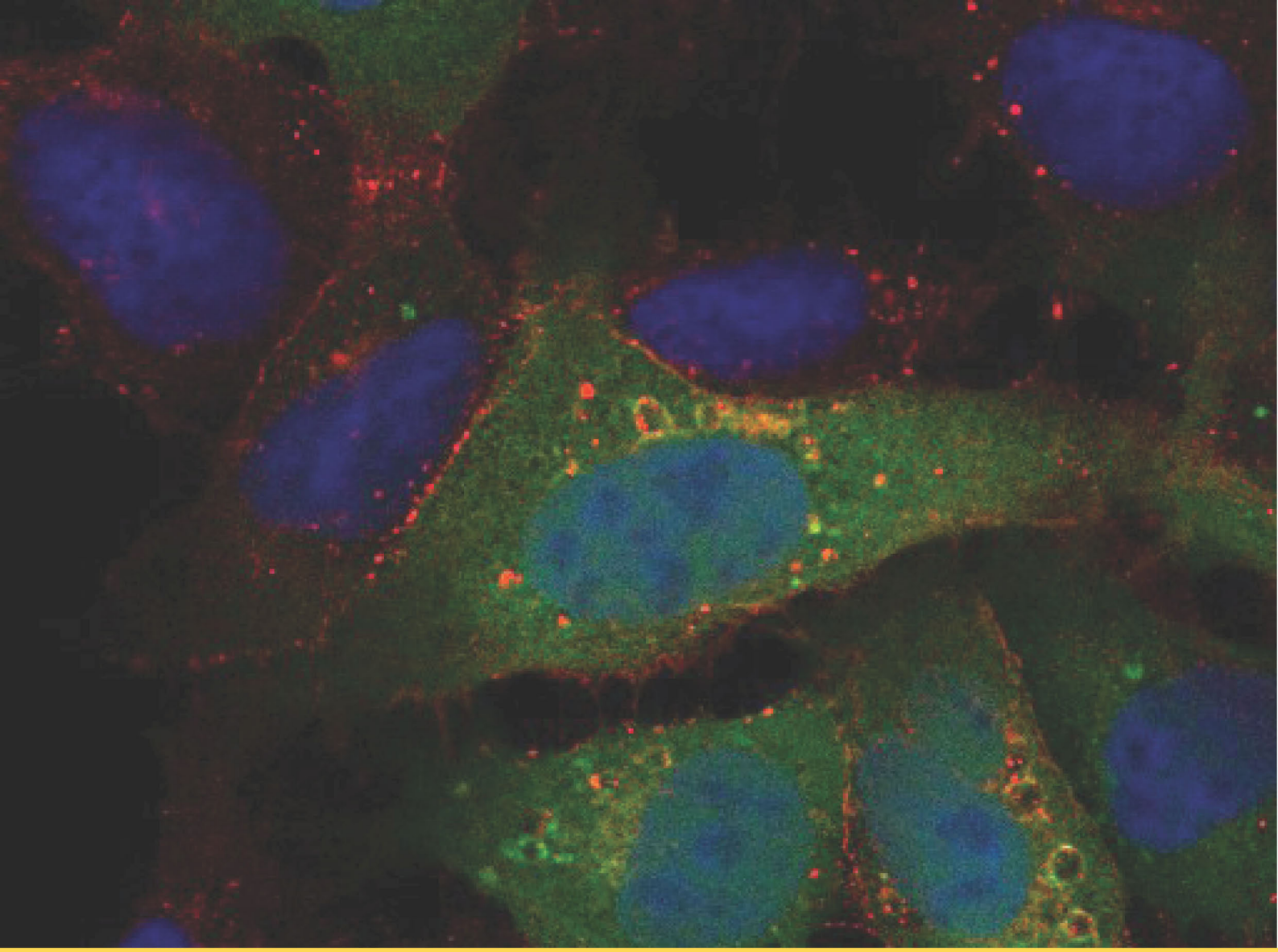

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Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra

Mestrado em Investigação Biomédica, Ramo de Oncobiologia

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ACRONYMS AND ABBREVIATIONS LIST

AMSH - Associated Molecule with the SH3 domain of STAM

ATG – autophagy-related gene

ATP – adenosine triphosphate

BSA – bovine serum albumin

CMA – chaperone-mediated autophagy

Cx – connexin

Cos7 - fibroblast-like cell line derived from monkey kidney tissue

DMSO – dimethyl sulphoxide

DN – dominante negative

DUB – deubiquitinating enzyme

ECL – enhanced chemiluminescence

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum associated degradation

ESCRTs - endosomal sorting complexes required for transport

FBS – fetal bovine serum

GFP – green fluorescent protein

HEK293 - Human Embryonic Kidney 293 cells

IP – immunoprecipitation

LAMP-2A – lysosome-membrane protein type 2A

LC3 – microtubule-associated protein 1 light chain 3

LIR - LC3-interacting region

MS – mass spectrometry

MVB - multivesicular body

NCS – newborn calf serum

PBS – phosphate buffered saline

PE – phosphatidylethanolamine

PFA – paraformaldehyde

PM – plasma membrane

PQC – protein quality control

QC – quality control

SDS – PAGE – sodium dodecylsulphate–polyacrylamide gel electrophoresis

SH3 - Src-homology domain 3

STAM - signal transducing adapter molecule

Ub - ubiquitin

UBPY - ubiquitin-specific processing protease Y

UPR – unfolded protein response

UPS – ubiquitin-proteasome system

WT – wild type

THESIS ORGANIZATION

This thesis is organized as a scientific paper. It contains five chapters preceded by an abstract in English and Portuguese.

The first chapter consists of an introduction to the subject. There is a general introduction to connexin 43 and its activity. The chapter also briefly describes the principal mechanisms involve in Cx43 ubiquitination and degradation by the ubiquitin-proteosome system (UPS) and the lysosomes-dependent degradation.

The second presents the major objectives of this study

The third chapter presents a detailed description of the materials and methods used in this work.

In the fourth chapter, the results obtained in this study are shown in figures and tables and are described in the text. This chapter presents data showing the role of DUBs in basal and starvation conditions. Furthermore, in the same chapter, we show the role of autophagy in the degradation of Cx43.

The fourth fifth comprises the discussion and the main conclusions of this thesis.

The sixth chapter lists all scientific references cited in this thesis by the order in which they appear in the text.

TABLE OF CONTENTS

ABSTRACT

Intercellular communication (IC) via gap junctions (GJ) plays a critical role in numerous cellular processes, including maintenance of tissue homeostasis, control of cell growth and differentiation, and embryonic development.

GJ are specialized cell-cell contacts formed by Connexins (Cx) that provide direct communication between adjacent eukaryotic cells. GJIC can be regulated at different levels, including channel gating, post-translational modifications of Cx and the amount of channels localized at the plasma membrane, in addition to internalization and degradation of GJ.

Regulation of GJIC by degradation of connexin 43 (Cx43) has been a matter of intense research over the last decades and both the proteosome and the lysosome have been implicated. Although some important achievements have been obtained in recent years, the molecular mechanisms associated with Cx43 degradation remain largely elusive.

Post-translational modification of Cx43 by ubiquitin is emerging as an important event in the regulation of connexin degradation. Indeed, ubiquitination of Cx43 has been established to be a triggering signal for degradation of GJ by both autophagy and endocytosis. Although the mechanism and molecular players associated with endocytic internalization of Cx43 have been described in detail, the molecular mechanisms and signaling pathways whereby Cx43 is degraded by autophagy, including Ub-mediated signaling, remain obscure. Results obtained in our lab demonstrated that Ub-dependent degradation of GJ by autophagy is mediated by the autophagy adaptor p62, which establishes a

bridge between Ub moieties attached to the substrate and LC3 that is embedded in autophagic vesicles. Furthermore, the pupative deubiquitination of Cx43, catalyzed by deubiquitinating enzymes (DUBs) has never been described, and may constitute an additional step in regulating the ubiquitinated profile of Cx43, and, consequently, its trafficking and degradation. Therefore a main aim of this work is to establish the role of DUBs in the regulation of Cx43 life-cycle.

Results obtained in this work show that Cx43 is a substrate for deubiquitination, modulated by AMSH and UBPY. Indeed, we demonstrated that inactivation of deubiquitinating enzymes, by overexpressing catalytically inactive mutated forms of AMSH and UBPY, leads to the increased degradation of Cx43. By immunofluorescence microscopy and IP assays, we demonstrate that Cx43 interacts with AMSH and UBPY, both intracellularly and at the PM. Moreover, impairing AMSH-mediated deubiquitination of Cx43, either, by overexpressing a catalytically inactive mutant form or by silencing AMSH, is shown to increase the internalization and degradation of Cx43, suggesting that AMSH-mediated deubiquitination of Cx43 stabilizes the protein, protecting it for internalization and degradation.

In accordance with a role of deubiquitination in rescuing Cx43 from degradation, we demonstrate that overexpression of the catalytically inactive mutants forms of both AMSH and UBPY accelerates degradation of Cx43 upon activation of autophagy. To the best of our knowledge, this is the first time deubiquitination of a membrane protein can prevent its degradation by autophagy.

Furthermore, we demonstrated that mutations in the LIR domain of Cx43, that modulates the interaction with LC3, stabilize the protein under basal conditions,

but promotes degradation of Cx43 in starved cells. This led us to propose a model in which degradation of Cx43 by autophagy may occur through different mechanisms: under basal physiological conditions Cx43 binds directly to LC3, through the LIR domain, while in stimuli-induced autophagy, ubiquitination of Cx43 recruits p62, that mediates the interaction with LC3. Altogether, the results obtained in this study demonstrate that deubiquitination of Cx43, mediated by AMSH and UBPY, constitutes an additional step in the regulation of GJ degradation, namely through autophagy.

RESUMO

A Comunicação intercelular (IC) por meio de Gap juntions (GJ), desempenha um papel fundamental em diversos processos celulares, incluindo a manutenção da homeostase dos tecidos, o controlo do crescimento e diferenciação celular e desenvolvimento embrionário.

GJ são contatos célula-célula especializados formados por conexinas (Cx) que permitem a comunicação direta entre células eucarióticas adjacentes. A GJIC pode ser regulada a diferentes níveis, incluindo a abertura e fecho dos canais, modificações pós-tradução de Cx, a quantidade de canais localizados na membrana plasmática, e também através da internalização e degradação das GJ.

A regulação da GJIC pela degradação da conexina 43 (Cx43) tem sido um assunto de intensa pesquisa nas últimas décadas e tanto o proteossoma como o lisossoma têm sido implicados neste processo. Apesar de alguns avanços importantes obtidos nos últimos anos, os mecanismos moleculares associados à degradação da Cx43 não são inteiramente conhecidos.

A ubiquitinação da Cx43 pela ubiquitina, é um evento importante na regulação da degradação da conexina. A ubiquitinação da Cx43 pode funcionar como um sinal para a degradação das GJ tanto por autofagia, como pela via endocítica. Embora o mecanismo molecular e os seus intervenientes associados à internalização endocítica da Cx43 têm sido descritos em detalhe, os mecanismos moleculares e as vias de sinalização através do qual a Cx43 é degradada por autofagia, incluindo a autofagia mediada pela ubiquitina,

permanecem obscuros. Os resultados obtidos no nosso laboratório mostraram que a ubiquitinação das GJ dependente da autofagia, é mediada pelo adaptador autofágico p62, que estabelece uma ponte entre o substrato ubiquitinado e a LC3, que está incorporado em vesículas autofágicas. Além disso, a deubiquitinação da Cx43, catalisada pelas enzimas deubiquitinantes (DUBs) nunca foi descrita, e pode constituir um passo adicional na regulação do perfil de ubiquitinação da Cx43, e, consequentemente, no seu trafego e degradação. Portanto, um dos principais objetivos deste trabalho é estabelecer o papel de DUBs na regulação do ciclo de vida da Cx43.

Os resultados obtidos neste trabalho mostram que a Cx43 é um substrato para deubiquitinação, mediada pela AMSH e pelo UBPY. De fato, foi demonstrado que a inativação das enzimas deubiquitinantes, por sobreexpressão de uma forma cataliticamente inactiva da AMSH e do UBPY, leva ao aumento da degradação Cx43. Por microscopia de imunofluorescência, e ensaios de imunoprecipitação (IP), demonstramos que a Cx43 interage com a AMSH e com o UBPY, tanto a nível intracelular como na membrana plasmática. Além disso, a inibição da deubiquitinação da Cx43, por sobreexpressão de forma cataliticamente inactiva da AMSH ou por siRNA silenciamento da proteína leva a um aumento da internalização e degradação da Cx43, o que sugere que a deubiquitinação da mediada pela AMSH protege as GJ da internalização.

