ALTERED CONNEXIN43 EXPRESSION AND FUNCTION IN CALRETICULIN DEFICIENT CELLS

By

Maliheh Aghasi

A Thesis
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Department of Biochemistry and Medical Genetics
Faculty of Medicine
University of Manitoba
and Division of Stroke and Vascular Disease
St. Boniface General Hospital Research Center
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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ABSTRACT

Gap junctions are channels between neighboring cells which facilitate cell-cell communication and influence different processes in the cells, including proliferation and differentiation. Connexins (Conx) are the building blocks of gap junctions. They are synthesized and folded in the endoplasmic reticulum and oligomerised on their way to the cell membrane. Conxs have a short half life and after internalization undergo degradation via both the proteasome and the lysosome. To date, the identities of the chaperones involved in the folding of Conx43 have not been reported.

Calreticulin (CRT), an endoplasmic reticulum resident chaperone, indirectly regulates expression and function of a variety of proteins in the cells. We hypothesized that CRT as an ER lectin-like chaperone is involved in the proper folding and maturation of Conx43 to ensure the formation of functional gap junctions at cell-cell junctions. Furthermore, we propose that CRT via its modulation of the ubiquitin-proteasome pathway can regulate the stability of gap junctions. We utilized wild type and calreticulin knockout mouse embryonic fibroblast cells. Our data showed that there was significantly higher Conx43 protein expression in the calreticulin knockout cells. We also showed that this increase in protein level was due to the activation of the Conx43 promoter. Interestingly, there was lower gap junction density at the cell-cell junction and a significant decrease in the gap junction function in the absence of calreticulin. Administration of Berfeldin A similar disrupted trafficking of Conx43 to cell surface in

both cell types. Inhibition of proteasome activity by MG132 resulted in increased Conx43 localization to the plasma membrane and an increase in the stability of Conx43 at gap junction plaques. Furthermore, inhibiting protein degradation via the proteasome resulted in a significant decrease in the Conx43 promoter activity. These observations suggest that, in the absence of CRT, Conx43 folding, maturation and transport is delayed and once in the membrane Conx43 rapidly undergoes degradation via the proteasome pathway. Interestingly, blocking the lysosomal activity also increased Conx43 protein level, but did not improve the stability of Conx43 at the plasma membrane in both cell types. Inhibition of glycosylation increased the stability of Conx43 at cell membrane of the *wt* and *crt-/-* cells. This effect was most apparent in the *crt-/-* cells. Overall our data suggest an indirect role for calreticulin in regulating the folding, maturation and degradation of Conx43 and also its expression at the mRNA level.

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A. REVIEW OF LITERATURE

I. Connexin

I-1. Introduction

In multicellular organisms, cell-cell communication can be facilitated via gap junctions. Gap junctions form channels between two adjacent cells leading to the direct communication between the cytoplasm of these cells. Opening of the gap junction channel allows the transfer of ions, metabolites and small messenger molecules (less than ~ 1 KDa) through the channels [1]. Gap junctions play important roles in embryonic development, cellular growth control and differentiation [2]. In the cardiovascular system, gap junctions allow a rapid cell-to-cell transfer of action potentials thus synchronizing the contractile activities of both cardiomyocytes and smooth muscle cells [3]. In neuronal cells, gap junctions allow for the simultaneous firing of nerve cells [4]. Gap-junctions remain highly conserved during evolution. In vertebrates gap junction are made of hexamers of Connexin molecules (Conx) [5], however lower organism such as *Drosophila* and *Caenorhabditis Elegans* lack Conx genes. Instead these organisms express innexins or pannexins which are member of the same family as Conx that share the four transmembrane topology but no sequence similarity [6]. Interestingly, three homologs of innexins have been identified in the genomes of mouse and human [4]. Moreover, the expression of two isoforms has been shown in the central nervous system of mice but their function is not yet known [4].

I-2. Structure

Gap junction channels are composed of two subunits known as connexon or hemichannels. The connexon from one cell joins another connexon of the adjacent cell to form the functional gap junction. Each individual connexon is made of six transmembrane proteins known as Connexins (Conx) (Figure 1) [7, 8].

To date, 20 different Conx genes (isoforms) have been identified in the mouse genome [9]. These isoforms are differentially expressed in various tissues. For example, Conx 37 is expressed in endothelial cells of mouse vascular system and it is not expressed in smooth muscle cells [10]. Vascular smooth muscle cells on the other hand express Conx40, 43 and 45 [11]. Conx43 is the most widely expressed member of the Conx family, and it has also been identified as the major gap junction protein in stem cells [12].

Conx has four transmembrane domains and two extra cellular loops which are highly conserved [1]. Extracellular loops of Conx form disulfide bonds which provide the proper conformation for Conx molecules. The extracellular loops of two adjacent Conx molecules form a non-covalent bond which establishes the docking of connexons that forming the gap junction channel with a 2–3 nm gap between cells [13]. Conx also contains a single loop, between the second and third transmembrane region of Conx, which resides in the cytoplasm [1]. The N-terminal and C-terminal domains of each Conx are located in the cytoplasm [1]. The cytoplasmic tail is highly divergent between isoforms of Conx and plays an important role in the regulation of function of gap junction due to the presence of a number of phosphorylation sites [1]. Cardiac Conxs have larger cytoplasmic tail compared to other Conxs [14]. The size of the cytoplasmic loop region

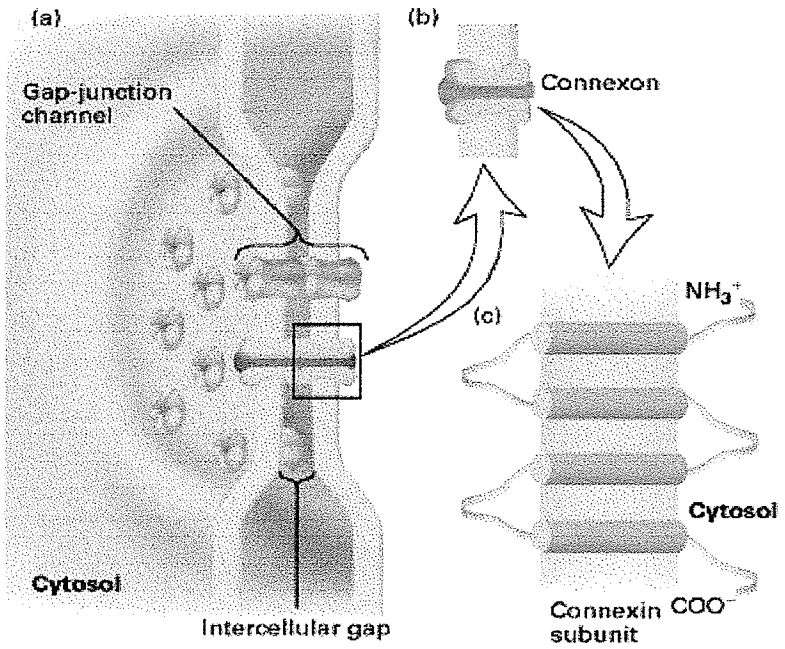

Figure1. A diagram showing the structure of a gap junction (a) connexon (b) and connexin (c) [7].

and the length of the carboxyl terminal tail are different among the various isoforms of Conxs [15].

Each gap junction is made of either homotypic or heterotypic channels. The homotypic intracellular channels are made by the same form of connexons from each of the adjacent cells, whereas heterotypic intercellular channels contain different connexons from each cell [16]. Not all of the Conx isoforms can form heterotypic channels. The formation of heterotypic channels depends on the interaction of the second extracellular loop of the Conx molecules with each other [16]. Furthermore, each connexon may contain either one type of Conx (homomeric), or different Conx misinforms (heteromeric) [17]. It has been suggested that this diversity of Conx channels plays a critical role in regulating their permeability. For example, homomeric connexons made of Conx32 are permeable to both cAMP and cGMP, but heteromeric connexon composed of Conx32 and Conx26 are only permeable to cAMP but not to cGMP [18].

I-3. Conx43 gene

Conx43 is expressed in several adult tissues such as, epithelium [19], lens [20, 21], myocardium [22, 23], myometrium [24] as well as other smooth muscle cells [11, 25]. The adult heart has been considered as an example of regional regulation of Conx43 expression with high Conx43 protein levels in the trabeculae accompanied by a low epicardial Conx43 levels [26]. In addition, Conx43 expression has also been shown to be important for proper embryonic development [23, 26, 27]. This wide distribution of Conx43 expression is regulated via several mechanisms involving both external stimuli and internal signal transduction pathways [28-31].

Data obtained from human, rat and mouse cells and tissue showed that Conx43 expression is regulated at transcription, splicing and translation stages [32]. The Conx43 gene was initially reported to contain two exons and a single intron [33, 34]. The second exon contains the complete coding sequence for Conx [34]. However, a recent report by Pfeifer *et al.* [32] showed that there is an additional four exons in the mouse Conx43 gene. This group also demonstrated the presence of 8 more novel 5′-UTRs in mouse Conx43 mRNA and suggested that these new sites play a role in the differential expression of Conx43 in different cell types [32]. In addition, the existence of an internal ribosome entry site (IRES) within the 5′-UTR (exon I) of Conx43 mRNA has been identified deletion of which suppressed Conx43 transcription [35]. These results illustrate a complex regulatory mechanism for controlling the expression of Conx43 protein.

The promoter region of Conx43 was initially cloned from a mouse genomic cDNA library [34]. Subsequently, the Conx43 promoter was isolated from rat [33] and human [36]. All the promoters contain AP-1 and AP-2 sites immediately upstream of translation initiation sites and this region is highly homologous between different species [33, 34]. Using a chloramphenicol acetyltransferase reporter gene assay, Chen *et al.,* showed the presence of a negative regulatory element (-102 to -92) and a positive regulatory element (-72 and -62) in the 100 bp upstream of the translation initiation signal [37]. Although they reported binding of nuclear proteins to this site, the identity of transcription factors binding to these sites is not known yet. Following the original cloning several studies have focused on identifying the transcriptional regulatory elements in Conx43 promoter. In 1999, Echetebu *et al.,* showed the presence of one AP-1, two SP1 sites, an Ets/NFkB consensus sequence and a TATA box all within the -164 to +148 region of the Conx43 gene [38]. They also showed that both Sp1 and AP-1 sites are necessary for the maximum

promoter activity of Conx43 in human myometrial cell cultures [38]. More recently, two more SP1 sites site has been identified in this region (-148 to -1) of the rat Conx43 gene [39]. Furthermore, a second AP-1 binding site has been described 1 Kb upstream of the transcription initiation site [40].

The AP-1 sites present in the Conx43 promoter have been shown to play a role in the regulation of Conx43 expression in the myometrium during labour [41, 42]. Different members of the Jun and Fos transcription factor family can bind to the AP-1 sites in the Conx43 promoter and regulate its function differentially during labour [42, 43]. Fos/Jun heterodimers have been shown to bind to the AP-1 site closer to the transcription initiation site and increase Conx43 promoter activity a process which requires the activation of Fra-2 [42]. In addition to direct role for binding of Fos/Fra to the AP-1 site, these proteins have been shown to form complexes with other groups of transcription factors such as the glucocorticoid receptor and cAMP response element-binding protein/activating transcription factor (for review see [44]). Stimulation of protein kinase C (PKC) in response to phorbol ester, increased Conx43 promoter activity in uterine smooth muscle cells an effect which was abolished by mutation of the AP-1 site [40]. Thus, illustrating a role for the AP-1 site and transcription factors which bind to it, such as c-Jun and c-Fos, in modulating PKC function [40]. Recently, the AP-1 sites in the Conx43 promoter were shown to be prone to methylation [45]. Methylation of this site resulted in disruption of binding of AP-1 proteins to this site and caused a significant decrease in the Conx43 promoter activity and mRNA transcription in non-small cell lung cancer [45]. Interestingly, a decreased level of Conx43 gene expression has been reported in many cancer cell lines and tumour tissue [46-49], it is not known if there is similarly increased methylation of Conx43 in other tumour cells.

In cardiomyocytes, Conx43 is regulated by a number of cardiac specific transcription factors such as Nkx2.5, Tbx5 and GATA4. Overexpression of Nkx2.5 in neonatal cardiomyocytes resulted in a two fold reduction in Conx43 promoter activity [39]. These cardiac transcription factors Nkx2.5, Tbx5 and GATA4 have been shown to act together with the ubiquitous SP1 transcription factor to modulate the activity of Conx40 promoter in the rat smooth muscle cell line A7r5 [50]. In addition, a Tbx5 family member, Tbx3, has been shown to be highly expressed in the cardiac conduction system and was able to repress the Conx40 promoter [51].

In a recent study on effect of protoncogenes on Conx43 expression, Carystons *et al.,* showed that H-Ras was able to increase both the level of Conx43 protein and mRNA in NIH3T3 fibroblast cells [52]. Furthermore, they found a new *cis*-element in the region between +149 and +158 downstream of the Conx43 transcription start site (RRConxE) which is important for this regulation [52]. They also demonstrated that c-Myc and HSP90 (the 90 KDa heat shock protein) form a complex which binds to this element resulting increased Conx43 promoter activity [52]. The involvement of c-Myc in regulation of Conx43 promoter activity supports the previous reports on the role of MEK1 inhibitor PD98059 on inhibition of Conx43 promoter activity [53].

I-4. Synthesis of Conx

The regulation of intercellular communication requires dynamic changes in the level of gap junction proteins. These changes are achieved by the tight regulation of Conx synthesis and turnover leading to continuous reconfiguration of gap junctions [54]. Conxs have a short half-life of between 1 to 5 hrs [20, 55]. The rate of Conx turnover depends on

the isoform of Conx and the type of cells which express it. As well as altered internal and external signal transduction pathways can affect connexin levels [56]. Conxs are synthesized in the endoplasmic reticulum (ER) where they are folded via an unknown chaperone and integrated into the ER membrane [57]. Conx undergoes oligomerization resulting in dimeric and tetrameric intermediates before forming the mature hexameric Conx [58]. Though the exact intracellular place of oligomerisation of Conx is unknown it has been thought that it is completed when the Conxs enter in to the Golgi apparatus [59]. In addition, oligomerization of Conx has been reported in different localization depending on the isoform of Conx and cell type [60]. For example, oligomers of Conx26 were observed in the secretory pathway, and disruption of the Golgi apparatus by Brefeldin A treatment did not block its transport to the cell membrane whereas Nocadazol (an inhibitor of tubulin polymerization) treatment inhibited this transport [61, 62]. On the other hand, oligomerisation of Conx43 and Conx32 were blocked by Brefeldin A and was not affected by Nocadazol treatment, this finding illustrated the importance of Golgi apparatus in the assembly of Conx43 and Conx32 [61, 62]. These data strongly suggest a role for both the Golgi and the microtubular network in trafficking of Conxs to the gap junctions. They also could explain the difference in the generation of gap junctions containing different Conx isoform under different physiological conditions.

Insertion of connexons into the plasma membrane takes place over large regions of the cell's surface [63]. Studies using GFP-tagged Conx43 have shown that the newly formed Conx are localized in transport intermediates such as secretory vesicles, necessary for the trafficking of Conx to the gap junction [62, 63]. In this process a connexon forming hemichannel dock with another hemichannel in the opposed membrane to form a cell-cell

channel [63]. Then the channels cluster to build a packed group of gap junction channels known as gap junction plaques which is required for gap junction function [64].

I-5. Conx post-translational modification

I-5.1. Phosphorylation

It has been documented in several studies that the newly synthesized Conx undergoes post-translational modifications such as phosphorylation, glycosylation and ubiquitination. Conx43 is synthesized as a 41 KDa protein which undergoes phosphorylation resulting in a shift to 45 KDa on the SDS-PAGE gels [65]. The phosphorylation sites of Conx are localized at its C-terminal tail [66]. Conx43 does not contain any phosphorylation sites in its intracellular loop or N-terminal region [65]. At least five kinases have been reported to phosphorylate Conx43 [67] ensuring a tight regulation of Conx43 phosphorylation in different tissue [68, 69]. Phosphorylation of Conx43 is not only important for its intracellular trafficking and channel assembly but also affects gap junction channel function as well as Conx turnover [70]. A recent study showed an increased level of phosphorylation of Conx43 in the CIN III lesions and cervical carcinomas resulting in the loss of cell-cell communication and altered tissue structure [71]. Therefore, identifying the signalling pathways and kinases involved in the various aspects of the life cycle of Conxs is an important step to understanding the molecular mechanisms responsible for the regulation of gap junction intercellular communication.

There is still no agreement about the exact subcellular compartment in which Conx phosphorylation occurs, although it has been agreed that Conx might be phosphorylated

mostly at the plasma membrane. In addition, pulse-chase studies have demonstrated that Conx43 phosphorylation can also occur in cytoplasmic organelles such as Golgi and carrier vesicles before Conx43 reaches the plasma membrane [72].

The phosphorylation of Conx might not be the only factor essential in the formation of gap junction channels. Indeed, Conx26, which is the only Conx with a short C-terminal tail, does not undergo phosphorylation but is still capable of forming functional channels [55]. Furthermore, truncated Conx43 that lacks most of the C-terminal amino acids can also form functional channels [14]. However, lack of Conx43 phosphorylation in these mutants interrupted the permeability and electrophysiological properties of these channels [14]. In addition, non-phosphorylated Conx43 has also been reported in the cell membrane, although they do not form a functional gap junction in gap junction-deficient S180 and L929 cells [73]. The site of phosphorylation is the key for its effect on Conx fate and function. The carboxyl terminus of Conx43 has more than 12 different motifs containing serine or tyrosine residues which may undergo phosphorylation by at least five different protein kinases [69]. Inhibition of phosphorylation of Conx43 at serine 325 (by casein kinase 1 inhibitor) leads to its accumulation in the intracellular space rather than the cell membrane [74]. On the other hand, phosphorylation of Conx43 at serine 255, in rat epithelial cells, is thought to be required for the closure of the gap junction as well as its internalization and degradation [69]. In general, effect of phosphorylation does not appear to be the only factor important for modifying the biological function of Conx43. Factors such as the Conx isoforms, the site of phosphorylation of Conx, the responsible kinases as well as the type of cells and experimental conditions may modulate the effects of phosphorylation on channel behaviours.

Conx43 phosphorylation and gap junctional intercellular communication are simultaneously regulated during the cell cycle. During the transit of cells from the G_0 to S phase, activation of PKC leads to increased Conx43 phosphorylation which causes less cell-cell communication [75]. Moreover, $p34^{cdc2}$ kinase-dependent phosphorylation of Conx43 decreases gap junction function during the G_2/M phase [76]. Furthermore, phosphorylation at serines 255 and 262 is enhanced during mitosis either through direct or indirect activation of $p34^{cdc2}$ [77]. Increased Conx43 phosphorylation at S368 during the S and G_2/M phases has been related to reduced gap junction assembly [78]. In a recent study, inhibition of Cdc25A phosphatase that is necessary for an accurate G_1-S transition in cell cycle was shown to provoke cell cycle arrest in G_1 as well as the loss of gap junction intercellular communication [79]. Interestingly, inhibitors of EGFR or MEK-1/2 reversed this result [79].

PKC is one of the major kinase families involved in Conx phosphorylation [69, 80, 81]. Its contribution to phosphorylation of Conx43 at S368 and S372 has been hypothesized to play an essential role in tumourgenesis [82]. PKC activity could regulate not only the assembly and degradation of gap junctions but also its function [75]. Generally, agents which induce PKC activation also enhance Conx43 phosphorylation, diminish gap junction communication and promote tumourgenesis however their effects are cell type dependent [67]. A well-known example of these tumour promoting factors is tumour-promoting phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), a common activator of PKC [83], that has been shown to disrupt gap junction intercellular communication in numerous cell types [84, 85]. TPA disrupting effects on intercellular communication are partially a PKC dependent event [69]. Recovery of gap junction

intercellular communication after prolonged TPA treatment in IAR20 rat liver cell lines has been correlated with the down regulation of the PKC activity due to increased PKC degradation [86]. TPA treatment has been shown to induce proteasome activity in IAR20 rat liver cells; in addition, a proteasomal inhibitor MG132, suppressed not only PKC activity but also the recovery of gap junction intercellular communication [87].

cAMP dependent protein kinase (PKA) has been shown to increase Conx43 expression as well as gap junction intercellular communication in mesangial cells after nitric oxide treatment [28]. In addition, elevated intracellular cAMP and activation of PKA increased phosphorylation of Conx43 at S364, which leads to enhance gap junction intercellular communication and produce significant biological changes in these cells [88]. The increase in size and number of gap junction is due to increased Conx43 synthesis and/or enhanced trafficking to the plasma membrane in these cells [88]. Furthermore, phosphorylation of Conx43 on S364 by PKA has also been shown to enhance the assembly of new gap junctions [88].

Another serine/threionine kinase, casein kinase 1 (CK1, mainly the δ isoform) can directly phosphorylate Conx43 at S325, S328 or S330 *in vitro* [74]. In addition, CK1 activity resulted in localization of Conx43 to the plasma membrane but gap junction formation was disrupted thus suggesting a role for CK1 mediated phosphorylation (S325, S328 or S330) in the correct assembly of gap junctions [74].

