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Regulation of Gap Junction Internalization and Degradation

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Regulation of Gap Junction Internalization and Degradation

by

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ABSTRACT

Gap junctions (GJs) are channels that traverse the plasma membrane of neighboring cells and provide direct intercellular communication (GJIC) in multi-cellular organisms. GJIC is a prerequisite for coordinated development, differentiation and tissue function; disruption of which can cause disease. Post-translational modification is essential for regulation of GJ trafficking, gating, internalization, and degradation. In the dissertation work presented here (1) I show the role of clathrin-mediated endocytosis (CME) on acute Cx43 (a GJ protein) GJ internalization. I treated primary pulmonary artery endothelial cells (pPAECs) with CME inhibitors after vascular endothelial growth factor (VEGF) stimulation and analyzed internalization patterns of GJs. VEGF treatment of endothelial cells leads to rapid internalization of GJs. I found that pharmacological inhibition of CME during VEGF treatment leads to GJs remaining in the plasma membrane, suggesting CME as the acute GJ internalization pathway. (2) I elucidated the ubiquitin-mediated regulatory mechanism of Cx43 GJ internalization and degradation. I utilized mutational analysis to identify two lysine residues that are K63-polyubiquitinated in the Cx43 C-terminal domain that are necessary for GJ internalization. Mutating these residues results in loss of K63-polyubiquitination, accumulation of GJs in the plasma membrane, and longer Cx43 protein half-life. My analysis also revealed a link between phosphorylations known to decrease GJIC (pS368, pS279/pS282, and pS255) and K63-polyubiquitination that regulates GJ internalization. (3) Additionally, I used knockdowns of key autophagy proteins and immuno-colocalization analysis to identify autophagy as the pathway that degrades internalized Cx43 GJs. I found that Beclin-1 and p62/SQSTM1 knockdowns leads to increased AGJs and decreased colocalization of Cx43 AGJs with the autophagic membrane protein, LC3. p62 (a ubiquitin

binding protein) colocalization of Cx43 at GJs and AGJs strongly suggests a link between ubiquitination of GJs and autophagic degradation. Additionally, I found that p62 and LC3 robustly colocalize with Cx43 in endogenous Cx43 expressing PAECs. Work during my Ph.D. contributed to four primary research articles (three published, one submitted), four review articles and one book chapter.

Chapter 1:

Introduction

1-1: Connexins and gap junctions

Connexins (Cxs) are four pass transmembrane proteins containing two extracellular loops, one intracellular loop and an intracellular N- and C- terminus. Six connexins oligomerize to form a connexon, or hemichannel, which is trafficked to the plasma membrane (**Figure 1**). Connexons from adjacent cells dock to form a double membrane spanning channel, or gap junction (GJ), that connects the cytosol of the two cells. This connection allows for the free passage of ions, small metabolites, and signaling molecules. GJ channels cluster to form paracrystalline arrays known as GJ plaques. In humans there are 21 known Cx proteins that are identified by molecular weight. Cx43 is ubiquitously expressed and the most studied within the Cx family. Mutations of Cx43 lead to heart conditions such as ischemia and heart failure (Fontes et al., 2012) as well as bone malformations such as Occulodentodigital Dysplasia (ODDD syndrome)(Batra et al., 2012). Diseased heart muscle shows signs of Cx43 GJ displacement from intercalated discs and downregulation of Cx43 expression (Dupont et al., 2001; Jongsma and Wilders, 2000; Kostin et al., 2003; Matsushita et al., 1999; Peters et al., 1993). Therefore, studying Cx43 regulation on a cellular level is crucial to understanding how intercellular communication is involved in both developmental and diseased states.

1-2: Cx43 post-translational modifications

Multiple regulatory mechanisms are known to impact the dynamics of Cx43, most notably post-translational modification by ubiquitination and phosphorylation. Ubiquitination is the addition of an 8.5 kDa protein onto a lysine of a target protein by an enzyme cascade (**Figure 2A**). Seven internal lysines and an N-terminal methionine allow for multiple ubiquitin moieties to link end-to-end to create complex chains that dictate the fate of the target protein (**Figure 2B**) (Komander and Rape, 2012). Cx43 ubiquitination has been described for 20 years (Laing and Beyer, 1995; Laing et al., 1997). However, little is known about how many ubiquitins are attached, which lysine residues they are attached to, and what pathways they signal within the cell.

Phosphorylation of Cx43 has been shown to be a regulatory mechanism for Cx43 trafficking, GJ assembly, gating and plaque internalization and degradation (King and Lampe, 2005; Laird, 2005; Lampe and Lau, 2004; Moreno, 2005; Solan and Lampe, 2005; Solan and Lampe, 2007; Solan and Lampe, 2009; Warn-Cramer and Lau, 2004). These phosphorylation events lead to conformational changes within the C-terminus of Cx43 that allow for regulatory protein binding. Numerous phosphorylation events by Akt (protein kinase B), PKA (protein kinase A) and CK1 (casein kinase 1) regulate Cx43 trafficking and assembly (Cooper and Lampe, 2002; Park et al., 2006; Park et al., 2007) (**Figure 3**). Phosphorylation by PKC (protein kinase C), CDC2 (cell division cycle protein 2), MAPKs, and Src regulate GJ channel closure, inhibition of GJIC, and GJ internalization (Kanemitsu et al., 1998; Lampe et al., 1998; Lampe et al., 2000; Leykauf et al., 2003; Petrich et al., 2002; Polontchouk et al., 2002; Saez et al., 1997; Sirnes et al., 2009; Solan and Lampe, 2007; Solan and Lampe, 2008).

1-3: Gap junction internalization

Removal of GJs from the plasma membrane requires internalization of the entire GJ into one cell as individual hemichannels are unable to physiologically uncouple once they form a GJ channel (Ghoshroy et al., 1995; Goodenough and Gilula, 1974). Internalized GJs are known as annular gap junctions (AGJs) or connexosomes (Falk et al., 2009; Gaietta et al., 2002; Jordan et al., 2001; Lauf et al., 2002; Piehl et al., 2007). This process of internalization requires the clathrin-mediated endocytosis (CME) machinery (Baker et al., 2008; Fong et al., 2013; Gumpert et al., 2008; Piehl et al., 2007) (**Figure 4,** steps 1-4). Additionally, cells can acutely internalize GJs in response to inflammatory agents or growth factors (Baker et al., 2008; Fong et al., 2014; Leithe and Rivedal, 2004a; Thuringer, 2004). Vascular endothelial growth factor (VEGF) is an angiogenic growth factor that is highly specific for endothelial cells and was found to lead to GJ serine phosphorylation and rapid internalization in endothelial cells (Nimlamool et al., 2015). Additionally, pathways of VEGF activation has been reported to transiently disrupt gap junction intercellular communication (GJIC) in endothelial cells via activation of MAPK and c-Src activity (Kevil et al., 1998; Suarez and Ballmer-Hofer, 2001) and lead to Cx43 tyrosine phosphorylation in rat coronary capillary endothelium (Thuringer, 2004).

1-4 Autophagic degradation of gap junctions

Autophagic degradation involves the targeted sequestration of large protein complexes and cytoplasmic organelles (He and Klionsky, 2009). Targeted proteins are recruited to a growing double membrane structure known as the phagophore, which completely engulfs its cargo and becomes an autophagosome. The outer membrane of the autophagosome fuses with a

lysosome, allowing for complete degradation of the internal membrane and all of its contents. Our lab and others have shown that Cx43 AGJs are degraded by autophagy under physiological (Fong et al., 2012), starvation (Bejarano et al., 2012; Lichtenstein et al., 2010) and diseased states (Hesketh et al., 2010) (**Figure 4,** steps 5-10). The process of autophagosomal degradation relies on the ubiquitin binding protein, p62, to interact with and sequester targeted cargo to autophagosomes. Work from our lab and others have verified the colocalization/interaction of p62 with Cx43, bridging GJ ubiquitination with autophagic degradation (Bejarano et al., 2012; Fong et al., 2012; Lichtenstein et al., 2010).

The chapters presented in this dissertation cover several steps of internalization and degradation of GJs. Chapters 2 and 3 contribute to steps 1-4 and Chapter 4 contributes to steps 5-10 in **Figure 4**.

Figures

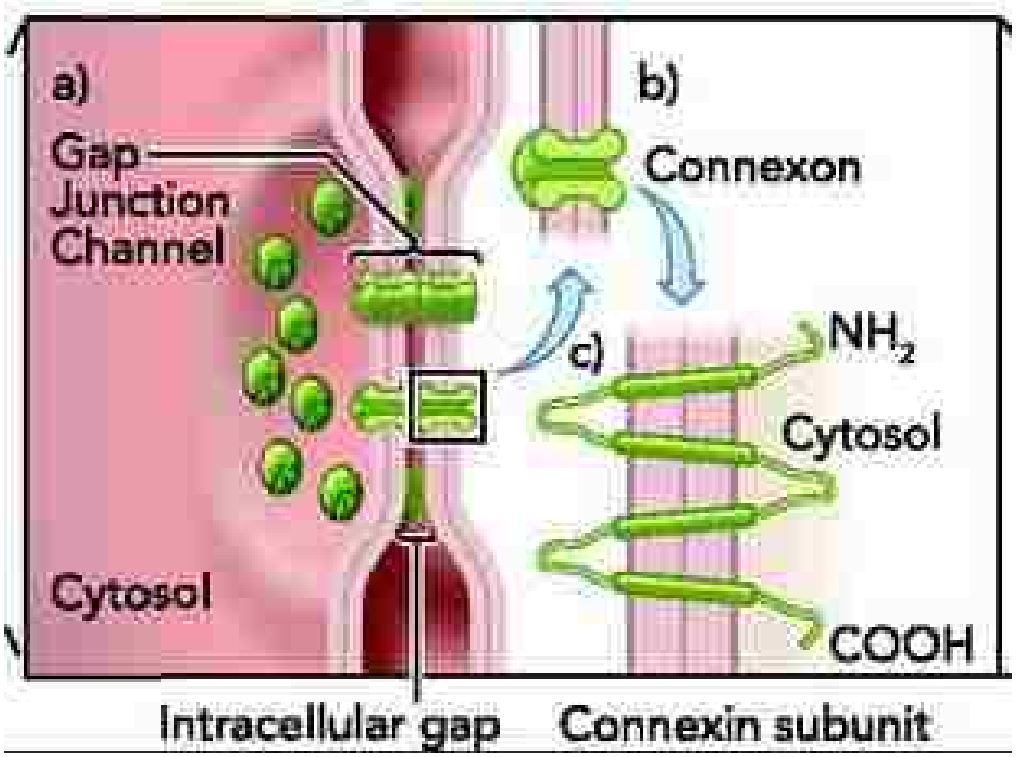


Figure 1: Gap junction structure. GJ channels form by the head-on docking of two hemichannels or "connexons," each assembled and trafficked to the PM by one of the two contacting cells. Connexons are assembled from six four-pass transmembrane proteins termed "connexins" (Cxs). Adapted from (Thévenin et al., 2013).

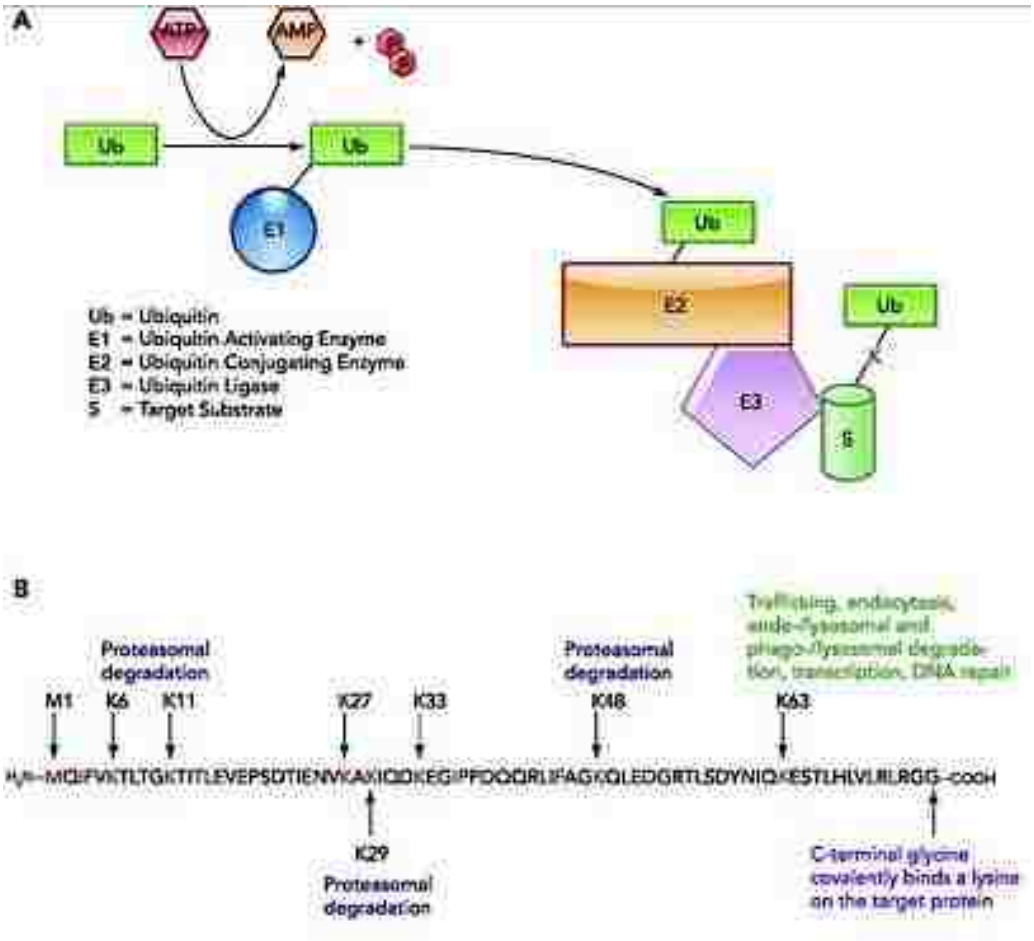


Figure 2: Ubiquitination. A) Ubiquitin targeting cascade. E1 (ubiquitin activating enzyme) binds to ubiquitin (Ub) in an ATP-dependent process. E1 transfers ubiquitin to E2 (ubiquitin conjugating enzyme), which moves the ubiquitin moiety to the lysine (K) of the target protein. E3 (ubiquitin ligase) is bound to the target protein and aids in the transfer of ubiquitin from E2 to the target substrate and in its covalent attachment. B) lysine residues of the ubiquitin amino acid sequence involved in the formation of polyubiquitin chains. Ubiquitin has 76 amino acids, eight of which are involved in forming polyubiquitin chains. Of the seven lysines (K), K6, K11, K29, and K48 linkages lead to proteasomal degradation, whereas K63 linkages lead to trafficking, endocytosis, endo-/lysosomal and phago-/lysosomal degradation, transcription, and DNA repair. The functions of K27 and K33 linkages have yet to be elucidated. In addition to the seven lysines, methionine 1 (M1) also can link ubiquitin moieties together to form linear chains. The COOH-terminal glycine (G) residue is responsible for the covalent linkage of the ubiquitin moiety to the lysine of the target protein. Adapted from (Thévenin et al., 2013).

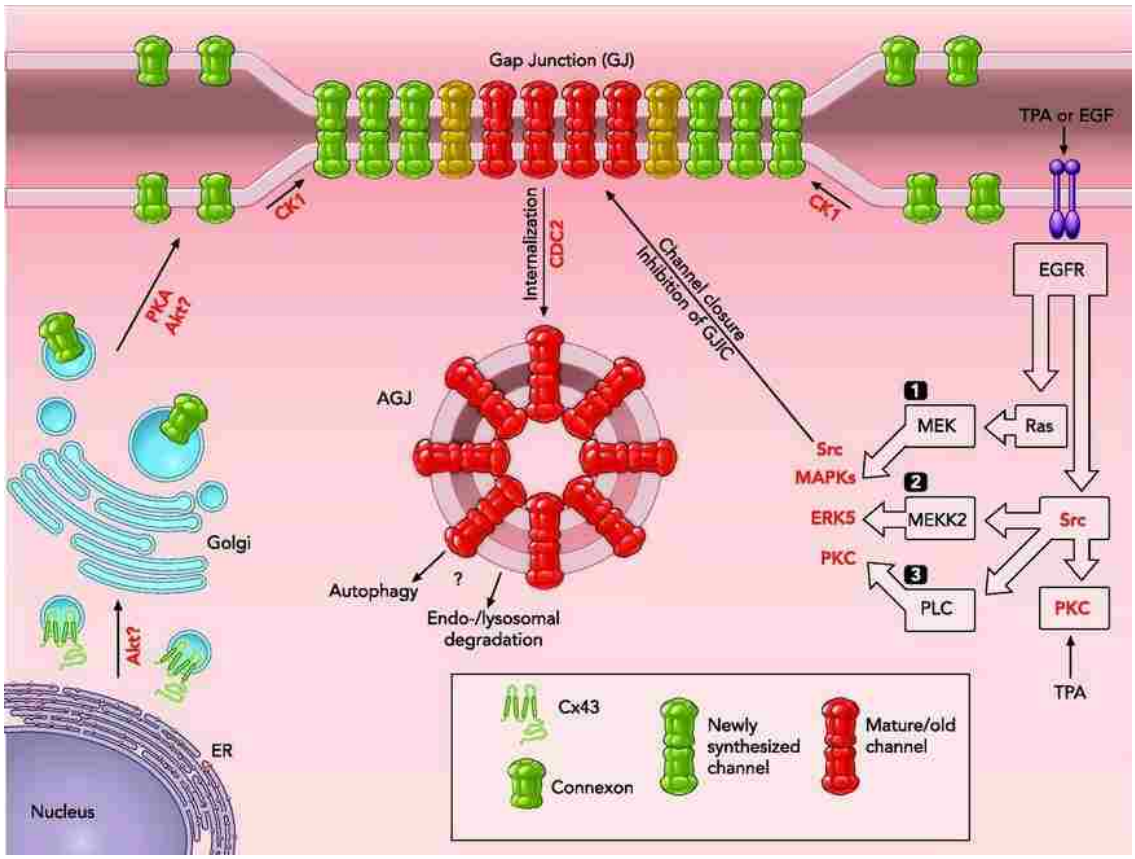


Figure 3: Kinases involved in the regulation of the Cx43 life cycle. Forward trafficking of Cx43 monomers from the ER to the Golgi apparatus may be regulated through Akt phosphorylation (and interaction with 14-3-3 protein) (*left*). Trafficking of connexons toward the PM is regulated through phosphorylation by PKA, whereas assembly of PM-localized connexons into newly synthesized channels in GJ plaques is regulated via CK1 phosphorylation. Src, PKC, and MAPK activation can be achieved through the activation of the EGF receptor (EGFR) in an EGF- or TPA-dependent manner (*right*). MAPKs (i.e., ERKs, JNKs, or p38) are activated through Ras, Raf, and MEK (*pathway 1*). Src can be activated directly by the EGFR, which in turn can activate ERK5 through MEKK2 (*pathway 2*). Src can also activate PKC through diacylglycerol (DAG) that is generated by phospholipase C (PLC)-mediated phosphatidylinositol 4,5-bisphosphate (PIP_2) cleavage (*pathway 3*). PKC can also be activated directly by TPA. Activated kinases (Src, MAPKs, ERK5, and PKC) regulate downregulation of GJIC, channel closure, and possibly internalization, formation of annular GJs (AJGs), and degradation through either autophagosomal and/or endo-/lysosomal pathways (*middle*). CDC2 phosphorylates Cx43 at the onset of mitosis, also leading to GJ internalization and degradation (Thévenin et al., 2013).

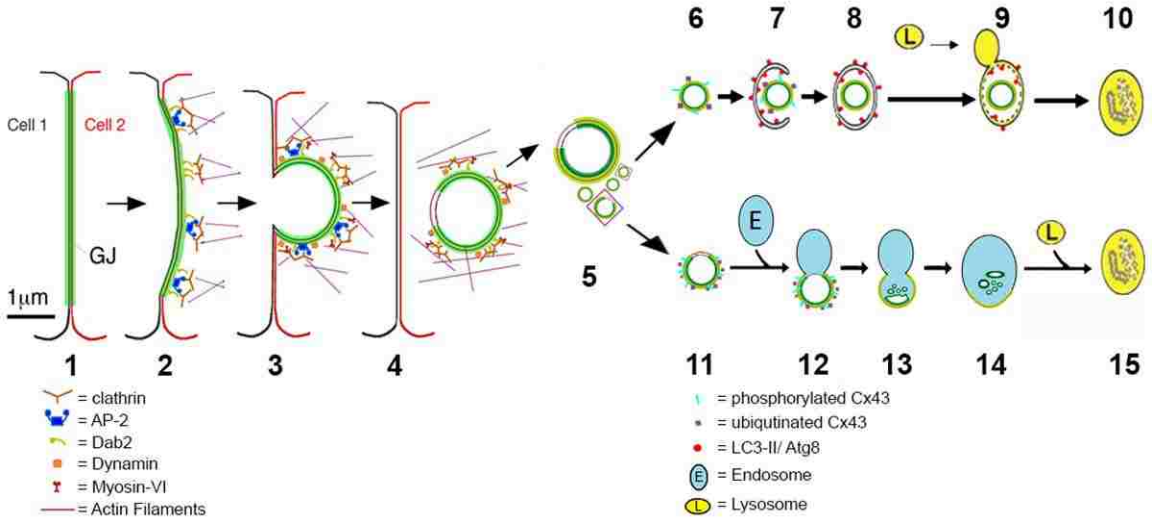


Figure 4: Schematic representation of the proposed steps that lead to GJ internalization (1-3), AGJ vesicle formation and fragmentation (4, 5), and AGJ vesicle degradation by phago-/lysosomal (6-10) and endo-/lysosomal (11-15) pathways based on the present and previous work by others and us (Fong et al., 2012).

Chapter 2:

VEGF-mediated internalization of Connexin43 GJs requires clathrin-mediated endocytosis

(from Nimlamool, Kells Andrews, and Falk, 2015)

Contribution: My contribution to (Nimlamool et al., 2015) is summarized in **Figure 5 and 6**. I was responsible for establishing an essential protocol to block clathrin-mediated endocytosis (CME) by pharmacological inhibition upon reviewer request. I used Alexa568-conjugated transferrin as a means to determine efficiency of endocytosis after addition of Dynasore, Pitstop 2, Ikarugamycin, and Hypertonic medium to endothelial cells that were pre-treated with vascular endothelial growth factor (VEGF). The resulting blockage of CME with the aforementioned CME inhibitors leads to decreased internalization of Cx43 GJs during VEGF treatment (**Figure 5**). The addition of this data to the manuscript allowed us to conclude that VEGF-induced internalization of Cx43 GJs relies on the CME pathway. **Figure 6** shows a positive control for Alexa568-conjugated transferrin uptake of cells under untreated, VEGF, and Hypertonic conditions. Alexa568-conjugated transferrin is taken up in cells via CME. Blockage of CME results in a loss of Alexa568-conjugated transferrin signal, as seen under hypertonic medium conditions (**Figure 6**).

2-1: Abstract

Gap junction intercellular communication (GJIC) is essential for multi-cellular life. Gap junctions (GJs) provide a physical connection to neighboring cells and allow for the passage of signals between cells. Several growth factors, including vascular endothelial growth factor (VEGF), have been reported to disrupt cell-cell junctions and to abolish GJIC. However,

the mechanism of decreased GJIC upon inflammatory mediators has not fully been elucidated. Here we show that acute internalization of GJs during VEGF stimulation is dependent on clathrin-mediated endocytosis (CME) using pharmacological inhibitors of CME.

2-2: Introduction

Aberrant function of GJs and reduction of cell-cell coupling via GJs has been associated with many pathological conditions, including cancer (Chang et al., 2003; Cronier et al., 2009; Leithe et al., 2006; Simon, 1999; Sulkowski et al., 1999). To maintain normal cell-to-cell communication, recruitment of newly synthesized GJ channels along the outer edge of GJ plaques and simultaneous removal of older channels from plaque centers are precisely regulated (Dunn and Lampe, 2014; Falk et al., 2009; Gaietta et al., 2002; Lauf et al., 2002; Rhett and Gourdie, 2011). Dynamic assembly and degradation of GJs correlates with the short half-life of Cx proteins of 1-5 hours determined *in situ* as well as in cultured cells (Beardslee et al., 1998; Berthoud et al., 2004; Falk et al., 2009; Fallon and Goodenough, 1981). A characteristic feature of GJs is that docked double-membrane spanning GJ channels cannot be separated into individual, single-membrane spanning hemi-channels (connexons) under physiological conditions (Ghoshroy et al., 1995; Goodenough and Gilula, 1974). However, cells can constitutively turn over GJs either by internalizing small double-membrane domains from central areas of plaques (Cone et al., 2014; Falk et al., 2009; Gaietta et al., 2002; Jordan et al., 2001), or by internalizing entire plaques or large portions of plaques (Piehl et al., 2007). Moreover, cells can acutely internalize GJs in response to inflammatory agents or growth factors (Baker et al., 2008; Fong et al., 2014; Leithe and Rivedal, 2004a;

Thuringer, 2004). Earlier studies have shown that GJ turnover utilizes the clathrin-mediated endocytosis (CME) machinery (Gilleron et al., 2011; Gumpert et al., 2008; Huang et al., 1996; Larsen et al., 1979; Naus et al., 1993; Nickel et al., 2013; Nickel et al., 2008; Piehl et al., 2007), and internalization results in the formation of cytoplasmic double-membrane GJ vesicles, termed annular gap junctions (AGJs) or connexosomes (reviewed in (Falk et al., 2014; Ogawa et al., 2005; Su and Lau, 2014; Thévenin et al., 2013).

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor that is highly specific for endothelial cells. VEGF-specific tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4, in lymphangiogenesis) activate several intracellular signaling pathways upon VEGF binding (de Vries et al., 1992). Proliferation and migration of endothelial cells is primarily mediated by VEGFR-2 (Waltenberger et al., 1994). Activation of VEGF receptors promotes phosphorylation of a number of downstream proteins in endothelial cells, including phospholipase Cγ (PLCγ) (Takahashi et al., 2001), phosphatidylinositol 3-kinase (PI3-kinase) (Qi and Claesson-Welsh, 2001), and guanine 5' triphosphate and (GTP)ase-activating protein (Suzuma et al., 2000). Furthermore, VEGF induces protein kinase C (PKC), which results in an increase in intracellular calcium ions, and stimulates inositol-1,4,5-triphosphate accumulation (Brock et al., 1991). VEGF has also been reported to transiently disrupt GJIC in endothelial cells via the activation of VEGFR-2, which activates MAPK and c-Src activity (Kevil et al., 1998; Suarez and Ballmer-Hofer, 2001). Additionally, in 2004 it was reported that the VEGF-induced disruption of GJIC correlated with the rapid internalization of Cx43 and its tyrosine phosphorylation in rat coronary capillary endothelium (Thuringer, 2004).

Potential mechanisms including post-translational modifications of Cx43 such as phosphorylation or ubiquitination that may be important for VEGF-induced inhibition of GJIC, and whether VEGF-mediated inhibition of GJIC involves GJ internalization have not been investigated. Elucidating the mechanisms that down-regulate GJIC in response to VEGF is important for e.g. understanding the biology of cancer development including changes in cell cycle progression, angiogenesis, and tumor cell metastasis. Here we use pharmacological inhibition of CME to show that the rapid internalization of Cx43 GJs is blocked when CME is inhibited.

2-3: Results

2-3-1: VEGF-induced GJ internalization is clathrin-mediated.

