Rita Rodrigues Sá Ferreira

Interaction between Cx43 and LC3 directs gap junctions to ubiquitin-independent autophagy degradation

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica do Doutor Henrique Girão e co-orientação do Doutor Steve Catarino apresentada à Faculdade de Medicina da Universidade de Coimbra.

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UNIVERSIDADE DE COIMBRA

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Abbreviations

3MA	3-methyladenine
AGJs	Annular gap junctions
AIM	Atg8-interacting motif
Akt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
Atg	Autophagy-related genes
BafA1	Bafilomycin A1
Bnip3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CHX	cycloheximide
CIP75	Ubiquitin-binding Cx43-interacting protein of 75kDa
CMA	Chaperone-mediated autophagy
Cvt	Cytoplasm-to-vacuole
Cx43	Connexin43
Dab2	Disabled-2
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
Eps15	Epidermal growth factor receptor pathway substrate 15
ER	Endoplasmatic reticulum
ERAD	Endoplasmic Reticulum associated degradation of proteins
ERK1/2	Extracellular-signal-regulated kinase 1/2
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
FIP-200	Focal adhesion kinase family-interacting protein of 200 kDa
FKBP12	FK506-binding protein of 12 kDa
FRAP	FKBP12-rapamycin associated protein
GABARAP	c-amino butyric acid receptor-associated protein
GAPs	Rab GTPase-activating proteins
GJ	Gap Junctions
GJIC	Gap Junction Intercellular Communication
HRP	Horseradish peroxidase
Hrs	Hepatocyte growth-factor regulated tyrosine kinase substrate
Hsc70	Chaperone heat shock cognate 70
IP	Immunoprecipitation
LAMP2A	Lysosome-associated membrane protein 2A
LC3	Microtubule-associated protein 1 light chain 3
LDS	LIR docking sites
LIR	LC3-interacting region
MA	Macroautophagy
MAPK15	Mitogen-activated protein kinase 15
MTOC	Microtubule organizing center
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MVB	multivesicular body
NBR1	Neighbor of BRCA1 gene 1
NDP52	Nuclear dot protein 52 kDa
Nedd4	Neural precursor cell expressed developmentally down-regulated protein 4
NT	N-terminal

ODDD	Oculodentodigital dysplasia
p62/SQSTM1	Sequestosome-1
PAS	Phagophore assembly site
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI3K-III	Class III phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PRU	Pleckstrin-like receptor for ubiquitin
PSSM	Position specific scoring matrix
PTM	Post-translational modifications
PY	Proline-rich region
RAFT1	Rapamycin and FKBP12 target-1
RAPT1	Rapamycin target-1
SDS-PAGE	Sodium dodecyl sulfate- plyacrilamide gel electrophoresis
SMART	Simple Modular Architecture Research Tool
TBC1D25	TBC1 Domain Family member 25
TBK1	Protein kinase TANK binding kinase 1
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween 20
TNF	Protein tumor necrosis factor
TP53INP2	Tumor protein 53-induced nuclear protein 2
TPA	Tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate
TRAF6	Receptor-associated factor 6
Tsg101	Tumor susceptibility gene 101
UBA	Ubiquitin-associated domains
Ubl	Ubiquitin-like
UIM	Ubiquitin-interacting motif
ULK	UNC-51-like kinase
UPS	Ubiquitin-proteasome system
WB	Western blot
ZO-1	Zonula occludens-1

Abstract

Connexin43 (Cx43) is a membrane protein present in gap junctions (GJ) of several tissues, including the heart, where they permit the metabolic and electrical coupling vital for heart contraction. Previous studies carried out in our lab showed that degradation of Cx43 by macroautophagy requires the autophagy adaptor p62, that links the ubiquitinated protein to the nascent autophagosome, through the microtubule-associated protein-1 light chain 3 (LC3). Proteins that interact with LC3, like p62, contain a short hydrophobic LC3-interacting region (LIR). We identified an amino acid sequence in the N-terminus of Cx43 that is consistent with the accepted form for a LIR motif. The main goal of this study was to identify the LIR domain in Cx43 and establish the relevance of the motif for the degradation of Cx43. We hypothesized that the presence of this motif could mediate an alternative form of Cx43 degradation by autophagy that is independent of ubiquitin and p62. Using Cx43 mutanted in the putative LIR domain and through immunoprecipitation assays we evaluated the involvement of the LIR in mediating the direct interaction between Cx43 and LC3 and the influence of p62 in this interaction. Moreover, to investigate the stability of the Cx43 LIR mutants, we performed cycloheximide-chase assays to determine their half-life in several conditions. To evaluate the effect of the mutations in the subcellular distribution of the protein we used immunofluorescence confocal microscopy. Our results showed that Cx43 interacts with LC3 and that mutations in the LIR motif of Cx43 abrogate this interaction. In addition, we demonstrated that these mutants are more stable than wild type Cx43, suggesting that the LIR motif is required for Cx43 degradation.

Altogether, the results obtained in this study establish a novel non-canonical pathway of degradation of Cx43 that implies the direct binding of Cx43 to LC3, without the need of ubiquitin and p62.

Resumo

A conexina 43 (Cx43) é uma proteína transmembranar presente nas *gap junctions* (GJ) de diversos tecidos, incluindo o coração onde permite o acoplamento metabólico e elétrico vital para o normal funcionamento do coração. Estudos prévios levados a cabo no nosso laboratório mostraram que a degradação da Cx43 por macroautofagia requer a proteína adaptadora p62, que liga proteínas ubiquitinadas ao autofagossoma, através da *microtubule-associated protein-1 light chain 3* (LC3). Proteinas que interagem com a LC3, como o p62, contém uma região hidrofóbica que interage com a LC3 (LIR). A Cx43 contém no seu N-terminal uma sequência de aminoácidos consistente com o estabelecido para um domínio LIR. O principal objectivo deste estudo é a caracterização do domínio LIR na Cx43 e estabelecer a relevância deste na degradação da Cx43 por macroautofagia. A presença deste domínio poderia então mediar a degradação da Cx43 por macroautofagia, de uma forma independente de ubiquitinação e do recrutamento do p62. Para tal, usamos construções de Cx43 mutadas neste possível domínio LIR e através de ensaios de imunoprecipitação avaliamos o envolvimento do LIR na interacção entre a Cx43 e a LC3 e a influência do p62 nesta interacção. Para comparar a estabilidade dos mutantes da Cx43 no dominio LIR, levamos a cabo ensaios de *cycloheximide chase* para determinar o tempo de meia vida da proteína, em diferentes condições. A avaliação da distribuição subcelular da proteina foi feita através de imunofluorescência. Os nossos resultados demostram que a Cx43 interage com a LC3 e que as muatações no domínio LIR levam a uma diminuição desta interacção. Além disso, estes mutantes mostram ser mais estáveis que a Cx43 não mutante, sugerindo que o motivo LIR é importante na degradação da Cx43.

Em suma, os resultados obtidos neste estudo sugerem uma nova via não-canónica para a degradação da Cx43, que implica a ligação direta entre Cx43 e LC3, sem a necessidade de ubiquitinação prévia e do recrutamento do adaptador p62.

1. Introduction

1.1 Connexins and gap junctions

Intercellular communication through gap junctions (GJ) is essential to maintain cellular homeostasis and to ensure the coordination and proper function of tissues and organs. Alterations in gap junction intercellular communication (GJIC) have been associated with several diseases, including heart disorders and even cancer [1],[2],[3]. GJ are hexameric channels formed by two docked hemichannels, also named connexons, from adjacent cells that allow the direct diffusion of ions and small molecules between cells. Each hemichannel is composed by an assembly of six units of a transmembrane protein called connexin (Figure 1). These proteins are essential in physiological processes such as the synchronized contraction of the heart muscle and in the development and differentiation of a variety of tissues [4]. Connexins are composed by four transmembrane domains, highly conserved between the different isoforms, two extracellular loops and three cytoplasmatic domains. The cytoplasmatic loop domain and the C-terminus of the protein present higher variability between connexin isoforms and are likely responsible for the functional differences between connexin species. The cytosolic localization of the N- and C-terminus of the protein also allows the interaction between connexins and several other proteins that can modulate the half-life, activity and functions of connexins. In humans, 21 connexin genes have been identified. They were named after their molecular weight but they can also be classified according to their sequence similarity into 5 groups: alpha, beta, gamma, delta, and epsilon gap junction proteins [3],[5].

The expression of connexins is tissue specific, depending on their physiological roles [6]. Several cells can express multiple connexins and consequently they may oligomerize into the same (homomeric) or mixed (heteromeric) connexons, although only certain combinations are allowed. Different types of connexons present differences in physiological properties like conductance and permeability [4].

1.1.1. Connexin43

Connexin43 (Cx43), a member of the α-connexin family, is the most widely expressed connexin, being found in tissues as diverse as the heart, lens, retina, skin, brain, kidney and bone marrow [7], [8]. Similar to other connexins, Cx43 is translated by ribosomes attached to the endoplasmatic reticulum (ER) and is thought to be co-translationally inserted into the ER membrane, where they acquire proper folding [1],[3],[9], although Cx43 does not oligomerize in the ER. After acquiring proper folding, it is transported to the trans-Golgi network where it oligomerizes into hexameric structures [9]. These connexons are then transported to the

plasma membrane, in a microtubule dependent or independent way, where they may remain uncoupled in the plasma membrane in the form of hemichannels or dock to hemichannels from an adjacent cell, creating intercellular channels. These channels aggregate and form plaques named GJ [1],[4]. One particular feature of Cx43 is its short half-life of 2-4h, when compared to other transmembrane proteins [7]. For this reason, the regulation of protein synthesis, degradation and trafficking is extremely important to maintain GJIC [3].
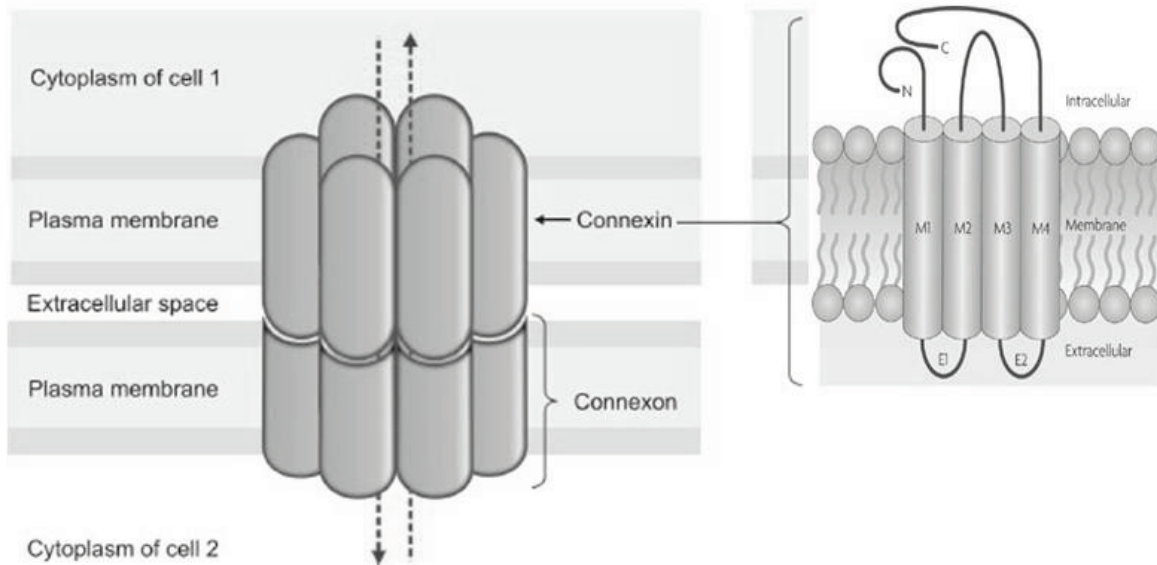
Figure 1 – Assembly of GJ and structure of connexins. GJ are formed by two docked connexons from adjacent cells, allowing direct exchange of ions and small molecules between them. Each connexon is composed by six connexin proteins. Connexins present four transmembrane domains, two extracellular loops and cytoplasmatic N-and C- terminals. (Adapted from Bloomfield SA, et al. 2009 and from Tripathi P et al. 1010).

1.2. Proteolytic pathways

Cellular protein levels are balanced by their rate of synthesis and degradation. In eukaryotic cells, proteins can be degraded by the ubiquitin-proteasome system (UPS) or through the autophagy pathway.

1.2.1. The Ubiquitin-Proteasome system

The UPS is the major proteolitic pathway for the degradation of cytosolic soluble proteins [10]. This ATP-dependent pathway has significant importance in several biological processes such as the regulation of the cell cycle, inflammatory response, immune response, protein misfolding and endoplasmic reticulum associated degradation of proteins (ERAD)[11].

Introduction

The UPS is a very complex and regulated pathway and its deregulation has been associated specially with neurodegenerative diseases [12].

The degradation of a protein by the UPS usually requires the attachment of a poly-ubiquitin chain to the substrate. Ubiquitin is a highly conserved small (8.5 kDa) regulatory protein, expressed in all eukaryotes [13]. The ubiquitination reaction evolves three enzymatic steps. First, the E1 ubiquitin-activating enzyme (ATP-dependent) activates ubiquitin by forming a covalent bond between the C-terminal end of ubiquitin and a cysteine residue in its active site. Then, the thioesterified ubiquitin is transferred to an E2 or ubiquitin-conjugating enzyme. In the final step, ubiquitin is transferred to the target substrate with the help of an E3 ubiquitin ligase that confers specificity to the system [14]. The ubiquitination of proteins generally occurs on lysine (K) residues and proteins can undergo mono-ubiquitination (conjugation of one ubiquitin monomer) or poly-ubiquitination (conjugation of a poly-ubiquitin chain). Poly-ubiquitination may occur through different types of ubiquitin chains, depending on which lysine residue ubiquitin is used. Mono-ubiquitination and the type of linkage formed by poly-ubiquitin chains can signal the protein for different fates. Indeed, ubiquitination is not always a signal for proteasomal degradation: it can recruit other factors to mediate various cellular responses such as signaling, gene regulation, endocytosis, macro-autophagy, and DNA repair [14]. For example, substrates linked via lysine 48 (K48) ubiquitin chains are targeted for proteasomal degradation, while polyubiquitination through lysine 63 (K63) is a signal for internalization of the protein and is also involved in targerting substrates to autophagy mediated degradation [15].