Phosphorylation of Conx43 on tyrosine residues has been shown to regulate gap junction communication [65, 89]. Both non-receptors tyrosine kinases such as v-Src [65] and receptor protein tyrosine kinases like EGF and PDGF [90] phosphorylate Conx43 on

tyrosine residues and regulate gap junctional communication. *In vitro* studies using purified protein showed that $Pp60^{v\text{-}Src}$ can phosphorylate Conx43 [91]. Furthermore, co-expression of $Pp60^{v\text{-}Src}$ and Conx43 in insect cells resulted in phosphorylation of Conx43 [92]

Using site directed mutagenesis Lin *et al.* [93] showed that v-Src phosphorylates Conx43 at Y247 and Y265. They also showed that Y265 phosphorylation was required for Y247 phosphorylation. These phosphorylation events results in the disruption of gap junction communication [93]. In addition to its role in tyrosine phosphorylation of Conx43, v-Src activation also mediates activation of MAP kinase which has been shown to disrupt gap junction intercellular communication [94]. Activation of both v-Src and MAPK has been shown to decrease the electrical coupling of the cells due to decreases in the channel open probability [95] and not a change in gap junction channel number or localization [93]. Furthermore, VEGF receptor 2 (Flk-1 or KDAR) tyrosine kinase activation cause a transient reduction in gap junction communication via the activation of both the c-Src tyrosine kinase and MAP kinase that are responsible for serine/threonine phosphorylation of Conx43 [94].

Activation of the EGF receptor tyrosine kinases by ligand binding results in a marked increase in the phosphorylation of Conx43, at serine residues [80]. This event triggers the rapid and transient disruption of gap junction communication which appears to be based on defects in individual channel activity rather than the number of existing gap junction plaques in the plasma membrane. This effect of EGF on Conx43 function was due to the activation of the downstream MAP kinase and phosphorylation of S255, S279, and S282 residues of Conx43 [80]. Interestingly, expression of Conx43 mutant (S255A,

S279A, and S282A) lacking MAPK phosphorylation sites in Conx43 knockout cells by transient gene transfer restored functional gap junctions in these cells. The function of these gap junctions, however, was unaffected after EGF receptor activation [96]. These data confirmed the involvement of S255, S279, and S282 residues in mediating EGF effects on gap junction communication. A recent report has demonstrated that these three serine residue of Conx43 could also be phosphorylated by ERK-1 and ERK-2 downstream of EGF activation [79].

Activated PDGF receptor disrupted gap junction intercellular communication via stimulation of the PKC and MAPK downstream effectors as well as additional signalling pathways such as of ERK1/2 in the mesangial cells of the kidney glomerulus [97]. Another receptor tyrosine kinase, FGF-2, has also been shown to increase phosphorylation of Conx43 on serine residues and decreased gap junction intercellular communication between cardiomyocytes [98]. FGF-2 did not affect Conx43 mRNA or protein localization at sites of intercellular contact. [99]

In the living cell, the functions of different processes are regulated by cycles of phosphorylation (mediated by a number of protein phosphatases). As described above Conx43 phosphorylation inhibits gap junction function, on the other hand inhibition of protein phosphatases such as protein phosphatase 1 and 2 alpha and 2 beta is also implicated in the regulation of Conx43 phosphorylation [100-103]. The role of each phosphatase seems to be dependent on the tissue and cell type studied. Studies about non-ischemic rat heart failure, which is characterized with increased level of PP2A, showed increased co-localization of PP2A with Conx43 and no change in co-localization with PP1[102]. These hearts also contained increased level of non-phosphorylated Conx43 and

reduced cell coupling [102]. This effect was abolished after okadaic acid treatment. However, in the ischemic heart and *in vitro* cardiomyocyte model both okadaic acid and calyculin A was shown to decrease ischemic induced dephosphorylation of Conx43 [104]. Furthermore, these authors reported that fostriecin (a PP2A specific inhibitor) had no effect on Conx dephosphorylation, emphasizing the role of PP1 in this experimental model [104].

I-5.2. Glycosylation

Conx43 does not contain a consensus glycosylation sequence [57]. Although the mature Conx is not a glycoprotein, some studies have demonstrated the effect of glycosylation on gap junction function [105]. Inhibition of glycosylation in MHD1 cells (Morris Hepatoma cell line) increased both the basal and cAMP induced gap junction intercellular communication [105]. This increase was due to an increase in the number of gap junction plaques (not mRNA level) [106]. On the other hand, high glucose treatment of bovine retinal endothelial cells led to loss of gap junction intercellular communication, decreased Conx43 protein level [107], enhanced degradation of Conx43, and decreased half-life of the protein. Inhibition of proteasome activity (MG132 or lactacystin) in addition to high glucose treatment prevented the decrease in Conx43 protein and restored gap junction intercellular communication, illustrating the role of proteasome dependent degradation in this process [107].

I-6. Connexin degradation

Two major pathways have been identified for the degradation of Conx: the lysosome and the proteasome (Figure 2) [108]. Internalization of gap junction plaques from the plasma membrane is a required and regulated process for Conx degradation [109, 110]. To examine the role of gap junction internalization in its degradation Jordan *et al.* used GFP-tagged Conx43 and live cell imaging [111]. These authors demonstrated that in Rat Kidney cells expressing GFP-Conx43 co-cultured with non-transfected cells (expressing endogenous Conx43) the internalized GFP-Conx was present only in one of the cells forming the gap junctions [111]. Electron microscopic studies [112] and cell fractionation experiments [113] have demonstrated that the internalized gap junctions are localized to endosomes, which then associate with the lysosomes. More recent studies show the importance of both the lysosomal and the proteasomal pathways in mediating Conx degradation [110, 114, 115].

The lysosomal pathway has been shown to be important for degradation of internalized gap junctions [46]. Inhibition of lysosome by chloroquine [116] or leupeptin [115] resulted in increased Conx43 immunoreactivity at the plasma membrane. Furthermore, inhibition of the lysosome by leupeptin resulted in accumulation of Conx43 in a human breast cancer cell line with no effect on gap junction communication [117]. In addition, the lysosome was shown to target degradation of newly synthesized Conx43 delivered from early secretory compartments and creates a lysosomal pool of Conx43 in a human breast tumour cell line which exhibited reduced dye coupling [117]. How Conx43 was delivered to lysosome is unclear, one possibility could be that unstable assembled gap junction is internalized quickly from the membrane to the endosome, or misfolded and

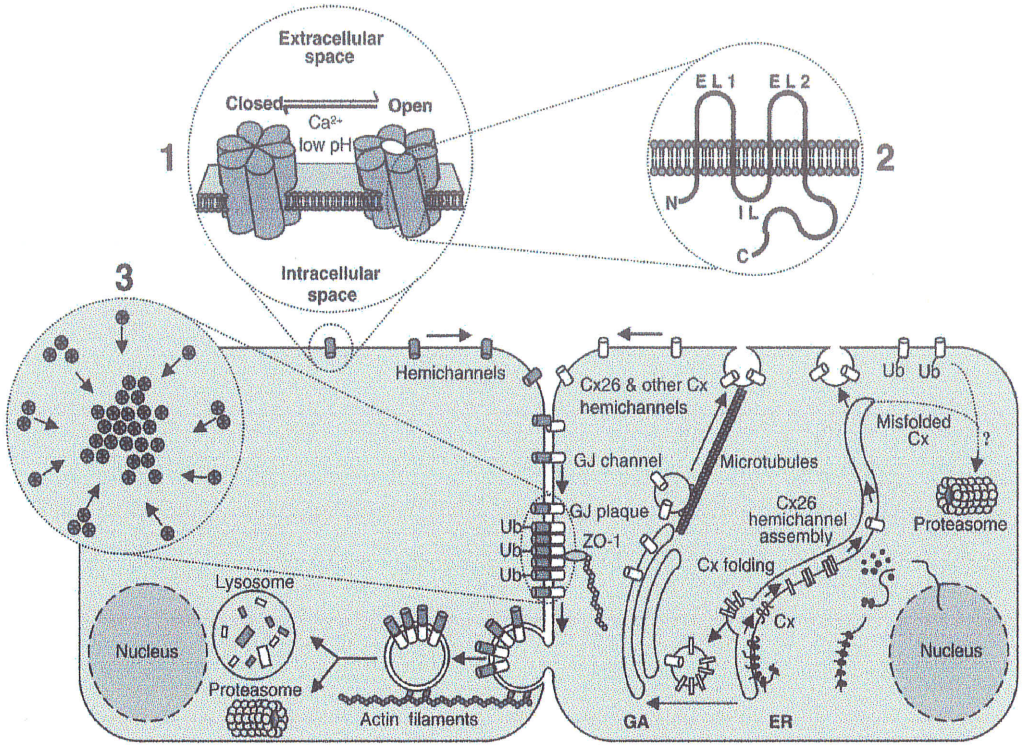

Figure 2. A diagrammatic representation of connexin expression, trafficking and degradation in a typical cell [108].

aggregated Conx were triggered for lysosomal degradation via the secretory pathway [118]. The latter pathway is more plausible due to the observation that accumulation of misfolded DsRed-tagged Conx43 occurs within the lysosome in HBL-100 cells, whereas wild-type Conx43 assembled into gap junctions and localized in the plasma membrane [118]. Interestingly, it has been demonstrated that generating a point mutation at tyrosine 286 of human Conx43 increased its stability at plasma membrane and led to increase gap junction communication in SKHep1 cell line in which degradation of Conx43 is mostly lysosome dependent [56]. In general, the molecular configuration of the Conx protein plays the most important role in its lysosomal degradation [110].

A role for the proteasome pathway in Conx degradation was originally suggested due to the observed extension of the Conx43 half life following the inhibition of proteasomal degradation in cultured cells [119] and the intact heart [120]. Furthermore, Conx43 ubiquitination which is required for its proteasomal degradation occurs at the plasma membrane before Conx43 internalization [121]. TPA can promote Conx43 ubiquitination leading to its internalization and proteasomal degradation [122].

The ubiquitin proteasome pathway is important for the degradation of misfolded proteins [8]. This process is important in proofreading of newly synthesized proteins and quality control. Inhibition of this pathway (via MG132 or lactacystin) results in accumulation of phosphorylated Conx43 and increased gap junctional communication [110, 114]. Furthermore, inhibition of the proteasome revealed the presence of a small population of Conx43 (and Conx32) which translocate from the ER to cytosol due to defect in their folding [123]. Interestingly, proteasomal degradation of Conx43 is enhanced upon phosphorylation by different ligands. Leithe *et al.*, showed that EGF induced hyperphosphorylation of Conx43 resulting in its proteasome dependent

degradation which can be blocked by MG132 [121]. Exposure to high glucose was reported to activate PKC leading to the phosphorylation and degradation of Conx43 via the proteasome pathway [107]. TPA, a well-known activator of PKC, has been shown to disrupt gap junction intercellular communication in numerous cell types by increasing the internalization and degradation of Conx43 in a proteasome dependent manner. For example, treatment of lens epithelial cells with TPA and proteasome inhibitors resulted in the accumulation of phosphorylated Conx43 in the cell membrane, increased intercellular communication through gap junction channels and extended half-life of the phosphorylated Conx43 [21]. In general, these data indicated that the rate of proteasomal degradation modulates the stability of phosphorylated Conx43 and affects Conx half-life by promoting its internalization from the cell surface [110]. More importantly, it has been demonstrated that not only the interaction of Conx43 with other molecules but also the conformational changes in Conx43 protein itself are responsible to mark the protein for internalization [124].

I-7. Regulation of Gap junction function

The previous concept that gap junctions are inert and passive pores between cells required only for the transit of ions or small molecules less than 1 KDa has greatly changed. Currently, gap junctions are referred to as a highly controllable filter that regulates the passage of molecular information between cells. For example, a new study showed the novel finding that non-hybridized and hybridized forms of siRNA are likely to be transferred between mammalian cells via Conx-specific gap junctions [125].

The most studied function of Conx43 is its contribution to the formation of gap junction channels to permit the passage of small molecules and ions between neighbouring cells [126]. The rate of Conx expression, folding and trafficking to the plasma membrane as well as internalization and degradation of gap junctions all affect the efficiency of gap junction intercellular communication [127]. The ability of gap junctions to transmit molecules between cells is regulated by a number of factors including intracellular pH [128], oxidative stress [129, 130], phosphorylation [103] and protein-protein interaction [126, 131, 132]. In addition, modulation of gap junction function by all the above factors is dependent of cell type studied. A role for change in intracellular pH on gap junction function was originally demonstrated using expression of Conx43 or Conx32 in paired *Xenopus* Oocytes [128]. These authors showed that the acidification of intracellular space increased gap junction intercellular communication when either Conx43 or Conx32 were expressed [128]. In the heart however, intracellular acidification was shown to uncouple gap junctions leading to the development of cardiac arrhythmias [133]. The proline rich residues in the C-terminus (residues 271-287) of Conx43 has been shown to be responsible for the acidification mediated uncoupling [134, 135].

Phosphorylation of Conx causes conformational change in the structure of the gap junction leading to its closure or opening depending on the stimulus, the isoform of Conx, and cell type. Phosphorylation of Conx32 or Conx40 by PKA was shown to increase gap junction intercellular communication [136]. Similarly Casein kinase I mediated phosphorylation of Conx50 in sheep lens increased gap junction coupling [137]. To date at least 12 sites of phosphorylation has been identified in the carboxy terminus of Conx43 which are extensively phosphorylated by five different protein kinases [69] (as described above).

Src mediated phosphorylation of tyrosine residues of Conx43 can inhibit gap junctional communication [65, 89]. Lin *et.al.*, showed phosphorylation of Y265 alone did not affect gap junction communication [93]. This elucidated the importance of phosphorylation of both the Y265 and Y247 for efficient inhibition of cell-cell communication. The effect of Src on gap junction was shown to be due to diminishing the open probability of the channel not its conductance [69, 138]. Treatment of the cells with lysophosphatidic acid has also led to phosphorylation of Conx43 on tyrosine residues (with no change in serine phosphorylation) and inhibited gap junction coupling [139]. The permeability of Conx43 hemichannels increases following phosphatase treatment [140].

Growth factors such as EGF, PDGF and VEGF regulate gap junction communication not only by affecting Conx phosphorylation but also via phosphorylation independent mechanisms. EGF treatment has been shown to activate MAPK leading to phosphorylation of Conx43 at serine residues and disrupt gap junction intercellular communication [95, 96]. However, longer treatment with EGF (2–3 hrs) elevated the level of cellular Conx43 protein causing an increase in gap junction communication [141]. In addition, activation of the EGF receptor, and MAPK by Vitamin K_3 (menadione) produced loss of gap junction intercellular communication in WB-F344 rat liver epithelial cells due to the increased phosphorylation of Conx43 at plasma membrane with no visible changes in the localization of Conx43 [142]. VEGF stimulation of endothelial cells also increased Conx43 phosphorylation and interrupted gap junction communication [94].

In addition to growth factors, treatment of cells with TPA [143] or activation of PKC [69, 138] has been shown to phosphorylated Conx43 and disrupt gap junction communication. The effect of PKC stimulation was mediated via a decrease in

conductance through the gap junction channel [69]. Furthermore, PKC mediated phosphorylation of chicken homologue of Conx46 led to a significant decrease in gap junction communication [54]. However, PKC stimulation in rat neonatal cardiomyocytes was reported to induce no changes [144] or increase gap junction conductance [138]. This discrepancy could be due to different in isoform of PKC involved in these cells. Erk1/2 activation by TPA has been shown to hyperphosphorylate Conx43 resulting in its internalization and decreased gap junction function [143]. In contrast to the above kinases, PKA mediated phosphorylation of Conx43 increases gap junction communication [22].

The first evidence of involvement of Ca^{2+}-Calmodulin in Conx and gap junction was elucidated when a direct interaction between Conx43 and Calmodulin was observed in hepatocytes [145]. However, these authors did not demonstrate any effect on gap junction communication. The Ca^{2+}-Calmodulin kinase II inhibitor, KN93, was able to disrupt the gap junctional conductance in auditory afferent nerve cells [146]. A role for Ca^{2+}-Calmodulin kinase II in the activation of gap junction was further illustrated in mouse spinal cord astrocytes [147]. Recently, Ca^{2+} dependent binding of Calmodulin to Conx50 has also been shown in lens [148]. This interaction between Calmodulin and Conx50 was shown to be required for channel assembly and gating properties [149]. To date, no data is available on the interaction of Calmodulin with Conx43.

Other factors affecting gap junction communication include mechanosensitivity. Mechanical stress was shown to open the Conx46 channel at negative potentials, and close them at a positive potential [150]. This mechanosensitivity of Conx46 hemichannels is essential for its physiological role as Conx46 is expressed at high levels in the lens and it is the major Conx forming gap junction in eye. Factors secreted by neighbouring cell can

also affect gap junction communication. For example, it has been reported that human marrow stromal cells in culture secret soluble factors that considerably improve astrocytic gap junction intercellular communication [151]. These factors not only trigger the elevated expression of Conx43 in astrocytes but also facilitate the function of gap junction [151]. Astrocytes are coupled via Conx-43 to form a cellular network that is essential in neuroprotection process by normalizing the level of K^+ and neurotransmitters via gap junctions [152].

In addition to the above factors, the rate of Conx protein turnover and their localization plays an important role in the regulation of gap junction intercellular communication. The gap junction intercellular communication deficiency in tumour cells that are able to express Conxs is reported to be due to the loss of localization of Conx to the plasma membrane [114]. This could be the result of Conx instability at the plasma membrane leading to internalization of Conx before formation of functional channel [114]. Alternatively, a defect in the trafficking of Conxs to the cell surface for gap junction assembly could reduce the number of gap junction plaques and reduce gap junction communication [110]. For example increasing the half life of Conx by inhibition of proteasome activity has been suggested to be efficient in promoting the gap junction intercellular communication in lens epithelial cells [21]. Moreover, high glucose treatment in bovine retinal endothelial cells led to enhanced internalization and degradation of Conx43 and decreased the gap junction intercellular communication [107]. On the other hand, impaired gap junction communication in human prostate cancer cell lines was shown to be due to a defect in trafficking of Conx to plasma membrane but not the inability to form gap junctions [153].

I-8. Proteins interacting with Conx

To date, several proteins have been reported to interact with Conx family members. Some of these proteins interact with a specific isoform of Conx. For example several members of the CCN (Cyr61/connective tissue growth factor/nephroblastoma-overexpressed) interact only with Conx43 in C6 glioma cells [154]. While zonula occludens-1 (ZO-1) bind to all types of Conxs [155-157]. Conx interactions with these proteins modulate Conx conformation and in the most cases lead to Conx degradation or altered gap junction communication [158, 159]. However, Conx interaction with some proteins appeared to be required for effective assembly of gap junction and channel opening maintenance.

Calmodulin interacts with Conx at an early stage of gap junction assembly. In a recent study, it has been observed that the Calmodulin antagonist W7 repressed oligomerization of Conx32 in an *in vitro* cell free transcription/translation system [160]. Interestingly, immunocytochemical studies of the transfected COS-7 cells with the truncated Conx32 that lack the Calmodulin binding site illustrated that these Conxs accumulated in intracellular spaces [160]. This observation supports the hypothesis that oligomerisation of Conx is a Calmodulin-dependent step. Close association between Calmodulin and Conx32 is further reported in HeLa cells expressing Conx32 and in *Xenopus* Oocytes [149]. In addition, a Ca^{2+} dependent interaction between Conx50 and Calmodulin in the lens was demonstrated recently using confocal co-localization and co-immunoprecipitation experiments [148].

The N-terminal region of ZO-1 was also shown to bind to the carboxyl terminal region of Conx43 [132]. In cardiomyocytes, the interaction of ZO-1 with Conx43 and the

tyrosine phosphorylation site which is the c-Src binding site were reported to be essential for regulation of Conx localization [161]. Both c-Src mediated tyrosine phosphorylation of Conx43 and c-Src binding to Conx43 via SH2-SH3 domains inhibited ZO-1 binding to Conx43 and its membrane localization [161]. In addition, diminished association of Conx43 to ZO-1 at low pH is due to an enhanced association between Conx43 and c-Src (dominant-negative) [124]. ZO-1 has been observed to bind to catenins in epithelial cells and cardiomyocytes [162]. The formation of a catenin-ZO-1-Conx43 complex is required for Conx43 transport to the plasma membrane during the assembly of gap junctions [163]. The establishment of an organized adherent junction is suggested to be a prerequisite for the subsequent progressive formation of gap junctions in cardiomyocyte and non-ephithelial cells [164]. Furthermore, ZO-1 has been known as a linker molecule between the cadherin/catenin complex and the cytoskeleton through direct interaction with alpha catenin and actin filaments in non-epithelial cells [165]. ZO-1 functions as a basic component in the cadherin-based cell adhesion system [165]. These data suggest that ZO-1 is not only a linker of Conx43 to cytoskeletal actin but it acts as a scaffold to hold Conx43 in a close connection with other associated proteins. In the heart interaction of Conx43 and alpha-spectrin, via ZO-1, has been proposed to be essential for the localization of Conx43 at the intercalated discs, and producing functional gap junctions [166]. Such interaction has been also reported between ZO-1, Conx46 and Conx50 in the lens [167] and Conx43 and Conx32 in liver cell lines [168]. Moreover, overexpression of the N-terminal domain of ZO-1 (cytosolic form) in Conx43 expressing cells trigger localization of Conx43 from the cell-cell border to the cytoplasmic region, leading to loss of electrical coupling [166].

Indeed antibodies that blocked the interaction of N-cadherin/catenin complexes with the actin cytoskeleton diminished the formation of adherens junction and inhibited the assembly of gap junctions [164]. Moreover, preventing the formation of adherens junctions by utilizing antisera against α-catenin, β-catenin, and ZO-1 prevented the assembly of gap junction in cardiomyocytes [163]. A role for cross-talk between Conx43, catenin and N-cadherin complex has been implicated in the regulation of the motor apparatus necessary for the neural crest cell movement [169]. ZO-1 binding with E-cadherin has also been reported at the cell surface in MDCK cells [162]. This interaction was mediated by the ability of catenins to recruit ZO-1 from the cytosol to the plasma membrane which is a process that could be blocked by decreasing the levels of E-cadherin [162].

