mutations are most common (Lamartine et al., 2000b) and have become the mutants of most research focus. It has been reported that while the G11R and A88V mutants were unable to form gap junctions at the cell surface of HeLa cells and keratinoctyes (Common et al., 2002), they exhibited abnormal hemichannel activity, as

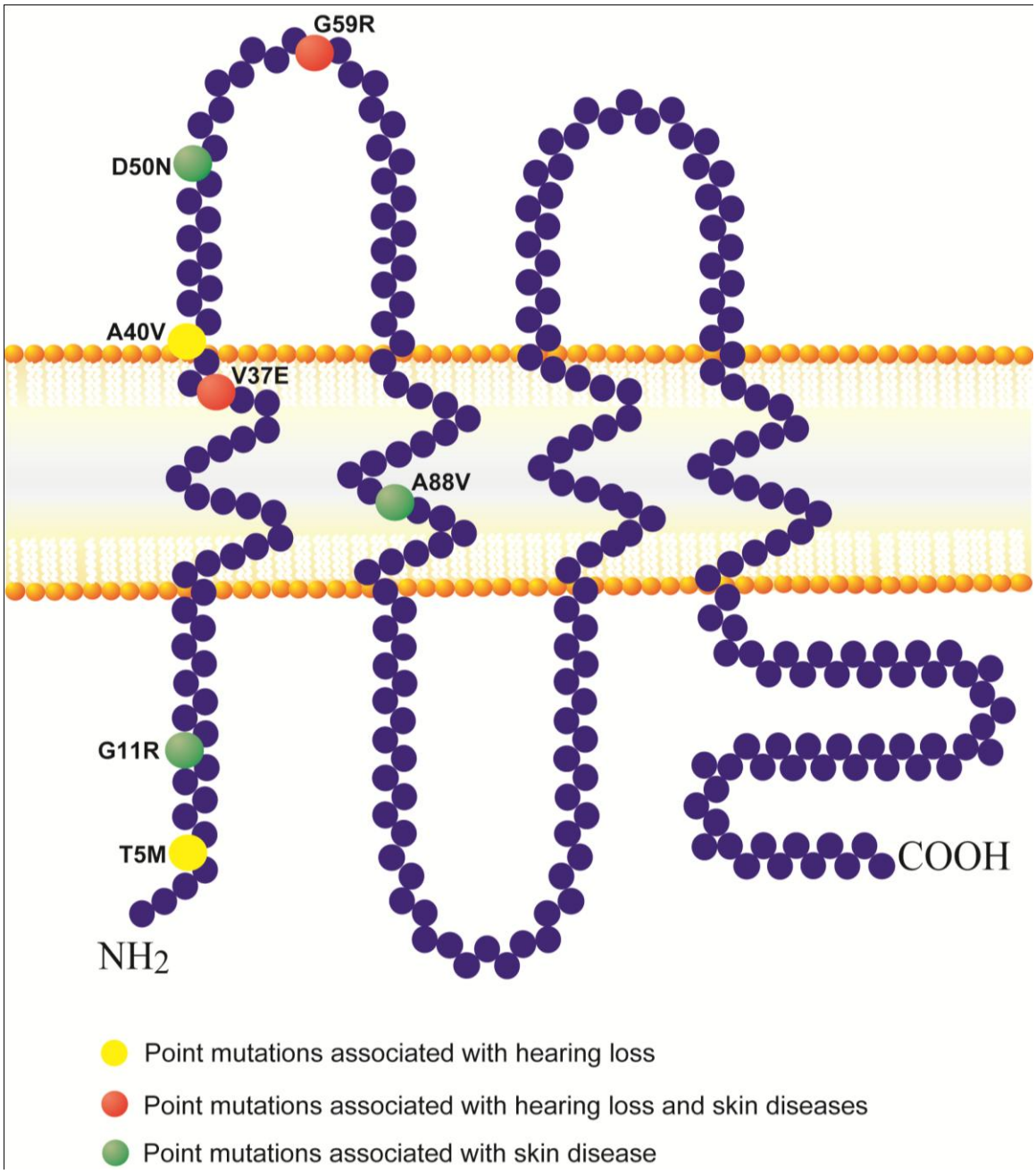

Figure 1.1: Hearing loss- and skin disease-linked Cx30 mutations

To date, seven Cx30 mutants have been reported to cause various skin diseases, hearing loss or a combination of both. These single amino acid substitution mutations are found within the 1st half of the Cx30 amino acid polypeptide.

shown by the release of ATP and subsequent cell death (Essenfelder et al., 2004). The amino terminal and 2nd transmembrane connexin domains, where G11R and A88V are located, have been suggested to play important roles in stabilizing intra-connexin interactions that dictate channel permeability, as has been reported for Cx26 (Maeda et al., 2009). Mutations in these critical domains may result in open hemichannels, increasing their permeability and causing them to leak important cellular molecules. Furthermore, premature cell death as a result of abnormal hemichannel activity may explain the PPK associated with Clouston syndrome, however further studies are necessary to determine how these particular point mutations are related to the development of this disease.

A novel G59R mutation (59th amino acid - glycine to asparagine) has been recently identified in the Cx30 gene, and the patient with this mutation presented with symptoms resembling Bart-Pumphrey and Vohwinkel syndromes (Nemoto-Hasebe et al., 2009). Bart-Pumphrey syndrome is characterized by hearing loss, knuckle pads, PPK and leukonychia (white discolouration of the nails) (Bart and Pumphrey, 1967), while Vohwinkel syndrome symptoms include mutilating keratoderma and the formation of constricting fibrous bands around fingers and toes, which result in their spontaneous auto-amputation (pseudoainhum) (Goldfarb et al., 1985). The patient harbouring the G59R Cx30 mutation experienced knuckle pads, severe hearing loss, mild PPK, and pseudoainhum of the toes (Nemoto-Hasebe et al., 2009). These two diseases have never before been linked to Cx30 mutations, as they are more frequently caused by mutations in Cx26 (Alexandrino et al., 2005; Bondeson et al., 2006; Maestrini et al., 1999; Marziano et al., 2003; Richard et al., 2004), thus the G59R Cx30 mutant remains poorly understood. However, D66H and G59A Cx26 mutants linked to Vohwinkel syndrome are localized mainly to intracellular compartments and are non-functional (Marziano et al., 2003; Thomas et al., 2004).

In addition to skin abnormalities, two particular Cx30 gene mutations have been linked to non-syndromic hearing loss (Grifa et al., 1999; Wang et al., 2011), and both act through distinct mechanisms. Wang et al. (2011) demonstrated that the A40V mutant (40th amino acid - alanine to valine) was retained in the ER and the Golgi apparatus, rendering the

mutant unable to form gap junction channels at the plasma membrane. This amino acid is highly conserved among many connexins including Cx26, Cx30, Cx30.3, Cx31 and Cx32, and is located in the 1st transmembrane domain suggested to be important for protein folding and stability (Maeda et al., 2009). This mutant may negatively affect overall K^+ recycling in the inner ear as it obviously impairs the formation of gap junction channels (Wang et al., 2011). Another mutation reported to affect K^+ recycling through Cx30-based gap junctions is the T5M mutation (5th amino acid – threonine to methionine) (Grifa et al., 1999), however its functional capacity remains controversial. Although the T5M mutation does not impair the trafficking of the protein to the plasma membrane of HeLa cells or keratinocytes (Common et al., 2002; Zhang et al., 2005), studies have demonstrated its inability to pass electrical currents and small molecules (Common et al., 2002; Grifa et al., 1999). As previously discussed, more recent research has suggested that T5M-based channels may only be defective in their capacity to pass larger molecules, indicating that metabolic coupling and not K^+ recycling impairment may be the cause of hearing loss related to this mutant (Schutz et al., 2010; Zhang et al., 2005). Importantly, the amino terminal domain has been suggested to play a critical role in channel gating and permeability (Maeda et al., 2009), and the T5M substitution may change which molecules or ions can permeate through the gap junction channels. Nevertheless, further characterization of all Cx30 mutants is critical for the mechanistic understanding of how each mutant is linked to a specific disease. In my thesis I will focus on four mutants (T5M, V37E, G59R and A88V) that cause skin disease and/or hearing loss.

1.11 Apoptosis

Apoptosis, also known as programmed cell death, is a highly regulated and complex three-stage process, that results in the eventual degradation and removal of damaged cells (Abraham and Shaham, 2004; Elmore, 2007; Saraste and Pulkki, 2000). In contrast to this controlled process, necrosis is an uncontrolled form of cell death characterized by cellular swelling, degradation of subcellular organelles, rupture of the cell membrane and leakage of intracellular components into the extracellular environment (Krysko et al., 2008). The initiation and commitment stages of apoptosis involve the activation/cleavage

of a number of proteases called caspases, which work together to subsequently cleave cellular substrates; characteristic of the degradation phase (Saraste and Pulkki, 2000). During the last apoptotic stage, a number of distinct morphological changes become visible. These include shrinkage of the cell as structural proteins are cleaved (Brown et al., 1997; Orth et al., 1996; Wen et al., 1997), activation of DNase enzymes which degrade DNA (Liu et al., 1997; Sakahira et al., 1998), fragmentation of the nucleus, cell detachment and the formation of apoptotic bodies which are later phagocytosed by neighbouring cells (Elmore, 2007; Kerr et al., 1972). The activation of caspases has been reported to occur mainly through two well-established apoptotic pathways, the extrinsic death receptor-mediated pathway and the intrinsic mitochondrial-mediated pathway (Elmore, 2007). In the intrinsic pathway, death stimuli activate a number of signalling cascades that directly or indirectly result in the release of cytochrome c from the mitochrondria, triggering the activation of caspase-9. In the extrinsic pathway, a number of death ligand-receptor interactions (Ashkenazi and Dixit, 1998; Marsters et al., 1998) trigger the activation of caspase-8 (Elmore, 2007). Both pathways specifically result in the downstream activation of caspase-3, which triggers the final degradation stage of apoptosis (Elmore, 2007).

1.12 Connexins in Cell Death

The role of connexins in cell survival and cell death is poorly understood, however studies suggest that connexins play critical roles in mediating the passage of survival and death signals between cells (Decrock et al., 2009b; Krysko et al., 2005; Vinken et al., 2006). In the epidermis, connexins play a role in differentiation-dependent programmed cell death known as cornification, which is distinct from apoptosis (Lippens et al., 2005), however connexins have also been implicated in apoptotic signalling. In particular, GJIC has been suggested to relay pro-apoptotic signals to coupled cells in a connexin type-dependent manner and increasing evidence suggests that hemichannels also play a role (Decrock et al., 2009a; Decrock et al., 2009b; Kalvelyte et al., 2003; Kameritsch et al., 2013). HeLa cells expressing Cx37, Cx40 or Cx43 were exposed to streptonigrin and α-Fas, which respectively trigger the intrinsic and extrinsic apoptotic pathways, and apoptosis was significantly higher in Cx43-expressing cells compared to empty vector

controls and Cx37-expressing cells. When gap junction function was inhibited with pharmacological agents, apoptosis was reduced in Cx43-expressing cells, providing evidence for the role of gap junctions in differentially mediating apoptotic signals (Kameritsch et al., 2013).

Many disease-linked connexin mutations result in trafficking defects that prevent the formation of gap junctions at the plasma membrane (Common et al., 2002; Di et al., 2002; Gottfried et al., 2002; Tattersall et al., 2009; Wang et al., 2011), while others alter gap junction channel or hemichannel function (Gerido et al., 2007; Grifa et al., 1999; Mese et al., 2011; Shao et al., 2012; Stong et al., 2006; Thomas et al., 2004). Additionally, a number of these mutations have been linked to cell death. For example, Cx26 mutations linked to KID syndrome induce cell death as a result of abnormal hemichannel activity (Gerido et al., 2007; Lee et al., 2009; Mhaske et al., 2013; Montgomery et al., 2004; Stong et al., 2006; Terrinoni et al., 2010), where hemichannels are inappropriately gated open under normal physiological conditions. In addition, G11R and A88V Cx30 mutants also exhibit leaky hemichannels, and released ATP may act on surrounding cells in a paracrine fashion to induce cell death (Essenfelder et al., 2004). When connexin mutations result in trafficking defects, mutant connexins are often retained in the ER or Golgi apparatus (Common et al., 2002; Tattersall et al., 2009; Wang et al., 2011). In particular, the accumulation of mutant connexin proteins in the ER has previously been shown to induce cell death through an ER stress-mediated pathway, as reported for a number of Cx31 mutations linked to EKV (Di et al., 2002; He et al., 2005; Tattersall et al., 2009). Further studies are necessary to dissect the mechanisms by which mutant connexins can induce cell death and ultimately cause disease.

1.13 ER Stress and the Unfolded Protein Response

The ER is a unique subcellular organelle that functions to synthesize and properly fold and modify newly synthesized proteins (Berridge, 2002). The ER contains a number of chaperone proteins, including calreticulin and calnexin, that are involved in the quality control process (Hirsch et al., 2004). Chaperone proteins aid in the process of protein packaging, and play an important role in sensing misfolded proteins, which they either attempt to fold, or target for degradation (Groenendyk and Michalak, 2005). The

accumulation of persistently misfolded proteins in the ER triggers ER stress and an adaptive cellular response known as the unfolded protein response (UPR) (Groenendyk and Michalak, 2005; Malhotra and Kaufman, 2007; Rasheva and Domingos, 2009). Glucose regulated protein 78 (GRP78) is a luminal ER chaperone protein normally bound to three UPR sensors known as inositol requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6) and double stranded RNA-activated protein kinase-like ER kinase (PERK) (Malhotra and Kaufman, 2007). GRP78 directly binds misfolded proteins and results in the co-ordinated activation of the UPR upon releasing these sensors (Bertolotti et al., 2000; Shen and Prywes, 2005). Activation of the IRE1 pathway primarily results in the induction of a number of genes involved in endoplasmic reticulum-associated degradation (ERAD), as well as an increase in lipid synthesis, which expands the size of the ER to accommodate new folding requirements (Berridge, 2002; Malhotra and Kaufman, 2007). The PERK pathway impedes protein translation and synthesis, and converges with the ATF6 pathway to up-regulate a number of chaperone proteins, as well as induce apoptosis (Teske et al., 2011).

The UPR occurs in a number of normal processes including plasma cell differentiation (Iwakoshi et al., 2003), muscle development (Nakanishi et al., 2005) and keratinocyte differentiation (Sugiura et al., 2009). In contrast, the UPR has also been implicated in a number of diseases including Alzheimer's disease (Imaizumi et al., 2001), Huntington's disease (Vidal et al., 2012), atherosclerosis (Zhou et al., 2005) and diabetes (Scheuner and Kaufman, 2008) to name a few. The UPR is also involved in normal lens development, however, autosomal dominant Cx50 mutants (G22R and S59P) that result in cataracts are retained in the ER and elevate the expression of UPR markers (Alapure et al., 2012). In addition, autosomal dominant (G12R, G12D, R42P, C86S and F137L) (Di et al., 2002; He et al., 2005) and recessive (L34P) (Gottfried et al., 2002) Cx31 mutants linked to EKV, exhibited trafficking defects that could manifest as an UPR. In fact, while all autosomal dominant Cx31 mutants induced cell death (Di et al., 2002; He et al., 2005), some were indeed reported to induce an ER stress-mediated UPR (Tattersall et al., 2009).

1.14 Hypothesis

We hypothesized that Cx30 mutations result in various skin diseases and non-syndromic hearing loss through several distinct mechanisms. To test this hypothesis, we characterized four Cx30 mutants (T5M, V37E, G59R and A88V) in connexin-deficient HeLa cells and GJIC-competent REKs, and examined the ability of each mutant to form functional gap junction channels and hemichannels at the cell surface. In addition, Cx30 mutants were assessed for their ability to induce cell death and evoke an unfolded protein response. Finally, trafficking-defective Cx30 mutants were co-expressed with wild-type Cx30 and Cx26 to determine if they could be rescued from intracellular compartments to the cell surface. Cx30 mutants were also examined for their ability to out-compete co-expressed wt Cx30 and Cx26, known as exhibiting dominant-negative and transdominant effects, respectively, causing the wt connexins to be retained inside the cell. The overall goal of these studies was to determine the mechanisms underlying each disease-linked Cx30 mutant.

1.15 Objectives

The specific objectives of this study were to:

1. Characterize the subcellular localization and function of T5M, V37E, G59R and A88V Cx30 mutants in REKs and HeLa cells.

2. Determine the cell death mechanisms activated by V37E and A88V Cx30 mutants.

3. Examine the potential dominant-negative and transdominant effects of Cx30 mutants on co-expressed wild-type Cx30 and Cx26.