De acordo com o papel da deubiquitinação da Cx43 na protecção desta contra a degradação, nós demonstramos que a sobreexpressão de ambos os mutantes cataliticamente inactivos da AMSH e do UBPY, acelera a degradação de Cx43 após activação da autofagia. Esta é a primeira vez que é demonstrado

que a deubiquitinação de uma proteína de membrana pode impedir a sua degradação por autofagia.

Além disso, demonstrou-se que os mutantes do domínio LIR de Cx43, que modula a interacção com LC3, estabilizam a proteína em condições basais, mas promovem a degradação da Cx43 em células em privação de nutrientes. Isso levou-nos a propor um modelo em que a degradação da Cx43 por autofagia pode ocorrer através de diferentes mecanismos: em condições fisiológicas basais a Cx43 liga-se directamente à LC3, através do domínio LIR, enquanto em estímulos induzidos por autofagia, a ubiquitinação de Cx43 recruta o p62, que medeia a interacção com LC3. No seu conjunto, os resultados obtidos neste estudo demonstram que a deubiquitinação de Cx43, mediada pela AMSH e pelo UBPY, constitui um passo adicional na regulação da degradação das GJ através da autofagia.

CHAPTER 1: INTRODUCTION

Intercellular communication is an important cellular function of multicellular organisms. In fact the evolution of increasingly fine tuned communication mechanisms between cells allowed for the development of more complex organisms, with specialized cells, tissues and organs. Cells can communicate with each other by releasing signaling molecules into the extracellular environment. These signaling molecules then travel through the extracellular medium until they reach their target cells, which can be located either in the immediate vicinity of the originating cell, as in the case of neurotransmitters, or over long distances, as in the case of hormones. After reaching their target cell, signaling molecules are recognized by specific receptors on the cell's surface, initiating signaling cascades that ultimately lead to specific cellular responses by inducing the expression of genes. However, not all intercellular communication relies on the release of signaling molecules into the extracellular environment. Cells can communicate directly with each other through gap junctions (GJ), channel structures that establish direct communication between the cytoplasm of adjacent cells. GJ channels are formed by the coupling of two hemichannels from adjacent cells. Each hemichannel, in turn, is formed by the oligomerization of six monomeric proteins. In vertebrates, the main protein family forming GJs are connexins; other hemichannel proteins include pannexins and their invertebrate homologues, the innexin protein family.

Typically, hundreds to thousands of GJ channels cluster into densely packed two-dimensional arrays, termed GJ plaques that can reach several square-

micrometers in size. In addition to providing intercellular communication, GJs, based on their characteristic double-membrane configuration, significantly contribute to physical cell-to-cell adhesion.

Gap junctions play a crucial role in regulating transmission and spreading of local signals, allowing the direct passage of small molecules inferior to 1KDa (such as ATP) between adjacent cells. The capacity to modulate the level of GJ-mediated intracellular communication (GJIC) and physical cell-to-cell adhesion is crucial for many physiological and pathological conditions. This local communication mechanism is also important in essential cellular processes such as proliferation, differentiation and apoptosis.

The function of GJ in the cell is of the utmost importance and, unsurprisingly GJ intercellular communication exists in nearly every mammalian cell type[1, 2]. In vertebrates, connexin gap junctions play an important role in tissue homeostasis, embryonic development, and coordinated contraction of excitable cells, normal cell growth and differentiation. Connexin gene mutations have been associated to multiple diseases [3]. Mutations in several connexins like Cx26, Cx30, Cx30.3 and Cx31 can give rise to sensorial hearing loss and hyperproliferative skin disorders [4], while oculodentodigital dysplasia (ODDD) is related to mutations in the Cx43 gene [5].

Connexins

All members of the connexin family contain four transmembrane domains, one cytosolic loop and two extracellular loops. Both the amino- and carboxyl-terminals of connexins are located in the cytosolic face of the plasma membrane. The transmembrane domains and extracellular loops are highly

conserved throughout the connexin family; however, many key functional differences between connexins stem from amino acid sequence differences in these domains.

There are two nomenclature systems for connexins proteins. The first system identifies connexins according to their predicted molecular weight in kDa, for example Cx43, Cx40, *etc.*(Cx = connexin). The second system identifies connexins according to their sequence homology, the length of their cytoplasmic domains and the order of discovery. Cx43 was the first connexin of the alpha family to be discovered, therefore it is named gja1. With this nomenclature, two more protein families have been discovered: beta (b) and gamma (c). Connexins unrelated to any of these families are classified as divergent connexins and identified as being part of the delta family (d).

Several tissues and cell types express two or more members of the connexin family. Cardiomyocites express Cx40, Cx43 and Cx45, while the lens expresses Cx43, Cx46 and Cx50. The expression of multiple members of the connexin family within the same cell type may allow for compensatory mechanisms to take place when a specific connexin protein is either mutated or lost. Several studies have demonstrated that the endogenous expression of many connexins were able to compensate for the deletion of a specific connexin gene. However, this does not mean that all connexins are redundant when present inside cells which express more than one type of connexin protein; in some cells types, a specific connexin protein is sometimes essential for the normal function of the cell, regardless of the presence of more members of the connexin family. One example of this happens in the lens, knockout of Cx46 results in the formation of cataracts while knockout of the Cx50 gene results in the formation of lens

growth defect and cataracts. The knockin of the Cx46 gene into the loci of the Cx50 gene is capable of preventing the formation of opacities in the lens, but does not rescue the growth defect, proving the requirement of Cx50 for normal function of the lens [6]. Of all the connexins family members, Cx43 is the most ubiquitously expressed and is endogenously expressed in at least 35 distinct tissues and 46 cell types include cardiomyocytes, keratinocytes, astrocytes, endothelial cells and smooth muscle cells [7].Unsurprisingly, Cx43 is also the most widely studies connexin protein.

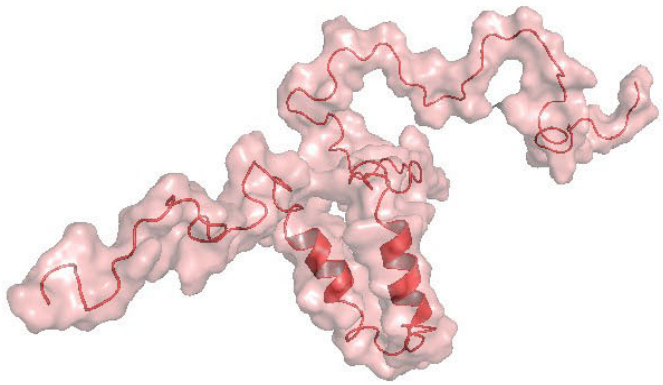

Figure 1: 3D structure of Cx43 [8].

Connexons

Each connexon contains six individual connexin proteins. Connexons can be composed by different Cxs (heteromeric) or identical Cxs (homomeric). GJs, in turn, can be formed by identical (homotypic) or different (heterotypic) connexons. Not all Cxs can interact with each other to form connexons: for example, Cx26 can form heteromeric channels with Cx30 and Cx32 but not with Cx40 [9]. One of the functions of Cxs inside a connexon is to provide selectivity to the gap junction. The ability of different connexins to form heteromeric connexons is not only dependent on direct compatibility between connexins, but

also regulated by the cellular environment. Selective connexin hetero-oligomerization can also take place within a cell. Vascular endothelium and smooth muscle cells express Cx37, Cx40 and Cx43, which can all hetero-oligomerize with each other. However, it was found that Cx37 was specifically excluded from myoendothelial junctions between endothelial cells and smooth muscle cells, while still being incorporated in gap junction channels connecting adjacent endothelial cells or adjacent smooth muscle cells [10]. The capacity of different connexons to assemble a heterotypic gap junction channel also varies with the connexin composition of each connexon. Heterotypic compatibility between connexins appears to be determined by the extracellular loops and the cytoplasmic loop of connexin proteins. The selectivity in heterotypic gap junction assembly is an important property of connexons, allowing cells expressing different connexins to remain structurally and functionally independent, which in turn allows for the presence of independent communication pathways within tissues or organs such as the retina or the brain. The permeability properties of gap junction channels are also directly affected by their connexin composition.

Assembly of oligomeric integral membrane proteins usually occurs in the organelle in which they are synthesized, however, connexon assembly can occur either in the endoplasmic reticulum (ER) or in the Golgi network, after which, they are transported to the plasma membrane. Depending on the tissue, some connexons may remain undocked forming a hemichannel at the plasma membrane, which connects the cytoplasm with the extracellular medium. Connexons will dock with other connexons from adjacent cells forming an intercellular pore. These pores will aggregate to form a gap junction plaque. Connexins have a short half-life (1-5 hours) when compared to other plasma

membrane proteins. GJ internalization also has a unique feature in that both sides of a GJ plaque are internalized into one of the two adjacent cells, forming structures termed annular gap junction. This annular GJ subsequently fuses with the lysosome where its contents are degraded.

Post-translational modification of Connexins.

Phosphorylation

Protein phosphorylation consists in the addition of a phosphate group (PO_4) to a protein residue. Dephosphorylation and phosphorylation of proteins is a very common post-translational modification, functioning as a molecular switch that modulates the activity of many proteins. The most common protein phosphorylation residues are serine and threonine, followed by tyrosine.

The phosphorylation of connexins occurs mostly in the carboxyl-terminal (CT). This post-translational modification has been implicated in many important metabolic events of connexin life. More is known about Cx43 phosphorylation than for all other connexin species. The phosphorylation of Cx43 has been implicated in Cx43 transport to the plasma membrane, as activation of PKA enhances transport of the protein to the plasma membrane and increases gap junctional communication. Activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) induces Cx43 phosphorylation and internalization, being also important for Cx43 channel gating [11]. Phosphorylation of Cx43 by mitogen-activated protein kinase (MAPK) has been implicated in channel gating by reducing the time Cx43 channels remain in the open state [12]. Furthermore, phosphorylation was shown to act as a stimuli for Cx43 proteasome-dependent

degradation in lens epithelial cells [13] , while both PMA and EGF (epidermal growth factor) induced phosphorylation of Cx43 has been shown to induce Cx43 ubiquitination and subsequent internalization [14, 15]. Lastly, phosphorylation of Cx43 is also involved in regulating the interaction of Cx43 with other proteins. For example, Cx43 phosphorylation has been shown to be important for its interaction with the E3 ligase Nedd4 (neural-precursor-cell-expressed developmentally down-regulated 4) [16].

Ubiquitination

Usually, ubiquitin is covalently conjugated to other proteins by forming a bond between the carboxy-terminal glycine on ubiquitin and the NH_2-group of a lysine residue in the substrate protein. The first evidence that ubiquitin was involved in the degradation of Cx43 came from studies by Laing and Beyer. Using the Chinese hamster ovary cell line CHO-ts120, which express a thermolabile E1 ubiquitin-activating enzyme, they found that Cx43 protein levels were increased under conditions in which the ubiquitin-activating enzyme was inhibited [17]. The expression of Cx43 observed under these conditions was associated with enlarged Cx43 gap junctions at the plasma membrane as well as more diffuse Cx43 localization in the cytoplasm. The data also suggested that Cx43 could be conjugated to ubiquitin [17]. More recently, it was shown that treatments that induce Cx43 internalization also induce ubiquitination of protein. In addition, the E3 ligase Nedd4 was shown to interact with Cx43, and consistent with this, our group has shown that this ligase mediates the ubiquitination of Cx43 through a mechanism that requires the PY motif (proline-rich motif) of Cx43 [18], and that the ubiquitination of Cx43 triggers the internalization and degradation of Cx43.

Our group also demonstrated that Eps15 binds to ubiquitinated Cx43, through its ubiquitin-interacting motifs (UIMs) and that this interaction is, at least in part, required for the internalization of Cx43. Furthermore, Cx43 ubiquitinylation has also been implicated in its trafficking from early endosomes to lysosomes, through a mechanism mediated by the ESCRT (Endossomal Sorting Complex Required for Transport) components Hrs and Tsg101. We have also shown that Cx43 ubiquitination and its subsequent recognition by Eps15 and p62 also mediates the degradation of Cx43 by macroautophagy.

The Ubiquitin-proteasome system

The ubiquitin-proteosome system (UPS) is the main proteolytic system involved in the selective degradation of soluble proteins. The UPS pathway is involved in a wide variety of cell functions and has been implicated in numerous cellular events where protein degradation is required either to dispose of obsolete proteins or to regulate various biological processes.

Degradation of a protein by the ubiquitin-proteasome pathway involves two different successive steps: the first step implies the covalent attachment of a polyubiquitin chain to the target protein; the second step involves recognition and degradation of the targeted protein by the 26S proteasome with the release of free and reusable ubiquitin.