The 26S proteasome is a large enzymatic complex (2.4 MDa) consisting of 33 different subunits organized into two sub-complexes: the catalytic 20S core particle and the 19S regulatory particle [16], [17].

Ubiquitinated proteins are docked to the 19S subunit through ubiquitin receptors Rpn10 and Rpn13 that recognize ubiquitin through small domains: a pleckstrin-like receptor for ubiquitin domain (PRU) in Rpn13 and ubiquitin-interacting motif (UIM) domains in Rpn10 and one or two ubiquitin-associated domains (UBA) in the detachable ubiquitin receptors [17]. The proteasome does not degrade ubiquitin. Instead, Rpn11 cleaves the isopeptide bond between ubiquitin and the subtract, promoting the recycling of the entire ubiquitin chain [11], [17]. This cleavage also allows the targeted protein to enter the 20S catalytic core. The 20S contains two beta-rings that form the catalytic cavity. Catalytic activity is conveyed by β1, β2 and β5 subunits that have caspase-like, trypsin-like and chymotrypsin-like activity, respectively. These subunits are responsible for the cleavage of the peptide bonds in the substrate protein [16].

1.2.2. Autophagy

Autophagy is a cellular process in which cytoplasmatic content, such as toxic protein aggregates, long lived proteins and excess or damaged organelles, are delivered to the lysosome for degradation [18]–[20]. The morphology and constitution of the autophagy machinery is highly conserved from yeasts to mammals [19]. There are three types of autophagy: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy (MA). CMA only occurs in mammalian cells and it is selective for proteins that present a specific pentapeptide KFERQ motif. The KFERQ motif is recognized by the chaperone heat shock cognate (Hsc70) that leads the substrate to the lysosomal membrane. Here, it binds to the receptor lysosome-associated membrane protein 2A (LAMP2A), followed by translocation to the lysosomal lumen where the protein is degraded [18], [19].

In microautophagy, the lysosome is responsible for the direct internalization of cytoplasmatic material, through invagination or protrusion of the lysosomal membrane. In terms of mechanism, little is known about microautophagy and most of our knowledge comes from studies in yeast [21].

Macroautophagy is a mechanism that involves the formation of a double-membrane structure, named autophagosome. First, the phagophore (double-membrane cup-shaped structure) engulfs portions of the cytoplasm and after fusion of the membranes the autophagosome is formed. Subsequently, these vesicles are transported through microtubules to lysosomes, where the fusion between these two structures occurs, and the sequestered material is degraded [22].

The autophagy pathway is upregulated in response to physiological stress conditions, such as nutrient and growth factor depletion, hypoxia, drug and radiation treatment [18]. In mammalian cells, autophagy also occurs at a basal level acting like a protein quality control system, promoting the clearance of normally occurring, misfolded, ubiquitinated proteins [22]. Autophagy has been associated with several human diseases, for instance cancer, certain neurodegenerative disorders and infectious diseases [18] and for that reason, this pathway is a potential therapeutic target for the improvement of these conditions [20].

1.2.2.1. The autophagy machinery

Studies in the yeast *Saccharomyces cerevisiae* led to the identification of more than 30 different autophagy-related genes (ATG) and many of these have mammalian orthologs [23]. These genes can be divided into four groups, according to their functions in the different stages of the autophagy pathway [18],[19]. Induction of autophagosome formation is

regulated by the Atg1/UNC-51-like kinase (ULK) complex (Atg1, Atg11, Atg13, Atg17, Atg29 and Atg31). Vesicle nucleation is mediated by the class III phosphatidylinositol 3-kinase (PI3K-III) complex (Vps34, Vps15, Vps30/Atg6/Beclin 1, and Atg14). Membrane delivery to the expanding phagophore is mediated by Atg9 and its cycling system (Atg2, Atg9 and Atg18). Finally, two ubiquitin-like (Ubl) conjugation systems: the Atg12 (Atg5, Atg7, Atg10, Atg12 and Atg16) and Atg8 (Atg3, Atg4, Atg7 and Atg8) conjugation systems play a role in vesicle expansion [24] (Figure 2).
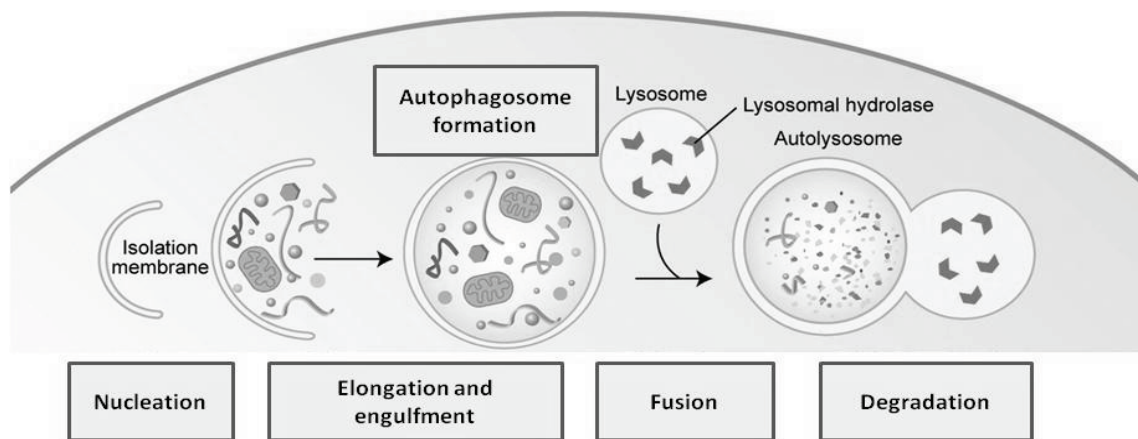
Figure 2 – The autophagy machinery. Autophagosome formation starts at the PAS, with the formation of the phagophore or isolation membrane (nucleation step). In the elongation step, the phagophore expands and culminates with autophagosome formation. This structure is responsible for the engulfment of the substrates targeted for degradation (elongation and engulfment steps). Fusion between the autophagosome and the lysosome forms an autophagolysosome, where the degradation finnaly occurs (fusion and degradation). (Adapted from Meléndez, A, 2009).

1.2.2.2. Autophagosome formation

It is widely accepted that in yeast autophagosome formation starts at the phagophore assembly site (PAS). This model was derived from studies in yeast that showed co-localization of proteins involved in the formation of the autophagosome at a single site in the cell [25]. The source of the membranes that compose the autophagosomes is still a matter of discussion. It is suggested that membranes from distinct locations, such as the endoplasmatic reticulum (ER), mitochondria and plasma membrane, may contribute to the formation of this vesicle [23]–[26].

The initiation step evolves the ULK complex, composed by ULK1/2, Atg13, FIP-200 (focal adhesion kinase family-interacting protein of 200 kDa) and Atg101. Under nutrient-rich conditions, Atg13 and ULK1/2 are phosphorylated and ULK kinase activity is inhibited. When

autophagy is induced, the dephosphorylation of Atg13 and ULK1/2 leads to the activation of FIP-200, which induces autophagosome formation.

The other complex implicated in the initiation step is the PI3K-III, containing Vps34, Beclin-1, Atg14 and Vsp15 (previously known as p150). Autophagosomal membranes contain a specific lipid molecule, phosphatidylinosositol-3-phosphate (PI3P), that is requires for the recruitment of proteins required for elongation of the phagophore. Vps34 is responsible for the phosphorylation of phosphatidylinositol (PI), producing PI3P [22], [23], [24], [25].

1.2.2.3. Phagophore elongation

The process of phagophore elongation that culminates in autophagosome formation is controlled by two ubiquitination-like reactions. In the first reactions, the ubiquitin-like protein Atg12 is conjugated to Atg5 by Atg7, which acts as an E1 ubiquitin-activating enzyme, and Atg10, that acts as an E2 ubiquitin-conjugating enzyme. The Atg5-Atg12 complex then conjugates with Atg16 (L1) and together they associate with phagophores. When the autophagosome is fully formed, the Atg5-Atg12-Atg16 (L1) complex dissociates [18], [23], [25]. The second ubiquitin-like reaction involves the conjugation of microtubule-associated protein 1 light chain 3 (LC3), called Atg8 in yeast, to the lipid phosphatidylethanolamine (PE). LC3 is translated in a precursor form that is cleaved at its COOH terminus to form the cytosolic LC3-I. This cytosolic form is covalentely conjugated to PE to form the membrane associated LC3-II, in a reaction envoluving Atg7 and Atg3, that act as E1-like and E2-like enzymes, respectively [26]. LC3-II is targeted to the membrane of the elongating phagophore and remains on autophagosomes until the fusion with the lysosome. After fusion, LC3-II that is faced to the cytoplasm can be delipidated by Atg4 and recycled, while LC3-II present in the internal surface of autophagosomes is degraded in the lysosome [18], [23], [25].

Furthermore, cross-talk between these two ubiquin-like systems has been reported. The Atg-12-Atg5-Atg16 (L1) complex can facilitate the conjugation of LC3-I to PE, by bringing LC3 to the site of lipidation. However, the mechanism responsible for targeting LC3 to specific membranes is yet to be identified [27].

LC3-II is the main protein used as a macroautophagy marker, as it specifically associates with the autophagosome membrane. LC3-II can mediate fundamental processes for expansion of the phagophore, such as membrane tethering and hemifusion [26].

1.2.2.4. Autophagosome fusion

Autophagosomes in mammalian cells can be formed at different sites in the cytoplasm. Transport along microtubules, in a dynein-dependent manner, leads the vesicles to the microtubule organizing center (MTOC), where lysosomes are enriched. Autophagosomes then tether, dock and fuse with lysosomes. Proteins such as the endosomal sorting complex required for transport (ESCRT), SNAREs, Rab7, and the class C Vps are involved in the fusion process [28]–[33]. This process is also dependent on proper lysosomal function. BafA1, an inhibitor of the lysosomal H+ATPase, prevents the fusion of autophagosomes with lysosomes as it interferes with the normal acidification of the lysosomal lumen [34].

1.2.2.5. Autophagy regulation

In mammalian cells, autophagy is regulated by several signaling pathways. The classical pathway involves the serine/threonine kinase, mammalian target of rapamycin (mTOR) that is known to inhibit autophagy [23]. However, TOR independent signaling pathways involved in autophagy signaling also exist [22].

The mTOR pathway [also known as rapamycin and FKBP12 target-1 (RAFT1), rapamycin target-1 (RAPT1), and FKBP12-rapamycin associated protein (FRAP)] is the main pathway that regulates autophagy signaling in eukaryotes. It regulates other cellular processes like transcription, translation, cell growth and proliferation, cytoskeletal reorganization and ribosome biogenesis. Several signals regulate the activity of mTOR, such as growth factors, insulin, nutrients, energy availability and cell stressors like hypoxia, osmotic stress, reactive oxygen species and viral infection [22].

In mammals, the mTOR pathway is organized in two functional complexes: rapamycin-sensitive mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These complexes share common components but perform different functions and phosphorylate different downstream substrates. mTORC1 consists of the catalytic subunit and it is responsible for the regulation of autophagy [22], [23]. Rapamycin, a lipophilic macrolide antibiotic, is an inhibitor of mTORC1 activity. It forms a complex with the immunophilin FK506-binding protein of 12 kDa (FKBP12), which then stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR . Under nutrient rich conditions, mTOR is associated with the Atg13-ULK1/2 complex that mediates their interaction with FIP200. This way, the autophagy pathway is inhibited. Under starvation conditions, mTOR dissociates from the complex and leads to the dephosphorylation of Atg13 and ULK1/2. This, in turn leads to the dephosphorylation-dependent activation of ULK1/2 and the consequent phosphorylation of Atg13,FIP200 and ULK1 itself, which triggers autophagy [18], [23].

Introduction

Nutrient deprivation causes a reduction in the cellular ATP levels and consequent energetic stress. In response, activation of autophagy is crucial to maintain cellular homeostasis. Adenosine monophosphate-activated protein kinase (AMPK) has been shown to sense changes in cellular energy levels triggering autophagy. AMPK can directly phosphorylate ULK1, leading to autophagy activation, or can act as a negative regulator of the mTOR signaling pathway [35].

1.2.2.6. Selective autophagy

Autophagy was initially described as a non-selective bulk process for degradation of proteins that was activated, in response to starvation. However, it is now clearly established that autophagy is able to degrade substrates in a selective manner. The removal of aggregated proteins, mitochondria, perixisomes, ribosomes, ER and pathogens by autophagy is highly selective and requires cargo recognition by the autophagy machinery [36]. Ubiquitination of substrates can lead to their recognition by the autophagy machinery and specific degradation in the lysosome. Degradation of protein aggregates requires ubiquitination and this modification also targets bacteria for lysosomal degradation [37]. Autophagy adaptors such as the mammalian p62/SQSTM1 (sequestosome-1) and Neighbor of BRCA1 gene 1 (NBR1), contain a carboxyl-terminal ubiquitin-associated domain (UBA) that binds to ubiquitinated cargo. These autophagic adaptors interact with LC3 (Atg8), through a LIR (LC3-interacting region) motif, leading the substrates to lysosomal degradation. Both NBR1 and p62 are degraded in the process [37]–[40] (Figure 3).
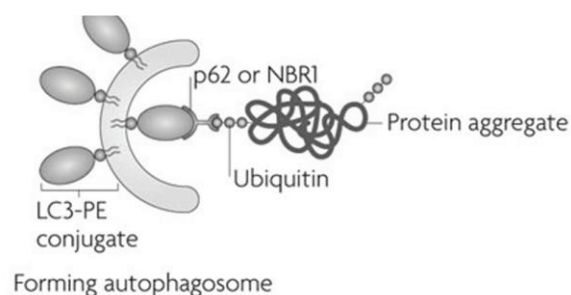
Figure 3 – Selective autophagy mediated by p62 or NBR1 Selective adaptors NBR1 or p62 are recruited to ubiquitinated protein aggregates. Through interaction with LC3, they bring the cargo to the forming autophagosome, which then fuses with the lysosome, leading to the degradation of the substrates. (Adapted from Tyedmers, J et al. 2010)

1.2.2.6.1. p62

The p62 protein or SQSTM1, is commonly found in inclusion bodies containing polyubiquitinated protein aggregates. It has been associated with several neurodegenerative

diseases, being found in Lewy bodies in Parkinson disease, neurofibrillary tangles in Alzheimer disease, and Huntingtin aggregates in Huntington disease [41]–[43].

p62 binds to monoubiquitin, K48-linked and K63-linked ubiquitin chains through its UBA domain. It is suggested that this protein has higher affinity for K63-linked chains and can serve as a shuttle for proteasomal degradation. However, K63-linked protein aggregates containing p62 can be cleared out by autophagy uptake [41]. Through the LIR domain, p62 binds directly to LC3 and to the related c-amino butyric acid receptor-associated protein (GABARAP) and GABARAP-like proteins, promoting the uptake of proteins for autophagy degradation and the formation of inclusion bodies [42], [43].