The ability of Conx to interact with caveolins (the structural proteins of caveolae) has been reported using co-localization, co-fractionation, and co-immunoprecipitation experiments [170]. Caveolin-1 has two distinct binding sites for Conx43 in the caveolin-scaffolding region (residues 82-101) and the C-terminal domain [170]. Caveolin binding with Conx43 recruits PKC to phosphorylate Conx thus regulating gap junction communication [171].

Another cytoskeletal protein which binds to Conx43 is tubulin (α and β) [131]. Sedimentation experiments in COS-7 cells have illustrated the direct interaction of Conx43 with tubulin [131]. The tubulin-binding motif is unique for Conx43 and it is not observed in other Conx family [131]. Disruption of microtubule structure in Rat-1 cells (by Nocadazol) had no effect on gap junction intercellular communication suggesting that Conx43-microtubule interaction does not play a significant role in the regulation of gap

junction function [172]. In addition, Conx43 interaction with α-tubulin as well as β-tubulin has been reported in a different study [132]. Therefore, the attachment of microtubule ends to gap junctions might support the properties of microtubules in intact cells.

A novel 85-KDa Conx43 interacting protein (CIP85) has been identified by *in vitro* interaction assays, *in vivo* co-immunoprecipitation and immunocytochemical experiments [159, 173]. This interaction is mediated through the two proline-rich regions in Conx43 protein [173]. CIP85 co-localized with the Conx43 at the plasma membrane and provoked the lysosomal degradation of Conx43 in HEK293 (human embryonic kidney cells) and Hela cells (human breast cancer cells) [159]. The exact role of this protein in the regulation of other Conxs is not known yet.

I-9. Conx in disease

Defects in gap junction properties and Conx expression have recently been documented in a variety of diseases, such as some forms of neuropathy [174], hereditary deafness [175], cataracts [176], skin disease [177], Charcot-Marie-Tooth disease [178], heart disease [179] and different types of cancer [48]. Conx43 has been identified as tumour suppressor gene both in cell culture and whole animal [47, 180, 181]. One suggested mechanism for prevention of tumour growth is via increasing gap junctional communication between normal and tumour cells [47]. This increased communication can improve the transfer of anti-proliferative signals to cancer cells, thus restoring the growth regulatory process and inhibiting improper proliferation [47]. Kaempferol has been reported to act as an anticancer agent by increasing the expression of Conx43 protein in a

tumourigenic colon cancer cell line that already expresses endogenous Conx43 mRNA [49]. However, it did not suppress tumourigenic colon cancer in cell lines that initially were not able to express Conx43 mRNA [49]. Intra hepatic metastasis of the hepatocellular carcinoma was correlated with the down regulation of Conx43 expression suggesting a role for conx43 in metastasis [182].

In the heart, a decrease in Conx43 expression and its dephosphorylation by protein phosphatase 2A has been proposed to uncouple cardiomyocytes during non-ischemic heart failure in rabbits and humans [102]. Furthermore, dephosphorylation of Conx40 and Conx43 by protein phosphatase 1B and subsequent impaired gap junction has been reported to be associated with an aberrant conduction system in patients with systolic dysfunction leading to arrhythmias and sudden death [29]. A role for Conx43 in the development of the inflammatory events in atheroma, as well as in the generation of overall inflammatory response in the arterial wall has also been shown [25]. Other Conxs also appear to be important in the immune system. For example, lipopolysaccharide (LPS) induced liver inflammation triggered a sudden down regulation of Conx 32 due to its rapid degradation [183].

II. Calreticulin

II-1. Introduction

CRT is as an endoplasmic reticulum (ER) resident protein that affects many cellular functions both in the ER and outside the ER. CRT was first isolated in 1974 by

Ostwald and Maclennan and purified based on its high affinity for Ca^{2+} [184]. The human [185] and mouse [186] CRT genes have been cloned and are 70% identical [186]. The CRT gene contains 9 exons and 8 introns [185, 186]. In the human, CRT gene is localized on chromosome 19 at locus p13.3-p13.2 while in mouse CRT gene is located on chromosome 8 [187]. There is no evidence for alternative splicing of the CRT mRNA [188] and the sole mRNA transcript of CRT is 1.9 KB. CRT protein is 46 KDa and contains 400 amino acids. At the amino acid level, CRT shares a high percentage of identity (about 90%) among human, rabbit, rat and mouse [188].

The expression of CRT is induced by depletion of Ca^{2+} stores, by Zn^{2+}, heat shock and amino acid deprivation (for review see [188]). CRT is a ubiquitous eukaryotic protein and many functions have been attributed to this protein. The two main functions of CRT are its lectin like chaperone activity and regulation of Ca^{2+} homeostasis [188]. Other functions which are attributed to CRT include regulation of gene expression and cell adhesion [187]. Here, I will discuss the functions of CRT relevant to my research.

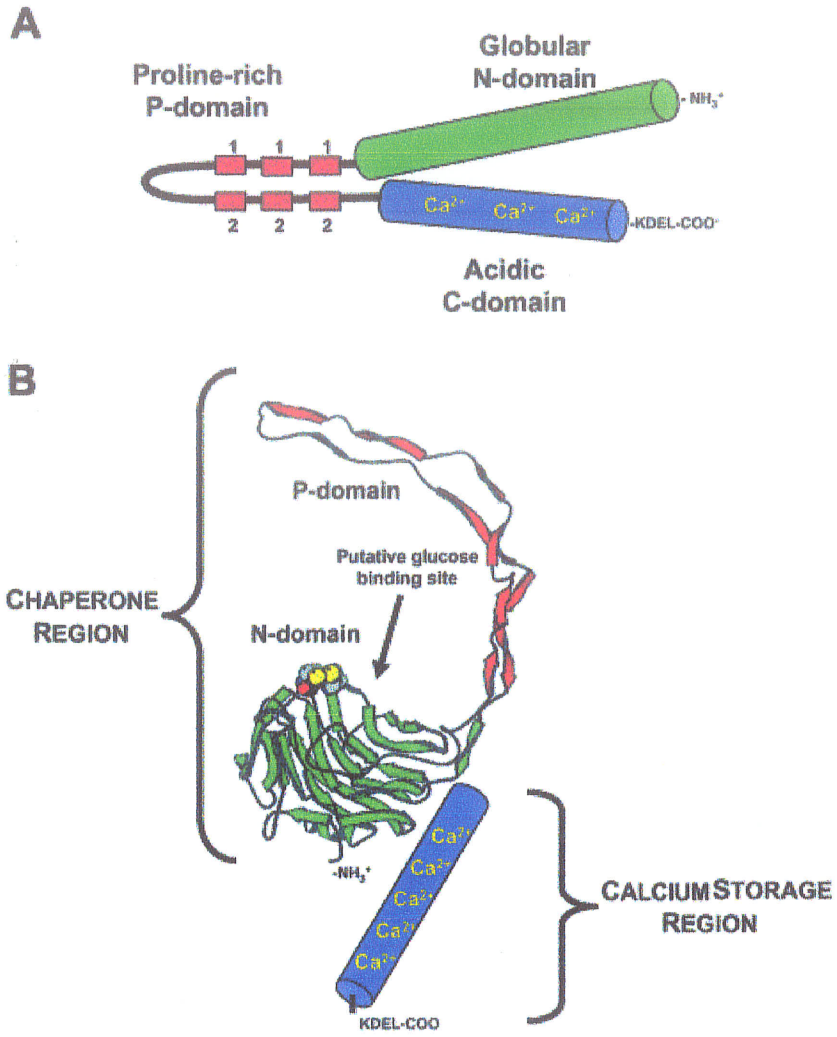

Figure 3. The putative three-dimensional model of CRT protein [118].

II-2. Structure

The CRT protein has been divided into at least three domains according to its structure: a globular N-domain with most conserved sequence of amino acid, a proline rich P-domain and a negatively charged (acidic) C-domain [189] (Figure 3). The N-domain is highly hydrophobic and has a globular structure consisting of eight anti-parallel β-strands [187]. This globular structure is similar to that of Calnexin and is involved in glucose binding [189] and therefore it is important in the lectin-like function of CRT. The N-domain contains 3 cysteine residues, two of them form an intermolecular disulphide bridge [190]. Moreover, the N-domain of CRT includes 5 histidine residues, which are responsible for Zn^{2+} binding [191] that causes significant conformational changes in CRT [192]. This alteration is essential for CRT interaction with the DNA-binding domain of glucocorticoid receptor [193, 194] or other nuclear receptors [195, 196]. Point mutations of the histidine residues of the N-domain of CRT showed that His^{153} is important in its folding and chaperone function of [197]. This mutation leads to the increased susceptibility of CRT to proteolysis (by trypsin) and confirms the role of N-domain in meduating the stability of CRT [197].

The P-domain of CRT is a proline-rich region. It forms an extended arm structure which consists of three short helices and three anti-parallel β-sheets [187]. The high concentration of proline, serine and threonine residues in P-domain make this region highly charged [198]. This region interacts with other chaperones (for example protein disulfide isomerase (PDI) and Erp57) [188]. The P-domain also contains two sets of repeated peptide sequences which are important for the lectin–like chaperone activity of CRT [199]. This domain is similar to Calnexin, containing the binding site to a

monoglycosylated glycoprotein [200]. Together the N-domain and P-domain encompass the chaperone region of CRT. The P-domain also contains a high affinity, low capacity Ca^{2+} binding site [201]. However, the P-domain does not contain a proper EF hand structure (a Ca^{2+} binding motif) [201].

The C-domain is highly acidic due to a large number of negatively charged amino acids such as aspartic and glutamic acid [199]. The C-domain binds Ca^{2+} with a high capacity and low affinity (over 25 mol Ca^{2+}/mol protein) [199]. This region is involved in the storage of Ca^{2+} in ER and it is important for regulation of interacellular Ca^{2+} homeostasis[187]. The C-domain also contains a C-terminal KDEL ER-retention sequence [199]. In addition, to the ER retention signal, the CRT protein starts with an ER signal sequence [188]. Both of these sequences are required to ensure localization of CRT to the ER. The ER localization of CRT is observed by immunolocalization techniques [202].

II-3. CRT as an ER chaperone

The ER contains the protein folding machinery of the cell. In the rough ER, ribosomes associate with mRNA and ER to ensure the delivery of the newly synthesized protein in to the ER lumen. Membrane proteins, secretory proteins and proteins requiring glycosylation are directed into the ER for their proper folding and formation of correct tertiary structure. ER contains a number of chaperones and modifying enzymes (glycosylasion, disulfide isomerase) which help in this process. As an ER chaperone, CRT helps to fold newly synthesized proteins in the ER. It has been shown that CRT functions in association with its membrane-bound homologue calnexin, as lectin-like molecular chaperones [203]. The majority of proteins that are translocated into the ER in eukaryotic

cells are *N*-glycosylated and it has been reported that *N*-Linked glycosylation is a highly conserved process in eukaryotic evolution [204]. CRT and calnexin contribute in a well-characterized series of binding and releasing to monoglucosylated structures ($Glc_1Man_{5-9}GlcNAc_2$) [205] generating a glycoprotein specific chaperone cycle [206]. In contrast to the other chaperone systems, which directly interact with polypeptide sequences, CRT and calnexin bind to monoglucosylated oligosaccharides of the unfolded glycoproteins [205]. This interaction depends on the activity of carbohydrate-modifying enzymes and lectins [207]. Glucosidase I and II are enzymes important for the removal of glucose residue thus releasing the newly synthesized protein from their association with CRT/calnexin [208]. On the other hand, if the newly synthesized protein is not folded properly the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT) will insert a glucose residue to the mannose site of the newly synthesized protein and the protein will re-enter the CRT/calnexin binding cycle [209]. The inhibition of interaction between glycoproteins and CRT/calnexin in cells defective in glucosidase I (II) or cells treated with glucosidase inhibitors, which block deglucosylation of the protein-bound $Glc_3Man_9GlcNAc_2$, confirms the importance of a single glucose residue for the CRT/calnexin binding [206]. In addition, it has been reported that the treatment of mouse embryonic fibroblast cells (MEF) cells with glucosidase inhibitor which prevent trimming of N-glycans to the monoglycosylated form, blocks the association of newly synthesized polypeptides with calnexin and CRT [210] and results in the accumulation, aggregation and retention of misfolded proteins in the ER, thus disrupting ER function (ER stress).

In the cycle of binding and release of the unfolded proteins from the chaperone, de-glucosylation by glucosidase II prevents the proteins from interacting with chaperone

and re-glucosylation by UGGT promotes binding to CRT/calnexin [211]. The re-glucosylating enzyme or UGGT recognizes only incompletely folded glycoproteins as substrates and does not re-glucosylate native structures or free oligosaccharides [212]. This unique capability of UGGT to differentiate between the native from non-native conformations of its glycoprotein substrates has been observed not only *in vitro*, but also in living cells [207]. In addition, UGGT prefers partially folded conformations as substrate rather than entirely denatured proteins. This observation suggests that this enzyme operates at late stages during glycoprotein folding [212]. In other words, after completing its interaction with CRT, the folded glycoprotein would be released from the CRT complex by the action of glucosidase II. Glucosidase II removes the terminal glucose from the glycoprotein [206]. As part of quality control process, incompletely folded proteins or misfolded ones would be targeted to UGGT [212] which will add the terminal glucose to the protein and inhibits transport of proteins that have not obtained a native conformation [213]. UGGT in collaboration with both CRT and calnexin are important for the synthesis and folding of many proteins such as cell surface receptors and transporters [203], and play an important role in directing the maturation and quality control processes of the early secretory pathway. [214]

The ER quality control machinery is essential for the removal of incompletely folded proteins and ensures that only the correctly folded and functional molecules are organized and exported to their specific localization in the cells [215]. Misfolded proteins which do not pass quality control are translocated to the cytosol for proteasomal degradation via a process called ER associated degradation (ERAD) [216]. A recent study showed that an oligosaccharide modification that is common to several distinct proteins,

rather than a specific amino acid sequence, functions as a signal for ER quality control to target the misfolded protein for degradation by the proteasome [217]. Furthermore, Yoshida *et al.,* have shown the existence of an E3 ubiquitin ligase selective for N-glycosylated polypeptides in neuronal cells [218]. This E3 ubiquitin ligase, named Fbx2, binds N-glycosylated peptides in the cytoplasm (following activation of ERAD) and targets them for degradation via the proteasome [218]. The UGGT system is able to recognize the glycan component of the misfolded protein and modify it to generate an asparagine-linked oligosaccharide binding to the lectin chaperone [216]. The ER Mannosidase-I will then modify this glycan and generate $Glc_1Man_8GlcNAc_2$ which delays the release of the glycoprotein substrates from lectin chaperones triggering their degradation of the protein by the 26S proteasome in the cytosol [216].

Interestingly, increased level of misfolded proteins in the ER stimulates the expression of chaperones in the ER. The accumulation of misfolded protein in ER results in a process termed the unfolded protein response (UPR). UPR has been observed in cultured cells after protein misfolding is induced by: heat shock, blocking the disulfide bonds, depletion of ER Ca^{2+}, or by preventing protein glycosylation [200]. Activation of UPR results in increased expression of chaperone which is necessary for cell survival and elimination of misfolded proteins from the ER [219]. Accumulation of toxic proteins that escape the quality control process may cause serious disease such as a heritable form of liver cirrhosis associated with plasma α1-antitrypsin deficiency [216], Cystic fibrosis [220], familial hypercholesterolemia [221], a heritable form of pulmonary emphysema [222] and Alzheimer`s disease [223].

CRT also contains a site capable of binding to polypeptide segments of unfolded glycoproteins [224], which raises the possibility that it posses a chaperone activity which is independent of its lectin binding ability. Indeed, Saito *et al.* have shown the formation of protein complexes between CRT and a non-glycosylated protein *in vitro* [225]. This interaction and inhibition of protein aggregation was improved upon ATP and Zn^{2+} addition [225]. The ability of calnexin to bind to non-glycosylated protein and prevent their accumulation has also been shown *in vitro* [226]. As with CRT, this interaction is enhanced in the presence of ATP [226]. Recently, the ability of *in vivo* binding of calnexin and CRT to non-glycosylated peptides has been shown too [227]. Although calnexin and CRT have the same lectin ligand specificity, calnexin is a membrane bound protein whereas CRT is soluble in the ER lumen. It has been shown that calnexin binds to membrane proximal glycans but CRT binds to glycans that appears deeper into the lumen [212]. Several studies confirmed the clear distinction between calnexin and CRT substrates. This fact has been validated strongly by observing the fate of CRT and calnexin transgenic mice. As calnexin deficient mice die at 8 to 10 days after birth with severe motor abnormalities [228]. Importantly, CRT cannot replace calnexin function to rescue these animals to survive. Moreover, CRT deficient mice also die but at embryonic stage (14.5-16.5 dpc) due to developmental defects [229] and calnexin, although overexpress din these mice, is not capable to replace the function of CRT [230].

II-4. CRT and Ca^{2+}

The ER is the main site for storage for Ca^{2+}. Stimulation of Ca^{2+} release from ER via the IP_3 receptor or Ryanodine receptor leads to a rapid increase the in cytosolic Ca^{2+}

concentrations which can activate multiple processes in the cell such as cell motility, proliferation, contraction, signal transduction and gene expression [231]. Enhanced transcriptional and translational cascades that result from increased Ca^{2+} concentration stimulates many processes including ER chaperone activity [232] as well as quality control in the ER [233]. Subsequently, these Ca^{2+}-dependent signals could be involved in regulating ER stress, the UPR and ERAD [233]. CRT knock out ES cells displayed disrupted integrin mediated Ca^{2+} signalling [198]. Thus the regulation of Ca^{2+} homeostasis is crucial for cells and cells utilize various strategies to tightly regulate intracellular free Ca^{2+} levels. CRT has been shown to be important for the regulation of Ca^{2+}-homeostasis [197, 234, 235].

CRT plays an important role in the regulation of Ca^{2+} homeostasis by binding to Ca^{2+} and storing it in the ER. As discussed above, CRT has two Ca^{2+} binding sites [188]. Overexpression of CRT increases the amount of intracellular Ca^{2+} stored [234, 236], however CRT deficient cells did not have a reduced capability to store Ca^{2+} in the ER [198, 229]. In addition, CRT overexpressing fibroblast cells did not show increased permeability to divalent cations after Ca^{2+} store depletion [234]. CRT can also stimulate the Ca^{2+} influx after Ca^{2+} store depletion [237, 238]. It has been proposed that CRT, due to its Ca^{2+} storage capacity within the ER, affects both the release of Ca^{2+} from the ER via inositol 1,4,5-trisphosphate ($InsP_3$) receptor mediated Ca^{2+} release and the transport of Ca^{2+} back into the ER through a Ca^{2+} pump known as sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [239]. These functions are probably due to protein/protein interaction of CRT with either SERCA2 or $InsP_3$ receptor, or they could be as a result of the lectin-like chaperone activity of CRT. Overexpression of CRT in *Xenopus* oocyte resulted in a complicated cytosolic Ca^{2+} signals (Ca^{2+} waves) after

$InsP_3$microinjection [240, 241]. Expression of CRT and SERCA2 increased Ca^{2+} release without the concomitant oscillation [241]. Moreover, the loss of Ca^{2+} handling in the ER in CRT deficient (*crt-/-*) cells interrupts the agonist-mediated Ca^{2+} release from the ER [229].

On the other hand, Ca^{2+} plays a regulatory role in CRT interaction with other chaperones, e.g. protein disulfide isomerase [188]. In addition, the Ca^{2+} binding domain in the P-domain of CRT binds to blood-clotting factors [188] and inhibits injury-induced restenosis [242]. Any interruption in Ca^{2+} homeostasis can induce the apoptotic pathways by regulating the function of proteins involved in this pathway [243]. Due to detrimental effect of uncontrolled changes in Ca^{2+} concentration in ER and cytosol, this process is controlled by several ion channels, pumps, Ca^{2+} binding proteins and signalling transduction pathways. For example, the anti-apoptotic protein Bcl-2 (either wild type or selectively localized to the ER) significantly inhibits InsP3-mediated calcium release from ER in WEHI7 cells [244]. Therefore, any alteration in CRT protein levels modifies the Ca^{2+} availability for release from ER and can affect apoptotic stimulation. These events can modulate Calcineurin (Ca^{2+}/Calmodulin-dependent serine/threonine phosphatase) activation which is responsible for stimulating several signal transduction pathways involved in apoptosis or survival [239]. Consequently, CRT deficient cells are more resistant to apoptosis [245] because they have less ER luminal Ca^{2+} and impaired p53 activity [246]. On the other hand overexpression of CRT increases the sensitivity of the cells to apoptosis [245, 247, 248].

II-5. CRT and cell adhesion

CRT has been shown to modulate cell adhesion by altering the expression and function of adhesion molecules [188, 249]. In 1997, Coppolino *et al.* showed that CRT is capable to bind to the tail of integrin *in vitro* and alter its function [198]. In general, increased expression of CRT correlates with an increase in cell adhesiveness and decreased CRT level correlates with reduced cell adhesion [250]. Due to the localization of CRT to the ER, it has been suggested that any effect of CRT on cell adhesion would be indirect via controlling the protein-tyrosine kinases activities and expression [249], modulation of gene expression of adhesion-related molecules and by affecting the integrin-dependent Ca^{2+} signalling [198].