The 26S proteosome

The 26S proteasome is a multicatalytic protease that breaks down proteins to produce small peptides. It is composed of two subunits: the 20S core particle subunit and two 19S regulatory cap subunits. The 20S core particle is a barrel-

shaped structure arranged as a stack of four rings, two α and two β, each of them composed of seven subunits. The 20S core particle is hollow and provides an enclosed environment in which proteins are degraded; openings at the two ends of the core allow for the entry of substrates. Catalytic sites are located in the β-rings. One important function of the 19S regulatory particle is to recognize polyubiquitinated proteins. A second function is to open an orifice which allows the substrate to enter the proteolytic chamber of the 20S core particle. In this subunit there are six different ATPases which are indispensable for functions that required metabolic energy like channel-opening and substrate-unfolding.

Ubiquitin conjugation cascade

Ubiquitin is a heat-stable and evolutionarily conserved 76 amino acid protein that is ubiquitously expressed in all eukaryotic cells. Most proteosomal substrates have to be polyubiquitinated in order to be properly recognized by the 26S proteosome. The prime tag for proteasomal degradation is a chain of four or more ubiquitin moieties covalently linked to a lysine residue(s) of the substract [19]. Ubiquitin has seven internal lysines (K6, K11, K27, K29, K33, K48 and K63) that can themselves be ubiquitinated to form polyubiquitin chains [20]. K48-linked polyubiquitin chains represent the canonical proteasomal degradation tag, but recently other chains were also identified as being able to target substrates for degradation in the protesome, these include K11 and K63-linked polyubiquitin chains [21].

Conjugation of ubiquitin to the substrate protein proceeds trough three steps. The first step involves the activation of ubiquitin by the ubiquitin-activating enzyme E1. In the second step, the activated ubiquitin moiety is transferred to

an E2 (ubiquitin-carrier protein or ubiquitin-conjugating enzyme). In the last step, the activated ubiquitin is transferred from the E2 enzyme to the target protein with the help of an E3 ubiquitin-protein ligase enzyme that specifically binds to the target substrate.

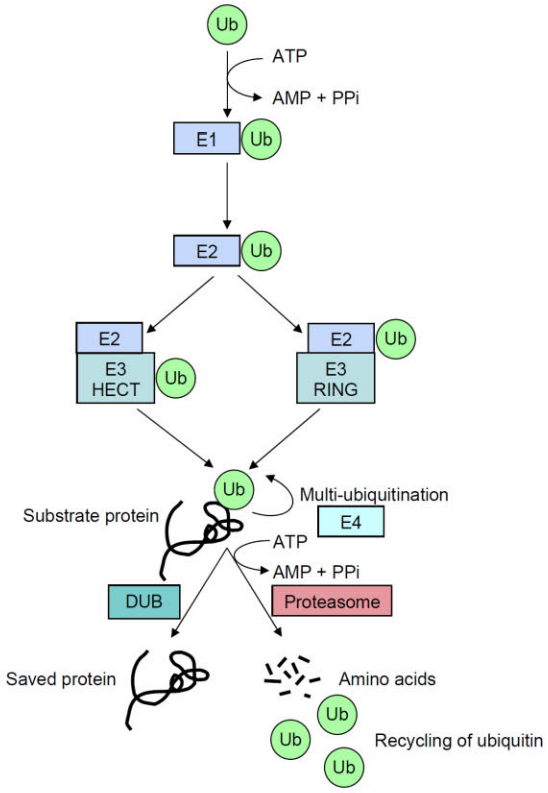

Figure 2. Schematic representation of the ubiquitination process that target proteins for degradation. An ubiquitin molecule (Ub) is first linked by its carboxy-terminal amino acid to an E1-activating enzyme (E1) through a high-energy bond on a cysteine residue, while consuming energy (ATP). Activated ubiquitin is then translocated to the E2-conjugating enzyme (E2). RING E3 ubiquitin ligases (E3 RING) catalyze the transfer of ubiquitin directly from E2 to the substrate, whereas HECT E3 enzymes (E3 HECT) accept activated ubiquitin from E2 before transferring it to the substrate. Following this first step, called monoubiquitination, the process may be repeated by some processive E2/E3 enzymes, or with the help of E4 enzymes and, finally, leads to polyubiquitination. Proteins targeted for degradation into amino acids can however be rescued by deubiquitining enzymes (DUB) [25].

The human genome encodes two ubiquitin-specific E1 activation enzymes, about 30 E2 conjugation enzymes, and more than 1000 E3 ligases providing a great versatility in substrate recognition and enabling diversity in ubiquitin chain linkages added to substrates [22-24] (figure 2).

Deubiquitinating enzymes

To maintain ubiquitin homeostasis, ubiquitin has to be recycled once a substrate has been committed to the degradative pathway. Therefore, ubiquitination is a reversible process that can be catalyzed through the action of deubiquitinating enzymes, also known as DUBs. DUBs are proteases that process ubiquitin or ubiquitin-like gene products, reverse the modification of proteins by ubiquitin or ubiquitin-like proteins, and remodel polyubiquitin chains on target proteins.

DUBs play several roles in the ubiquitin pathway. They are essential for activating newly expressed ubiquitin. DUBs are also implicated in the recycling of ubiquitin that may have been trapped by the reaction of small cellular nucleophiles with the thiol ester intermediates that are formed during the ubiquitin process [26]. DUBs also play a major role in reversing the modification of proteins by ubiquitin-like conjugation. Lastly, DUBs are also responsible for the recycling of monomeric ubiquitin from free polyubiquitin chains that are synthesized by the conjugating machinery or that have been released from target proteins by other DUBs.

DUBs contain additional ubiquitin-binding domains as well as several protein-protein interaction domains. These domains enable the binding and recognition of different ubiquitin chain linkages and can also direct the assembly of

multiprotein complexes that localize DUBs and assist in substrate selection [27, 28].

The human genome encodes ~90 DUBs, which can be grouped into five different families. Of these proteins, 79 are predicted to be active. The largest of the five families is the ubiquitin-specific proteases (USP) family (~55 members). The USPs as well as the ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs) and members of the Josephin family are cysteine proteases. The fifth DUB family comprises a group of Zn^{2+} metalloproteases that are referred to as the JAB1/MPN/MOV34 metalloenzymes (JAMMs, also known as MPN^{+}) [29].

The substrate specificity of DUBs is determined by sub-cellular localization, specific binding interaction and the preference of the catalytic domain for particular types of ubiquitin chain linkages. The principal of DUB chain linkage specificity was first established for the endossomal JAMM family member Associated Molecule with the SH3 domain of STAM (AMSH, officially known as STAMBP) and, later, for the closely related AMSH-like protein (AMSH-LP, officially known as STAMBPL1), both of which are selective for K63 linked ubiquitin chains [29].

Autophagy (Lysosomal-dependent degradation)

In the past decade there has been an explosion of research on a fundamental cell biology pathway called autophagy (Greek for “self-eating”). Increasing evidence suggests that the deregulation of autophagy may contribute to a broad spectrum of mammalian diseases [30]. The best-characterized signal for the activation of autophagy is nutrient deprivation. When nutrients are insufficient,

this pathway allows a cell to break down its own components, including proteins and organelles and recycle important molecules. It is a very important protein quality control (PQC) system that represents the adaptation of a single cell to starvation/nutrient deprivation, allowing the cell to survive until there is food available in the medium [31, 32]. In unicellular organisms such as yeasts, starvation response is one of the primary functions of autophagy, but in fact this function extends up to humans. For example, every day autophagy is upregulated between meals in organs such as the liver to maintain its metabolic functions, supplying amino acids and energy through catabolism [33].

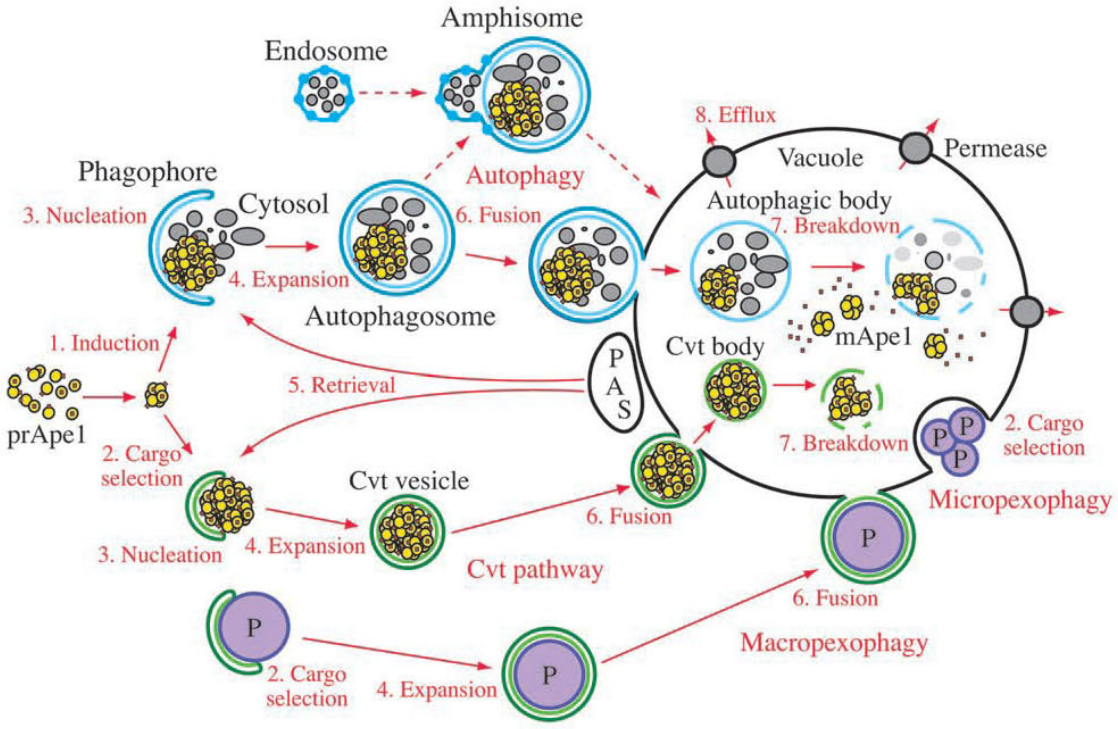

Figure 3 - Schematic overview of autophagy related processes. Non-specific autophagy can be separated into seven steps: (1) Induction; (3) Vesicle nucleation; (4) Vesicle expansion and completion; (5) Retrieval; (6) Fusion; (7) Breakdown; (8) Efflux. Specific types of autophagy include an additional step (2) of cargo recognition and packaging. The cytoplasm to vacuole targeting (Cvt) pathway and pexophagy are two examples of specific autophagy [34].

To date, at least three types of autophagic pathways have been described, which differ in their routes to lysosomes: macroautophagy (also commonly called "autophagy"), microautophagy and chaperone-mediated autophagy. Variants of each type of autophagy have been described and named to indicate the cargo preferentially degraded: mitophagy (autophagy of mitochondria), pexophagy (autophagy of peroxisomes), lipophagy (autophagy of lipid droplets), and aggregophagy (autophagy of aggregates) [21].

The essential component of these proteolic systems is the lysosome, a single membrane vesicle that contains in its lumen a large variety of cellular hydrolases including proteases, lipases, glycosidades, and nucleotidases [35].

Macroautophagy

Macroautophagy is one of the major degradative pathways in eukaryotic cells, and it is the only one with the capacity to degrade entire organelles [34]. This mechanism of degradation can be divided into eight steps (figure 3). These steps begin with induction, a signal transduction event that triggers the nucleation of sequestering membrane or phagophore (figure 4), which leads to the formation of a cytosolic double-membrane vesicle that sequesters cytoplasm, the hallmark of macroautophagy. Autophagy is initiated when autophagy-related gene 12 (ATG12) is conjugated to ATG5 resulting in the formation of an oligomeric ATG5-ATG12-ATG16L complex.

Yeast have a single ATG8 protein, but mammals have two subfamilies of ATG8 homologues made up of at least three MAP1 light chain 3 (LC3A, -B and –C) and four gamma-aminobutyrate receptor associated proteins (GABARAP) and GABARAP-like proteins (GABARAPL1-3) [36].

LC3 is the best characterized of these proteins and is regarded as a functional homolog of yeast ATG8 in autophagy [37]. LC3I is activated by Atg7, transferred to Atg3, and finally conjugated to PE. The LC3-PE conjugate is designated LC3-II. The carboxyl terminal Gly of LC3 is also essential for the formation of a thio-ester bond with the active site Cys residues on Atg7 and Atg3, as well as being essential for the formation of an amide bond with PE. LC3-I is localized in the cytosol and LC3-II is localized to autophagosemes [38]. LC3-II on the cytoplasmic surface of autophagosomes is delipidated by Atg8 to recycle LC3-I for further autophagosome formation [37]. This reaction is similar to ubiquitination as it is catalyzed by an E1-like enzyme (Atg7) and an E2-like enzyme (Atg3) [25]. LC3-II proteins are also involved in membrane biogenesis of autophagosomes via their membrane fusion activity [38]. Autophagosomes are formed by closure of the phagophore into a double-membrane vesicle [24].