1.16 References

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Chapter 2

2 Skin diseases and non-syndromic hearing loss linked to Cx30 mutations arise through several distinct mechanisms

Connexin 30 (Cx30), a member of the large gap junction protein family, plays a role in the homeostasis of the epidermis and inner ear through gap junctional intercellular communication (GJIC). Here, we investigated the underlying mechanisms of four autosomal dominant Cx30 gene mutations linked to hearing loss and/or various skin diseases. First, the T5M mutant linked to non-syndromic hearing loss formed functional gap junction channels and hemichannels, similar to wild-type Cx30. The loss-of-function V37E mutant associated with keratitis-ichthyosis-deafness (KID) syndrome was retained in the endoplasmic reticulum and significantly induced unfolded protein response (UPR)-mediated apoptosis. The G59R mutant linked to Vohwinkel and Bart-Pumphrey syndromes was retained primarily in the Golgi apparatus and exhibited loss of gap junction channel and hemichannel function. Lastly, the A88V mutant related to Clouston syndrome also significantly induced apoptosis, primarily through an UPR-independent mechanism. Collectively, we discovered that four unique Cx30 mutants cause disease through different mechanisms that likely include their selective dominant-negative and transdominant effects on co-expressed connexins, highlighting the overall complexity of connexin-linked diseases and the importance of GJIC in disease prevention.

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2.1 Introduction

Gap junctions are clusters of specialized intercellular channels that regulate the direct exchange of ions and various hydrophilic cellular metabolites smaller than 1000 Da, a process known as gap junctional intercellular communication (GJIC) (Alexander and Goldberg, 2003). Two inter-docked connexons (hemichannels), one from each of two apposing cells, form a functional gap junction channel. Each connexon is composed of six oligomerized connexin (Cx) subunits, and to date, the connexin family consists of 21 members in humans (Sohl and Willecke, 2003; Sohl and Willecke, 2004). Interestingly, while the primary function of gap junction channels is to facilitate intercellular communication, hemichannels have also been reported to exist and function at the cell surface in an undocked state, permitting the transfer of molecules between extracellular and intracellular environments (Anselmi et al., 2008; Burra and Jiang, 2011; Tong et al., 2007). Hemichannels formed from single or multiple types of connexins are termed homomeric and heteromeric, respectively, and gap junction channels are characterized as homotypic or heterotypic according to whether their channels are composed of the same or different connexons (Burra and Jiang, 2011). Channel composition depends on the connexin expression profile of each cell and tissue type, as well as the natural compatibility of the connexins to intermix (Beyer et al., 2001; Burra and Jiang, 2011; Laird, 2006).

Connexins are highly expressed in virtually all tissues in the human body, and GJIC plays an essential role in the regulation of cellular and physiological processes including proliferation, differentiation, apoptosis, growth and development (Alexander and Goldberg, 2003; Choudhry et al., 1997; Decrock et al., 2009; Kumar and Gilula, 1996; McLachlan et al., 2007). In the inner ear and the skin, proper connexin expression and function directly relate to the maintenance of cochlear homeostasis and epidermal differentiation, respectively (Langlois et al., 2007; Wangemann, 2006; Zhao et al., 2006). In the cochlea, Cx26, Cx29, Cx30, Cx31 and Cx43, found in the epithelial and connective tissue gap junction networks, play a crucial role in sound transduction. Cx26, and possibly other connexins, are thought to be involved in the recycling of K^+ through the supporting cells back to the endolymphatic space, for potential re-entry into sensory cells

when activated by an acoustic stimulus (Kikuchi et al., 2000; Nickel and Forge, 2008). Interestingly, at least seven connexins, including Cx26, Cx30 and Cx43, are temporally and spatially expressed at the protein level in the human epidermis with overlapping distribution in the various non-cornified epidermal strata (Di et al., 2001; Kretz et al., 2004). While GJIC plays an important role in epidermal differentiation, it is also critical to the wound healing process (Churko and Laird, 2013; Langlois et al., 2007). Here we focus on Cx30, which in humans, is predominantly expressed in the inner ear and epidermis (Di et al., 2001; Nickel and Forge, 2008).

Connexin mutations have been linked to a number of different diseases ranging from developmental disorders to congenital cataracts (Laird, 2006). Mutations in the genes encoding Cx26, Cx30, Cx30.3 and Cx31 in particular have been linked predominantly to hearing loss and various skin diseases (Di et al., 2001). Importantly, mutations in Cx30 and Cx26, the most predominant connexins in the inner ear (Hoang Dinh et al., 2009), are the leading cause of nearly half the cases of inherited prelingual non-syndromic hearing loss (Chang et al., 2009; Schutz et al., 2010; Wang et al., 2011). In particular, seven distinct single amino acid substitutions in the 1st half of the coding sequence of Cx30 are responsible for hearing loss and/or skin disease. The T5M and A40V mutations have been linked to non-syndromic hearing loss, as no other tissues or organs where Cx30 is expressed were affected (Grifa et al., 1999; Wang et al., 2011). Interestingly, hidrotic ectodermal dysplasia (HED), commonly known as Clouston syndrome, is a skin disease distinctly linked to G11R (Chen et al., 2010; Common et al., 2002; Zhang et al., 2003), V37E (Common et al., 2002; Jan et al., 2004; Smith et al., 2002), D50N (Baris et al., 2008) and A88V (Common et al., 2002; Essenfelder et al., 2004) Cx30 mutations. This rare disease has a founder effect within the French-Canadian population and is characterized by palmoplantar hyperkeratosis (PPK), nail dystrophies, and partial to complete alopecia (Kibar et al., 2000; Zhang et al., 2003). In some patients, other symptoms like ocular and craniofacial abnormalities, hearing loss and abnormal sweating and cardiac findings have been reported (Christianson and Fourie, 1996; Fraser and Der Kaloustian, 2001; Lamartine et al., 2000). Interestingly, one patient harbouring a V37E Cx30 mutation was diagnosed with keratitis-ichthyosis-deafness (KID) syndrome, which is commonly associated with Cx26 mutations. This patient experienced Clouston

syndrome-like symptoms, but also hearing impairment and vascularising keratitis (Jan et al., 2004). Finally, a G59R Cx30 mutation results in the development of classical Vohwinkel syndrome and Bart-Pumphrey syndrome (Nemoto-Hasebe et al., 2009), which are also diseases most commonly caused by mutations in Cx26 (Bakirtzis et al., 2003; Jan et al., 2004; Richard et al., 2004). Both syndromes result in PPK and sensorineural hearing loss, however, Bart-Pumphrey syndrome can be distinguished by the formation of knuckle pads, while patients with Vohwinkel syndrome develop constriction bands that cause spontaneous auto-amputation of the digits (pseudoainhum) (Bakirtzis et al., 2003; Richard et al., 2004).

Previously, studies on a few of the Cx30 disease-linked mutations have revealed reduced or abolished gap junction function, as the majority of mutant proteins are retained in subcellular compartments (Common et al., 2002; Essenfelder et al., 2004; Wang et al., 2011). Typically, most connexins follow the traditional secretory pathway by folding in the endoplasmic reticulum (ER), oligomerizing into connexons either in the ER or Golgi apparatus and by employing microtubules for efficient trafficking to the plasma membrane (Laird, 2006). Disruptions to any stage of this connexin transport process can have detrimental cellular effects and commonly result in connexin-linked diseases (Laird, 2006). In particular, ER stress can result from the accumulation of misfolded proteins in the ER, which has been reported to trigger a process known as the unfolded protein response (UPR) (Malhotra and Kaufman, 2007). Typically, quality control mechanisms involving chaperones in the ER facilitate the re-folding of misfolded proteins for export out of the ER (Groenendyk and Michalak, 2005). The UPR involves the activation of three ER membrane-bound sensors that work collectively to attenuate mRNA translation and protein synthesis, increase the folding capacity of the ER through the up-regulation of chaperone proteins, increase lipid synthesis and force misfolded proteins through an ER-associated degradation (ERAD) pathway to relieve ER stress and maintain cellular homeostasis (Malhotra and Kaufman, 2007). Failure of these processes to reduce ER stress results in the induction of apoptosis (Groenendyk and Michalak, 2005; Rasheva and Domingos, 2009).

Currently, it is poorly understood how Cx30 mutations manifest into syndromic and non-syndromic diseases involving the skin and cochlea. In the present study, we characterized four different Cx30 mutants linked to non-syndromic hearing loss, Clouston syndrome, KID syndrome and Vohwinkel/Bart-Pumphrey syndromes to gain critical insight into the mechanisms behind these distinct disease manifestations. Our results indicate that the T5M mutant associated only with non-syndromic hearing loss formed functional gap junction channels, while the skin disease-linked mutants were primarily retained in intracellular compartments, reducing channel function. In addition, since we showed that the V37E and A88V mutants, linked to KID syndrome and/or Clouston syndrome, induced cell death in keratinocytes, we investigated the potential mechanisms of cell death and discovered that both mutants induced apoptosis. In particular, we propose that the V37E mutant evoked an UPR which drove cells into apoptosis, while the A88V mutant may exert its effects through a different mechanism. Finally, we demonstrated that skin disease-linked mutants are not rescued by co-expressed wild-type (wt) Cx30 or Cx26 in rat epidermal keratinocytes (REKs), and in particular, the V37E and A88V mutants may have a dominant-negative effect on these co-expressed connexins. Collectively, these studies demonstrate the complexity of the mechanisms involved in connexin-linked diseases, as the T5M Cx30 mutant exhibited intercellular channel and hemichannel function, yet still causes disease. Additionally, the V37E mutant highlights Cx30 mutant-driven UPR and apoptosis as a previously unreported mechanism of how Cx30 mutations may cause disease.

2.2 Materials and Methods

2.2.1 Generation of cDNA constructs

Mouse Cx30 complementary DNA (cDNA) encoded within the pBluescript vector, kindly provided by Dr. C. C. Naus (UBC, Vancouver, BC), was digested with *Xho*I and *Not*I to remove the green fluorescent protein (GFP) cDNA sequence as described by Thomas et al. (2004). Cx30 mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer's instructions. The following primer pairs were used to create the Cx30 mutations. The nucleotide change is underlined in each case:

T5M: sense, 5'-GCACGATGGACTGGGGGATGCTGCACACCGTCATCGG-3'; antisense, 5'-CCGATGACGGTGTGCAGCATCCCCCAGTCCATCGTGC-3';

V37E: sense, 5'-CCGAGTCATGATCCTAGAGGTGGCTGCCCAG-3'; antisense, 5'-CTGGGCAGCCACCTCTAGGATCATGACTCGG-3';

A88V: sense, 5'-CTTTGTGTCTACCCCAGTCCTGTTGGTGGCCATGC-3'; antisense, 5'-GCATGGCCACCAACAGGACTGGGGTAGACACAAAG-3'.

The G59R mutant was purchased from Norclone Biotech Laboratories (London, ON). All mutations were validated by sequencing, indicating the presence of the mutation and ensuring that no other mutations were introduced.

To create GFP-tagged constructs and red fluorescent protein (RFP)-tagged Cx30 (Cx30-RFP), polymerase chain reaction (PCR) was performed to introduce *Xho*I and *Eco*RI restriction sites to the 5' and 3' ends of the Cx30 and Cx30 mutant sequences, respectively, effectively removing the stop codon. Following digestion with *Xho*I and *Eco*RI restriction enzymes, the PCR products were cloned into the pEGFP-N1 (Clontech, Palo Alto, CA) and pTagRFP-N (Cedarlane, Burlington, ON) vectors to produce GFP-tagged constructs and Cx30-RFP, respectively. All constructs were sequenced for verification.

2.2.2 Cell culture and transient transfections

GJIC-competent REKs, kindly provided by Dr. Vincent C. Hascall (Cleveland Clinic, Cleveland, OH) and connexin-deficient HeLa cells (ATCC, Manassas, VA) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (Invitrogen, Burlington, ON), in a humidified incubator maintained at 37°C with 5% CO_2 as previously described (Maher et al., 2005). Cells were passed once they reached 80-100% confluency using 0.25% trypsin-ethylene-diamine-tetraacetic acid (trypsin-EDTA) (Invitrogen), and were cultured in 35 mm or 60 mm plastic tissue culture dishes for all experimental procedures. Prior to transfection, all cells were grown to 65-80% confluency, and those destined for immunolabeling or cell death assays were also

grown on glass coverslips. Cells were transfected with 2-6 µg of DNA using Lipofectamine 2000 (Invitrogen) in the presence of low serum optimal minimal essential medium (Opti-MEM) (Invitrogen) as previously described (Penuela et al., 2007), or using the Polyplus JetPRIME-mediated transfection kit (VWR International, Mississauga, ON) according to the manufacturer's instructions. As controls, cells were exposed to transfection reagents and the appropriate media without the addition of any DNA, and are referred to in the following experiments and figures as wt. As a second control, a population of cells was also transfected with a vector encoding free GFP. Co-transfections were performed using the Polyplus JetPRIME transfection method by mixing 1µg of Cx30- or Cx26-RFP and 1 µg of each GFP-tagged Cx30 mutant. All transfections were terminated after 24 hours. Positive controls for the induction of ER stress or apoptosis included cells plated in parallel, treated for 24 hours with 2 µg/mL tunicamycin (Tm) or 0.5 µg/mL staurosporine (Stauro), respectively (both from Sigma Aldrich, St Louis, MO).

2.2.3 Immunolabelling

Cells grown in monolayer on glass coverslips were fixed with 10% neutral buffered formalin (NBF) (EMD Millipore, Billerica, MA) for 25 minutes at room temperature. In a humidified chamber, fixed cells were blocked for 30-45 minutes at room temperature in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) (Santa Cruz Biotechnology, Dallas, TX) to prevent non-specific antibody binding and 0.1% Triton X-100 (Sigma Aldrich) for permeabilization. Endogenous or ectopic connexin expression and localization was detected by labeling with rabbit anti-Cx43 (1:400, Sigma Aldrich) or anti-Cx30 (1:500, Invitrogen) antibodies for 1 hour. In some cases, cells were labeled with mouse anti-protein disulfide isomerase (PDI) (1:500, Enzo Life Sciences, Farmingdale, NY) to denote the position of the ER, mouse anti-Golgi matrix protein 130 (GM130) (1:500, BD Transduction Laboratories, Mississauga, ON) to demarcate the Golgi apparatus or rabbit anti-cleaved caspase-3 (1:1000, Cell Signaling Technology, Danvers, MA) to denote cells undergoing apoptosis. Cells were then incubated with secondary AlexaFluor555 or AlexaFluor488 conjugated antibodies (1:500, Invitrogen) for 45-60 minutes, and stained for 5-10 minutes with Hoescht 33342 (1:1000, Invitrogen)

to denote the nuclei. Cells were mounted on glass microscope slides with Airvol mounting medium. Cells co-transfected with Cx30- or Cx26-RFP and GFP-tagged Cx30 mutants were not immunolabeled, but were stained with Hoescht 33342 and mounted as described above. All slides were stored at 4°C with minimal exposure to light. Slides were imaged using a Zeiss LSM 410 confocal microscope (Thornwood, NY) equipped with a 63X lens as previously outlined by Thomas et al. (2007).

2.2.4 Microinjection assays

In order to test Cx30 and mutant gap junction function, REKs and HeLa cells ectopically expressing Cx30 or mutants were microinjected with 10 mM Alexa Fluor 350 hydrazide (Invitrogen) using an automated Eppendorf FemtoJet microinjection system (Mississauga, ON), as previously described (Huang et al., 2013). For each biological replicate of GFP-, Cx30-, T5M-, V37E- and G59R-expressing cells, ~15-20 cells were microinjected and the incidence of dye transfer to surrounding cells was recorded. Images were acquired using a Leica DM IRE2 inverted epifluorescent microscope (Richmond Hill, ON), equipped with a Hamamatsu digital camera (Bridgewater, NJ) and OpenLab 5.5.3 Imaging Software (Lexington, MA). HeLa cells expressing the A88V mutant could not be microinjected due to the fact that cells were already undergoing cell death and had a permeable cell membrane, however in the case of REKs, 5-10 cells were microinjected in each replicate. REKs and HeLa cells were microinjected and processed in the same manner to ensure that cells were well coupled and connexin-deficient, respectively. A one-way analysis of variance statistical test (ANOVA) and Tukey's post hoc test were performed on the averages of 3 biological replicates (N=3), and values represent the mean percent incidence of dye transfer ± standard error of the mean (SEM).

2.2.5 Dye uptake hemichannel assays

To assess Cx30 and mutant hemichannel function, dye uptake assays were performed as previously described by Tong et al. (2007), with some modifications. Briefly, HeLa cells plated at about 20-30% confluency as single isolated cells were transfected with 1.5 µg of DNA as described above using Lipofectamine 2000. Regular extracellular solution (ECS) and divalent cation free-ECS (DCF-ECS) were made with 25 mM D-glucose, and

cells were incubated with 500 µL of solution containing 0.15 mM propidium iodide (PI) (Invitrogen). Groups of single isolated Cx30- or mutant-expressing cells were analyzed for their ability to uptake PI under physiological (ECS) and no Ca^{2+} or Mg^{2+} (DCF-ECS) conditions. Isolated GFP-positive cells were quantified in the assay, and neighbouring cells attached by a single long cytoplasmic projection were also included. Approximately 60 cells were recorded for each biological replicate. Images were taken under a 20X lens using the Leica microscope and OpenLab software. The number of cells that exhibited dye uptake was recorded as a percentage of the total number of GFP-positive cells examined, and a two-way ANOVA was performed on the averages of 4 biological replicates (N=4). Values represent the mean percentage of GFP-positive isolated cells that exhibited dye uptake ± SEM.