After treatment of primary pulmonary artery endothelial cells (PAECs) endogenously expressing Cx43 with VEGF for 15 min, Nimlamool et al. 2015 found a rapid internalization of Cx43 GJs from the plasma membrane (Nimlamool et al., 2015). The observed GJ internalization occurred in response to VEGF-treatment triggered by the phosphorylation of Cx43 in GJs at serines 368, 279/282, 255, and 262. Previous studies in our and other laboratories have revealed that Cx43 GJs are internalized utilizing the clathrin-mediated endocytosis (CME) machinery (Baldwin and Thurston, 2001; Fong et al., 2013; Fong et al., 2014; Gilleron et al., 2011; Gumpert et al., 2008; Nickel et al., 2013; Piehl et al., 2007). We therefore reasoned that VEGF-induced Cx43 GJ internalization is also clathrin-mediated. It has been established that hypertonic conditions can efficiently prevent the budding of clathrin-coated vesicles from the plasma membrane and that this eventually will significantly inhibit clathrin-mediated endocytosis (Ferrara, 2004; Hansen et al., 1993; Heuser and

Anderson, 1989; Wu et al., 2001). We therefore inhibited CME by exposing PAECs to hypertonic culture medium (0.45 M sucrose) before and during VEGF-exposure. As shown in **Figure 5A, c**, and quantified in **Figure 5B**, VEGF-induced Cx43 GJ internalization was almost completely inhibited under these hypertonic culture conditions when compared to normal conditions (**Figure 5A, a-c, B**). Since transferrin receptor endocytosis is known to be dependent on clathrin-mediated endocytosis (Motley et al., 2003), Alexa568-conjugated transferrin uptake was used as a control to verify that our conditions successfully blocked clathrin-mediated endocytosis. Significant inhibition of Alexa568-transferrin uptake was observed under these conditions as well (**Figure 6**). Similar results were obtained when VEGF-treated cells in addition were incubated with other known CME-inhibitors such as dynasore, a dynamin-specific small molecule inhibitor (Macia et al., 2006), Pitstop 2, a small molecule inhibitor that inhibits both clathrin-dependent and independent endocytic processes (Dutta et al., 2012), or Ikarugamycin, a macrocyclic antibiotic also known to block CME (Luo et al., 2001), also resulting in significant inhibition of GJ internalization (**Figure 5A, d-f**). These results suggest that VEGF-mediated GJ internalization is also clathrin driven.

2-4: Discussion

These results demonstrate that VEGF stimulation of endothelial cells causes Cx43 GJ internalization via CME. In a larger context, these results combine with those from Nimlamool et al. 2015 to demonstrate that VEGF-treatment induces the phosphorylation of Cx43 at serines 255, 262, 279/282, and 368 by MAPK and PKC that correlates with GJIC inhibition and Cx43-based GJ internalization (Nimlamool et al., 2015).

Integrity of blood vessel walls must be maintained precisely to prevent the leakage of plasma components and blood cells into surrounding tissues, while simultaneously permitting extravasation of leukocytes and other immune cells at sites of inflammation. GJs are one type of dynamic intercellular junction located at cell-cell contacts that regulate the selective permeability of endothelial cells. VEGF is a potent endothelial cell mitogen with a significant role in regulating angiogenesis (Ferrara, 2004). VEGF has been reported to disrupt GJIC in endothelial cells (Kevil et al., 1998; Suarez and Ballmer-Hofer, 2001; Thuringer, 2004); however, underlying molecular mechanisms were not characterized. We investigated cellular and molecular effects of VEGF-induced GJ reorganization and characterized relevant signaling pathways in primary pulmonary artery endothelial cells (PAECs) that express high levels of endogenous Cx43 and VEGF receptors. Hypertonic conditions and incubation of cells in Dynasore, Pitstop 2, or Ikarugamycin, known potent inhibitors of CME (Dutta et al., 2012; Hansen et al., 1993; Heuser and Anderson, 1989; Luo et al., 2001; Macia et al., 2006; Wu et al., 2001), significantly inhibited internalization (**Figure 5, 6**). This suggests that VEGF-mediated GJ internalization is also clathrin driven, as has been characterized before for GJ internalization occurring under various other conditions (Baker et al., 2008; Cone et al., 2014; Fong et al., 2013; Gilleron et al., 2009; Gumpert et al., 2008; Nickel et al., 2013; Piehl et al., 2007).

This data contributes to the first study that reports that Ser255, Ser262, Ser279/282, and Ser368, well-known Cx43 MAPK and PKC target sites, are specifically phosphorylated in response to VEGF stimulation (Nimlamool et al., 2015), and that phosphorylation on all or on a subset of these sites induces CME-mediated GJ internalization (**Figure 5,6**).

Stimulation with growth factors such as VEGF, EGF and PDGF, inflammatory mediators such as thrombin and endothelin, and with phorbol esters (TPA and derivatives, analogs of the second messenger molecule diacylglycerol [DAG] that activate PKC), or ischemia, wounding or oncogene activation are all known to efficiently down-regulate GJIC (Kevil et al., 1998; Pahujaa et al., 2007; Postma et al., 1998; Sirnes et al., 2009; Solan and Lampe, 2014; Suarez and Ballmer-Hofer, 2001; Thuringer, 2004; Warn-Cramer and Lau, 2004). For some stimulants (EGF, VEGF, thrombin, endothelin, PDBu, a TPA derivative) concomitant GJ internalization has now been shown (Baker et al., 2008; Cone et al., 2014; Fong et al., 2014; Leithe and Rivedal, 2004a). Based on these observations, we postulate that inhibition of GJIC in general is accompanied by GJ internalization that is initiated by specific Cx43 C-terminal phosphorylation events as described above. Elucidating in detail the molecular signals that trigger GJ internalization and turnover will be critical for understanding the many essential and dynamic roles of GJs in physio-/pathology.

2-5: Materials and methods

2-5-1: Materials

Recombinant human vascular endothelial growth factor-165 (VEGF-165, VEGF-A) was purchased from Millipore (Cat. No. GF315, Billerica, MA, USA). Dynasore was from Tocris Bioscience (Cat. No. 2897), Pitstop 2 was from Abcam (Cat. No. AB120687), and Ikarugamycin was from Biomol (Cat. No. El313). Rabbit polyclonal anti-Cx43 antibody (Cat. No. 3512). Hoechst 33342, DAPI, and Alexa568-conjugated Transferrin (Cat. No. T23365) were from Invitrogen (Grand Island, NY, USA).

2-5-2: Cell culture

Primary porcine pulmonary artery endothelial cells (PAECs) were purchased from Cell Applications (Cat. No. P302, San Diego, CA). PAECs were cultured in DMEM supplemented with 10% FBS, 1.0% L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified atmosphere at 37°C, 5% CO2. Confluent cells were incubated in serum-free medium for 3 hours before exposure to VEGF.

2-5-3: Immunofluorescence

PAECs were grown on glass cover slips coated with poly-L-lysine and then were left untreated or treated with 100 ng/ml VEGF. Cells were fixed and permeabilized with -20°C-cold methanol for 10 min, blocked with 10% FBS/PBS for 30 min, and incubated with 1:200 of an anti-Cx43 polyclonal antibody at 4°C, overnight. After three PBS washes, cells were incubated with 1:500 secondary antibodies (Alexa488-conjugated goat anti-rabbit, and Alexa568-conjugated goat anti-mouse) for 1 h at RT. Cell nuclei were counter-stained with 1μg/ml Hoechst 33342, or 1μg/ml DAPI. Cells were mounted using Fluoromount-G™ (SouthernBiotech, Cat. No. 0100-01). Observations were performed on a Nikon EclipseTE 2000-U inverted fluorescence microscope equipped with 40x and 60x, Plan-Apochromat, NA1.4 oil-immersion objectives (Nikon, Tokyo, Japan). Quantitative image analyses were performed using ImageJ software (National Institutes of Health, USA).

2-5-4: Endocytosis inhibition assays

PAECs grown to confluency on poly-L-lysine coated cover glasses were treated with 100 ng/ml VEGF for 15 min at 37°C in Hepes-buffered and serum-free DMEM without or with

the presence of 0.45M sucrose. To inhibit CME, cells were treated with 100 ng/ml VEGF for 15 min at 37°C in serum-free DMEM containing 30 μM Pitstop 2, 80 μM Dynasore, or 4 μM Ikarugamycin. Cells were fixed and immuno-stained with anti-Cx43 antibodies and visualized using fluorescence microscopy. Transferrin was used as a positive control to determine efficiency of CME inhibition. Cells were incubated with 50 μg/ml Alexa568-conjugated transferrin in the absence or presence of 0.45M sucrose (for 45min) or pharmacological CME inhibitors (for 15min) at 37°C. Cells were then fixed and mounted. Immunofluorescence images were captured as described above.

2-5-5: Statistical analyses

Unpaired student t-tests were performed to analyze intracellular and plasma membrane fluorescence intensities (**Figure 5**). Data are presented as mean ± SD. In all analyses, a p-value ($p^*<0.05$, $p^{**}<0.01$, and $^{***}p<0.001$) was considered statistically significant. NS is depicted for non-significant results.

Figures

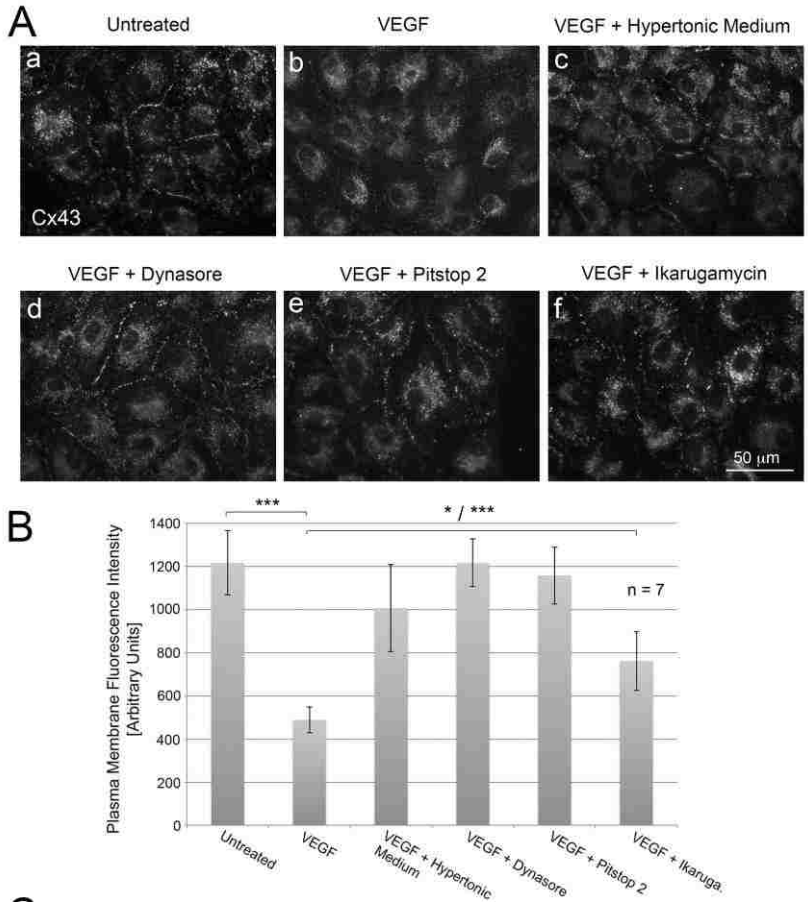


Figure 5: VEGF induces Cx43 GJ internalization via clathrin-mediated endocytosis (CME). **(A)** PAECs were (a) untreated, or treated with VEGF for 15 min at 37˚C in Hepes-buffered, serum-free DMEM without (b) or with (c) 0.45M sucrose (Hypertonic Medium, a potent inhibitor of endocytosis), or with inhibitors of CME (d) 80 µM Dynasore, (e) 30 µM Pitstop 2, or (f) 4 µM Ikarugamycin before fixation and immuno-staining cells for Cx43. Representative images acquired with identical camera settings are shown. **(B)** Quantitative analyses of plasma membrane localized Cx43 GJ fluorescence of 7 independent measurements. Note significant inhibition of Cx43 GJ internalization in cells in which CME was blocked (published in (Nimlamool et al., 2015)).

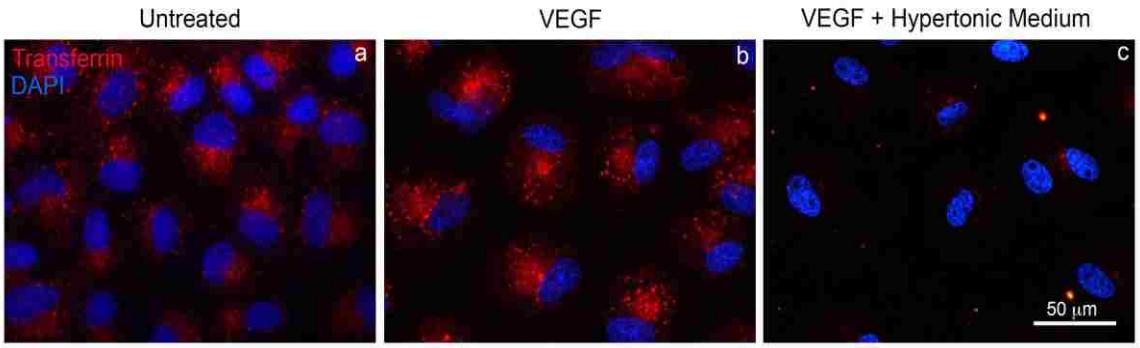


Figure 6: Hypertonic Medium efficiently inhibits clathrin-mediated endocytosis. PAECs were left untreated (a), or treated with VEGF for 15 min at 37˚C in Hepes-buffered serum-free DMEM, without (b) or with 0.45M sucrose (c) (a potent inhibitor of CME) before incubation with Alexa568-conjugated transferrin (red, a protein whose uptake is known to be CME dependent), and fixation. Cell nuclei were stained with DAPI (blue). Note drastically reduced uptake of transferrin in cells placed in hypertonic medium (published in (Nimlamool et al., 2015)).

Chapter 3:

Phosphorylation-induced K63-polyubiquitination of Connexin 43 is essential for gap junction internalization

(Kells Andrews and Falk, in review)

3-1: Abstract

Gap junctions (GJs) assembled from connexin (Cx) proteins play a pivotal role in cell-cell communication by forming channels that connect the cytosol of adjacent cells. Connexin43, the best-studied Cx, is ubiquitously expressed in vertebrates. While phosphorylation is known to regulate multiple aspects of GJ function and turnover, much less is known about the role ubiquitination plays in these processes. Here we demonstrate that Cx43 in GJs and internalized GJs is K63-polyubiquitinated on K264 and K303. Relevant Cx43 K to R mutants exhibited impaired internalization. Intriguingly, ubiquitin-deficient Cx43 mutants accumulated as hyper-phosphorylated polypeptides, indicating that phosphorylation triggers subsequent ubiquitination. Phospho-specific Cx43 antibodies revealed that upregulated phosphorylation affected serines 368, 279/282, and 255, well-known regulatory PKC and MAPK phosphorylation sites. Together, these novel findings demonstrate that Cx43 in GJs is K63-polyubiquitinated, ubiquitination is necessary for GJ internalization, and that K63-polyubiquitination is induced by Cx phosphorylation as is typical for phosphodegron-regulated protein degradation.

3-2: Introduction

Ubiquitination, the addition of a small, 76 amino acid, 8.5kDa protein to a target protein is known to play multiple functions, from influencing protein structure and function to

trafficking and especially protein degradation (Komander and Rape, 2012; Nguyen et al., 2013; Ravid and Hochstrasser, 2008). Multiple types of ubiquitination (mono- and several different types of poly-ubiquitination) are known to execute these numerous functions (Komander and Rape, 2012). Ubiquitin (Ub) is covalently attached to lysines (K) on target proteins by an enzyme cascade consisting of an E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzyme (Chen and Sun, 2009). Ub can also be removed from its target via proteases termed deubiquitinases (DUBs). Ub has seven internal lysines (K6, K11, K27, K29, K33, K48 and K63), all of which (and also the N-terminal methionine) are capable of forming linkages to the C-terminal glycine of subsequent Ub moieties, forming polyubiquitin (polyUb) chains. The two best studied polyUb chains are K48-polyUb, which is known to lead to proteasomal degradation, and K63-polyUb, which -besides other functions- is known to signal endo- and phago-lysosomal degradation (Komander and Rape, 2012). Ub-mediated degradation is often regulated via protein phosphorylation on a specific site (termed phosphodegron). Phosphorylation on one or more residues induces substrate changes (conformational or other) allowing the subsequent ubiquitination of target lysines that then triggers the degradation of the phosphorylated/ubiquitinated protein (Nguyen et al., 2013; Ravid and Hochstrasser, 2008). Ubiquitination of gap junction (GJ) proteins, termed connexins (Cxs), has also been reported (Girao et al., 2009; Laing and Beyer, 1995; Laing et al., 1997; Leithe and Rivedal, 2004b; Ribeiro-Rodrigues et al., 2015) however published results are inconsistent making it difficult to reliably judge the types of ubiquitination and the role they may play in GJ function.

Connexins (Cxs) are the four pass transmembrane protein components of GJs that serve as a pathway for intercellular communication by physically coupling cells together and allowing

the passage of small metabolites, signaling molecules, and ions. Cxs have two extracellular loops, one intracellular loop, and an intracellular N- and C-terminus. Six Cxs oligomerize to form a hemichannel or connexon, which is trafficked to the plasma membrane (PM) and docks with a hemichannel of an adjacent cell forming the complete GJ channel. Accrual of multiple channels at the PM into clusters or two-dimensional arrays forms typical GJ plaques. Interestingly, once hemichannels dock at the PM, they can no longer be physiologically separated (Goodenough and Gilula, 1974). Therefore, in a process that requires the clathrin-mediated endocytic (CME) machinery, one of the two adjacent cells invaginates the GJ plaque forming an annular gap junction (AGJ) vesicle or connexosome in the cytoplasm of one of the coupled cells (Falk et al., 2009; Gaietta et al., 2002; Jordan et al., 2001; Lauf et al., 2002; Piehl et al., 2007). The AGJ is then trafficked for degradation by autophago-lysosomal (under physiological, pathological, and starvation conditions) (Bejarano et al., 2012; Fong et al., 2012; Hesketh et al., 2010; Lichtenstein et al., 2010) and possibly endo-lysosomal pathways under TPA (12-O-Tetradecanoylphorbol 13-Acetate, a Diacylglycerol analog) treatment (Fykerud et al., 2012; Leithe et al., 2009). In humans, there are 21 different Cx proteins, which are identified by molecular weight. Cx43 is the best-studied Cx and is expressed in most tissues. Mutations in Cx43 have been linked to such cardiac diseases as heart failure, ischemia, and hypertrophy (Fontes et al., 2012) and the developmental bone malformation Occulodentodigital Dysplasia (ODDD) (Batra et al., 2012). The regulation of Cx43 trafficking and turnover is therefore imperative for the study of human health and disease.

Phosphorylation of Cx43 is well established and has been shown to be a regulatory mechanism for Cx43 trafficking, GJ assembly, gating, plaque internalization, and degradation

(Falk et al., 2016; Solan and Lampe, 2014; Thévenin et al., 2013). Cx43 phosphorylation by Akt (protein kinase B), PKA (protein kinase A), and CK1 (casein kinase 1) is known to up-regulate gap junction intercellular communication (GJIC), whereas phosphorylation by PKC (protein kinase C), CDC2 (cell division cycle protein 2), MAPKs, and Src down-regulate GJIC by closing GJ channels and internalizing GJ plaques (Falk et al., 2014; Kanemitsu et al., 1998; Lampe et al., 1998; Lampe et al., 2000; Leykauf et al., 2003; Nimlamool et al., 2015; Petrich et al., 2002; Polontchouk et al., 2002; Saez et al., 1997; Sirnes et al., 2009; Solan and Lampe, 2007; Solan and Lampe, 2008). However, whether phosphorylation is linked to Cx43 ubiquitination has not been established.

Cx43 ubiquitination was initially discovered by Laing and Beyer in 1995 and characterized as a signal leading to Cx43 degradation by the proteasome (Laing and Beyer, 1995). Later, the same group published evidence suggesting that ubiquitination plays a role in both proteasomal and lysosomal degradation of Cx43 (Laing et al., 1997). Afterward, it was reported that Cx43 likely is modified by multiple mono-ubiquitinations (Girao et al., 2009; Leithe and Rivedal, 2004b), while more recent evidence suggests that Cx43 interacts with AMSH (associated molecule with the SH3 domain of STAM) (Ribeiro-Rodrigues et al., 2015). AMSH is a DUB with specificity toward K63-polyubiquitinated proteins (McCullough et al., 2004; Sato et al., 2008), suggesting that Cx43 may also become K63-polyubiquitinated (Ribeiro-Rodrigues et al., 2015). It is possible that the different discovered types of Cx43 ubiquitination distinguish the degradation of single, misfolded Cx polypeptides (by the 26S proteasome) from the degradation of GJs and AGJs (by phago- and endo-lysosomal pathways). However a rigorous analysis of where and when ubiquitination occurs on Cxs and at which stages of its 'life-cycle' (Cx polypeptides, connexons, GJs, AGJs) has not been

performed; nor is it known what signals trigger potential Cx43 ubiquitination and whether Cx43 ubiquitination may have additional functions beyond its proteasomal degradation.

Here, we used Ub-specific antibodies and generated Cx43 lysine to arginine mutations to investigate whether Cx43 ubiquitination occurs in GJs, what type of ubiquitination may occur, and whether Cx43 ubiquitination may be used as a signal for GJ internalization and degradation. We found that two juxtaposed ubiquitination sites in the C-terminus of Cx43 become K63-polyubiquitinated, ubiquitination is required for constitutive GJ internalization, and ubiquitination is triggered by phosphorylation on well-known Cx43 regulatory serine residues. These findings suggest that a phosphodegron regulates GJ internalization and degradation.

3-3: Results

3-3-1: Cx43 is K63-polyubiquitinated in gap junctions and internalized gap junctions

To determine whether and what type of ubiquitination may occur on Cx43 in GJs, we immunostained endogenously expressed Cx43 in primary porcine pulmonary artery endothelial cells (pPAECs) with antibodies directed against Cx43 and different forms of Ub (**Figure 7**). FK2 antibodies, which recognize both monoUb and polyUb, colocalized with Cx43 at GJ plaques (insert 1, marked with arrows) and AGJs (insert 2, marked with arrowheads) as shown in **Figure 7A**. This suggests that Cx43 in GJs and AGJs can become ubiquitinated. To address this question further we co-immunostained Cx43 in pPAECs with antibodies that detected only polyubiquitinated proteins (FK1). We found that colocalization of Cx43 GJ plaques and AGJs with antibodies for polyUb alone was also evident (**Figure 7B**). This result indicates that Cx43 in GJ plaques and AGJs can become

polyubiquitinated. To test whether the detected polyubiquitin modification is K63-based (or at least K63-based polyubiquitination may occur in GJs) we again used co-immunostaining in pPAECs with an antibody that detects only K63-polyUb. Again, robust colocalization of GJs and AGJs with the K63-polyUb antibody indicates that Cxs in GJs and AGJs can become K63-polyubiquitinated (**Figure 7C**). Our finding correlates with the enzyme specificity of the E3 ubiquitin ligase, Nedd4-1 (neural precursor cell expressed developmentally down-regulated protein 4-1), which preferentially K63-polyubiquitinates its substrates (Kim et al., 2007), and has been found to ubiquitinate Cx43 (Girao et al., 2009; Leykauf et al., 2006). Our data also correlates with recent biochemical evidence suggesting that Cx43 interacts with the K63 polyUb specific DUB, AMSH (Ribeiro-Rodrigues et al., 2015).

To further verify K63-polyubiquitination of Cx43 in GJs and AGJs, we performed Triton X-100 (TX-100) insolubility assays with endogenously Cx43 expressing pPAECs and exogenously Cx43 expressing MDCK cells (not expressing endogenous Cxs) (Dukes et al., 2011) to separate the TX-100 insoluble (GJs, AGJs) from the TX-100 soluble (Cxs, connexons) fractions based on a previously described method (Musil and Goodenough, 1991). Prior to lysis, cells were left untreated or treated with 20μM PR-619, a pan-DUB inhibitor, for 1.5 hours. TX-100 soluble and insoluble fractions were analyzed via western blot and probed for Cx43 (**Figure 8A**). Both TX-100 soluble and insoluble fractions contained a triplet of Cx43 corresponding to the fastest migrating form of Cx43 (P_0) and the slower-migrating phosphorylated forms of Cx43, commonly referred to as P_1 and P_2. TX-100 insoluble fractions contained an extra band migrating at about 70kDa that was more prominent when DUBs were inhibited, indicating a potential ubiquitinated form of Cx43 in

the GJ fraction (**Figure 8A, arrow)**. When TX-100 insoluble fractions were probed for monoUb/polyUb (FK2), polyUb (FK1), and K63-polyUb (HWA4C4), a typical ladder of ubiquitinated higher molecular weight proteins was observed along with a single band migrating at about 70kDa (**Figure 8B, arrows**). Since these 70kDa bands were more prominent in both the GJ and Ub blots when DUBs were blocked (**Figures 8A, B**), it suggests that blocking deubiquitination leads to accumulation of Cx43 ubiquitination at GJs. To provide further evidence that the 70kDa band indeed represents K63-polyubiquitinated Cx43, K63-polyubiquitinated proteins were immunoprecipitated from PAEC cell lysates using the K63-polyUb specific antibody (HWA4C4) and probed for Cx43 after western blot (**Figure 8C, right panel**). Again, a Cx43 band around 70kDa was prominent after immunoprecipitation, showing that Cx43 appears to be K63-polyubiquitinated **(Figure 8C, arrow)**. In addition to the ubiquitinated form of Cx43, the non-ubiquitinated P0, P1/P2 triplet (besides other undetermined bands) was observed after immunoprecipitation migrating at the typical ~40kDa molecular weight range, indicating that not all connexins within a connexon or a GJ are ubiquitinated. pPAEC cell lysates probed for K63-polyubiquitinated proteins and Cx43 were analyzed in control (**Figure 8C, left panel**).

To examine whether K63-polyubiquitination also occurs in GJs when Cx43 was expressed exogenously, MDCK cells were transfected transiently with wild type Cx43 cDNA, lysed and fractionated as described above for pPAECs 24 hours post transfection. The TX-100 insoluble pellet was then used for K63-polyUb and Cx43 immunoprecipitations. Bands corresponding to K63-polyubiquitinated Cx43 were detected in the GJ-containing pellet fraction when either Cx43 was precipitated and probed with K63-polyUb specific antibodies (**Figure 8D, left panel**) or K63-polyubiquitinated proteins were precipitated and probed

with Cx43 specific antibodies (**Figure 8D, right panel**). Protein A-Sepharose beads alone and immunoprecipitation reactions without antibodies analyzed in parallel did not precipitate any proteins. Along with our immuno-colocalization data, these results provide compelling evidence indicating that Cx43 in GJs and AGJs in both endogenously and exogenously Cx43 expressing cells is K63-polyubiquitinated.