The autophagosomes often form at the cell periphery and move linearly toward the perinuclear region at the microtubule organizing center (MTOC) area where lysosomes are localized. The fusion of autophagosomes with late endosomes or lysosomes is the final step in this process that results in the degradation of their contents [24].

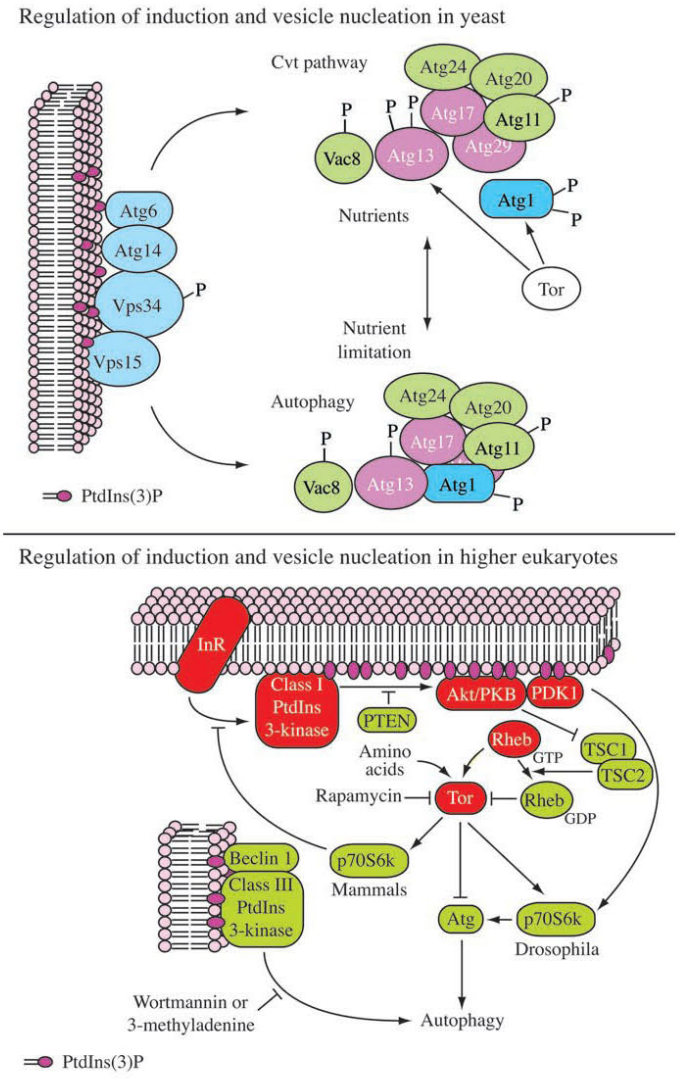

Figure 4. Machinery involved in signal transduction events that induce autophagy, and nucleate formation of the sequestering vesicle. In yeast (top), the Atg1 kinase is part of a complex that is composed of proteins that are relatively specific for autophagy (Atg17, Atg29 and possibly Atg13) or the Cvt pathway (Atg11, Atg20, Atg24 and Vac8). The phosphorylation of at least Atg13 is nutrient dependent and the highly phosphorylated form appears to have a lower affinity for Atg1. The Atg1 and Atg13 proteins appear to be phosphorylated in a Tor- and protein kinase A-dependent manner. In eukaryotes (bottom), Tor is regulated by a class I PtdIns 3 kinase that acts through a regulatory cascade of kinases and phosphatases to inhibit autophagy. In this case, in addition to Atg proteins, other targets of Tor such as p70S6 kinase also contribute to the modulation of autophagy [34].

Selective macroautophagy

Macroautophagy was initially described as a form of indiscriminate degradation by which cytosolic substrates are degraded “in bulk”. However, although this may still be true for soluble cytosolic proteins that are trapped along with other cargo as the autophagosomes form, selective autophagy determines the selective degradation of organelles, bacteria, ribosomes, specific proteins, and protein aggregates. In this PQC system, proteins acting as receptors, such as p62 and NBR1, that bind directly to LC3, play an important role in recognizing substrates for degradation [24,26].

Autophagic degradation mediated by p62 is dependent on an 11-amino acid long, linear motif (LC3-interacting region; LIR) mediating an interaction with LC3/GABARAP family proteins [36]. NBR1 has a similar domain architecture as p62 and shares several key features with p62. These proteins bind ubiquitin via the C-terminal UBA domain, interact with ATG8 family proteins via LIR, mediate aggregate formation and are found in inclusion bodies associated with human pathologies. The levels of P62 and NBR1 are regulated by autophagy and do not seem to be influenced by proteosomal degradation. The continuous autophagy of these two proteins is the basis for their ability to act as cargo receptors for selective autophagy of ubiquitinated substrates, though p62 and NBR1 are also involved in cellular processes that are apparently unrelated to autophagy. In these situations selective autophagy can be an important mechanism to regulate their levels.

The list of proteins that use a LIR motif to interact with Atg8 homologues is expanding. When attached, LIR is positioned in the interface between the N-terminal arm and the C-terminal Ub-like domain of the ATG8 protein. Central in

LIR is the W/Y-X-X-L/I motif that interacts with conserved residues in the hydrophobic pocket of the Ub-like domain. The complexity of the binding surface and the sequence variation between different human ATG8 family members makes it likely that proteins will be found, which contain a LIR that specifically interacts with only a subgroup of these proteins [36].

Microautophagy

Microautophagy processes also involve sequestration of a portion of cytoplasm; however, this occurs at the surface of the lysosome/vacuole [34]. In microautophagy the process of degradation is very quick, because the sequestration of cargo occurs directly at the surface of the lysosomes by invaginations of the lysosomal membrane. This pathway can be detected under basal conditions in differents cell types, but there is currently no information as to whether this process can be further upregulated under specific cellular conditions.

Chaperone-mediated autophagy

In contrast to macroautophagy and microautophagy, CMA, so far, only has been observed in mammals [40]. All CMA substrates described until now are soluble proteins [26], that are selectively targeted, one-by-one, to lysosomes and are then translocated across the lysosomal membrane into the lumen where they are degraded by resident proteases [26]. Substrates for this pathway are cytoplasmic proteins that contain in their amino acid sequence pentapeptide motifs biochemically related to the sequence KFERQ and is dependent on the molecular chaperone Hsc70. When exposed (e.g. during protein misfolding or

disassembly of protein complexes) this sequence is recognized by the cytoplasmic form of the chaperone Hsc70 through a process that can be modulated by associated co-chaperones, leading to the delivery of substrate protein to a receptor at the lysosomal membrane. This receptor was identified as the lysosomes-associated protein-2 (LAMP-2A) that binds to CMA substrates facilitating, in conjuction with the lysosomal form of Hsc70, their translocation into the lysosomal lumen where substrates are degraded. Moreover, chaperone-mediated autophagy does not involve the degradation of lipids or organelles [37].

All cells contain basal CMA activity, but it can be further upregulated in response to different stressors including prolonged starvation and conditions leading to protein damage, such as oxidative stress. This pathway depends on the accessibility of this motif to Hsc70, suggesting that conformational changes in the substrate protein, posttranslational modifications, or changes in interacting proteins that usually mask the motif, could be triggers for CMA degradation [26.] These can explain the small number of CMA substrates that have been indentified so far.

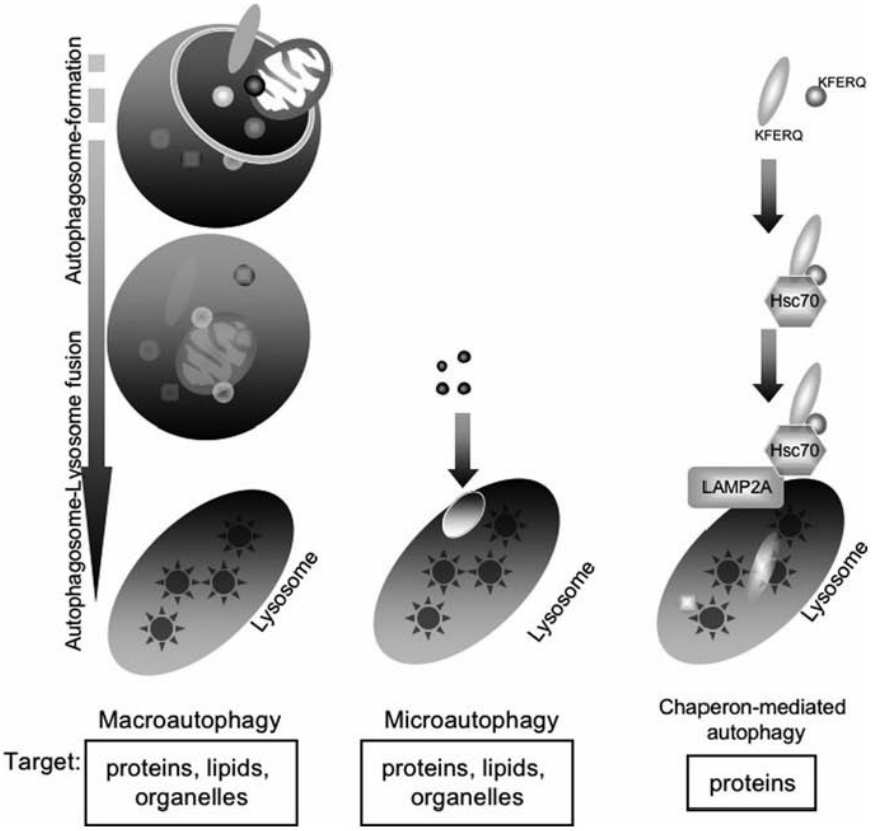

Figure 5. Macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is classical "autophagy." During macroautophagy, dynamic membrane formation and fusion occur, including autophagosome formation and fusion. Most autophagy-related genes (Atg) contribute to autophagosome formation. Microautophagy is another type of autophagy, during which the lysosomal membrane is invaginated into the lysosome to engulf cytoplasmic components directly. Chaperone-mediated autophagy is specific for KFERQ motif-containing proteins. The latter proteins are recognized by Hsc70 and translocated into lysosomes by LAMP2A, a lysosomal membrane protein. Macroautophagy and microautophagy degrade proteins, lipids, and organelles, whereas chaperone mediated autophagy degrades proteins only. LAMP2A, lysosomes associated protein-2A [37].

CHAPTER 2: AIMS

Cx43 has long been known to be a substrate for ubiquitination, however the role of deubiquitinating enzymes in Cx43 regulation has never been described before. Thus, one of the main objectives of this work is to evaluate the role of deubiquitination in the regulation of Cx43.

Once we have established that DUBs catalyze the deubiquitination of Cx43, we further evaluate the role that overexpression of catalytically inactive forms of AMSH and UBPY have in Cx43 modulation in conditions where autophagy is activated.

It is known that LIR motifs on substrate proteins are recognized by the autophagy adaptor LC3 which, in turn, targets them for degradation by autophagy. Thus, another aim of this work is to study the role of the LIR motif present on Cx43's amino-terminal in regulating Cx43 stability during starvation.

CHAPTER 3: MATERIAL AND METHODS

Antibodies and reagents

Goat anti-Cx43, anti-tubulin and anti-GAPDH polyclonal antibodies (Cat No AB1600) were obtained from SICGEN (Cantanhede, Portugal). Mouse monoclonal P4D1 antibodies against ubiquitin were obtained from Covance (CA, USA). Rabbit polyclonal antibodies against AMSH and UBPY were obtained from Cell Signalling Technology (MA, USA). Mouse monoclonal against Cx43 were obtained from Abcam. Rebbit polyclonal antibody against LC3 were obtained from Termo.

Cell culture and transfections

Cos7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% newborn calf serum (NCS), 50µg/ml penicillin, 50µg/ml streptomycin at 37°C with 5% CO_2. Transient transfections of cells were performed with Lipofectamine 2000 (invitrogen), according to manufacturer's recommendations.

