DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

To beat or not to beat: detrimental autophagy contributes to gap junctions degradation in ischemic heart

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Henrique Girão (Universidade de Coimbra) e sob a orientação interna da Doutora Margarida Castro (Universidade de Coimbra)

Tânia Sofia Martins Marques

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O trabalho apresentado nesta dissertação foi realizado no Instituto de Imagem Biomédica e Ciências da Vida (IBILI) da Faculdade de Medicina da Universidade de Coimbra, sob a orientação do Doutor Henrique Girão, ao abrigo dos projectos PTDC/SAU-ORG/119296/2010 e PEST-C/SAU/UI3282/2013-COMPETE (Fundação para a Ciência e Tecnologia – FCT), no âmbito do Mestrado em Biologia Celular e Molecular (MBCM) da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Abbreviations

Akt	protein kinase B
ANOVA	analysis of variance
AMPK	adenosine monophosphate-activated protein kinase
AP-1	adaptor protein 1
AP-2	adaptor protein 2
Atg	autophagy-related gene
Atg1	autophagy-related gene 1
AVN	atrio-ventricular node
Baf	Bafilomycin A1
CDC2	cyclin-dependent kinase 2
CHX	cycloheximide
CIP75	Cx43-interacting protein of 75kDd
CK1	casein kinase 1
CL	citoplasmic loop
CQ	C-terminal
CT	chloroquine
Cx	connexin
DMEM	Dulbecco's Modified Eagle Medium
DTT	dithiothreitol
DUB	deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
ECG	electrocardiography
EGF	epidermal growth factor
EL	extracellular loop
Eps15	epidermal growth factor substrate 15
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
ESCRT	endosomal sorting complex required for transport
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GJ	gap junctions
GJIC	gap junction intercellular communication
GSK-3β	glycogen synthase kinase 3β
HRP	horseradish peroxidase
Hrs	hepatocyte growth-factor regulated tyrosine kinase substrate
Hsp90	heat shock protein 90
IDs	intercalated discs
IGF-1	insulin growth factor 1
IP	immunoprecipitation
I/R	ischemia/reperfusion
LC3	microtubule-associated protein 1 light chain 3
LIR	LC3-interacting region
MAPKs	mitogen-activated protein kinases
MCB	methyl-β-cyclodextrin
MI	myocardial ischemia

mTOR	mammalian target of rapamycin
NBR1	neighbor of BRCA1
Nedd4	neural precursor cell expressed developmentally down-regulated protein 4
NT	N-terminal
OA	okadaic acid
ODDD	oculodentodigital dysplasia
p62/SQSTM1	sequestosome 1
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI	phosphatidylinositol
PI3KIII	phosphatidylinositol 3-kinase class III
PKA	protein kinase cAMP-dependent
PKC	protein kinase C
PM	plasma membrane
PMSF	phenylmehanesulphonyl fluoride
PTM	post-translational modifications
ROS	reactive oxygen species
SAN	sino-atrial node
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Smurf1	smad ubiquitination regulatory factor 1
Smurf2	smad ubiquitination regulatory factor 2
Sp1	specificity protein 1
Src	proto-oncogene tyrosine-protein kinase
TBS-T	Tris-buffered saline-Tween 20
Tbx5	T-box 5
TEM	transmission electron microscopy
TGN	trans-Golgi network
TM	transmembrane
TOM	translocase of the outer mitochondrial membrane
TPA	tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate
TRIM21	tripartite motif-containing protein 21
TSC	tuberous sclerosis complex
TSG101	tumor susceptibility gene 101
Ub	ubiquitin
UBD	ubiquitin binding domains
ULK1	UNC-51-like kinase 1
WB	Western blot
WWP1	WW domain-containing E3 ubiquitin protein ligase 1
ZO-1	zonula occludens-1
3-MA	3-methyladenine