Overexpression of CRT in retinal pigment epithelial cells elevated levels of vinculin expression [250]. In addition, L fibroblast cells overexpressing CRT had increased mRNA levels of N-cadherin [249]. Interestingly, any change in the level of CRT was shown to modulate phosphorylation of □-catenin [249]. □-catenin is a part of cadherin-mediated adhesion complex structure, a member of the Armadillo protein family and a member of the Wnt/Wingless signalling transduction pathway [251]. Tyrosine phosphorylation of β-catenin modulates its stabilization in junctional complexes [252]. Cells that overexpress CRT exhibit increased adhesiveness and show reduced tyrosine phosphorylation of β-catenin and increased membrane localization of β-catenin compared to the wild type cells [249]. Protein phosphorylation on tyrosine has been identified to have a significant function in mediating cell adhesion [253]. This finding suggests the presence of a novel pathway which is involved in CRT-dependent modification of tyrosine

phosphorylation and through which the ER signals to the plasma membrane to modulate cell adhesiveness [249].

Another example of CRT effects on cell adhesion is CRT binding to thrombospondin (TSP). TSP is an extracellular matrix protein that exists in both soluble and insoluble-extracellular matrix bound form. CRT interaction with TSP is required for TSP function to stimulate focal adhesion disassembly [254]. Zn^{2+}- and Ca^{2+}-dependent interactions support CRT binding to TSP via a binding site in the N-terminal region of the N-domain of CRT [255].

II-6. Calreticulin and diseases

There is currently no direct evidence for a link between CRT and a specific human pathology. However, impaired ER functions such as protein folding and modulation of Ca^{2+} homeostasis could result in severe pathology. There is a correlation between function of CRT and appearance of these diseases [189]. For example, deficiencies in protein folding is identified as a significant cause for development of such severe diseases as Alzheimer's disease, prion and neurodegenerative diseases, lysosomal storage disease, cystic fibrosis, α1-antitrypsin deficiency, myeloperoxidase deficiency, and defects in carbohydrate metabolism [212].

CRT has been identified as a crucial protein in cardiac development in mice [229] and it could also be vital for the appropriate development of the human heart as well. CRT is elevated in early embryonic heart development but its expression decreases sharply after birth [229]. Increasing the post-natal expression of CRT in the heart of transgenic mice

has been reported to cause severe arrhythmias and a complete atrio-ventricular (AV) conduction block [236]. Increased CRT could be a reason of complete heart block in children [189].

The association of CRT with Systemic Lupus Erythematosus (SLE) has been reported [256]. The serum of SLE patients contains elevated levels of circulatory anti-CRT antibodies [257]. To date, it is not clear how CRT, which is normally located in the ER, is released to the extracellular space and blood of these patients. It has been suggested that in the course of SLE development CRT is released to the extracellular space due to an ongoing necrotic and apoptotic process in the patient [258]. The released CRT in the blood then can induce (activate) the immune-response in the SLE patient [259]. The other hypothesis is that viral infection can induce the expression of CRT [260]. This hypothesis stems from the observation of increased viral infection (cytomegalo virus) in some SLE patients [261].

III. Transgenic mice

III-1. Conx expression in transgenic mice

Recent studies using transgenic mice have shown the importance of Conx for the normal function of different organs. The observation that Conx46 deletion results in deformities in the lens formation [262] or Conx26 deletion causes embryo malformation due to decreased trans-placental uptake of glucose [263] indicates the importance of Conxs during development. In addition, Conx40, Conx37, Conx43 and Conx45 are known as "cardiovascular" Conxs and display significant phenotypic heterogeneity between man and mouse.

Gene targeted deletion of Conx43 caused neonatal lethality and the newborn pups died immediately after birth due to malformation of the conotruncal region of the right ventricle [27]. These mice also have an obstructed cardiac outflow track which is associated with infundibular pouches [27]. The formations of infundibular pouches are important in the regulation of coronary vasculogenesis. Thus, the data from the Conx43 deficient mice indicates a crucial role for Conx43 in formation of gap junctions required for coronary vasculogenesis and vascular remodelling [264]. It also implies a correlation between Conx43 down-regulation and arrhythmias [265]. In the cardiovascular system, deletion of Conx40 causes partial atrioventricular conduction block [266] and the Conx40 deficient mice are more prone to arrhythmias [267]. However Conx37 knockout mice were viable and had normal heart function [268]. Interestingly, the $Conx37^{-/-}/Conx40^{-/-}$ double deficient mice also could not survive soon after birth as a result of vascular abnormalities with pronounced dilatation of blood vessels [269]. The $Conx40^{-/-}/Conx43^{+/-}$

mice on the other hand, demonstrated aberrant cardiac conduction and morphogenesis triggered in neonatal death in most of cases [270]. These results offer an understanding of the function of different members of the Conx gene family during mouse development. Although, Conxs have their unique functions in the tissue were they are expressed, they can largely compensate for the loss of the other family members to a degree.

Interestingly, overexpression of Conx43 in the heart resulted in heart malformations such as right ventricular defects, abnormal development of surface coronary vasculature and enlargement of the conotruncal region of the right ventricle [271]. These mice also had defects in the cranial neural tube. The sudden death in these mice has been attributed to heart defects [271]. These observations demonstrate that the precise level of Conx43 expressions and function plays a vital role in the heart development.

III-2. CRT expression in transgenic mice

Gene targeted deletion of CRT in mouse is embryonic lethal by 14.5-16 days post-coitus [229]. The homozygous CRT null embryos had defects in heart morphology including a decrease in the thickness of the ventricular wall in and intertrabecular recesses [229]. On the other hand, overexpression of CRT in the heart postnatally resulted in a decrease in systolic function, sinus bradycardia and prolonged atrioventricular node conduction followed by complete heart block and sudden death 3 weeks after birth [236].

Interestingly, overexpression of CRT in the heart resulted in a reduction in the levels of Conx 40 and Conx43 proteins and mRNAs and protein [236]. Furthermore, western blot analysis of these hearts showed that the amount of both phosphorylated and

non-phosphorylated Conx43 was significantly decreased [236]. These observations suggest that CRT might play an important role in the regulation of Conx gene expression or it might regulate the folding of Conx43 protein and assembly of gap junctions.

B. RATIONALE

Conxs are the fundamental building blocks of the gap junction channels which are important for the communication between neighbouring cells. Gap junction communication is important in many different processes in the multi-cellular organism, including cell proliferation and differentiation. Thus, the process of assembly and disassembly of gap junctions is highly regulated. One point of regulation is the rate of synthesis and maturation of Conx. Conxs have a very short half life and their correct folding is important for trafficking and function. Conxs are synthesized in the ER and transported via the Golgi apparatus to the cell membrane. Many reports have suggested a role for a chaperone in the synthesis and maturation of Conx. However, to date, the identity of this chaperone is not known. CRT is an ER resident chaperone which has been shown to regulate the folding and maturation of many proteins such as MHC class I, InsP3 receptor and bradykinin receptor. CRT can also modulate the function of other chaperones in the ER. Furthermore, our lab has recently showed a role for CRT in the regulation of the ubiquitin proteasome pathway. Therefore, this study was carried out to test the following hypothesis.

C. HYPOTHESIS

CRT as an ER lectin-like chaperone is involved in the proper folding and maturation of Conx43 to ensure the formation of functional gap junction at cell-cell junctions. Furthermore, we propose that CRT via its effect on modulation of the ubiquitin-proteasome pathway can regulate the stability of gap junction and Conx degradation.

D. MATERIALS AND METHODS

I. Material

The rabbit polyclonal antibody to Conx43 was a generous gift from Dr E. Kardami (Department of Human Anatomy and Cell Science, University of Manitoba). The rabbit polyclonal antibody to actin and mouse monoclonal anti-tubulin as well as leupeptin, Saponin, Tunicamycin, cycloheximide (CHX) and luciferase were purchased from Sigma-Aldrich (Oakville, ON). DMEM, Opti-MEM, Collagen and Lipofectamine2000 were purchased from Invitrogen (Burlington, ON). The Vectashield mounting medium with DAPI was from Vector Laboratories (Burlingame, CA). Texas-Red labelled anti-concavalinA, Brefeldin A, Rhodamine Dextran and Lucifer yellow were purchased from Molecular Probes (Burlington, ON). MG132, Wortmanin, were obtained from Calbiochem (Mississauga, ON). Enhanced chemiluminescence detection system (ECL) was purchased from SantaCruz (California). In addition, all secondary antibodies e.g. Horse Radish Peroxidase (HRP), Texas-red and FITC-conjugated goat anti rabbit secondary antibody were obtained from Jackson Laboratories (Bar Harbor, Maine). Qiagen maxi prep plasmid purification was from Qiagen (Mississauga, ON). Formaldehyde and other general chemicals were from Fisher Scientific (Ottawa, ON).

II. Cell culture

Wild type (*wt*) and CRT knockout (*crt-/-*) cells were used in this study. 14-day-old mouse embryos were used to isolate the mouse embryonic fibroblast cells (MEF) as

described previously [246]. These cells were regularly cultured in Dulbaco Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS).

III. Cell Treatment

To examine changes in the Conx synthesis, post-translational modification, transport to plasma membrane and its degradation in response to different pharmacological agents, cells were seeded on Petri dishes and allowed to attach over night. In some experiments, glass cover slips were added to the culture plates. To inhibit protein synthesis, cells were treated with media containing 10 μg/ml of CHX for 4 hr following [110]. In order to disrupt the Golgi apparatus, cells were treated with media containing 2 μg/ml of Brefeldin A [115] for 4 hr. To block the glycosylation process in the cells 4 μg/ml of Tunicamycin was added to the media and cells were incubated for 6 hrs 10 μl [106].

As described earlier Conx43 is degraded via both proteasomal and lysosomal degradation pathways. To examine the role of proteasomes in degradation of Conx43, cells were treated with 20μg/ml MG132 for 4 hr [115]. To inhibit lysosomal protein degradation pathway, Leupeptin was added at a final concentration of 100 μg/ml to media [115, 121] for 6 hr incubation at 37°C.

Cells were then washed 3 times with 1% phosphate-buffered saline (PBS) followed by immunoblot, immunocytochemical localization of Conx43 as well as reporter gene assay as would be described later (below). To inhibit MAPK signalling pathway, cells were starved for 24 hr (serum free media) and then were treated with media

containing 10% FBS and 50μM PD98059 a MEK or MAPK/ERK kinase kinase 1 inhibitor [272]. Then cells were lysed and reporter gene assay were carried out.

IV. Immunoblotting

For analysis of protein expression of Conx43, cells were cultured until they reached 80% confluency in Petri dishes and then each plate was washed with ice-cold PBS. Cells were lysed in New RIPA buffer containing 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 10 mM Na4P2O7, 30 mM NaF, 0.5% NaDoc, 0.1% SDS 1% NP4O and 1% Triton x 100, as protease inhibitor cocktail, as well as two phosphatase inhibitors Sodium Fluoride (30 mM) and activated Vanadium Chloride (1 mM). An aliquot of the cell lysates was used for quantification of protein content using a BioRad Dc-Protein Assay Kit (Bio-Rad, Hercules, CA). After protein assay 30 μg proteins were mixed with SDS electrophoresis sample buffer (10 mM Tris, pH 6.8, 15% w/v glycerol, 3% w/v SDS, 0.01% w/v Bromphenol blue and 5% v/v 2-mercaptoethanol) and resolved on 7.5% SDS polyacrylamide gel (SDS-PAGE). Proteins were then transferred to the nitrocellulose membranes using a semi-dry transfer method.

For Western blot analysis, the nitrocellulose membranes were incubated in PBS containing 5% skim milk powder for 1 hr at room temperature to block any non-specific binding. Rabbit anti-Conx43 antibody (1:2000) was then added to the membrane and incubated for 2 hrs at room temperature. The membrane was then washed 3 times with PBS containing 0.05 % Tween (10 min each). Subsequently the membrane was incubated for 1 hr with HRP conjugated anti-rabbit secondary antibody (1:10000). Enhanced chemiluminescence detection system (ECL) was used as a substrate and protein bands

were visualized on a Fluor-S-Max MultiImager system (BioRad, Hercules, CA). To ensure equal loading of proteins blots were first stripped by incubation for 1 hr in a stripping buffer containing β-mercaptoethanol at 55°C followed by extensive wash with PBS to remove any trace-amount of β-mercaptoethanol. The blot was then re-blocked with 5% skim milk powder followed by incubation with rabbit anti-actin (1:1000) or mouse anti-tubulin (1:200) antibodies for 2 hr at room temperature. Protein bands were quantified by Fluor-S-Max machine and the optical densities of the bands were measured with Quantity One program. All results are representative of at least three independent experiments. The western blot data are presented as a ratio of Conx43 to the loading controls.

V. Immunocytochemistry

To examine the localization of Conx43 and its trafficking, immunocytochemical studies using fluorescent antibodies were carried out. The *wt* and *crt-/-* cells were plated on glass cover slips at 70% confluency and incubated for 24 hrs for the cells to form monolayers. Cells were then treated as described above with media containing MG132; Brefeldin A; MG132 and Brefeldin A; CHX; MG132 and CHX; tunicamycin; MG132 and tunicamycin or leupeptin. One group of cover slips were not treated and labelled as controls. In few experiments, the effect of starvation on Conx43 localization was also investigated using immunocytochemistry. In brief, *wt* and *crt-/-* cells were starved for 24 hrs to eliminate protein synthesis. Starved cells were then divided in to 4 groups: control (non-treated), MG132, Brefeldin A or MG132 and Brefeldin A treatment as described above. At the end of each treatment coverslips were washed with PBS and cells were fixed

in 4% formaldehyde in PBS for 10 min. Coverslips were then washed by 3 subsequent changes of 0.1% saponin (permeabilizing agent) in PBS. Non-specific sites were then blocked with 0.1 % saponin in PBS and 2% milk powder for 1 hr. Rabbit anti-Conx43 antibody (1:2000) was added to each coverslip and incubated for 2 hrs at room temperature. Coverslips were then stained with either FITC-conjugated goat anti-rabbit or Texas-Red conjugated goat anti-rabbit secondary antibody at 1:70 dilutions for 1hour at room temperature. Each coverslip was co-stained with Texas-Red labelled anti-concavalin A in (1:100 dilution) to determine the co-localization of Conx43 with the ER. Finally, all coverslips were mounted on slides using Vectashield mounting media with DAPI to stain the nucleus. Fluorescent images were obtained using a Zeiss Axioplan 2 microscope equipped with a digital camera.

VI. Transient transfection

Transient transfection was used for the reporter gene assays. The mouse pConx1686-luc containing the Conx43 promoter (1.8kb) upstream of luciferase reporter gene was a kind gift from Dr. S Lye (University of Toronto). This plasmid was used to examine the changes in Conx43 promoter activity. A pcDNA3.1 β-galactosidase plasmid (β-gal) was used as a control for transfection efficiency. Both of these plasmids were transformed in to *E. coli* (*DH5α* strain) and individual clones were isolated and confirmed by restriction enzyme digestion. One colony of each plasmid was then expanded and super-coiled DNA was purified by Qiagen column chromatography.

MEF cells (*wt* and *crt-/-*) were seeded in 6-well plates at a density of 400,000 cells per well and left overnight. For transfection, 2 μg of pConx1686-luc and 2μg of β-gal

plasmid were used for each well. Transient transfection was carried out using a mixture of 10 µl Lipofectamine 2000 and 4µg total DNA in Opti-MEM media. The mixture was left for 20 min at room temperature. The DNA/Lipofectamine 2000 mixture was then added to the cells and incubated for 4 hrs at 37°C. At the end of the incubation Opti-MEM media was replaced with DMEM containing 10% FBS and cells were incubated for 48 hrs followed by lysis and reporter gene assays.

In a few experiments, cells were treated with MG132 or PD98059 to investigate the role of proteasome or MEK activation on the Conx43 promoter. For MG132 treatment, 40 hr after transfection, the media was changed to DMEM containing 10 % FBS and 20 µg/ml MG132 and cells were incubated for 4 hrs. For PD98059 treatment, 20 hrs after transfection media was changed to DMEM alone (starvation media). After 24 hrs, plates were divided to two groups. First group was control starved and received the same starvation media for another 12 hrs. The media of the second group was changed to DMEM containing 10% FBS and 50 µM PD98059 and incubated at 37°C for 12 hrs. Cells were then lysed and used for reporter gene assay.

VII. Reporter Gene Assay

To study changes in Conx43 promoter activity, luciferase reporter gene assays were carried in out as described previously [246]. Briefly, transfected cells (*wt* and *crt-/-*) were lysed in 100 µl NP40 lysis buffer containing 100 mM Tris pH 7.8, 0.5% NP40 and 50 mM DTT. Cells were incubated in the lysis buffer for 10min; the cell lysates were centrifuged briefly to precipitate insoluble particles. To measure the luciferase reporter gene activity in these cells, 20 µl of each sample was aliquoted in duplicate and incubated

with a buffer containing 20 mM Tricine, 1.07 mM MgCO3, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin and 530 μM ATP. The luciferase activity was then measured using a Lumate Luminometer. For the β-galactosidase assay, 20 μl of cell lyaste was mixed with 20 μl O-Nitrophenyl β-D-galactopyranoside (ONPG) and 80 μl H2O in a 96 well plate and incubated for 1 hr at 37°C. The activity of β-galactosidase was measured by a microplate reader at an optical density of 420 nm (OD 420). The results were then presented as a ratio of the Luciferase activity to the β-gal activity of each sample in duplicate. Data was then presented as mean ± standard error (SE) using Microsoft Excel.

VIII. Scrape Loading/Dye Transfer Assay

To study intercellular communication via gap junction channel, a dye transfer assay was performed [114]. *wt* and *crt-/-* cells were seeded on 18 mm-diameter collagen coated glass coverslips. Cells were incubated in DMEM containing 10% FBS till they formed a confluent monolayer. Coverslips were then rinsed with PBS followed by the addition of PBS containing 0.1% Lucifer yellow (LY) (a gap junction permeable dye) and 0.1% of the gap junction-impermeant compound Rhodamine-Dextran. The monolayer cell was then scraped using a scalpel blade. Cells were incubated in the dye mixture for exactly 3 min and then quickly rinsed three times with PBS. Cells were immediately fixed for 20 min at room temperature with 4% Formaldehyde. Coverslips were then mounted on slides using Vectashield mounting media. Dye transfer between the cells was then visualized by fluorescence microscopy.

IX. Statistics

All results are representative of at least three experiments. Data are expressed as the mean ± SE. Statistical variations between two samples were calculated by using Student's T-test and a P value <0.05 was considered as statistically significant. Bar graphs were plotted using the EXCEL program.

E. RESULT

I. Conx43 expression and localization.

I-1. Conx43 protein level in crt-/- cells

CRT is an ER chaperone which mediates the folding of many proteins. To examine the role of CRT in the expression of Conx43, we utilized MEF cells isolated from the *wt* and *crt-/-* mice. Figure 4 A shows a significant increase in the expression of total Conx43 protein in the *crt-/-* cells as compared to the *wt* cells. To determine if the observed change in Conx43 protein was due to the absence of CRT, we examined Conx43 expression in the *crt-/-* cells which were stably transfected with CRT (CRT-*crt-/-*, Figure 4A, B). As demonstrated in Figure 4A, lane 3, re-introduction of CRT into *crt-/-* cells decreased the total Conx43 protein level. The Conx43 level in the CRT-*crt-/-* was still higher than the *wt* (Figure 4A), this observation is due to the fact that CRT-*crt-/-* cells express less CRT as compared to the *wt* cells (Figure 4B). Therefore, these data suggest that there is an inverse correlation between CRT protein level and Conx43 expression.

Phosphorylation is an important post-translation modification of the Conx43 protein. Figure 5A shows that both the phosphorylated and non-phosphorylated Conx43 are increased *crt-/-* cells. Changes in the level of Conx43 (both phosphorylated and non-phosphorylated) was quantified and presented in Figure 5B. Interestingly, the level of phosphorylated Conx43 was increased by 30% in the *crt-/-* as compare to the *wt* cells. However, the level of non-phosphorylated level of Conx43 in the *crt-/-* was increased

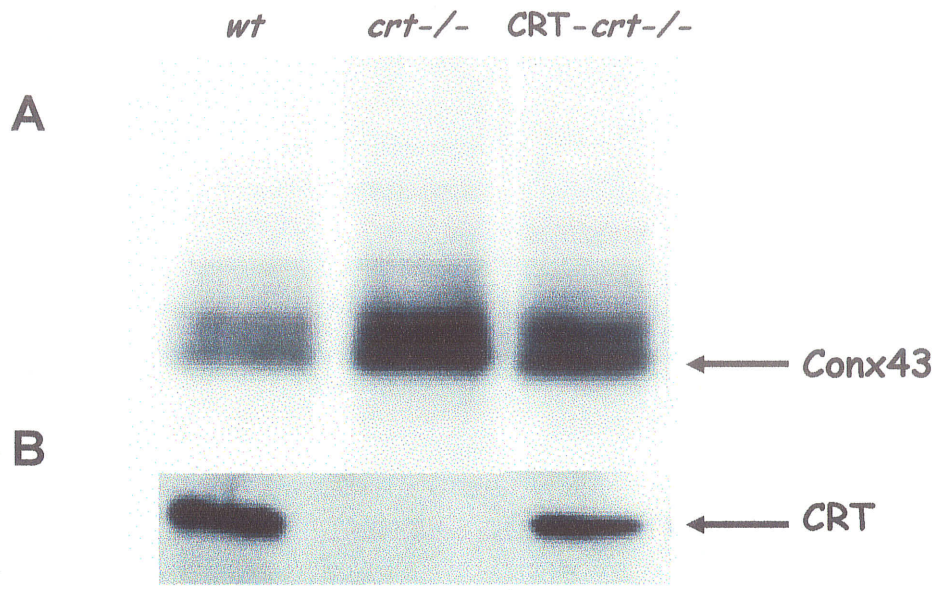

Figure 4. Western blot analysis of Connexin43 (Conx43) protein in cells with altered level of calreticulin (CRT) expression. Cells were cultured as described in the "Materials and Methods" followed by lysis in New RIPA buffer. 30 µg of total proteins from , wild type (*wt)*, CRT knockout (*crt-/-*) and *crt-/-* transfected with CRT cell lysates were separated on a 10 % SDS-PAGE, transferred to nitrocellulose membranes and probed with rabbit anti-Conx43(A) and goat anti-CRT (B).