HEK293-Cx43 cells constitutively expressing V5-Cx43 [17] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 g/mL streptomycin and 8 µg/ml blasticidin) and maintained at 37 ºC under 5% CO_2. Transient transfections of cells were performed with Lipofectamine 2000 (Invitrogen), according to manufacturer's recommendations

Plasmid constructions

Plasmids expressing GFP-wtAMSH, GFP-AMSH-D348A, GFP-wtUBPY and GFP-UBPY-C786S were kindly provided by Dr. Silvie Urbé (University of Liverpool, UK). Cx43 cDNA was cloned into a modified pENTR GFP C2 vector [41]. Plasmids expressing V5-Cx43 were generated by cloning the Cx43 cDNA into a pENTR vector containing the V5 tag. Site-directed mutagenesis was performed to generate the V5-Cx43W4A, V5-Cx43L7A and V5-Cx43W4A,L7A mutants from the V5-Cx43 vector.

siRNA-mediated knockdown

siRNA targeting AMSH (s20852 (CAUCCUCUAUAACAAGUAUtt) or s20853 (GAGUUGAGAUUAUCCGAAUtt)) and a non-targeting control sequence were obtained from Ambion (Silencer Select Pre-designed siRNA). Cells were grown until they reach 40-50% confluency. siRNA was complexed with Lipofectamine 2000 (Invitrogen), according to manufacturer's recommendations, and added to the cells medium to a final concentration of 20 nM. AMSH knockdown was achieved by transfecting twice at intervals of 24 hours, and experiments were performed 24 hours after the second transfection.

Immunoprecipitation and Western blotting

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 10 minutes. The samples were then centrifuged at 10,000g for 10 minutes, and the supernatants used for immunoprecipitation. Briefly, protein G was incubated with goat polyclonal antibodies directed against Cx43 while protein A was incubated with monoclonal antibodies directed against AMSH. Non-specific antibodies were used as controls. Incubations proceeded for 1 hour, at 4 ºC, followed by incubation with supernatants, for 3 hours at 4 ºC. The samples were then centrifuged and the protein G/A-sepharose sediments washed 3 times in an appropriate washing buffer (500 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3), resuspended in Laemmli buffer and denatured at 100 ºC, for 5 minutes.

For Western blot analysis of the immunoprecipitated proteins, samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with appropriate antibodies. Inputs represent about 5% of the total amount of protein in the lysates before immunoprecipitation.

Immunofluorescence

Indirect immunofluorescence was performed following conventional procedures. Cells were grown on coverslips, fixed for 10min in either ice-cold methanol or 4% formaldehyde in PBS, blocked and permeabilized (1% BSA, and 0.01% Triton X-100), and then incubated with the primary monoclonal against Cx43 or polyclonal antibodies against AMSH or UBPY(Santa-Cruz Biotechnology), proceeded for 1 hour at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for 1 hour at room temperature. The specimens were rinsed in PBS and mounted with MOWIOL 4-88 Reagent (Calbiochem). 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. The images were collected by confocal microscopy using a Zeiss LSM 710.

Statistical analysis

Data present in this manuscript are representative of at least three independent experiments. Data are expressed as means ± S.D. Comparison between groups was performed with an ANOVA test.

CHAPTER 4: RESULTS

AMSH and UBPY interact with Cx43 and modulate the ubiquitination levels of the protein

Although initially described as a tag for proteosomal degradation of cytosolic proteins, more recent studies have demonstrated that ubiquitin acts as a signal for internalization and sorting of a variety of cargo receptors, thus acting as a link between the cargo and components of the endocytic and sorting machinery. Indeed the attachment of Ub to membrane proteins has been shown to trigger the internalization and/or degradation of various channels and receptors. Accordingly, results obtained in our laboratory and others, demonstrated that gap junction protein Cx43 is a substrate for ubiquitination, which in turn, promotes its internalization and degradation. Moreover, we demonstrated that Nedd4 mediated ubiquitination of Cx43 not only modulates the endocytosis of GJ but is required to direct degradation of GJ by autophagy.

It has been recently established that not only the attachment but also the removal of ubiquitin moieties, catalyzed by DUBs, contributes to modulate the ubiquitination levels and topology of polyubiquitin chains attached to a substrate. Some DUBs have been implicated in the regulation of the intracellular trafficking of various membrane proteins, being AMSH and UBPY the most well characterized DUBs associated with the endocytic pathway. Therefore we hypothesized that the Ub-mediated signal, attached to Cx43, could be modulated through deubiquitination, catalyzed by AMSH and UBPY, thus constituting an additional level of regulation of GJIC. To address this

question, we first used a strategy that involves the overexpression of either the WT (AMSH WT and UBPY WT) or the catalytically inactive mutants of AMSH (AMSH-D348A) and UBPY (UBPY-C786S) after which we evaluated the levels of ubiquitin attached to Cx43 through IP assays, using antibodies against Cx43, followed by Western blot, using an antibody against ubiquitin. The results presented in figure 6 (A) show that the overexpression of AMSH WT and UBPY WT slightly reduces the levels of ubiquitinated Cx43 with no significant changes in the total levels of Cx43. Whereas, cells overexpressing the mutated forms of AMSH or UBPY present a robust increase of Ub attached to Cx43. These results strongly suggest that these two DUBs catalyze deubiquitination of Cx43. Furthermore we evaluated the interaction between Cx43 and these DUBs, by staining the same membranes, with antibodies directed against the enzymes. The results presented in figure 6 show that both the WT and the catalytically inactive forms of AMSH and UBPY interact with Cx43. To identify the subcellular structures where these interactions occur, as well as the subcellular redistribution caused by overexpression of WT or mutated forms of DUBs, we performed immunofluorescence confocal microscopy using antibodies directed against AMSH, Cx43 and UBPY. Regarding AMSH, the results demonstrate that the WT form of the enzyme is homogenously distributed in the cytoplasm of cells, while the mutated form of AMSH accumulates in large intracellular vesicles. More importantly, both forms of AMSH colocalized with Cx43 both at the plasma membrane and in intracellular vesicles, as shown in figure 7 (A). Concerning UBPY, and similar to what we observed for AMSH, Cx43 colocalized with UBPY both intracellularly and at the PM. Moreover, in cells overexpressing the mutated form of UBPY Cx43 is entrapped in large

intracellular vesicles that likely correspond to MVBs, as shown in figure 7 (B). Altogether, the results obtained up to this point demonstrate that both AMSH and UBPY interact with Cx43 and modulate its ubiquitination levels.

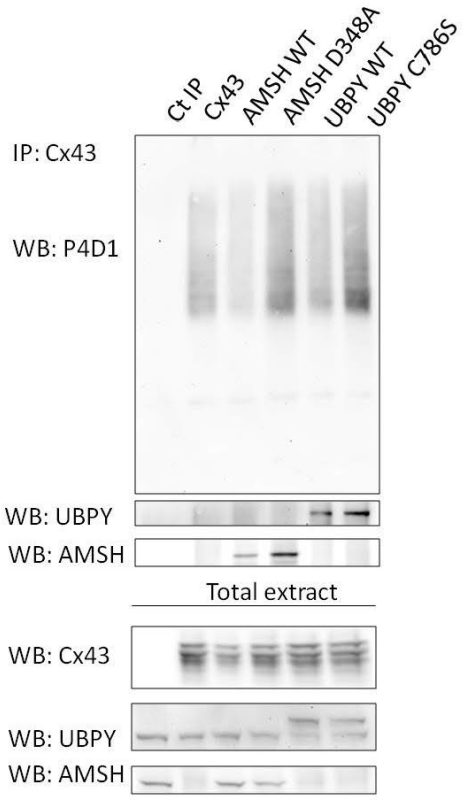

Figure 6: AMSH and UPBY interact with Cx43 and mediate its deubiquitination. Lysates of Cos7 cells transfected with either GFP-AMSH WT, GFP-AMSH-D348A, GFP-UBPY WT or GFP-UBPY-C786S were immunoprecipitated with goat polyclonal antibodies against Cx43 and the precipitates analysed by Western blot using monoclonal antibodies against Cx43 or Ub (P4D1) or rabbit polyclonal antibodies against AMSH or UBPY. Both forms of AMSH and UBPY are readily co-immunoprecipitated with Cx43, however only expression of the dominant negative form of AMSH and UBPY leads to increased levels of Cx43 ubiquitination.

Since ubiquitination of Cx43 has been associated with internalization and degradation of GJ, it is conceivable to suggest that DUBs constitute an additional step in the regulation of the intracellular trafficking of Cx43.

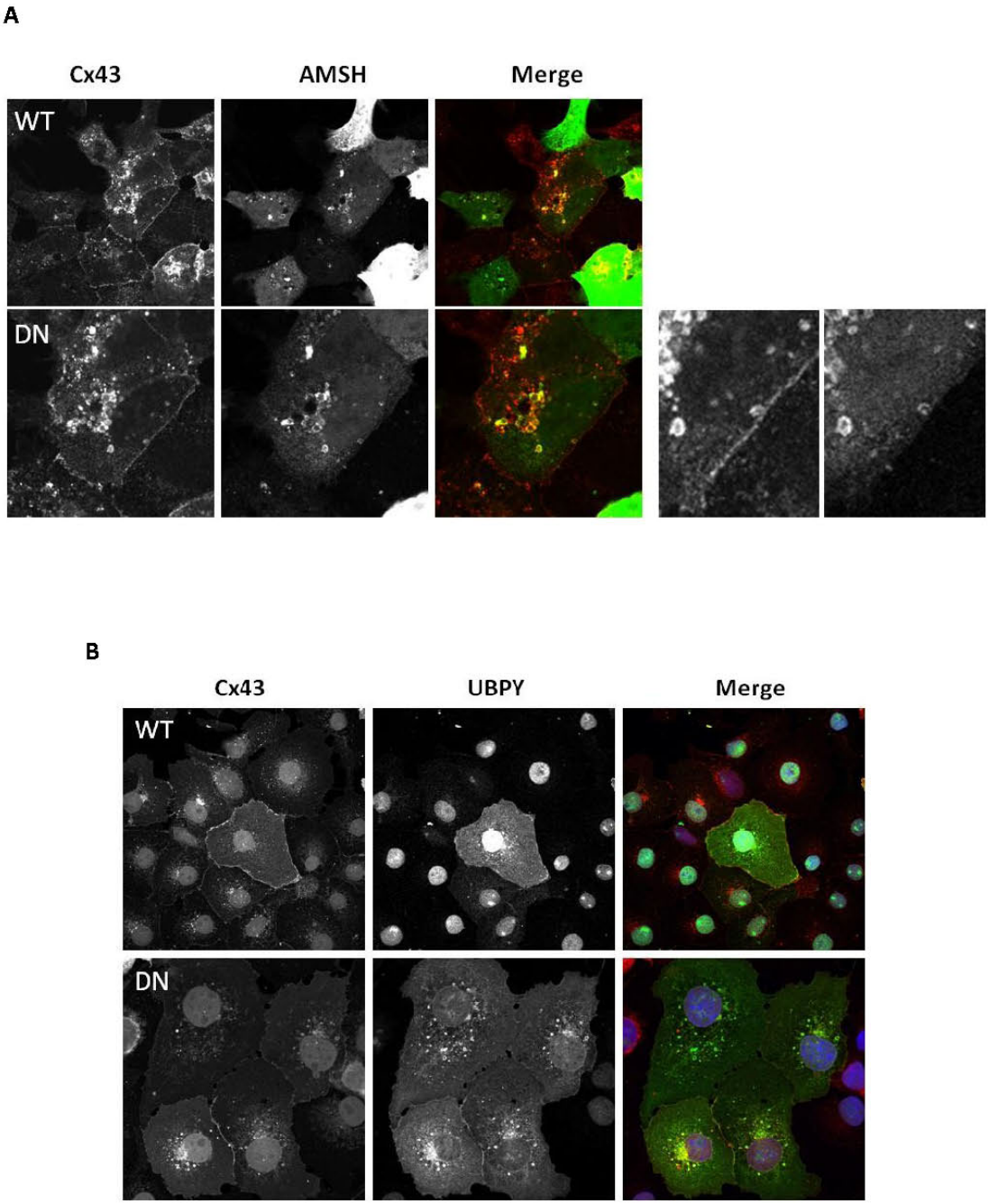

Figure 7: AMSH and UPBY colocalize with Cx43. (A) and (B) Cos7 cells transfected with either GFP-AMSH, GFP-AMSH-D348A (DN), GFP-UBPY-C786S (DN), and GFP-UBPY WT, were fixed and stained with polyclonal antibodies against each DUB and monoclonal antibodies against Cx43. Cx43 can be found colocalized with AMSH and UBPY, both at the plasma membrane and in intracellularly, likely in vesicles.

AMSH activity regulates the endosomal sorting and degradation of Cx43

The ubiquitination of Cx43 has been shown to regulate the turnover of Cx43 at different stages of the Cx43 life-cycle, including internalization, sorting of Cx43

into MVBs and degradation through macroautophagy. Since we showed in the previous section that AMSH and UBPY catalyzed the deubiquitination of Cx43, we hypothesized that these DUBs may play a role in regulating the degradation rate of Cx43. Although both enzymes demonstrated an effect upon Cx43, AMSH presented a more robust and significant effect. For this reason, in the next step of this study we investigated the involvement of AMSH in regulating Cx43 half-life. For this purpose, cells expressing either the WT or the mutated form of AMSH were subjected to a cycloheximide (CHX)-chase assay, in which protein synthesis is inhibited with 50 μg/mL of CHX, for different periods of time. The total amount of Cx43 was further analyzed by Western blot using antibodies directed against Cx43 and the density of the detected bands measured and plotted in a graph. The results presented in figure 8 (A) show that the overexpression of AMSH-D348A results in a slight decrease in the half-life of endogenous Cx43 from around 2.5 hours to 2 hours. On the other hand, the overexpression of AMSH WT results in a biphasic pattern, in which the protein is stabilized in the first 2 hours, after which the levels of Cx43 decrease to 50% of the initial amount at 4 hours, with a similar degradation rate as controls.