Resumo

A contracção coordenada do músculo cardíaco depende em larga medida de uma eficiente comunicação intercelular entre os cardiomiócitos. Esta comunicação é maioritariamente mediada por *gap junctions* (GJ), que são canais transmembranares formados por seis subunidades de uma proteína denominada conexina (Cx), e que permitem uma rápida e anisotópica propagação do impulso eléctrico. Assim, uma correcta e eficiente comunicação entre os cardiomiócitos é vital para assegurar o normal funcionamento do coração. Desta forma, mecanismos que afectem o ciclo de vida da Cx43, a principal Cx presente nas GJ do ventrículo esquerdo, e consequentemente afectem a comunicação intercelular podem ter implicações muito profundas na função cardíaca. Efectivamente, várias cardiomiopatias têm sido associadas a distúrbios na comunicação intercelular mediada por GJ, os quais têm sido parcialmente atribuídos a um aumento da degradação da Cx43. Apesar da sua importância, os mecanismos moleculares associados à degradação da Cx43, nomeadamente no coração em isquémia, permanecem por esclarecer. Resultados recentes do nosso grupo demostraram que a Cx43 é um substrato da autofagia, uma via proteolítica que se sabe estar activada durante a isquémia. Com base nestes resultados, e uma vez que a sua relevância fisiopatológica permanece indeterminada, o principal objectivo do presente estudo é esclarecer o papel da autofagia na degradação da Cx43 induzida pela isquémia nos cardiomiócitos. Através do uso de linhas celulares de cardiomiócitos (HL-1) e culturas organotípicas de tecido cardíaco, demostrámos que a isquémia leva a uma degradação da Cx43 que pode ser parcialmente revertida pela inibição química e genética da via autofágica, nomeadamente através do silenciamento do p62. O envolvimento da autofagia foi confirmado também através de ensaios de co-localização e co-imunoprecipitação, nos quais demonstrámos que a Cx43 interage com as proteínas da maquinaria da autofagia, nomeadamente com a LC3, p62 e NBR1. Adicionalmente, demonstrámos que a degradação da Cx43 na reperfusão é mais acentuada que em períodos equivalentes de isquémia e, mais importante, que a degradação da Cx43 em isquémia ou isquémia/reperfusão assenta em diferentes mediadores moleculares. Usando inibidores farmacológicos da AMPK ou o silenciamento da Beclina 1, os resultados obtidos mostram que durante os períodos iniciais de isquémia, a degradação da Cx43 é mediada maioritariamente pela AMPK, enquanto que durante períodos mais tardios de isquémia ou durante a reperfusão, a Beclina 1 é o principal mediador. Ao contrário do observado nas abordagens envolvendo linhas celulares ou culturas organotípicas, onde se observa uma degradação acentuada da Cx43 em isquémia, no modelo de Langendorff, apenas a reperfusão é capaz de induzir uma degradação robusta da proteína, enquanto a isquémia resulta numa ligeira degradação da Cx43 só nos períodos mais prolongados. Desta forma, os resultados presentes neste trabalho constituem novas provas relativamente aos mecanismos moleculares associados à degradação da Cx43 no coração em isquémia, o que poderá constituir uma ferramenta poderosa para o desenvolvimento de novas estratégias moleculares com vista à preservação da função cardíaca na isquémia

Abstract

Gap junction intercellular communication (GJIC) between cardiomyocytes is essential for coordinated cardiac muscle contraction, which involves rapid anisotropic impulse propagation through connexin (Cx)-containing channels. Therefore, disturbing mechanisms that affect the turnover of Cx43, the main GJ protein present in the left ventricle, has profound effects on heart function. Accordingly, several cardiomyopathies have been related to dysfunctional GJIC, partially attributed to increased Cx43 degradation. Nevertheless, the molecular mechanisms underlying ischemia-induced degradation of Cx43 remain largely undefined. Therefore, the main objective of this study was to establish whether autophagy mediates degradation of Cx43 in cardiomyocytes subjected to ischemia. Using both HL-1 cells and organotypic heart slices we showed that ischemia-induced Cx43 degradation can be reverted by chemical and genetic inhibition of autophagy, namely through the silencing of p62. The involvement of autophagy was further confirmed by co-localization and co-immunoprecipitation assays in which we demonstrated that Cx43 interacts with proteins of the autophagy machinery, such as LC3, p62 and NBR1. Strikingly, we showed that degradation of Cx43 in ischemia or reperfusion relies upon different molecular players. Indeed, degradation of Cx43 during early periods of ischemia depends on AMPK, whereas in late periods of ischemia and in reperfusion it relies on Beclin 1. Contrarily to the observed in the cell line and in organotypic cultures, in the *ex vivo* model, the Langendorff heart, only reperfusion was able to induce a robust degradation of Cx43, while no-flow ischemia results in a slight degradation of the protein, only after prolonged times. Altogether, this data provides new evidence regarding the molecular mechanisms whereby Cx43 is degraded in the ischemic heart, which may contribute for the development of new strategies that aim to preserve cardiac function in ischemia.