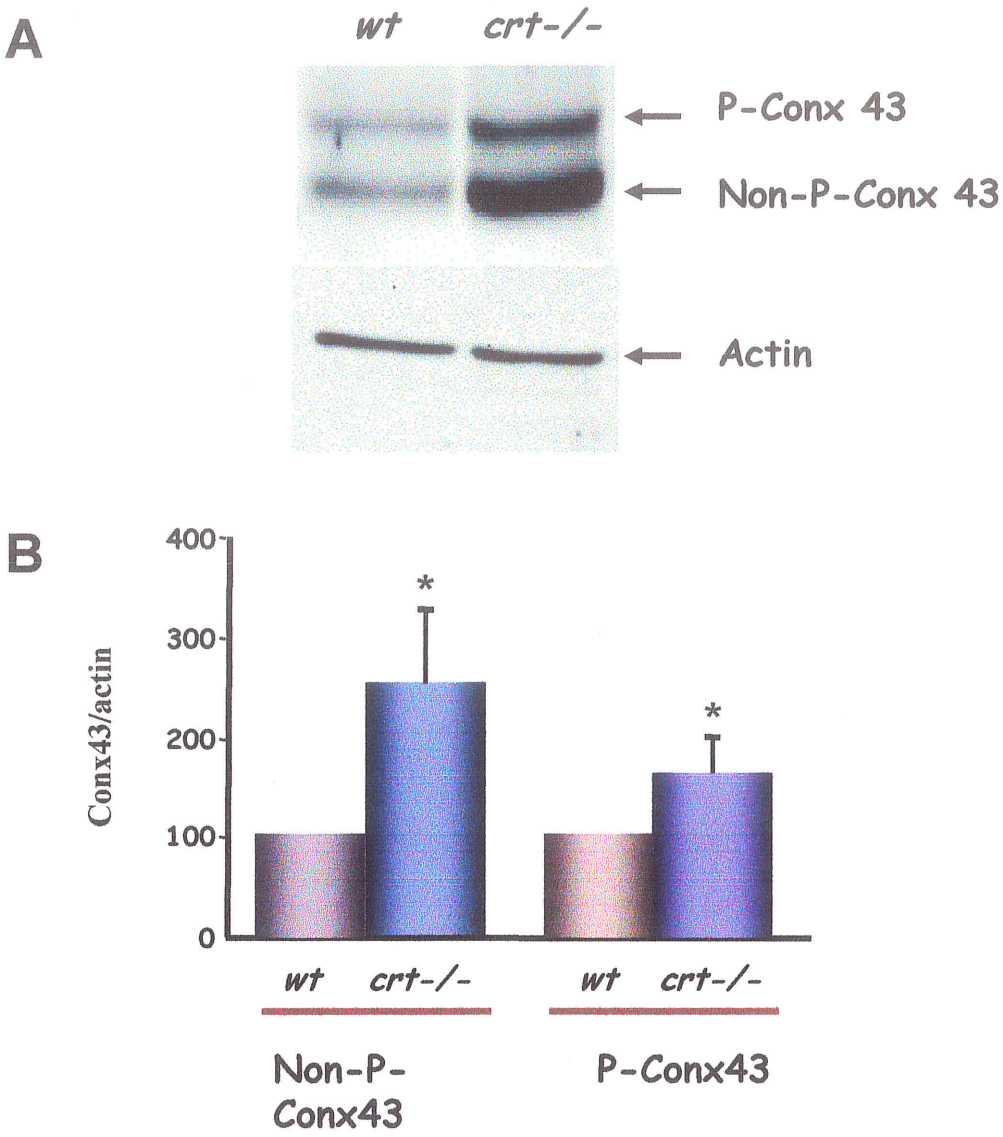

Figure 5. Western blot analysis showing the level of phosphorylated conx43(P-Conx43) and non-phosphorylated conx43 (Non-P- Conx43) in *wt* and *crt-/-* cells. 30 μg of total proteins from wt and *crt-/-* cell lysates were separated on 7 %SDS page, transferred to nitrocellulose membranes and probed with antibodies to Conx43 (1 : 2000) and actin (1: 1000)as control (A). The intensity of bands were quantified using Quantity One program as described in "Materials and Methods" (B). Bar graph representing the ratio of P-Conx43 or Non-PConx43 to actin in the *wt* and *crt-/-* cells. Data is the mean + standard error (SE) of 8 independent experiments.

* $P<0.05$ significantly different as compare to the *wt*.

more than 70% as compared to the *wt* cells. This difference could result in a defect in the localization or function of Conx43.

I-2. Conx43 localization

Immunocytochemical localizations of Conx43 in the *wt* and *crt-/-* cells are shown in Figure 6. Conx43 is localized in the cell membrane and intracellular location of both cell types (Figure 6). Furthermore, despite the higher Conx43 protein expression in the *crt-/-* cells, these cells contain less Conx43 staining at the cell-cell junction (Figure 6, compare *crt-/-* to *wt*). The intracellular localization of Conx43 inside the cells is located to one side of nucleus in a structure ressembling the Golgi apparatus (Figure 6, *wt*) and ER.

II. Conx43 function

To determine the effect of higher Conx43 protein expression in the *crt-/-* cells on gap junctional communication, scrape loading/dye transfer assay was carried out as described in "Materials and Methods". Figure 7 shows that Rodamine-Dextran, which is a gap junction-impermeant molecule with molecular mass of 10 KDa, stays confined to a single row of cells immediately bordering the wound in both cell types. On the other hand, Lucifer yellow (LY), which is a small florescent molecule and passes through functional gap junction, travels away from the wound area only in the *wt* cells (Figure 7A, C). Interestingly, the fluorescent signal from the LY overlaps with the red Rodamine-Dextran signal in the *crt-/-* cells (Figure 7B, D) indicating that gap junction function is

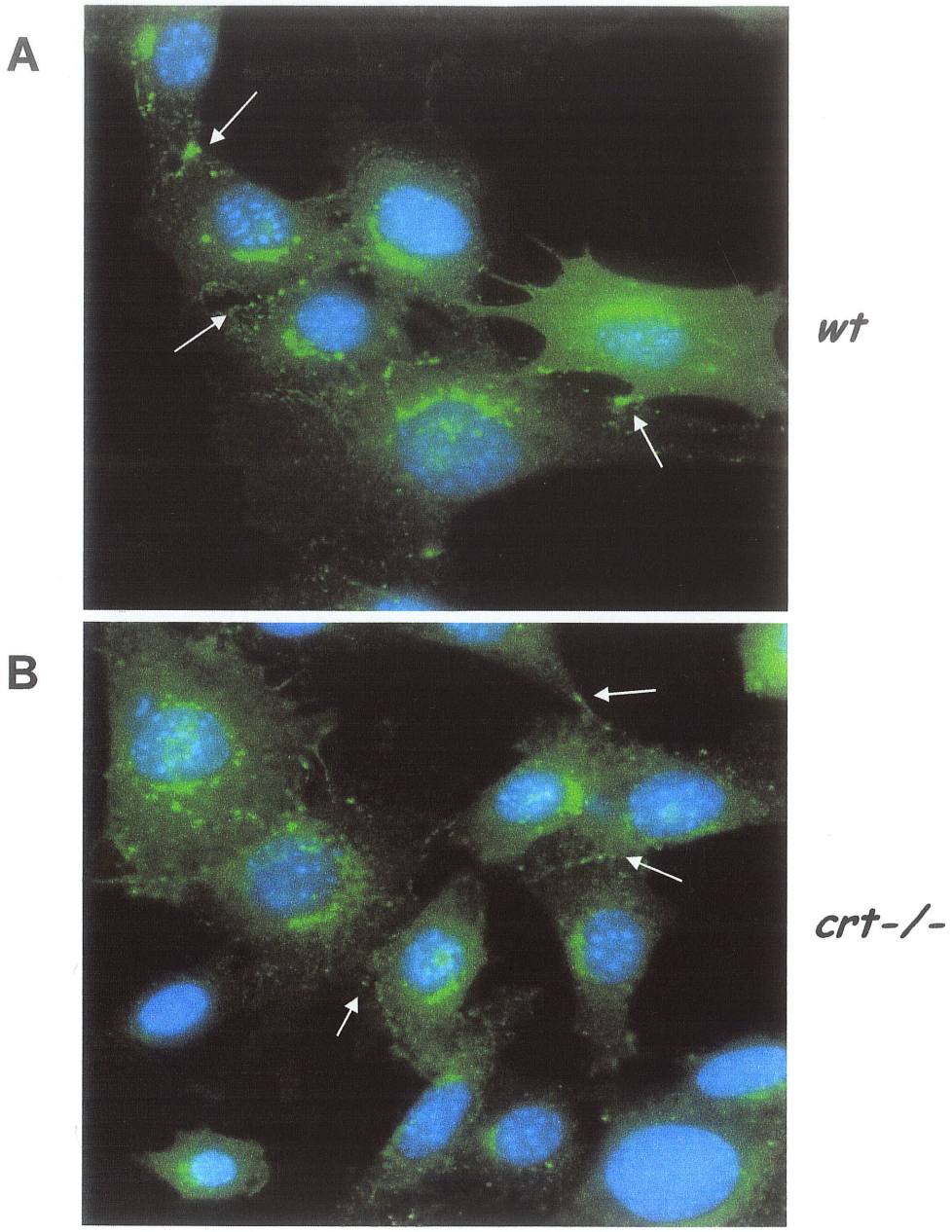

Figure 6. Immunocytochemical localization of Conx43 in the *wt* and *crt-/-* cells. *wt and crt-/-* MEF cells were fixed with 4% formaldehyde, immunostained with anti-Conx43 antibody (1 : 2000) followed by an FITC conjugated secondary antibody. Nuclei are stained with DAPI (blue). Images visualized using a Zeiss Axiovert fluorescence microscope.

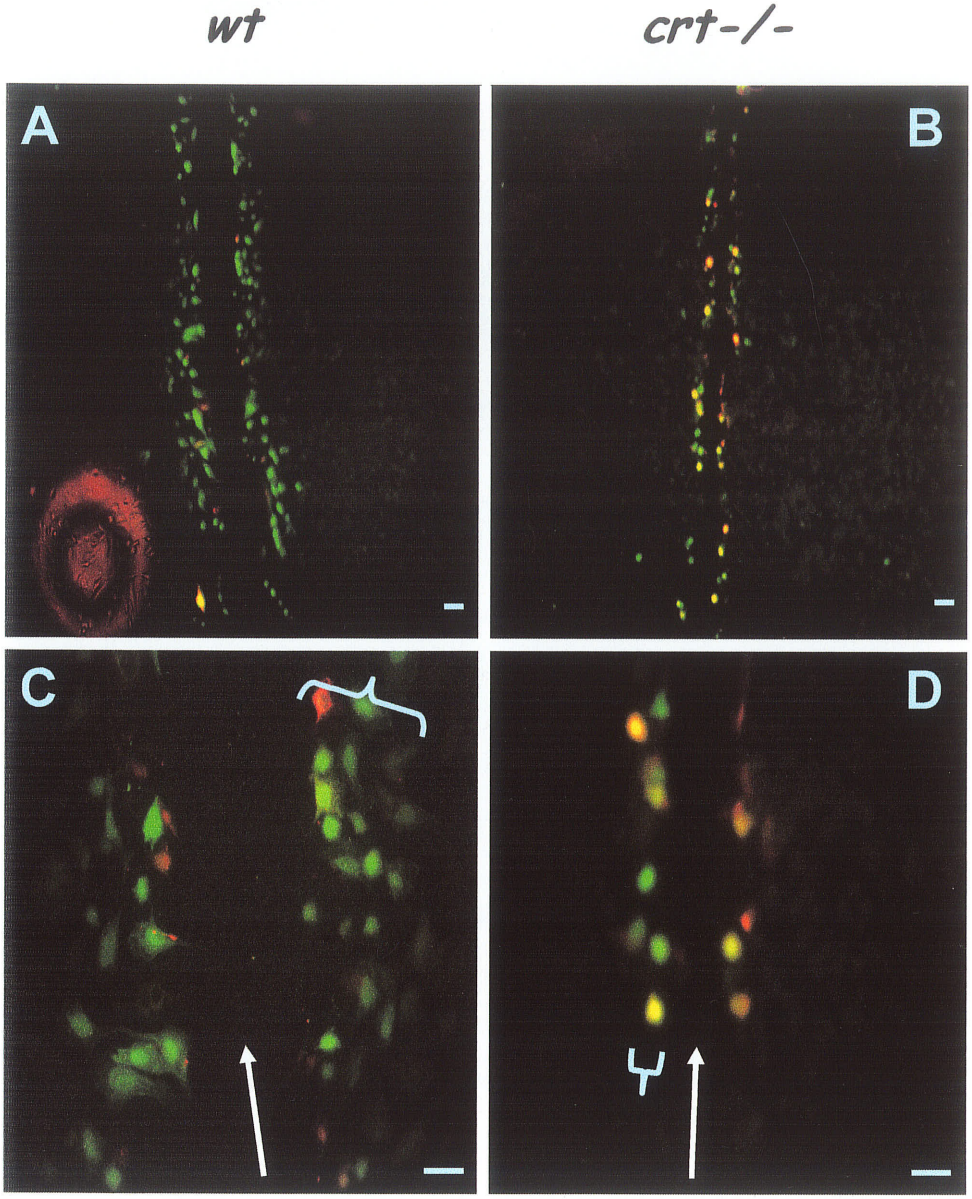

Figure 7. Changes in cell-cell communication via gap junction in the *crt-/-* MEF cells as compared to the *wt* cells. *wt* and *crt-/-* cells were plated on collagen coated coverslips. Gap junction function was analyzed using a scrape loading assay as described in the "Materials and Methods". Lucifer yellow (green fluorescence) is gap junction permeable, and is transported from the wounded cells away from the scrape site in the *wt* cells (A, C). In the *crt-/-* cells, however LY is not dispersed (B, D). Rhodamine-Dextran (red fluorescent) labels the wounded cells only in both the *wt* and *crt-/-* cells. Arrow indicates the scrape (wound) area.

compromised in these cells. Therefore, despite the higher level of Conx43 protein in the *crt-/-* cells the gap junction communication is inhibited in these cells.

III. Conx43 promoter activity in the crt-/- cells

The increase in Conx43 protein could be due to either increased gene expression or decreased protein degradation. Thus, we were interested to address these possibilities. First to examine changes in Conx43 gene expression we used a pConx1686-luc plasmid in which the Conx43 promoter (1.8 kb) is cloned upstream of the luciferase reporter gene. This plasmid was co-transfected with β-gal plasmid in to the *wt* and *crt-/-* cells as described in "Materials and Methods". Figure 8 shows that there is a doubling of the Conx43 promoter activity in the *crt-/-* cells as compared to the *wt* cells. Thus, the increase in the Conx43 protein level in the absence of CRT could be explained by the activation of Conx43 gene transcription and subsequent translation.

IV. Conx43 protein degradation pathways

As described earlier, Conx43 protein undergoes rapid turnover. This protein is the substrate for degradation via two pathways, the ubiquitin-proteasome pathway and lysosomal pathway. Recently, our lab has shown a significant increase in the ubiquitin-Proteasome pathway in *crt-/-* cells. Therefore in this study we examined both of these pathways using commercially available inhibitors.

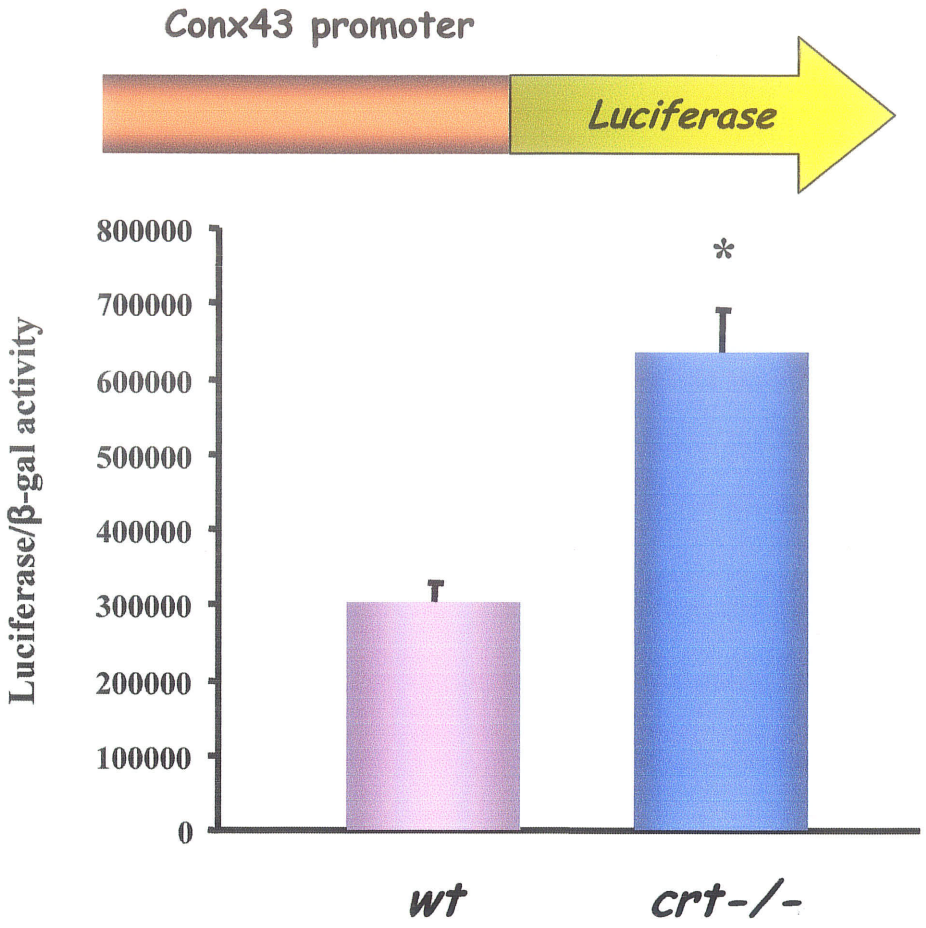

Figure 8. Conx43 promoter activity in the *wt* and *crt-/-* MEF cells. Cells were co-transfected with Conx43-Luc plasmid and ß-gal plasmid, as described in "Material and Method" Cell lysate were prepared 42 hours after transfection and used for luciferase and ß-gal assay. Bar graph is presented as the ratio of luciferase activity divided by ß-gal activity of the same samples. Data are mean + SE of 10 independent experiments carried out in duplicates.
* $P<0.05$ significantly different as compared to the *wt*.

IV-1. Proteasome degradation pathways

Figure 9A illustrates the effect of proteasome inhibition (by MG132) on Conx43 protein level. MG132 treatment induced a significant increase in Conx43 protein in both *wt* and *crt-/-* cells (Figure 9B). Interestingly, the Conx43 protein level following proteasome inhibition was still significantly higher in the *crt-/-* cells (Figure 9A and B). Furthermore, there was no change in the percentage increase of Conx43 protein between the *wt* and *crt-/-* cells (Figure 9B). These data confirm the role of proteasome in Conx43 degradation in both cell lines and suggest that the increase in proteasome activity in the *crt-/-* cell is not the direct cause of the altered Conx43 protein level seen in these cells.

IV-2. Immunolocalization of Conx after proteasome inhibition

Previous reports have shown that inhibition of proteasome results in accumulation of Conx43 in the cell membrane and increased gap junction communication [114]. To investigate the effect of proteasome inhibition on Conx43 stability and localization at cell membrane, *wt* and *crt-/-* cells were treated with MG132 for 4 hr as described in "Material and Methods". Figure 10 shows that proteasome inhibitor treatment leads to increased Conx43 at the gap junctions in the treated *crt-/-* cells. Furthermore, we observed accumulation of Conx43 signal at some vesicular structures inside the cells in both the treated *wt* and *crt-/-* cells (Figure 10C, D). There were more vesicular structures in the *crt-/-* cells (Figure 10B) as compared to the *wt* cells (Figure 10C).