In addition to the UBA and LIR domains, p62 also presents other protein-protein interactive-motifs, such as the PB1 motif that binds atypical protein kinase C (PKC); ZZ finger, a binding site for the RING finger protein tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6); and NLS, NES, and two PEST sequences for rapid protein degradation. This may suggest that p62 plays several roles in the regulation of cell signaling and homeostasis of the cellular protein content [43].

1.3. The LIR motif

In mammals, the Atg8 family of proteins includes two subfamilies: the LC3 proteins LC3A, LC3B and LC3C and the GABARAP family GABARAPL1 and GABARAPL2. LC3B is the most prevalent and extensively studied Atg8 protein [44], [45].
Proteins that interact with LC3 present a short-hydrophobic LIR domain in their sequence. In yeasts, this region is referred as the Atg8-interacting motif (AIM). The first LIR sequence was identified in p62 and in yeast Atg19 [42].

The accepted form for a LIR motif consists of a core consensus WxxL (Tryptophan (W), Leucine (L)) sequence, preceded N-terminally by negatively charged residues (Glutamic acid (E), Aspatic acid (D),Serine (S) or Threonine (T)). Two hydrophobic pockets (W and L pockets) of the LIR docking sites (LDS) on Atg8 proteins accommodate the aromatic and aliphatic amino acids of the LIR domain [44], [45] (Figure 4). In the first position, W is the residue that binds with higher affinity, although Phenylalanine (F) and Tyrosine (Y) residues can also be found at this position [46], [47]. The lower affinity towards F and Y can be compensated by the presence of acidic residues and/or S and T phosphorylating sites preceding the core motif. These can be involved in electrostatic interactions with the basic residues present in the N-terminal extension and the Ubl domain of LC3 [44], [45]. Interaction with the Atg8 family can also be LIR-independent. Several proteins interact with LC3 in a manner that is not affected by mutations in the LDS [48].
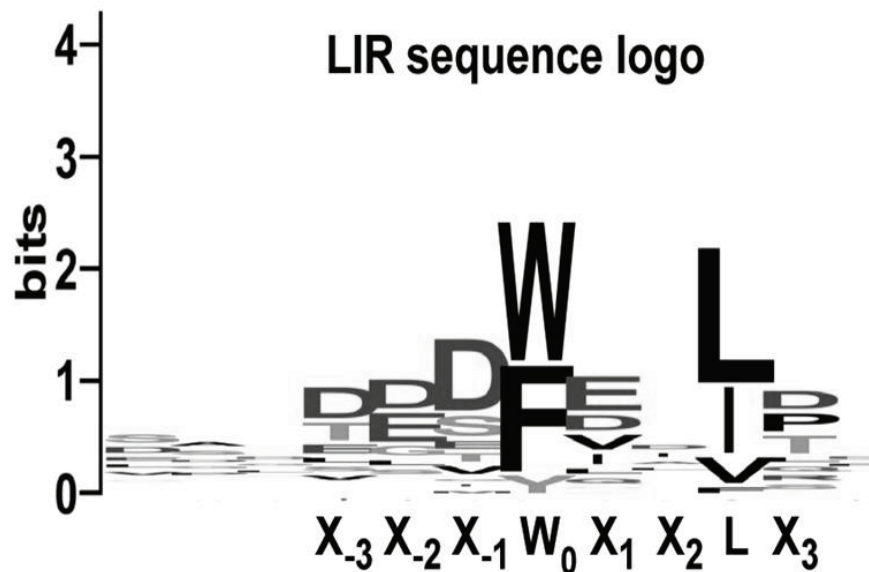
Figure 4 – LIR motif consensus sequence. Sequence logo represents the amino acid residues present at each LIR motif position. The stacks overall height represents the sequence conservation at that position, while the height of the symbols indicates the relative frequency of that amino acid at that position. The consensus form for a LIR motif consists of a WxxL sequence, preceded mostly by D residues in the N-terminal. (Adapted from Birgisdottir, Â, 2013).

1.3.1. Autophagy-related proteins containing LIR motifs

Several members of the autophagy machinery contain LIR domains in their sequence. Both the mammalian ULK complex and the yeast equivalent Atg1 kinase complex present canonical LIR motifs, that can facilitate their association with the autophagosome. ULK1 complex components such as yeast Atg13 and mammal FIP200 also contain LIR motifs. Proteins of the ULK complex preferentially interact with the GABARAP subfamily rather than LC3 proteins [46].

The yeast E2-like enzyme Atg3 undergoes LIR-mediated interaction with Atg8 and is essential for the yeast-specific cytoplasm-to-vacuole (Cvt) pathway but dispensable for starvation-induced autophagy. It mediates the conjugation of Atg8 to PE [49]. The components of the Atg8 lipidation system (Atg4, Atg5, Atg7, Atg16L) all interact with LC3. Among them, Atg4B is responsible for processing Atg8 precursors and Atg8-PE. Crystal structures of the Atg4-LC3B complex indicate conformational changes upon binding that facilitates access of LC3B to the catalytic core of Atg4 [50].

Several LIR-containing proteins are associated with autophagosomes and other vesicles [45]. The tumor protein 53-induced nuclear protein 2 (TP53INP2 also known as DOR) exits the nucleus in response to cellular stress or the activation of autophagy. On early

autophagosomes, it interacts with LC3B through a LIR motif and mutations in this motif lead to reduced transcriptional activity, impairment of nuclear exit in response to autophagy activation and disruption of autophagy [51].

Regarding membrane trafficking in autophagy, Rab GTPases are responsible for recruitment of regulators of intracellular membrane trafficking. The Rab GTPase-activating proteins (GAPs) negatively regulate the activity of Rab GTPases. One example is TBC1 Domain Family member 25 (TBC1D25) that through interactions between its LIR motif and Atg8 regulates the fusion between autophagosomes and lysosomes [52]. Other GAPs were shown to interact with Atg8 proteins, such as 14 of the TBC (Tre2, Bub2, and Cdc16) domain-containing Rab-GAPs. Particularly, TBC1D5 contains two LIR motifs, both required for Atg8 binding and to bring the endosome to the autophagosome, via its C-terminal LIR motif [53].

When it comes to autophagy signaling, mitogen-activated protein kinase 15 (MAPK15) controls both basal and starvation-induced autophagy. It can be found in autophagosomes through an interaction between its LIR motif and Atg8 proteins, leading to the stimulation of the formation of autophagic compartments [54].

As said before, selective autophagy adaptors have the ability to bind LC3. After the identification of the LIR motif in the adaptor p62, other proteins were found to be autophagy adaptors, such as NBR1, nuclear dot protein 52 kDa (NDP52) and optineurin, all of these containing LIR motifs [55]–[57] (Figure 5).
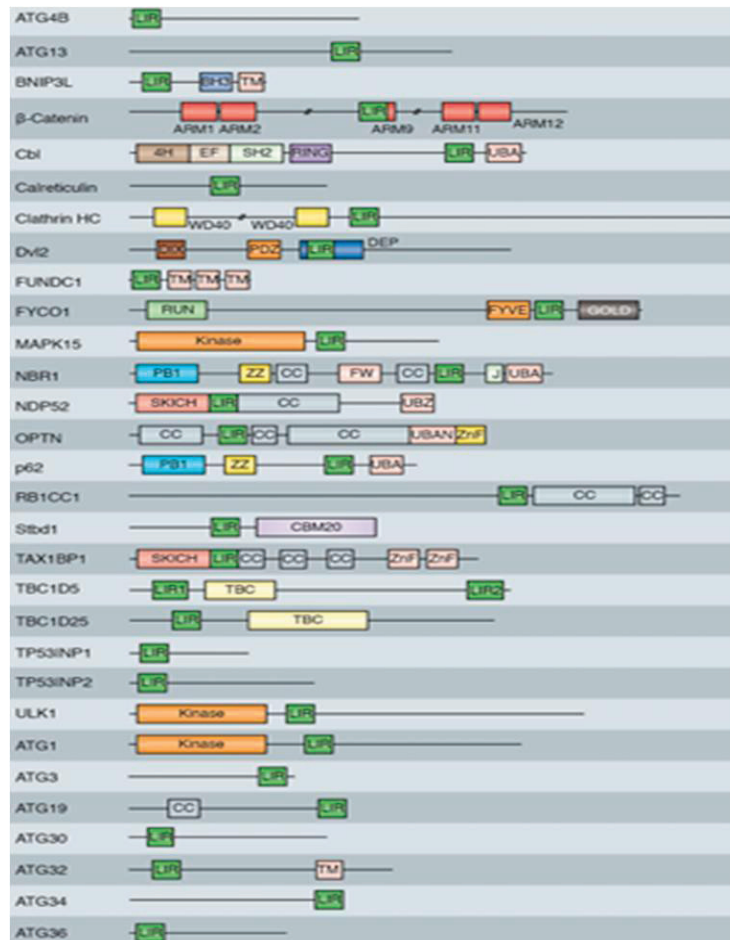
Figure 5 – LIR containing proteins. Table representing the domain arquitecture of LIR containing proteins. (Adapted from Wild, P et al. 2014)

1.3.2. Regulation of LIR-mediated interactions by phosphorylation

In 25% of the identified LIR domains, S or T residues are found directly preceding the aromatic amino acid. Thus, it is thought that phosphorylation may regulate the binding affinity of the LIR motif [45]. Phosporylation of the autophagy receptor optineurin by the protein kinase TANK binding kinase 1 (TBK1) on S-177 enhances LC3 binding affinity and autophagy clearance of cytosolic *Salmonella enteric.* This effect was reverted by mutation of the LIR motif of optineurin [57]. The phosphorylation of the mitophagy receptor BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3) on the S residues flanking its LIR motif promotes binding to LC3, resulting in mitochondrial clearance [58].

1.3.3. Identification of LIR motifs

The identification of the first LIR motif on SQSTM1/p62 was accomplished by a combination of deletion mapping and point mutation techniques to identify a 22-residue peptide responsible for the binding to LC3. Using sequences from SQSTM1 homologs, the authors identified conserved residues always flanking a tryptophan residue [42]. Further studies with atomic detail structure data for the mouse and human SQSTM1-LC3 interaction narrowed the LC3 recognition sequence to a short tetrapeptide of the general form WXXL (X stands for any residue), with the W and L residues interacting with 2 distinct hydrophobic pockets of LC3 [59], [60]. The description of this region was enhanced to X-3X-2X-1[WY]X1X2[LIV], where at least one of X-3X-2X-1, X1 and/ or X2 are acidic residues [61]. The identification of LIR motifs in proteins from the ULK complex led to the redefinition of the LIR motif expression to [DE][DEST][**WFY**][DELIV]X[**ILV**], through comparison with 26 previously described LIR motifs [46].

With the increased data describing LIR motifs and the availability of complete eukaryotic genomes, a bioinformatical approach for the identification of LIR containing proteins was delevoped. iLIR is a web server that analyses aminoacid sequences and identifies possible LIR motifs in a protein. Using the amino acid sequence, iLIR searches for short sequences consistent with a refined sensitive regular expression pattern of an extended LIR motif and retrieves characterized protein domains from the Simple Modular Architecture Research Tool (SMART) database for the query. Also, it scores the possible LIR motifs against a custom position specific scoring matrix (PSSM) and identifies subsequences that can potentially undergo other protein interactions, overlapping with the detected LIR motifs [62].

1.4. Degradation of Cx43

The high turnover of Cx43 is determined by its synthesis and degradation rates and the control of these rates is essential for the regulation of GJIC. The main pathways for degradation of Cx43 are the proteasomal, endosomal and autophagosomal pathways.

1.4.1. Signals involved in the degradation of Cx43

Connexins can undergo different post-translational modifications (PTM), including phosphorylation, ubiquitination, SUMOylation, methylation, acetylation, nitrosylation and glutamate γ-carboxylation. Phosphorylation and ubiquitination of Cx43 is involved in the internalization and degradation of GJ plaques [63].

1.4.1.1. Phosphorylation of Cx43

Cx43 presents at least 19 phosphorylation sites, either on S or Y residues and is phosphorylated by several kinases, such as protein kinase B (Akt), protein kinase A (PKA), PKC, extracellular-signal-regulated kinase 1/2 (ERK1/2) and Src. This PTM is responsible for the regulation of intracellular traffic, assembly and turnover of the protein and affects the interaction of Cx43 with other regulatory proteins [6]. Akt has been shown to phosphorylate Cx43, controlling gap junction stability and traffic of Cx43. Phosphorylation on the S373 residue of Cx43 by Akt mediates its interaction with zonula occludens-1 (ZO-1), stabilizing Cx43 at the plasma membrane [64]. Src phospohorylates Cx43 on Y247 and Y265 sites, promoting dowregulation of GJIC and causing gap junction disassembly [65], [66]. PKC can phosphorylate Cx43 at the S368 and S372 sites, leading to channel closure and reduced half-life of Cx43 [6]. This PTM can signal subsequent modifications on the proteins. Epidermal growth factor (EGF) induces hyperphosphorylation of Cx43, leading to its ubiquitination, internalization and degradation [67]. Also, PKC mediated phosphorylation on S368 can facilitate the ubiquitination of the protein and degradation of Cx43 by the UPS [68].