2.2.6 TUNEL assays

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays were performed using an ApopTag® Red *In Situ* Apoptosis Detection Kit (EMD Millipore) as per the manufacturer's instructions, with a few modifications. Briefly, control and mutant-expressing cells grown in monolayer were fixed in 10% NBF, permeabilized for 10 minutes with 0.5% Triton X-100 in PBS, and subsequently washed with 1X PBS. Following the incubation period with terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C, cells were washed with working strength Stop/Wash buffer twice for 5 minutes each wash. Nuclei were stained with Hoescht 33342 and mounted. Images were obtained using the Leica microscope and OpenLab Software with a 63X oil immersion objective lens. For each biological replicate of transfected cells and controls, 10-15 images were taken of random areas. The number of ApopTag positive cells was recorded as a percentage of the total cell number per image and a one-way ANOVA and Tukey's post hoc test were performed on the averages of 3 biological replicates (N=3). Values represent the mean percentage of apoptotic cells/total number of cells per image ± SEM.

2.2.7 Western blotting

Cell lysates were collected from cultures using a Triton-based extraction buffer [1% Triton X-100 (Sigma Aldrich), 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM

ethylene glycol tetraacetic acid (EGTA), 0.5% nonyl phenoxypolyethoxylethanol (NP-40), 100 mM NaF, 100 mM sodium orthovanadate and one proteinase inhibitor mini-EDTA tablet (Roche Applied Science, Laval, QC)] adjusted to pH 7.4 as previously described by (Stewart et al., 2013). Extractions were repeated 3 times, and protein lysate concentrations were quantified using a bicinchoninic acid (BCA) protein determination kit (Thermo Scientific, Rockford, IL). Total protein lysates of 50 µg were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked using 5% Blotto Non-Fat Dry Milk (Santa Cruz Biotechnology) with 0.05% Tween 20 (Sigma Aldrich) in PBS (PBS-T) for 30-60 minutes, and subsequently incubated overnight at 4°C with rabbit anti-Cx30 (1:750-1000, Invitrogen), rabbit anti-Cx43 (1:5000, Sigma Aldrich), goat anti-glucose regulated protein 78 (GRP78) (1:500, Santa Cruz Biotechnology), mouse anti-C/EBP homologous protein (CHOP) (3 µg/mL, Abcam, Toronto, ON) and mouse anti-activating transcription factor 4 (ATF4) (5 µg/mL, Abcam) primary antibodies. Gel loading controls included probing for the levels of β-tubulin using mouse anti-β-tubulin primary antibody (1:10000, Sigma Aldrich). Blots probed for Cx30 were counterstained with mouse anti-GFP antibodies (1:2500, EMD Millipore) to validate that the GFP tag was attached to Cx30 and Cx30 mutants. After several washes with PBS-T, blots were then incubated with secondary anti-rabbit and anti-goat Alexa Fluor 680 (1:5000, Invitrogen) and anti-mouse IRdye 800 (1:5000, LI-COR Biosciences, Lincoln, NE) secondary antibodies for 45-60 minutes. Following more PBS-T washes, blots were scanned and densitometry measurements were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). Each signal was normalized to its β-tubulin loading control in the same lane, and the wt/β-tubulin or Cx30/β-tubulin outcome value was set to 1. Unpaired t-tests were performed on the fold change averages of these values from 3 distinct sets of protein lysates to minimize variation between blots as well as between mutant lysates within each blot. Values represent fold change ± SEM.

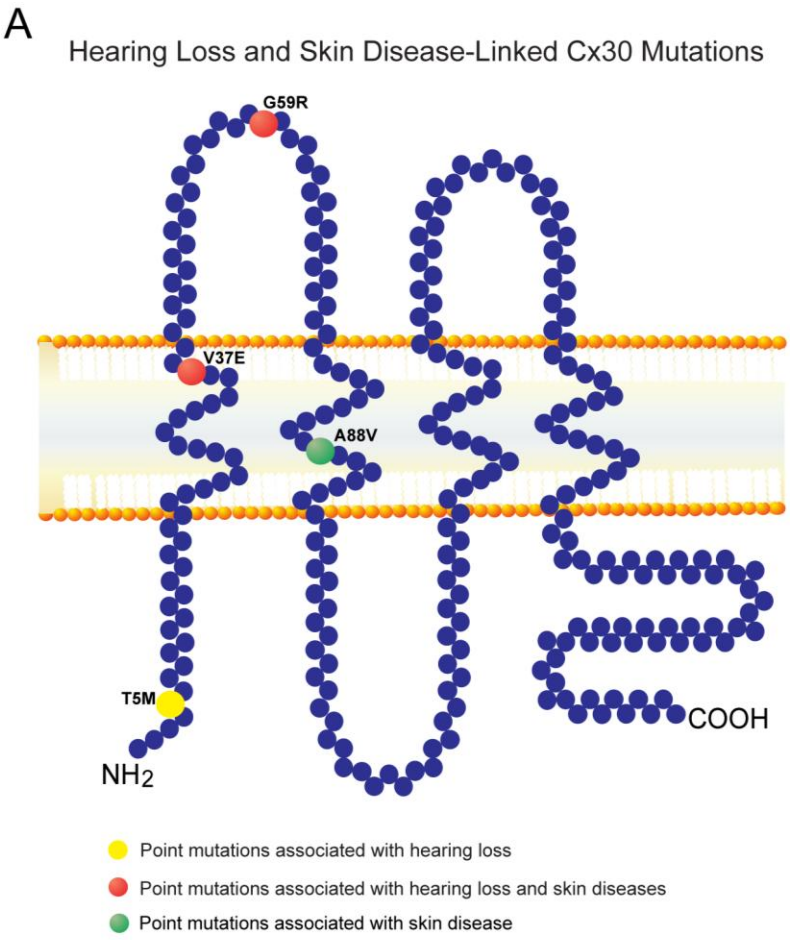
2.3 Results

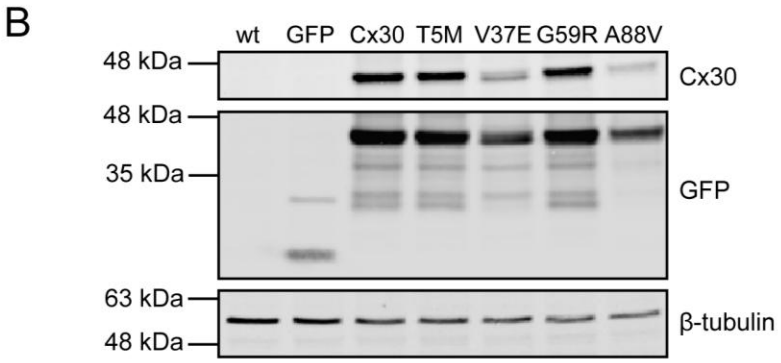
2.3.1 Cx30 mutations linked to skin disease and non-syndromic hearing loss, and their differential ectopic expression and localization in REKs

Cx30 is a 261 amino acid gap junction protein that exhibits the topological structure of a typical connexin, with four transmembrane domains, two extracellular loops, one intracellular loop and cytoplasmic-exposed amino and carboxy termini **(Fig. 2.1A)**. The location of four distinct mutations that cause skin disease and/or hearing loss are all located within the 1st half of the Cx30 protein **(Fig. 2.1A)**.

Western blot analysis was used to determine the expression levels of GFP-tagged T5M, V37E, G59R and A88V mutants in REKs. Quantification of densitometry values revealed that V37E-GFP and A88V-GFP protein levels were ~35% and ~50% lower, respectively, compared to the relative expression of Cx30-GFP (P<0.01) **(Fig. 2.1B, C)**. In contrast, the non-syndromic hearing loss-linked T5M and Vohwinkel syndrome-linked G59R mutants exhibited expression levels similar to that of Cx30-GFP.

To compare the localization profiles of the mutant forms of Cx30, we examined GFP-tagged Cx30 mutants **(Fig. 2.2A)** and untagged Cx30 mutants **(Fig. 2.2B, C)** in REKs. Consistent with previous reports, the T5M mutant formed gap junction-like plaques at the cell surface similar to wt Cx30 (Common et al., 2002), while the V37E and A88V Clouston syndrome-linked mutants remained in intracellular compartments (Common et al., 2002; Essenfelder et al., 2004) **(Fig. 2.2A, B)**. Immunolabeling for ER-resident chaperone PDI revealed that the Clouston syndrome/KID syndrome-linked V37E mutant was retained in the ER **(Fig. 2.2A)**. A88V mutant-expressing cells appeared to be entering a cell death pathway as they exhibited small fragmented nuclei, and since they lacked PDI staining, it remains unclear whether a population of this mutant resided in the ER. The novel Vohwinkel syndrome-linked G59R mutant (Nemoto-Hasebe et al., 2009) also showed an intracellular localization profile **(Fig. 2.2A, B)**, and immunolabeling for GM130 revealed its localization largely in the Golgi apparatus **(Fig. 2.2C)**. Interestingly, while we have never observed V37E plaques at the interface between apposing cells, a population of the G59R and A88V mutants successfully trafficked to the cell surface


A
Hearing Loss and Skin Disease-Linked Cx30 Mutations
G59R
V37E
A88V
T5M
NH2
COOH
Point mutations associated with hearing loss
Point mutations associated with hearing loss and skin diseases
Point mutations associated with skin disease


B
wt GFP Cx30 T5M V37E G59R A88V
48 kDa
48 kDa
35 kDa
63 kDa
48 kDa
Cx30
GFP
β-tubulin

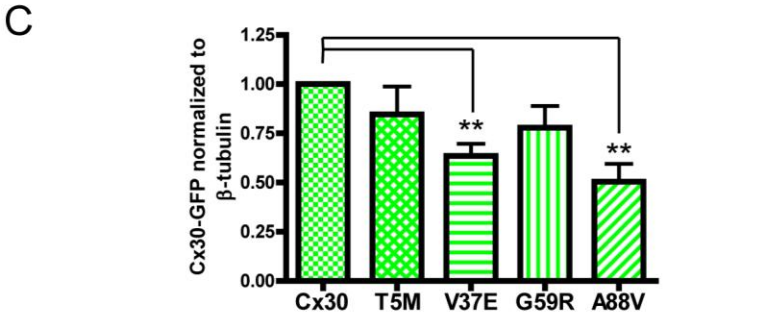

C
Cx30-GFP normalized to β-tubulin
1.25
1.00
0.75
0.50
0.25
0.00
**
**
Cx30
T5M
V37E
G59R
A88V

Figure 2.1: Cx30 mutations linked to hearing loss and skin diseases, and their differential ectopic expression in REKs

(A) Schematic diagram of Cx30 depicting four mutations associated with hearing loss (yellow), hearing loss plus skin disease (orange) and skin disease (green). **(B)** Western blot analysis was used to detect the levels of GFP-tagged Cx30 and Cx30 mutants when ectopically expressed in REKs. Blots were probed with anti-Cx30, anti-GFP or anti-β-tubulin antibodies. **(C)** Anti-GFP labeling revealed significantly lower levels of GFP-tagged V37E and A88V mutants compared to the levels of wild-type Cx30. Values represent fold change ± SEM (unpaired t-test, $^{**}P<0.01$, N=3).

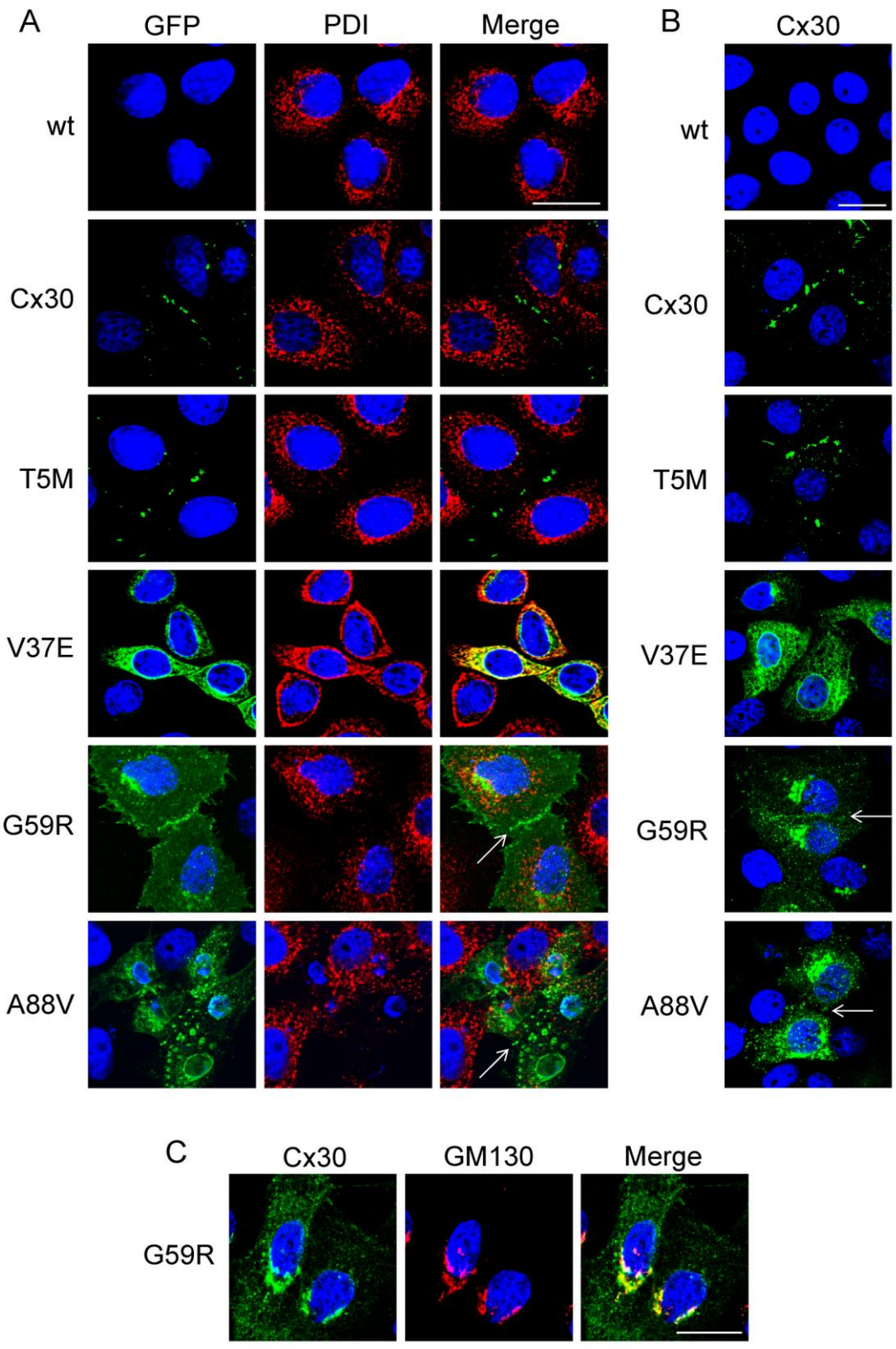

A
GFP
PDI
Merge
B
Cx30
wt
Cx30
T5M
V37E
G59R
A88V
C
Cx30
GM130
Merge
G59R

Figure 2.2: Skin disease-linked Cx30 mutants have impaired abilities to form gap junction plaques. **(A)** Wild-type (wt) REKs and REKs ectopically expressing GFP-tagged Cx30 or Cx30 mutants (green) were immunolabeled for protein disulfide isomerase (PDI) (red) to denote the endoplasmic reticulum (ER). Nuclei were stained with Hoescht 33342 (blue). Cx30 and the T5M mutant formed punctate gap junction-like structures at the cell-to-cell interface, while V37E co-localized with PDI. The G59R and A88V mutants were primarily localized in intracellular compartments, however a population of the mutants did reside at the cell surface (white arrows). PDI staining was absent in A88V mutant-expressing cells, and GFP-positive cells without nuclei represented dead "ghost" cells. Scale bar = 20µm. **(B)** Immunolabeling REKs for Cx30 (green) revealed that untagged mutants exhibited similar localization profiles as GFP-tagged mutants. Similarly, populations of the G59R and A88V mutants localized to the cell surface (arrows). Scale bar = 20µm. **(C)** REKs ectopically expressing the G59R mutant were double-labeled for Cx30 (green) and Golgi matrix protein 130 (GM130) (red), which revealed that the G59R mutant co-localized with the Golgi apparatus. Scale bar = 20µm.

(Fig. 2.2A, B, arrows). Cx30 and Cx30 mutants all similarly localized within the cell regardless of the presence or absence of the GFP tag **(Fig. 2.2A-C)**.

2.3.2 V37E and G59R skin disease-linked mutants exhibit loss of gap junction function

Since the T5M mutant and a population of the G59R mutant formed punctate gap junction-like structures at the cell surface in REKs, we hypothesized that these mutants may form functional gap junctions. Mutant-expressing GJIC-deficient HeLa cells were microinjected with Alexa 350 to assess whether any of the mutants restored GJIC. As expected, Cx30 gap junction channels readily facilitated dye transfer ($P<0.001$), while wt and free GFP-expressing cells showed no significant dye transfer to surrounding cells **(Fig. 2.3A, B)**. Interestingly, the T5M mutant exhibited dye transfer in ~90% ($P<0.001$) of cells injected **(Fig. 2.3A, B)**, indicating that this mutant was functional. No dye transfer was observed in microinjected HeLa cells expressing the V37E and G59R mutants **(Fig. 2.3A)**. A88V-expressing cells were not included in these functional studies, as they could not be microinjected due to their porous cell membranes caused by cell death (data not shown).