To further confirm the role of AMSH in regulating the degradation of Cx43, we performed CHX-chase assays in cells previously transfected with siRNA directed against AMSH for 48 hours. Results presented in figure 8 (B) show that both of the siRNA directed against AMSH significantly reduced the endogenous levels of the protein.

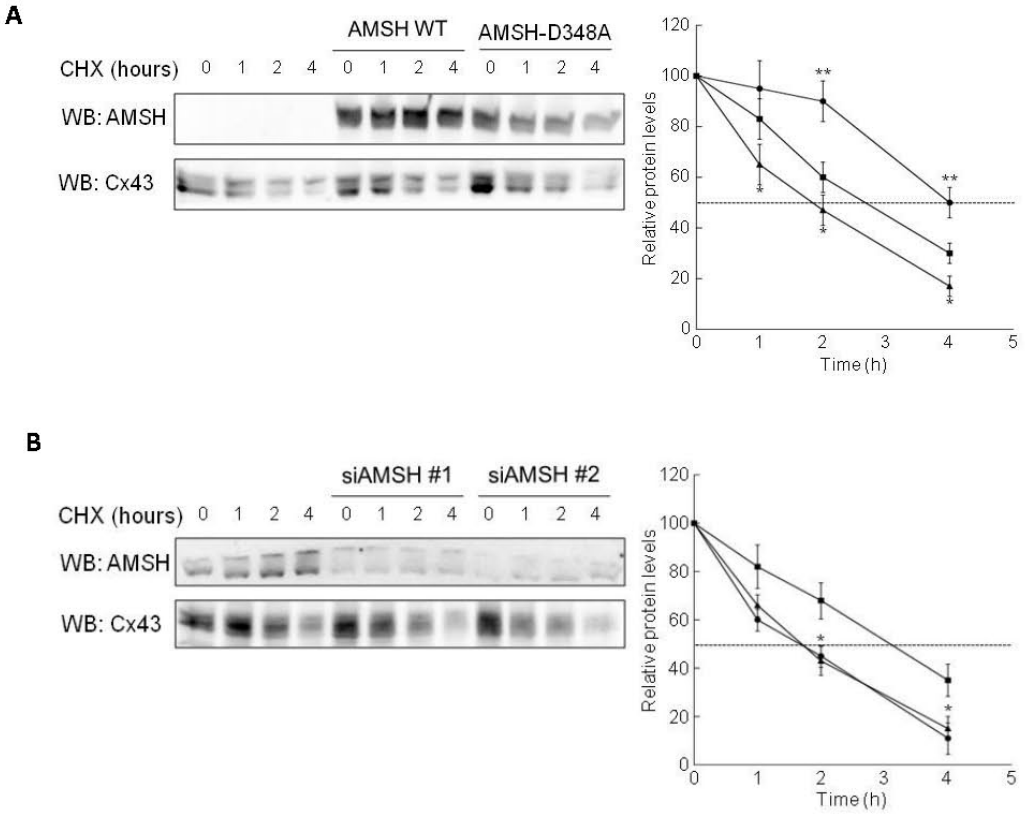

Figure 8: AMSH regulates the half-life of Cx43. (A) HEK293-Cx43 cells transfected with either GFP-AMSH WT or GFP-AMSH-D348A were incubated with 50 μg/ml CHX for 0, 1, 2 and 4 hours. Lysates were then analyzed by Western blot using goat polyclonal antibodies directed against Cx43 or rabbit polyclonal antibodies against AMSH. The intensity of the Cx43 bands was measured and plotted in a graph (Squares = controls; Circles = AMSH WT; Triangles = AMSH-D348A). Expression of AMSH WT increased the half-life of Cx43 from 2.5 hours to 4 hours, while the expression of AMSH-D348A reduced the half-life of Cx43 to a little less than 2 hours. The values are the averages of three individual experiments ± SD. Asterisks indicate statistically significant differences from controls (* $p < 0.05$; ** $p < 0.01$). (B) HEK293-Cx43 cells transfected with siRNA directed against AMSH were incubated with 50 μg/ml CHX for 0, 1, 2 and 4 hours. Lysates were then analyzed by Western blot using goat polyclonal antibodies directed against Cx43 or rabbit polyclonal antibodies against AMSH. The intensity of the Cx43 bands was measured and plotted in a graph (Squares = controls; Circles = siAMSH#1; Triangles = siAMSH#2). Depletion of AMSH decreased the half-life of Cx43 from 3 hours to 1.5 hours. The values are the averages of three individual experiments ± SD. Asterisks indicate statistically significant differences from controls (* $p < 0.05$).

Furthermore, the half-life of Cx43 decreases from 3 hours in the controls, to around 1,5 hours in cells depleted of AMSH. Since both knockdown of AMSH and overexpression of the catalytically inactive mutated form of the protein result in an increased degradation rate of Cx43, taken together these results strongly suggest that AMSH protects Cx43 from degradation, most likely by catalyzing its deubiquitination.

Nutrient deprivation induces variations in the levels of Cx43 over time.

Although initial studies associated degradation of Cx43 with the endocytic pathway, very recently results have shown that degradation of Cx43 also occurs through autophagy, through a process that depends on the previous ubiquitination of Cx43.

Although various studies demonstrated that activation of macroautophagy, by starvation, leads to degradation of Cx43, in all of these approaches the levels of Cx43 were determined following 6/8 hours of serum deprivation. However, it's possible that the cell response to serum deprivation is not linear and can vary during the time course of starvation, likely involving the selective and orchestrated recruitment and detachment of binding partners over time. Moreover, we hypothesized that this dynamic, including the regulated recruitment of specific proteins, may modulate the ubiquitination of Cx43.

To address this question we evaluated the amount of Cx43 as well as its ubiquitination levels in different time points of starvation. The results in Figure 9 (A) show an increase in the levels of Cx43 in the first 4 hours of starvation after which the levels decrease in the following 4 hours. Moreover, the results

presented in figure 9 (A) show a conversion of LC3-I into LC3-II, demonstrating that macroautophagy is activated after 1 hour of serum deprivation.

Since Ub is known to signal degradation of Cx43 by autophagy, we further evaluated the ubiquitination levels of Cx43 at the different time points of starvation.

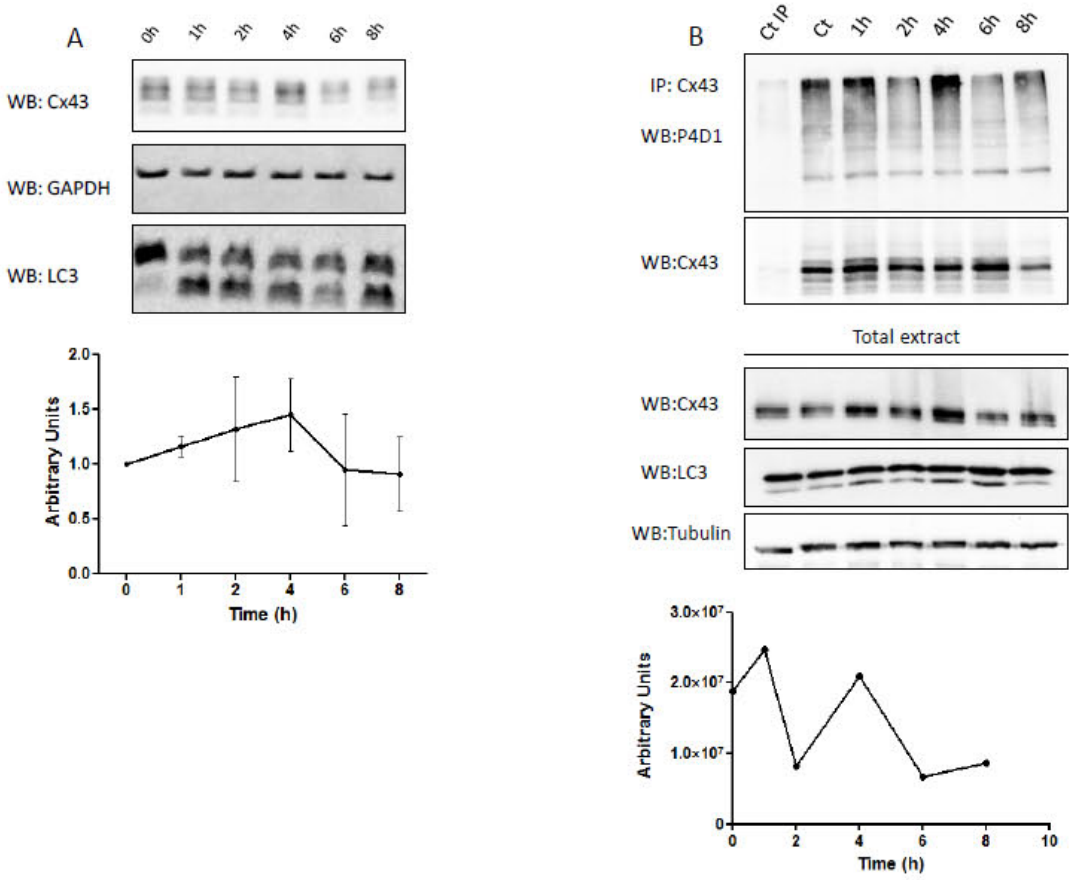

Figure 9: Variations in the levels of Cx43 during starvations. (A) Cos7 cells were transfected with Cx43 and serum-deprivated for 0, 1, 2, 4, 6 and 8 hours. Lysates were analyzed by Western Blot using goat polyclonal antibodies against Cx43 and GAPDH, and rabbit polyclonal antibodies against LC3. Cx43 levels increase reaching a maximal peak at 4 hours, decreasing afterwards. The values are the averages of four individual experiments ± SD. (B) Cos7 cells transfected with Cx43 were serum-deprivated for 0, 1, 2, 4, 6 and 8 hours. Cell lysates were immunoprecipitated with goat polyclonal antibodies against Cx43. The precipitates were analyzed by Western blot using monoclonal antibodies against ubiquitin (P4D1) and polyclonal antibody against Cx43. The levels of ubiquitinated Cx43 change over time.

Thus, we immunoprecipitated Cx43 using polyclonal antibodies, followed by Western blot and probing with antibodies against ubiquitin, which recognizes polyubiquitinated and monoubiquitinated proteins. The results presented in figure 9 (B) show that ubiquitination of Cx43 dramatically increases in the first hour of starvation, after which the levels of Ub attached to Cx43 drop to control levels in the second hour. This pattern of increased ubiquitination by followed by a decrease in ubiquitination in the following hours, suggests a cyclic pattern of ubiquitination/deubiquitination and degradation.

AMSH and UBPY activity regulates the degradation of Cx43 by macroautophagy.

The results presented above suggest that the deubiquitination of Cx43 has a protective role towards the protein, preventing its degradation. Therefore it is conceivable to suggest that by removing ubiquitin, the "degradation tag", DUBs protect Cx43 from degradation by autophagy.

To test this hypothesis, we transfected Cos7 cells with a plasmid encoding either the wild type (AMSH WT and UBPY WT) or a catalytically inactive mutant form of AMSH and UBPY (AMSH-D348A / UBPY-C786S), after which the cells were incubated either in the presence (control) or absence (starvation) of serum. The results presented in figure 10 (A) show that overexpression of AMSH WT doesn't significantly affect the starvation-induced degradation of Cx43, while in cells overexpressing the mutated form of the enzyme, the levels of Cx43 decrease faster, as compared with the control.

The same tendency was observed for UBPY, in which the overexpression of the mutated form leads to an increased degradation of Cx43 by autophagy.

Since impairment of the DUBs results in faster starvation-induced degradation of Cx43, these results suggest that deubiquitination of Cx43 prevents its degradation by autophagy.