3-3-2: Mutating a set of lysines in the Cx43 C-terminus leads to an accumulation of mutant Cxs and GJs in the plasma membrane

To determine whether ubiquitination of Cxs in GJs plays a role in GJ internalization and which lysines in Cx43 GJs become ubiquitinated, we transiently transfected HeLa cells with rat Cx43 constructs containing a set of C-terminal lysine (K) to arginine (R) mutations (called K/R) in order to block ubiquitination. We focused on mutating lysines in the C-terminus of Cx43 as this domain is highly post-translationally modified and is known to interact with numerous binding partners (Thévenin et al., 2013). Mutant 6K/R, generated via mutagenesis, contains six lysines within the C-terminus (K258, K264, K287, K303, K345, and K346) mutated to arginines (**Figure 9A,** boxed blue and green). Mutant 3K/R contains the central lysines K264, K287, and K303 mutated to arginines (**Figure 9A,** boxed green only). 24 hours post transfection, cells were fixed and immunostained using Cx43 antibodies. Both mutants trafficked to the plasma membrane and assembled into GJ plaques with equivalent efficiency compared to wild type, however cells expressing either 6K/R or 3K/R mutants had noticeably larger GJ plaques compared to wild type (**Figure 9B, left**). Total GJ length and number per cell pair was measured using ImageJ, and when averaged confirmed that both mutants had significantly larger and more GJ plaques in their PMs that

also was reflected by a significant increase in total pixel number. The average GJ size/number and corresponding fluorescence intensity per cell pair significantly increased approximately 1.5-2 fold in both mutants (6K/R and 3K/R increased to $8.60x10^6 \pm 0.65$ and $10.20x10^6 \pm 0.85$, respectively; n=3) when compared to wild type ($5.35x10^6 \pm 0.58$) (**Figure 9B, right**). Interestingly, the 6K/R and 3K/R mutants gave similar results, implying that the additional peripheral residues in the 6K/R mutant (K258, K345, and K346) do not become ubiquitinated. A comparable result was obtained when equal amounts of cell lysates were analyzed and probed for Cx43 protein content in Cx-deficient MDCK cells expressing wild type and mutant Cx43 (**Figure 9C**). MDCK cells were used here and in the following experiments due to higher transfection efficiency compared to HeLa cells. Quantification of 6K/R and 3K/R mutants resulted in 8.88 ± 2.70 and 7.71 ± 2.95 fold increases in total Cx43 polypeptide amounts compared to wild type, respectively (**Figure 9C**). Intriguingly, both mutant proteins accumulated as hyper-phosphorylated forms (compare the pronounced appearance of all three Cx forms, P0, P1 and P2 in the mutants that are barely visible in the wild type Cx43 under these exposure conditions), suggesting that ubiquitination of Cx43 in GJs is required for GJ internalization, blocking ubiquitination impairs GJ internalization, and ubiquitination is induced by Cx43 phosphorylation.

3-3-3: Cx43 K/R mutants exhibit significantly longer half-lives and accumulate as hyper-phosphorylated polypeptides

To further investigate the inhibitory effect of mutating lysine residues on GJ internalization, we analyzed the half-lives of K/R mutants expressed in Cx-deficient MDCK cells with pharmacological inhibition of protein translation. Furthermore, we generated all possible

double and single mutants of lysines 264, 287, and 303 (termed K264/303R, K264/287R, K287/303R, K264R, K287R, and K303R) to determine if one or more lysines become ubiquitinated and are necessary to inhibit GJ internalization. 24 hours after transient transfection protein translation was blocked by treatment with 50μg/ml of cycloheximide. Cells were lysed 0, 1, 2, 3, 4, and 6 hours post cycloheximide addition and total wild type and mutant Cx43 protein levels were analyzed using western blots (**Figure 10**). The half-life for wild type Cx43 was determined to be approximately 2.3 hours, correlating with the known, short 1-5 hours half-live described previously for Cx43 (Beardslee et al., 1998; Falk et al., 2009; Fallon and Goodenough, 1981) (**Figure 10A, top panel**). Unlike wild type, the protein levels of Cx43 in both 6K/R and 3K/R at 6 hours post cycloheximide treatment had only decreased to 75% of starting Cx43 levels (**Figure 10A, middle and bottom panels)**. Using a linear fit, the half-lives of 6K/R and 3K/R mutants were extrapolated as app. 15.7 and 13.1 hours, respectively, making the mutant half-lives 4-5 times longer than that of wild type Cx43. Again, 6K/R and 3K/R half-lives were not significantly different from one another, further suggesting that lysines 258, 345, and 346 are not targets for Cx43 ubiquitination in GJs, and play no direct role in GJ internalization. Expression of double-mutants (K287/303R, K264/303R, and K264/287R) resulted in a significantly increased half-live of all three mutants (app. 11.4, 6.6, and 7.7 hours, respectively), suggesting that at least two separate lysine residues become K63-polyubiquitinated (**Figure 10B**). Finally, expressing the single lysine residue mutants again resulted in extended protein half-lives for K264 and K303 mutants (4.5 hours for each), while the half-life of the K287 mutant corresponded to the protein half-life of wild type Cx43 (2.5 hours) (**Figure 10C**). As already shown in **Figure 10C**, relevant mutants (all except K287R) showed higher Cx43 protein

levels compared to wild type Cx43 and accumulated as hyper-phosphorylated variants (**compare Figure 9C with Figure 10**). Taken together these results identified two juxtaposed lysine residues in the Cx43 C-terminal domain (K264 and K303) that apparently become K63-polyubiquitinated in Cx43 in GJs to mediate GJ internalization.

3-3-4: Immunoprecipitation of Cx43 mutants confirms K63-polyubiquitination of K264 and K303 on Cx43 in GJs

To confirm that K264 and K303 are indeed the residues that become K63-polyubiquitinated in Cx43 in GJs, we again expressed Cx43 wild type and relevant double and single K/R mutants (K264/303R, K264R, K287R, and K303R) in MDCK cells. Cells were fractionated into soluble (Cx, connexon) and pellet fractions (GJ, AGJ) using TX-100 extraction. Then, wild type and mutant Cx43 polypeptides were immunoprecipitated from the pellet and probed for the presence of K63-polyUb modification with K63-polyUb-specific (HWA4C4) antibodies (**Figure 11**). Resulting Western blots showed that wild type as well as Cx43 K287R mutant were K63-polyubiquitinated as indicated by the higher molecular weight band pattern (marked with asterisks in **Figure 11B**) (consistent with previously detected Cx43 patterns shown in **Figure 8**), while K264 and K303 double and single mutants were not. Control lanes consisting of lysate plus Protein A-Sepharose beads, beads alone, or beads plus Cx43 antibody also did not precipitate any K63-polyubiquitinated proteins. Amounts of total Cx43 and of K63-polyUb proteins (with α-tubulin as a loading control) present as starting material in the cell lysates were analyzed in control (**Figure 11A**). Taken together, these analyses confirm K63-polyubiquitination of K264 and K303 of Cx43 in

GJ/AGJs. Our findings correlate with recent mass spectrometry evidence suggesting ubiquitination on Cx43 lysines 9 and 303 (Wagner et al., 2011).

3-3-5: Preventing ubiquitination on K264 and K303 leads to hyper-phosphorylation of serines 368, 279/282, and 255

As Cx43 ubiquitination-deficient lysine mutants accumulate as hyper-phosphorylated polypeptides, phosphorylation at specific sites in Cx43 appears to precede Cx43 ubiquitination in GJs. Phosphorylation of degrons allowing subsequent protein ubiquitination is a well-known signaling event that regulates protein degradation (Nguyen et al., 2013; Ravid and Hochstrasser, 2008). To further investigate which residues in Cx43 GJs are phosphorylated preceding ubiquitination, we analyzed and quantified phosphorylation levels of S368, S279/S282, S255, and S262, specific Cx43 phosphorylation events known to down-regulate GJ mediated cell-to-cell communication (Thévenin et al., 2013). MDCK cells were lysed 24 hours post transfection with wild type or K/R mutant constructs, and analyzed by western blots using Cx43 phospho-specific antibodies (**Figure 12**). Amounts of phosphorylated S368 (pS368), S279/S282 (pS279/pS282), and S255 (pS255) Cx43 polypeptides were significantly increased in all double and single K/R mutants with the exception of the non-ubiquitinated K287R mutant. Increased phosphorylation of S262 (pS262) was observed only in a few mutants (some mutants containing 287, and/or 303 K/R exchanges), suggesting that mutating K287 may cause additional GJ internalization-impairing side effects (see discussion). Taken together, these results show that phosphorylation of S368, S279/S282, and S255 is significantly increased in all Cx43 mutants that harbor lysine to arginine exchanges on K264 and/or K303, strongly suggesting that phosphorylation on

one, or a combination of these serine residues, induces K63-polyubiquitination of Cx43 in GJs.

3-4: Discussion

Ubiquitination is a post-translational modification that plays an important role in regulating protein degradation. Particularly, the additions of K48-linked and K63-linked polyubiquitin chains are well-known signals for targeting proteins and protein-complexes to either proteasomal or endo-/phago-lysosomal degradation (Komander and Rape, 2012). Here, we report for the first time the identification of two juxtaposed lysine residues in the C-terminus of Cx43, K264 and K303, which become K63-polyubiquitinated in GJs and internalized AGJs, and that K63-polyubiquitination is required for efficient GJ internalization. Our additional discovery that Cx43 ubiquitin-deficient lysine mutants accumulated as hyper-phosphorylated proteins in the plasma membrane further indicates that Cx43 K63-polyubiquitination is triggered by Cx43 phosphorylation. Use of phospho-specific Cx43 antibodies showed that hyper-phosphorylation occurred on S368, S279/S282, and S255, well characterized PKC and MAPK targets known to down-regulate GJIC (Fong et al., 2014; Kanemitsu et al., 1998; Lampe et al., 1998; Lampe et al., 2000; Leykauf et al., 2003; Nimlamool et al., 2015; Petrich et al., 2002; Polontchouk et al., 2002; Saez et al., 1997; Sirnes et al., 2009; Solan and Lampe, 2007; Solan and Lampe, 2008).

K63-polyubiquitinated Cx43 migrated as a band of approximately 70 KDa molecular weight and as higher molecular weight complexes on SDS-PAGE gels (**Figures 8, 11**). Although ubiquitinated proteins are known to not necessarily migrate on SDS-PAGE gels according to their molecular weight (Hospenthal et al., 2015), polyubiquitin chains in general are believed

to consist of at least 4 Ubs (Ravid and Hochstrasser, 2008). Four Ubs plus the mol. weight of Cx43 add up to 77 kDa, which is relatively close to the migration pattern of the prominently detected band, suggesting that the K63-linked polyubiquitin chain added to Cx43 conforms to established criteria.

Ubiquitination of Cx43 in order to facilitate its degradation has been known for some time (Girao et al., 2009; Laing and Beyer, 1995; Laing et al., 1997; Leithe and Rivedal, 2004b). Originally, ubiquitination of Cx43 was discovered as a signal for proteasomal degradation (Laing and Beyer, 1995), thus likely regulating the degradation of mis-folded or aberrantly oligomerized Cx43 polypeptides shortly after their biosynthesis in the ER. The later discovery that ubiquitination may also play a role in the lysosomal degradation of Cx43 (Laing et al., 1997) suggested that ubiquitination may also play a role in the degradation of oligomerized Cx43 complexes (connexons, GJ channels, and GJ plaques). However, the type of ubiquitination, the lysine residues that become ubiquitinated, and the signals that may induce Cx43 ubiquitination in GJs to aid in their internalization and degradation remained elusive. It is now well known that the type of Ub linkage conveys Ub signal specificity (Ikeda et al., 2010; Komander and Rape, 2012; MacGurn et al., 2012). Specifically, exposure of a hydrophobic patch on Ub and flexibility of the Ub C-terminus rely on the type of polyUb chain conformation (e.g. K48-linked versus K63-linked polyUb chains) and thus determine Ub signal specificity (Ye et al., 2012). Different E3 ligases add Ubs in a chain specific manner and thus determine the fate of the target protein by different cellular degradation machineries. Nedd4-1, Wwp1, and Smurf2 (all three HECT ligases) as well as Trim21 (a RING ligase) have been described to interact with Cx43 (Basheer et al., 2015; Chen et al., 2012; Fykerud et al., 2012; Leykauf et al., 2006). Of these, Nedd4-1 has been

found to preferentially interact with K63-polyubiquitinated proteins (Kim and Huibregtse, 2009) and to interact with ^{283}PPGY^{286} in Cx43 via its WW2 domain (Leykauf et al., 2006) (see **Figure 13A**) suggesting that this E3 ligase induces K63-polyubiquitination on Cx43 to aid in GJ internalization and degradation. Indeed, phosphorylation of S279/S282 *in vitro* was recently found to increase the binding affinity of this E3 ligase for its Cx43 binding domain (Spagnol et al., 2016), further supporting the concept that Nedd4-1 K63-polyubiquitinates Cx43 in GJs to mediate their internalization and degradation.

Cx43 domains known to be exposed to the cytoplasm when oligomerized into GJ channels (intracellular/I-loop and C-terminal [CT] domain) contain a large number of lysine residues (11 in the I-loop, 9 in the CT) that are all potential targets for ubiquitination (see **Figure 9A**). However, as all regulatory phosphorylation events occur in the Cx43-CT, we hypothesized that ubiquitination - if occurring in GJs – would occur in the CT as well. In addition, the C-terminal region juxtaposed to the fourth transmembrane domain is known to harbor a microtubule binding site (Giepmans et al., 2001) and when mutated will likely interfere with Cx trafficking (Wayakanon et al., 2012). We thus left the lysines located within this region (K234, K237, and K241) untouched as well. Both mutants, with the 6 remaining C-terminal lysines mutated (6K/R), and the mutant with the 3 central lysines mutated (3K/R; K264, K287, K303) behaved indistinguishable, resulting in significantly increased mutant protein half-lives, the accumulation of hyper-phosphorylated Cx43 polypeptides, and larger GJ plaques in the plasma membrane (**Figures 9, 10**), suggesting that one or more of these 3 lysine residues become K63-polyubiquitinated. Generating and examining all possible double and single mutants then indicated that two of the three lysine residues, K264 and K303, can become K63-polyubiquitinated. The result of Cx43 protein half-lives of

K264 and K303 single mutants were not as long as protein half-lives of the double lysine mutants (4.5 hours compared to 6.6 -11.4 hours, see **Figure 10C, Table**) argues that ubiquitination of only one lysine is not sufficient for normal GJ turnover (2.3 hours). Indeed, K63-polyubiquitination of K264 and K303 single mutants was not detected using K63-polyUb specific antibodies (**Figure 11**), suggesting that mutating one lysine residue may inhibit K63-polyubiquitination of Cx43 in GJs altogether.

Phosphorylation of Cx43 by various kinases (PKC, CDC2, MAPKs, and Src) is well known to down-regulate GJIC by closing GJ channels (Fong et al., 2014; Kanemitsu et al., 1998; Lampe et al., 1998; Lampe et al., 2000; Leykauf et al., 2003; Nimlamool et al., 2015; Petrich et al., 2002; Polontchouk et al., 2002; Saez et al., 1997; Sirnes et al., 2009; Solan and Lampe, 2007; Solan and Lampe, 2008) however a link between phosphorylation and ubiquitination has not been demonstrated. More recent findings from our lab also provided evidence for a link between Cx43 phosphorylation and GJ internalization. Specifically, phosphorylation of serines 368, 279/282, 255 (and in some cases 262) by PKC and MAPKs in response to epidermal- (EGF) and vascular endothelial growth factor (VEGF) stimulation has been linked to acute GJ internalization in mouse embryonic stem cells and pPAECs (Fong et al., 2014; Nimlamool et al., 2015). Additionally, S279/S282 phosphorylation-deficient Cx43 mutants were shown to stabilize GJ plaques in human pancreatic tumor cells (Johnson et al., 2013), and upon EGF stimulation in HeLa cells (Schmitt et al., 2014). Recently, Solan and Lampe (Solan and Lampe, 2015) and we (Falk et al., 2016) suggested a kinase program consisting of PKC, MAPKs, and Src that hierarchically phosphorylate Cx43 on serines 368, 279/282 (and potentially 255 and 262), and tyrosine 247, respectively to spatiotemporally regulate GJ internalization. Of these events, phosphorylation of S368 is of particular interest

as it has been found to preferentially localize to the center of GJ plaques (Cone et al., 2014), the region where GJ channel internalization occurs (Falk et al., 2009; Gaietta et al., 2002; Lauf et al., 2002). Furthermore, S368 phosphorylation is known to be dependent on S365 dephosphorylation (termed "gate keeper"), an event known to trigger a large conformational change of the Cx43-CT that affects the upstream region that harbors the above described ubiquitination sites (Solan and Lampe, 2007). Phosphorylation occurring at specific sites in a protein resulting in conformational changes allowing subsequent ubiquitination is a well-established signaling event known to regulate protein degradation (termed a phosphodegron) (Ravid and Hochstrasser, 2008; Wayakanon et al., 2012). As our data show that phosphorylation of S368, S279/S282, and S255 occur prior to ubiquitination, and preventing ubiquitination on relevant lysine residues results in the build-up of hyper-phosphorylated mutant Cx43 protein and GJs in the plasma membrane, our findings suggest that similarly, phosphorylation of Cx43 induces K63-polyubiquitination that then triggers GJ internalization and degradation. At this time, we do not know if all three phosphorylation events are required. However as S365 de-phosphorylation/S368 phosphorylation is linked to conformational Cx43-CT rearrangements (Solan and Lampe, 2007) and S279/S282 phosphorylation is linked to enhancing Nedd4-1 binding (Spagnol et al., 2016), phosphorylation on at least these two sites appear to be linked directly to Cx43 K63-polyubiquitination.

How might K63-polyubiquitination of Cx43 induce GJ internalization and degradation? Previously, we and others have demonstrated that Cx43 in GJs and internalized GJs interacts with a protein, p62/SQSTM1, which sequesters AGJs to autophago-lysosomal degradation (Bejarano et al., 2012; Fong et al., 2012; Lichtenstein et al., 2010). p62/SQSTM1 interacts

specifically with K63-polyubiquitinated substrates via its UBA (ubiquitin associated) domain (Seibenhener et al., 2004) to sequester targets for autophagosomal degradation (Bjorkoy et al., 2005; Pankiv et al., 2007). Depleting cells of autophagy-associated proteins such as LC3, Beclin1, Atg5/Atg12, and p62/SQSTM1 by RNAi significantly impaired GJ degradation (Bejarano et al., 2012; Fong et al., 2012; Lichtenstein et al., 2010). Thus, it is likely that Cx43 K63-polyubiquitination sequesters internalized GJs for degradation. In addition, as described above, we found that Cx43 binds AP-2 at two sites in its C-terminal domain to recruit clathrin and internalize GJs (Fong et al., 2013; Piehl et al., 2007). However, clathrin may also be recruited by another group of adaptor proteins, termed clathrin associated sorting proteins, or CLASPs that specifically bind via a Ub-interacting motif (UIM) to a polypeptide sequence that is exposed in K63-polyubiquitin chains (Traub and Bonifacino, 2013). One such alternative adaptor, called Eps15, was found to bind to Cx43 and has been proposed to facilitate GJ internalization of GJs (Catarino et al., 2011; Girao et al., 2009). Thus, K63-polyubiquitination may also allow Eps15 to bind to Cx43, recruit clathrin and internalize GJs as an alternative to AP-2 (Falk et al., 2016; Falk et al., 2014).

Taken together, our findings show that the two post-translational modifications (phosphorylation succeeded by K63-polyubiquitination) need to occur on Cx43 polypeptides in GJs to successfully internalize and turn over GJ channels and plaques; a combination of amino acid modifications that is typical for phosphodegron-mediated protein degradation mechanisms (Nguyen et al., 2013; Ravid and Hochstrasser, 2008). Blocking K63-polyubiquitination prevents efficient GJ internalization and leads to the accumulation of hyper-phosphorylated Cx43 and GJs in the plasma membrane (schemed in **Figure 13B**). As E3-Ub specific small molecule inhibitors are available, their administration may prevent the

loss of GJs from intercalated discs as is typical for a number of acute heart diseases (Fontes et al., 2012).

3-5: Materials and Methods

3-5-1: cDNA constructs and mutagenesis

Full-length wild type rat Cx43 cDNA was cloned into pEGFP-N1 vector (Clontech) as previously described (Falk, 2000). To generate full length untagged wild type Cx43, an authentic TAA stop codon was re-introduced (Fong et al., 2013). Untagged rat Cx43 mutant 3K/R and 9K/R cloned into pcDNA3.1 were generously provided by Vivian Su and Alan Lau (Natural Products and Cancer Biology Program, Cancer Research Center of Hawaii, Honolulu, HI 96813) (Su et al., 2010). Untagged 6K/R Cx43 mutant was generated by restoring K234, K237, and K241 from 9K/R by site-directed mutagenesis and adding a BglII restriction site for confirmation of mutagenesis. Double and single K/R mutants were generated by restoring one or two of the remaining lysines 264, 287, and 303 from the Cx43 mutant 3K/R using Quick Change Mutagenesis method (Stratagene, Santa Clara, CA). Forward recovery mutagenesis primers were as follows (arginine recovered codons are underlined, BglII site in bold): **R234_237_241K recovery**, 5'-G CTC TTC TAC GTC TTC TTC AAA GGC GTT AAG GAT CGC GTG AAG GGA **AGA TCT** GAT CC-3'; **R264K recovery**, 5'-CCA TCA AAA GAC TGC GGA TCT CCA AAA TAC GCC TAC TTC AAT GGC-3'; **R287K recovery**, 5'-CCT ATG TCT CCT CCT GGG TAC AAG CTG GTT ACT GGT GAC AGA AAC AAT TCC-3'; and **R303K recovery**, 5'-CC TCG TGC CGC AAT TAC AAC AAG CAA GCT AGC GAG CAA AAC TGG-3'. PCR mutagenesis reactions were generated using proofreading *Pfu* Ultra II polymerase (Cat. No. 600670-51;

Stratagene). Digestion of template cDNA with *Dpn*1 restriction endonuclease (Cat. No. R0176-S; New England Biolabs, Ipswich, MA) was followed by transformation into chemically competent DH5α *E. coli* cells (Cat. No. 18258-012; Invitrogen). Sequencing was performed on all cDNA constructs to confirm the presence of intended mutations.

3-5-2: Cell culture and transient transfections

HeLa (gap junction deficient; Cat. No. CCL-2; American Type Culture Collection, Manassas, VA), Madine-Darby Canine Kidney (MDCK) (gap junction deficient; Cat. No. NBL-2; American Type Culture Collection), and primary porcine Pulmonary Artery Endothelial Cells (pPAECs) (expressing Cx43 and other Cxs, gap junction proficient; Cat. No. P302; Cell Applications, San Diego, CA) were maintained in low glucose Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. SH30021.01; HyClone, Logan, UT) supplemented with 50 I.U/ml penicillin and 50μg/ml streptomycin (Cat. No.30-001-Cl; Corning, Manassas, VA), 2mM L-glutamine (Cat. No. 25-005-C1; Mediatech, Manassas, VA), and 10% Fetal Bovine Serum (Cat. No. S11150; Atlanta Biologicals, Flowery Branch, GA) at 37˚C, 5% CO_2, and 100% humidity. Cells were washed with 1x PBS and trypsinized with 0.25% trypsin with 2.21mM EDTA (Cat. No. 25-053-Cl; Corning) for passage. 24-48 hours after passing, 60-80% confluent HeLa and MDCK cells were transiently transfected with wild type and mutant constructs using Superfect (Cat. No. 301307; Qiagen, Hilden, Germany) or Lipofectamine2000 (Cat. No. 11668019; Invitrogen, Carlsbad, CA) reagents, respectively, according to manufacturer's recommendations.

3-5-3: Immunofluorescence staining and image analyses

pPAECs and HeLa cells were grown on pretreated poly-L-Lysine (Cat. No. P8920; Sigma, St. Louis, MO) coated glass coverslips in low glucose DMEM at 37°C, 5% CO_2, and 100% humidity. Cells were fixed in 3.7% formaldehyde/PBS and permeabilized with 0.2% Triton X-100 (TX-100) (Cat. No. 3929-2; VWR, Radnor, PA) in PBS. Cells were blocked in 10% FBS/PBS for 30 minutes at room temperature (RT) and incubated with primary rabbit polyclonal anti-Cx43 antibodies (Cat. No. 3512; Cell Signaling, Danvers, MA) diluted 1:500 in 10% FBS/PBS at 4°C overnight. Additionally, pPAECs were incubated with primary mouse monoclonal anti-monoUb and polyUb (FK2) (Cat. No. PW8810; Enzo, Farmingdale, NY), polyUb (FK1) (Cat. No. PW8805; Enzo), or K63-polyUb (clone HWA4C4) (Cat. No. PW0600; Enzo) antibodies diluted 1:200 in 10% FBS/PBS at 4°C overnight. Cells were incubated in secondary antibodies (goat anti-rabbit Alexa Fluor488 and goat anti-mouse Alexa Fluor568 (Cat. No. A11008 and A11031, respectively; Molecular Probes/Invitrogen, Eugene, OR) diluted 1:200 or 1:500 in 10% FBS/PBS, respectively, for 1 hour at RT. Cells were also stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Cat. No. D1306; Molecular Probes). Coverslips were rinsed in distilled water and mounted using Fluoromount-G (Cat. No. 0100-01; Southern Biotechnology, Birmingham, AL). pPAEC and HeLa cells were imaged using a Nikon Eclipse TE2000E wide-field inverted fluorescence microscope equipped with 40x NA 1.3 Plan Fluor and 60x NA 1.4 Plan-Apochromat oil-immersion objectives (Nikon Instruments, Melville, NY) and a forced-air cooled Photonics CoolSnap® HQ CCD camera (Roper Scientific, Duluth, GA). Images were acquired using MetaVue software version 6.1r5 (Molecular Devices, Sunnyvale, CA). To quantify GJ plaque size/number, GJ plaques were outlined using the freeform tool in ImageJ version 1.43u (National Institutes of Health, Bethesda, MD) and total pixel

number/area was quantified for 73, 98 and 83 cell pairs, expressing Cx43 wild type, 6K/R, and 3K/R, respectively.

3-5-4: Triton X-100 solubility assays

Prior to lysis, pPAECs were treated with 20μM of the pan-DUB inhibitor, PR-619, (Cat. No. 662141; EMD Millipore, Billerica, MA) for 1.5 hours and cultured at 37°C, 5% CO_2 and 100% humidity. To separate Cx proteins and connexons from plasma membrane GJs, TX-100 solubility assays were performed based on a method described by Musil and Goodenough (Musil and Goodenough, 1991). MDCK cells transiently transfected with wild type or Cx43 K/R constructs or pPAECs were used. 24 hours post transfection (MDCKs), or 48 hours after seeding (pPAECs), cells were briefly washed in ice cold PBS. Cells were lysed on ice for 15 min in 400μL pre-chilled lysis buffer containing 50mM Tris-HCl, pH7.4 (Cat. No. BP 153-1; Fisher Bioreagents, Fair Lawn, NJ), 150mM NaCl (Cat. No. S671-3; Fisher Chemicals, Fair Lawn, NJ), 1mM EDTA (Cat. No. 161-0729; Biorad, Hercules, CA), 0.5% sodium deoxycholate (Cat. No. D6750; Sigma), 1% Triton X-100 (Cat. No. VW3929-2; VWR), and 1% Igepal (Cat. No.18896; Sigma). The buffer was supplemented with the following protease and phosphatase inhibitors: 1mM protease inhibitor cocktail (Cat. No. P8340; Sigma), 1mM β-glycerol phosphate (Cat No. 157241; MP Biomedicals, LLC, Solon, OH), 1mM sodium orthovanadate (Cat. No. 450234; Sigma), 20mM N-ethylmalemide (Cat. No. E1271; Sigma), and 10mM 1,10-phenanthroline monohydrate (Cat. No. AC130130050; ACROS Organics, NJ). Lysates were transferred to Eppendorf® tubes and centrifuged at 10,000xg for 5 min at RT to pre-clear cell lysates. 180μL of each resulting supernatant was centrifuged at 100,000xg in a Beckman Coulter Airfuge® ultracentrifuge for 10 minutes.