Next, we assessed the ability of each Cx30 mutant to form functional hemichannels by observing the incidence of PI dye uptake in cells under normal ECS and no Ca^{2+} or Mg^{2+} DCF-ECS conditions, the latter of which induces hemichannels to open (Lai et al., 2006; Stout et al., 2002). Under ECS conditions, Cx30 and T5M, V37E and G59R mutant hemichannels remained closed as expected, indicating that the mutants did not exhibit leaky or abnormal activity **(Fig. 2.4A)**. Under DCF-ECS conditions, ~90% of single isolated T5M-expressing cells exhibited dye uptake, closely resembling the ~95% incidence observed for cells expressing Cx30, both of which were significantly higher than that observed under control ECS conditions ($P<0.001$) **(Fig. 2.4A, B)**. Collectively, these functional studies indicated that V37E and G59R mutants were loss-of-function mutants while the T5M mutant showed similar functional channel properties to Cx30.

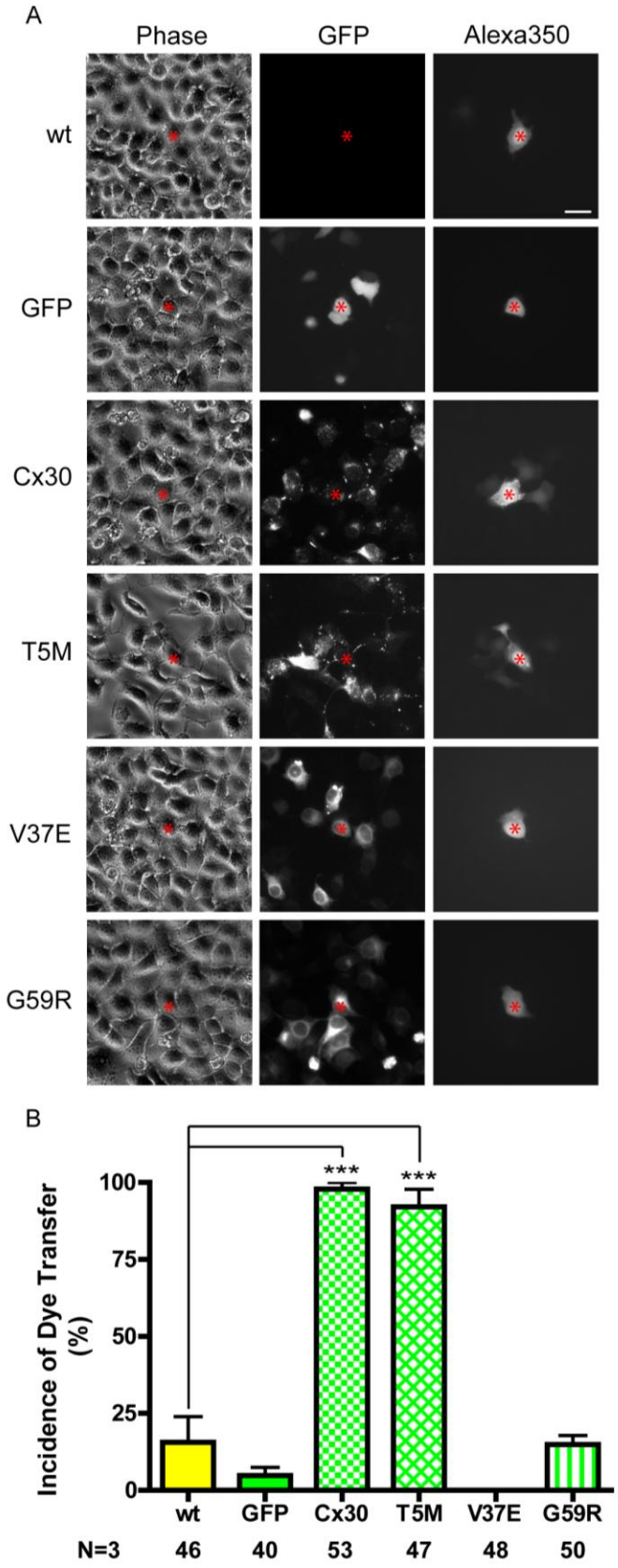

A
Phase
GFP
Alexa350
wt
GFP
Cx30
T5M
V37E
G59R
B
Incidence of Dye Transfer (%)
100
75
50
25
0
***
***
wt
GFP
Cx30
T5M
V37E
G59R
N=3
46
40
53
47
48
50

Figure 2.3: Skin disease-linked V37E and G59R mutants exhibit loss of gap junction function when ectopically expressed in HeLa cells

(A) Free GFP-, Cx30-GFP- and mutant-GFP-expressing HeLa cells (denoted in phase-contrast images) were imaged for the presence of GFP and microinjected with Alexa 350 dye (red asterisks). Scale bar = 20µm. **(B)** Relative to wild-type (wt) control cells, cells expressing Cx30 or the T5M mutant exhibited significantly greater incidences of dye transfer than those expressing the V37E or G59R mutants. Values represent the mean percent incidence of dye transfer ± SEM (one-way ANOVA, ***P<0.001, N=3). Total numbers of injected cells are presented along the bottom of the figure.

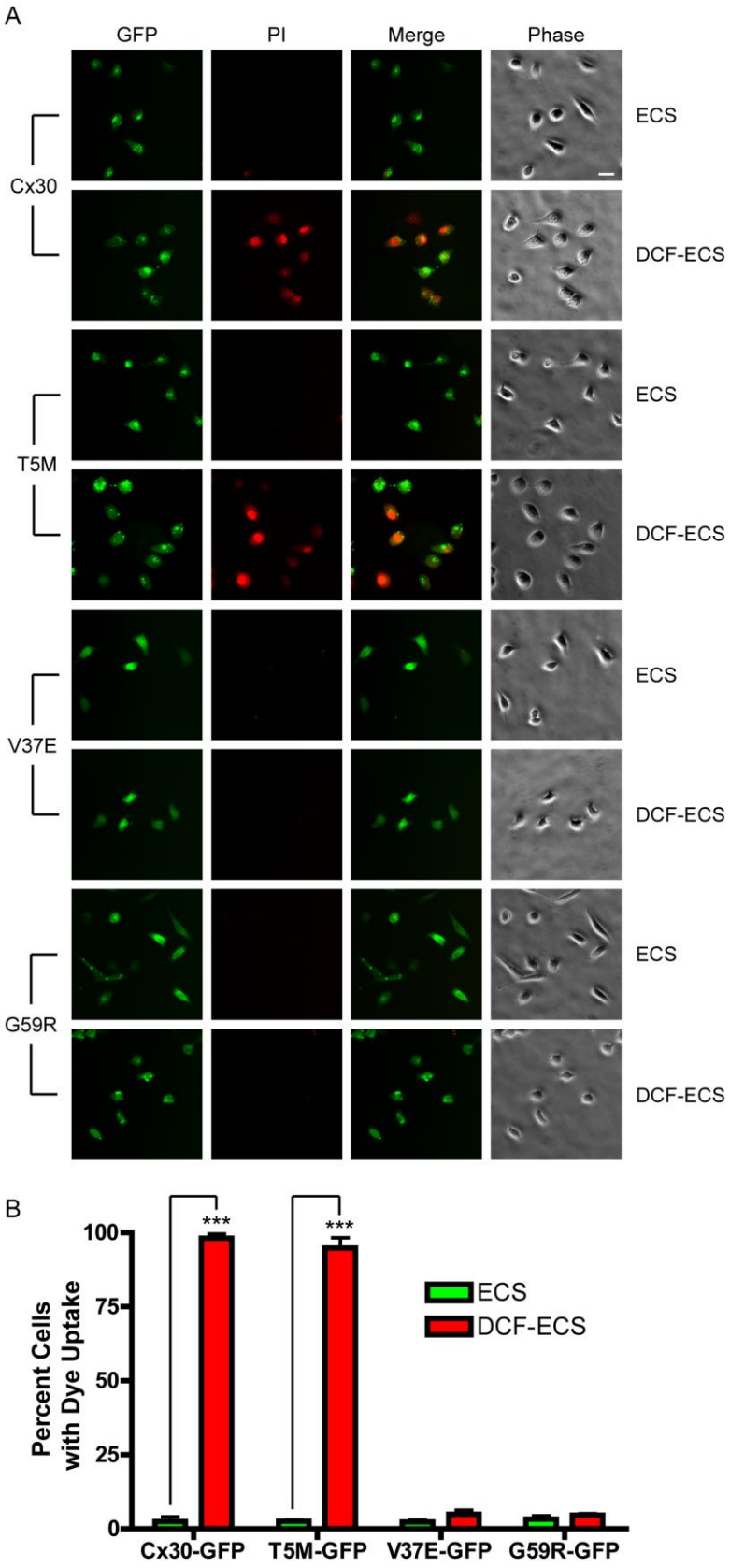

A
GFP
PI
Merge
Phase
Cx30
ECS
DCF-ECS
T5M
ECS
DCF-ECS
V37E
ECS
DCF-ECS
G59R
ECS
DCF-ECS
B
Percent Cells with Dye Uptake
100
75
50
25
0
***
***
ECS
DCF-ECS
Cx30-GFP
T5M-GFP
V37E-GFP
G59R-GFP

Figure 2.4: The hearing loss-linked T5M mutant exhibits hemichannel activity in HeLa cells that mimics Cx30

(A) Single isolated Cx30-GFP- or mutant-GFP-expressing HeLa cells (denoted in phase-contrast images) were incubated with propidium iodide (PI) in normal extracellular solution (ECS) or divalent cation free ECS (DCF-ECS). Cells were imaged for the presence of GFP (green) and PI uptake (red). Scale bar = 20µm. **(B)** Cells expressing the V37E and G59R mutants did not exhibit PI uptake under DCF-ECS conditions, while those expressing Cx30 or the T5M mutant exhibited significant hemichannel activity. Values represent the mean percentage of GFP-positive isolated cells that exhibited PI uptake ± SEM (two-way ANOVA, ***P<0.001, N=4).

2.3.3 V37E and A88V mutants reduce Cx43-based coupling in REKs by potentially affecting Cx43 plaque formation

In Cx43-rich REKs engineered to express GFP-tagged Cx30 or the T5M and G59R mutants, Cx43 was frequently co-localized with Cx30 mutants at cell-to-cell interfaces. Cx43-based gap junctions appeared less frequently between cells expressing the V37E and A88V mutants **(Fig. 2.5A)**, however the total protein levels of Cx43 remained unchanged **(Fig. 2.5B, C)**. Total Cx43 levels were only slightly decreased in T5M mutant-expressing cells compared to those expressing Cx30 ($P<0.05$).

To determine whether Cx30 mutants affected Cx43-based GJIC, REKs were microinjected with Alexa 350 and the incidence of dye transfer was recorded. Wt REKs exhibited 100% dye transfer, and no significant differences were observed in REKs expressing free GFP, Cx30-GFP, or the GFP-tagged T5M and G59R mutants. Cell pairs or clusters expressing the V37E and A88V mutants exhibited decreased Cx43-mediated dye transfer ($P<0.01$) **(Fig. 2.6A, B)**.

2.3.4 V37E and A88V mutants induce apoptosis

The ectopic expression of the V37E and A88V mutants induced some degree of cell death in REKs as early as 18 hours post-expression with the majority of A88V-expressing cells dying within 48 hours (data not shown). To determine the mechanism of cell death induced by these mutants, control and mutant-expressing REKs were immunolabeled with anti-cleaved caspase-3, a marker of the committed stage of apoptosis (Saraste and Pulkki, 2000). Interestingly, some V37E- and A88V-expressing REKs expressed cleaved caspase-3, indicating that these cells were undergoing apoptosis **(Fig. 2.7)**. To further validate this finding, TUNEL assays representing the degradation stage of apoptosis (Saraste and Pulkki, 2000) were performed on Cx30- and mutant-expressing REKs. V37E and A88V Cx30 mutants significantly induced apoptosis, as ~30% and ~35% of total cells respectively were ApopTag positive ($P<0.001$) compared to wt REKs of which ~2% were ApopTag positive **(Fig. 2.8A, B)**. Cells treated for 24 hours with staurosporine, a strong protein kinase inhibitor (Chae et al., 2000), served as

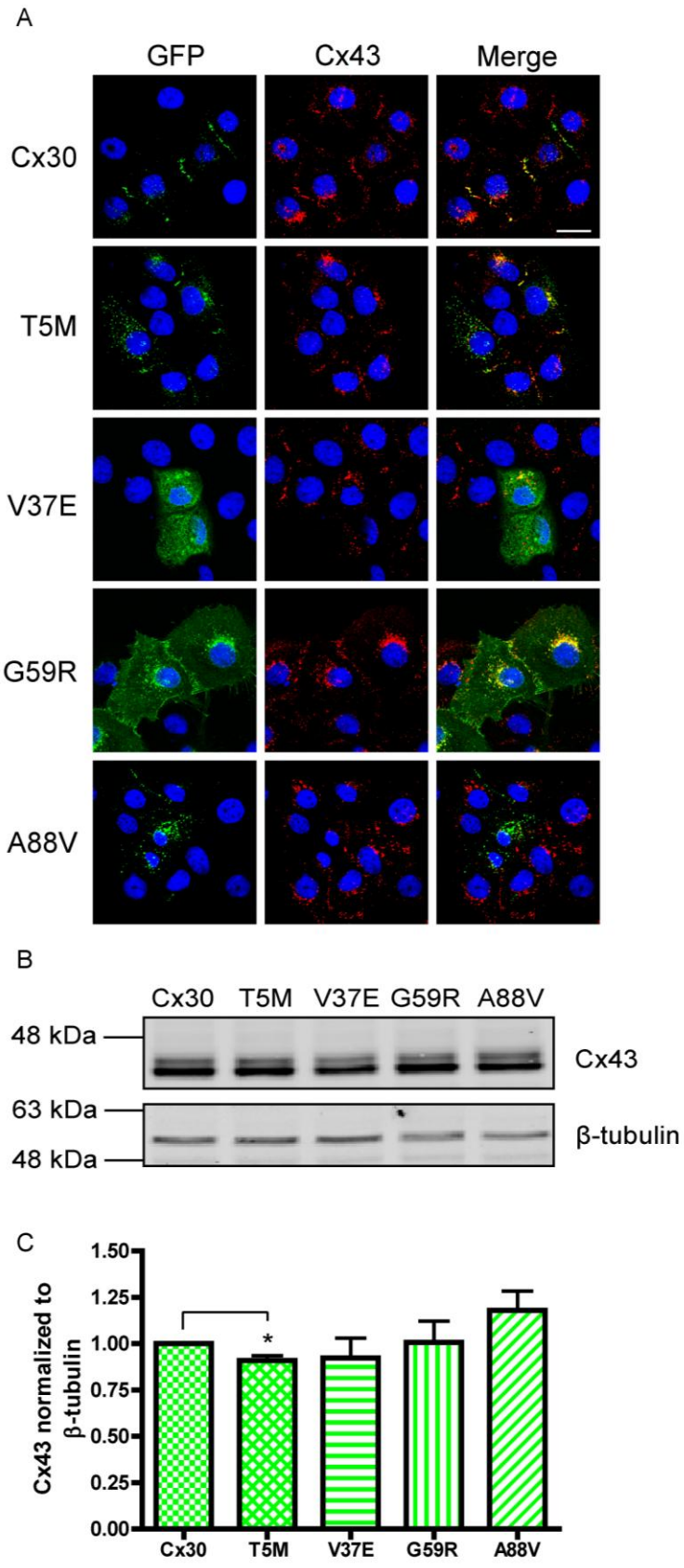

A
GFP
Cx43
Merge
Cx30
T5M
V37E
G59R
A88V
B
Cx30
T5M
V37E
G59R
A88V
48 kDa
Cx43
63 kDa
48 kDa
β-tubulin
C
Cx43 normalized to β-tubulin
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
Cx30
T5M
V37E
G59R
A88V

Figure 2.5: Skin disease-linked V37E and A88V mutants may reduce Cx43 plaque formation in REKs, although total levels of Cx43 remain unchanged

(A) Cx30-GFP- and Cx30 mutant-GFP-expressing REKs (green) were immunolabeled for Cx43 (red) and stained with Hoescht 33342 (blue) to denote cell nuclei. Cx43 plaques were localized at the cell surface between REKs expressing Cx30 or the T5M and G59R mutants. V37E- and A88V-expressing REKs did not exhibit Cx43 plaque formation between apposing cells. Scale bar = 20µm. **(B)** Western blot analysis was used to detect total levels of Cx43 in Cx30- and mutant-expressing cells when normalized to β-tubulin. **(C)** Total levels of Cx43 were slightly lower in REKs expressing only the T5M hearing loss-linked mutant and remained unchanged in V37E, G59R and A88V mutant-expressing cells. Values represent fold change ± SEM (unpaired t-test, **P*<0.05, N=3).