1.4.1.2. Ubiquitination of Cx43

The first evidence that Cx43 could be ubiquitinated was provided in 1995 in a study using the Chinese hamster ovary cell line CHO-ts20, which expresses a thermolabile E1 ubiquitin-activating enzyme. They found that in conditions in which the enzyme was inative, there was an accumulation of Cx43, suggesting a role for the UPS in the degradation of Cx43 [69].

As mentioned before, phosphorylation is an important regulator of ubiquitination. The EGF-induced phosphorylation of Cx43, mediated by the MAPK pathway, induces the ubiquitination of the protein [70], [71].

The tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is an activator of the PKC and MAPK pathways and promotes the ubiquitination, internalization and degradation of Cx43 [72].

Neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) is an E3 ubiquitin ligase that has been shown to interact with Cx43, in a process modulated by the phosphorylation of Cx43. Nedd4 contains three WW domains that bind to the proline-rich region (PY) of Cx43 and directly catalyzes the ubiquitination of the protein [73] .

The pathway that is followed by ubiquitinated proteins is determined by the different polyubiquitin chains attached to it and by the different adaptor proteins that interact with

them. Ubiquitinated Cx43 localized at the plasma membrane is recognized by the endocytic adaptor epidermal growth factor substrate 15 (Eps15), which contains an ubiquitin-interacting motif (UIM), mediating the internalization of Cx43 [74]. The autophagy adaptor p62/SQSTM1 also interacts with ubiquitinated Cx43 and mediates the degradation of internalized Cx43 [63], [75].

1.4.2 Proteasomal degradation

As mentioned above, the first evidence that Cx43 could be ubiquitinated also suggested a role for the UPS in the degradation of Cx43 [69]. Subsequent studies have proven that treatment of different Cx43-expressing cell lines with proteasomal inhibitors increases Cx43 levels and decreases its rate of degradation [76]–[79].

Cx43 can undergo degradation through the ERAD [63], a protein quality control system that ensures that proteins that did not acquire proper folding in the ER are labeled for degradation in the proteasome. Abnormal proteins are recognized by the ER proteins, retranslocated into the cytosol, ubiquitinated and then dedraded by the UPS [80]. As proteasomal inhibition results in the accumulation of Cx43, ERAD may also be involved in the degradation of this protein. Further studies using ER stress inducers to prevent proper folding of Cx43, such as dithiothreitol (DTT), enhanced dislocation from the ER into the cytoplasm and elevated proteasomal degradation in response to ER stress [78].

The Ub-binding Cx43-interacting protein of 75kDa (CIP75) interacts with Cx43 localized at the membrane of the ER and is essential for the ERAD degradation of Cx43. It plays a role in translocating Cx43 from the ER to the proteasome [81]. Although CIP75 interacts with ubiquitinated Cx43, it was demonstrated that it can also bind to non-ubiquitinated Cx43, suggesting an ubiquitin-independent pathway for the ERAD degradation of Cx43 [82].

1.4.3. Endo-lysosomal pathway

The removal of old GJ involves the internalization of double membrane structures called connexosomes or annular gap junctions (AGJs) that are targeted for degradation [3]. The endocytosis of GJ involves the recruitment of clathrin, mediated by the clathrin-adaptors AP-2 and Disabled-2 (Dab2), and the GTPase dynamin [83], [84]. Endocytosis of GJ is also dependent on intact actin filaments and actin retrograde motor myosin VI [85]. After internalization, GJ degradation takes place in the lysosome. However, internalized Cx43 can be recycled back to the plasma membrane, through the Rab11 pathway with the participation of dynamin2 [84].

As shown by our lab, ubiquitination of gap junctional Cx43 recruits Eps15, promoting the internalization of GJ [74]. After the endocytosis of Cx43, hepatocyte growth-factor

regulated tyrosine kinase substrate (Hrs) and the tumor susceptibility gene 101 (Tsg101), are responsible for directing Cx43 towards the lysosome, preventing the recycling of the protein. Depletion of Hrs or Tsg101 leads to the accumulation of phosphorylated and ubiquitinated Cx43 in early endosomes, thus preventing lysosomal degradation [86].

1.4.4. Autophagosomal pathway

The first studies of autophagic degradation of Cx43 showed the enclosure of Cx43 in structures containing the autophagosomal marker LC3 and co-localization with the autophagic adaptor p62. Furthermore, inducing autophagy through starvation led to a significant decrease in Cx43 levels, which can be counteracted by inhibition of the lysosome (using cloroquine) and knockdown of Atg5 [87].

The pool of Cx43 in plasma membrane gap junction plaques is targeted for autophagic degradation. In a study using Cx43-GFP expressing HeLa cells, co-localization between Cx43 localized in the AGJs and autophagosomal markers LC3 and p62 was observed. Inhibition of autophagy by siRNA knockdown of multiple proteins required for the autophagic process (Beclin-1/Atg6, LC3B, LAMP-2, p62) or the pharmacological inhibition of autophagy [with 3-methyladenine (3MA), wortmannin, or BafA1] led to an increase of total levels of Cx43-GFP [88].

A study carried out in our lab demonstrated that macroautophagy has a regulatory role in GJIC, in an ubiquitin dependent manner. Inducing autophagy through starvation led to an enrichment of Cx43 in autophagic compartments and to a decrease in Cx43 present at the plasma membrane, decreasing the half-life of gap junctional Cx43. Starvation treatments also induced Cx43 ubiquitination. Nedd4-mediated ubiquitination of Cx43 was demonstrated to be required for the recruitment of Eps15 to GJ and consequent internalization and degradation of Cx43. This study also demonstrated that Eps15 interacts with LC3 but not with p62, which suggests that it functions as an intermediary between ubiquitinated Cx43 and the autophagy machinery [75] (Figure 6).
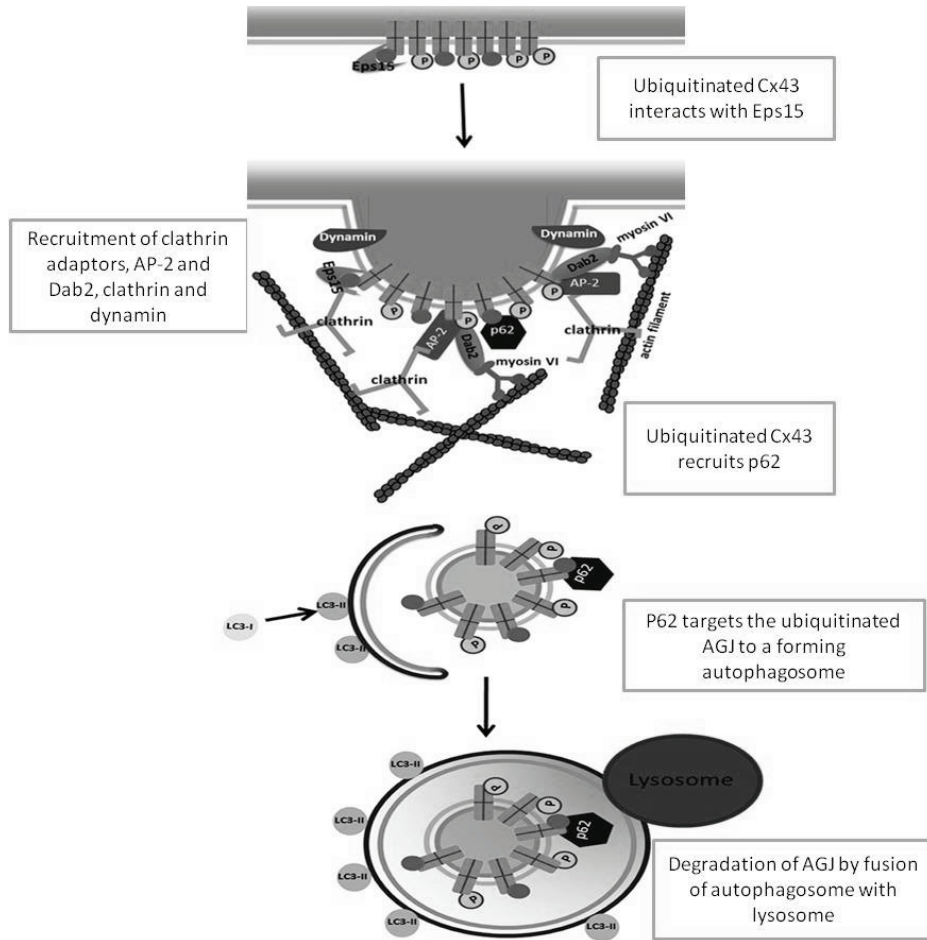
Figure 6 – Degradation of Cx43 by the autophagosomal pathway. Phosphorylated Cx43 is ubiquitinated by Nedd4, which recruits Eps15. Phosphorylation and/or ubiquitination of Cx43 leads to the recruitment of the clathrin adaptors, AP-2 and Dab2, clathrin and dynamin to endocytose the GJ plaque. Ubiquitinated Cx43 recruits p62 and targets the ubiquitinated protein to the forming autophagosome. Finally, degradation of Cx43 occurs after fusion of the autophagosome with the lysosome. (Adapted from Falk, M.M., et al., 2014)

2. Objectives

Cx43 is the most widely expressed human connexin. In the heart, Cx43 has a preponderant role in maintaining proper GJIC that allows synchronized and coordinated heart beating. Impairment of GJIC has been associated with several cardiac diseases such as heart failure and myocardial ischemia. Due to its short half life, regulated turnover of Cx43 is crucial for maintaining the activity and stability of GJ. Our lab has previously shown that Nedd4-mediated ubiquitination of Cx43 is responsible for the recruitment of the autophagy adaptor p62 and consequent degradation of Cx43 by macroautophagy. However, depletion of p62 does not completetly abolish the interaction between Cx43 and LC3, seggesting that alternative pathways modulate this interaction. p62 is responsible for bringing the complex to the autophagosome, through the interaction with LC3. Proteins that interact with LC3 contain a LIR motif, with a WxxL consensus sequence, N-terminally preceded by negatively charged residues. Cx43 presents a WSAL sequence in the N-terminal domain preceded by a residue of aspartic acid, consistent with the accepted form for a LIR domain (Figure 7), suggesting that interaction between Cx43 and LC3 can occur directly, without the need of p62. Therefore, the main goal of this project is to validate the presence of a LIR motif in Cx43 and characterize its involvement in modulating the direct interaction between Cx43 and LC3. Autophagy is a degradation mechanism that maintains cellular homeostasis under basal conditions by degrading toxic protein aggregates and damaged organelles. In response to stimuli such as nutrient depletion, autophagy is upregulated. For that reason, in this project we will also evaluate the influence of starvation in the binding between Cx43 and LC3. As mentioned before, ubiquitination and the adaptor p62 are responsible for bringing the protein to the autophagosome for lysosomal degradation. Our hypothesis is that Cx43 can be targeted for macroautophagy degradation without need of prior ubiquitination and p62 connection. Thus, we intend to prove that the LIR-mediated interaction is capable of bringing Cx43 to the autophagosome directly.

Altogether, this projects aims to unravel a new form of degradation for Cx43 and the establishment of a new LC3 interacting protein.
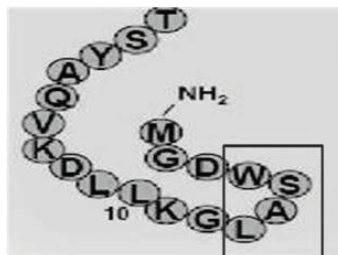

Figure 7 – N-terminal of Cx43 The underlined WSAL sequence corresponds to the putative LIR domain of Cx43.

3. Materials and Methods

3.1. Chemicals

All chemicals, unless otherwise stated were purchased from Sigma-Aldrich. Bafilomycin A1 (BafA1) was from Bioaustralis.

3.2. Cell culture and treatments

HEK293A and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 100 g/mL streptomycin) and maintained at 37 ºC under 5% CO_2.

Protein synthesis was inhibited by the addition of 50μg/mL cycloheximide (CHX) [89]. Lysosome-dependent degradation was inhibited by the addition of 50 nM BafA1 and proteasome inhibition was achieved by the use of 10 μM MG-132. Autophagy was activated through starvation by removing the cell medium and culturing the cells in DMEM without FBS for 4h of incubation.

3.3. Cell culture transfections

One day before transfection, HEK293A and HeLa cells were plated in 10 cm^2 or 4 cm^2 multiwell plates, so that HEK293A were 90-95% confluent and HeLa cells were 80% confluent at the time of transfection.

Plasmids expressing V5-Cx43, V5-Cx43-W4A, V5-Cx43-L7A and V5-Cx43-W4AL7A were generated by subcloning Cx43 and V5 into a pENTR vector. Plasmids expressing mCherry-p62 were generated by subcloning mCherry-p62 into a pEGFP-C1 vector, where GFP was replaced with mCherry, kindly provided by Sascha Martens lab, Max F. Perutz laboratories. Plasmids expressing LC3 were generated by subcloning LC3 into a pEGFP C1 vector.

3.4. siRNA mediated knockdown

siRNA targeting p62 (**s16960** and **s16961**) obtained from Ambion, Life Technologies (Silencer Select Pre-designed siRNA), was used to perform siRNA-mediated knockdown of p62 in HeLa cells. One day before transfection, HeLa cells were plated in 10 cm^2 so that they were 80% confluent at the time of transfection. siRNA was then complexed with Lipofectamine 200 (Invitrogen), according to the manufacture's recommendations, and added to the cells medium to a final concentration of 45pmol. Knockdown of p62 was achieved by transfecting once, 48h prior to the experiments.

3.5. Western Blot (WB)

After treatments, cells were rinsed twice with phosphate buffered saline (PBS), collected immediately in 50µL of 4x Laemmli Buffer (250mM Tris-HCl (pH 6.8), 35% (v/v) glycerol, 20% (v/v) β-mercaptoethanol, 8% (m/v) SDS, 0.03% (m/v) bromophenol blue) and boiled for 5min at 95ºC.