For western blot analysis, 100,000xg supernatants (TX-100 soluble fractions) were mixed with 4x SDS sample buffer and pellets (TX-100 insoluble fractions) were resuspended in 2x SDS sample buffer and boiled for 5 minutes.

3-5-5: SDS-PAGE and Western blot analyses

Immunoprecipitation, protein half-life, and phosphorylation analyses samples were loaded onto 10% SDS-PAGE minigels (Biorad). Proteins were transferred onto nitrocellulose membranes and blocked for 1 hour at RT in 5% non-fat dry milk/TBST or 5% bovine serum albumin (BSA) (Cat. No. A7906; Sigma)/TBST. Antibodies were diluted in 5% BSA/TBS as follows: rabbit anti-Cx43, rabbit anti-K63-polyUb (Cat. No. 05-1308; EMD Millipore), rabbit anti-Cx43 pS279/pS282, rabbit anti-Cx43 pS255, rabbit anti-Cx43 pS262 (Cat. No. sc-12900-R, sc-12899-R and sc-17219-R, respectively; Santa Cruz, Dallas, TX) and rabbit anti-Cx43 pS368 (Cat. No. 3511S; Cell Signaling) at 1:2000, and mouse anti-α-tubulin primary antibodies at 1:5000. Blots were incubated with primary antibodies at 4°C overnight, then washed three times with TBST. Secondary HRP-conjugated goat anti-rabbit or goat anti-mouse antibodies (Cat. No. 81-6520 or 81-6120, respectively; Zymed, San Francisco, CA) were diluted 1:5000 and secondary HRP-conjugated mouse anti-rabbit light chain specific antibodies (Cat. No. 211-032-171; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were diluted 1:15,000. Blots were incubated in secondary antibodies for 1 hour at RT. Protein bands were detected using Pierce® ECL2 Western Blotting Substrate (Cat. No. 80196; Thermo Scientific, Rockford, IL) and KODAK® BioMax® Light Autoradiography Film (Cat. No. 1788207; Carestream Health, Rochester, NY). Cx43

protein amounts were quantified using NIH ImageJ and normalized to the corresponding α-tubulin intensities.

3-5-6: Cx43 protein half-life analyses

24 hours post transfection, MDCK cells were treated with 50μg/ml cycloheximide (Cat. No. C655; Sigma) for 0, 1, 2, 3, 4, and 6 hours at 37°C, 5% CO_2 and 100% humidity. Cells were lysed in 1x SDS sample buffer at each time point and boiled for 5 minutes. Samples were separated on 10% SDS-PAGE gels using western blot protocols described below. Blots were probed with rabbit anti-Cx43 antibody, then stripped with stripping buffer and stringently washed in TBS/0.1% Tween20 (Cat. No. BP337; Fisher Bioreagents) (TBST) before re-probing with mouse anti-α tubulin antibodies (Cat. No. 9026; Sigma). Cx43 protein intensities were quantified using NIH ImageJ and normalized to the corresponding α-tubulin intensities.

3-5-7: Immunoprecipitations

For immunoprecipitation in PAECs, cells were washed in ice cold PBS and lysed in fresh lysis buffer without TX-100 for 15 minutes on ice. Cell lysates were transferred to Eppendorf® tubes and centrifuged at 10,000xg for 5 min at RT to pre-clear cell lysates. For immunoprecipitation in MDCK cells after TX-100 solubility assay, 100,000xg pellets (TX-100 insoluble fraction) from MDCK cells transiently transfected with Cx43 wild type or K/R mutants were resuspended in fresh lysis buffer without TX-100 and sonicated 6 times (1 second/pulse), with 5 minute intervals on ice in between pulses. Immunoprecipitation samples were incubated with rabbit anti-Cx43 or mouse anti-K63-polyUb antibodies

adsorbed to protein A-Sepharose beads (Cat. No. 3391; Sigma) for 2 hours at RT while rotating. After incubation, beads were washed in lysis buffer and immunoprecipitated protein was eluted from beads with 2x SDS sample buffer and boiled for 5 minutes.

3-5-8: Statistical analyses

One-way ANOVA analyses with Post-Hoc Bonferroni corrections were performed on data sets including GJ plaque size measurements (**Figure 12B**), Cx43 wild type and mutant protein levels (**Figure 12C**), and amounts of phosphorylated Cx43 in K/R mutants (**Figure 15**) in SPSS software. Unpaired student's t-tests were performed to analyze Cx43 protein half-lives (**Figure 13**) in Excel. All data are presented as mean ±SEM. In all analyses, p-values of $*p<0.05$, $**p<0.005$, and $***p\leq0.0005$ were considered statistically significant.

Figures

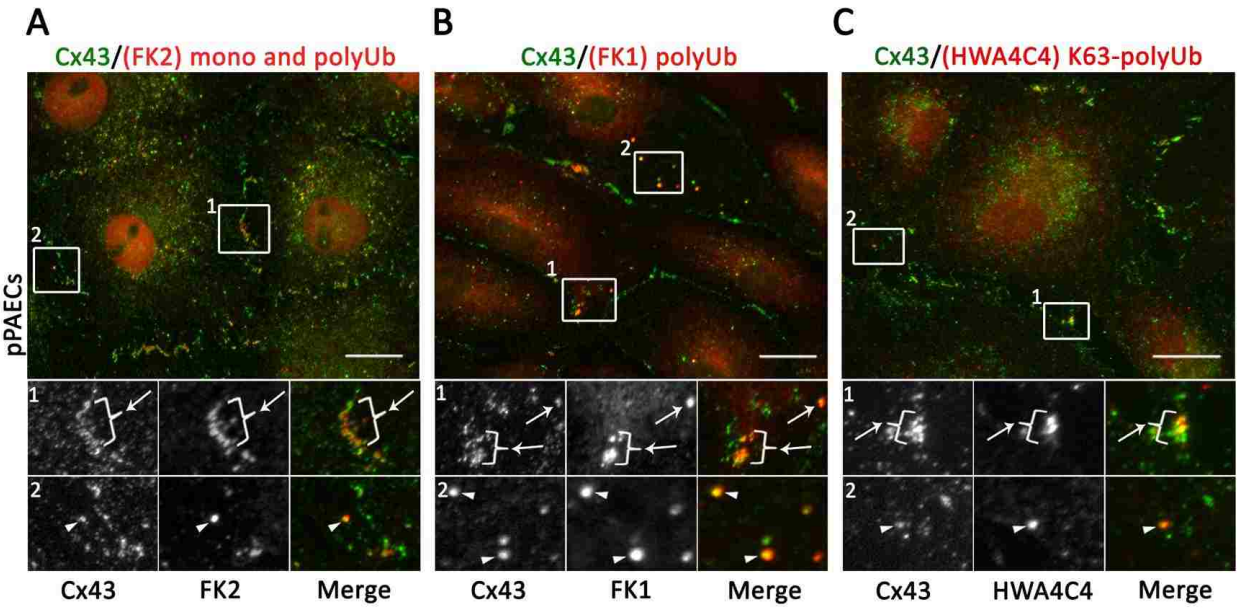


Figure 7: Cx43 is K63-polyubiquitinated in GJs and AGJs. Endogenously Cx43-expressing primary pulmonary artery endothelial cells (pPAECs) were immunostained with antibodies toward Cx43 (green) and ubiquitin specific antibodies (red). Below each panel, magnified insets highlight GJs (in brackets and arrows) and AGJs (arrowheads). (A) Cx43 antibodies robustly colocalized with monoUb and polyUb antibodies (FK2) at GJs and AGJs, suggesting that Cx43 ubiquitination may regulate GJ internalization. (B) The polyUb antibody (FK1) and Cx43 antibody also colocalized at GJs and AGJs, providing evidence that polyubiquitination plays a role in GJ internalization. (C) Cx43 antibodies also colocalized with K63-polyUb antibodies (HWA4C4) at GJs and AGJs, suggesting that K63-polyubiquitination of Cx43 regulates GJ internalization and degradation. Scale bar = 20μm.

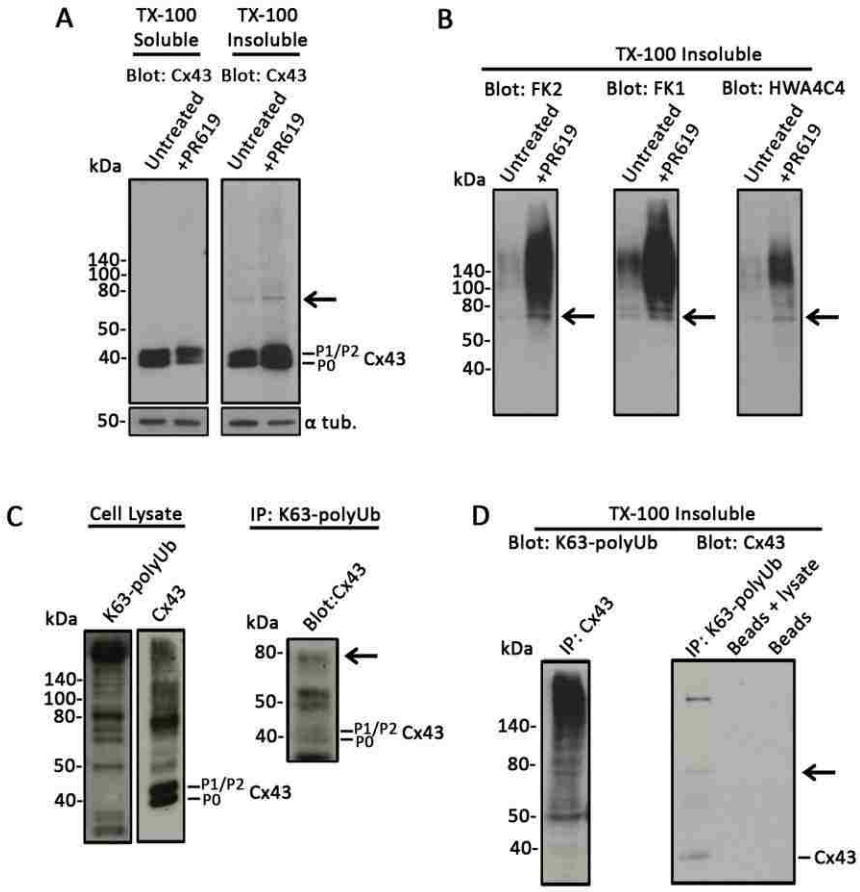


Figure 8: Cx43 is K63-polyubiquitinated in TX-100 insoluble fractions. (A) Endogenously Cx43-expressing pPAECs were untreated or treated with 20μM DUB inhibitor, PR-619, for 1.5 hours prior to lysis. Cells were lysed in buffer containing TX-100 and separated into TX-100 insoluble (GJ/AGJ) and soluble (Cx, connexons) fractions by ultracentrifugation. Fractions were analyzed via western blot and probed using Cx43, mono/polyUb (FK2), polyUb (FK1), and K63-polyUb (HWA4C4) antibodies. A band suggestive of ubiquitinated Cx43 was evident at about 70kDa in the TX-100 insoluble fraction (arrow). (B) TX-100 insoluble fractions probed with mono/polyUb (FK2), polyUb (FK1), and K63-polyUb (HWA4C4) antibodies also show the band around 70kDa, indicative of Cx43 ubiquitination (arrows). Ubiquitination (70kDa and higher molecular weight) was more pronounced in DUB inhibited fractions. (C) Immunoprecipitation of K63-polyubiquitinated proteins from pPAEC lysates shows that Cx43 is among the K63-polyubiquitinated proteins. The band around 70kDa corresponds with the TX-100 pattern observed upon blockage of DUBs (arrow). Note that non-ubiquitinated Cx43 is also immunoprecipitated, indicating that not all connexins within a GJ/channel are ubiquitinated. (D) Immunoprecipitations of Cx43 and K63-polyubiquitinated proteins from TX-100 insoluble fractions derived from MDCK cells transiently expressing Cx43 were probed for K63-polyUb and Cx43, respectively. Resulting blots show the 70 KDa band (arrow), as well as higher molecular weight bands of K63-polyubiquitinated Cx43 along with unmodified Cx43.

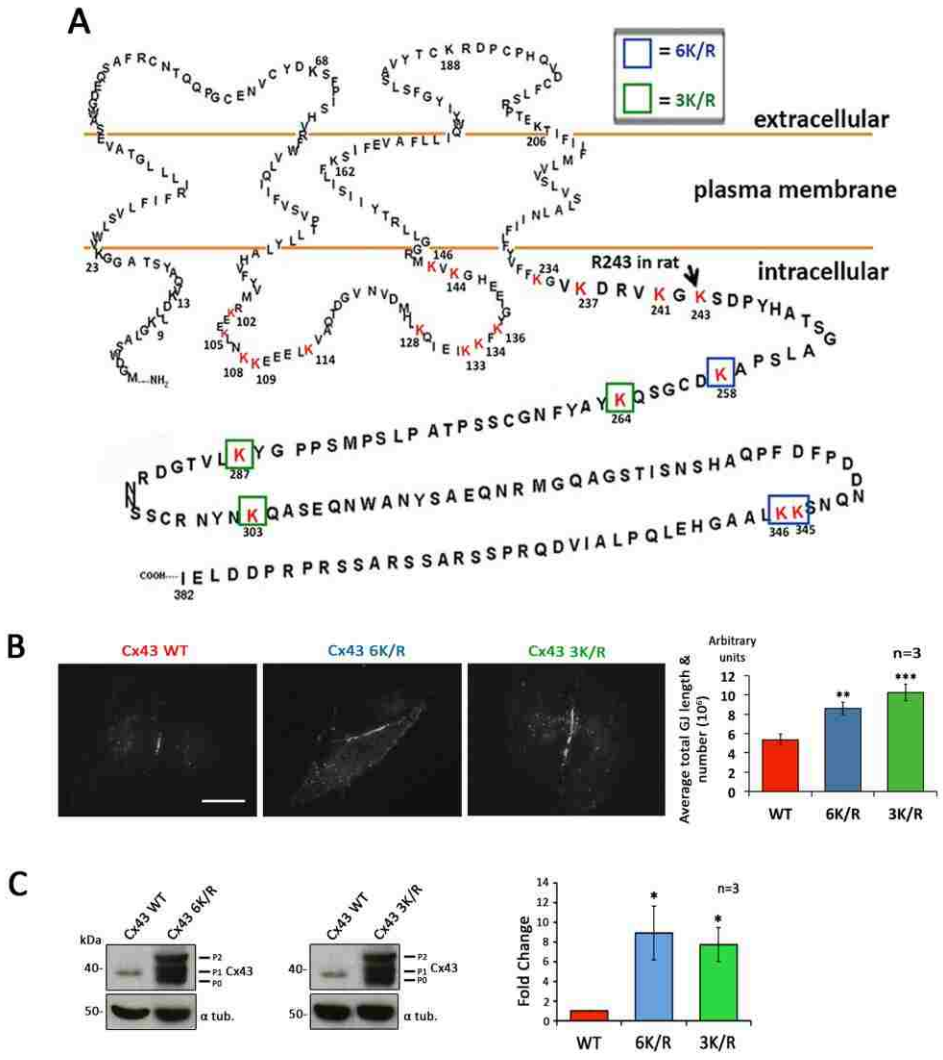
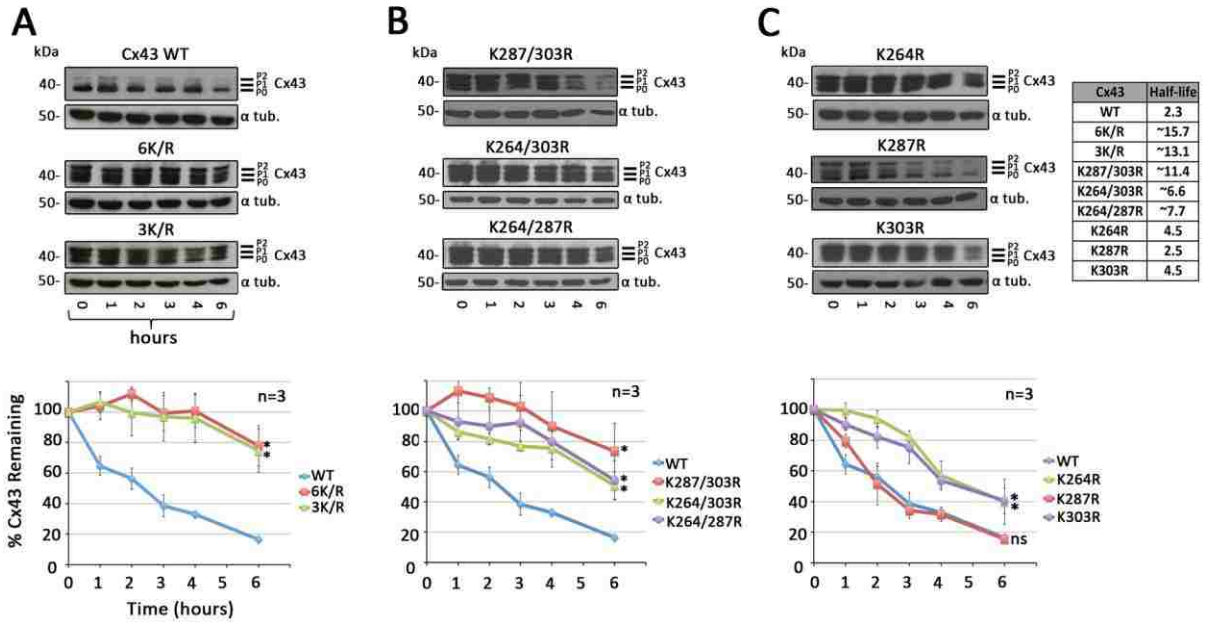


Figure 9: Cx43 mutants with a set of lysine residues mutated to arginines accumulate as hyper-phosphorylated Cxs and GJs in the PM. **(**A) Amino acid sequence of human Cx43 with lysines (K) in the intracellular loop and the C-terminus (potentially accessible to ubiquitination in GJs) highlighted in red. Lysines mutated to arginines (R) in the C-terminus of Cx43 are boxed in blue and green for mutant 6K/R and green only for mutant 3K/R. (B) Cx-deficient HeLa cells were transfected with wild type or K/R constructs and immunostained with rabbit anti-Cx43 primary and Alexa488 conjugated secondary antibodies. GJs were outlined and corresponding pixel counts were used to determine GJ length and number. Both K/R mutants show significantly larger and more GJs compared to wild type. p-values ≤0.05 were considered significant. Scale bar = 20µm. (C) Total Cx43 protein levels of wild type, 6K/R and 3K/R mutants exogenously expressed in MDCK cells were analyzed by western blot. Phosphorylation of Cx43 leads to multiple electrophoretic forms, commonly known as P_0, P_1 and P_2. In both mutants app. 8 times the number of Cx43 protein accumulated including a large amount of hyper-phosphorylated (P_1, P_2) variants compared to wild type.



Cx43	Half-life
WT	2.3
6K/R	~15.7
3K/R	~13.1
K287/303R	~11.4
K264/303R	~6.6
K264/287R	~7.7
K264R	4.5
K287R	2.5
K303R	4.5

Figure 10: Cx43 K/R mutants with critical lysine residues mutated to arginines have significantly extended protein half-lives. MDCK cells transfected with Cx43 wild type or mutant constructs were treated with 50µg/ml cycloheximide for 0, 1, 2, 3, 4, or 6 hours followed by cell lysis and western blot analyses. (A) Quantification of Cx43 protein levels reveal a half-life of Cx43 wild type of 2.3 hours, with app. 20% of starting Cx43 protein remaining after 6 hours of cycloheximide treatment. Mutants 6K/R and 3K/R had significantly increased half-lives extrapolated to 15.7 and 13.1 hours, respectively. (B) Double K/R mutants all had significantly extended half-lives. (C) Mutating 264 and 303 -together or independent of one another- also resulted in increased half-lives, whereas the half-live of the K287R mutant was unaffected (2.5 hours). α-tubulin was probed as a loading control. Extrapolated half-lives were calculated using a linear fit curve.

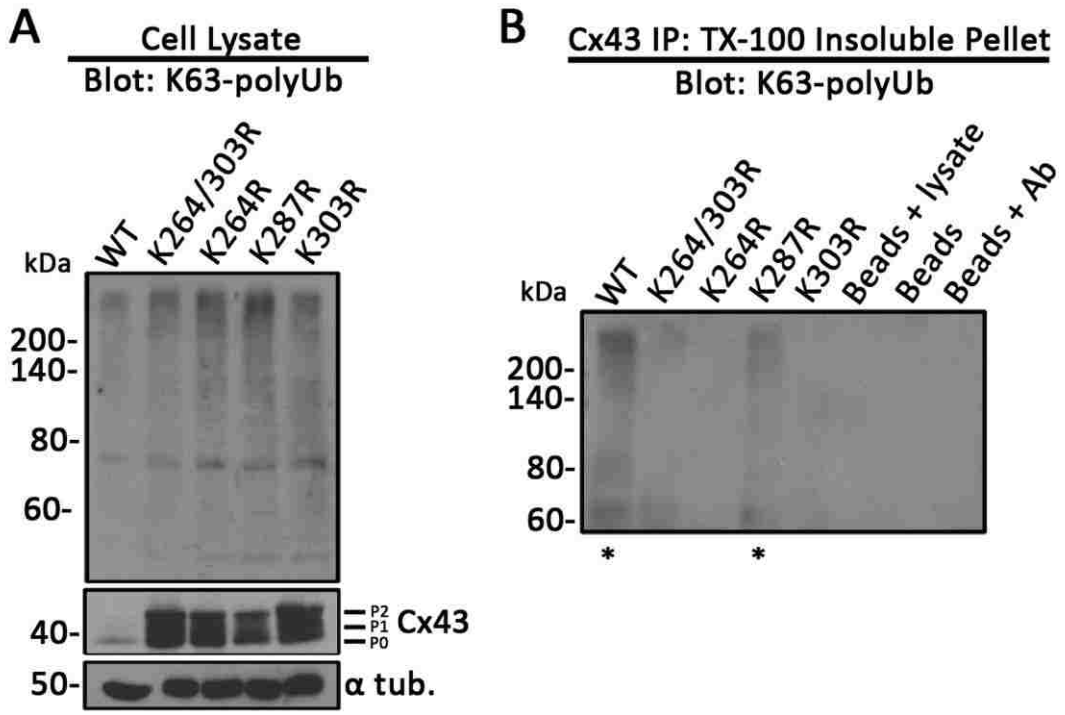


Figure 11: K264R and K303R mutants no longer get K63-polyubiquitinated. Cx43 antibodies were used to immunoprecipitate Cx43 from MDCK cells transfected with wild type, K264R, K287R, K303R and K264/303R constructs. Cells were lysed in TX-100 containing buffer and separated into soluble and insoluble fractions. Samples were analyzed via western blot and probed for K63-polyUb. (A) Cell lysates were probed for K63-polyubiquitination, Cx43 and α-tubulin as a loading control. (B) Immunoprecipitation of the pellet fraction with Cx43-specific antibodies reveals the higher molecular weight band pattern typical of ubiquitination when probed with the K63-polyUb-specific antibodies. None of the mutants, except K287R, showed a similar higher molecular weight pattern indicating that both K264 and K303, but not K287, become K63-polyubiquitinated.

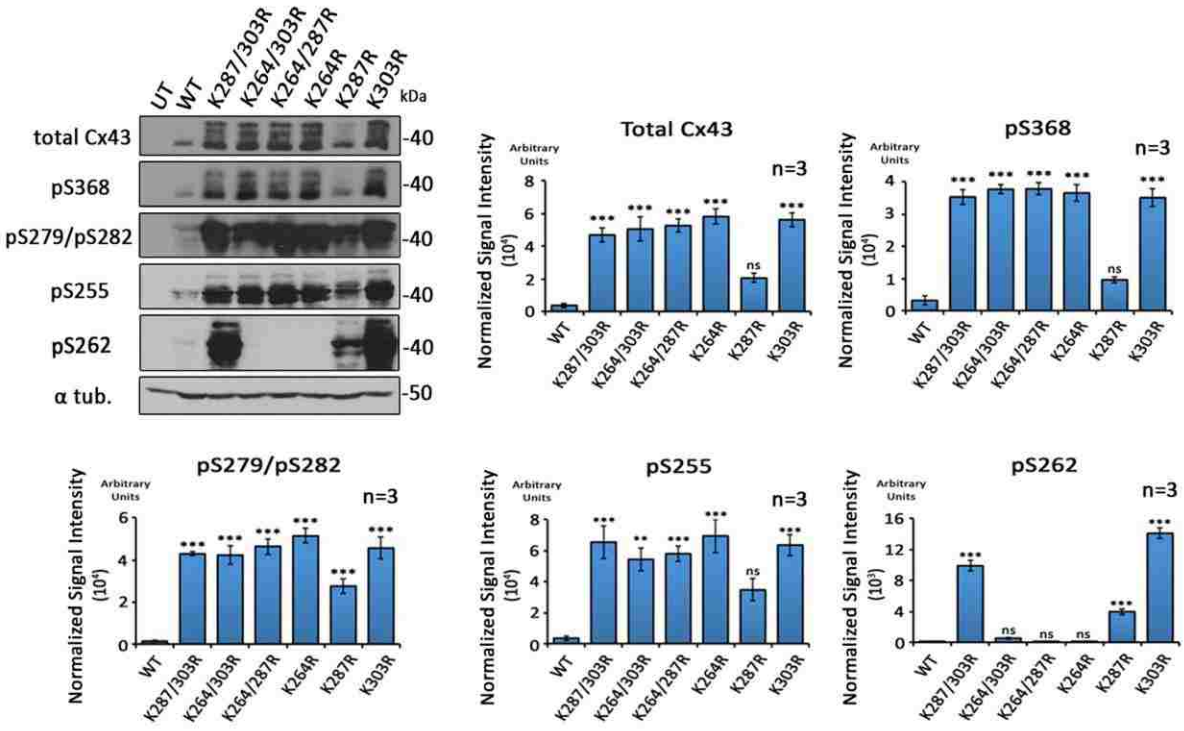


Figure 12: Cx43 K/R mutant proteins are hyper-phosphorylated on S368, S279/S282, and S255. Wild type, single and double K/R mutants were analyzed via western blot and probed using antibodies directed against total Cx43, and with Cx43-phosphospecific antibodies directed against phosphorylated S368 (pS368), S279/S282 (pS279/pS282), S255 (pS255), and S262 (pS262); Cx43 phosphorylation events known to down-regulate GJIC. α-tubulin was probed as a loading control. Densitometry analyses of Cx43 were normalized to α-tubulin. Significant increases in pS368, pS279/p282, and pS255 were detected whenever K264 and K303 were mutated. Accumulation of Cx43 phosphorylated on serine 262 (pS262) was only detected in a few mutants (mutants containing K287R and/or K303R).