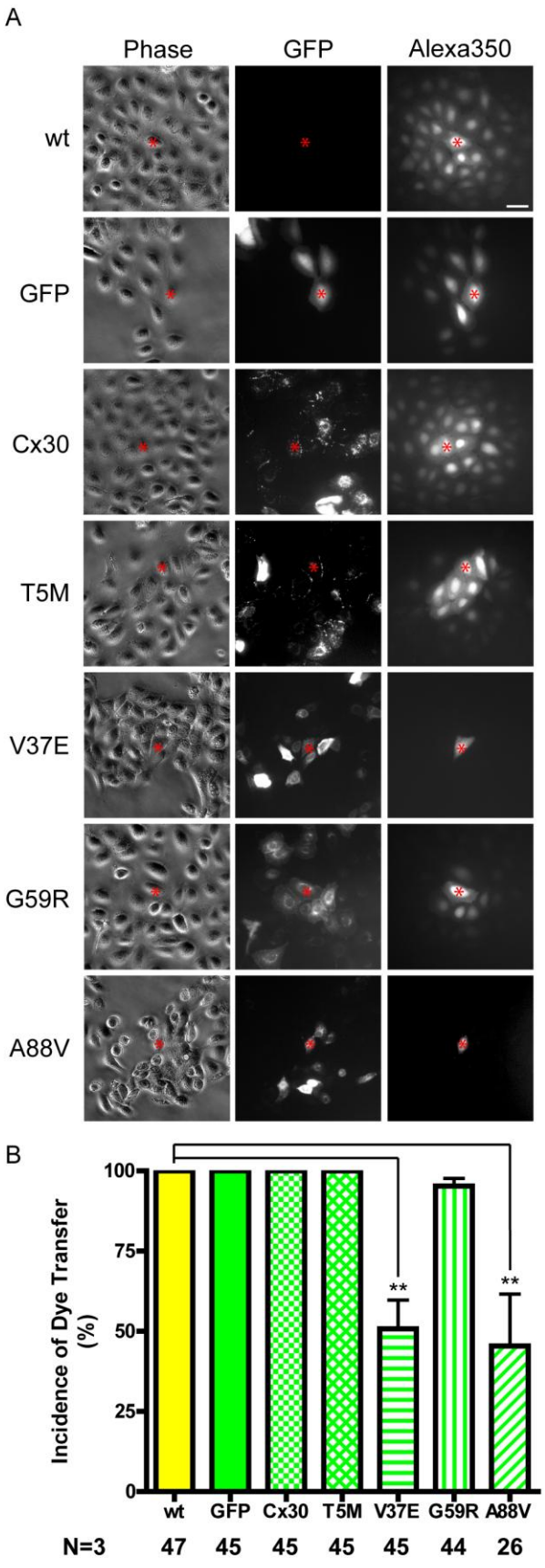

A
Phase
GFP
Alexa350
wt
GFP
Cx30
T5M
V37E
G59R
A88V
B
Incidence of Dye Transfer (%)
100
75
50
25
0
**
**
wt
GFP
Cx30
T5M
V37E
G59R
A88V
N=3
47
45
45
45
45
44
26

Figure 2.6: V37E and A88V skin disease-linked mutants reduce Cx43-based gap junction coupling in REKs.

(A) Wild-type (wt) and free GFP-, Cx30-GFP- and mutant-GFP-expressing REKs (denoted by phase-contrast images) were imaged for the presence of GFP and microinjected with Alexa 350 dye (red asterisks). Scale bar = 20µm. **(B)** Relative to wt controls, cells expressing the V37E and A88V mutants exhibited significantly lower incidences of Cx43-mediated dye transfer. Values represent the mean percent incidence of dye transfer ± SEM (one-way ANOVA, $^{**}P<0.01$, N=3). Total numbers of injected cells are presented along the bottom of the figure.

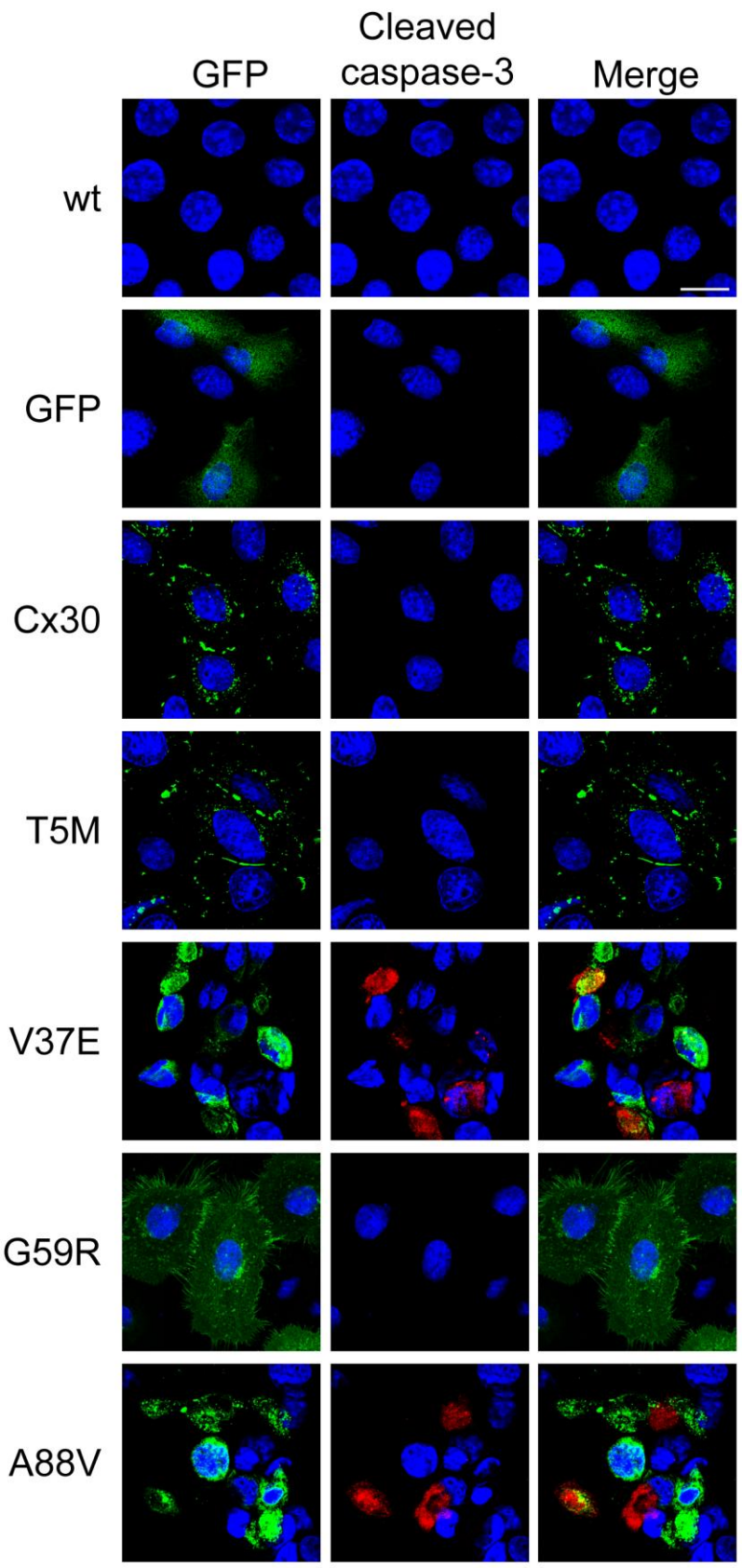

GFP
Cleaved caspase-3
Merge
wt
GFP
Cx30
T5M
V37E
G59R
A88V

Figure 2.7: REKs expressing V37E and A88V mutants express cleaved caspase-3. Wild-type (wt) or free GFP-, Cx30-GFP- and mutant-GFP-expressing REKs (green) were immunolabeled for cleaved caspase-3 (red) and nuclei were stained with Hoescht 33342 (blue). Cells expressing the V37E and A88V mutants also expressed the apoptotic marker cleaved caspase-3. Scale bar = 20 μm.

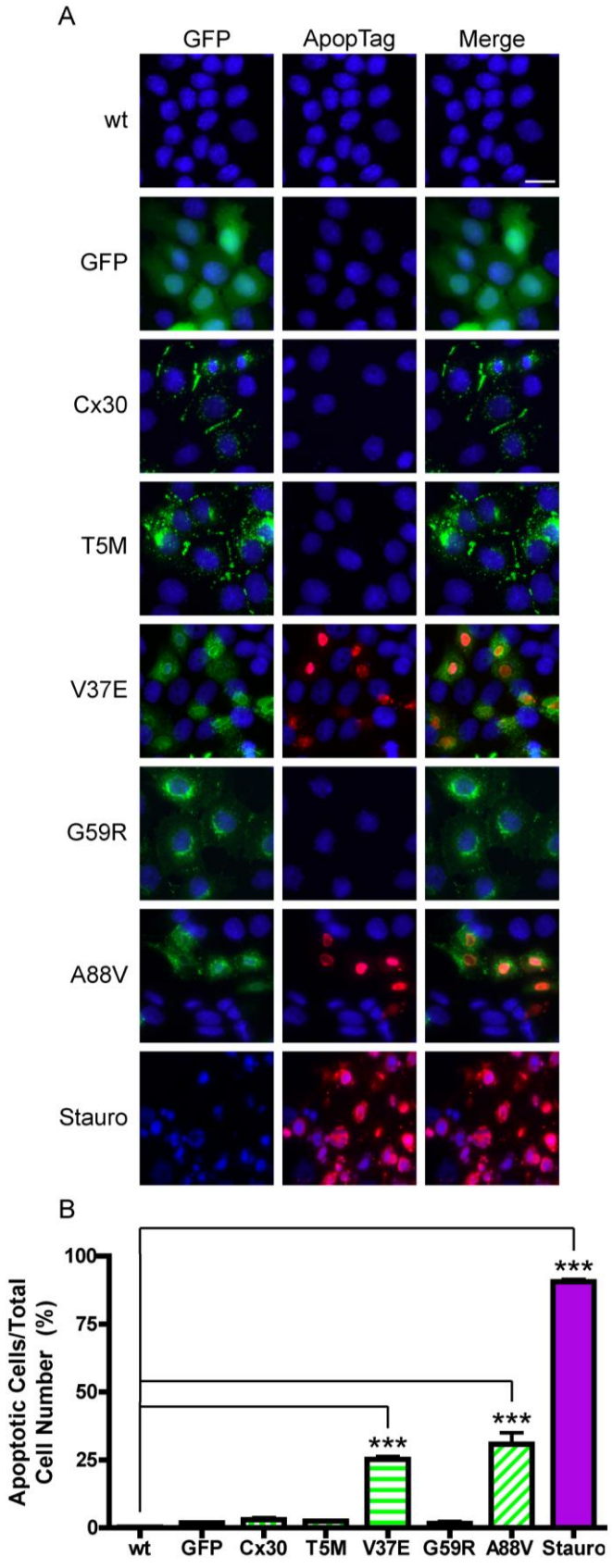

A
GFP
ApopTag
Merge
wt
GFP
Cx30
T5M
V37E
G59R
A88V
Stauro
B
Apoptotic Cells/Total Cell Number (%)
100
75
50
25
0
***
***
***
wt
GFP
Cx30
T5M
V37E
G59R
A88V
Stauro

Figure 2.8: Ectopic expression of V37E and A88V mutants induces apoptosis in REKs. (A) TUNEL assays were performed on wild-type (wt), free GFP-, Cx30-GFP- and mutant-GFP-expressing REKs (green). Nuclei were stained with Hoescht 33342 (blue) and apoptotic cells are indicated by ApopTag staining (red). Staurosporine (Stauro) treatment served as an inducer of apoptosis. Scale bar = 20 µm. **(B)** The expression of V37E and A88V mutants, as well as staurosporine treatment, significantly induced apoptosis in REKs compared to controls. Values represent the mean percentage of apoptotic cells/total number of cells per image ± SEM (one way ANOVA, ***P<0.001, N=3).

positive controls for the assay, as ~90% of total cells were apoptotic (P<0.001) **(Fig. 2.8A, B)**.

To determine whether apoptosis was triggered by an ER stress-mediated UPR, Western blot analyses of REK cell lysates were performed to detect changes in the levels of markers involved in different stages and pathways of the UPR. REKs treated with tunicamycin (Tm), an ER stress inducer that blocks N-glycosylation of proteins (de Freitas Junior et al., 2011), served as positive controls for UPR markers. GRP78 expression was significantly higher in cells expressing GFP, Cx30 and the G59R mutant (P<0.05), which revealed that a slight ER stress response was common to the transfection process and not specific to the presence of the Cx30 mutants **(Fig. 2.9A)**. ATF4 was mildly up-regulated in cells expressing GFP (P<0.05) and the A88V mutant (P<0.05), although most predominantly in cells expressing the V37E mutant (P<0.001) **(Fig. 2.9B)**. As a control, cells treated with Tm exhibited significantly increased ATF4 protein levels (P<0.01) **(Fig. 2.9B)**. Finally, CHOP was significantly up-regulated in V37E mutant-expressing cells (P<0.05) and in Tm-treated cells (P<0.01) **(Fig. 2.9C)**.

2.3.5 V37E and A88V mutants may exhibit dominant-negative and transdominant effects on wt Cx30 and Cx26 when co-expressed in REKs

To determine whether skin disease-linked Cx30 mutants that were localized to intracellular compartments could be rescued to the cell surface, REKs were engineered to express Cx30-RFP or Cx26-RFP simultaneously with GFP-tagged Cx30 mutants. Alone, Cx30-RFP formed gap junctions **(Fig. 2.10)**, which facilitated the transfer of Alexa 350 in HeLa cells and REKs (data not shown). Cx30-GFP and T5M-GFP showed distinct co-localization with Cx30-RFP, with limited intracellular localization **(Fig. 2.10)**. Wt Cx30 did not appear to rescue the V37E mutant to the cell surface **(Fig. 2.10)**. Although the G59R and A88V mutants showed some co-localization with wt Cx30 at the cell surface, the majority of these mutants were localized in intracellular compartments, indicating lack of a trafficking rescue **(Fig. 2.10)**. Notably, the V37E and A88V mutants may exhibit partial dominant-negative effects on wt Cx30, as a large population of Cx30-RFP was retained inside the cell as compared to situations where Cx30-RFP was expressed

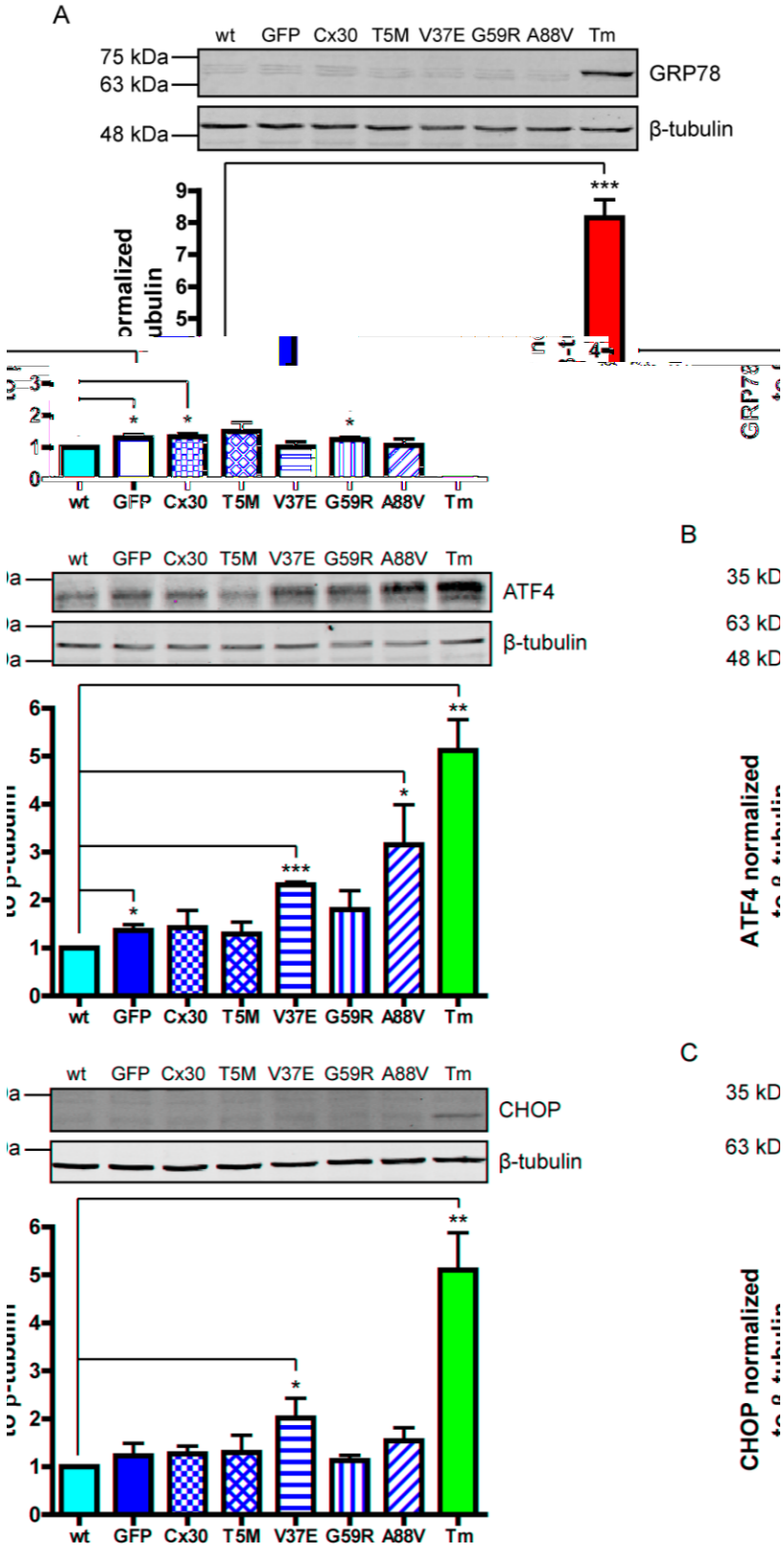

A
wt GFP Cx30 T5M V37E G59R A88V Tm
75 kDa
63 kDa
GRP78
48 kDa
β-tubulin
wt GFP Cx30 T5M V37E G59R A88V Tm
B
ATF4
β-tubulin
35 kD
63 kD
48 kD
ATF4 normalized
C
CHOP
β-tubulin
35 kD
63 kD
CHOP normalized