For WB analysis, proteins were separated by electrophoresis on a 10-15% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE). After electrophoresis, proteins were electro-transferred to nitrocellulose membranes. After transfer, membranes were blocked with 5% (m/v) nonfat milk in Tris-buffered saline (TBS) (50 mM Tris (pH 7.6), 150 mM NaCl,) Tween-20 0.01% (v/v) (TBS-T), for at least 30min under agitation, at room temperature. Membranes were incubated with primary antibodies diluted in TBS-T supplemented with 5% non-fat milk, overnight at 4°C and subsequently incubated with horseradish peroxidase (HRP) conjugated secondary antibodies with agitation for 1h, at room temperature. The immunoreactive bands were visualized with enhanced chemiluminescence (ECL) reagent, revealed by scanning blots using a VersaDoc (Bio-Rad Life Science) imaging system. Densitometric analyses were performed using the Image Lab 4.1 Software.

3.6. Immunoprecipitation (IP)

Cells were rinsed with PBS at 4 ºC, resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3) supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF, 10 mM iodoacetamide, and incubated on ice during 10min. The samples were then centrifuged at 10,000g for 10min, and the supernatants used for immunoprecipitation. Briefly, the supernatants were incubated with antibodies directed against a specific protein for 3h, at 4 ºC. Supernatants with no antibodies were used as controls. Then, supernatants were incubated with protein G for 1 hour, at 4 ºC.

Protein G-sepharose sediments were washed 2 times in an appropriate washing buffer (500 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3), 1 time in PBS, resuspended in Laemmli buffer and denatured at 95 ºC, for 5 minutes.
Western blot analysis of the immunoprecipitated proteins was then performed. Inputs represent about 5% of the total amount of protein in the lysates before immunoprecipitation.

3.7. Triton X-100 fractionation assay

The detergent solubility assay with 1% Triton X-100 was performed essentially as described previously by others [90]. Cells were resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3) supplemented with protease inhibitor

cocktail (Roche), 2 mM PMSF and 10 mM iodoacetamide. Samples were then ultracentrifuged at 100,000g for 50min and the supernatant recovered (Triton X-100 soluble fraction). The detergent-insoluble pellets were resuspended in lysis buffer supplemented with 0.1% SDS (Triton X-100 insoluble fraction) and sonicated. Laemmli buffer was then added to the Triton X-100 soluble and insoluble fractions and denatured at 100 ºC for 5min before SDS-PAGE analysis.

3.8. Biotinylation of cell surface proteins

HEK293A cells grown on 10 cm^2 multiwell plates were rinsed twice with 2 mL of ice-cold PBS containing 0.5 mM MgCl2 and 1 mM CaCl2, followed by the addition of 1 mL of the same ice cold solution containing 1 mg/mL of freshly added Sulfo-NHS-SSbiotin (Pierce, Rockford, IL, USA). After 30min at 4 ºC, to stop subcellular trafficking, the medium was discarded and the plates were washed three times with PBS containing 0.5 mM MgCl2, 1 mM CaCl2 and 100 mM glycine. The cells were scraped in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, supplemented with protease inhibitor cocktail, 2 mM PMSF, 10 mM iodoacetamide, pH 7.5). After 15min on ice the cells were sonicated and the homogenates were centrifuged at 14.000 rpm, for 10min. Samples were then transferred to 1.5 mL Eppendorf microfuge tubes containing 25 µL of Neutravidin beads (Pierce, Rockford, IL, USA). After 2h incubation at 4 ºC under agitation, the beads were washed four times with lysis buffer. The final pellets were resuspended in 20 µL 2x Laemmli buffer and denatured at 95 ºC, for 5min. The beads were pelleted and the solubilised proteins were separated by SDS PAGE, transferred to nitrocellulose membranes and probed with antibodies directed against Cx43.

3.9. Immunofluorescence

HEK293A cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS. The samples were then washed with PBS, permeabilised with 0.2% v/v Triton X-100 in PBS and blocked with goat serum (1:10) for 20min prior to incubation with primary antibodies. Incubation with antibodies proceeded for 1h at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for 1h at room temperature. The specimens were rinsed in PBS and mounted with MOWIOL 4-88 Reagent (Calbiochem). Nuclei were stained with DAPI. All solutions were made in 0.2% w/v BSA (Sigma) containing 0.02% sodium azide (Sigma) in PBS. For controls primary antibodies were omitted. Images were collected by confocal microscopy using a Zeiss LSM 710

3.10. Antibodies

Table 1: List of primary and secondary antibodies used for the WB and confocal microscopy analysis

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
anti-Cx43	goat polyclonal	AB0016-500	WB/IP	1:2500/ 1:500	SICGEN (Cantanhede, Portugal)
anti-Cx43	mouse monoclonal	610062	IF	1:25	BD Transduction Laboratories (CA, USA)
anti-LC3	rabbit polyclonal	pa116931	WB	1:500	Thermo Fisher Scientific (Waltham, MA, USA)
anti-p62	rabbit polyclonal	5114S	WB	1:1000	Cell Signalling Technology (MA, USA)
anti-Ubiquitin	mouse monoclonal	P4D1	WB	1:1000	Covance (Princeton, NJ, USA)
anti-GAPDH	goat polyclonal	AB0049	WB	1:2500	SICGEN (Cantanhede, Portugal)
anti-Calnexin	goat polyclonal	AB0041	WB	1:2500	SICGEN (Cantanhede, Portugal)
anti-GFP	goat polyclonal	AB0020	WB	1:1000	SICGEN (Cantanhede, Portugal)
anti-goat IgG-HRP	rabbit	61-1620	WB	1:5000	Life Technologies (Carlsbad, CA)
anti-rabbit - HRP	goat	656120#	WB	1:5000	BioRad (Hercules, CA, USA)
anti-mouse - HRP	goat	626520#	WB	1:5000	BioRad (Hercules, CA, USA)
alexa 568 anti-rabbit	goat	A-11011	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)

3.11. Statistical analysis

Results were analyzed using GraphPad Prism (GraphPad Prism 6.0 software, La Jolla, CA, USA). They are presented as mean ± standard deviations of the mean (SD). All the results are representative of at least three independent experiments. Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. Differences were considered significant at $p < 0,05$.

4. Results

The WxxL sequence accepted for a LIR domain is present at the N-terminal (NT) of Cx43, in the WSAL form. Comparisons between the amino acid sequences in the N-terminal of 20 human connexins reveals that the WxxL sequence is highly conserved between isoforms, highlighted in Figure 8. To confirm that the WSAL sequence in the NT of Cx43 constitutes a putative LIR domain, that mediates the binding to LC3, we used a mutated form of Cx43 in which the aminoacids tryptophan (W) and leucin (L) were replaced by alanine (A), Cx43-W4AL7A, to serve as comparison towards wild type Cx43 (Cx43-wt).

| Amino acid number (β) | | | | | | 1 | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
Amino acid number (α)						1	2	3	4	5	6	7	8	9	10	11	12	13		14	15	16	17	18	19	20	21	22	23
GJB7	Cx25	-	-	-	M	-	S	W	M	F	L	R	D	L	L	S	G	-	V	N	K	Y	S	T	G	T	G	W
GJB2	Cx26	-	-	-	M	-	D	W	G	T	L	Q	T	I	L	G	G	-	V	N	K	H	S	T	S	I	G	K
GJB6	Cx30	-	-	-	M	-	D	W	G	T	L	H	T	F	I	G	G	-	V	N	K	H	S	T	S	I	G	K
GJB4	Cx30.3	-	-	-	M	-	N	W	A	F	L	Q	G	L	L	S	G	-	V	N	K	Y	S	T	V	L	S	R
GJB3	Cx31	-	-	-	M	-	D	W	K	T	L	Q	A	L	L	S	G	-	V	N	K	Y	S	T	A	F	G	R
GJB5	Cx31.1	-	-	-	M	-	N	W	S	I	F	E	G	L	L	S	G	-	V	N	K	Y	S	T	A	F	G	R
GJB1	Cx32	-	-	-	M	-	N	W	T	G	L	Y	T	L	L	S	G	-	V	N	R	H	S	T	A	I	G	R
GJA4	Cx37	-	-	-	M	G	D	W	G	F	L	E	K	L	L	D	Q	-	V	Q	E	H	S	T	V	V	G	K
GJA5	Cx40	-	-	-	M	G	D	W	S	F	L	G	N	F	L	E	E	-	V	H	K	H	S	T	V	V	G	K
GJA1	Cx43	-	-	-	M	G	D	W	S	A	L	G	K	L	L	D	K	-	V	Q	A	Y	S	T	A	G	G	K
GJA3	Cx46	-	-	-	M	G	D	W	S	F	L	G	R	L	L	E	N	-	A	Q	E	H	S	T	V	I	G	K
GJA8	Cx50	-	-	-	M	G	D	W	S	F	L	G	N	I	L	E	E	-	V	N	E	H	S	T	V	I	G	R
GJA9	Cx59	-	-	-	M	G	D	W	N	L	L	G	D	T	L	E	E	-	V	H	I	H	S	T	M	I	G	K
GJA10	Cx62	-	-	-	M	G	D	W	N	L	L	G	G	I	L	E	E	-	V	H	S	H	S	T	I	V	G	K
GJC3	Cx30.2/Cx31.3	-	-	-	M	-	C	G	R	F	L	R	R	L	L	A	E	-	E	S	R	R	S	T	P	V	G	R
GJC1	Cx45	-	-	-	M	-	S	W	S	F	L	T	R	L	L	E	E	-	I	H	N	H	S	T	F	V	G	K
GJC2	Cx47	M	T	N	M	-	S	W	S	F	L	T	R	L	L	E	E	-	I	H	N	H	S	T	F	V	G	K
GJD3	Cx31.9	-	-	-	M	G	E	W	A	F	L	G	S	L	L	D	A	-	V	Q	L	Q	S	P	L	V	G	R
GJD2	Cx36	-	-	-	M	G	E	W	T	I	L	E	R	L	L	E	A	A	V	Q	Q	H	S	T	M	I	G	R
GJD4	Cx40.1	-	-	-	M	E	G	V	D	L	L	G	F	L	I	I	T	-	L	N	C	N	V	T	M	V	G	K
Consensus					M	X	D	W	X	F	L	X	X	L	L	X	X	-	V	X	X	H	S	T	X	X	G	K

Figure 8 – Amino acid sequences of the N-terminal of 20 human connexins. The consensus sequence for the N-terminal of connexins includes a WxxL sequence, consistent with the accepted form for a LIR domain. (Adapted from Beyer, E. et al., 2012).

4.1. The putative LIR domain on Cx43 regulates the stability of the protein

We started by analyzing the influence of this mutation on the half-life of Cx43. CHX-chase assays were performed, in which following transfection with Cx43-wt or Cx43-W4AL7A, protein synthesis is inhibited, with 50 μg/mL of CHX, during 2 or 4h. Samples were then analyzed by WB using antibodies directed against Cx43. The results presented in Figure 9 show that the mutated form of Cx43 (Cx43-W4AL7A) is slightly more stable than the wild type form of the protein.
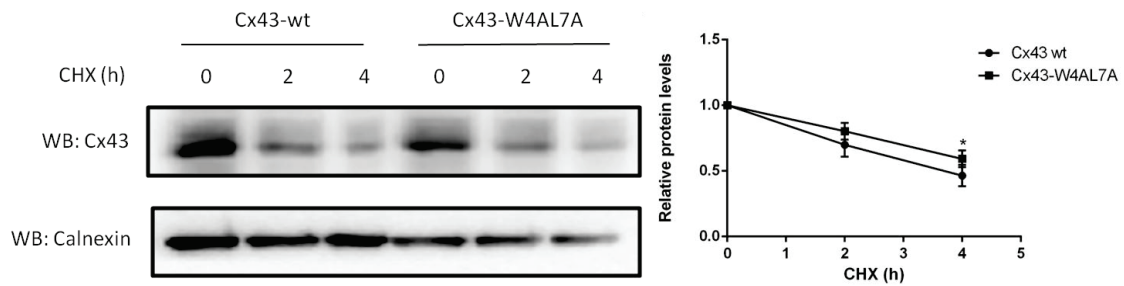
Figure 9 – Cx43 mutated in the putative LIR domain presents higher stability. HEK293A cells were transfected with wt or Cx43 mutated in the WSAL sequence and were incubated with 50µg/mL CHX for 0 ,2 and 4h. Lysates were then analyzed by WB using antibodies directed against Cx43. Calnexin was used as an internal control. The values presented are the average of four individual experiments ± SD. * indicates statistically significant differences from control ($p < 0.05$).

4.2. Cx43-W4AL7A is present at the plasma membrane

Being Cx43 a membrane protein, it was important to confirm if the mutation in the putative LIR domain had any influence on the translocation of the protein to the membrane. We assessed the presence of Cx43-W4AL7A at the plasma membrane by different experimental approaches: cell surface biotinylation assays and Triton X-100 insolubility. Through the biotinylation assays, we show that Cx43-W4ALTA is present at the plasma membrane (Figure 10A), most likely in the form of hemichannels. To evaluate the presence of Cx43 in GJ we performed Triton X-100 extraction assays, since Cx43 localized in GJ is not accessible to biotin. The results presented in Figure 10B demonstrate that Cx43-W4AL7A is present in the insoluble fraction, which corresponds to Cx43 localized at the GJ. The levels of Cx43-wt and mutated Cx43 in the plasma membrane are similar in both experiments, suggesting that the mutation does not affect the ability of Cx43 to localize at the plasma membrane.
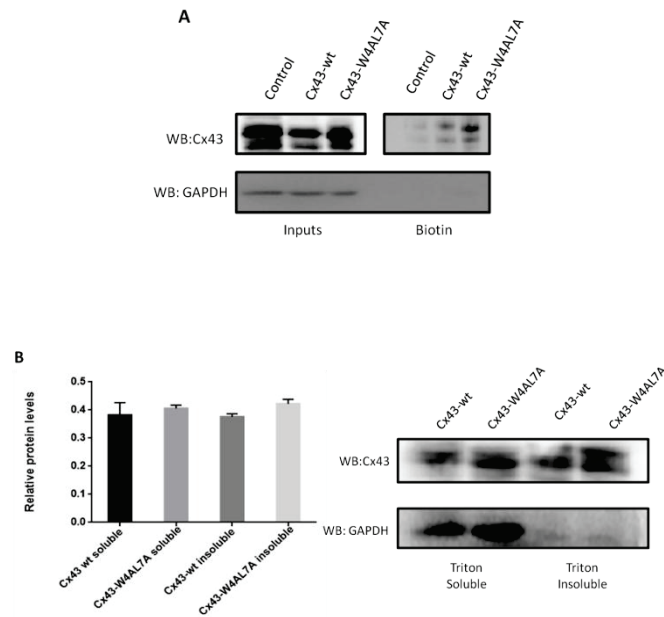
Figure 10 – Cx43 mutant is present at the plasma membrane. A) The biotinylated fraction of cell lysates was precipitated with NeutrAvidin beads, and further analyzed by WB using antibodies against Cx43. GAPDH was used as a negative control for the biotinylated fraction. B) HEK293A cells were transfected with wt or mutant Cx43 and cell lysates were subjected to subcellular fractioning with 1% Triton X-100. Triton X-100-soluble and –insoluble fractions were further analyzed by WB, using antibodies against Cx43. GAPDH was used to differentiate the Triton X-100-soluble from the -insoluble fraction.