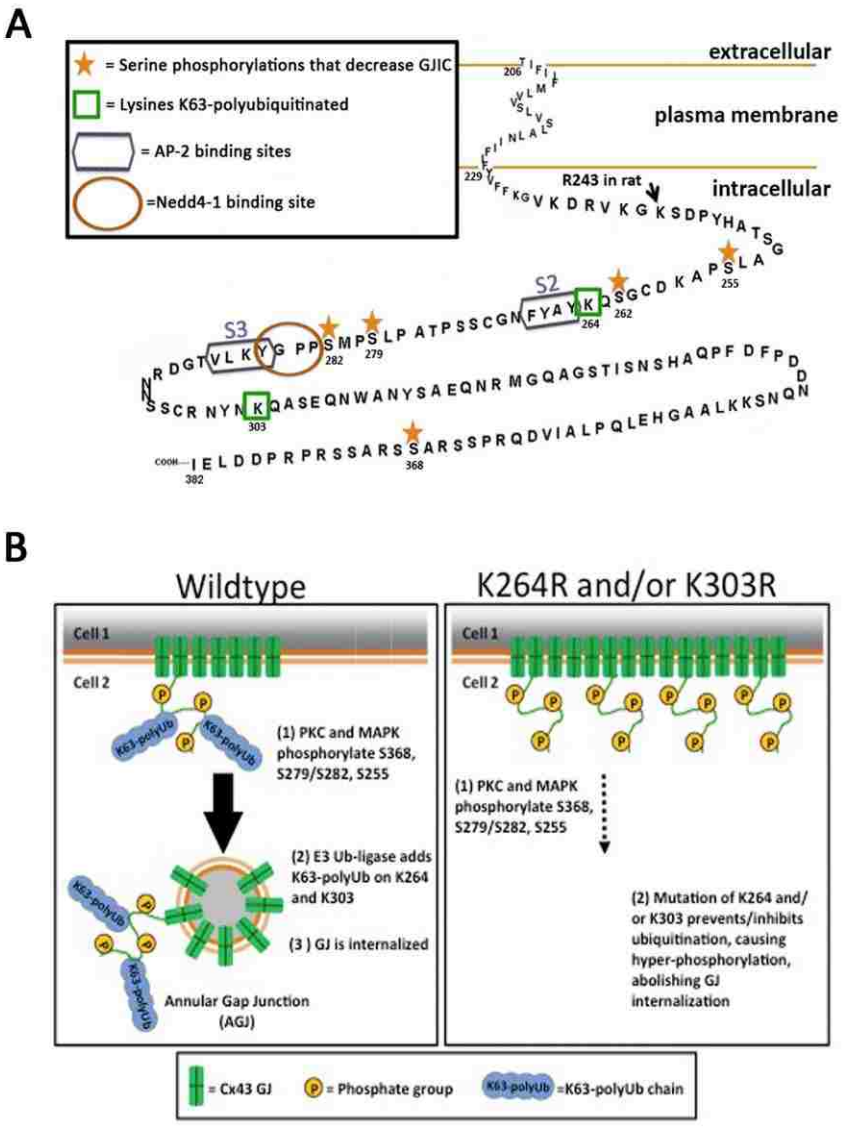


Figure 13: Schematic of K63-polyUb-mediated internalization of Cx43 GJs. (A) C-terminal tail of Cx43 showing the relevant E3-Ub ligase, Nedd4-1 binding site (brown circle), the AP-2/clathrin binding sites (purple boxes), the K63-polyubiquitinated lysine residues (green boxes), and the phosphorylated serine residues that are phosphorylated preceding to Cx43 K63-polyubiquitination (golden stars). Note that ubiquitination occurs juxtaposed (to the left and right) of the Nedd4-1 and AP-2/clathrin binding sites. (B) Phosphorylation of Cx43 by Src and MAPK on S368, S279/S282, S255 and possibly by other kinases on additional residues leads to Nedd4-1 binding, K63-polyubiquitination, and AP-2/clathrin binding, resulting in GJ internalization. Mutating either K264 or K303 prevents ubiquitination, causes hyper-phosphorylation and abolishes GJ internalization. Preventing ubiquitination on both residues K264 and K303 further exacerbates internalization.

Chapter 4:

Cx43 GJs are degraded by autophagy

(from John T. Fong, Rachael M. Kells, Anna M. Gumpert, Jutta Y. Marzillier, Michael W. Davidson, and Matthias M. Falk, 2012)

Contribution: My contribution to (Fong et al., 2012) is summarized in **Figure 14, 15, and 16**. My essential contribution upon reviewer request was the knockdown of Beclin-1/ATG6 and p62/SQSTM1 in HeLa cells and the imaging and quantification of Cx43 AGJ, LC3 positive autophagosomes and the % colocalization between them. My finding of significant decreases in LC3 positive autophagosome colocalization with AGJs in Beclin-1/ATG6 and p62/SQSTM1 knockdowns compared to wild type shows that autophagy is required for Cx43 AGJ degradation (**Figure 15**). Additionally, I contributed western blots of relative protein amounts to confirm Beclin-1/ATG6 and p62/SQSTM1 knockdowns in HeLa cells (**Figure 14**). I pursued co-immunolocalization studies in endogenous Cx43 expressing primary pulmonary artery endothelial cells (pPAECs) to corroborate this novel degradation process with the data in HeLa cells. Immunostaining in pPAECs shows that LC3 colocalizes with Cx43 at AGJs and internalized portions of GJ plaques. Staining with p62 and Cx43 shows robust colocalization of p62 (a ubiquitin binding protein) with Cx43 at both GJs and AGJs (**Figure 16**). This intriguing finding suggests the interaction of Cx43 with p62 prior to GJ internalization; indicating that Cx43 ubiquitination is necessary for internalization.

4-1: Abstract

Gap junction intercellular communication (GJIC) is essential for multi-cellular life. Gap junctions (GJs) provide a physical connection to neighboring cells and allow for the passage of signals between cells. The components of GJs, termed connexins (Cx), oligomerize to form connexons, or hemichannels, that dock with hemichannels of neighboring cells to form the full GJ. Whole GJs internalize into annular gap junctions (AGJs). The mechanism of AGJ degradation has not fully been elucidated. Here we show that knocking down key autophagy proteins, Beclin-1 and p62, in HeLa cells leads to decreases in LC3-positive autophagosome colocalization with AGJs and increases in AGJ number. We also show through co-immunolocalization studies in endogenous Cx43 expressing primary pulmonary artery endothelial cells (pPAECs) that Cx43 robustly colocalizes with p62 (a ubiquitin binding protein) at both GJs and AGJs.

4-2: Introduction

Direct cell-to-cell communication is a pivotal cellular function of multi-cellular organisms. It is established by gap junction (GJ) channels, which bridge apposing plasma membranes of neighboring cells. Typically, hundreds to thousands of GJ channels cluster into densely packed two-dimensional arrays, termed GJ plaques that can reach several square-micrometers in size. In addition to providing intercellular communication, GJs, based on their characteristic double-membrane configuration, significantly contribute to physical cell-to-cell adhesion. The ability to modulate (up- and down-regulate) the level of GJ-mediated intercellular communication (GJIC), and of physical cell-cell adhesion is as important as the basic ability of GJ formation itself; and is for example crucial for many physiological and pathological conditions, including cell migration during development and wound healing,

mitosis, apoptosis, leukocyte extravasation, ischemia, hemorrhage, edema, and cancer metastasis.

GJ channels are assembled from a ubiquitously expressed class of four-pass trans-membrane proteins, termed connexins, with connexin 43 (Cx43) being the most abundantly expressed connexin type. Six connexin polypeptides oligomerize into a ring to form a hexameric structure with a central hydrophilic pore, called hemi-channel or connexon. Once trafficked to the plasma membrane, two connexons, one provided by each of two neighboring cells, dock head-on in the extra-cellular space to form the complete trans-membrane GJ channel. Recruitment of additional GJ channels along the outer edge then enlarges the channel plaques, while simultaneous removal of older channels from plaque centers balances GJ channel turnover (Falk et al., 2009; Gaietta et al., 2002; Lauf et al., 2002).

While GJ channels can open and close (gate) to regulate electrical and chemical cell-cell coupling, GJ channel gating does not provide a means for modulating cell-to-cell adhesion, or for plasma membrane GJ channel renewal. Moreover, docked GJ connexons were found to be inseparable under physiological conditions, (Ghoshroy et al., 1995) posing potential challenges to these cellular functions. How is then the removal of GJ channels from the plasma membrane achieved?

We reported previously that cells appear to continuously internalize and turn over their GJs via a combined endo-/exocytic process that utilizes clathrin-mediated endocytosis components (Baker et al., 2008; Gilleron et al., 2008; Gumpert et al., 2008; Piehl et al., 2007) GJ internalization generates characteristic cytoplasmic double-membrane GJ vesicles, termed earlier annular GJs (AGJs) or connexosomes, preferentially in one of two coupled cells. We and others further found that internalization is highly efficient and regulated, for example in

response to natural inflammatory mediators such as thrombin and endothelin, (Baker et al., 2008) well-known inhibitors of GJIC; (Blomstrand et al., 2004; Postma et al., 1998; Spinella et al., 2003; van Zeijl et al., 2007) and under pathological conditions as e.g. in the failing canine ventricular myocardium (Hesketh et al., 2010). Continuous, as well as spontaneous internalization of GJ channels as complete, double-membrane spanning protein structures is supported by the fundamental observation that connexons, once docked, are inseparable under physiological conditions, and by a short half-life of Cxs and GJ channels of only 1-5 hours (Beardslee et al., 1998; Berthoud et al., 2004; Falk et al., 2009; Fallon and Goodenough, 1981; Gaietta et al., 2002). A similar internalization of GJs into cytoplasmic AGJ vesicles has been observed by others in cells in culture, as well as *in situ* in tissues (Ginzberg and Gilula, 1979; Hesketh et al., 2010; Hesketh et al., 2009; Jordan et al., 2001; Larsen et al., 1979; Leach and Oliphant, 1984; Mazet et al., 1985).

Previous studies identified proteasomal, endo-/lysosomal, and to a lesser extent phago-/lysosomal degradation pathways in the regulation of GJ stability and connexin protein degradation (Hesketh et al., 2010; Laing et al., 1997; Leach and Oliphant, 1984; Leithe and Rivedal, 2004b; Musil et al., 2000; Pfeifer, 1980; Qin et al., 2003). We thus embarked on investigating, on a molecular level, the fate of internalized AGJ vesicles. Following internalization, AGJ vesicles were observed to move away from the plasma membrane and to translocate deeper into the cytoplasm in a process that involves actin-filaments and the retrograde actin motor protein myosin VI (myo6) (Piehl et al., 2007). Here we report that GJs, following internalization into cytoplasmic AGJ vesicles, are targeted to autophagosomes via the ubiquitin-binding protein, p62/SQSTM1, and are degraded by autophagy.

4-3 Results

4-3-1: RNAi-mediated knockdown of Beclin-1 and p62/SQSTM1-protein significantly reduced AGJ/phagosome colocalization.

To further support assumptions made in Fong et al. 2012 that internalized AGJ vesicles are degraded by autophagy, we depleted cells of autophagy-relevant proteins using RNA-interference (RNAi) technology. We depleted cells of Beclin-1/(Atg6) and p62/SQSTM1 and investigated its impact on AGJ vesicle degradation and AGJ vesicle/autophagosome colocalization. As part of a class III PI3-kinase complex, Beclin-1 (the mammalian homolog of the yeast protein Atg6) is crucial for mediating the localization of autophagic machinery-proteins to pre-autophagosomal structures, and Beclin-1 has been identified as a key-protein required for isolation-membrane nucleation (Kihara et al., 2001). The Ub-binding protein p62, also named sequestosome 1 (SQSTM1), has been found instrumental in recognizing and targeting ubiquitinated cytoplasmic protein complexes to autophagic degradation, (Bjorkoy et al., 2005; Ding and Yin, 2008; Pohl and Jentsch, 2009) and to interact directly with LC3/(Atg8) (Pankiv et al., 2007). We performed three independent sets (n=3) of knockdown (KD) experiments in HeLa cells transiently expressing Cx43-GFP. The levels of each of these proteins were significantly depleted as verified by Western blot. This assay demonstrated a significant reduction of both proteins in the HeLa KD cells (**Figure 14**). RNAi-oligonucleotide transfection-efficiency was confirmed in control experiments to be above 90%, using a fluorescently labeled siGLO RISC-free oligonucleotide (not shown) (Gumpert et al., 2008).

Staining Beclin-1/(Atg6) KD and SI-control cells with LC3-specific antibodies followed by quantitative analyses revealed that a significantly smaller portion of AGJ vesicles (60.8+/-

21.8%) colocalized with LC3-positive phagosomes in the Beclin-1 KD cells (7.6+/-4.6%, n=3, p<0.001) when compared to SI-control cells (18.9+/-2.2%, n=3) (**Figure 15B**, panel 3). Similar results (69.5+/-21.1% reduction) were obtained for p62/SQSTM1 KD cells (5.5+/-3.5%, n=3, p<0.001) (**Figure 15B**, panel 3). As expected, an increased number of AGJs (**Figure 15B**, panel 1), and a decreased number of autophagosomes (**Figure 15B**, panel 2) were detected in all of these KD experiments as well. Representative LC3-immunofluorescence/Cx43-GFP images of Beclin-1 and p62/SQSTM1 KD, and SI-control cells, are shown in **Figure 15A**. LC3-positive Cx43-GFP AGJ vesicles are marked with arrows in the inserts. Taken together, these results further support the assumption that under normal conditions (no induction or inhibition of autophagy or of any other cellular protein degradation pathway) internalized GJs are degraded by autophagy.

4-3-2: LC3 and p62/SQSTM1 colocalize with Cx43 in primary PAE cells indicating autophagosomal degradation of AGJs in endogenously Cx43-expressing cells.

Given that most analyses described in Fong et al. 2012 were either done in transiently or stably Cx43-GFP/YFP expressing HeLa cells, we wanted to know whether autophagy-specific proteins would also co-localize with endogenously expressed Cx43. Cytoplasmic AGJ vesicles had been seen before *in situ* under normal conditions inside autophagosomes, (Leach and Oliphant, 1984; Pfeifer, 1980) suggesting that autophagy might be the generic degradation pathway for internalized GJs. We stained endogenously Cx43 expressing primary pulmonary artery endothelial cells (PAECs), that we had characterized before for thrombin/endothelin-mediated GJ internalization for Cx43, LC3, and p62/SQSTM1 using specific antibodies and qualitatively and quantitatively analyzed potential Cx43-LC3/p62

colocalization. Representative images are shown in **Figure 16**. Significant colocalization of both autophagic proteins with Cx43 was observed (**Figure 16A**, panels 1 and 2), even at stringent threshold settings (set to 120 of a maximum intensity of 250 arbitrary units**; Figure 16B**, panels 1 and 2). Interestingly, while LC3 appeared to exclusively colocalize with Cx43-AGJ vesicles located deep in the cytoplasm and occasionally juxtaposed to plasma membrane GJs, p62/SQSTM1 protein appeared to localize with both plasma membrane GJs as well as cytoplasmic AGJ vesicles; consistent with a role of p62 in sequestering ubiquitinated Cx43 to autophagosomes and LC3 as a specific autophagy marker (**Figure 16A**, panels 1 and 2, marked with arrows in inserts 1 and 2).

4-4 Discussion

While previous studies identified preferentially proteasomal and endo-/lysosomal degradation pathways in the regulation of GJ stability and connexin degradation, our study provides novel molecular and mechanistic insights into the autophagic degradation of endocytosed GJs. We observed significant colocalization of AGJ vesicles with marker proteins specific for autophagosomal degradation (LC3/Atg8), or autophagosomal cargo sequestration (p62/SQSTM1) ((Fong et al., 2012)and **Figures 15-16**). Using pharmacological inhibition of key autophagy proteins shows that Cx43 GJs are degraded by autophagy. Upon knockdown of Beclin-1 and p62/SQSTM1 in Cx43-GFP expressing HeLa cells, a significant reduction of LC3-positive AGJ vesicles was shown (**Figure 15A, B**). Colocalization between autophagic proteins, GJs and AGJ vesicles was observed in primary PAE cells, endogenously expressing Cx43 protein (**Figure 16**). Together, these findings strongly suggest that under normal physiological conditions, internalized AGJ vesicles (similarly to

cellular organelles cytoplasmic protein aggregates, and other superfluous macromolecular protein-complexes such as the mitotic midbody ring) are cleared from the cytoplasm and degraded by autophagy (Bjorkoy et al., 2005; Gutierrez et al., 2004; Nakagawa et al., 2004; Ogawa et al., 2005; Pohl and Jentsch, 2009).

Remarkably, although autophagic degradation of GJs had been described in several classical ultrastructural analyses of various cells and tissues *in situ*, including heart, dermis, and liver, (Leach and Oliphant, 1984; Mazet et al., 1985; Pfeifer, 1980; Severs et al., 1989) not much attention was attributed to this GJ degradation pathway. The recent observation that GJs appear to be internalized and degraded by autophagy in the failing ventricular myocardium (Hesketh et al., 2010) lends further importance to this GJ degradation pathway.

Substantial research over the past decade has indicated that autophagy represents a common lysosome-based cellular degradation pathway specifically designed to remove and degrade protein aggregates, multi-protein complexes, organelles and invading pathogens from the cytoplasm (Bjorkoy et al., 2005; Hung et al., 2009; Pohl and Jentsch, 2009; Ravikumar et al., 2008). Recent studies have further shown that protein aggregates, such as the ones formed by huntingtin and β-amyloid protein, and cellular structures such as the midbody ring (a cytokinesis left-over multi-protein complex) are all degraded by autophagy (Bjorkoy et al., 2005; Hung et al., 2009; Pohl and Jentsch, 2009; Ravikumar et al., 2008). Clearly, these cellular structures are degraded by autophagy independent of starvation.

Conjugation of ubiquitin (Ub) moieties to proteins has been recognized as a signal for both proteasomal targeting (addition of K48-linked poly-Ub chains), and more recently also as a sorting signal towards internal vesicles of the late endocytic pathway (addition of multiple mono-Ub moieties or of K63-linked poly-Ub chains) which ultimately leads to degradation

by lysosomes (Hicke, 2001; Hicke and Dunn, 2003; Schnell and Hebert, 2003; Shih et al., 2002; Stahl and Barbieri, 2002). The latter includes Ub-conjugation that acts as an internalization signal for clathrin-mediated endocytosis (Belouzard and Rouille, 2006; Geetha et al., 2005). In this process, multiple mono-Ub moieties are attached to the target protein and are recognized by specific clathrin-mediated endocytic machinery protein-components that associate with a subset of Ub-binding proteins, specifically Epsin1 and Eps15(Girao et al., 2009; Hawryluk et al., 2006). Further work has shown that the protein p62/SQSTM1 recognizes and interacts via its UBA-domain with poly-ubiquitinated proteins (Ciani et al., 2003; Seibenhener et al., 2004; Wilkinson et al., 2001) and delivers poly-ubiquitinated (K63-linked) oligomeric protein complexes to the autophagic degradation pathway (Bjorkoy et al., 2005; Pankiv et al., 2007). Ubiquitination of Cx43-based GJs has been described previously, (Girao et al., 2009; Kjenseth et al., 2010; Leithe et al., 2009; Leithe and Rivedal, 2004b; Leithe et al., 2012) and is consistent with our own unpublished fluorescence analyses (data not shown). The finding that Cx43-based GJs can become ubiquitinated, the known affinity of p62/SQSTM1 for ubiquitinated protein complexes, its colocalization with plasma membrane GJs in PAECs (**Figure 16**), and its apparent involvement in targeting AGJ vesicles for autophagic degradation (**Figure 15**), suggests that ubiquitination of Cx43, besides serving as a likely signal for GJ internalization, also serves as a likely signal for targeting AGJ vesicles to autophagic degradation.

4-5 Materials and methods

4-5-1: Cell culture, cDNA constructs, transient and stable transfections

Human epitheloid cervix carcinoma cells (HeLa, American Type Culture Collection [ATCC], Cat. No. CCL2,) were maintained in a humidified atmosphere containing 5% CO_2 at 37°C in low glucose (1000mg/l) Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, Cat. No. SH30021.01). Medium was supplemented for a final concentration of 10% with fetal bovine serum (FBS, Atlanta Biologicals, Cat. No. S11050), 2mM L-glutamine (Thermo Scientific, Hyclone, Cat. No. SH30034.01, stock 200mM) and 50 I.U/ml penicillin and 50µg/ml streptomycin (Cellgro, Cat. No. 30-001-CI). Porcine primary pulmonary artery endothelial cells (PAECs) were isolated and cultured as described in reference (Baker et al., 2008).

Fluorescent protein-tagged Cx43-GFP construct was described previously (Falk, 2000; Gumpert et al., 2008; Shaner et al., 2008). For transfections cells were grown on glass cover-slips coated with poly-L-lysine (Sigma-Aldrich, Cat. No. P8920) for 24hrs prior to transfection and allowed to reach 70-80% confluency. Cells were single and double-transfected with cDNAs using SuperFect® Transfection Reagent (Qiagen, Cat. No. 301307) according to manufacturer's directions. Cells were observed 20-24hrs post transfection, and transfection efficiencies between 15% and 30% were considered appropriate for qualitative and quantitative experiments.

4-5-2: siRNA duplexes and knock-down procedures

All RNAi oligonucleotides (oligos) were purchased from Dharmacon RNA Technologies and transfected into HeLa cells using Oligofectamine (Invitrogen, Cat. No. 12252011) according to manufacturer's recommendations, and as described in reference (Gumpert et al., 2008). Oligonucleotides directed against Beclin-1/(Atg6) (siGENOME SMARTpool,

Cat. No. M-010552-01), p62/SQTM1 (siGENOME SMARTpool, Cat. No. M-010230-00-0005), as well as a non-targeting RISC-activating duplex control (siControl, Cat No. D-001210-01-05) were used. RNAi-transfection efficiency was tested using a fluorescently labeled, non-targeting control oligonucleotide (siGLO RISC-Free, Cat. No. D-001600-01-05) and was established to be ≥ 90% efficient (Gumpert et al., 2008). 48hrs post oligo transfection cells were transfected with Cx43-GFP cDNA using SuperFect® Transfection Reagent (Qiagen, Cat. No. 301307), as recommended by the manufacturer. Cells were assayed 72hrs post oligo transfection and 20-24hr into Cx43 expression for KD efficiency by Western blot analyses (**Figure 14**). To evaluate whether Beclin-1 and p62 KD would result in a reduced number of LC3-positive AGJ vesicles, KD and SI-control cells were stained with LC3-specific antibodies (**Figure 15**).

4-5-3: Stains, antibodies and immunofluorescence analyses

mouse monoclonal anti-LC3 recognizing both, LC3-I and LC3-II of the human splice-variants LC3A, B, and C (MBL International Corp., Cat. No. M115-3), rabbit polyclonal Beclin-1 (Cell Signaling, Cat. No. 3738), mouse monoclonal anti-3/p62 Lck ligand (BD Biosciences, Cat. No. 610832), and rabbit polyclonal anti-Cx43 (Sigma, Cat. No. 6219) antibodies were used at dilutions of 1:50–1:250 in 10% FBS/PBS. Secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 568 (Molecular Probes/Invitrogen, Cat. No. A11036 and A11004), or Alexa Fluor 488 (Molecular Probes/Invitrogen, Cat. No. A21206) respectively, were used at 1:100 –1:200 dilutions in 10% FBS/PBS.

Cells were fixed and permeabilized either in 3.7% formaldehyde for 15min followed by permeabilization in 0.1% Triton X-100 for 20min at RT. Cells were washed three times in

phosphate-buffered saline (PBS) between all steps. Following blocking with 10% FBS in PBS (20min), cells were incubated with primary and secondary antibodies (diluted in blocking solution) for 60min at RT each, then for 30sec with DAPI (10μg/ml), rinsed with PBS and diH_2O, and mounted using Fluoromount G (Southern Biotechnology, Cat. No. 0100-01). Knockdown of proteins was evaluated by comparing quantitative fluorescence intensity signals measured along lines on images taken under identical exposure conditions from treated and mock-treated cells. Fluorescence colocalization signals in **Figure 16** were quantified using the colocalization tool of the Zeiss LSM 510 ZEN 2008 (Version 5.0.0.267) software package.

4-5-4: Immunoblot analyses

Verification of protein knockdown (KD) efficiency was assessed by Western-blotting 72hrs post-RNAi transfection. Cells were lysed in standard 4x SDS-PAGE sample buffer and boiled for 5min. Biotinylated protein ladder (Cell Signaling Technology, Cat. No. 7727) was used to determine the molecular weight of relevant protein bands. Samples were resolved on 12% Bis/Acrylamide (1:29) gels and transferred onto nitrocellulose membranes (pore size 0.2μm, Whatman, Optitran BA-S83, Cat. No. 10439396). Following blocking in 5% dry milk in TBST, membranes were incubated in primary antibodies (1:1000 in 5% BSA in TBST) for 3hrs at RT or o/n at 4°C. Membranes were washed in TBST for 15min and incubated with horseradish peroxidase (HRP)–conjugated anti-mouse and anti-rabbit secondary antibodies (1:5000 in 5% BSA in PBST, Zymed Laboratories, Cat. No. 81-6520 and 81-6120) for 3hrs at RT or o/n at 4°C. Immuno-reactive bands were detected using Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare, Cat. No. RPN2132). Membranes

were washed in TBST for 15 minutes, stripped in stripping buffer (Boston Bioproducts, Cat. No. BP-96) at 65°C for 30 minutes, followed by a stringent wash in TBST. Membranes were re-blocked with 5% dry milk in TBST and re-probed with mouse monoclonal antibodies directed against α-tubulin (Sigma, Cat. No. T9026) or b-tubulin (Developmental Studies Hybridoma Bank, clone E7) (1:5000 in 5% BSA in PBS) to serve as a loading control. Signals were quantified by scanning developed films using SCION software (NIH).

4-5-5: Microscopy and quantitative image analyses

Wide-field fluorescence microscopy was performed on a Nikon Eclipse TE 2000E inverted fluorescence microscope equipped with 40x Plan Fluor (numerical aperture [NA] 1.3), 60x and 100x Plan Apochromat (NA 1.4) oil immersion objectives; a forced-air-cooled Photometics CoolSnap HQ charge-coupled device camera (Roper Scientific), and a ProScan II motorized stage (Prior Scientific). Images were captured, analyzed, and processed using MetaVue software version 6.1r5 (Molecular Devices) and Adobe Photoshop (Adobe Systems). Fluorescence colocalization analyses were performed on a Zeiss Axiovert 200 M inverted fluorescence microscope (Carl Zeiss) equipped with an LSM510 META scan head and a 63x Apochromat oil-immersion objective (NA 1.4). Argon-ion and Helium-Neon lasers were used to generate the 488- and 543-nm excitation lines, and pinholes were typically set to 1 airy unit. Images were acquired using four-line mean averaging in separated channels to avoid bleed-through and LSM510 META 3.0 software.

4-5-6: Statistical analyses

AGJ vesicles and GJ plaques were measured and counted on images taken of knockdown, and control cells in at least three independent experiments each. Only clearly recognizable GJs (a line of fluorescent puncta, or elongated plaques located between cell pairs), and AGJs (bright fluorescent spherical structures located in the cytoplasm and ≥0.5 μm in diameter) as defined in detail in Piehl et al.(Piehl et al., 2007) and Gumpert et al. (Gumpert et al., 2008) were considered. Only spherical, clearly LC3-positive vesicular structures were considered as autophagosomes, and were counted as well. Participation of selected proteins and pathways in GJ internalization and subsequent degradation was evaluated by manually counting the number of Cx43-GFP expressing cells and by counting the number, and measuring the size of AGJ vesicles, GJ plaques, and LC3-positive autophagosomes. For statistical analyses, the total number of AGJ vesicles per experiment was divided by the number of cell pairs positive for Cx43-GFP expression and clearly coupled by GJs as described (Gumpert et al., 2008; Piehl et al., 2007). To compare the Beclin-1 and p62/SQSTM1 KD cells with the SI-control cells, the number of AGJs, autophagosomes, and AGJ/autophagosome colocalization was determined for each cell pair. For each experiment, the ratio of AGJ vesicles colocalizing with LC3-positive structures was then formed over the total number of AGJs and of autophagosomes that were counted per cell pair. For statistical analyses ratios of Beclin-1 and p62 KD cell pairs (99 and 40) were compared to the ratios obtained in SI-control cells (99). In all analyses, a P-value ≤0.05 was considered statistically significant." Statistical analyses were performed using Microsoft Excel's "*Students* T-test: Two samples assuming equal or unequal variances" function of the data analysis package.