Figure 2.9: Assessment of the unfolded protein response in mutant-expressing REKs. Western blot analysis was used to detect the levels of unfolded protein response (UPR) markers glucose regulated protein 78 (GRP78) (A), activating transcription factor 4 (ATF4) (B) and C/EBP homologous protein (CHOP) (C) in wild-type (wt) and GFP-, Cx30- and mutant-expressing REKs when normalized to β-tubulin. Normalized wild-type (wt) values were set to 1. Protein lysates from tunicamycin (Tm)-treated cells served as an inducer of the UPR. Blots were also probed with anti-β-tubulin antibody. **(A)** GRP78 was elevated in cells expressing free GFP, Cx30 or the G59R mutant (*P<0.05), and was highly up-regulated in Tm-treated cells (***P<0.001). **(B)** ATF4 was up-regulated in free GFP- and A88V-expressing cells (*P<0.05), and more markedly elevated in V37E-expressing cells (***P<0.001) and Tm-treated controls (**P<0.01). **(C)** CHOP was significantly up-regulated in V37E-expressing and Tm-treated cells only (*P<0.05 and **P<0.01). Values represent fold change ± SEM (unpaired t-test, N=3).

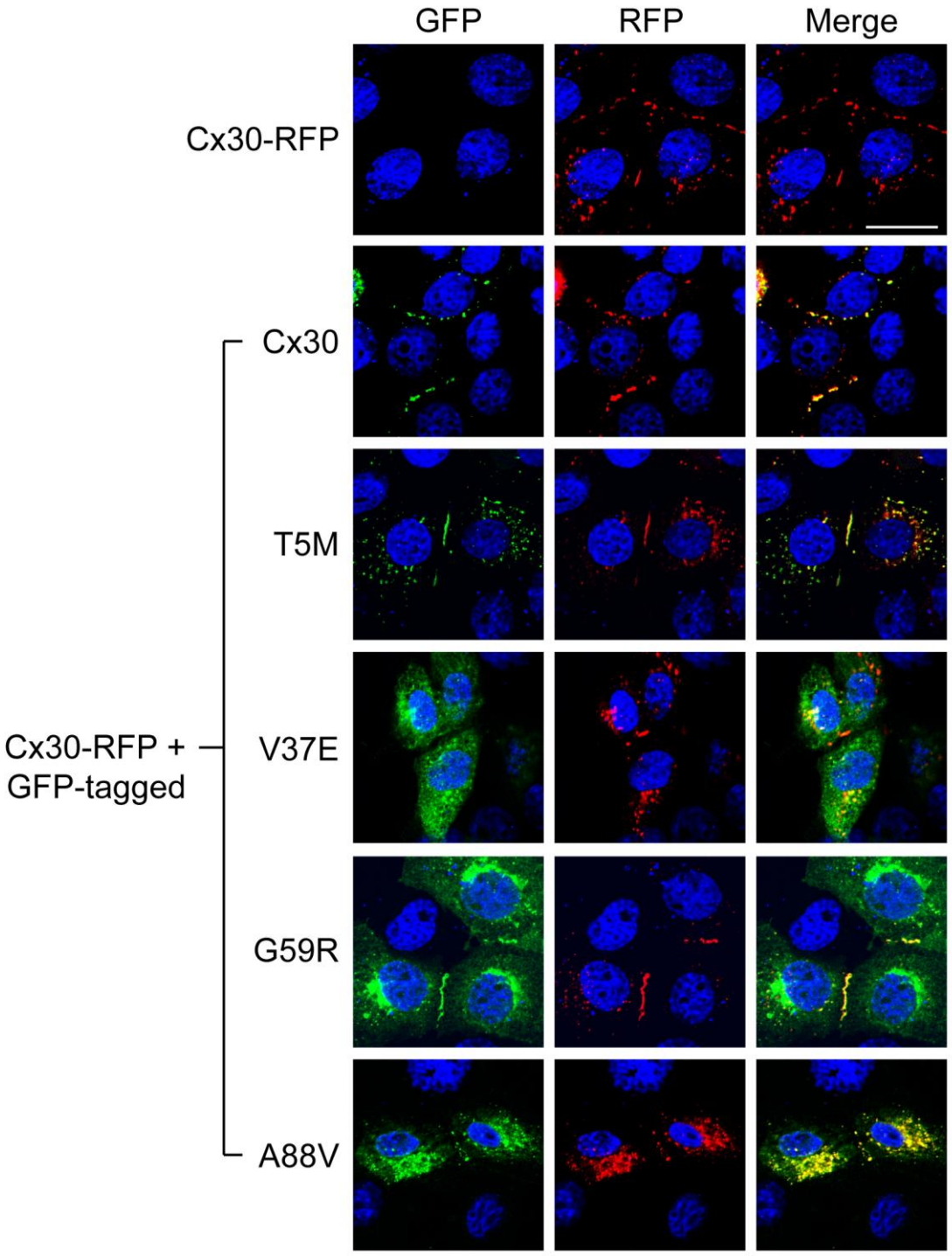

GFP
RFP
Merge
Cx30-RFP
Cx30-RFP +
GFP-tagged
Cx30
T5M
V37E
G59R
A88V

Figure 2.10: V37E and A88V Cx30 mutants exhibit a dominant-negative effect on ectopically-expressed Cx30 in REKs. REKs co-expressing Cx30-RFP (red) together with GFP-tagged Cx30, T5M, V37E, G59R or A88V mutants (green) were stained with Hoescht 33342 (blue) to denote the cell nuclei. Cx30, the T5M mutant and populations of the G59R and A88V mutants distinctly co-localized with Cx30-RFP at the cell surface. Wild-type Cx30 failed to rescue mutant trafficking as the V37E, and the majority of the G59R and A88V mutants, were primarily localized to intracellular compartments. V37E and A88V Cx30 mutants exhibited a dominant-negative effect on wild-type Cx30, as Cx30-RFP was retained inside the cell and particularly co-localized with the A88V mutant. Scale bar = 20μm.

alone **(Fig. 2.10)**. In particular, the A88V mutant and wt Cx30 exhibited overlapping co-localization in a distinct subcellular compartment. We also observed similar results in HeLa cells, however, the V37E mutant exhibited some gap junction-like structures at the cell surface that co-localized with wt Cx30 **(Fig. 2.11)**. Alone, Cx26-RFP formed gap junctions in REKs **(Fig. 2.12)**. Cx30-GFP, T5M-GFP and G59R-GFP also showed distinct localization with Cx26-RFP in REKs, however the V37E, A88V and the majority of the G59R mutant remained in intracellular compartments, indicating that wt Cx26 did not rescue the trafficking of these mutants **(Fig. 2.12)**. However, the V37E and A88V mutants also may exhibit transdominant effects on Cx26, as the majority of Cx26-RFP was retained inside the cell compared to when Cx26-RFP was expressed alone **(Fig. 2.12)**.

2.4 Discussion

In the present study, we first determined that the T5M mutant linked to non-syndromic hearing loss exhibited similar properties to Cx30, as it formed functional gap junctions and hemichannels, while skin disease-linked mutants exhibited impaired gap junction formation and function. In particular, the V37E mutant linked to KID syndrome was retained in the ER and triggered UPR-mediated apoptosis, as indicated by the up-regulation of ATF4 and CHOP. In contrast, the G59R mutant associated with Vohwinkel and Bart-Pumphrey syndromes was primarily retained in the Golgi apparatus, however, did not induce cell death. The A88V mutant linked to Clouston syndrome remained primarily in intracellular compartments, triggered the up-regulation of ATF4 and significantly induced apoptosis, although primarily through a UPR-independent mechanism. Finally, we determined that skin disease-linked mutants retained in intracellular compartments were not rescued to the cell surface by co-expressed Cx43, Cx30 or Cx26, and the V37E and A88V mutants exhibited dominant-negative and transdominant effects on the trafficking of these connexins to the cell surface. Thus, we clearly demonstrated the overall complexity of connexin-linked diseases, as each disease-linked Cx30 mutant causes disease via a different mechanism.

In order to evaluate the link between Cx30 mutants and disease, we used spontaneously immortalized, newborn REKs, which have previously been reported to express messenger

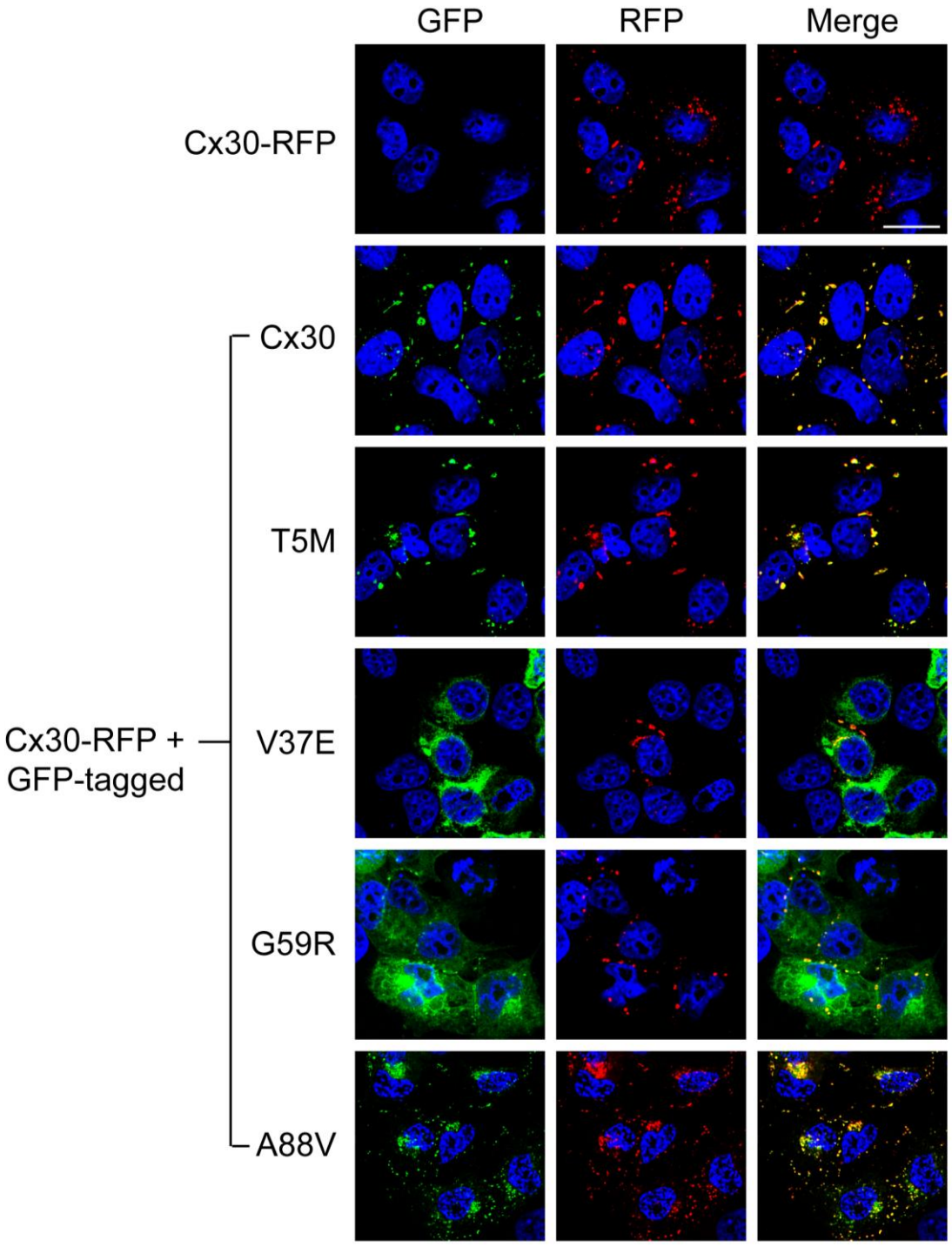

GFP
RFP
Merge
Cx30-RFP
Cx30-RFP +
GFP-tagged
Cx30
T5M
V37E
G59R
A88V

Figure 2.11: Cx30 may partially rescue the trafficking of V37E and A88V mutants in HeLa cells. HeLa cells co-expressing Cx30-RFP (red) together with GFP-tagged Cx30, T5M, V37E, G59R or A88V mutants (green) were stained with Hoescht 33342 (blue) to denote the cell nuclei. Cx30, T5M and populations of the V37E, G59R and A88V mutants distinctly co-localized with Cx30-RFP at the cell surface, however all skin disease-linked mutants were primarily intracellularly localized. Scale Bar = 20μm.

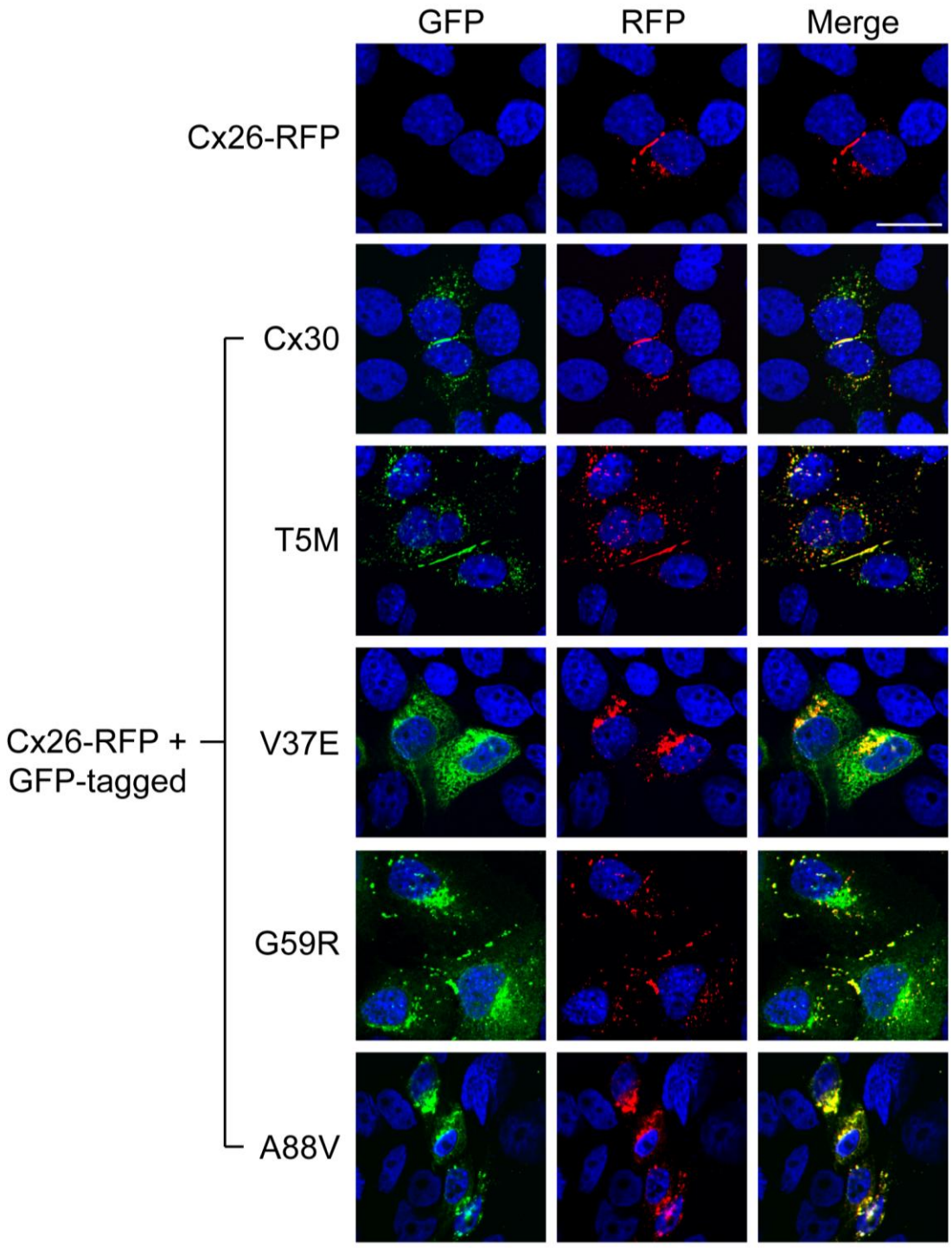

GFP
RFP
Merge
Cx26-RFP
Cx30
T5M
V37E
G59R
A88V
Cx26-RFP +
GFP-tagged

Figure 2.12: V37E and A88V Cx30 mutants exhibit a dominant-negative effect on ectopically-expressed Cx26 in REKs. REKs co-expressing Cx26-RFP (red) together with GFP-tagged Cx30, T5M, V37E, G59R or A88V mutants (green) were stained with Hoescht 33342 (blue) to denote the cell nuclei. Cx30, the T5M mutant and a population of the G59R mutant distinctly co-localized with Cx26-RFP at the cell surface. Wild-type Cx26 failed to rescue skin disease-linked mutant trafficking as the V37E, A88V and the majority of the G59R mutants were primarily localized to intracellular compartments. V37E and A88V Cx30 mutants exhibited a (trans)dominant-negative effect on wild-type Cx26, as Cx26-RFP was retained inside the cell and particularly co-localized with the A88V mutant. Scale bar = 20μm.