4.3. Interaction between Cx43 and LC3 is reduced when the putative LIR domain is mutated

According to our model the WSAL sequence in the N-terminal of Cx43 constitutes a putative LIR and mediates its interaction with LC3. To confirm that this sequence was indeed a LIR domain, we evaluated the interaction between LC3, and either the wild type or three different Cx43 mutants (Cx43-W4A, Cx43-L7A and Cx43-W4AL7A), using immunoprecipitation assays. For that, HEK293A cells were co-transfected with Cx43 (wt or mutant) and GFP-LC3 after which Cx43 was immunoprecipitated and the amount of LC3 that was bound to Cx43 determined using antibodies against GFP. The results presented on Figure 11 show that Cx43-wt interacts with LC3, and that this interaction is highly reduced when the WSAL sequence is mutated. In addition, both Cx43-wt and the mutated forms of Cx43 are ubiquitinated, showing that the mutations performed on Cx43 do not affect the ubiquitination of the protein.
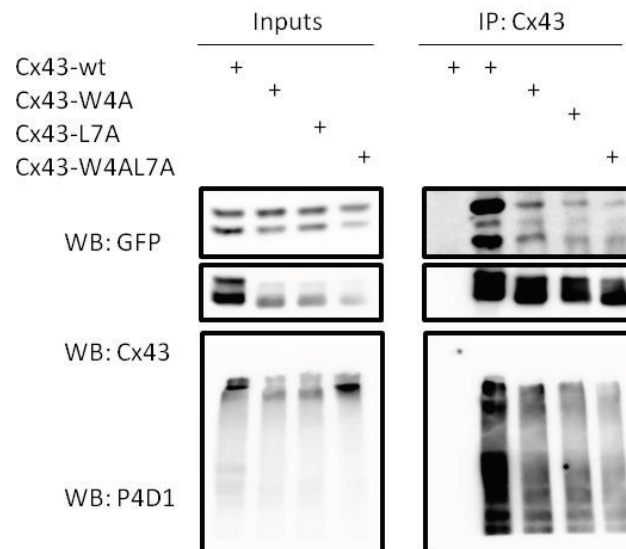
Figure 11 – Mutations in the LIR motif of Cx43 impairs the interaction of Cx43 with LC3. HEK293A cells were transfected with wt or Cx43 mutated in the WSAL sequence and GFP-LC3. Immunoprecipitation assays were performed using antibodies against Cx43. Cell lysates and precipitates were analyzed by WB using antibodies against GFP, Cx43 and P4D1.

4.4. Cx43 co-localizes with LC3

The results presented above demonstrate that Cx43 can interact with LC3, via LIR motif localized in the NT of Cx43. In a subsequent stage, we intended to evaluate where this interaction occurs. For that we used immunofluorescence to assess the subcellular localization of both Cx43 and LC3, in HEK293A cells transfected with Cx43-wt or Cx43-W4AL7A and GFP-LC3. The images were collected by confocal microscopy. Interestingly, we observed that Cx43-wt not only co-localizes with LC3 intracellularly but also at the plasma membrane (Figure 12). Regarding the Cx43-W4AL7A mutant, as expected, the levels of co-localization at the perinuclear region were diminished, as compared with the wt, whereas at the plasma membrane the co-localization was virtually null.
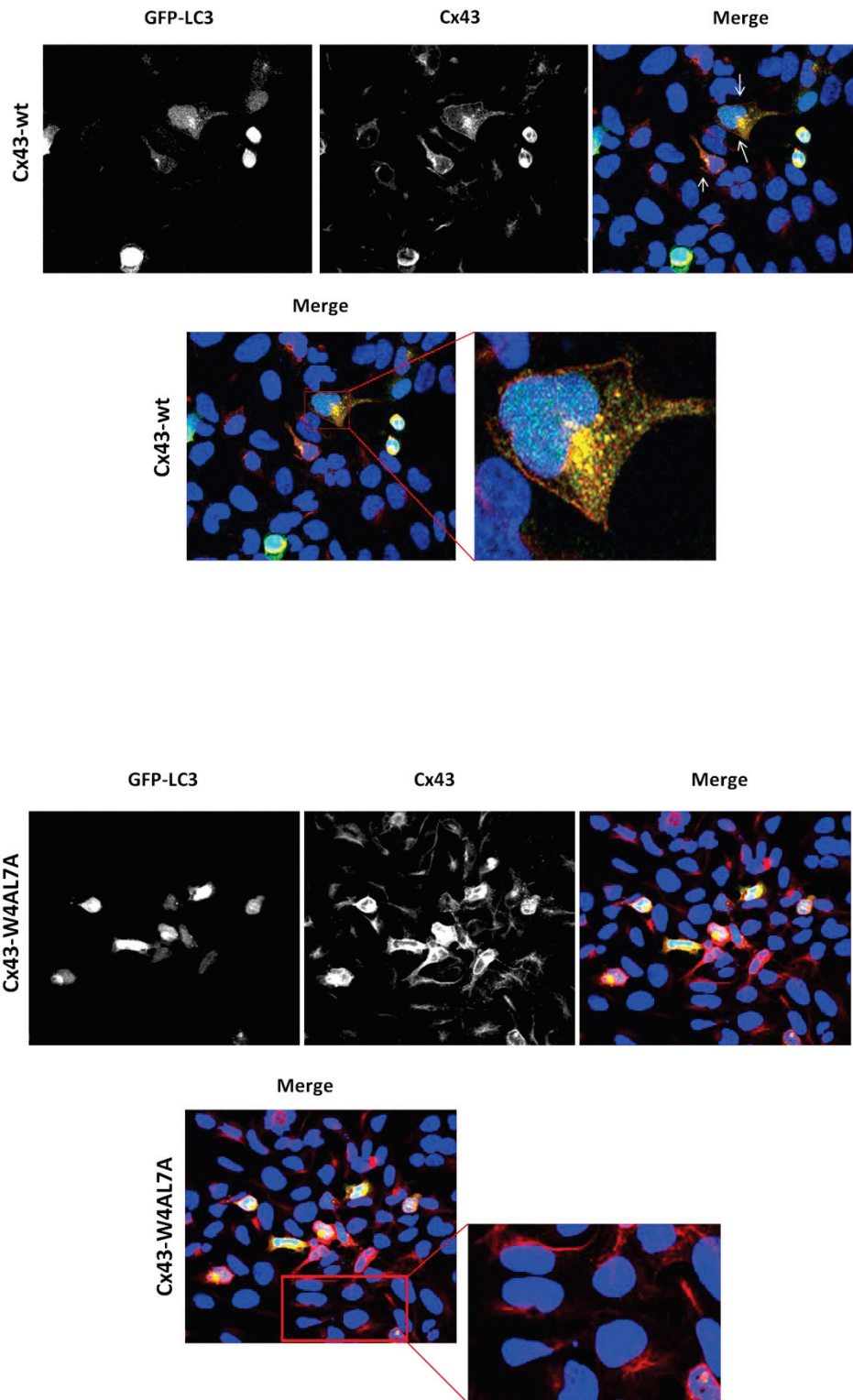
Results

Figure 12 – Cx43-wt co-localizes with LC3 at the plasma membrane. HEK293A cells were transfected with Cx43 (wt or mutated) and GFP-LC3, fixed with 4% PFA for 10 min and used for immunofluorescence using specific antibodies directed against Cx43. Nuclei were stained with DAPI.

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Results

4.5. The putative LIR domain on Cx43 stabilizes the protein in starvation conditions

Starvation is used as a mechanism to activate autophagy. Having analyzed the influence of the WSAL mutation in the stability of Cx43 in basal conditions, we proceeded to evaluate the role of the LIR motif in starvation-induced degradation of Cx43. For that, we induced autophagy in HEK293A cells transfected with either Cx43-wt or Cx43-W4AL7A through 4 hours of serum deprivation. The results in Figure 13 demonstrate that the mutation on the putative LIR domain stabilizes the protein in autophagy-induced conditions. While the levels of Cx43-wt decrease about 45% after 4 hours of starvation, the amount of Cx43-W4AL7A is not significantly altered when autophagy is triggered by serum removal.
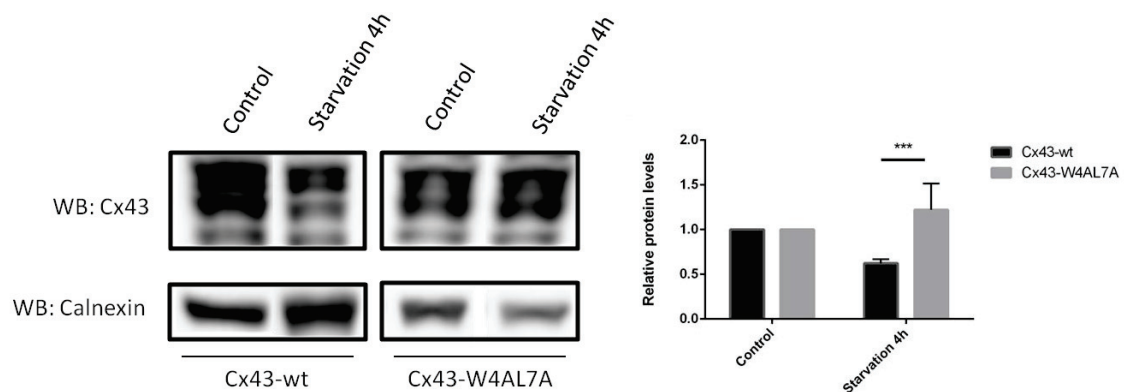

Figure 13 – Cx43 mutated in the putative LIR domain presents higher stability during starvation. HEK293A cells were transfected with wt or Cx43 mutated in the WSAL sequence. Cells were submitted to 4 hours of Starvation. Lysates were then analysed by WB using antibodies directed against Cx43. Calnexin was used as an internal control. The values presented are the average of four individual experiments ± SD. *** indicates statistically significant differences from control (p< 0,001).

4.6. Starvation leads to an increase in the interaction between Cx43 and LC3

Once we have established that mutations in the putative LIR motif protect Cx43 from starvation-induced degradation, we subsequently evaluated if the interaction between Cx43 and LC3 still occurs in these conditions and to what extent. For that, HEK293A cells were transfected with Cx43 (wt or mutated) and GFP-LC3 and submitted to 4h of starvation. Following immunoprecipitation we could observe (Figure 14) that activation of the autophagy pathway through starvation leads to an increase in the interaction between Cx43-wt and LC3. As for Cx43-W4AL7A, the interaction with LC3 is still highly decreased in starvation conditions.
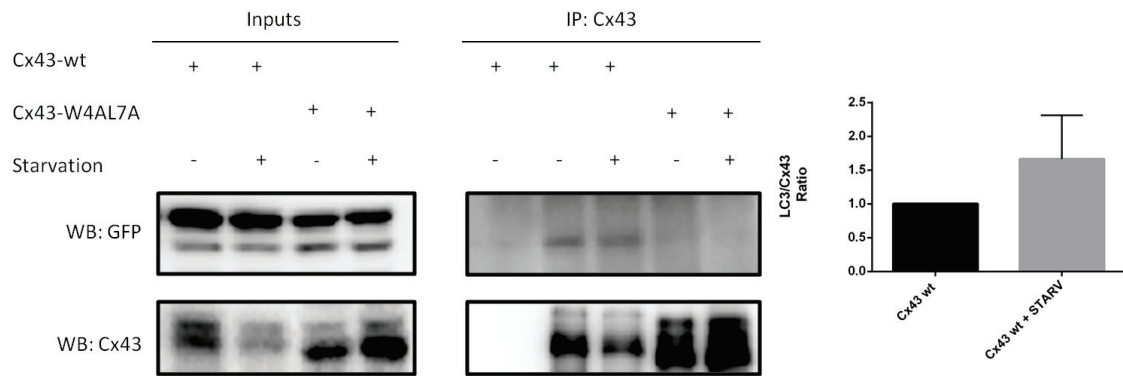
Results

Figure 14 – Starvation increases the interaction between Cx43 and LC3. HEK293A cells were transfected with wt Cx43 mutated in the WSAL sequence and LC3 and submitted to starvation for 4h. Immunoprecipitation assays were performed using antibodies against Cx43. Cell lysates and precipitates were analyzed by WB using antibodies against GFP and Cx43. The values presented are the average of three individual experiments ± SD.