Figures

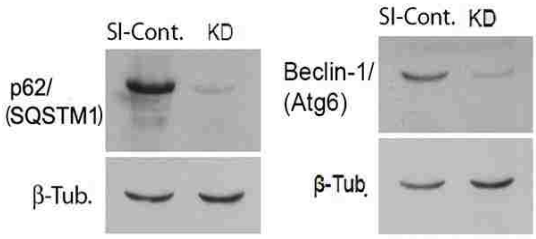


Figure 14: Efficient RNAi-mediated depletion of autophagy-relevant proteins monitored by Western blot. HeLa cells were transfected with SI-control RNA-oligonucleotides, or with RNA-oligos targeting the autophagy-relevant proteins, Beclin-1/(Atg6) and p62/SQSTM1 (KD). 48 hours later cells were transfected with Cx43-GFP cDNA and analyzed for KD efficiency 72 hours after oligo-transfection. Target protein-depletion assayed by Western blot analyses in SI-control and RNAi-KD cells (published in and adapted from (Fong et al., 2012)).

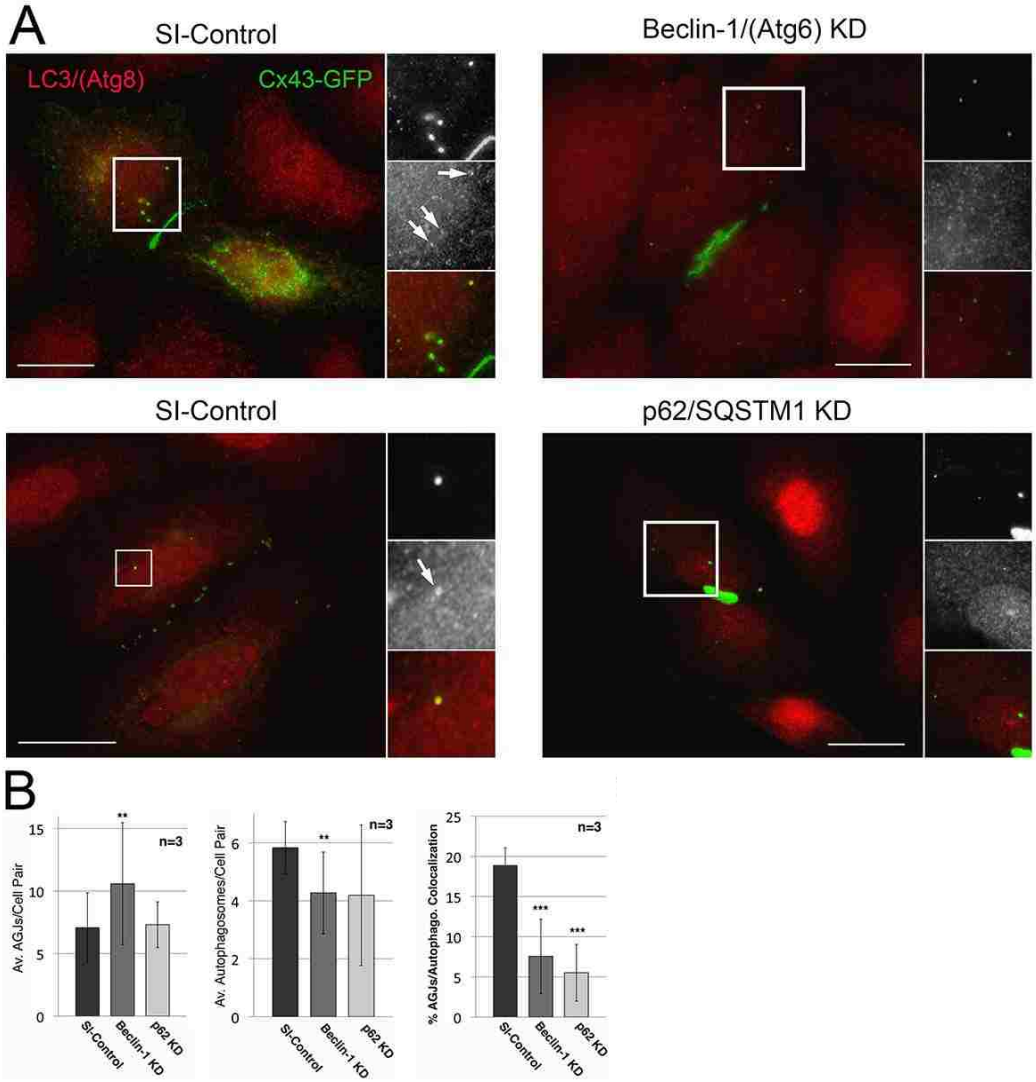


Figure 15: RNAi-mediated depletion of key-autophagic proteins inhibits degradation of AGJ vesicles and decreases the number of AGJ-containing LC3-labled phagosomes. HeLa cells were transfected with SI-control RNA-oligonucleotides, or with RNA-oligos targeting the autophagy-relevant proteins Beclin-1/(Atg6) and p62/SQSTM1 (KD). 48 h post oligo transfection cells were transfected with Cx43-GFP cDNA and 24 h later, total number of AGJs (in C), and colocalization with LC3-positive phagosomes (in A and B) was analyzed. (A) Beclin-1 and p62 KD and SI-control cells were stained with anti-LC3 antibodies 72 hours post oligo, and 24 hours post Cx43-GFP cDNA transfections. Representative immuno-fluorescence images are shown. Individual and merged fluorescence signals of the boxed areas are shown at higher magnification on the right. Colocalization of Cx43-GFP AGJ vesicles and LC3-positive phagosomes was observed preferentially in the SI-control cells (marked with arrows). Bars = 20 mm. (B) Quantitative analyses of AGJ vesicles (panel 1), LC3-positive autophagosomes (panel 2), and colocalizing AGJ/autophagosomes (panel 3)

revealed a significant increase of AGJs, a significant decrease of autophagosomes, and significantly reduced AGJ/autophagosome colocalization in the Beclin-1 and p62 KD knockdown cells in all three independent experiments (** = $p<0.01$; *** = $p<0.001$) ((published in and adapted from (Fong et al., 2012)).

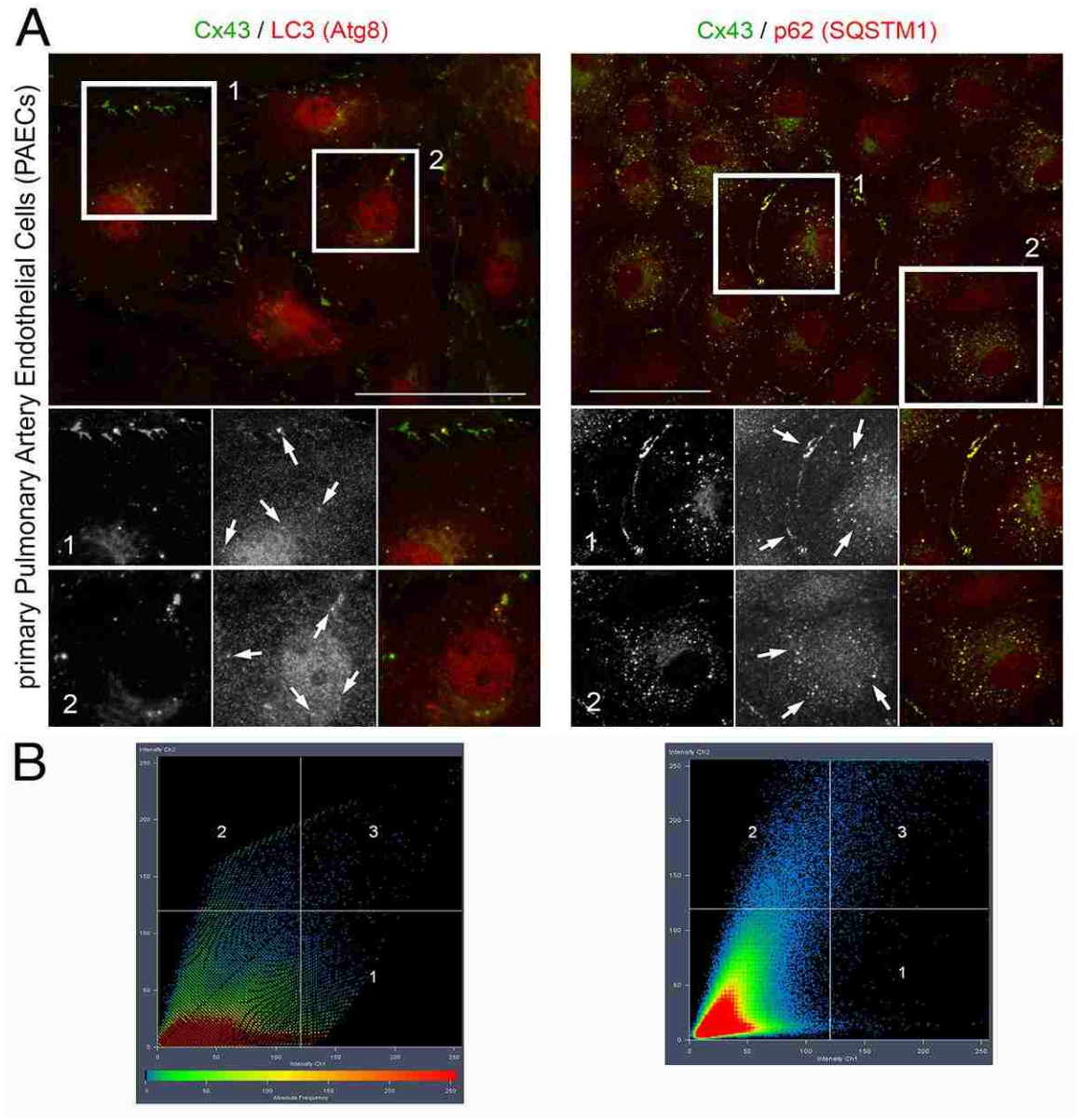


Figure 16: LC3 and p62/SQSTM1 protein colocalize with endogenously expressed Cx43-based GJs and AGJ vesicles. Endogenously Cx43 expressing porcine primary pulmonary artery endothelial cells (PAECs) were stained for Cx43, LC3, and p62/SQSTM1 using specific mono- and polyclonal antibodies, and potential Cx43-LC3/p62 colocalization was qualitatively and quantitatively analyzed. (A) Representative merged fluorescence images of confluent PAECs are shown. Individual and merged fluorescence signals of the boxed areas are shown below at higher magnification. Significant colocalization of LC3 with AGJ vesicles, and of p62 with AGJ vesicles and individual plasma membrane GJs was observed, even at stringent threshold settings (marked with arrows). Bars = 20 mm. (B) Quantitative scatter-blot colocalization analyses of the images shown in (A) with applied maximum intensity threshold settings of 120 (of maximally 250 arbitrary units; white lines) indicated (published in (Fong et al., 2012)).

Chapter 5:

Conclusions and Future Perspectives

5-1: Conclusions

This dissertation describes three related aspects of Cx43 regulation during GJ internalization and degradation. Chapter 2 describes my identification of clathrin-mediated endocytosis (CME) of Cx43 GJs under VEGF stimulation **(Figures 5 and 6**). Stimulation of endothelial cells with the inflammatory mediator VEGF causes phosphorylation of Cx43 on S368, S279/S282, S255, and S262, residues that are known to cause GJIC downregulation (Nimlamool et al., 2015). My observations are in accordance with previous findings showing VEGF-mediated activation of the MAPK signaling pathway that were characterized in aortic endothelial cells (Kroll and Waltenberger, 1997). Our lab and others (Kevil et al., 1998; Suarez and Ballmer-Hofer, 2001; Thuringer, 2004) report a rapid inhibition of GJIC in response to VEGF that correlates with activation of several kinases, including MAPK. At least 7 different kinases (Akt, CK1, PKA, PKC, MAPK, CDC2, Src) of different signal transduction pathways have been characterized that phosphorylate numerous regulatory C-terminal serine and tyrosine residues of Cx43 at various stages of its life cycle. Of these, PKC, MAPK, CDC2 (during mitosis) and Src have been reported to induce GJ channel closure and inhibition of GJIC (Kanemitsu et al., 1998; Lampe et al., 2000; Leykauf et al., 2003; Petrich et al., 2002; Polontchouk et al., 2002; Sirnes et al., 2009; Solan and Lampe, 2007; Solan and Lampe, 2014; Solan et al., 2007; Thévenin et al., 2013). A recent related study in our laboratory showed that treating mouse embryonic stem cell colonies (which express endogenous Cx43) with epidermal growth factor (EGF) also resulted in a significant

inhibition of intercellular communication (GJIC) and activation of MAPK and PKC signaling cascades to phosphorylate serines 262, 279/282, and 368 of Cx43, leading to Cx43-based GJ endocytosis (Fong et al., 2014). This study, as well as additional studies in phorbol ester-treated (PKC-activated) Cos7 cells (Cone et al., 2014), and in GJ assembly-impaired BxPC3 and Capan-1 pancreatic cancer cells (Johnson et al., 2013) also describe the serine phosphorylation-mediated internalization of Cx43 GJs. Thus, phosphorylation on well-recognized C-terminal regulatory Cx43 amino acid residues by a series of different kinases emerges as an early molecular signal that triggers GJ internalization.

Integrity of blood vessel walls must be maintained precisely to prevent the leakage of plasma components and blood cells into surrounding tissues. Internalization for these purposes would be required under conditions where cells must rapidly internalize GJs in order to uncouple from neighboring cells for division or allowing trans-endothelial migration of immune cells from the blood to areas of tissue damage. Elucidating the mechanisms that down-regulate GJIC in response to VEGF is important for understanding the biology of cancer development including changes in cell cycle progression, angiogenesis, and tumor cell metastasis. Chapter 2 in this dissertation provides a mechanism for such physiological processes that involves not only the internalization process (CME) but together with Nimlamool et al. 2015 connects specific phosphorylations required to downregulate Cx43 GJs upon stimulation with VEGF (pS368, pS279/S282, pS255, and ps262). These results further our understanding of how, for example, angiogenesis occurs within the body. VEGF in the extracellular environment stimulates phosphorylation of Cx43 GJs, which results in rapid internalization via CME. This downregulation of GJs then allows cells to uncouple from one another for new blood vessel formation.

Chapter 3 in this dissertation connects Chapters 2 and 4 to form a larger story involving the phosphorylation, ubiquitination, internalization and subsequent degradation of Cx43 GJs. In Chapter 3, I describe the elucidation of lysines 264 and 303 which are K63-polyubiquitinated on Cx43 GJs prior to internalization. This mechanism ties in with the findings from Chapter 4, that Cx43 GJs are degraded by autophagy, in that p62 is likely binding to this K63-polyubiquitinated Cx43 at GJs and sequestering it for autophagic degradation once internalized (**Figure 17**). Intriguingly, this chapter also reveals that the phosphorylation of Cx43 on serines 368, 279/282, and 255 occur prior to ubiquitination. These phosphorylations correlate with results presented earlier in Chapter 2 that show that phosphorylation is required for CME-mediated internalization of Cx43 GJs after VEGF stimulation (**Figure 5, 6** and (Nimlamool et al., 2015)). Together with Chapters 2 and 3, these findings have lead us and others in the field to conclude that regulation of Cx43 requires a "kinase program" (Falk et al., 2016; Solan and Lampe, 2015), suggesting that specific phosphorylations occur sequentially in order for proper regulation of the Cx43 lifecycle.

Additionally, work in Chapter 3 relates to the previous findings from our lab showing that CME internalization of Cx43 GJs requires the interaction with clathrin and the clathrin adaptor, AP-2 (Fong et al., 2013; Piehl et al., 2007). Sufficient internalization of Cx43 GJs requires both S2 (^{263}YAYF^{268}) and S3 (^{286}YKLV^{289}) binding motifs within the Cx43 C-terminus. Importantly, since K287 lies within the critical S3 binding site for AP-2 access to Cx43, it is likely that blocking this interaction (Chapter 3) adversely impacts the AP-2 mediated CME process. Therefore, clathrin may also be recruited by another group of adaptor proteins, termed clathrin associated sorting proteins, or CLASPs, that specifically

bind via a Ub-interacting motif (UIM) to a polypeptide sequence that is exposed in K63-polyubiquitin chains (Traub and Bonifacino, 2013). One such alternative adaptor, called Eps15, was found to bind to Cx43 and has been proposed to facilitate GJ internalization (Catarino et al., 2011; Girao et al., 2009). Thus, K63-polyubiquitination may also allow Eps15 to bind to Cx43, recruit clathrin, and internalize GJs as an alternative to AP-2 (Falk et al., 2016; Falk et al., 2014; Girao et al., 2009) (**Figure 17**).

Chapter 4 describes my work in elucidating the degradation of internalized GJs by autophagy. As shown in (Baker et al., 2008; Fong et al., 2013; Gumpert et al., 2008; Nimlamool et al., 2015; Piehl et al., 2007) and Chapter 2, the process of GJ internalization requires CME machinery under baseline and acute internalization conditions. Docked GJ channels cannot be separated into connexons under physiological conditions, posing potential challenges to GJ channel renewal and physical cell-cell separation (Ghoshroy et al., 1995; Goodenough and Gilula, 1974). This poses additional challenges once the internalized GJ, or annular GJ (AGJ), must be degraded as the structure is a double membraned vesicle with docked GJs. Degrading this kind of a cellular structure would require more than proteasomal degradation (misfolded protein degradation). My work in Chapter 4 and Fong et al. 2012 shows that AGJs are degraded by autophagy under unstarved, physiological conditions. My work shows that the autophagic proteins Beclin-1/ATG6 and p62/SQSTM1 are necessary for the sequestration of AGJs as knockdown of each protein lead to increases in AGJ number and decreases in colocalization of AGJs with the autophagosomal marker LC3 (**Figures 14, 15** and (Fong et al., 2012)). To add a more physiological perspective to my work I also used co-immunolocalization studies of Cx43 GJs and p62 in primary pulmonary artery endothelial cells (pPAECs) to corroborate this novel degradation process with the data

in HeLa cells presented in Chapter 4 and Fong et al. 2012 (**Figure 16** and (Fong et al., 2012)). The findings from our lab show baseline degradation of Cx43 AGJs by autophagy and is one of the first to elucidate autophagy as the degradative fate of Cx43 internalized GJs under physiological conditions (Fong et al., 2012; Hesketh et al., 2010). In addition, my co-immunolocalization findings in Chapter 4 show that p62 colocalizes with Cx43 at GJ and at AGJs (**Figure 16**). This finding was also described biochemically by others in the field (Bejarano et al., 2012; Lichtenstein et al., 2010). p62/SQSTM1 interacts specifically with K63-polyubiquitinated substrates via its UBA (ubiquitin associated) domain (Seibenhener et al., 2004) to sequester targets for autophagosomal degradation (Bjorkoy et al., 2005; Pankiv et al., 2007). My finding of p62 colocalizing with Cx43 at GJs in Chapter 4 suggests the interaction of Cx43 with p62 prior to GJ internalization (**Figure 17**). In this way, p62 provides a connection between phosphorylations required to internalize GJs, such as in Chapter 2, and the degradation of internalized GJs (Chapter 4). Physiologically, autophagy has been observed as a degradation pathway for internalized GJs in the failing ventricular myocardium (Hesketh et al., 2010). Cx43 GJs are prominent at intercalated discs in myocardium, which is associated with altered Cx43 expression and regulation in heart disease (Dupont et al., 2001; Fontes et al., 2012; Kostin et al., 2003; Peters et al., 1993). In accordance with results from Chapter 2 it is feasible to speculate that MAPK/PKC-induced phosphorylations leading to decreased GJIC and Cx43 GJ internalization would result in AGJs that are degraded by autophagy (**Figure 17**).

This dissertation ties together several aspects of Cx43 GJ phosphorylation (Chapter 2) and ubiquitination (Chapter 3) required for GJ internalization. Additionally, since the K63-polyubiquitin binding protein, p62, binds to Cx43 at GJs (Chapter 4) and Cx43 at GJs is

K63-polyubiquitinated (Chapter 3), my results together show that p62 binds to K63-polyubiquitinated Cx43 at GJs and upon internalization sequesters the ubiquitinated AGJs to the autophagosomal machinery for degradation. Taken together, these results provide a more comprehensive understanding of GJ internalization and degradation that starts with specific phosphorylations regulating the event. S368 phosphorylation is known to be dependent on S365 dephosphorylation (termed "gate keeper"), an event known to trigger a large conformational change of the Cx43 C-terminus that affects the upstream region which harbors the above described ubiquitination sites (Solan and Lampe, 2007). PKC-mediated phosphorylation on S368 may serve as the lead phosphorylation event that then triggers subsequent MAPK-mediated phosphorylation on serines 255, 262, and 279/282 to allow E3 Ub ligases, AP-2/Eps15, and clathrin to access juxtaposed Cx43 binding sites (Fong et al., 2013; Thévenin et al., 2013) resulting in GJ internalization (**Figure 18**). These events are categorized as early and late, corresponding to early trafficking and docking of open channels to the outer region of the GJ plaque (**Figure 18,** steps 1-3) and later closure of the gap junctions as they move toward the center of the GJ plaque and eventually are internalized (**Figure 18,** steps 4-5). These early and late events are proposed to correspond to the conformational change in the C-terminus initiated by S365 dephosphorylation (**Figure 19**). Early events occur on residues located juxtaposed to the C-terminal ZO-1 binding site and late occur on residues located juxtaposed to the S2, S3 AP-2 (Eps 15)/clathrin binding sites (**Figure 19**). Findings from Chapters 2, 3, and 4 are depicted in **Figure 17, 18,** and **19** where Cx43 in GJs is first phosphorylated (Chapter 2) and ubiquitinated (Chapter 3) prior to internalization via clathrin-mediated endocytosis, then finally degraded by autophagy (Chapter 4). The findings presented in this dissertation

provide a mechanism for Cx43 GJ regulation that is crucial for many physiological and pathological conditions, including cell migration during development and wound healing, mitosis, apoptosis, ischemia, hemorrhage, edema, cancer metastasis, and heart disease.

5-2 Future Perspectives

Chapter 3 describes the identification of K63-polyubiquitination on Cx43 GJs at lysine residues 264 and 303. To further verify that the link between GJ internalization and AGJ autophagic degradation is K63-polyubiquitionation of Cx43, future analyses would involve immunoprecipitation of K/R mutants with p62. Since K264 and K303 within the C-terminus of Cx43 are K63-polyubiquitinated, it would be expected that mutating these sites would abolish interaction of Cx43 and p62 whereas mutation of K87 should retain the interaction as wild type. Additionally, co-immunolocalization studies would show the lack of colocalization between p62 and Cx43 at GJs and AGJs and result in increased AGJ number as seen in Chapter 4. Another interesting aspect of Cx43 ubiquitination is that four E3 ligases are described to interact with and ubiquitinate Cx43; Nedd4-1, Wwp1, Smurf2, and Trim21 (Basheer et al., 2015; Chen et al., 2012; Fykerud et al., 2012; Leykauf et al., 2006). So far, only Nedd4-1 has been shown to directly interact with the ^{283}PPGY^{286} motif in the C-terminus of Cx43 (Leykauf et al., 2006). It is unknown where these remaining three E3 ligases (Wwp1, Smurf2 and Trim21) bind within the C-terminus of Cx43 and if they ubiquitinate Cx43 with the same K63-linked polyUb chain. Current literature suggests that Trim21 builds K63-polyUb chains *in vitro* in response to infection (McEwan et al., 2013) while Wwp1 chain building specificity in toll-like receptor (TLR) inflammation is suggested to be K48-linkage specific (Lin et al., 2013). Further investigation into the role of Trim21,

Wwp1, and Smurf2 in the ubiquitination of Cx43 GJs would require identification of each E3 ligase binding motif within the C-terminus of Cx43. Trim21 and Wwp1 both belong to the same family as Nedd4-1 and have multiple WW binding domains that bind to PPxY motifs in target proteins (Scheffner and Kumar, 2014) whereas the binding specificity of Trim21 is less well known. Mutation of each E3 ligase binding motif and immunoprecipitation analysis with the C-terminus of Cx43 would determine where each binds. Additionally, an analysis of the downstream effects of each E3 ligase would shed more light onto the regulation of Cx43 ubiquitination. Co-immunolocalization studies would provide evidence for interaction of E3 ligases at each stage of the Cx43 life cycle (Cx, connexon, GJ, AGJ). This would also be accomplished by knockdown or mutation of each DUB followed by analysis of Cx43 GJ/AGJ ubiquitination by immunoprecipitation. It is feasible to speculate that some E3 ligases are responsible for ubiquitination of misfolded Cx43 whereas others are required for modifying GJs.

Another interesting finding in this dissertation work is that I identified a phosphodegron mechanism of Cx43 GJ regulation involving serine 368, 279/282, and 255 phosphorylations occurring prior to ubiquitination of Cx43. These phosphorylations lead to downregulation of GJIC and are increased in Cx43 ubiquitin mutants K264R and K303R. This regulation is consistent with a phosphodegron model in which phosphorylation of specific sites leads to ubiquitination. My finding is supported by recent evidence that phosphorylation on S279/S282 enhances the binding affinity of the E3 ligase, Nedd4-1, to the C-terminus of Cx43 (Spagnol et al., 2016). This added layer of regulation emphasizes the complex orchestration that must occur in physiological GJ turnover. To more definitively show that phosphorylations of Cx43 on serines 368, 279/282, and 255 are required prior to

ubiquitination, analysis of K63-polyubiquitin patterns of serine phospho-dead and phospho-mimetic mutants would be conducted. Analyses would entail expression of the above-mentioned serines as either phospho-dead (S/A) or phospho-mimetic (S/D or E) Cx43 mutants on residues 368, 279/282, and 255 and immunoprecipitation of Cx43 to determine if K63-polyubiquitination still occurs. The expectation is that a loss of phosphorylation in S/A mutants would results in loss of K63-polyubiquitination because phosphorylation is not present to "prime" GJs for ubiquitination. Conversely, it is expected that S/D or E mutations would result in K63-polyubiquitination, potentially in higher amounts than wild type and/or a faster rate of internalization. Another direction of these findings would incorporate combinations of phosphorylation and ubiquitin mutations in transgenic mice to determine the effects on development and tissue physiology. Evidence that the C-terminus of Cx43 is crucial for development is shown in transgenic mice expressing Cx43K258Stop as heterozygous mice have altered Cx43 channel function, intercalated disc organization and longer Cx43 half-life (Maass et al., 2007). An *in vivo* model using ubiquitin and phospho-mimetic/dead mutations would provide a more comprehensive view of GJ turnover. One interesting aspect would be the effect of such mutations on the development of heart tissue as Cx43 has been known to be affected in diseased hearts (Kostin et al., 2003).

In conclusion, these findings greatly contribute to the broader understanding of mechanisms that regulate GJ internalization and degradation. They provide a comprehensive explanation of how GJ phosphorylation, ubiquitination, internalization, and autophagic degradation are linked together. Relevant findings of two of these chapters have been published and one will be published.