RNA (mRNA) for 9 connexins, including Cx30 (Maher et al., 2005), and are phenotypically similar to basal keratinocytes given their ability to differentiate and stratify (Langlois et al., 2007; Maher et al., 2005; Thomas et al., 2007). At the protein level, REKs abundantly express Cx43, and only express Cx26 upon differentiation (Maher et al., 2005). The fact that REKs did not express detectable levels of Cx30 allowed us to express and track both GFP-tagged and untagged versions of Cx30 and mutants. Similar localization profiles and function were observed for all GFP-tagged and untagged Cx30 and Cx30 mutants, strongly suggesting that the presence of GFP on the C-terminal tail did not affect the properties of Cx30, similar to what has been reported for Cx26 (Marziano et al., 2003) and Cx43 (Jordan et al., 1999).

2.4.1 The non-syndromic hearing loss-linked T5M mutant

The T5M mutant is one of only two Cx30 mutants linked specifically to non-syndromic hearing loss (Grifa et al., 1999; Wang et al., 2011). Consistent with our results, the T5M mutant was previously found to form gap junctions (Common et al., 2002), however, its functional capacity was controversial. *In vitro* electrophysiological studies previously showed that the T5M amino acid substitution drastically altered electrical coupling (Grifa et al., 1999), while other reports showed restrictions in the transjunctional molecules that can pass through T5M channels (Common et al., 2002; Schutz et al., 2010; Zhang et al., 2005). In our study, we demonstrated that both T5M-based hemichannels and gap junction channels were functional to the passage of sizable molecules (e.g. prodium iodide, Alexa 350), raising the question as to why this mutant causes hearing loss.

The answer may be linked to subtle changes in the N-terminal domain of Cx30 where the ‘threonine’ to ‘methionine’ substitution occurs. Cx30 shares 76% sequence homology with Cx26 (Grifa et al., 1999), and while the crystal structure of Cx30 has not yet been resolved, the crystal structure of Cx26 has elucidated that the amino terminal tail lines the gap junction channel pore, creating a funnel that restricts channel selectivity based on molecular size, flexibility, charge and charge distribution (Harris, 2007; Kwon et al., 2011; Maeda et al., 2009). In the non-sensory cells of the inner ear, Cx26 and Cx30 intermix to form heteromeric and heterotypic channels (Ahmad et al., 2003; Forge et al., 2003b; Marziano et al., 2003; Yum et al., 2007), and are also capable of forming

functional hemichannels (Gossman and Zhao, 2008; Zhao et al., 2005). Through extrapolation, a mutation of the highly conserved hydrophilic T5 Cx30 amino acid (Grifa et al., 1999) may alter the permeability properties of both homotypic Cx30 and heterotypic Cx26/Cx30 channels. Another possibility may be how the T5M mutant affects Cx26, as the role of Cx30 in hearing remains controversial (Boulay et al., 2013; Miwa et al., 2013), and increasing evidence suggests that Cx26 and Cx30 are co-regulated (Boulay et al., 2013). Expression levels of Cx26 were dramatically reduced in Cx30 knock-out (Boulay et al., 2013) and in Cx30 T5M knock-in mice that also exhibited decreased levels of Cx30 (Schutz et al., 2010). Importantly, in the inner ear, this mutant may be affecting Cx26 levels, potentially reducing the frequency of heteromeric and heterotypic channel formation necessary for K^+ recycling, however further investigation is necessary to clearly understand this relationship.

2.4.2 The loss-of-function V37E mutant linked to Clouston and KID syndromes

Although previously considered to be linked distinctly to Clouston syndrome (Common et al., 2002; Smith et al., 2002), the V37E mutant has now also been implicated in KID syndrome (Jan et al., 2004). Other than the fact that the V37E mutant was retained in an unknown intracellular compartment (Common et al., 2002), little was known about this mutant. Here, we conclusively show that the V37E mutant was retained within the ER, where it was possibly surveyed by molecular machinery as part of cellular quality control (Kleizen and Braakman, 2004). Furthermore, this loss-of-gap junction channel and hemichannel function mutant acted in a (trans)dominant-negative fashion on co-expressed Cx43, Cx26 and Cx30 and significantly induced UPR-mediated apoptosis in REKs, as indicated by up-regulated levels of ATF4 and CHOP.

The V37E mutant is positioned in the 1st transmembrane domain of Cx30. According to the crystal structure of Cx26, the 1st transmembrane domain is the major pore-lining helix involved in prominent intra-connexin interactions with all other transmembrane domains, which stabilizes the basic structure of the connexin subunit (Maeda et al., 2009). In addition, the V37 amino acid is located in a valine-valine-alanine-alanine (VVAA) motif conserved between Cx26 and Cx30 (Maeda et al., 2009; Smith et al., 2002), and as

demonstrated by the V37I Cx26 mutant, mutations within this particular motif reduce hexamer formation and channel function (Jara et al., 2012). Importantly, the V37E Cx30 mutation involves a more unique and critical substitution of a hydrophobic 'valine' with an acidic 'glutamic acid' residue, which we propose alters critical intra-connexin interactions and Cx30 stability, resulting in improper folding and protein accumulation in the ER. In addition, various Cx26 mutants linked to Vohwinkel syndrome and PPK have been reported to exhibit dominant-negative and transdominant effects on other connexins, including Cx30 and Cx43 (Forge et al., 2003b; Marziano et al., 2003; Rouan et al., 2001). While V37E Cx30 may exhibit these effects on co-expressed wt Cx30, Cx26 and Cx43, it is also possible that V37E-mutant expressing cells undergoing apoptosis are internalizing these connexins, as has been reported for Cx43 (Kalvelyte et al., 2003).

When the V37E mutant was expressed in keratinocytes, it induced apoptotic cell death through the activation of the UPR. The UPR is a protective cellular mechanism regulated by luminal ER chaperone GRP78 (Malhotra and Kaufman, 2007), and is involved in normal keratinocyte differentiation (Sugiura et al., 2009) as well as normal lens development (Alapure et al., 2012). Of note, mutations in Cx31 and Cx50 linked to erythrokeratoderma variabilis (EKV) and cataracts, respectively, induce an abnormal ER stress-mediated UPR (Alapure et al., 2012; Tattersall et al., 2009) which results in extensive cell death (Di et al., 2002; He et al., 2005). While the expression of the V37E Cx30 mutant did not induce the up-regulation of GRP78, which may occur upon the activation of the ATF6 pathway (Berridge, 2002), the V37E mutant significantly induced the expression of ATF4 and CHOP involved in the PERK pathway of the UPR. The induction of ATF4 and CHOP results in the activation of cell death-initiating caspases, including caspase-3, through the mitochondrial-dependent intrinsic cell death pathway (Galehdar et al., 2010; Groenendyk and Michalak, 2005; Malhotra and Kaufman, 2007). Although we identified the first Cx30 mutant to trigger UPR-mediated apoptosis, the apparent lack of GRP78 involvement suggests that further studies are necessary to determine if other cell death initiating pathways may also be involved.

2.4.3 The loss-of-function G59R mutant linked to Vohwinkel/Bart-Pumphrey syndromes

Here, a previously uncharacterized loss-of-function Cx30 G59R mutant was found to occasionally form gap junction plaques, but was mainly localized to the Golgi apparatus. This is consistent with findings for Vohwinkel syndrome-linked G59A and D66H Cx26 mutants (Bakirtzis et al., 2003; Marziano et al., 2003; Thomas et al., 2004), suggesting that mutations in the 1st extracellular domain of Cx30 and Cx26 may lead to similar disease phenotypes. Supporting the essential role of the 1st extracellular loop, the N45K Cx26 mutation located in this domain causes Bart-Pumphrey syndrome (Richard et al., 2004). The evolutionarily conserved 1st extracellular loop of Cx26 (and by extension Cx30) has been suggested to play a role in voltage gating (Tang et al., 2009; Verselis et al., 2009), and more importantly, inter-connexin and inter-connexon interactions (Maeda et al., 2009). Therefore, the Cx30 G59R mutant may result in defective connexin oligomerization, which occurs primarily in the ER and Golgi apparatus for several connexins including Cx32, Cx26, Cx43 and Cx46 (Das Sarma et al., 2002; Evans et al., 1999; Koval et al., 1997; Musil and Goodenough, 1993). Reduced channel function at the cell surface may also indicate defective hemichannel docking, highlighting the importance of this domain in channel formation and function linked to hearing loss and skin diseases.

2.4.4 The A88V mutant linked to Clouston syndrome

The A88V mutant is one of four mutations linked to Clouston syndrome. Previous studies localized the A88V mutant to intracellular compartments, although it could be partially rescued to the cell surface when co-expressed with wt Cx30 (Common et al., 2002; Essenfelder et al., 2004). In contrast, we demonstrated that a population of the A88V mutant formed gap junction-like structures at the cell surface and negatively affected Cx43-based gap junction coupling and the trafficking of both wt Cx26 and Cx30. In addition, the A88V mutant was previously found to exhibit abnormal hemichannel activity associated with adenosine triphosphate (ATP) release and subsequent cell death (Essenfelder et al., 2004). We extended these studies by demonstrating that the A88V mutant significantly induced apoptosis, potentially accounting for the observed dominant-

negative and transdominant effects as suggested for the V37E mutant, as well as induced the up-regulation of UPR protein ATF4. While this suggests that the A88V mutant could be activating a slight UPR, we propose that it results in cell death and disease primarily via a UPR-independent pathway.

Clearly, the A88 amino acid is critical for Cx30 hemichannel and channel function. Cx30 forms voltage-gated hemichannels (Valiunas and Weingart, 2000), which are normally closed under physiological conditions and open in response to low extracellular concentrations of Ca^{2+} and Mg^{2+} (De Vuyst et al., 2007; Tong et al., 2007; Verselis and Srinivas, 2008). Importantly, leaky hemichannels resulting in cell death have been reported for a number of other connexin mutations (Gerido et al., 2007; Lee et al., 2009; Mese et al., 2011; Stong et al., 2006), and also for the A88V Cx26 mutation linked to KID syndrome (Mhaske et al., 2013). The crystal structure of Cx26 suggests that part of the 2^{nd} transmembrane domain also lines the channel pore and is involved in intra-connexin interactions with other domains that dictate specific protein conformation, including the amino terminus (Kwon et al., 2011; Maeda et al., 2009). By analogy, it is possible that the A88V Cx30 mutation affects protein folding and stability, which may result in the retention of a population of this mutant in the ER and trigger a slight ER stress-mediated up-regulation of UPR proteins like ATF4. However, we propose that it primarily alters important interactions that move the amino terminal domain away from the cytoplasmic entrance of the pore (Kwon et al., 2011; Maeda et al., 2009) to favour abnormal hemichannel activity. Therefore, we propose that leaky A88V Cx30 hemichannels primarily induced apoptosis in REKs.

In conclusion, our present study characterized four Cx30 mutations linked to skin disease (A88V), hearing loss (T5M) and combinations of both (V37E and G59R). Each mutation results in disease manifestations through distinct mechanisms ranging from a mutant that exhibits characteristics similar to wild-type Cx30 (T5M), to mutants that induce apoptosis through activation of the UPR pathway (V37E, A88V) or potentially via abnormal hemichannel activity (A88V). Importantly, we discovered the first Cx30 mutation to induce UPR-mediated apoptosis, which may provide insight into how patients with the V37E mutation may develop KID syndrome and not Clouston syndrome, which is linked

to the A88V mutation. Thus, both V37E and A88V mutants cause similar skin phenotypes and induce apoptosis, but through different mechanisms. In addition, the loss-of-function G59R mutant causes yet another skin disease phenotype, a combination of Vohwinkel and Bart-Pumphrey syndromes. While all of these Cx30 autosomal dominant gene mutations cause syndromic and non-syndromic disease by different mechanisms, future studies will be needed to determine the key role of co-regulated connexins in the cochlea and epidermis.

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Chapter 3

3 General Discussion

Effective GJIC plays an important role in regulating a number of cellular processes that contribute to normal tissue function and homeostasis, and defective GJIC has been linked to a number of diseases (Laird, 2006). Alterations in GJIC are caused by mutations in connexins, the building blocks of hemichannels and gap junction channels (Bruzzone et al., 1996; Goodenough et al., 1996). Even single amino acid substitutions may affect any aspect of connexin trafficking, channel formation, channel function or overall connexin stability. Important to this particular study, defective GJIC in the epidermis and the cochlea results in various skin diseases and hearing loss (Nickel and Forge, 2008; Rabionet et al., 2002; Thomas et al., 2003; Zhao et al., 2006), as proper intercellular communication contributes to the continuous process of epidermal differentiation as well as sound transduction (Kikuchi et al., 2000; Langlois et al., 2007; Richard, 2000; Zhang et al., 2005). In particular, Cx30 is highly expressed in both the inner ear and the epidermis (Di et al., 2001; Forge et al., 2003a; Kretz et al., 2004; Nickel and Forge, 2008), and Cx30 mutations result in skin disease, hearing loss or syndromic diseases involving both (Baris et al., 2008; Chen et al., 2010; Essenfelder et al., 2004; Grifa et al., 1999; Jan et al., 2004; Nemoto-Hasebe et al., 2009; Smith et al., 2002; Wang et al., 2011; Zhang et al., 2003).

We investigated four different Cx30 mutants (T5M, V37E, G59R and A88V), each located in a specific protein domain, and hypothesized that each mutant results in disease through a distinct mechanism **(Table 3.1)**. By examining the subcellular localization and function of each mutant in GJIC-competent REKs and connexin-deficient HeLa cells, we discovered that the T5M mutant linked to non-syndromic hearing loss formed functional gap junctions and hemichannels between apposing cells, while the V37E and G59R mutants that cause KID syndrome and Vohwinkel/Bart-Pumphrey syndromes, respectively, were retained in intracellular compartments and were non-functional. In particular, the G59R mutant localized to the Golgi apparatus, with some evidence of gap junction formation at the cell surface, while the V37E mutant was localized only to the

	T5M Non-syndromic hearing loss	V37E Clouston/KID syndromes	G59R Vohwinkel/Bart Pumphrey syndromes	A88V Clouston syndrome
Localization	Gap Junctions	ER	Golgi Apparatus Gap Junctions ER	Intracellular Gap Junctions
Gap Junction Function	YES	NO	NO	?
Hemichannel Function	YES	NO	NO	?
Induces apoptosis in REKs	NO	YES	NO	YES
Induces UPR	NO	ATF4 CHOP	NO	ATF4
Rescued by Cx30	N/A	NO Dominant Negative	NO	NO Dominant Negative
Rescued by Cx26	N/A	NO Dominant Negative	NO	NO Dominant Negative

Table 3.1: Summary of Cx30 mutants linked to skin diseases and non-syndromic hearing loss.

This table outlines four Cx30 mutants linked to non-syndromic hearing loss as well as Clouston, Vohwinkel, Bart-Pumphrey and KID syndromes in terms of their localization, function, ability to induce UPR-mediated apoptosis and propensity to exhibit dominant-negative/transdominant effects on ectopically co-expressed wild type connexins.

ER. In addition, we determined that the A88V mutant associated with Clouston syndrome, as well as the V37E mutant, significantly induced apoptosis in REKs. Further examination revealed that V37E mutant expression resulted in the up-regulation of UPR-related proteins, ATF4 and CHOP, while A88V mutant expression resulted in a less pronounced up-regulation of ATF4 only. When examining the effects of Cx30 mutants on other connexins in REKs, the V37E and A88V mutants impaired the delivery of wild-type Cx26 and Cx30 to the cell surface, potentially contributing to how these mutants cause different disease characteristics.