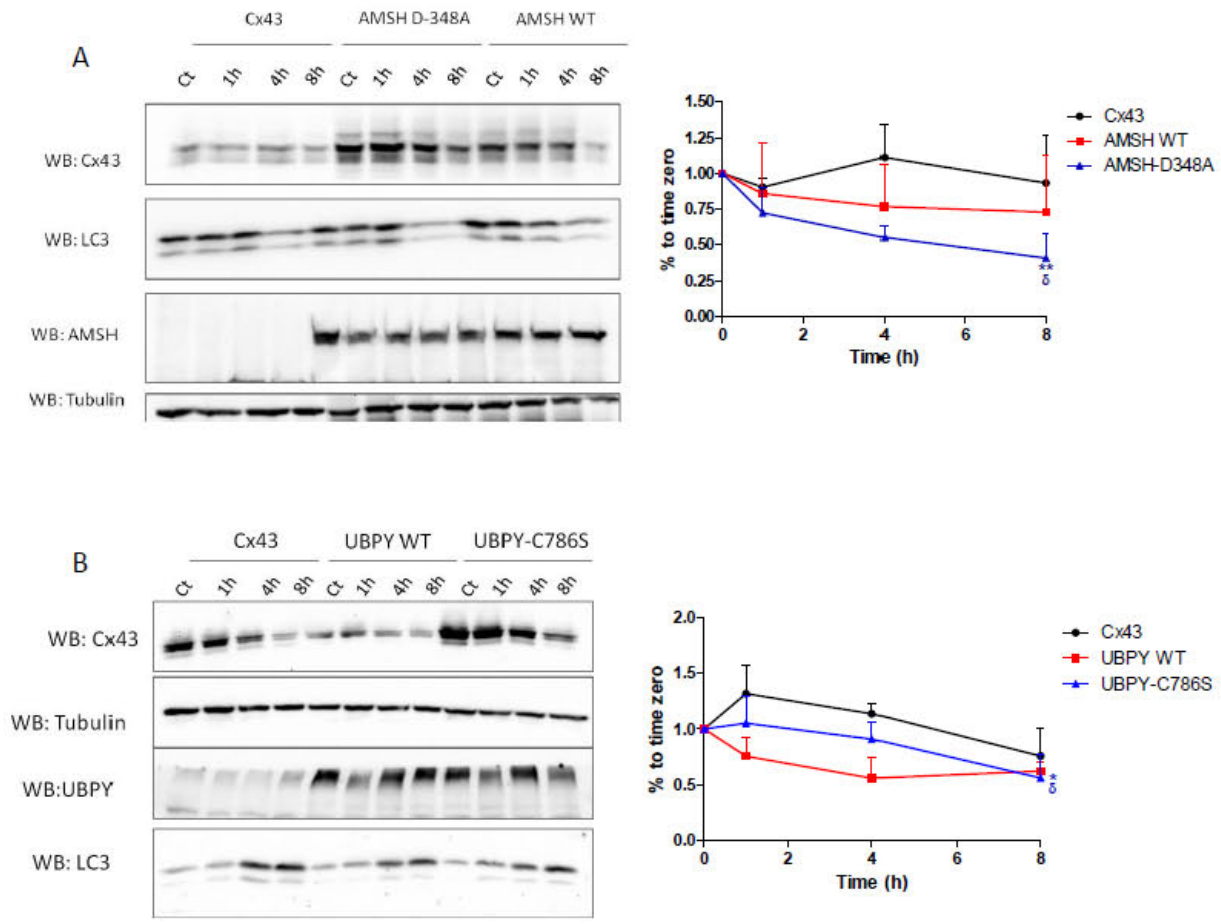

Figure 10: AMSH and UBPY modulate the degradation of Cx43 by autophagy. (A) Cos7 cells transfected with either GFP-AMSH WT or GFP-AMSH-D348A and Cx43 were incubated with medium without serum for 0, 1, 4, and 8 hours. Lysates were then analyzed by Western blot using goat polyclonal antibodies directed against Cx43, rabbit polyclonal antibodies against AMSH, LC3 or mouse monoclonal antibodies against tubulin. The intensity of the Cx43 bands was measured and plotted in a graph. The expression of AMSH-D348A leads to decreased the levels of Cx43 during starvation. The values are the averages of three individual experiments ± SD. Significant differences with treated cells are indicated with asterisks (* $p < 0.05$ to time 0h; ** $p < 0.01$ to time 0h; δ< 0,05 time 1h) (B) Cos7 cells transfected with either GFP-UBPY WT or GFP-UBPY-C786S and Cx43 were serum-deprivated for 0, 1, 4, and 8 hours. Lysates were

then analyzed by Western blot using goat polyclonal antibodies directed against Cx43, rabbit polyclonal antibodies against UBPY, LC3 or mouse monoclonal antibodies against tubulin. The intensity of the Cx43 bands was measured and plotted in a graph. The expression of UBPY-C786S induced a faster degradation rate of Cx43 in response to starvation. The values are the averages of three individual experiments ± SD. Significant differences with treated cells are indicated with asterisks (* $p < 0.05$ to time 0h; ** $p < 0.01$ to time 0h; δ< 0,05 time 1h)

The LIR domain of Cx43 mediates its constitutive degradation through autophagy.

It is well established that autophagic cargo receptors, such as p62 and NBR1, bind ATG8 and its homologues, such as LC3, suggesting that ATG8 family proteins provide an entry point for docking of autophagic cargo. In addition to mammalian p62 and NBR1, also Atg19, which is involved in selective autophagy in the cytoplasm to vacuole targeting (Cvt) pathway in yeast, uses a LIR to interact with Atg8. The list of proteins that uses a LIR motif to interact with Atg8 homologues is expanding [36]. Previous data from our lab demonstrated that degradation of Cx43 by autophagy depends on the ubiquitination of Cx43 and its further interaction with the adaptor p62.

In this scenario, the existence of a LIR motif in Cx43 would be redundant, since p62 establishes the bridge between Cx43 and LC3. However, most of the studies devoted to the study of degradation of Cx43 by autophagy were obtained in cells subjected to a stimulus, such as starvation. Moreover, studies by Matthias M. Falk's [50] group show that silencing p62 results in only a partial reduction of Cx43 degradation, suggesting that other mechanisms are somehow involved.

Therefore we hypothesize that under basal conditions, a putative LIR motif in Cx43 could allow a direct interaction with LC3, even in the absence of

ubiquitination and p62. Indeed, we found an LIR motif WxxL in the amino terminal of Cx43.

Since this LIR motif is likely to mediate the degradation of Cx43 by autophagy, we hypothesized that a mutation in this region would stabilize the protein. To accomplish this task we performed point mutations in the amino acids in position 4 and 7, by replacing tryptophan and leucine for alanine (figure 11).

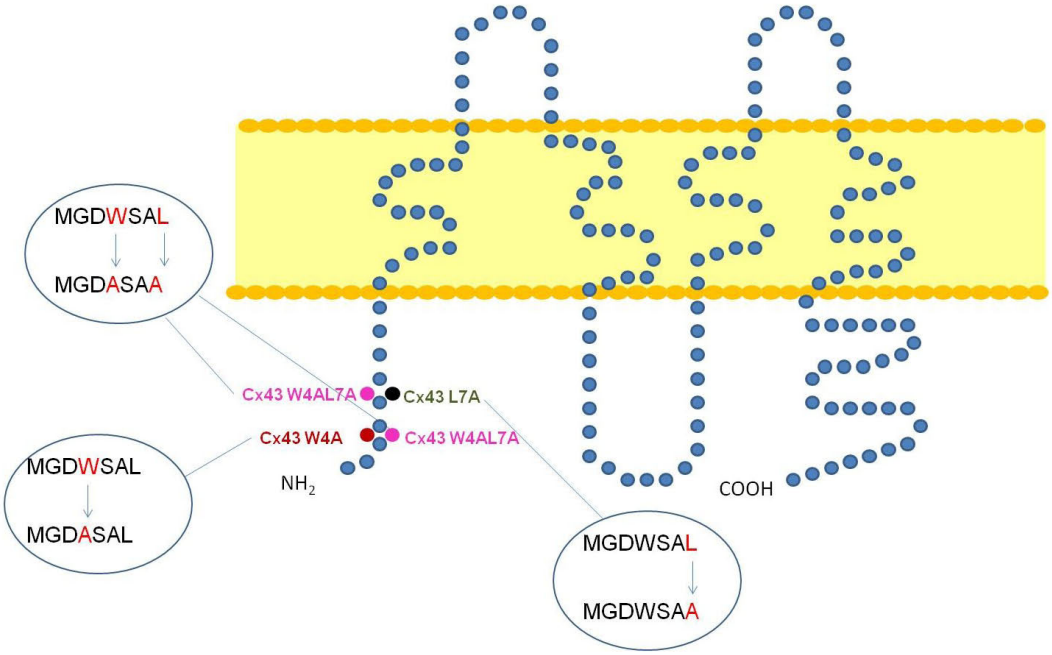

Figure 11: schematic representation of Cx43 and its mutations in the LIR motif. Both amino acids triptophane and leucine were replaced for alanine, to form three different Cx43 mutants.

To evaluate the half-life of these mutants we performed a CHX-chase. The results presented in figure 12 (A) show that a double mutation in LIR (V5-Cx43W4A,L7A) stabilizes Cx43, as demonstrated by the presence of higher levels of Cx43 after 4hours of treatment with CHX, when compared with WT and single mutated forms (V5-Cx43W4A and V5-Cx43L7A) of Cx43.

According to our model, the LIR motif is important in basal conditions, where it modulates the interaction with LC3, thus controlling the incorporation of Cx43

into autophagic vesicles. On the other hand, in cells subject to a stimulus, Ub attached to Cx43 recruits p62 that in turn mediates the interaction with LC3.

To test this hypothesis we evaluated the stability of Cx43 mutated in the LIR motif in cells incubated in the absence of serum, to induce macroautophagy. The results presented in figure 12 (B) show that mutations in the LIR motif does not protect Cx43 from autophagy degradation induced by starvation, thus suggesting that interaction of Cx43 with LC3 in these circumstances is modulated by p62.

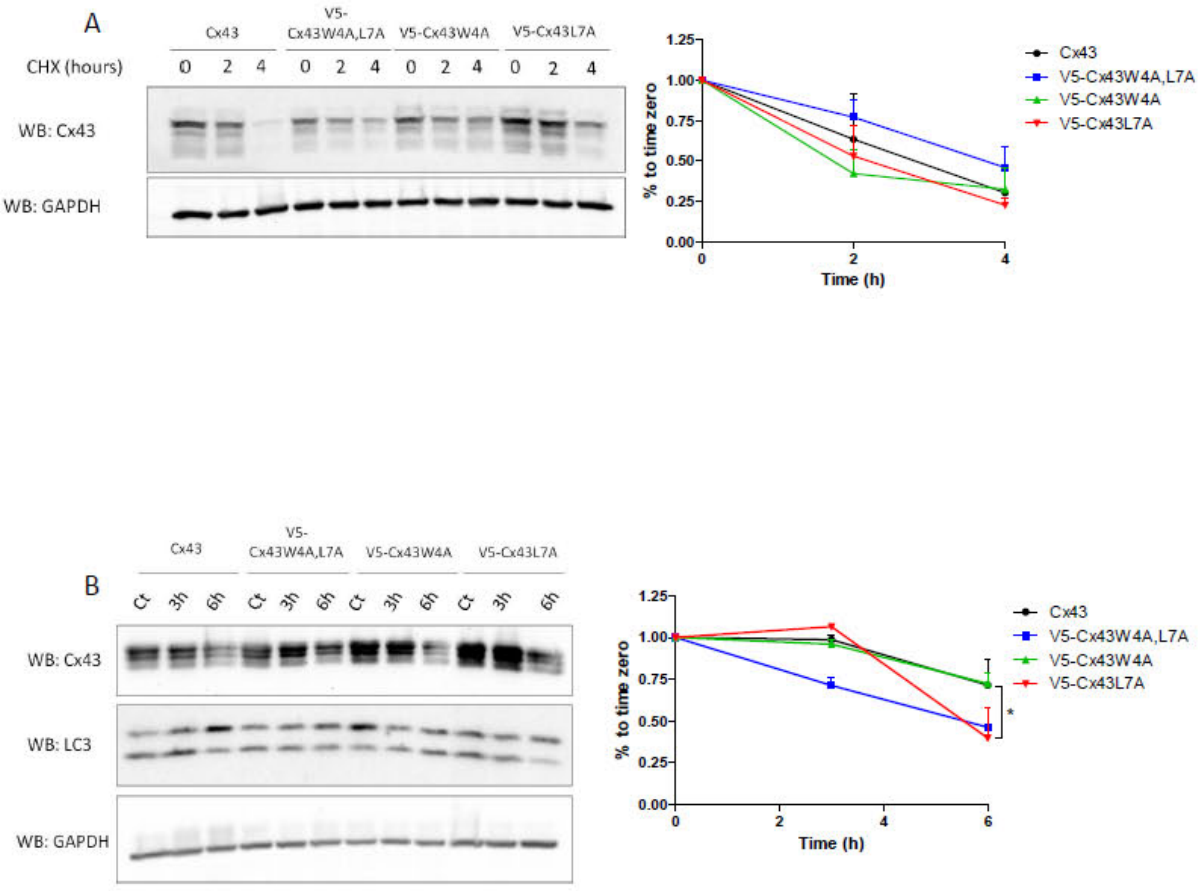

Figure 12: the LIR motif modulates the stability of the protein. (A) Cos7 cells were transfected with V5-Cx43, V5-Cx43W4A,L7A, V5-Cx43W4A and V5-Cx43L7A, and incubated with 50 μg/ml CHX for 0, 1, 2 and 4 hours. Lysates were then analyzed by Western blot using goat polyclonal antibodies directed against Cx43 or mouse monoclonal antibodies against tubulin. The V5-Cx43W4A,L7A mutant has a more marked decreased in the amount of Cx43, compared with the others mutant forms. (B) Cos7 cells transfected with Cx43 wild type (WT) or

mutated V5-Cx43W4A,L7A, V5-Cx43W4A and V5-Cx43L7A, and maintained in the presence or absence of serum as indicated, during 0, 3 and 6 hours of starvation. Lysates were then analyzed by Western blot using goat polyclonal antibodies directed against Cx43, rabbit polyclonal antibodies against LC3 or mouse monoclonal against tubulin. The intensity of the Cx43 bands was measured and plotted in a graph. Mutations in the LIR motif of Cx43 do not protect Cx43 from degradation during starvation. The values are the averages of three individual experiments ± SD. Asterisks indicate statistically significant differences from controls (* $p < 0.05$ between V5-Cx43L7A and Cx43). .