Figures

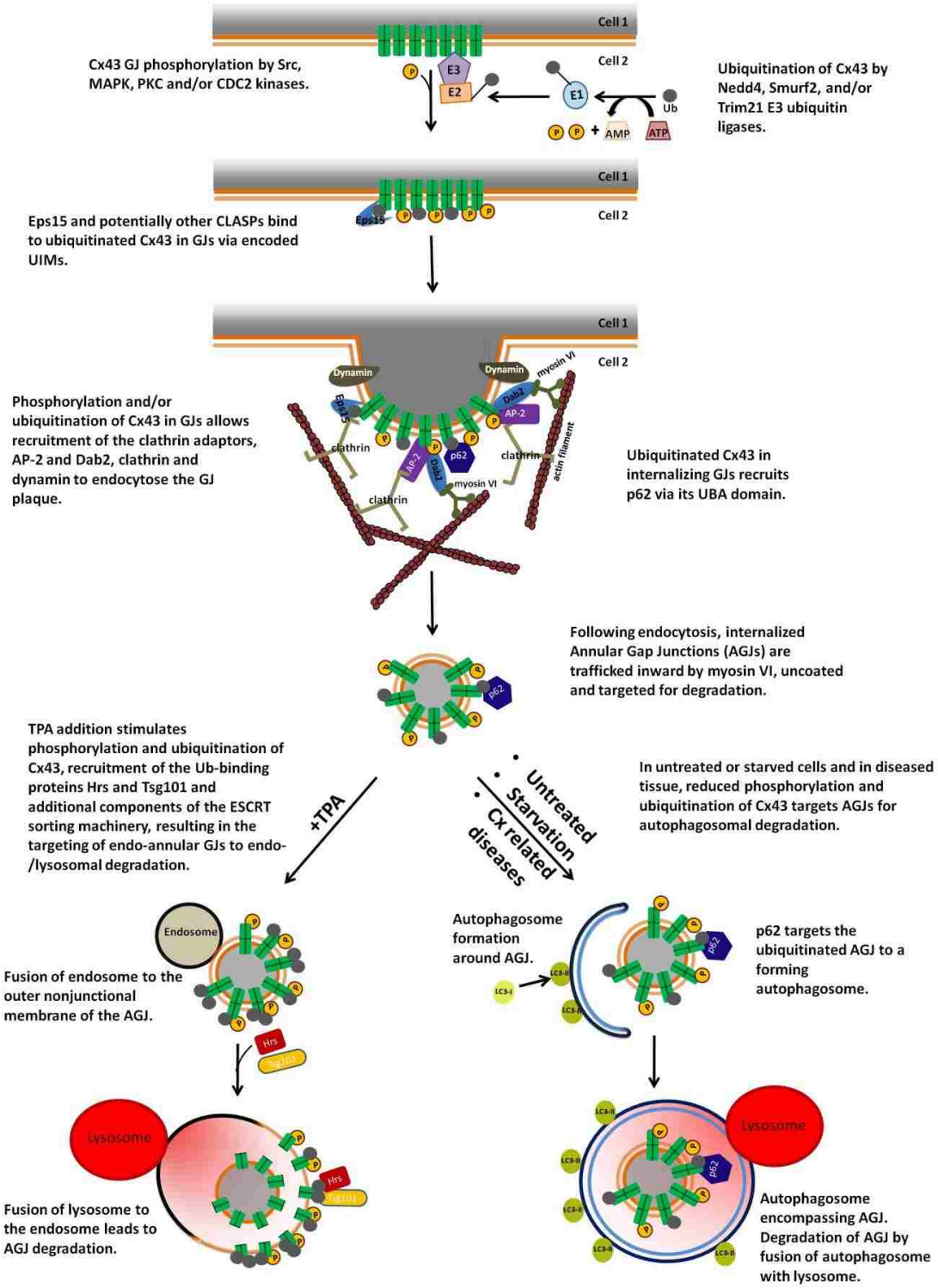


Figure 17: Schematic representation of the signals participating in the proposed steps that

lead to gap junction internalization, formation of annular gap junctions in the cytoplasm of the acceptor cell, and annular gap junction degradation through the phago-/lysosomal or the endo-/lysosomal pathway based on studies published in the literature. (Abbreviations are: AGJ, annular gap junction; CLASPs, clathrin-coat-associated proteins; ESCRT, endosomal sorting complexes required for transport; GJ, gap junction; UBA, ubiquitin-associated domain; UIMs, ubiquitin-interacting motifs (Falk et al., 2014).

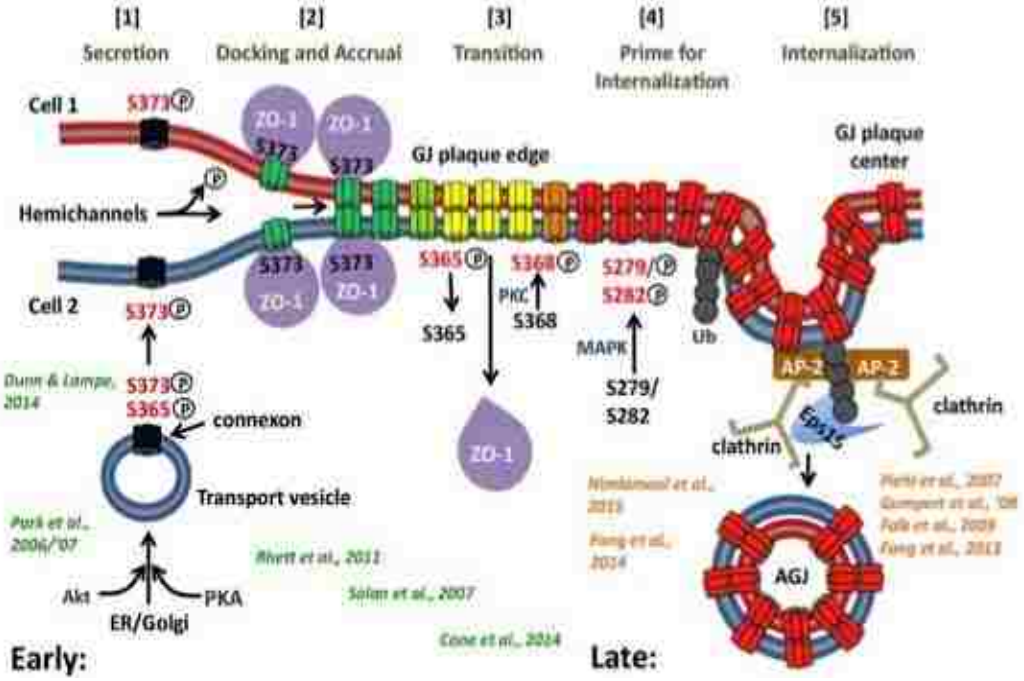


Figure 18: Scheme depicting the molecular signals in the Cx43-C-terminal domain hypothesized to regulate gap junction assembly and internalization based on our own (orange) and colleagues' (green) findings. Steps [1– 5] trigger and coordinate the transition from functional (green) into non-functional, internalization-prone gap junction channels (yellow, orange) that then are primed via post-translational modifications to allow interaction with clathrin components to mediate their internalization (red) (Falk et al., 2016).

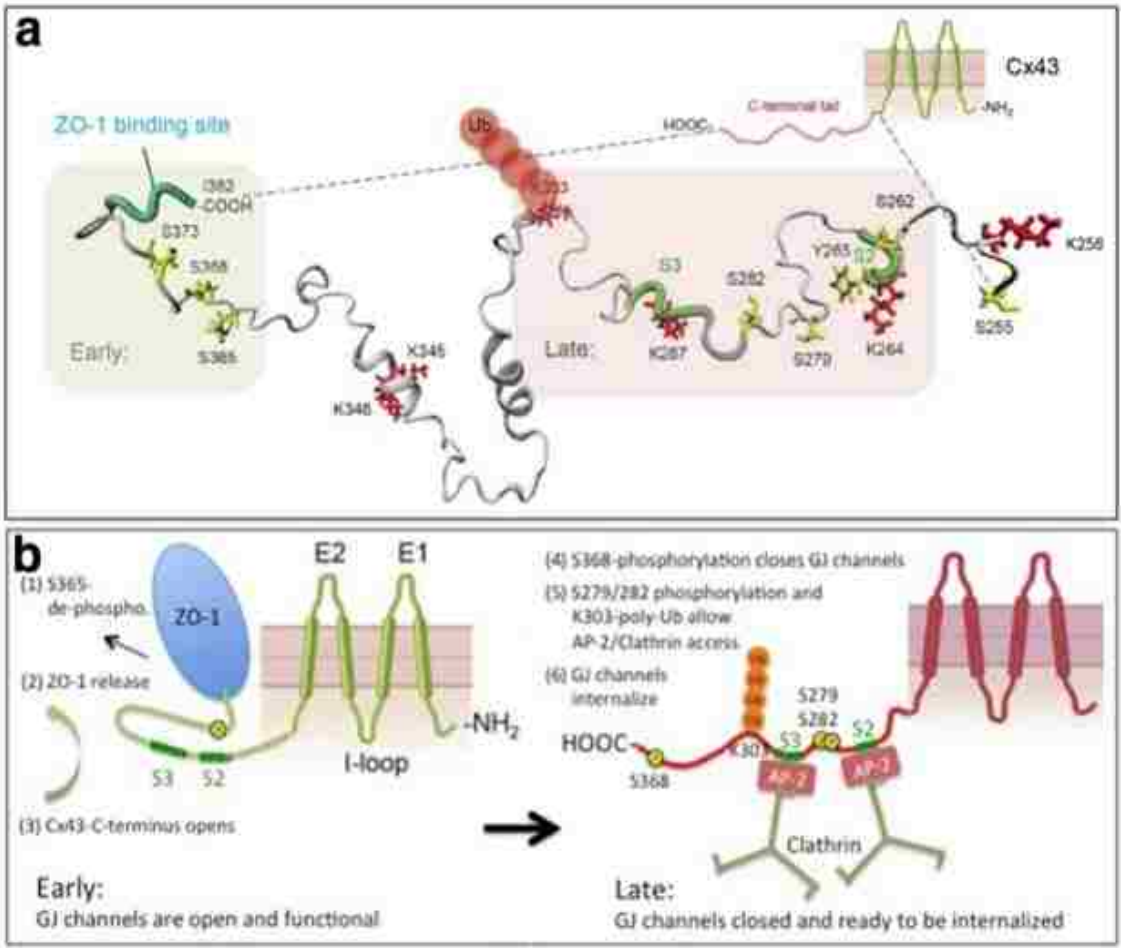


Figure 19: Scheme depicting how access of clathrin to Cx43 might be regulated. a Interestingly, all proposed Cx43 modifications relevant to Cx43 gap junction internalization cluster into two domains, 'early' occurring on residues located juxtaposed to the C-terminal ZO-1 binding site (shaded green), and 'late' occurring on residues located juxtaposed to the S2, S3 AP-2 (Eps 15)/clathrin binding sites (shaded red). The lowest energy 3D solution NMR structure of the Cx43-CT revealing the location of critical residues solved by Sorgen and colleagues [117] is shown. b We propose that a conformational change of the Cx43-C-terminal domain (CT) triggered by serine 365 de-phosphorylation [116] opens up the Cx43-CT allowing MAPK to access and phosphorylate S279/282 (and eventually also S262 and S255); and E3-ubiquitin ligases to bind to and ubiquitinate Cx43 (presumably on lysine 303) to promote AP-2 (and/or Eps15) to access the YXXΦ -binding motifs (S2, 265YAYF268; S3,286YKLV289), recruit clathrin and internalize gap junctions/central gap junction plaque portions (Falk et al., 2016).

References

Baker, S.M., N. Kim, A.M. Gumpert, D. Segretain, and M.M. Falk. 2008. Acute internalization of gap junctions in vascular endothelial cells in response to inflammatory mediator-induced G-protein coupled receptor activation. *FEBS letters*. 582:4039-4046.

Baldwin, A.L., and G. Thurston. 2001. Mechanics of endothelial cell architecture and vascular permeability. *Crit Rev Biomed Eng*. 29:247-278.

Basheer, W.A., B.S. Harris, H.L. Mentrup, M. Abreha, E.L. Thames, J.B. Lea, D.A. Swing, N.G. Copeland, N.A. Jenkins, R.L. Price, and L.E. Matesic. 2015. Cardiomyocyte-specific overexpression of the ubiquitin ligase Wwp1 contributes to reduction in Connexin 43 and arrhythmogenesis. *J Mol Cell Cardiol*. 88:1-13.

Batra, N., R. Kar, and J.X. Jiang. 2012. Gap junctions and hemichannels in signal transmission, function and development of bone. *Biochimica et biophysica acta*. 1818:1909-1918.

Beardslee, M.A., J.G. Laing, E.C. Beyer, and J.E. Saffitz. 1998. Rapid turnover of connexin43 in the adult rat heart. *Circ Res*. 83:629-635.

Bejarano, E., H. Girao, A. Yuste, B. Patel, C. Marques, D.C. Spray, P. Pereira, and A.M. Cuervo. 2012. Autophagy modulates dynamics of connexins at the plasma membrane in a ubiquitin-dependent manner. *Mol Biol Cell*. 23:2156-2169.

Belouzard, S., and Y. Rouille. 2006. Ubiquitylation of leptin receptor OB-Ra regulates its clathrin-mediated endocytosis. *EMBO J*. 25:932-942.

Berthoud, V.M., P.J. Minogue, J.G. Laing, and E.C. Beyer. 2004. Pathways for degradation of connexins and gap junctions. *Cardiovasc Res*. 62:256-267.

Bjorkoy, G., T. Lamark, A. Brech, H. Outzen, M. Perander, A. Overvatn, H. Stenmark, and T. Johansen. 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol*. 171:603-614.

Blomstrand, F., L. Venance, A.L. Siren, P. Ezan, E. Hanse, J. Glowinski, H. Ehrenreich, and C. Giaume. 2004. Endothelins regulate astrocyte gap junctions in rat hippocampal slices. *Eur J Neurosci*. 19:1005-1015.

Brock, T.A., H.F. Dvorak, and D.R. Senger. 1991. Tumor-secreted vascular permeability factor increases cytosolic Ca2+ and von Willebrand factor release in human endothelial cells. *Am J Pathol*. 138:213-221.

Catarino, S., J.S. Ramalho, C. Marques, P. Pereira, and H. Girao. 2011. Ubiquitin-mediated internalization of connexin43 is independent of the canonical endocytic tyrosine-sorting signal. *Biochem J*. 437:255-267.

Chang, E.H., G. Van Camp, and R.J. Smith. 2003. The role of connexins in human disease. *Ear Hear*. 24:314-323.

Chen, V.C., A.R. Kristensen, L.J. Foster, and C.C. Naus. 2012. Association of connexin43 with E3 ubiquitin ligase TRIM21 reveals a mechanism for gap junction phosphodegron control. *J Proteome Res*. 11:6134-6146.

Chen, Z.J., and L.J. Sun. 2009. Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell*. 33:275-286.

Ciani, B., R. Layfield, J.R. Cavey, P.W. Sheppard, and M.S. Searle. 2003. Structure of the ubiquitin-associated domain of p62 (SQSTM1) and implications for mutations that cause Paget's disease of bone. *J Biol Chem*. 278:37409-37412.

Cone, A.C., G. Cavin, C. Ambrosi, H. Hakozaki, A.X. Wu-Zhang, M.T. Kunkel, A.C. Newton, and G.E. Sosinsky. 2014. Protein kinase Cdelta-mediated phosphorylation of Connexin43 gap junction channels causes movement within gap junctions followed by vesicle internalization and protein degradation. *J Biol Chem*. 289:8781-8798.

Cooper, C.D., and P.D. Lampe. 2002. Casein kinase 1 regulates connexin-43 gap junction assembly. *J Biol Chem*. 277:44962-44968.

Cronier, L., S. Crespin, P.O. Strale, N. Defamie, and M. Mesnil. 2009. Gap junctions and cancer: new functions for an old story. *Antioxid Redox Signal*. 11:323-338.

de Vries, C., J.A. Escobedo, H. Ueno, K. Houck, N. Ferrara, and L.T. Williams. 1992. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science*. 255:989-991.

Ding, W.X., and X.M. Yin. 2008. Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. *Autophagy*. 4:141-150.
Dukes, J.D., P. Whitley, and A.D. Chalmers. 2011. The MDCK variety pack: choosing the right strain. *BMC cell biology*. 12:43.
Dunn, C.A., and P.D. Lampe. 2014. Injury-triggered Akt phosphorylation of Cx43: a ZO-1-driven molecular switch that regulates gap junction size. *J Cell Sci*. 127:455-464.
Dupont, E., T. Matsushita, R.A. Kaba, C. Vozzi, S.R. Coppen, N. Khan, R. Kaprielian, M.H. Yacoub, and N.J. Severs. 2001. Altered connexin expression in human congestive heart failure. *J Mol Cell Cardiol*. 33:359-371.
Dutta, D., C.D. Williamson, N.B. Cole, and J.G. Donaldson. 2012. Pitstop 2 is a potent inhibitor of clathrin-independent endocytosis. *PloS one*. 7:e45799.
Falk, M.M. 2000. Connexin-specific distribution within gap junctions revealed in living cells. *J Cell Sci*. 113 (Pt 22):4109-4120.
Falk, M.M., S.M. Baker, A.M. Gumpert, D. Segretain, and R.W. Buckheit, 3rd. 2009. Gap junction turnover is achieved by the internalization of small endocytic double-membrane vesicles. *Mol Biol Cell*. 20:3342-3352.
Falk, M.M., C.L. Bell, R.M. Kells Andrews, and S.A. Murray. 2016. Molecular mechanisms regulating formation, trafficking and processing of annular gap junctions. *BMC cell biology*. 17 Suppl 1:22.
Falk, M.M., R.M. Kells, and V.M. Berthoud. 2014. Degradation of connexins and gap junctions. *FEBS letters*. 588:1221-1229.
Fallon, R.F., and D.A. Goodenough. 1981. Five-hour half-life of mouse liver gap-junction protein. *J Cell Biol*. 90:521-526.
Ferrara, N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*. 25:581-611.
Fong, J.T., R.M. Kells, and M.M. Falk. 2013. Two tyrosine-based sorting signals in the Cx43 C-terminus cooperate to mediate gap junction endocytosis. *Mol Biol Cell*. 24:2834-2848.
Fong, J.T., R.M. Kells, A.M. Gumpert, J.Y. Marzillier, M.W. Davidson, and M.M. Falk. 2012. Internalized gap junctions are degraded by autophagy. *Autophagy*. 8:794-811.
Fong, J.T., W. Nimlamool, and M.M. Falk. 2014. EGF induces efficient Cx43 gap junction endocytosis in mouse embryonic stem cell colonies via phosphorylation of Ser262, Ser279/282, and Ser368. *FEBS letters*. 588:836-844.
Fontes, M.S., T.A. van Veen, J.M. de Bakker, and H.V. van Rijen. 2012. Functional consequences of abnormal Cx43 expression in the heart. *Biochimica et biophysica acta*. 1818:2020-2029.
Fykerud, T.A., A. Kjenseth, K.O. Schink, S. Sirnes, J. Bruun, Y. Omori, A. Brech, E. Rivedal, and E. Leithe. 2012. Smad ubiquitination regulatory factor-2 controls gap junction intercellular communication by modulating endocytosis and degradation of connexin43. *J Cell Sci*. 125:3966-3976.
Gaietta, G., T.J. Deerinck, S.R. Adams, J. Bouwer, O. Tour, D.W. Laird, G.E. Sosinsky, R.Y. Tsien, and M.H. Ellisman. 2002. Multicolor and electron microscopic imaging of connexin trafficking. *Science*. 296:503-507.
Geetha, T., J. Jiang, and M.W. Wooten. 2005. Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Mol Cell*. 20:301-312.
Ghoshroy, S., D.A. Goodenough, and G.E. Sosinsky. 1995. Preparation, characterization, and structure of half gap junctional layers split with urea and EGTA. *J Membr Biol*. 146:15-28.
Giepmans, B.N., I. Verlaan, T. Hengeveld, H. Janssen, J. Calafat, M.M. Falk, and W.H. Moolenaar. 2001. Gap junction protein connexin-43 interacts directly with microtubules. *Curr Biol*. 11:1364-1368.
Gilleron, J., D. Carette, C. Fiorini, M. Benkdane, D. Segretain, and G. Pointis. 2009. Connexin 43 gap junction plaque endocytosis implies molecular remodelling of ZO-1 and c-Src partners. *Communicative & integrative biology*. 2:104-106.
Gilleron, J., D. Carette, C. Fiorini, J. Dompierre, E. Macia, J.-P. Denizot, D. Segretain, and G. Pointis. 2011. The large GTPase dynamin2: a new player in connexin 43 gap junction endocytosis, recycling and degradation. *The international journal of biochemistry & cell biology*. 43:1208-1217.

Gilleron, J., C. Fiorini, D. Carette, C. Avondet, M.M. Falk, D. Segretain, and G. Pointis. 2008. Molecular reorganization of Cx43, Zo-1 and Src complexes during the endocytosis of gap junction plaques in response to a non-genomic carcinogen. *J Cell Sci*. 121:4069-4078.
Ginzberg, R.D., and N.B. Gilula. 1979. Modulation of cell junctions during differentiation of the chicken otocyst sensory epithelium. *Dev Biol*. 68:110-129.
Girao, H., S. Catarino, and P. Pereira. 2009. Eps15 interacts with ubiquitinated Cx43 and mediates its internalization. *Exp Cell Res*. 315:3587-3597.
Goodenough, D.A., and N.B. Gilula. 1974. The splitting of hepatocyte gap junctions and zonulae occludentes with hypertonic disaccharides. *J Cell Biol*. 61:575-590.
Gumpert, A.M., J.S. Varco, S.M. Baker, M. Piehl, and M.M. Falk. 2008. Double-membrane gap junction internalization requires the clathrin-mediated endocytic machinery. *FEBS letters*. 582:2887-2892.
Gutierrez, M.G., S.S. Master, S.B. Singh, G.A. Taylor, M.I. Colombo, and V. Deretic. 2004. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell*. 119:753-766.
Hansen, S.H., K. Sandvig, and B. van Deurs. 1993. Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. *J Cell Biol*. 121:61-72.
Hawryluk, M.J., P.A. Keyel, S.K. Mishra, S.C. Watkins, J.E. Heuser, and L.M. Traub. 2006. Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. *Traffic*. 7:262-281.
He, C., and D.J. Klionsky. 2009. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet*. 43:67-93.
Hesketh, G.G., M.H. Shah, V.L. Halperin, C.A. Cooke, F.G. Akar, T.E. Yen, D.A. Kass, C.E. Machamer, J.E. Van Eyk, and G.F. Tomaselli. 2010. Ultrastructure and regulation of lateralized connexin43 in the failing heart. *Circ Res*. 106:1153-1163.
Hesketh, G.G., J.E. Van Eyk, and G.F. Tomaselli. 2009. Mechanisms of gap junction traffic in health and disease. *J Cardiovasc Pharmacol*. 54:263-272.
Heuser, J.E., and R.G. Anderson. 1989. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol*. 108:389-400.
Hicke, L. 2001. Protein regulation by monoubiquitin. *Nature reviews. Molecular cell biology*. 2:195-201.
Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol*. 19:141-172.
Hospenthal, M.K., T.E. Mevissen, and D. Komander. 2015. Deubiquitinase-based analysis of ubiquitin chain architecture using Ubiquitin Chain Restriction (UbiCRest). *Nature protocols*. 10:349-361.
Huang, X.-D., M. Horackova, and M.L. Pressler. 1996. Changes in the expression and distribution of connexin 43 in isolated cultured adult guinea pig cardiomyocytes. *Experimental cell research*. 228:254-261.
Hung, S.Y., W.P. Huang, H.C. Liou, and W.M. Fu. 2009. Autophagy protects neuron from Abeta-induced cytotoxicity. *Autophagy*. 5:502-510.
Ikeda, F., N. Crosetto, and I. Dikic. 2010. What determines the specificity and outcomes of ubiquitin signaling? *Cell*. 143:677-681.
Johnson, K.E., S. Mitra, P. Katoch, L.S. Kelsey, K.R. Johnson, and P.P. Mehta. 2013. Phosphorylation on Ser-279 and Ser-282 of connexin43 regulates endocytosis and gap junction assembly in pancreatic cancer cells. *Mol Biol Cell*. 24:715-733.
Jongsma, H.J., and R. Wilders. 2000. Gap junctions in cardiovascular disease. *Circ Res*. 86:1193-1197.
Jordan, K., R. Chodock, A.R. Hand, and D.W. Laird. 2001. The origin of annular junctions: a mechanism of gap junction internalization. *J Cell Sci*. 114:763-773.
Kanemitsu, M.Y., W. Jiang, and W. Eckhart. 1998. Cdc2-mediated phosphorylation of the gap junction protein, connexin43, during mitosis. *Cell Growth Differ*. 9:13-21.
Kevil, C.G., D.K. Payne, E. Mire, and J.S. Alexander. 1998. Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. *Journal of Biological Chemistry*. 273:15099-15103.
Kihara, A., Y. Kabeya, Y. Ohsumi, and T. Yoshimori. 2001. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep*. 2:330-335.
Kim, H.C., and J.M. Huibregtse. 2009. Polyubiquitination by HECT E3s and the determinants of chain type specificity. *Mol Cell Biol*. 29:3307-3318.

Kim, H.T., K.P. Kim, F. Lledias, A.F. Kisselev, K.M. Scaglione, D. Skowyra, S.P. Gygi, and A.L. Goldberg. 2007. Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. *J Biol Chem.* 282:17375-17386.

King, T.J., and P.D. Lampe. 2005. Temporal regulation of connexin phosphorylation in embryonic and adult tissues. *Biochimica et biophysica acta*. 1719:24-35.

Kjenseth, A., T. Fykerud, E. Rivedal, and E. Leithe. 2010. Regulation of gap junction intercellular communication by the ubiquitin system. *Cell Signal.* 22:1267-1273.

Komander, D., and M. Rape. 2012. The ubiquitin code. *Annu Rev Biochem.* 81:203-229.

Kostin, S., M. Rieger, S. Dammer, S. Hein, M. Richter, W.P. Klovekorn, E.P. Bauer, and J. Schaper. 2003. Gap junction remodeling and altered connexin43 expression in the failing human heart. *Mol Cell Biochem.* 242:135-144.

Kroll, J., and J. Waltenberger. 1997. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. *Journal of Biological Chemistry*. 272:32521-32527.

Laing, J.G., and E.C. Beyer. 1995. The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. *J Biol Chem*. 270:26399-26403.

Laing, J.G., P.N. Tadros, E.M. Westphale, and E.C. Beyer. 1997. Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. *Exp Cell Res*. 236:482-492.

Laird, D.W. 2005. Connexin phosphorylation as a regulatory event linked to gap junction internalization and degradation. *Biochimica et biophysica acta*. 1711:172-182.

Lampe, P.D., W.E. Kurata, B.J. Warn-Cramer, and A.F. Lau. 1998. Formation of a distinct connexin43 phosphoisoform in mitotic cells is dependent upon p34cdc2 kinase. *J Cell Sci*. 111 (Pt 6):833-841.

Lampe, P.D., and A.F. Lau. 2004. The effects of connexin phosphorylation on gap junctional communication. *Int J Biochem Cell Biol*. 36:1171-1186.

Lampe, P.D., E.M. TenBroek, J.M. Burt, W.E. Kurata, R.G. Johnson, and A.F. Lau. 2000. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol*. 149:1503-1512.