To further confirm the influence of the LIR motif in Cx43 degradation, we performed experiments inhibiting lysosomal degradation. Cells were incubated for 4h with BafA1 to inhibit the lysosome. Starvation was also used for 4h to activate autophagy and induce the lysosomal degradation of Cx43. As we can see in Figure 15, Cx43-wt levels decrease in starvation conditions, an effect that is reverted by lysosomal inhibition with BafA1. However, the Cx43 mutant does not accumulate in the presence of BafA1, suggesting that the mutant is insensitive to BafA1. It is well established that the one of the cardinal features of autophagy activation is the conversion of LC3-I into membrane-associated LC3-II by conjugation with PE. Thus, the conversion of LC3-I into LC3-II is widely used as a measure of autophagy activity. In accordance with previous studies, serum deprivation results in the increase in the ratio LC3-II/LC3-I, demonstrating that the autophagy pathway is being activated in our experimental conditions. To further evaluate the autophagy flux, we analyzed the ratio LC3-II/LC3-I, in cells subjected or not to serum deprivation (starvation), either in the presence or absence of the lysosome inhibitor Baf1A. As we can observe in Figure 15, both in cells overexpressing either Cx43-wt or Cx43-W4AL7A, the ratio LC3-II/LC3-I increases in starvation, being this value even higher in the presence of Baf1A, meaning that mutations in the putative LIR motif of Cx43 do not affect autophagy activity. Besides the levels of LC3, we also analyzed the levels of autophagy adaptor p62 to confirm autophagy activation. The levels of p62 increase in starvation conditions and in the presence of BafA1.
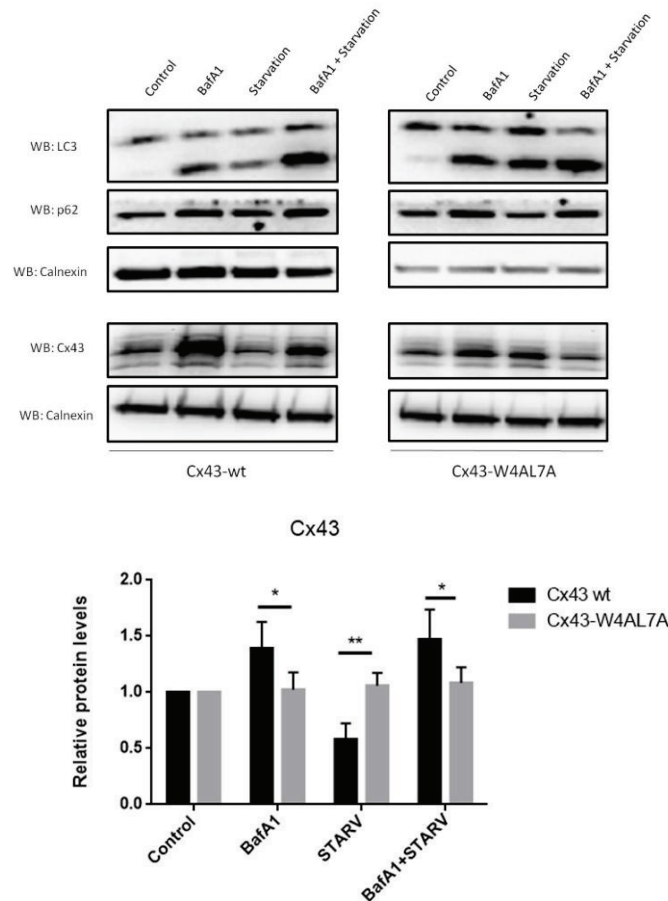
Figure 15 – Cx43-W4AL7A is less degraded by autophagy. HEK293A cells were transfected with wt or Cx43 mutated in the WSAL sequence and submitted to starvation and incubated with 50nM of BafA1 for 4h. Cell lysates were analyzed by WB using antibodies against LC3, Cx43 and p62. Calnexin was used as an internal control. The values presented are the average of three individual experiments ± SD. * and ** indicate statistically significant differences from control (p< 0.05 and p < 0.01, respectively).

4.7. Overexpression of p62 leads to a decrease in the interaction between Cx43 and LC3

Canonically, the substrates are sorted into autophagy vesicles through the interaction with p62, that establishes a bridge between the ubiquitinated substrate and LC3 anchored to the autophagosomal membrane, through its LIR motif. We have previously demonstrated that degradation of Cx43 by starvation- or ischemia-induced autophagy requires ubiquitination of Cx43 and its subsequent recognition by p62 [87]. The existence of a LIR motif in Cx43 envisions a new mechanism for direct targeting of Cx43 to the autophagosome that is independent of p62 and ubiquitination. To evaluate whether the amount of p62 could somehow affect the levels of binding between p62 and LC3, we overexpresed p62 and determined the influence of this protein in the Cx43-LC3 interaction. Using the HEK293A cell line transfected with Cx43 (wt

or mutated), GFP-LC3 and mCherry-p62, we performed immunoprecipitation assays and evaluated the amount of p62 and LC3 that is being co-immunoprecipitated with Cx43 in each condition. As demonstrated in Figure 16, the presence of higher levels of p62 leads to a decrease in the interaction between Cx43-wt and LC3. On the other hand, the interaction between Cx43-W4AL7A and LC3 does not seem to be affected by the presence of overexpressed p62 that is, higher levels of p62 do not promote the interaction between Cx43-W4AL7A and LC3, which suggests that in this cell line the binding of Cx43 to LC3 occurs predominantly through the LIR motif. Importantly, both Cx43-wt and Cx43-W4AL7A interact with p62, suggesting that the interaction of Cx43 with p62 occurs upstream of the recruitment to the autophagosomes and binding to LC3.
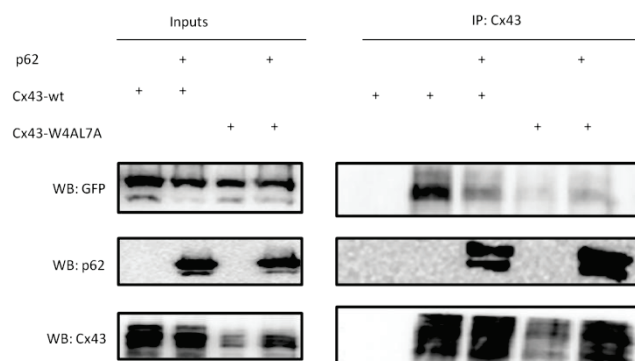

Figure 16 – Overexpression of p62 decreases the interaction between Cx43 and LC3. HEK293A cells were transfected with wt or Cx43 mutated in the WSAL sequence and p62. Immunoprecipitation assays were performed using antibodies against Cx43. Cell lysates and precipitates were analyzed by WB using antibodies against GFP, Cx43 and p62.

4.8. Overexpression of p62 produces a major decrease on Cx43-W4AL7A half life

To further analyze the influence of p62 in the degradation rate of Cx43 mutated in the LIR domain, we performed CHX chase assays to determine the half-life of Cx43, in the presence of higher levels of p62. For that, HEK293A cells were transfected with mutated or wild type Cx43 and mCherry-p62. Comparing with results presented in Figure 9, in which we evaluated the half-life of Cx43-wt and Cx43-W4AL7A, in the presence of endogenous levels of p62, the data shown in Figure 17 demonstrate that the overexpression of p62 leads to a faster degradation of Cx43-W4AL7A, whereas the turnover of Cx43-wt is not significantly altered. Indeed, in constitutive conditions, the mutation of LIR motif slightly stabilizes Cx43, whereas in the presence of high levels of p62 the turnover of the mutated form of Cx43 increases.
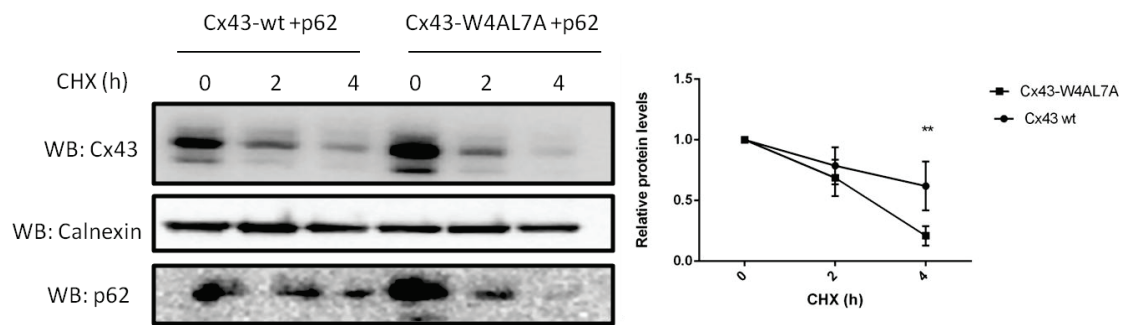
Figure 17 – Overexpression of p62 decreases the half life of mutant Cx43. HEK293A cells were transfected with wt or Cx43 mutated in the WSAL sequence and p62 and were incubated with 50µg/mL CHX for 0 ,2 and 4h. Lysates were then analysed by WB using antibodies directed against Cx43 and p62. Calnexin was used as an internal control. The values presented are the average of three individual experiments ± SD. ** indicates statistically significant differences from control ($p < 0.01$).

4.9. Higher levels of p62 in HeLa cells increase the stability of Cx43-W4AL7A

In an effort to confirm our hypothesis in another cell line, we replicated our experiments in the HeLa cell line. First we assessed the half-life of Cx43-wt and Cx43-W4AL7A, with CHX-chase assays. Contrary with HEK293A cell line, the results obtained in the HeLa cell line show that the wild type form of Cx43 is marginally more stable that Cx43 mutated in the WSAL domain (Figure 18).

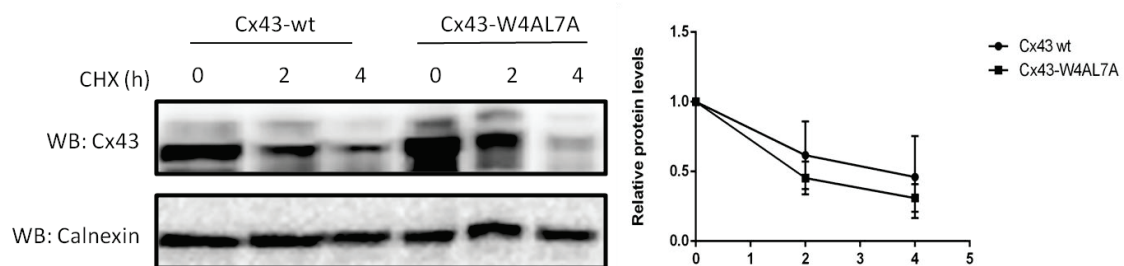**Figure 18 - Cx43 mutated in the putative LIR domain presents lower stability in HeLa cells.** HeLa cells were transfected with wt or Cx43 mutated in the WSAL sequence and were incubated with 50µg/mL CHX for 0, 2 and 4h. Lysates were then analyzed by WB using antibodies directed against Cx43. Calnexin was used as an internal control. The values presented are the average of three individual experiments ± SD.

Since we had shown that the levels of p62 affect the stability of Cx43-W4AL7A, we compared the amount of p62 in the two cell lines. Surprisingly, the levels of p62 in HeLa cells are much higher than in the HEK293A cell line (Figure 19).
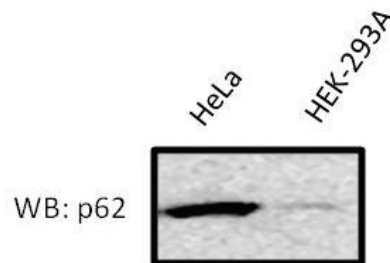

Figure 19 – HeLa cell line presents higher levels of p62. Cell lysates from HeLa and HEK293A cell lines were collected and the same amount of protein was used for WB analysis, using antibodies against p62.

4.10. Interaction between Cx43 and LC3 is diminished when silencing p62 in HeLa cells

Given the results presented above, it is conceivable that the difference in p62 expression levels in the two cells lines affect the interaction between LC3 and Cx43, and the dependence on the LIR motif. To address this question, we proceeded to evaluate the interaction between Cx43 and LC3 in the HeLa cell line. As with the HEK293A cells, the results presented in Figure 20 demonstrate that, in HeLa cells, the binding of Cx43 to LC3 is diminished when the LIR motif of Cx43 is mutated (compare lane 2 and lane 4 in the right-side image). Given the difference in p62 levels between the HeLa and HEK-293A cell lines, we also investigate the interaction between Cx43 and LC3, in cells silenced for p62, using siRNA mediated knockdown of p62. For that, HeLa cells were simultaneously co-transfected with Cx43-wt, GFP-LC3 and siRNA targeting p62. As we can observe in figure 20, the interaction between Cx43-wt and LC3 is partially impaired when p62 is silenced. As expected, for the mutated form of Cx43, the interaction with LC3 is decreased (compare the lanes 2 and 3, of the right-side image of Figure 20). Altogether, these results suggest that in HeLa cells the interaction between LC3 and Cx43 is mediated both by p62 and LIR motif, since when either mechanisms are impaired the binding Cx43-LC3 decreases. On the other hand, in HEK293A cells, with lower levels of p62, this interaction mainly relies on the LIR motif.
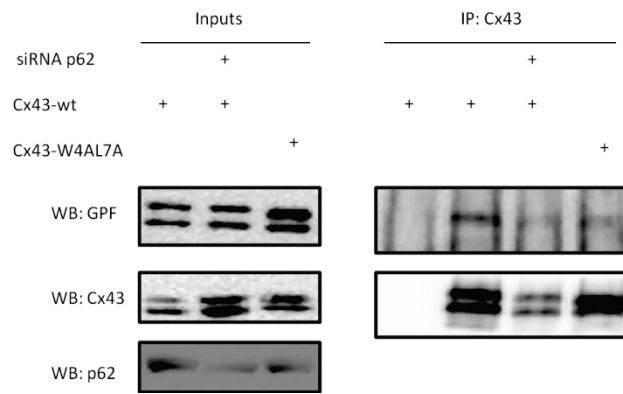
Figure 20 – Silencing p62 decreases the interaction between Cx43 and LC3. HeLa cells were transfected with wt or mutated Cx43 in the WSAL sequence, GFP-LC3 and siRNA against p62. Immunoprecipitation assays were performed using antibodies against Cx43. Cell lysates and precipitates were analyzed by WB using antibodies against GFP, Cx43 and p62.

5. Discussion

GJIC maintenance is essential for the proper function of tissues and organs, by allowing electrical and metabolic coupling between cells. Indeed, the impairment of GJIC has been shown to lead to several disorders, which makes this a process that needs to be highly controlled. Several biological processes have been shown to be involved in the regulation od GJIC, including the regulation of synthesis, degradation and trafficking of the GJ protein Cx43, which is crucial to preserve GJIC. Given the short half-life of Cx43, the degradation of this protein plays a major role in the regulation of GJIC. Results previously obtained in our lab demonstrated that degradation of Cx43 by the autophagosomal pathway requires the ubiquitination of the protein, followed by the recruitment of p62 that targets the protein to autophagosomes for degradation. Indeed, on one hand, p62 recognizes the ubiquitin moieties attached to the substrate and, on the other hand, also binds to LC3, through its LIR motif. Therefore, p62 acts as an adaptor that brings ubiquitinated substrates to nascent autophagic vesicles. Remarkably, a sequence consistent with a LIR motif is found in the N-terminal of Cx43, which suggests that the protein may bind directly to LC3 without the need for p62 thus, envisioning a novel mechanism for Cx43 degradation.