Cx30 and Cx26 share 76% sequence homology (Grifa et al., 1999), allowing for inferences to be made about the function of Cx30 domains based on what has been reported for Cx26, as its crystal structure has recently been determined (Kwon et al., 2011; Maeda et al., 2009). First, the T5 amino acid is located in the flexible amino terminal domain that lines the channel pore, restricting the pore diameter and channel permeability. Normally in a Cx26 hemichannel, a funnel-stabilizing, circular network of hydrogen bonds forms between the A2 amino acids and their neighbouring T5 amino acids, however, a threonine to methionine substitution (T5M) that introduces a sulphur-containing amino acid, most likely disrupts this network and alters channel permeability (Maeda et al., 2009). Given that cells *in vivo* are expected to co-express wild-type Cx30 and the T5M mutant, Cx30-containing hemichannels could be composed of all wild-type Cx30 or T5M subunits as well as a mixture of both, which may explain why some studies have shown that the T5M mutant is capable of passing electrical current but not metabolic molecules (Schutz et al., 2010; Zhang et al., 2005), while others have described it as completely non-functional (Grifa et al., 1999). Although we did not examine the electrical coupling capacity of the T5M mutant, our results indicate that the T5M mutant was permeable to Alexa 350, a small molecular dye. Secondly, the V37 amino acid is located in the 1st transmembrane domain, which plays a critical role in protein conformation and stability (Maeda et al., 2009). The V37E Cx30 mutant may alter interactions within a connexin subunit, resulting in an unstable protein conformation that prevents its export out of the ER, and triggers the UPR that we observed in REKs. A similar mutant in Cx26, V37I linked to non-syndromic hearing loss, exhibited reduced gap junction function and formed non-functional hemichannels (Jara et al., 2012),

indicating that the 1st transmembrane domain is not only important for protein stability, but also channel function at the cell surface. Thirdly, the G59 amino acid is located in the 1st extracellular loop, which has been suggested to be involved in oligomerization and hemichannel docking. In particular, hydrogen bonds form between the N54 and L56 amino acids in apposing connexins, as well as between apposing G57 amino acids (Maeda et al., 2009), stabilizing the docked hemichannel conformation. Similar to the G59R Cx30 mutant, the G59R, G59S and G59A Cx26 mutants result in many of the same symptoms (Alexandrino et al., 2005; Heathcote et al., 2000; Leonard et al., 2005), and consistent with our results for G59R Cx30, studies have shown that the G59A Cx26 mutant was non-functional and retained primarily in the Golgi apparatus (Marziano et al., 2003; Thomas et al., 2004). The G59R Cx30 mutant introduces a relatively large amine/amide-containing side chain compared to glycine, which could affect steric interactions involved in oligomerization and hemichannel docking, resulting in its retention in the Golgi and loss of function at the cell surface. Lastly, the A88 amino acid is located in the 2nd transmembrane domain, which may partially dictate protein stability, oligomerization and channel permeability (Maeda et al., 2009). The A88V Cx30 mutant may disrupt normal protein conformation and result in some retention in the ER, potentially accounting for the slight UPR we observed. While the localization of the A88V mutant remains unclear, we demonstrated that a population successfully trafficked to the cell surface. We were unable to perform functional studies with the A88V Cx30 mutant in HeLa cells or REKs, as its expression rapidly induced apoptosis in both cell lines. In previous studies, the A88V Cx30 Clouston syndrome-linked and A88V Cx26 KID syndrome-linked mutants both formed leaky hemichannels and resulted in cell death (Essenfelder et al., 2004; Mhaske et al., 2013), highlighting the importance of the A88 amino acid in channel permeability and function. Collectively, connexin point mutations may displace important interactions necessary to maintain proper GJIC. In particular, Cx26 and Cx30 mutations alter epidermal and cochlear homeostasis, resulting in various syndromic skin diseases and hearing loss.

Previous studies suggest that K^+ recycling through GJIC is important for hearing (Hibino and Kurachi, 2006; Kikuchi et al., 2000; Zhang et al., 2005; Zhao et al., 2006). Most gap junction channels in the cochlear epithelial and connective tissue gap junction systems

are composed of Cx26 and Cx30, which are capable of forming mixed channels (Ahmad et al., 2003; Forge et al., 2003a; Forge et al., 2003b; Marziano et al., 2003; Yum et al., 2007; Zhao and Santos-Sacchi, 2000). A great deal of research focuses on the involvement of these connexins in sound transduction, as mutations in Cx26 and Cx30 account for nearly 50% of all prelingual non-syndromic hearing loss cases (Ahmad et al., 2007; Chang et al., 2008; Cohen-Salmon et al., 2002; Schutz et al., 2010). During cochlear development in mice, the absence of Cx30 or the ablation of Cx26 from the epithelial gap junction network had no effect on cochlear morphology, indicating that GJIC may not play a critical role in the developing cochlea (Cohen-Salmon et al., 2002; Teubner et al., 2003). However, further studies revealed that both types of mice were deaf due to the lack of normal endolymphatic K^+ levels and EP, which resulted in sensory cell and supporting cell apoptosis. Initially, these studies elucidated the importance of both Cx26 and Cx30 in sound transduction, rather than cochlear development, however more recent studies indicate that the role of Cx30 remains controversial. Increasing evidence supports the notion that Cx26 and Cx30 are co-regulated, as both genes are located within 50 kilobases of each other on chromosome 13 (Ortolano et al., 2008). In particular, the down-regulation of Cx26 in *Gjb6*$^{-/-}$mice (referred to as Cx30$^{-/-}$ mice in this thesis), originally described by Teubner et al. (2003), was suggested to be the cause of hearing loss (Ahmad et al., 2007; Boulay et al., 2013; Ortolano et al., 2008), implying that Cx26 expression rather than Cx30 expression is critical for hearing. However, Chang et al. (2008) described that while Cx30$^{-/-}$ mice exhibited normal ionic coupling due to sufficient expression of Cx26, biochemical coupling was drastically reduced, indicating that Cx30 channels may be necessary for the proper transfer of metabolites or secondary messengers. Schutz et al. (2010) generated a T5M Cx30 mutant knock-in mouse, which upon investigation, was found to be mildly deaf and exhibit decreased expression of both Cx26 and Cx30 in the cochlea. The T5M mutation was inserted by homologous recombination into mouse embryonic stem cells, and its expression was controlled by the endogenous Cx30 promoter (Schutz et al., 2010). Consistent with our results in REKs, T5M Cx30 trafficked to the cell surface of cochlear supporting cells. However, in contrast, functional studies indicated that while ionic coupling was normal, biochemical coupling was reduced (Schutz et al., 2010), similar to Cx30$^{-/-}$ mice described

by Chang et al. (2008). In addition, mixed gap junction channels have been reported to facilitate the transfer of molecules that would not normally permeate through their homotypic counterparts (Ayad et al., 2006; Beyer et al., 2001; Yum et al., 2007), and Cx26/Cx30 mixed channels are able to pass biochemical molecules twice as fast as their homotypic Cx26 or Cx30 relatives (Sun et al., 2005), favouring the notion that Cx30 may play an important role in hearing through the formation of mixed channels. Collectively, the involvement of Cx30 in hearing remains controversial.

3.1 Conclusions and Future Directions

In conclusion, connexin mutations that affect trafficking, channel formation, channel function and protein expression, can drastically affect GJIC and disrupt normal tissue function and homeostasis. In particular, our study demonstrated how specific Cx30 mutations linked to non-syndromic hearing loss (T5M) and various skin diseases (V37E, G59R and A88V), resulted in disease through distinct mechanisms. However, future studies are essential for expanding our knowledge of how Cx30 mutants affect certain tissues and are linked to specific diseases, while the normal functioning of Cx30 maintains tissue homeostasis and facilitates disease prevention.

To further establish the roles of ER stress and the UPR in apoptosis linked to the V37E and A88V Cx30 mutants, we would first examine the expression levels of a number of ER-resident chaperone proteins, including GRP94, calreticulin and PDI, which are induced during ER stress as a result of the UPR (Berridge, 2002). Since we discovered that ATF4 is up-regulated by the V37E and A88V mutants, we would also further dissect the potential role of the PERK pathway by investigating whether the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) was phosphorylated, an event preceeding the up-regulation of ATF4 that results in transient mRNA translation attenuation to alleviate ER stress (Malhotra and Kaufman, 2007). In addition, investigation of the IRE1 and ATF6 pathways of the UPR would indicate if the expression of V37E and A88V mutants simultaneously activated multiple arms of the UPR. Collectively, these studies would contribute to our knowledge of how these particular mutants are linked to different disease characteristics.

In our studies, Cx30 mutants were transiently expressed in REKs, and we discovered that the V37E and A88V mutants rapidly induced cell death as early as 18 hours following transfection. All of our studies were carried out 24 hours after transfection, and since A88V mutant-expressing cells in particular were undergoing extensive cell death at this time, it was difficult to perform localization and functional studies. Therefore, the generation of an inducible expression system would allow us to perform a number of time course localization and functional studies with this particular mutant, as well as the V37E mutant, immediately following expression and prior to cell death. Using this system may also help to establish a clearer image of the UPR pathways associated with the V37E and A88V mutants.

While these future directions would allow us to examine Cx30 mutants at the cellular level, more relevant models would significantly contribute to understanding the mechanisms underlying Clouston, Vohwinkel, Bart-Pumphrey and KID syndromes. REKs are unique in their ability to stratify into 3D organotypic epidermis, a model system previously used to observe the expression levels and roles of certain connexins during epidermal differentiation, as well as the effects of mutant connexins on the formation of distinct epidermal strata (Langlois et al., 2007; Maher et al., 2005; Thomas et al., 2007). Previously, Thomas et al. (2007) observed the effects of the D66H and G59A Cx26 mutants, linked to Vohwinkel syndrome and PPK, on the growth and differentiation of organotypic epidermis. As previously mentioned, we demonstrated that the G59R Cx30 mutant behaves similar to the G59A Cx26 mutant in REKs. Thus, it would be beneficial to observe whether the G59R Cx30 mutant would affect epidermal differentiation, as the G59A Cx26 mutant had no effect on the differentiation of organotypic epidermis (Thomas et al., 2007). Since this technique requires the generation of stable mutant-expressing keratinocytes (Langlois et al., 2007; Thomas et al., 2007), our model would not be suitable for V37E and A88V Cx30 mutants that induced apoptosis in REKs as early as 18 hours post-transfection.

Cx26 mutants linked to Vohwinkel syndrome (D66H) and lethal KID syndrome (S17F and G45E) have previously been studied in mouse models (Bakirtzis et al., 2003; Mese et al., 2011; Schutz et al., 2011). In engineered mutant mice, the S17F mutant was

conditionally expressed (Schutz et al., 2011), while the D66H and G45E mutants were expressed only in epidermal keratinocytes (Bakirtzis et al., 2003; Mese et al., 2011). While homozygous S17F Cx26 mice were not viable, heterozygous mice were smaller than their wt littermates and exhibited increased epidermal proliferation of tail and foot skin (Schutz et al., 2011). D66H Cx26 mutant mice exhibited keratoderma shortly after birth as a result of thickened cornified layers and extensive apoptosis, indicating premature programmed keratinocyte cell death (Bakirtzis et al., 2003). Mice harbouring the G45E Cx26 mutant were less viable and developed a number of hyperproliferative skin abnormalities, which were associated with abnormal hemichannel activity in cultured mutant primary keratinocytes (Mese et al., 2011). These mouse models elucidated the effects of Cx26 mutants on overall epidermal differentiation and function in an *in vivo* setting. Therefore, the generation of V37E, A88V and even G59R Cx30 knock-in mutant mouse models, in a similar fashion as described by Schutz et al. (2010), would provide critical insight into the mechanisms behind Cx30-linked skin diseases. In particular, since we demonstrated that V37E and A88V Cx30 mutants induced cell death in REKs, generation of these mice would allow us to observe how premature cell death affects epidermal integrity and differentiation in a more relevant model system. Importantly, the G59R mutant mouse may also provide us with insight into why patients experience pseudoainhum. Furthermore, while the T5M Cx30 knock-in mouse (Schutz et al., 2010) contributes to our knowledge of how the T5M mutant is linked to non-syndromic hearing loss, little is known about the mechanisms underlying hearing loss in Vohwinkel, Bart-Pumphrey and KID syndromes caused by Cx30 mutations. Developing these knock-in mutant mice would expand our knowledge of how these mutations disrupt GJIC involved in normal cochlear and epidermal homeostasis in an *in vivo* setting, and would contribute to determining the functional roles of Cx30 in hearing and epidermal differentiation.

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Curriculum Vitae

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1: Kelly, J.J., **Berger, A.C.**, DeMelo, V., Shao, Q., Jagger, D.J., and Laird, D.W. (July 2013). Mutations in Cx30, an unusually stable connexin at the cell surface, cause skin disease via distinct mechanisms. ***International Gap Junction Conference 2013.*** Charleston, South Carolina, USA

2: Amy Berger, Qing Shao and Dale W. Laird. (February 2013). Connexin30 Mutants May Cause Skin Diseases through the Induction of Cell Death Pathways. ***Gap Junction Research Forum 2013.*** The University of Western Ontario, London, Ontario, Canada

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1: Penuela S, Kelly JJ, Churko JM, Barr K, **Berger AC**, Laird DW. (July 2013). Pannexin 1 regulates wound healing and early events in skin development. ***International Gap Junction Meeting 2013.*** Charleston, South Carolina, USA

2: Penuela S, Churko J, Barr K, **Berger A**, and Laird DW. (March 2013). ***London Health Research Day 2013.*** London Convention Centre, London, Ontario, Canada

3: Amy Berger, Qing Shao and Dale W Laird. (December 2012). Connexin30 Mutants May Cause Skin Diseases through the Induction of Cell Death Pathways. ***London Health Research Day 2013.*** London Convention Centre, London, Ontario, Canada

4: Amy Berger, Qing Shao and Dale W Laird. (December 2012). Connexin30 Mutants May Cause Skin Diseases through the Induction of Cell Death Pathways. ***American Society for Cell Biology Meeting.*** San Francisco, California, USA

5: Silvia Penuela, Jared Churko, Kevin Barr, **Amy Berger** and Dale W. Laird. (December 2012). The channel protein Pannexin1 regulates early events in skin development. ***American Society for Cell Biology Meeting.*** San Francisco, California, USA

6: Amy Berger, Qing Shao and Dale W Laird. (November 2012). Connexin30 Mutants May Cause Skin Diseases through the Induction of Cell Death Pathways. ***Physiology and Pharmacology Research Day 2012.*** The University of Western Ontario, London, Ontario, Canada

7: Silvia Penuela, Laszlo Gyenis, Amber Ablack, Jared Churko, **Amy Berger**, David W. Litchfield, John D. Lewis and Dale W. Laird. (April 2012). Pannexin1 Knockdown in Metastatic Melanoma Cells Induces Re-Differentiation Into a Melanocytic Phenotype Decreasing Tumor Size and Metastasis In Vivo. ***Experimental Biology Meeting.*** San Diego, California, USA

8: Amy Berger and Dale W Laird. (March 2012). Characterization of Connexin 30 Mutants Linked to Clouston Syndrome and Non-Syndromic Deafness. ***London Health Research Day 2012***. London Convention Centre, London, Ontario, Canada

9: Amy Berger and Dale W Laird. (November 2011). Characterization of Connexin 30 Mutants Linked to Clouston Syndrome and Non-Syndromic Deafness. ***Physiology and Pharmacology Research Day 2011.*** The University of Western Ontario, London, Ontario, Canada

10: Silvia Penuela, Jared Churko, Laszlo Gyenis, **Amy Berger**, Kevin Barr, David W. Litchfield and Dale W. Laird. (August 2011). Pannexin1 Knockdown in Metastatic Melanoma Cells Induces Partial Cell Re-differentiation into a Melanocytic Phenotype. ***International Gap Junction Conference***. Ghent, Belgium

11: Amy Berger and Dale W Laird. (2011). Characterization of Connexin 30 Mutants Linked to Clouston Syndrome and Non-Syndromic Deafness. ***Undergraduate Honors Thesis Poster.*** The University of Western Ontario, London, Ontario, Canada

12: Penuela S, Gyenis L, Churko J, **Berger A**, Litchfield DW and Laird DW. (October 2010). Pannexin1 regulates the re-differentiation of mouse melanoma cells into a melanocytic phenotype. ***Department of Anatomy and Cell Biology Research Day 2010.*** The University of Western Ontario, London, Ontario, Canada

13: Penuela, S., **Berger, A.,** and D.W. Laird. (September 2010). The channel forming glycoprotein Pannexin1 regulates the redifferentiation of mouse melanoma cells into a melanocytic phenotype. ***EMBO Meeting.*** Barcelona, Spain

Extracurricular/Volunteer Experiences:

2013	**Relay for Life 2013 –** Fundraising Event for Cancer Research London, Ontario, Canada
2012 - 2013	**Wolf Orthopaedic Biomechanics Laboratory** Fowler Kennedy Sports Medicine Clinic The University of Western Ontario, London, Ontario, Canada *Volunteer* Managing Research Associate: Ian Jones
2008 - present	**Dance Extreme Inc.** London, Ontario, Canada *Competitive Dance Instructor* Certifications: Acrobatic Arts Module 1, Acrobatic Arts Module 2