To examine if the appearance of these vesicular structure was due to the accumulation of Conx43 destined for degradation and not due to increased synthesis we

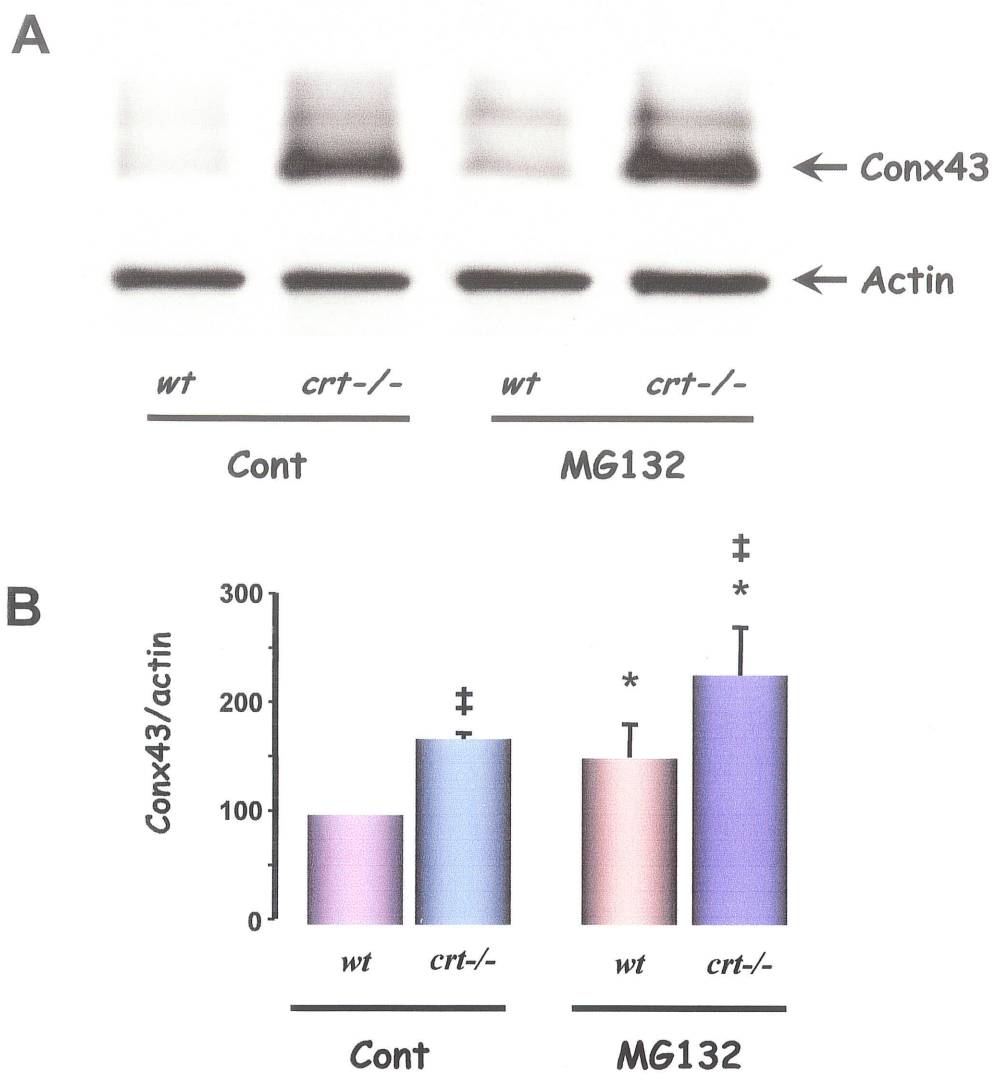

Figure 9. Effects of proteasomal inhibitor (MG132) on total Conx43 protein in the *wt* and *crt-/-* cells. *wt* and *crt-/-* MEF cells were treated with 20 μg MG132 for 4 hr or remained non-treated. After treatment cells were lysed and 30 μg proteins of cell lysates were separated and 7% SDS-PAGE, as described in "Materials and Methods". The blot was then stripped and re-probe with antibody against actin as a loading control. (A) is a representative western blot showing Conx43 protein level in control and MG132 treated cells. The intensity of the bands corresponding to Conx43 and actin was quantified using Quantity One program. (B) Bar graph presented as the ratio of Conx43 (total protein) to actin. Values are mean + SE of 4 independent experiments carried out in duplicates.
* $P<0.05$ significantly different as compared to the non-treated cells.
‡ $P<0.05$ significantly different as compared to the *wt* cells.

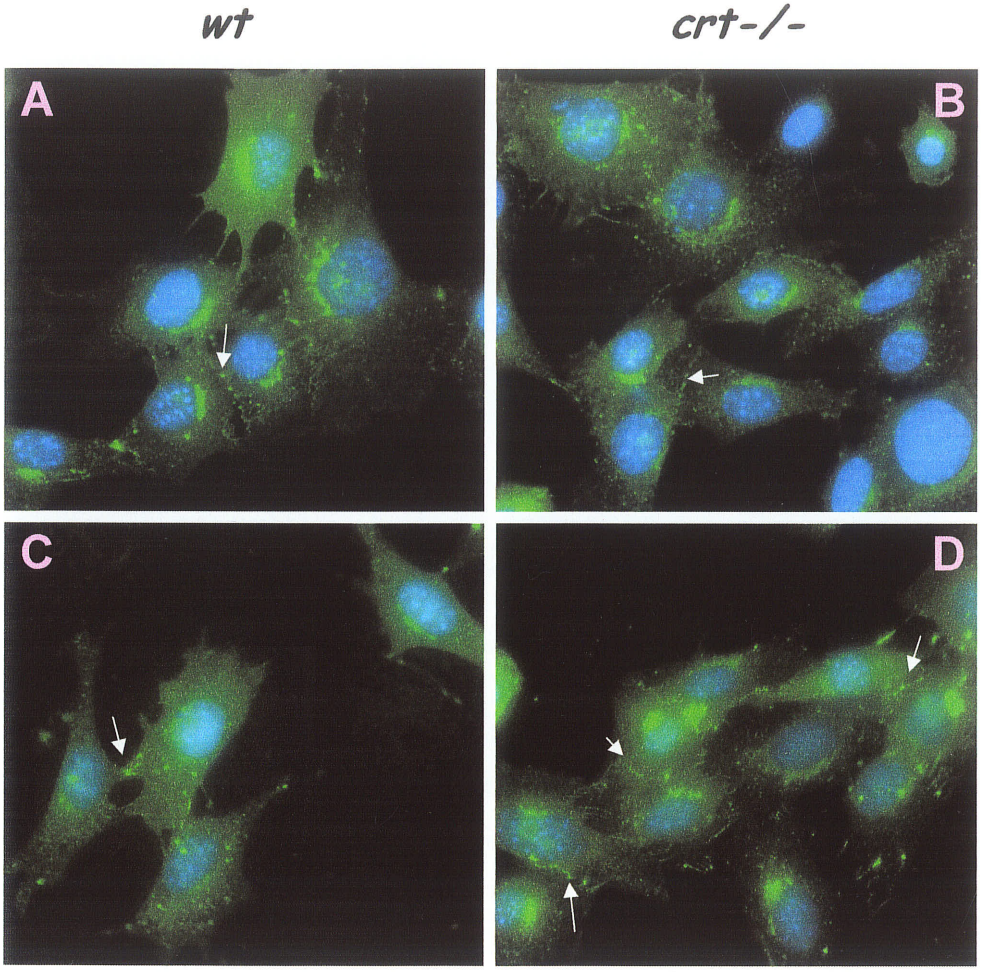

Figure 10. Immunocytochemical staining showing effect of inhibition of proteasome activity (MG132) on Conx43 localization in the *wt* and *crt-/-* cells. Cells were non-treated (A, B) or treated with MG132 (20 μg) for 4 hr (C, D) followed by fixation and staining with anti-Conx43 (1: 2000) as described in "Materials and Methods". Images visualized using a Zeiss Axiovert fluorescence microscope.

treated the cells with CHX to block *de novo* protein synthesis. Figure 11A and B shows that treatment of the *wt* and *crt-/-* cells with CHX for 4 hrs significantly decreased Conx43 protein expression. CHX treatment led to a significant reduction of Conx43 localization at the cell membrane (Figure 11A). However, in the *crt-/-* cell the Conx43 localization was not decreased, on contrary we observed a stronger signal at cell-cell junction (Fig our 11B). Treatment of these cells with both CHX and MG132 resulted in accumulation of Conx43 at vesicular structures inside the cytosol of the *wt* and the *crt-/-* cells (Figure 11C, D). This treatment again resulted in more Conx43 signal at cell-cell junctions in the *crt-/-* (Figure 11D) as compared to the *wt* cells (Figure 11C). These data indicate that inhibition of proteasome (by MG132 treatment) reduces Conx43 internalization from the cell membrane and blocks its degradation but does not affect the synthesis of new proteins. The higher amount of Conx43 observed in the *crt-/-* cells following CHX and MG132 treatment (Figure 11B, D) also reflects the fact that initially these cells have elevated level of Conx43 protein expression.

IV-3. Effect of proteasome inhibition on Conx43 promoter activity

To further investigate the effect of proteasome inhibition on Conx43 gene expression we measured Conx43 promoter activity following MG132 treatment. Figure 12 demonstrates that inhibition of proteasome activity resulted in a significant decrease in Conx43 promoter activity in the *crt-/-* cells. Interestingly, MG132 had no effect on Conx43 promoter activity in the *wt* cells (Figure 12). This observation confirms that the significant increase in Conx43 protein level observed in the *crt-/-* cells (Figure 9) is due to the inhibition of proteasome mediated protein degradation and not increased Conx gene

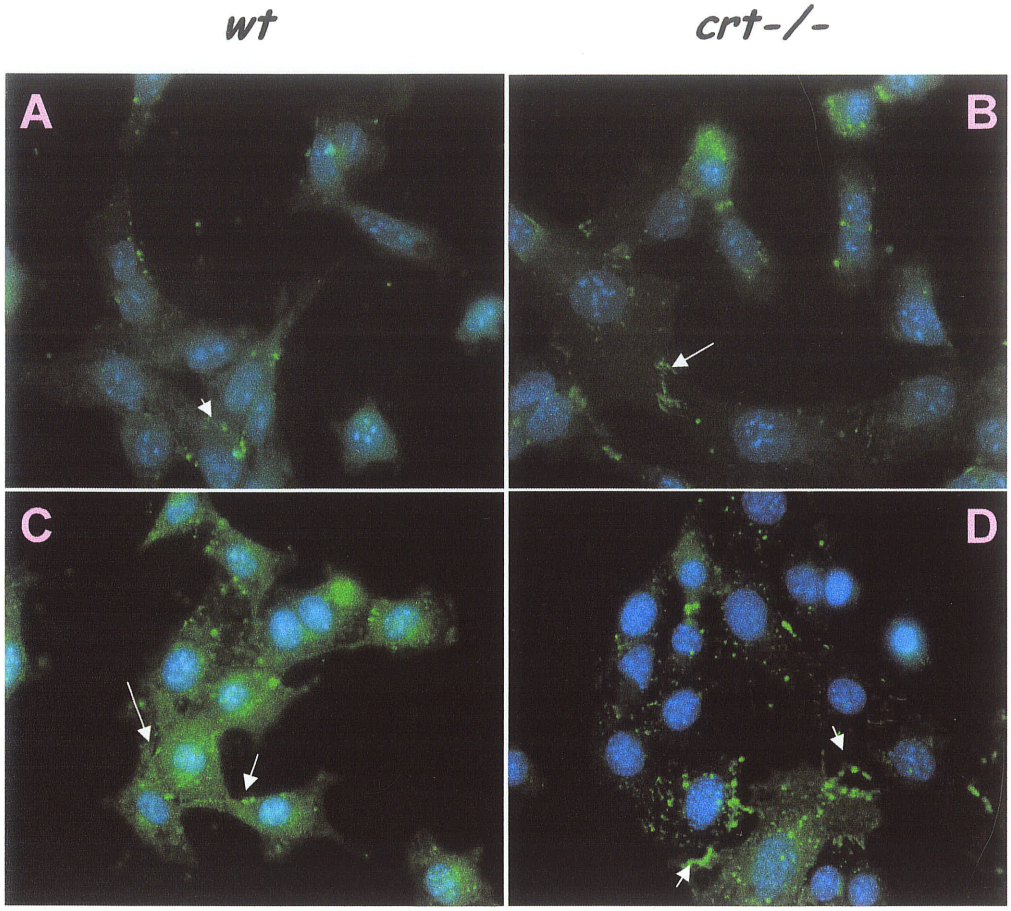

Figure 11. Immunocytochemistry showing the effect of inhibition of protein synthesis and protein degradation on Conx43 localization in the *wt* and *crt-/-* cells. MEF cells were treated with 10 µg/ml of cycloheximide (CHX) for 8 hr (A, B), or a combination of with MG132 (20 µg) CHX (10 µg/ml) for 8hr (C, D). Cells were fixed with 4% formaldehyde, immunostained with anti-Conx43 antibody (1 to 2000) followed by an FITC-conjugated secondary antibody .Images visualized using a Zeiss Axiovert fluorescence microscope. The amount of Conx43 staining significantly decreased following CHX treatment.

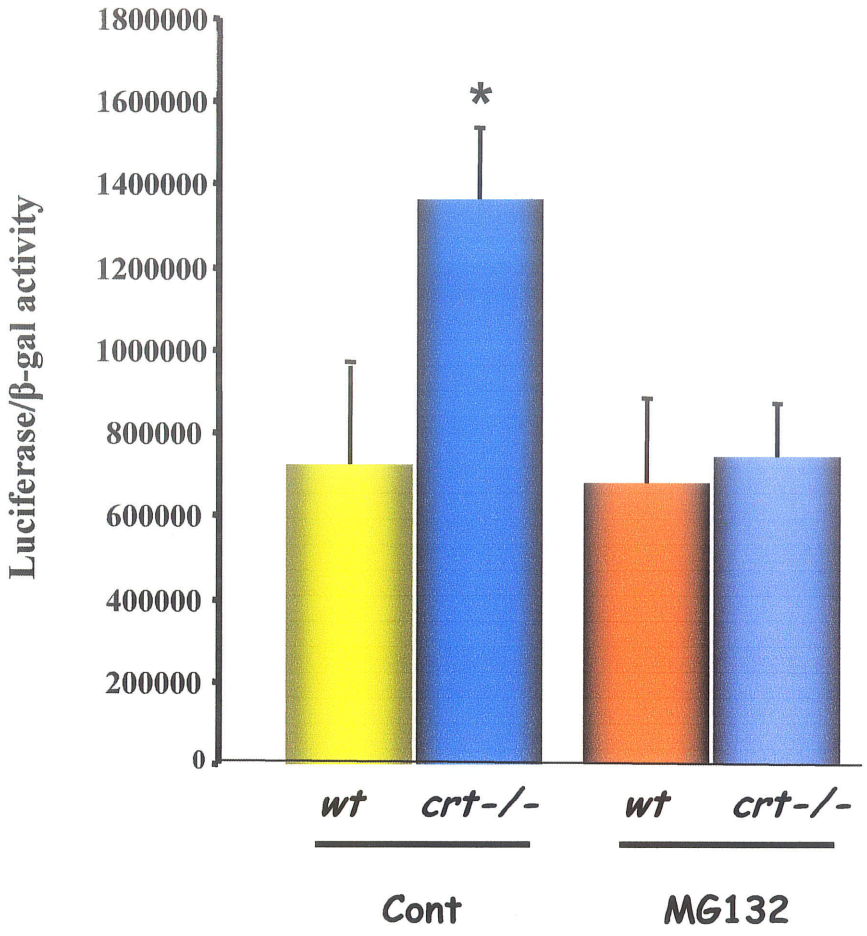

Figure 12. Effect of MG132 treatment on Conx43 promoter activity. Cells were co-transfected with Conx43-Luc and ß-gal plasmid. 40 hr after transfection MG132 (20 μg) was added to one set of *wt* and *crt-/-* cells and the rest were left non-treated as controls. All cells were lysed 4 h after treatment and luciferase and ß-gal assays performed as described in "Materials and Methods". (B) Bar graph presented as the ratio of luciferase to ß-gal values. Data are mean + SE of 9 independent experiments carried out in duplicates.

* $P < 0.05$ significantly different as compared to the *wt*.

expression. Figure 12 also demonstrates that the increased accumulation of Conx43 at the cell-cell junction in MG132 treated cells (immunocytochemical data shown in Figures 10 and 11) is not due to increase gene activity.

IV.4. Conx43 protein and Lysosome degradation pathways

Lysosome disruption by leupeptin results in the accumulation of Conx43 protein in both cell types (Figure 13A). Treatment of the *wt* cells with leupeptin increases Conx43 protein levels close to those of untreated *crt-/-* cells (Figure 13B). Leupeptin treatment induced a 2.5 fold increase in total Conx43 protein level in the *crt-/-* cells as compared to the non-treated *crt-/-* cells (Figure 13B). However, this treatment induced a 100% increase in the amount of total Conx43 in the *wt* cells compared to the non-treated *wt* cells (Figure 13B). Comparing the effect of leupeptin on the phosphorylation of Conx43 reveals that in contrast to MG132, leupeptin increased both the phosphorylated and non-phosphorylated Conx43 protein levels in the *crt-/-* cells as compared to the *wt* cells (Figure 14A and 14B). While MG132 only increased the level of non-phosphorylated Conx43 significantly in the *crt-/-* cells as compared to the *wt* cells (Figure 14A). On the other hand, the changes in the level of phosphorylated and non-phosphorylated Conx43 in the *wt* cells was only modest following leupeptin treatment (Figure 14A and 14B).

IV-5. Conx43 Immunocytochemistry after treatment with Lysosome inhibitors

Effect of lysosome inhibition (Leupeptin) on Conx43 localization was studied in the *wt* and *crt-/-* cells. Figure 15C and 15D shows that disruption of lysosomes abolishes

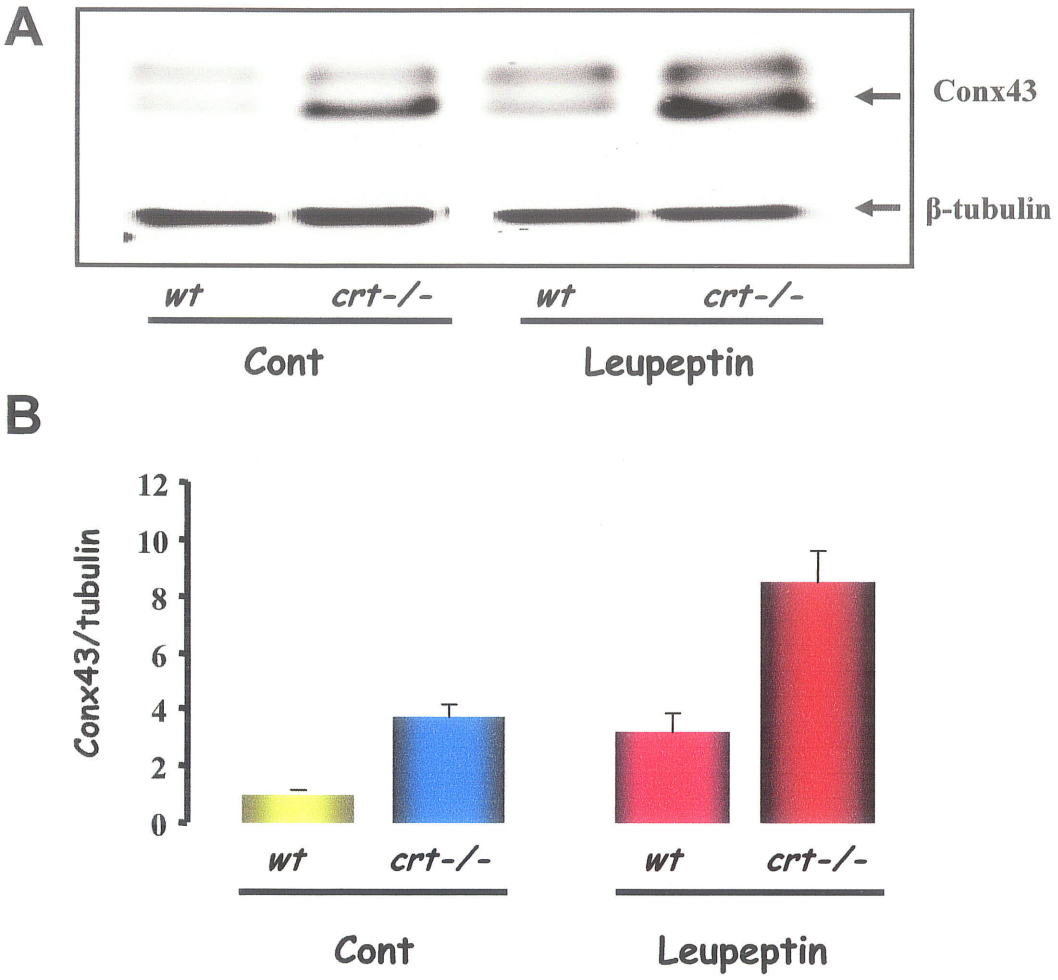

Fig 13. Effects of lysosomal inhibitor treatment on Conx43 protein stability in the *wt* and *crt-/-* cells. 30 μg of proteins of *wt* and *crt-/-* cells from non-treated cells and cells treated with Leupeptin (100 μg/ml) for 6 hr were resolved on a 7% SDS-PAGE, followed by western blot after being transferred to nitrocellulose membranes by semidried system, probed with antibodies to Conx43 (1:2000) and tubulin (1:100) as control. A) A representative western blot with Conx43 antibody of treated cells. The intensity of Conx43 bands (total phosphorylated and non-phosphorylated) and tubulin bands was quantified using Quantity One program. B) Bar graph showing the mean of one western blots.