CHAPTER 5: DISCUSSION AND CONCLUSIONS

Gap junctions (GJ) are cellular structures which allow direct cell-to-cell transfer of signaling molecules by forming plaques of channels that bridge apposing membranes of neighboring cells. The role of GJ mediated intracellular communication (GJIC) for all aspects of multicellular life, including coordination of development, tissue function, and cell homeostasis, has been well documented. The assembly and degradation of these membrane channels is a complex process that includes biosynthesis of the connexin subunit proteins on endoplasmatic reticulum (ER) membranes, oligomerization of compatible subunits into hexameric hemichannels (connexons), delivery of the connexons to the plasma membrane (PM), head-on docking of compatible connexons in the extracellular space at distinct localizations, arrangement of channels into spatially dynamic and temporally organized GJ channel plaques, as well as internalization of GJs into the cytoplasm followed by their degradation. Although regulation of GJIC can occur at different stages, the study of the mechanisms whereby Cxs are degraded has deserved particular attention from the scientific community devoted to this field of research. Pioneer results obtained in our lab, more than 10 years ago, ascribed a role to ubiquitin in the degradation of Cx43. However, by that time, the involvement of ubiquitin in the degradation of membrane proteins was a very novel concept. Indeed, ubiquitin was initially identified to be required for breakdown of short-lived cytosolic protein in the proteosome. It is now well accepted that Ubiquitin serves as a post-translational

modifier and is involved in the regulation of a variety array of cellular processes including the cell cycle, endocytosis, and DNA repair [43].

Ubiquitin signals are generated through covalent attachment of ubiquitin molecules to target a protein in a process known as ubiquitination. In this process, ubiquitin is first activated at the expense of ATP and transferred to the active site Cys residue of an ubiquitin-activating enzyme (E1). The activated ubiquitin is then transferred to the active site of a family of ubiquitin-carrier or ubiquitin conjugating enzymes (E2s) [43]. The last step is catalyzed by ubiquitin-ligases (E3s), where the carboxyl group of the C-terminal Gly residue of ubiquitin is ligated to the ε-amino group of an internal Lys or α-amino group of the N-terminal Met residue in the target protein [43]. More recent studies conducted in our lab established that Nedd4 mediated ubiquitination of Cx43 triggers the internalization and degradation of GJ channels. Moreover, it was shown that incorporation of ubiquitinated Cx43 into the channels localized at the plasma is required for the recruitment of the endocytic adaptor Eps15 that further drives the removal of channels from the plasma membrane.

Besides internalization, ubiquitination is also a process that is necessary for intracellular sorting of membrane proteins, through the interaction with multiple endossomal sorting complexes required for transport (ESCRTs), which dictate cargo selection and the production of vesicles that bud inside from the limiting membrane of the sorting endosome [42]. This process creates a multivesicular body (MVB), which can directly fuse with lysosomes.

As with many other pos-translational protein modifications, ubiquitination is a reversible process accomplished through the action of deubiquitinating enzymes (DUBs), proteases specific for the isopeptide bond that links ubiquitin chains, and ubiquitin to substrate proteins. In the human genome there are approximately 90 active DUBs. Regarding the endocytic pathway, numerous studies have demonstrated that DUBs mainly act at the endosome to regulate the fate of internalized receptors and consequently their signaling outputs.

Although Cx43 has long been known to be a substrate for ubiquitination, the deubiquitination of the protein has never been described before. Therefore, a main objective of this study was to evaluate the role of deubiquination in the regulation of the intracellular trafficking and final fate of Cx43. To accomplish this task we investigated the involvement of two DUBs, AMSH and UBPY, previously reported to be associated with the endocytic pathway. The results obtained in our work show, for the first time, that Cx43 is a substrate of these enzymes, which catalyze the deubiquitination of the protein. Indeed, we demonstrated that overexpression of a catalytically inactive mutant of both AMSH and UBPY results in the accumulation of the ubiquitinated form of Cx43. Moreover, IP assays and colocalization studies show that these DUBs interact with Cx43, both in intracellular vesicles and at the plasma membrane. These results are particularly evident in cells overexpressing the catalytically inactive mutant, where we observed an accumulation of Cx43 in cytoplasmic vesicles, suggesting that intracellular sorting of Cx43 is compromised. Since sorting of membrane proteins in MVBs requires deubiquitination of proteins, it is likely that an impairment of deubiquitination activity entraps Cx43 at the MVBs. To test this hypothesis specific markers of MVBs, such as CD63 and Tsg101 might be

used in future studies. In addition to its accumulation in intracellular vesicles, the data obtained in this study shows that Cx43 accumulates at the plasma membrane in cells overexpressing the catalytically inactive mutant of both AMSH and UBPY, suggesting that deubiquitination of Cx43 prevents the internalization of GJs. As far as we know, evidence for the of deubiquitination of membrane proteins at the plasma membrane are very scarce and constitute a major and striking finding of this study, which goes beyond the field of Connexin research.

Although both AMSH and UBPY demonstrated to mediate the deubiquitination of Cx43, the results obtained with AMSH were more robust and reproducible For this reason, in a subsequent stage of the study we evaluated the involvement of AMSH in controlling the half-life and internalization rate of Cx43. Consistent with the role of Ubiquitin as a "degradation signal", we observed a slight decrease in the half-life of Cx43, from 2,5 hours to 2 hours, in cells overexpressing the catalytically inactive form of AMSH. However, regarding the wild-type, a biphasic pattern is observed, in which Cx43 is stabilized in the first 2 hours, followed by a rapid decrease to half of the initial amount in the next 2 hours. To further confirm that AMSH regulates the half-life of Cx43, we used a siRNA strategy. In accordance with data obtained from the overexpression experiments,, the silencing of AMSH results in a faster decrease of Cx43.

Taken together, these results show, for the first time that the deubiquitination of the Cx43, catalyzed by AMSH, constitutes an additional step in the regulation of Cx43 life-cycle and GJIC.

Previous studies carried out in our lab showed that GJs are degraded by autophagy, through a mechanism that requires prior Nedd4-mediated

ubiquitination of Cx43 [45]. Macroautophagy is only one of the major degradative pathways in eucaryotic cells, and it's the only with the capacity to degrade entire organelles. Given the unusual large size of internalized GJs structures (200-500 nm), called annular GJ or connexosomes, autophagy is the degradative pathway that better fits in the degradation mechanism of GJs.

Autophagy is a survival catabolic process that is activated by different stimuli, such as ER stress, nutrient deprivation, hypoxia, reactive oxygen species, DNA damage, protein aggregates, damaged organelles or intracellular pathogens. Therefore, autophagy can be seen as a repair and quality control mechanism, eliminating damaged proteins and organelles that accumulate during stress and aging [46], as well as beneficial for the energetic balance in the cell, given that the recycling of amino acids that resulting from protein breakdown in lysosomes serves to sustain protein synthesis and to obtain adenosine triphosphate (ATP) through the entry of amino acids in the Krebs cycle.

Once we have established that DUBs catalyze the deubiquitination of Cx43, preventing its degradation, and knowing that degradation of Cx43 by autophagy requires ubiquitination of the protein, we further evaluated the putative role of DUBs in protecting Cx43 from degradation by autophagy. The results obtained in this study demonstrate that overexpression of the catalytically inactive forms of AMSH or UBPY enhances the degradation of Cx43 induced by starvation, used to activate macroautophagy. Indeed, we show that cells overexpressing the catalytically inactive forms of AMSH or UBPY, incubated for 8 hours, in the absence of serum, present lower levels of Cx43, than control cells transfected either with GFP or the wild type form of the enzymes, suggesting that

deubiquitination of Cx43 protects the protein from autophagy degradation. To test this hypothesis in the future, we will investigate the effect of autophagy inhibitors, such as 3-MA, in this process. If an impairment of deubiquitination activity results in an increased degradation of Cx43 by autophagy, it is expected that 3-MA will prevent this process, with a concomitant accumulation of ubiquitinated Cx43.

Moreover, the same experimental approach should need to be performed with siRNA directed against AMSH and UBPY in order to confirm their involvement in such a process.

Starvation-induced autophagy is considered a nonselective, bulk degradation process. However, several evidence suggests that selective, highly regulated autophagy can target a variety of substrates for degradation. Selective autophagy is mediated by autophagic cargo receptors or adaptors, such as p62, that has been shown to facilitate docking of ubiquitinated substrates to the autophagosome. Recently, a mammalian protein, NBR1 (neighbor of BRCA1 gene) was also described as a cargo receptor which cooperates with p62 in the autophagic clearance of certain substrates [48]. Both of these proteins share a similar overall domain organization, with a LIR (LC3-interacting region) motif. This LIR motif mediates the linkage between the targeted ubiquitinated protein with LC3, being this interaction required for the autophagic degradation of p62/NBR1-containing structures. Therefore, it is conceivable that proteins that carry LIR motifs can be degraded by autophagy even in the absence of p62. Published data showed that silencing of p62 only partially prevents degradation of Cx43 by autophagy [50], suggesting that alternative partners or mechanisms

may be involved. In this context, we hypothesized that a putative LIR motif in Cx43 could mediate p62-independent autophagy degradation of Cx43. In fact, we could find a LIR motif WxxL in the WT Cx43 localized in the N-Terminal of Cx43, between the tryptophan in position four and leucine in position seven. According to our model a mutated LIR motif stabilizes the protein. To address this question we determined the half-life of three different LIR mutants (V5-Cx43W4A,L7A, V5-Cx43W4A and V5-Cx43L7A), using CHX-chase assays. The results obtained show that the double mutant Cx43W4A,L7A is more stable, in comparison with the wild type Cx43. Indeed, after 2 hours of incubation with CHX, the amount of the WT and the mutants Cx43W4A and Cx43L7A decrease 50%, whereas the Cx43W4A,L7A only decreases less then 25%. To further investigate the importance of the LIR motif in the degradation of Cx43 upon activation of autophagy, we incubated transfected cells with the plasmids encoding either the WT or the LIR-mutated forms of Cx43, in the absence of serum. Surprisingly, under these circumstances, the mutations in the LIR motif do not prevent degradation of Cx43. Instead, the double mutant presented an increased degradation in comparison with the WT, suggesting that it induced autophagy and that the LIR motif does not play a major role. Although these results need further confirmation, at this point they suggest that under basal conditions, the LIR motif mediates the direct interaction of Cx43 with LC3 and, consequently, the degradation of Cx43, while in stimuli-induced autophagy, such as starvation, where an increased ubiquitination of Cx43 occurs, p62 assumes a preponderant role, mediating the interaction with LC3. To test this hypothesis, in the future we will compare the interaction between LC3 either the WT or the LIR mutants of Cx43, in the presence or absence of serum. If our

hypothesis is correct, we expect to observe a decrease in the interaction between LC3 and LIR-mutated Cx43 only in basal condiions, while in starved cells this interaction is not significantly affected. To further address the shift from LIR to p62, in stimuli-induced autophagy, we will silence p62. In this case we expect that interaction between LC3 and Cx43 is not affected in basal conditions, but is disrupted in cells subject to serum deprivation.

A biological model that could be comparative to these data is the heart failure induced by cardiac ischemia. In ischemia condition the levels of ubiquitin and autophagy are increased, which leads us to propose that the amount of p62 is increased, and so, leads to degradation of Cx43. Further work needs to be performed to assess whether treatments targeting Cx43 DUBs can help protect heart tissue from the negative effects of ischemia.

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