1. Introduction

Intercellular communication through gap junctions (GJ) is essential to maintain organism homeostasis since it ensures the flow of information among neighboring cells, being the basis of integration and coordination of cell actions within tissues and organs. GJ-mediated intercellular communication (GJIC) between adjacent cells relies on intercellular channels that allow the passage of small metabolites and ions between the cytoplasm of connected cells, essential to accomplish the metabolic and electric cellular coupling required to regulate development, cell growth and differentiation. GJ are formed by the docking of two hemichannels, also termed connexons, being each hemichannel formed by the assembly of six subunits of a transmembrane protein called connexin (Cx) [1].

1.1. Structure and synthesis of Cx43

The human and mouse genomes encode 21 and 20 Cxs, respectively, that can be classified into 5 groups (alpha, beta, gamma, delta, and epsilon GJ proteins), according to its gene sequence homology [1, 2]. Although Cx expression is considered tissue and/or cell type-specific, some Cxs, such as Cx32 (Gap-junction beta-1, GJB1) and Cx43 (Gap-junction alpha-1, GJA1) are widely expressed in a variety of tissues. Moreover, different types of Cxs can be simultaneously expressed in the same cell. Within a certain tissue, the differential expression of more than one type of Cx is tightly regulated, both in their amount and specific localization. This specific pattern of Cx expression within the tissues must be preserved in order to maintain proper cell coupling and hemichannel function, since differential Cx composition partially determines the conductivity and selectivity of the channels [2]. Regarding Cx43, the most abundant Cx in the human body, it is largely expressed in the brain, heart, bone, cartilage, kidney, eye and skin, among other tissues.

Given its wide expression, as well as its importance in the context of the present work, we will focus on Cx43. Similarly to other membrane proteins, Cx43 is synthesized on endoplasmic reticulum (ER)-bound ribosomes and, in parallel to its synthesis, it is inserted into the ER along which its proper conformation is acquired [2]. Structurally, Cx43 is formed by a cytoplasmic N- and C-terminal (NT and CT, respectively), a cytoplasmic loop (CL), two extracellular loops (EL) and four α-helical transmembrane (TM) domains, which are highly conserved among the different Cx isoforms [3] (Figure 1).