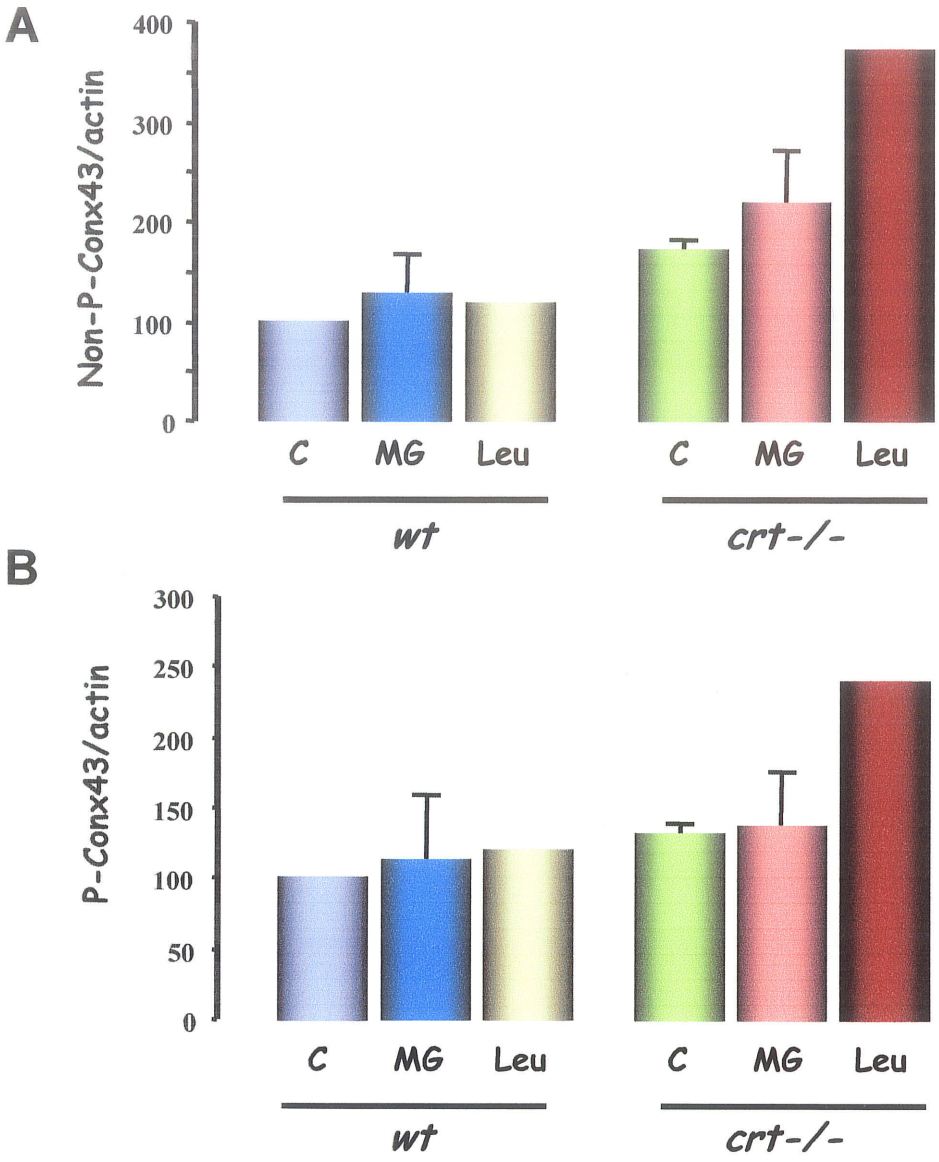

Figure 14: Effect of proteasome and lysosome inhibitors on non-phospho-Conx43 (A) and phospho-Conx43 (B) in the *wt* and *crt-/-* cells. Western blot of Conx43 expression in control non-treated, MG132 and leupeptin treated cells were carried out as described in "Materials and Methods". The bands corresponding to P-Conx43m And non-P-Conx43 were quantified using Quantity One program. Bar graph shows mean + SE of 6 experiments for MG132 and one experiments for leupeptin. B) shows that MG132 treatment resulted in no change in the level of P-Conx43 in both the *wt* and *crt-/-* cells. However, leupeptin significantly increased P-Conx43 in both cells .

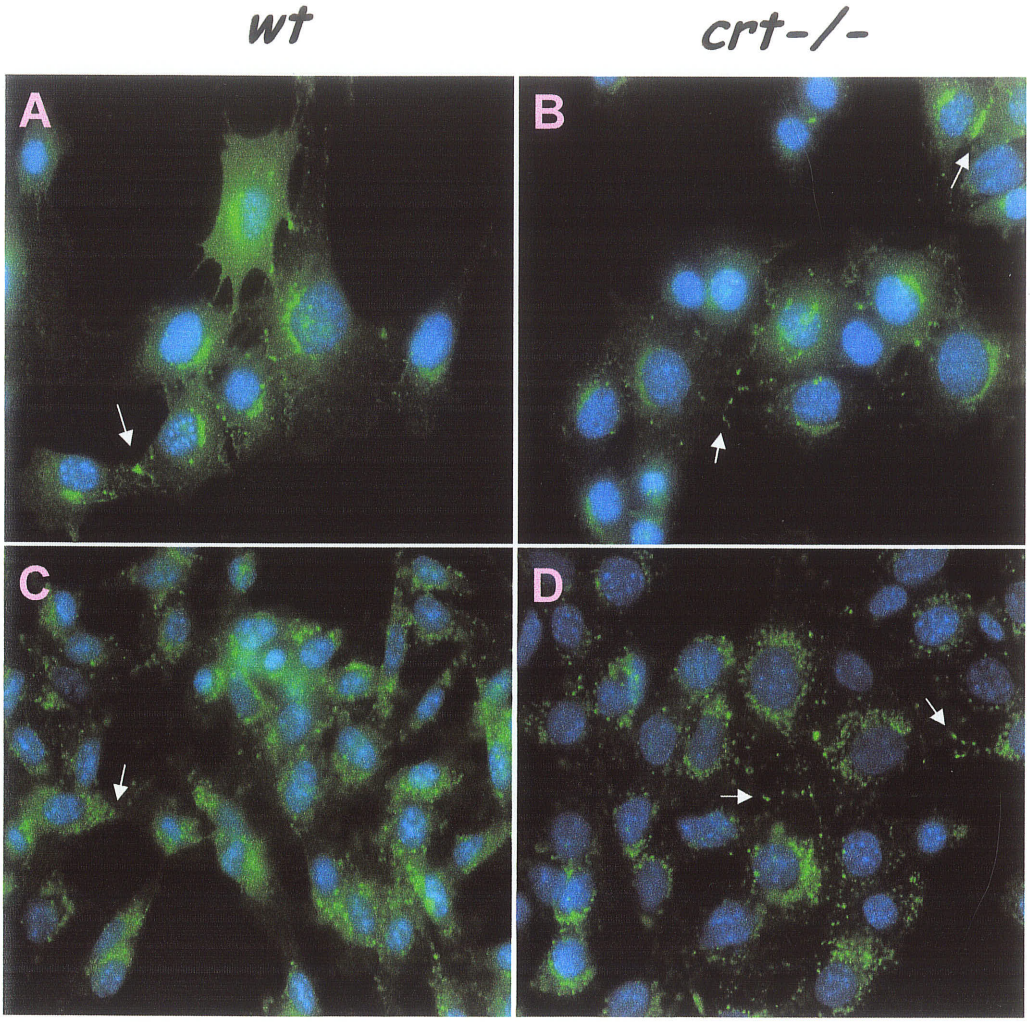

Figure 15. Immunocytochemical localization of Conx43 in the *wt* and *crt-/-* cells following treatment with the leupeptin. *wt* and crt-/- cells were treated with leupeptin for 6 hr, followed by fixation and immunostaining with Conx43 antibody as described in "Material and Methods". Images visualized using a Zeiss fluorescence microscopy. Leupeptin treatment resulted in accumulation of Conx43 in intracellular vesicles in both the *wt* and *crt-/-* cells (C, D). There was very little Conx43 at the cell-cell junction in the treated cells (C, D) as compared to the non-treated cells (A, B).

the localization of Conx43 to the cell membrane of the *wt* and *crt-/-* cells. Following leupeptin treatment Conx43 protein was localized to a punctuate pattern (Figure 15C, D). This pattern in the *wt* does not resemble the ER and it is probably corresponds to the lysosomal structure (Figure 15C). However, in the *crt-/-* some of the staining resembles the ER (Figure 15D) and could suggest of Conx43 is partially trapped in the ER in addition to lysosome in these cells. Further studies need to be carried out to confirm this prediction.

V. Conx43 and Golgi

Conx43 is synthesized in the ER lumen then it is transported to the Golgi apparatus where it is modified and processed through the vesicular transport system to arrive at the cell membrane. To test effect of disruption of the Golgi apparatus on Conx43 protein synthesis, cells were treated with Brefeldin A as described in "Material and Methods". Figure 16 shows the level of both non-phosphorylated and phosphorylated Conx43 after Berfaldin A treatment. Although following Brefeldin A treatment the level of non-phosphorylated Conx43 is still significantly higher in the *crt-/-* cells compared to *wt* cells (Figure 16), there is a dramatic decrease in the level of phosphorylated Conx43 in both of these cell lines (Figure 16 lanes 3 and 4). This observation suggests that the Golgi apparatus is important for the proper phosphorylation of Conx43 and CRT (which is present in ER) does not affect this process. Furthermore, disruption of Golgi function abolishes the membrane localization of Conx43 protein in both the *wt* and *crt-/-* cells (Figure 17C, D). Brefeldin A treatment for 4 hrs result in a diffuse punctuates staining

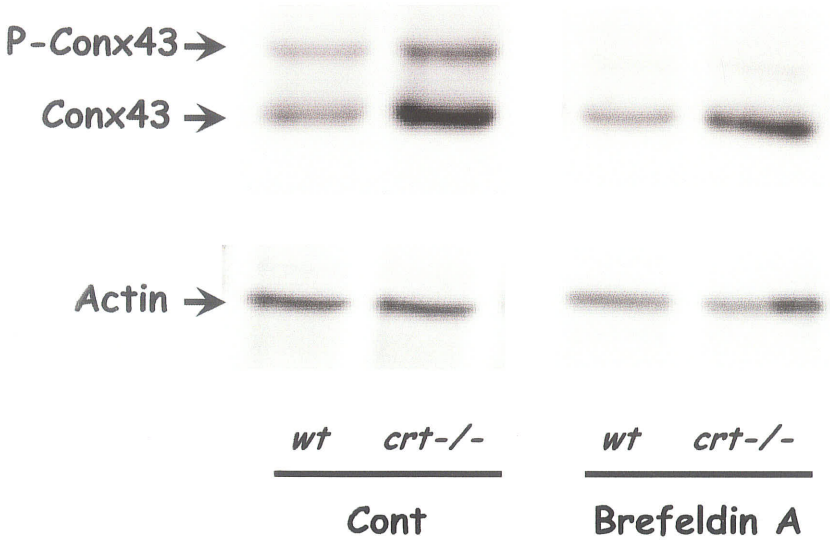

Figure 16. Western blotting analysis of Conx43 protein level in the *wt* and *crt-/-* cells following disruption of Golgi apparatus by (Brefeldin A). 30 μg proteins of *wt* and *crt-/-* from treated (Brefeldin A) and non-treated control were resolved on 7% SDS-PAGE. Western blot was carried out with anti-Conx43 as described in "Materials and Methods".

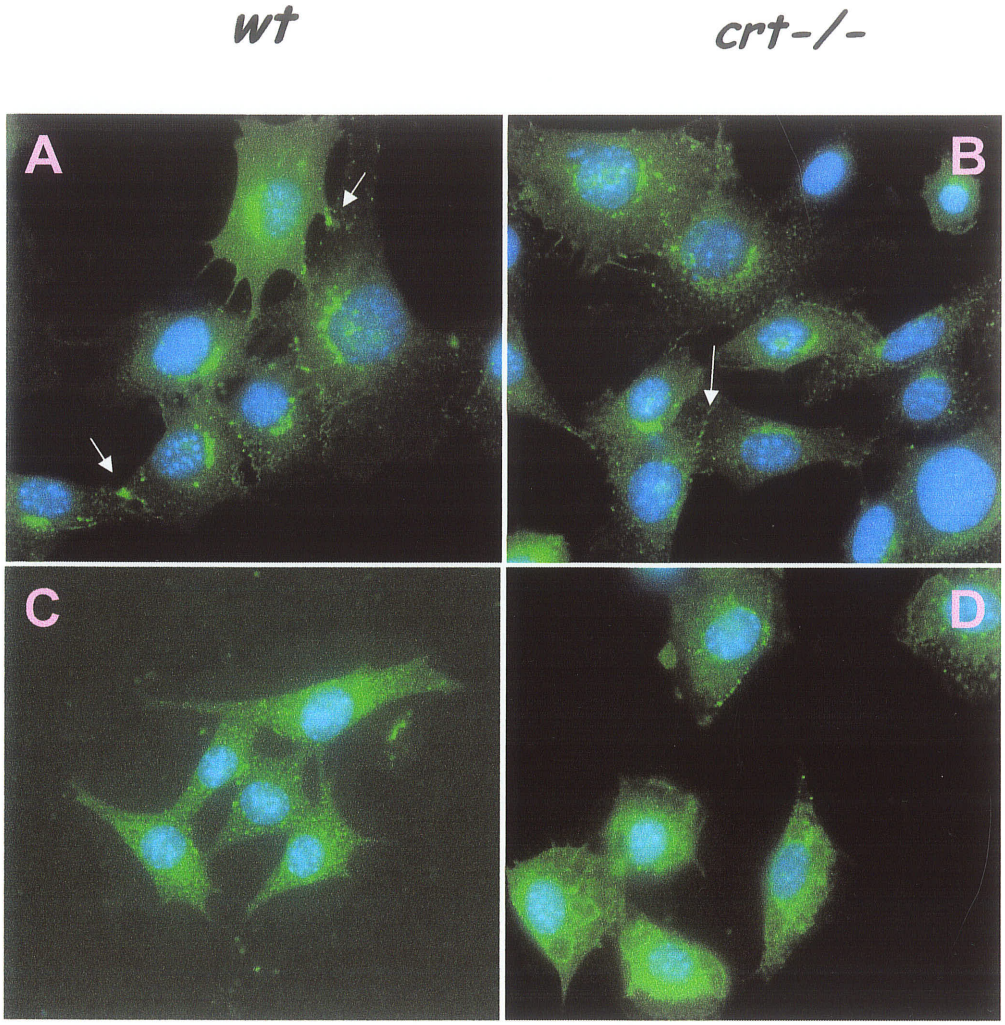

Figure 17. Immunocytochemical localization of Conx43 protein in the *wt* and *crt-/-* cells following disruption of Golgi apparatus (by Brefeldin A). Cells were cultured on glass coverslips, then treated with Brefeldin A (C, D) or left untreated (A, B) for 4 hr followed by staining with Conx43 antibody as described in "Materials and Methods". C, D) Show treatment with Brefeldin A disrupted the translocation of Conx43 protein from the ER to the cell membrane.

with Conx43 antibody in both cell types (Figure 17C, D). However, the size of these punctuate structures after Brefeldin A treatment seems smaller than the structures seen following leupeptin or MG132 treatment (compare Figure 17C, D and 15C, D and 10C, D). Interestingly, disruption of Golgi and inhibition of proteasome simultaneously resulted in appearance of some Conx43 protein signal at the cell-cell junction of both the *wt* and *crt-/-* cells (Figure 18C, D). However, the Conx43 signal at the membrane of *crt-/-* cells was stronger compared to the *wt* cells (Figure 18B). In addition to the membrane staining, the treated *crt-/-* cells contained the same vesicular staining (Figure 18D) seen following MG132 treatment (Figure 10D).

VI. Effects of glycosylation on the Conx43 internalization

Inhibition of glycosylation following Tunicamycin treatment increased Conx43 localization to the cell membrane of the *wt* and *crt-/-* cells (Figure 19B). However, the Conx43 signal at the cell membrane was higher in the *crt-/-* cells (Figure 19B *wt, crt-/-*) after tunicamycin treatment. Inhibition of proteasome activity and glycosylation at the same time resulted in further accumulation of Conx43 at the cell membrane of the *crt-/-* cells (Figure 19C) accompanied by large cytosolic vesicular structure (Figure 19C, *crt-/-*). On the other hand, *wt* cells showed less Conx43 at the membrane and in the cytosolic vesicles (Figure 19C, *wt*) following tunicamycin and MG132 treatment.

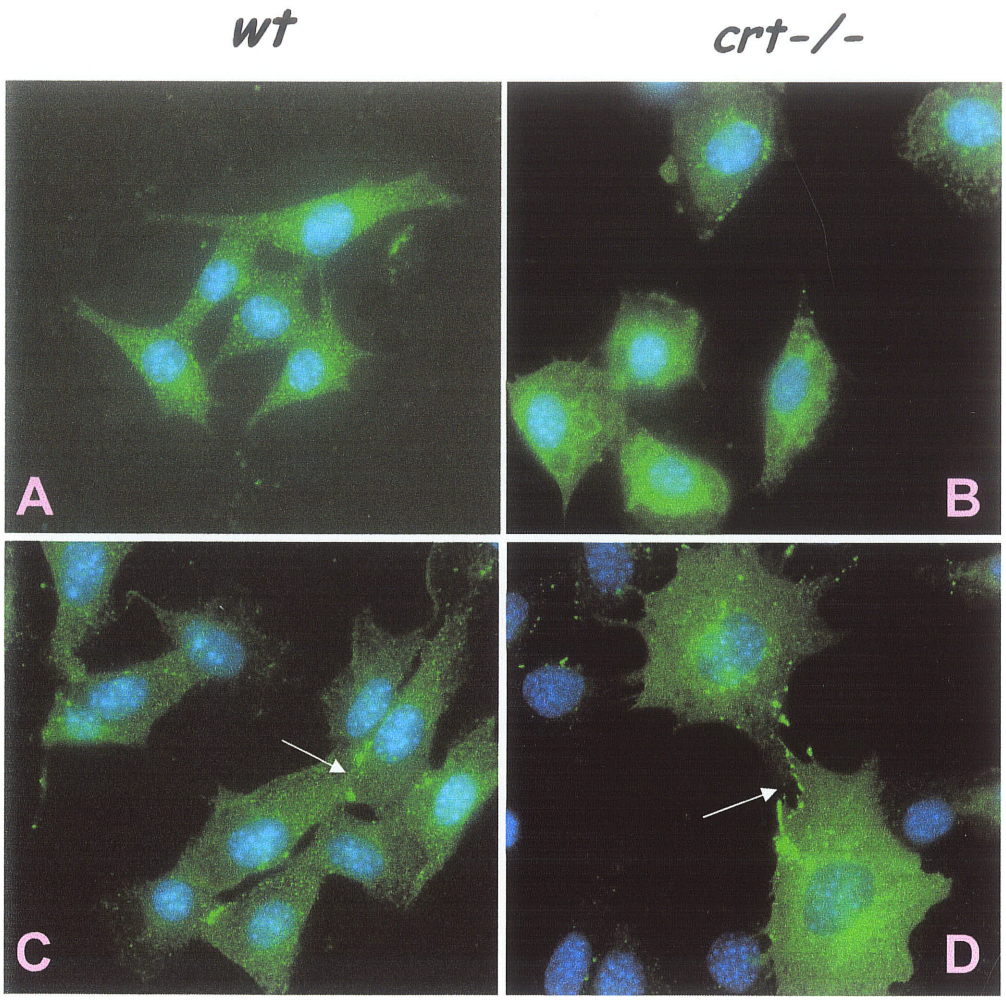

Figure 18. Immunocytochemical localization of Conx43 protein in the *wt* and *crt-/-* cells following disruption of Golgi and inhibition of proteasome activity. *Wt* and *crt-/-* cells were treated with either Brefeldin A (A, B) or Brefeldin A and MG132 (C, D) for 4 hrs followed by immunofluorescence staining with Conx43 antibody as described in "Materials and Methods".

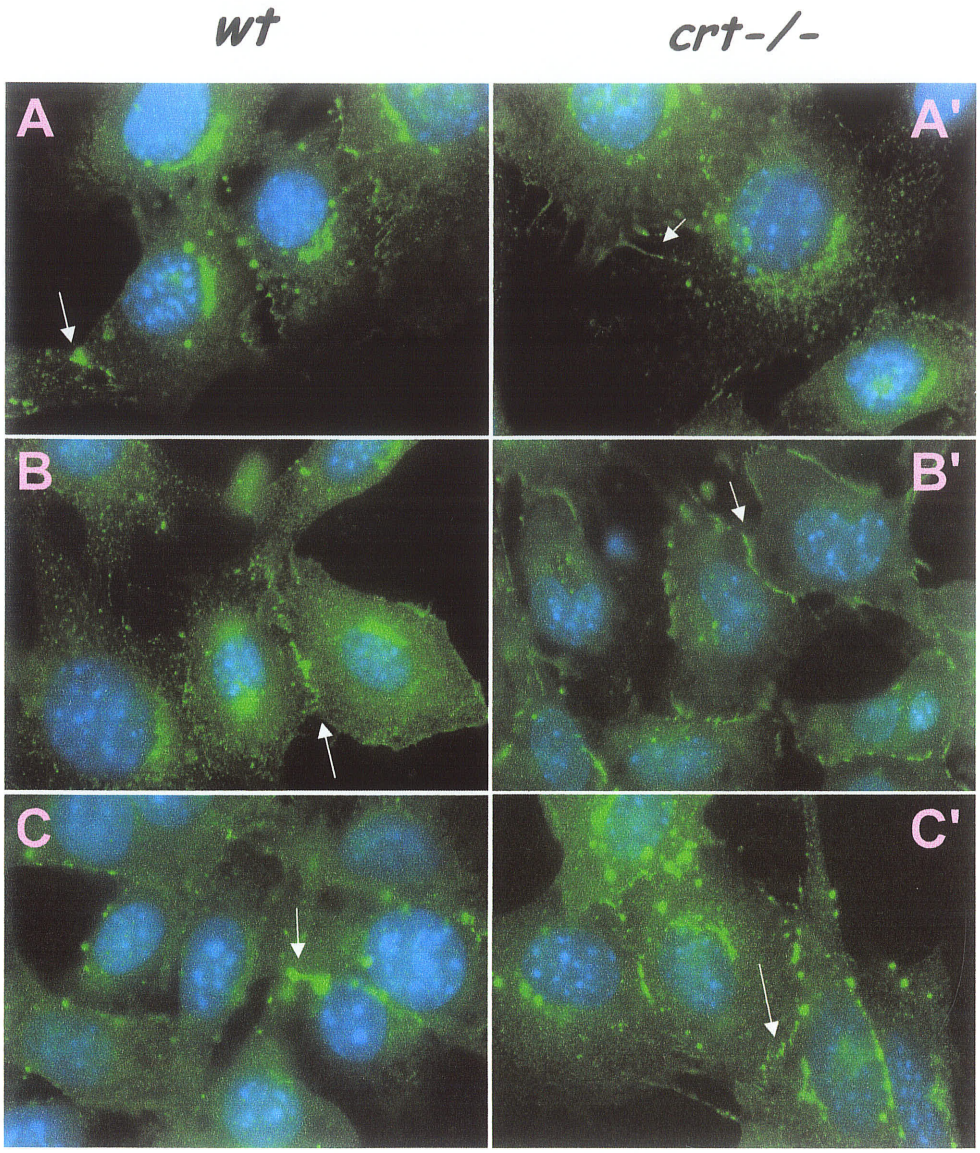

Figure 19. Inhibition of glycosylation (by tunicamycin) alters gap junctional localization of Conx43 in the *wt* and *crt-/-* cells. *wt* and *crt-/-* cells were untreated (A, A'), treated with tunicamycin (B, B'), co-treated with MG132 and tunicamycin (C, C'). Cells were then fixed and stained with anti-Conx43 antibody as described in "Materials and Methods". Images visualized using a Zeiss fluorescence microscope. Treatment with Tunicamycin resulted in a significant accumulation of Conx43 in the cell-cell junction of the *crt-/-* cells.