Therefore, a main objective of this study was to identify and characterize the putative LIR motif on Cx43. To address this question we used forms of Cx43 mutated in the sequence compatible with a LIR motif, after which we evaluated the importance of this motif in mediating the interaction with LC3.

The results presented in this thesis demonstrate, for the first time, the presence of a LIR motif in Cx43. Through immunoprecipitation assays carried out in the HEK293A cell line we demonstrated that Cx43 mutated in the putative LIR motif loses the ability to interact with LC3. Additionally, we showed that the Cx43 mutant showed higher stability, especially when the autophagy pathway is activated through starvation, suggesting that impairment of the LIR motif prevents Cx43 from being degraded by this pathway. The use of the autophagy inhibitor BafA1 revealed that the mutation on the LIR motif seems to make the protein less sensitive to lysosomal inhibition. Furthermore, we evaluated the role of p62 in the interaction between Cx43 and LC3 through overexpression and silencing of p62. Immunoprecipitation assays showed that the levels of p62 do not overcome the reduction of the interaction between Cx43 and LC3, due to mutation on the LIR motif. Importantly, mutation on the LIR motif does not impair the binding to p62. In addition, CHX chase assays performed in cells overexpressing p62 demonstrated that the mutant form of Cx43 is degraded faster, whereas degradation of the

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wild type Cx43 is not affected. These results are similar to those obtained with HeLa cells, where the endogenous expression of p62 is higher.

In a first stage of this work we assessed the role of the LIR motif in the turnover of Cx43. For that we evaluated the half life of Cx43-wt and Cx43-W4AL7A in HEK293A cells and observed that mutation of the LIR motif stabilizes the protein, ascribing a role to the LIR motif in the degradation of Cx43. Since Cx43 is a resident plasma membrane protein, we investigated if the W4AL7A mutation did not prevent the protein from being delivered to the cell surface. The biotinylation and Triton X-100 extraction assays demonstrated the presence of Cx43-W4AL7A at the plasma membrane. The interaction between Cx43-wt and LC3 was confirmed by immunoprecipitation, as used previously in other studies to assess interactions with LC3 [46], [91], [92]. For this assay, two other Cx43 mutants were also used: Cx43-W4A and Cx43-L7A, each of them mutated in one of the essential amino acids for the LIR domain, to provide stronger evidences for this interaction. Cx43 interacts with LC3 and mutations on the LIR motif highly reduce this interaction, strongly supporting the hypothesis that Cx43 possesses a LIR motif that mediates its interaction with LC3. Also, the fact that the Cx43 mutants are ubiquitinated and interact with p62 proves that the alterations performed do not affect the ability of the protein to be ubiquitinated, recognized by p62 and be degraded by the canonical mechanism for autophagy degradation. Furthermore, we could observe co-localization between Cx43 and LC3 at the plasma membrane, but not with the Cx43 mutant. This envisions a model in which interaction of Cx43 with LC3 at the plasma membrane constitutes an early stage of GJ degradation by autophagy.

Mutations on Cx43 have been associated with oculodentodigital dysplasia (ODDD), an autosomal-dominant human disorder where patients display symptoms of congenital craniofacial deformities, anomalies of the teeth and eyes and limb deformities. There are 72 known Cx43 mutations linked to ODDD and approximately 20 of these mutations lead to completely or almost completely nonfunctional GJ. However, many of these mutants can be found at the plasma membrane, where they assemble into these dysfunctional GJ. The L7V mutation on the N-terminal of Cx43 is one of the identified mutations linked to ODDD, leading to non-functional GJ [7], [93]. This mutation is localized on the putative LIR motif of Cx43, and opens up the possibility that defects in Cx43 degradation by autophagy may be one of the underlining causes of ODDD. Therefore, it is conceivable that the impairment of the direct binding between LC3 and mutated Cx43 reduces the degradation of Cx43 and leads to the accumulation of a non-functional Cx43 with consequences at the level of GJIC.

Previous studies performed in our lab and others demonstrated that inducing autophagy through starvation leads to the degradation of Cx43 by macroautophagy [75], [77],

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[87], [88]. Therefore, we analyzed the possible role for the LIR domain of Cx43 in conditions where autophagy is activated. We observed that the Cx43 mutant is not degraded to the same extent as Cx43-wt in these conditions. This result suggests that the LIR motif on Cx43 is important for targeting the protein to the autophagy machinery. We propose that the presence of the mutation on Cx43 prevents the protein from binding to LC3 and the protein is not directed to the autophagosome. In accordance, Cx43-W4AL7A is not responsive to the activation of the autophagy pathway. The continued synthesis of the protein together with the lack of response to the starvation treatment causes the accumulation of the protein in these conditions. The importance of this domain in starvation conditions was also verified by the increase in the Cx43-LC3 interaction after 4h of nutrient deprivation. In these conditions, higher levels of Cx43 are sent to lysosomal degradation when autophagy is induced, due to the increase of this interaction. However, the interaction between Cx43-W4AL7A and LC3 is impaired even when autophagy is activated. This result together with the stabilization of the mutant during starvation, led us to suggest that the LIR motif is important for the degradation of Cx43 during starvation. In agreement, the stabilization of Cx43 by LIR mutations occurs mainly in stress conditions, being this effect virtually null under resting conditions. Thus, to confirm this effect, we inhibited the autophagy pathway using BafA1, an inhibitor of the vacuolar H$^+$ATPase, which prevents the acidification of the lysosome and does not allow the fusion between autophagosomes and lysosomes [34]. Activation of the autophagy pathway and incubation with BafA1 causes the accumulation of Cx43-wt. However, Cx43-W4AL7A does not accumulate to the same extent as Cx43-wt. The non accumulation of the mutated form of Cx43 in the presence of the lysosome inhibitor reinforces the idea that the LIR motif is important for targeting the protein for lysosomal degradation. Since the mutation on the LIR motif diminishes the interaction between Cx43 and LC3, and the protein is not targeted to the autophagosome, BafA1 treatment does not lead to the accumulation of the protein. Most likely, the protein is being degraded by another pathway, possibly the UPS, since the ubiquitination of the mutant still occurs. To test this hypothesis further studies are required to evaluate the effect of proteasome inhibitors.

Previous studies stated that plasma membrane Cx43 could function as a negative regulator of the autophagy pathway by inhibiting autophagosome biogenesis. Cx43, under basal conditions, sequesters Atg16 and the initiation complex PI3K in an inactive status at the plasma membrane, negatively regulating autophagy. Under starvation conditions, Atg14 is recruited to the Cx-Atg complex and, together with Atg9, triggers the internalization of Cx43 and the inhibitory effects is released. [94]. However, they did not evaluate the interaction between Cx43 and LC3. Proteins containing LIR motifs can work as adaptors to bring proteins

to the autophagosome for degradation. For example, NBR1 was found to link ubiquitinated cargo to the autophagosome through interaction with LC3 [55]. In mitophagy, a selective form of autophagy that leads to the degradation of mitochondria, AMBRA1 was identified as a new LIR-containing protein and its interaction with LC3 facilitates mitochondrial clearance by bringing damaged mitochondria to the autophagosome [91]. The identification of a LIR motif on Cx43 suggests that, on the onset of starvation, LC3 could be recruited to Cx43 to induce its degradation. Alternatively, Cx43 may have a role in the targeting of proteins to autophagosomes, by functioning as a scaffold for the assembly of the autophagy machinery at the plasma membrane. It has been recently described in yeast that in the initial stages of nutrient deprivation, many integral membrane proteins undergo endocytosis and degradation in vacuoles, through the MVB pathway. This is thought to provide yeast with an initial supply of amino acids to synthesize the vacuolar proteins that are required for the subsequent bulk degradation of cytoplasmatic components during sustained starvation [95]. It is conceivable that a similar mechanism occurs with Cx43, where on the onset of nutrient deprivation, the protein recruits autophagy components to the plasma membrane, where it assists in the degradation of plasma membrane proteins. It will be interesting to test this hypothesis in future studies by investigating if the LIR motif of Cx43 is required for the starvation-dependent degradation of interacting plasma membrane proteins.

This non-canonical mechanism for the degradation of Cx43 which we are proposing in this study does not require prior ubiquitination by Nedd4 and binding to the adaptor p62 to recruit LC3. To address this question we evaluated the influence of the levels of p62 in the half-life of Cx43 and in the Cx43-LC3 interaction. The overexpression of p62 in HEK293A cells that present low endogenous levels of p62 results in a slight decrease in the interaction between Cx43-wt and LC3 suggesting that the autophagy adaptor may function as a competitor with LC3 for the direct binding to Cx43. It is possible that Cx43 binds to both LC3 and p62, being the protein degraded by both canonical and non canonical pathways. Although Cx43-W4AL7A binds to overexpressed p62, the interaction with LC3 remains diminished. Moreover, cells overexpressing p62 present a higher rate of degradation of the mutant, while the half-life of Cx43-wt remains approximately the same. Altogether, these results led us to propose that the presence of higher levels of p62 may facilitate the targeting of the mutant protein, that does not bind LC3, to the proteasome for degradation, as p62 has been described to assist in the delivery of substrates to the 26S proteasome [96]. Furthermore, the overexpression of p62 in cells may lead to the formation of large p62 cytoplasmatic bodies, also positive for ubiquitin. These aggregates are normally caused by the aggregation of ubiquitinated misfolded mutant proteins, as in the case with Lewy bodies in Parkinson's disease, neurofibrillary tangles in

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Alzheimer's disease and the huntington aggregates in Huntington's disease [97]. The formation of these aggregates when higher levels of p62 are present can explain the increased interaction between Cx43 and p62 and the higher rates of degradation of the Cx43 mutant.

After the confirmation of our hypothesis in the HEK293A cell line, we tried to replicate our results in another cell line in order to reinforce our results. Surprisingly, using the HeLa cell line our results were not concordant with the previous results from the HEK293A cell line. In HeLa cells, the CHX chase assay demonstrated that the Cx43 WSAL mutant has a shorter half life than Cx43-wt. The contrast in these results can be explained by differences in the composition of the cell lines, namely the content of proteins involved in autophagy. Analyzing the levels of some autophagy related proteins we came across with a remarkable difference in the levels of p62 between these cell lines. HeLa cells present much higher levels of p62 than the HEK293A cell line. Accordingly, the results obtained with the overexpression of p62 in HEK293A cells and in the HeLa cell line showed that the interaction between the mutant and LC3 is still highly diminished, despite the higher levels of p62. Silencing p62 in the HeLa cell line demonstrated that the interaction between Cx43-wt and LC3 still occurs but is reduced when p62 is silenced, presumably because p62 is no longer recruiting ubiquitinated Cx43 to LC3. Importantly, despite the high levels of endogenous p62 present in these cells, the interaction between LC3 and Cx43-W4AL7A is still much lower than the interaction with Cx43-wt. However, although this interaction is almost completely abolished in HEK-293A cells, in HeLa cells it is possible to observe a weak interaction between the mutant and LC3, which could be explained by the presence of high levels of p62 in HeLa cells that might mediate the formation of the complex LC3-p62-Cx43.

According to our results, degradation of Cx43 can also happen through an ubiquitin and p62-independent mechanism in basal conditions. Ubiquitination is an ATP dependent post-translational modification that has a major role in regulating protein degradation. The complexity of the ubiquitin system, provided by the variety of ubiquitin chains and the three enzymatic steps needed to attach these ubiquitin chains, makes the ubiquitin-dependent degradation pathways highly regulated [14]. This novel mechanism for degradation of Cx43 that we are proposing could have a major impact in situations where ubiquitination is impaired or a rapid degradation of Cx43 is required, in order to hinder GJIC. In this case ,the direct binding between Cx43 and LC3 could be a faster mechanism for targeting the protein for autophagy degradation, since it bypasses any need for ubiquitination, which may be relevant in conditions where ATP availability is reduced, as in the case of nutrient deprivation.

Furthermore, the LIR motif of Cx43 is highly conserved throughout the connexin family (Figure 8), suggesting that this mechanism of degradation may be a general mechanism for the

degradation of most connexin family members. However, future work is needed to ascertain this hypothesis.

In this study, we only describe the interaction between Cx43 and one member of the Atg8 family (LC3-B). However, LIR-containing proteins can also interact with other members of the Atg8 family, such as the GABARAP subfamily and LC3-A and LC3-C. Autophagy adaptor NBR1 has been shown to interact with GABARAPL-1 and proteins from the ULK complex (ULK1, ULK2, Atg13 and FIP200) also interact with the GABARAP subfamily [47], [46]. In the future, it will be interesting to study the possible affinity between Cx43 and other Atg8 family proteins and the possible role for this interaction.

6. Conclusions

Cx43 has a crucial role in the maintenance of GJIC, allowing the exchange of ions and small molecules between adjacent cells. The deregulation of this process leads to several diseases, including heart failure and myocardial ischemia. Cx43 presents a short half-life and for this reason, the regulation of the turnover of the protein is essential for maintaining the activity and stability of GJ. Therefore, it is important to understand the pathways that lead to the degradation of Cx43.

The results obtained in this study suggest a new mechanism for the degradation of Cx43 by macroautophagy. Degradation of Cx43 by autophagy has been described to require prior ubiquitination and the adaptor p62 to bring the protein to the autophagosome. However, here we report a direct interaction between Cx43 and LC3, suggesting a new pathway for the degradation of Cx43, independent of ubiquitin and p62. In this non-canonical pathway for Cx43 degradation, the protein is able to bind directly to LC3, being targeted directly to the autophagosome. This novel pathway for degradation of Cx43 likely takes place in basal conditions, together with the ubiquitin-mediated degradation pathway.

The relevance of this ubiquitin- and p62-independent mechanism for the degradation of Cx43 is still poorly understood and further studies are necessary to clarify the importance of this motif in the maintenance of GJIC.

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