Larsen, W.J., H.N. Tung, S.A. Murray, and C.A. Swenson. 1979. Evidence for the participation of actin microfilaments and bristle coats in the internalization of gap junction membrane. *J Cell Biol.* 83:576-587.

Lauf, U., B.N. Giepmans, P. Lopez, S. Braconnot, S.C. Chen, and M.M. Falk. 2002. Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. *Proc Natl Acad Sci U S A*. 99:10446-10451.

Leach, D.H., and L.W. Oliphant. 1984. Degradation of annular gap junctions of the equine hoof wall. *Acta Anat (Basel).* 120:214-219.

Leithe, E., A. Brech, and E. Rivedal. 2006. Endocytic processing of connexin43 gap junctions: a morphological study. *Biochem J*. 393:59-67.

Leithe, E., A. Kjenseth, S. Sirnes, H. Stenmark, A. Brech, and E. Rivedal. 2009. Ubiquitylation of the gap junction protein connexin-43 signals its trafficking from early endosomes to lysosomes in a process mediated by Hrs and Tsg101. *J Cell Sci*. 122:3883-3893.

Leithe, E., and E. Rivedal. 2004a. Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43. *J Cell Sci.* 117:1211-1220.

Leithe, E., and E. Rivedal. 2004b. Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment. *J Biol Chem*. 279:50089-50096.

Leithe, E., S. Sirnes, T. Fykerud, A. Kjenseth, and E. Rivedal. 2012. Endocytosis and post-endocytic sorting of connexins. *Biochimica et biophysica acta*. 1818:1870-1879.

Leykauf, K., M. Durst, and A. Alonso. 2003. Phosphorylation and subcellular distribution of connexin43 in normal and stressed cells. *Cell Tissue Res*. 311:23-30.

Leykauf, K., M. Salek, J. Bomke, M. Frech, W.D. Lehmann, M. Durst, and A. Alonso. 2006. Ubiquitin protein ligase Nedd4 binds to connexin43 by a phosphorylation-modulated process. *J Cell Sci*. 119:3634-3642.

Lichtenstein, A., P.J. Minogue, E.C. Beyer, and V.M. Berthoud. 2010. Autophagy: a pathway that contributes to connexin degradation. *J Cell Sci*. 124:910-920.

Lin, X.-W., W.-C. Xu, J.-G. Luo, X.-J. Guo, T. Sun, X.-L. Zhao, and Z.-J. Fu. 2013. WW domain containing E3 ubiquitin protein ligase 1 (WWP1) negatively regulates TLR4-mediated TNF-α and IL-6 production by proteasomal degradation of TNF receptor associated factor 6 (TRAF6). *PloS one*. 8:e67633.

Luo, T., B.L. Fredericksen, K. Hasumi, A. Endo, and J.V. Garcia. 2001. Human immunodeficiency virus type 1 Nef-induced CD4 cell surface downregulation is inhibited by ikarugamycin. *Journal of virology*. 75:2488-2492.

Maass, K., J. Shibayama, S.E. Chase, K. Willecke, and M. Delmar. 2007. C-terminal truncation of connexin43 changes number, size, and localization of cardiac gap junction plaques. *Circulation research*. 101:1283-1291.

MacGurn, J.A., P.C. Hsu, and S.D. Emr. 2012. Ubiquitin and membrane protein turnover: from cradle to grave. *Annu Rev Biochem*. 81:231-259.

Macia, E., M. Ehrlich, R. Massol, E. Boucrot, C. Brunner, and T. Kirchhausen. 2006. Dynasore, a cell-permeable inhibitor of dynamin. *Developmental cell*. 10:839-850.

Matsushita, T., M. Oyamada, K. Fujimoto, Y. Yasuda, S. Masuda, Y. Wada, T. Oka, and T. Takamatsu. 1999. Remodeling of cell-cell and cell-extracellular matrix interactions at the border zone of rat myocardial infarcts. *Circ Res*. 85:1046-1055.

Mazet, F., B.A. Wittenberg, and D.C. Spray. 1985. Fate of intercellular junctions in isolated adult rat cardiac cells. *Circ Res*. 56:195-204.

McCullough, J., M.J. Clague, and S. Urbe. 2004. AMSH is an endosome-associated ubiquitin isopeptidase. *J Cell Biol*. 166:487-492.

McEwan, W.A., J.C. Tam, R.E. Watkinson, S.R. Bidgood, D.L. Mallery, and L.C. James. 2013. Intracellular antibody-bound pathogens stimulate immune signaling via the Fc receptor TRIM21. *Nature immunology*. 14:327-336.

Moreno, A.P. 2005. Connexin phosphorylation as a regulatory event linked to channel gating. *Biochimica et biophysica acta*. 1711:164-171.

Motley, A., N.A. Bright, M.N. Seaman, and M.S. Robinson. 2003. Clathrin-mediated endocytosis in AP-2–depleted cells. *The Journal of cell biology*. 162:909-918.

Musil, L.S., and D.A. Goodenough. 1991. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J Cell Biol*. 115:1357-1374.

Musil, L.S., A.C. Le, J.K. VanSlyke, and L.M. Roberts. 2000. Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J Biol Chem*. 275:25207-25215.

Nakagawa, I., A. Amano, N. Mizushima, A. Yamamoto, H. Yamaguchi, T. Kamimoto, A. Nara, J. Funao, M. Nakata, K. Tsuda, S. Hamada, and T. Yoshimori. 2004. Autophagy defends cells against invading group A Streptococcus. *Science*. 306:1037-1040.

Naus, C.C., S. Hearn, D. Zhu, B.J. Nicholson, and R.R. Shivers. 1993. Ultrastructural analysis of gap junctions in C6 glioma cells transfected with connexin43 cDNA. *Experimental cell research*. 206:72-84.

Nguyen, L.K., W. Kolch, and B.N. Kholodenko. 2013. When ubiquitination meets phosphorylation: a systems biology perspective of EGFR/MAPK signalling. *Cell communication and signaling : CCS*. 11:52.

Nickel, B., M. Boller, K. Schneider, T. Shakespeare, V. Gay, and S.A. Murray. 2013. Visualizing the effect of dynamin inhibition on annular gap vesicle formation and fission. *J Cell Sci*. 126:2607-2616.

Nickel, B.M., B.H. DeFranco, V.L. Gay, and S.A. Murray. 2008. Clathrin and Cx43 gap junction plaque endoexocytosis. *Biochemical and biophysical research communications*. 374:679-682.

Nimlamool, W., R.M. Kells Andrews, and M.M. Falk. 2015. Connexin43 phosphorylation by PKC and MAPK signals VEGF-mediated gap junction internalization. *Mol Biol Cell*. 26:2755-2768.

Ogawa, M., T. Yoshimori, T. Suzuki, H. Sagara, N. Mizushima, and C. Sasakawa. 2005. Escape of intracellular Shigella from autophagy. *Science*. 307:727-731.

Pahujaa, M., M. Anikin, and G.S. Goldberg. 2007. Phosphorylation of connexin43 induced by Src: regulation of gap junctional communication between transformed cells. *Exp Cell Res*. 313:4083-4090.

Pankiv, S., T.H. Clausen, T. Lamark, A. Brech, J.A. Bruun, H. Outzen, A. Overvatn, G. Bjorkoy, and T. Johansen. 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem*. 282:24131-24145.

Park, D.J., T.A. Freitas, C.J. Wallick, C.V. Guyette, and B.J. Warn-Cramer. 2006. Molecular dynamics and in vitro analysis of Connexin43: A new 14-3-3 mode-1 interacting protein. *Protein Sci*. 15:2344-2355.

Park, D.J., C.J. Wallick, K.D. Martyn, A.F. Lau, C. Jin, and B.J. Warn-Cramer. 2007. Akt phosphorylates Connexin43 on Ser373, a "mode-1" binding site for 14-3-3. *Cell Commun Adhes*. 14:211-226.

Peters, N.S., C.R. Green, P.A. Poole-Wilson, and N.J. Severs. 1993. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. *Circulation*. 88:864-875.

Petrich, B.G., X. Gong, D.L. Lerner, X. Wang, J.H. Brown, J.E. Saffitz, and Y. Wang. 2002. c-Jun N-terminal kinase activation mediates downregulation of connexin43 in cardiomyocytes. *Circ Res*. 91:640-647.

Pfeifer, U. 1980. Autophagic sequestration of internalized gap junctions in rat liver. *Eur J Cell Biol*. 21:244-246.

Piehl, M., C. Lehmann, A. Gumpert, J.P. Denizot, D. Segretain, and M.M. Falk. 2007. Internalization of large double-membrane intercellular vesicles by a clathrin-dependent endocytic process. *Mol Biol Cell*. 18:337-347.

Pohl, C., and S. Jentsch. 2009. Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. *Nat Cell Biol*. 11:65-70.

Polontchouk, L., B. Ebelt, M. Jackels, and S. Dhein. 2002. Chronic effects of endothelin 1 and angiotensin II on gap junctions and intercellular communication in cardiac cells. *FASEB J*. 16:87-89.

Postma, F.R., T. Hengeveld, J. Alblas, B.N. Giepmans, G.C. Zondag, K. Jalink, and W.H. Moolenaar. 1998. Acute loss of cell-cell communication caused by G protein-coupled receptors: a critical role for c-Src. *J Cell Biol*. 140:1199-1209.

Qi, J.H., and L. Claesson-Welsh. 2001. VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase. *Exp Cell Res*. 263:173-182.

Qin, H., Q. Shao, S.A. Igdoura, M.A. Alaoui-Jamali, and D.W. Laird. 2003. Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. *J Biol Chem*. 278:30005-30014.

Ravid, T., and M. Hochstrasser. 2008. Diversity of degradation signals in the ubiquitin-proteasome system. *Nature reviews. Molecular cell biology*. 9:679-690.

Ravikumar, B., S. Imarisio, S. Sarkar, C.J. O'Kane, and D.C. Rubinsztein. 2008. Rab5 modulates aggregation and toxicity of mutant huntingtin through macroautophagy in cell and fly models of Huntington disease. *J Cell Sci*. 121:1649-1660.

Rhett, J.M., and R.G. Gourdie. 2011. The perinexus: A new feature of Cx43 gap junction organization. *Heart Rhythm*. 9:619-623.

Ribeiro-Rodrigues, T.M., S. Catarino, M.J. Pinho, P. Pereira, and H. Girao. 2015. Connexin 43 ubiquitination determines the fate of gap junctions: restrict to survive. *Biochemical Society transactions*. 43:471-475.

Saez, J.C., A.C. Nairn, A.J. Czernik, G.I. Fishman, D.C. Spray, and E.L. Hertzberg. 1997. Phosphorylation of connexin43 and the regulation of neonatal rat cardiac myocyte gap junctions. *J Mol Cell Cardiol*. 29:2131-2145.

Sato, Y., A. Yoshikawa, A. Yamagata, H. Mimura, M. Yamashita, K. Ookata, O. Nureki, K. Iwai, M. Komada, and S. Fukai. 2008. Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature*. 455:358-362.

Scheffner, M., and S. Kumar. 2014. Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 1843:61-74.

Schmitt, M., K. Leykauf, E. Reinz, H. Cheng, A. Alonso, and J. Schenkel. 2014. Mutation of human connexin43 amino acids s279/s282 increases protein stability upon treatment with epidermal growth factor. *Cell biochemistry and biophysics*. 69:379-384.

Schnell, D.J., and D.N. Hebert. 2003. Protein translocons: multifunctional mediators of protein translocation across membranes. *Cell*. 112:491-505.

Seibenhener, M.L., J.R. Babu, T. Geetha, H.C. Wong, N.R. Krishna, and M.W. Wooten. 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. *Mol Cell Biol*. 24:8055-8068.

Severs, N.J., K.S. Shovel, A.M. Slade, T. Powell, V.W. Twist, and C.R. Green. 1989. Fate of gap junctions in isolated adult mammalian cardiomyocytes. *Circ Res.* 65:22-42.
Shaner, N.C., M.Z. Lin, M.R. McKeown, P.A. Steinbach, K.L. Hazelwood, M.W. Davidson, and R.Y. Tsien. 2008. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat Methods*. 5:545-551.
Shih, S.C., D.J. Katzmann, J.D. Schnell, M. Sutanto, S.D. Emr, and L. Hicke. 2002. Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat Cell Biol*. 4:389-393.
Simon, A.M. 1999. Gap junctions: more roles and new structural data. *Trends Cell Biol*. 9:169-170.
Sirnes, S., A. Kjenseth, E. Leithe, and E. Rivedal. 2009. Interplay between PKC and the MAP kinase pathway in Connexin43 phosphorylation and inhibition of gap junction intercellular communication. *Biochem Biophys Res Commun*. 382:41-45.
Solan, J.L., and P.D. Lampe. 2005. Connexin phosphorylation as a regulatory event linked to gap junction channel assembly. *Biochimica et biophysica acta*. 1711:154-163.
Solan, J.L., and P.D. Lampe. 2007. Key connexin 43 phosphorylation events regulate the gap junction life cycle. *J Membr Biol*. 217:35-41.
Solan, J.L., and P.D. Lampe. 2008. Connexin 43 in LA-25 cells with active v-src is phosphorylated on Y247, Y265, S262, S279/282, and S368 via multiple signaling pathways. *Cell Commun Adhes*. 15:75-84.
Solan, J.L., and P.D. Lampe. 2009. Connexin43 phosphorylation: structural changes and biological effects. *Biochem J*. 419:261-272.
Solan, J.L., and P.D. Lampe. 2014. Specific Cx43 phosphorylation events regulate gap junction turnover in vivo. *FEBS letters*. 588:1423-1429.
Solan, J.L., and P.D. Lampe. 2015. Kinase programs spatiotemporally regulate gap junction assembly and disassembly: Effects on wound repair. *Seminars in cell & developmental biology*. 50:40-48.
Solan, J.L., L. Marquez-Rosado, P.L. Sorgen, P.J. Thornton, P.R. Gafken, and P.D. Lampe. 2007. Phosphorylation at S365 is a gatekeeper event that changes the structure of Cx43 and prevents down-regulation by PKC. *The Journal of Cell Biology*. 179:1301-1309.
Spagnol, G., M. Al-Mugotir, J.L. Kopanic, S. Zach, H. Li, A.J. Trease, K.L. Stauch, R. Grosely, M. Cervantes, and P.L. Sorgen. 2016. Secondary structural analysis of the carboxyl-terminal domain from different connexin isoforms. *Biopolymers*. 105:143-162.
Spinella, F., L. Rosano, V. Di Castro, M.R. Nicotra, P.G. Natali, and A. Bagnato. 2003. Endothelin-1 decreases gap junctional intercellular communication by inducing phosphorylation of connexin 43 in human ovarian carcinoma cells. *J Biol Chem*. 278:41294-41301.
Stahl, P.D., and M.A. Barbieri. 2002. Multivesicular bodies and multivesicular endosomes: the "ins and outs" of endosomal traffic. *Sci STKE*. 2002:pe32.
Su, V., and A.F. Lau. 2014. Connexins: mechanisms regulating protein levels and intercellular communication. *FEBS letters*. 588:1212-1220.
Su, V., R. Nakagawa, M. Koval, and A.F. Lau. 2010. Ubiquitin-independent proteasomal degradation of endoplasmic reticulum-localized connexin43 mediated by CIP75. *J Biol Chem*. 285:40979-40990.
Suarez, S., and K. Ballmer-Hofer. 2001. VEGF transiently disrupts gap junctional communication in endothelial cells. *J Cell Sci*. 114:1229-1235.
Sulkowski, S., M. Sulkowska, and E. Skrzydlewska. 1999. Gap junctional intercellular communication and carcinogenesis. *Pol J Pathol*. 50:227-233.
Suzuma, K., K. Naruse, I. Suzuma, N. Takahara, K. Ueki, L.P. Aiello, and G.L. King. 2000. Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. *J Biol Chem*. 275:40725-40731.
Takahashi, T., S. Yamaguchi, K. Chida, and M. Shibuya. 2001. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. *EMBO J*. 20:2768-2778.
Thévenin, A.F., T.J. Kowal, J.T. Fong, R.M. Kells, C.G. Fisher, and M.M. Falk. 2013. Proteins and Mechanisms Regulating Gap Junction Assembly, Internalization and Degradation *Physiology*. 28:93-116.

Thuringer, D. 2004. The vascular endothelial growth factor-induced disruption of gap junctions is relayed by an autocrine communication via ATP release in coronary capillary endothelium. *Ann N Y Acad Sci*. 1030:14-27.

Traub, L.M., and J.S. Bonifacino. 2013. Cargo recognition in clathrin-mediated endocytosis. *Cold Spring Harbor perspectives in biology*. 5:a016790.

van Zeijl, L., B. Ponsioen, B.N. Giepmans, A. Ariaens, F.R. Postma, P. Varnai, T. Balla, N. Divecha, K. Jalink, and W.H. Moolenaar. 2007. Regulation of connexin43 gap junctional communication by phosphatidylinositol 4,5-bisphosphate. *J Cell Biol*. 177:881-891.

Wagner, S.A., P. Beli, B.T. Weinert, M.L. Nielsen, J. Cox, M. Mann, and C. Choudhary. 2011. A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol Cell Proteomics*. 10:M111 013284.

Waltenberger, J., L. Claesson-Welsh, A. Siegbahn, M. Shibuya, and C.H. Heldin. 1994. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem*. 269:26988-26995.

Warn-Cramer, B.J., and A.F. Lau. 2004. Regulation of gap junctions by tyrosine protein kinases. *Biochimica et biophysica acta*. 1662:81-95.

Wayakanon, P., R. Bhattacharjee, K. Nakahama, and I. Morita. 2012. The role of the Cx43 C-terminus in GJ plaque formation and internalization. *Biochemical and biophysical research communications*. 420:456-461.

Wilkinson, C.R., M. Seeger, R. Hartmann-Petersen, M. Stone, M. Wallace, C. Semple, and C. Gordon. 2001. Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nat Cell Biol*. 3:939-943.

Wu, X., X. Zhao, L. Baylor, S. Kaushal, E. Eisenberg, and L.E. Greene. 2001. Clathrin exchange during clathrin-mediated endocytosis. *J Cell Biol*. 155:291-300.

Ye, Y., G. Blaser, M.H. Horrocks, M.J. Ruedas-Rama, S. Ibrahim, A.A. Zhukov, A. Orte, D. Klenerman, S.E. Jackson, and D. Komander. 2012. Ubiquitin chain conformation regulates recognition and activity of interacting proteins. *Nature*. 492:266-270.

Curriculum Vitae

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Talks

- Ubiquitination and phosphorylation as regulatory mechanisms of Cx43 gap junction turnover. Rachael Kells Andrews. Sept 16, 2014. Graduate student seminar series, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.
- Ubiquitin: Its role in Cx43 gap junction internalization and degradation. Rachael Kells. March 3, 2013. Graduate student seminar series, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.

Publications

- Kells Andrews, R.M., and Falk, M.M. Phosphorylation-induced K63-polyubiquitination of Connexin 43 is essential for gap junction internalization (in review).

- Falk, M.M., Fisher, C.G., Kells Andrews, R.M., and Kowal, T.J. (2016). Imaging gap junctions in living cells. In D. Bai & J. Saez (Eds.), Gap Junction Channels and Hemichannels (pp. 21-62). Boca Raton, FL: Taylor & Francis Group.

- Falk, M.M., Bell, C.L., Kells Andrews, R.M., and Murray, S.A. (2016). Molecular mechanisms regulating formation, trafficking and processing of annular gap junctions. BMC Cell Biol, 17(Suppl 1), 22.

- Nimlamool, W., Andrews, R.M.K., and Falk, M. (2015). Connexin43 phosphorylation by PKC and MAPK signals VEGF-mediated gap junction internalization. Mol Biol Cell, 26(15), 2755-2768.

- Falk, M.M., Kells, R.M., and Berthoud, V.M. (2014). Degradation of connexins and gap junctions. FEBS Lett, 588(8), 1221-1229.

- Fong, J.T., Kells, R.M., and Falk, M.M. (2013). Two tyrosine-based sorting signals in the Cx43 C-terminus cooperate to mediate gap junction endocytosis. Mol Biol Cell, 24(18), 2834-2848.

- Thévenin, A.F., Kowal, T.J., Fong, J.T., Kells, R.M., Fisher, C.G., & Falk, M.M. (2013). Proteins and mechanisms regulating GJ assembly, internalization and degradation. Physiology, 28(2), 93-116.

- Falk, M.M., Fong, J.T., Kells, R.M., O'Laughlin, M.C., Kowal, T.J., & Thévenin, A.N. (2012). Degradation of endocytosed gap junctions by autophagosomal and endo-/lysosomal pathways: A perspective. J Membr Biol, 245(8), 465–476.

- Fong, J., Kells, R., Gumpert, A., Davidson, M., Marzillier, J., & Falk, M. (2012). Internalized gap junctions are degraded by autophagy. Autophagy, 8(5), 794–811.

Poster Presentations

- Kells Andrews, R.M. and Falk, M.M. 2016. Ubiquitination and phosphorylation as regulatory mechanisms of Cx43 gap junction turnover. Oct 14th, 4th Biophysical Society Pennsylvania Network Meeting, Lehigh University, Bethlehem, Pennsylvania.

- Margraf, R.A., Kells Andrews, R.M. and Falk, M.M. 2016. Distribution of Phosphorylated Connexin 43 in Gap Junctions: Implications for Gap Junction Internalization. April 21st, Lehigh Valley Molecular and Cell Biology Symposium. DeSales University, Center Valley, Pennsylvania.

- Kells Andrews, R.M. and Falk, M.M. 2016. Ubiquitination and phosphorylation as regulatory mechanisms of Cx43 gap junction turnover. Feb 4th, Graduate Student Open House, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.

- Kells Andrews, R.M. and Falk, M.M. 2015. Ubiquitination and phosphorylation as regulatory mechanisms of Cx43 gap junction turnover. Jan 29th, Graduate Student Open House, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.

- Kells Andrews, R.M. and Falk, M.M. 2014. Ubiquitination and phosphorylation as regulatory mechanisms of Cx43 gap junction turnover. Dec. 8th, American Society for Cell Biology, Philadelphia, Pennsylvania.

- Kells, R.M., and Falk, M.M. 2014. Ubiquitination and phosphorylation as regulatory mechanisms of Cx43 gap junction turnover. May 12th, Delaware Membrane Protein Symposium, Newark, Delaware.

- Kells, R.M. and Falk, M.M. 2014. Ubiquitination and phosphorylation as regulatory mechanisms of Cx43 gap junction turnover. Jan 3rd, Graduate Student Open House, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.

- Kells, R.M., Fong, J.T., and Falk, M.M. 2013. Ubiquitin: its role in the internalization and autophagic degradation of Cx43 gap junctions. July 14th, International Gap Junction Conference, Charleston, South Carolina.

- Kells, R.M, Fong, J.T. and Falk, M.M. 2013. Ubiquitin: it's role in Cx43 gap junction internalization and degradation. April 29th, Delaware Membrane Protein Symposium, Newark, Delaware.

- Kells, R.M., Fong, J.T. and Falk, M.M. 2013. Ubiquitin: it's role in Cx43 gap junction internalization and degradation. March 5th, CAS research symposium, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.

- Kells, R., Fong, J., Gumpert, A., Davidson, M., Marzillier, J., and Falk, M. 2012. Internalized gap junctions are degraded by autophagy. Feb 14th, Graduate Student Open House, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.

- Kells, R., Fong, J., Gumpert, A., Davidson, M., Marzillier, J., and Falk, M. 2012. Internalized gap junctions are degraded by autophagy. May 14th, Delaware Membrane Protein Symposium, Newark, Delaware.

- Kells, R., Fong, J., Gumpert, A., Davidson, M., Marzillier, J., and Falk, M. 2012. Internalized gap junctions are degraded by autophagy. Jan 26th, Graduate Student Open House, Dept. Biol. Sci., Lehigh University, Bethlehem, Pennsylvania.

- Kells, R., Fong, J., Gumpert, A., Davidson, M., Marzillier, J., and Falk, M. 2011. Internalized gap junctions are degraded by autophagy. Dec 3rd, American Society for Cell Biology, Denver, Colorado.

- Kells, R., Coons, R., Bergsten, S., and Light, T. 2010. AFLP Analysis of O. obscurus and O. rusticus crayfish to determine hybridization. April 20th, Celebration of Student Research Conference, Shippensburg, Pennsylvania.

- Burke, K., Kells, R., Kohler, E., and Light, T. 2008. An investigation into hybridization between two related species of crayfish of the same genus in Central Pennsylvania. April 19th, Mid-Atlantic Ecology Conference. Wilkes Barre, Pennsylvania.

Professional Conferences

- 2016 4th Biophysical Society Pennsylvania Network Meeting. Bethlehem, Pennsylvania
- 2016 Advancing Technology for Business Growth Biotech and Biomedical Engineering. May 18th. Lehigh University - Ben Franklin TechVentures Building. Bethlehem, Pennsylvania
- 2016 Lehigh Valley Molecular and Cell Biology Symposium. April 21st. Center Valley, Pennsylvania

- 2014 American Society for Cell Biology 54th Annual Meeting. Dec. 6th-10th. Philadelphia, Pennsylvania
- 2014 Delaware Membrane Protein Symposium. May 12th. Newark, Delaware
- 2013 International Gap Junction Conference. July 13th-18th. Charleston, South Carolina
- 2013 Delaware Membrane Protein Symposium. April 29th. Newark, Delaware
- 2012 Biophysical Society Pennsylvania Network Meeting. Sept. 14th. Bethlehem, Pennsylvania
- 2012 Delaware Membrane Protein Symposium. May 14th. Newark, Delaware
- 2011 American Society for Cell Biology 51st Annual Meeting. Dec. 3rd -7th. Denver, Colorado
- 2010 American Society for Cell Biology 50th Annual Meeting. Dec. 10th -15th. Philadelphia, Pennsylvania
- 2008 Mid-Atlantic Ecology Conference, April 19th, Wilkes Barre, Pennsylvania

Professional Affiliations

- Student Member, Lehigh Valley Molecular and Cell Biology Society, since 2016.
- Pre-doctoral Student Member, The American Society for Cell Biology, since 2010.

Honors and Awards

2016 Marjorie Nemes Fellowship

Spring 2016

Awarded on a competitive basis to recognize exceptional students who have demonstrated excellence in research and have made significant progress toward their Ph.D.

2014-2015 GAANN (Graduate Assistance in Areas of National Need) Fellowship

Spring 2014-Fall 2015

Assists graduate students with excellent records who demonstrate financial need and plan to pursue the highest degree available in their course study at the institution in a field designated as an area of national need.

2013 Nominee for Lindau Nobel Laureate Meeting

Lehigh University's nominee to meet with the 2013 Nobel Prize winners in Medicine or Physiology, Randy Scheckman, Tom Suedhof, and Jim Rothman in Lindau, Germany. This prestigious event allows students from all over the world to meet Nobel Laureates and discuss the latest topics in science.

2010 Biotechnology Support Fund Award

Awarded to the senior student who demonstrates outstanding academic achievement in the classroom as indicated by attaining the highest Quality Point Average in the Biotechnology Concentration.

Undergraduates Mentored

Rachel A. Margraf
Spring 2015-present

Michael B. Ricci
Spring 2015

Community Outreach

GRaD Expo 2016
May 21st, 2016, Alumni Weekend
Lehigh University, Bethlehem, Pennsylvania

Lehigh Valley Science and Engineering Fair
March 12th, 2016
Lehigh University, Bethlehem, Pennsylvania

Broughal Middle School Bio fair
March 15th, 2014, March 20th, 2015, March 23rd, 2016
Broughal Middle School, Bethlehem, Pennsylvania