VII Conx43 in the serum-starved CRT-/- cells

VII-1. Serum starvation effects on the Conx43 promoter activity

To validate the hypothesis that growth factors are important in the regulation of Conx43 promoter activity, two set of experiments were carried out. First, the Conx43 promoter activity was measured in the *crt-/-* and *wt* cells 4 and 24 hrs after serum starvation as described in "Material and Methods". Figure 20 show that 4 hr starvation significantly increases Conx43 promoter activity in the *wt* cell only, while no effect was observed in the *crt-/-* cells. A longer starvation time (24 hrs), however, resulted in a significant decrease in the Conx43 promoter activity of the *wt* and *crt-/-* cells (Figure 20). Although 24 hrs starvation had a dramatic effect on Conx43 promoter activity in *wt*, it did not alter Conx43 promoter activity in the *crt-/-* cells. The percent change between the 24 hrs starved *wt* and *crt -/-* cells was similar to non-starved control cells (Figure 20).

VII-2. MAPK pathway and Conx43 promoter activity

The MAP kinase pathway is one of the signal transduction pathways downstream of many growth factors. This pathway has been reported to regulate Conx43 gene expression. Thus we examined the effect of inhibition of a branch of this pathway (MEK/ERK), on Conx43 promoter activity. The *wt* and *crt-/-* cells were transfected with Conx43 promoter and β-gal plasmid as described in "Material and Methods". These cells were then treated with PD98059. PD98059 inhibits MEK kinase activity thus blocking MAPK signalling transduction pathway. Figure 21 shows that PD98059 reduces Conx43 promoter activity by 2 fold in both *wt* and *crt-/-* cells. The percent difference between the

wt and *crt-/-* cells is the same in the treated and non-treated cells. Therefore, MAP kinase signalling pathway (in particular MEK) is not mediating the activation of Conx43 gene observed in the absence of CRT. Further studies needs to be carried out to detect the regulatory mechanisms involved in the differential Conx43 expression in the presence and absence of CRT.

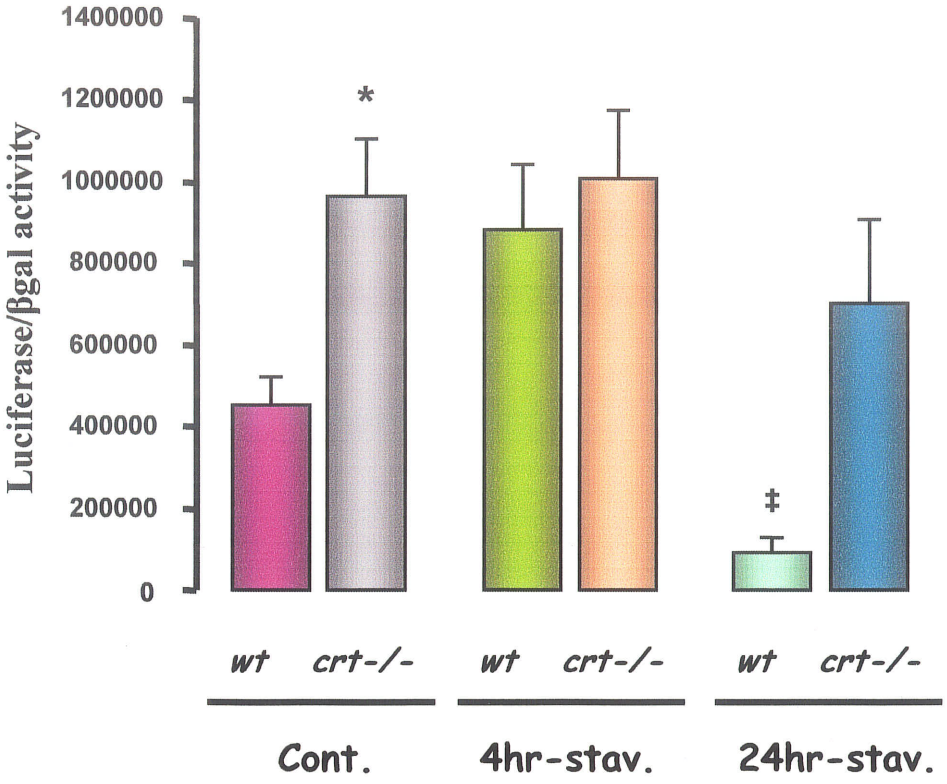

Figure 20. Serum starvation alters Conx43 promoter activity in both the *wt* and *crt-/-* cells. Cells were co-transfected with Conx43 promoter-Luc and ß-gal plasmid. Cells were serum starved for 4 hrs or 24 hrs as described in the "Materials and Methods". Bar graph presented as the ratio of luciferase to ß-gal activity. Values are mean +SE of 5 individual experiments carried out in duplicates.

* $P<0.05$ significantly different as compared to the *wt.*

‡ $P<0.05$ significantly different as compared to the cont. non-treated cells.

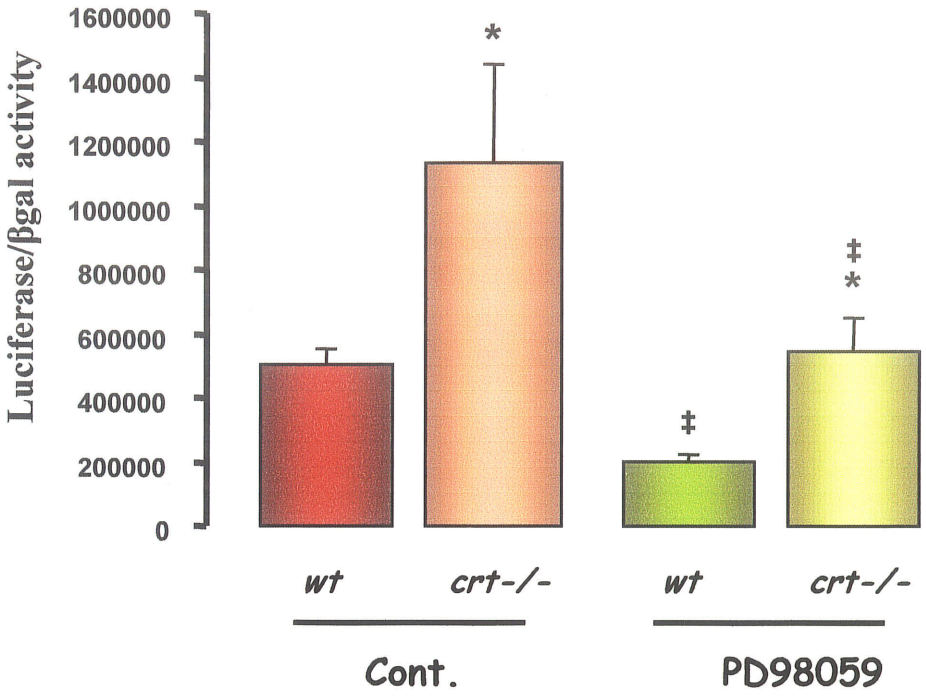

Figure 21. Effect of MEK inhibition on Conx43 promoter activity in the *wt* and *crt-/-* cells. Cells were co-transfected with 2 μg of Conx43 promoter-Luc and ß-gal plasmids. Cells were then starved for 24 hrs followed by incubation with media containing 10%FBS (control) or 10%FBS and PD98059 (MEK inhibitor) for 12 hr as described in the "Materials and Methods". Bar graph presenting the ratio of luciferase to ß-gal activity. Values are the mean +SE of 3 individual experiments carried out in duplicates.

*P<0.05 significantly different as compared to the wt.

‡ P<0.05 significantly different as compared to the cont. non-treated cells.

F. DISCUSSION

CRT is an ubiquitous ER chaprone and Ca^{2+} binding protein. Homozygous CRT deletion in the mouse is embryonic lethal due to defects in the heart development. Electron-microscopic analysis of the hearts from the CRT deficient embroys showed a change in the morphology of the cell-cell junction. Altered cell-cell junction was proposed to be one of the factors contributing to the malformation of *crt-/-* heart. Gap junctions are intercellular channels important in the formation of cell-cell junctions. In the myodardium, Conx43 is the main component of gap junction structure [23]. In addition to Conx43, the heart expresses Conx40 and 45 which are mainly found in conduction system [270, 273]. The significance of gap junction function in the heart development has been elucidated in studies utilizing gene targeted deletion of Conxs [27, 268, 270]. Reports of altered Conx expression in patients with various heart disorders such as non-ischemic heart failure, arrhythmias and sudden death due heart dysfunction [102, 265, 267, 274] further support the importance of Conx in cardiovascular system physiology.

Conx43 is synthesized in the ER and passes through the Golgi apparatus on its way to the membrane [57, 63]. It has a rapid rate of turnover, 1-5 hrs depending on the tissue [20, 55]. Therefore, it has been postulated that the newly synthesized Conxs associate with an ER chaperone to facilitate their folding and transport to the cell membrane. In the current study, we examined the expression and localization of Conx43 in the *wt* and *crt-/-* cell. We predicted that CRT acts as a chaperone for Conx43 and postulated that in the absence of CRT the level of Conx43 will be decreased or the protein will be misfolded and not properly localized to the cell-cell junction (cell membrane). Interestingly, we

observed a significant increase in the expression of Conx43 protein in the absence of CRT (Figure 4 and 5) which was due to a two fold increase in the Conx43 promoter activity (Figure 8). This increase in the protein level was observed for both phosphorylated and non-phosphorylated forms of Conx43 (Figure 5). We further, demonstrated that the increase in Conx43 did not increase gap junction formation in the *crt-/-* cells (Figure 6). Previous reports have shown that folding of Conx to a defined cofiguration as well as its proper oligomerization is required for appropriate function of gap junction [275]. These results, therefore suggested that CRT might be important for the folding and maturation of Conx43 protein and its absence leads to the formation of non-functional gap junctions. Indeed, similar defects in the folding and maturation of bradykinin receptor [235] and MHC class I [276] has been reported previously.

Formation of gap junctions from the malformed Conx43 protein in the *crt-/-* cells results in non-functional gap junctions. Indeed we have shown the lack of dye-coupling in the *crt-/-* cells (Figure 7B and D) postulating a defect in the assembled gap junctions. Decreased gap junction function despite the high expression of Conx43 has been reported previously [126]. These defects have been attributed to either formation of a defective channel [114], a defect in phosphorylation of the Conx proteins [21, 103], a defect in internalization of the protein [109, 110, 127], or an increase in the ubiquitination of Conx followed by proteasomeal degradation [110, 119]. Recent unpublished data from our lab showed a signifcant increase in the ubiqutin-proteasome pathway in the *crt-/-* cells (Uvarov and Mesaeli). In the surrent study we have shown a significant increase in the phosphorylation of Conx43 in the *crt-/-* cells (Figures 5, 9, 13).

The ubiquitin-proteasome pathway is an important regulatory mechanism in the cell that ensures the removal of both unfolded proteins and many regulatory proteins with

short half life. The involvement of this protein degradation pathway in the regulation of Conx43 level has been demonstrated in several studies [54, 110, 114, 116, 119, 122]. The addition of ubiquitin to the Conx43 protein leads to its internalization and degradation or recycling, thus regulating gap junction function [81]. In our study, we showed that treatment of the *wt* and *crt-/-* cells with a proteasome inhibitor (MG132) resulted in the accumulation of Conx43 at the cell-cell junction in both cell types (Figure 10). However, the level of gap junctions in the *crt-/-* cells was higher than in the *wt* cells (Figure 10D and C respectively). Furthermore, the accumulation of Conx43 at cell-cell junction was signifcantly higher in MG132 treated *crt-/-* cells as compared to the non-treated cells (Figure 10D and C). Previously, Rivedal *et.al.,* showed that ubiquitination of Conx43 occurred at the plasma membrane before its internalization and degradation [81]. The MG132 treated cells resulted in increased Conx43 staining in vesicular structures in the cytosol (Figure 10), albeit the size of the vesicles were larger in the *crt-/-* cells (Figure 10D). The appearance of these vesicular structure could be due to aggregation of the internalized Conx43 which are normaly destined for proteasomal degradation. The presence of larger Conx43 stained vesicles in the *crt-/-* cells could be due to the fact that these cells contain more Conx43 and inhibition of proteasome can cause accumulation of more proteins in these cell. Figure 9 shows that MG132 treatment increased Conx43 protein level in the *crt-/-* cells which supports this prediction. Recent data from our lab has demonstrated a significant increase in the activity of the ubiqutin-proteasome pathway in the CRT deficient cells (Uvarov and Mesaeli). Furthermore, inhibition of proteasome led to the accumulation of ubiquitinated proteins in the *crt-/-* cells as compared to the *wt* cells (Uvarov and Mesaeli). We predict that in the *crt-/-* cells the rate of degradation of Conx43 by proteasome is faster, thus inhibiting this pathway will cause a significant decrease in

the rate of Conx43 degradation and the appearnece of more Conx43 protein at the cell-cell junction. To date, the details of regulation of Conx43 life cycle via ubiquitination, internalization and degradation are not completely understood.

The second pathway for Conx degradation is the lysosome [46, 56, 108, 115, 116]. Lysosomes are thought to be imporatnt in the removal of Conxs not only from the cell membrane but also from the transport vesicle trafficking Conx from the Golgi apparatus to the cell membrane [118]. Thus disruption of lysosome function should remove all of the Conx43 protein from the membrane and prevent the maturation of newly synthesized Conx43. Indeed, leupeptin treatment was able to completely abolish Conx43 localization at the gap junctions in both the *wt* and *crt-/-* cells (Figure 15C, D). Conx43 localization in the *crt-/-* cells treated with leupeptin appeared in a punctate pattern similar to the ER patten, however whether this staining co-localizes with the ER needs further investigation. Interestingly, leupeptin treatment resulted in a large increase in the total Conx43 protein level in both cell types (Figure 13) however the level of phosphorylated form of Conx43 was increased only in the *crt-/-* cells (Figure 14B).

In the CRT deficent cells, the observed increase in the cell membrane localization of Conx43 following proteasome treatment (Figure 10D) and the accumulation of Conx43 signal to the ER of the leupeptin treated cells (Figure 15D) could be due to un-affected synthesis of new Conx43 protein in these cells. Inhibiting the protein synthesis by CHX treatment resulted in a significant decrease in the level of Conx43 in both cell type (Figure 11A, B). However, the cell membrane localization of Conx43 in the *crt-/-* cells was not increased (Figure 11B). Furthermore, the combined inhibition of protein synthesis and degradation did not alter the pattern of Conx43 signal in both of the cell types (compare Figure 11C, D with Figure 10C, D). Interestingly disruption of the Golgi appratus

following Brefeldin A treatment with or without MG132 had similar effect in the *wt* and the *crt-/-* cells (Figure 17B, C and Figure 18). Moreover we showed that interruption of glycosylation (by tunicamycin) significantly increased the localization of Conx43 in the cell membrane of both cell types (Figure 19B). This finding is in accordance with a previous report showing increased gap junction formation and communication between same cells or different types of cells following inhibition of glycosylation [106]. These authors also suggested a role for type of oligosaccharide moiety on the cell membrane for the formation of gap junction between different cell types [106]. Figure 19B also shows that the changes we observed in the localization of Conx43 (Figure 19A) in the *crt-/-* cells are not due to altered glycosylation. However, we showed that these defects could be due to change in the proteasome activity as illustrated by a difference in the accumulation of Conx43 at the gap junction of the *wt* and *crt-/-* cells following the inhibition of proteasome and glycosylation (Figure 19C). Collectively these data suggests that in the absence of CRT the trafficking of Conx43 from the ER to the Golgi apparatus and glycosylation process is not altered. However, from the current study we can not conclude if there were any changes in the rate of Conx43 trafficking. Our data from the proteasome inhibition illustrate that the change in the Conx43 protein in the absence of CRT is due to decreased stability of this protein.

In our study, we demonstrated that the effect of CRT on Conx43 is not only at the protein level but at the Conx43 mRNA. This finding is an interesting observation which requires futher investigation. An indirect role for CRT in modulating gene expression has been demonstrated previously. Altered CRT expression has been shown to modulate steroid receptor mediated gene expression [193-195, 277], adhesion protein expression [249, 250] and integrin-dependent gene expression [195, 196]. CRT has also been shown

to regulate the activity of different protein kinases such as protein-tyrosine kinase [249] and Akt kinase activity (Jalali and Mesaeli manuscript under revision) [248]. To elucidate the mechanism of regulation of Conx43 in the *crt-/-* cells, we examined the effect of serum starvation and MEK inhibitor treatment on Conx43 promoter activity. Serum deprivation resulted in a biphasic change in the Conx43 promoter (an initial increase and a later decrease) in the *wt* cells, while there were no significant changes in the Conx43 promoter activity in the *crt-/-* cells. The changes in the level of Conx43 promoter activity in the *wt* could be due to the gradual decreased in effects of growth factors on this promoter. However more research needs to be carried out to address this fact. Interestingly, inhibition of Ras/Raf/MEK (part of the MAPK pathway) resulted in the inhibition of the Conx43 promoter activity in both cell types. This observation is in accordance with a study showing activation of Conx43 promoter following Ras overexpression and the ability of MEK inhibitor (PD98059) to block this response in the NIH3T3 cell line [52]. This report showed binding of c-Myc and HSP90 to a novel consensus sequence present in the region +149 to +158 down stream of the transcription initiation signal. This binding induced the Conx43 promoter activity in a RAS-Raf-MAPK mediated manner [52]. Whether there are any changes in the Ras or c-Myc expression in the *crt-/-* cells is currently not known, however recent report from our lab showed a significant increase in the insulin receptor activity and its associated signal transduction pathway in these cells (Jalali and Mesaeli manuscript under revision). The Ras/Raf/MEK pathway is one of the down stream singling pathways of insulin receptor [278, 279].

As discussed above the modulation of proteasome activity can regulate Conx43 protein level in the *crt-/-* cells (Figure 10). In addition, we have demonstrated a significant decrease in the Conx43 promoter activity in the CRT deficient cells following MG132

treatment (Figure 12) with no effect in the *wt* cells. In fact, the level of Conx43 promoter activity following MG132 treatment was not different between the *crt-/-* cells and the *wt* cells. To date, little is known about how proteasome activity might regulate Conx43 gene expresion. It is plausible that in the CRT deficeint cells, which have a high proteasome activity, an inhibitory trasncriptional factor (or regulatory protein) is actively degraded leading to an increse in the Conx43 promoter activity. Thus inhibiting the proteasome activity will restere this inhibitory factor leading to the suppression of Conx43 gene expression.

In conclusion, in the current study we have shown a significant increase in Conx43 expression and promoter activity in the absence of CRT function. This increase however did not translate into increase in the gap junction communication, we postulated that this block is due to uncoupling of the gap junctions. We also presented some evidence for change in the stability of Conx43 protein at the cell membrane due to the increased rate of proteasome activity in the CRT deficient cells.

G. SUMMARY AND CONCLUSIONS

1. Conx43 protein expression and promoter activity is significantly higher in the *crt-/-* cells.
2. Conx43 is localized to the ER and the membrane of the *wt* and *crt-/-* cells.
3. Gap junction communication is disrupted in the *crt-/-* cells.
4. Proteasome activity regulates Conx43 internalization and stability at the cell membrane and Conx43 promoter activity.
5. Inhibition of lysosome results in the accumulation of Conx43 in the internal granules of *wt* and *crt-/-* cells and increase the Conx43 protein level (inhibit its degradation).
6. Disruption of Golgi abolished Conx43 transport to the membrane and prevented the formation of gap junction.
7. Inhibition of glycosylation also increases the number of gap junctions plaques at the cell membrane in the *crt-/-* cells.

H. FUTURE DIRECTION

The current study is the first examination of changes in the expression and gene regulation of Conx43 in the CRT deficient cells. There are many questions which need to be addressed. Future studies are needed to address whether there are any changes in the rate of Conx43 turnover in the CRT deficient cells, changes in Conx43 phosphorylation leading to its degradation via the proteasome and more a detailed analysis of Conx43 promoter activation.

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