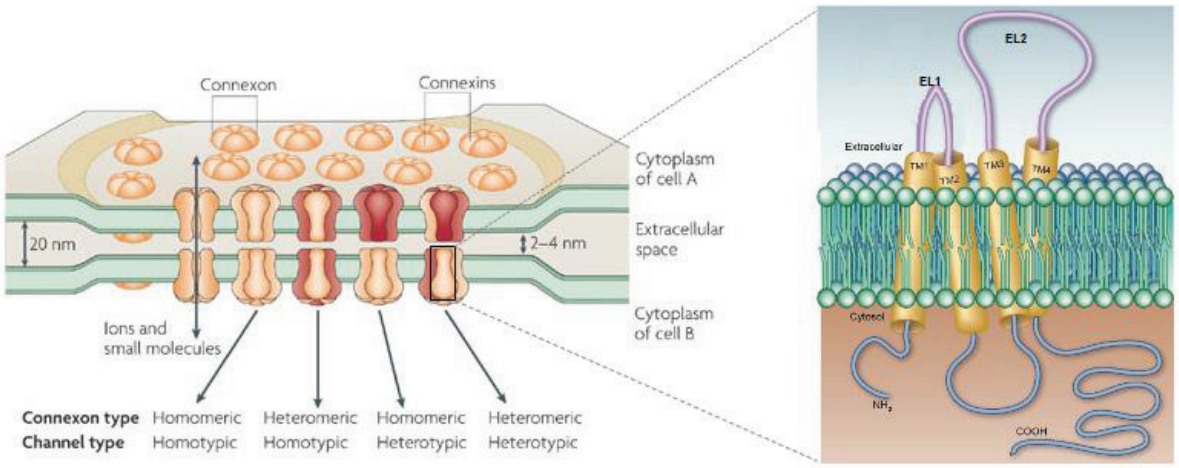

Figure 1 – Structure of Cx43 and assembly of GJ. Cx43 is an integral transmembrane protein. Cxs are assembled in groups of six to form hemichannels (or connexons) and two hemichannels then combine to form a GJ. Hemichannels can be composed by groups of the same (homomeric) or mixed (heteromeric) Cxs. Homotypic or heterotypic GJ are resultant of the assembly of identical or mixed connexons, respectively. GJ allow the passage of ions and small molecules, mediators of intercellular communication, between neighboring cells. Transmembrane (TM) domains, extracellular loops (EL1 and EL2) and NH_2 and COOH-terminal tails of Cx43 are illustrated. (Adapted from Bloomfield SA, et al. 2009 and from Chun-hong X, et al. 2012)

After leaving the ER, Cx43 is transported into the trans-Golgi network (TGN) where it oligomerizes into hexameric structures. Depending on the Cx type, oligomerization of Cxs can occur sequentially throughout the ER till they reach the TGN [4]. The resultant connexons may be translocated to the plasma membrane (PM) by budding and fusion of membrane vesicles, in a microtubule-dependent or independent manner. Once connexons are inserted in the PM, they can function as hemichannels, or can form GJ after docking with connexons of neighboring cells [1]. The hemichannels that arrive to the PM are usually closed, however they can open under certain circumstances allowing the passage of metabolites, such as ATP and ions, between the cytoplasm of adjacent cells and the extracellular medium. In cells that express multiple Cxs, these may co-oligomerize into the same (homomeric) or mixed (heteromeric) connexons, but only certain combinations are allowed. Similarly, identical connexons may dock forming homotypic intercellular channels or, if the docking occurs between connexons containing different Cxs, they can form heterotypic channels. As well, only some assembly combinations are allowed, however the diversity of intercellular channels that can be assembled is extremely large, thus contributing to the selectivity of the channels. Addition of new arriving Cxs to GJ can occur through lateral diffusion of connexons at the PM with its concomitant accretion at the edges of pre-existing plaques. Additionally, removal of subunits from the center of the plaques is required to maintain GJ turnover and the renewal of the plaque, however it is not clear how the selective removal of Cxs/connexons/intercellular channels might occur [1].

Besides its role upon GJIC, Cx43 has been also associated with non-junctional functions. Indeed, it is important to notice that Cx43 is not only localized at the PM, in hemichannels and GJ, but its presence both in the nucleus and in the mitochondria has also been established. The existence of a putative nuclear targeting sequence in Cx43CT, likely explains the presence of the full length Cx43 or its overexpressed CT at the nucleus, apparently functioning as a gene regulator. Accordingly, Cx43CT has been related with control of the cell cycle and inhibition of cell growth [5].

Recent reports regarding the role of Cx43 localized in the mitochondria have shown that heat shock protein 90 (Hsp90) and translocase of the outer mitochondrial membrane (TOM) are the Cx43-interacting partners responsible for its transport and insertion in the inner mitochondrial membrane [6]. The role of mitochondrial Cx43 is not clearly defined, however it has been implicated in pathological processes, such as endothelial dysfunction, and also in cardioprotection, through enhanced ischemic preconditioning response [7, 8].

1.2. Regulation of GJIC

For normal cell and tissue function, both in physiological and pathological conditions, fine-tuning and maintenance of intercellular communication are essential. Regulation of GJIC can occur at different levels at the Cxs life cycle, which include synthesis, trafficking, gating and degradation [9]. Indeed, a main feature of Cx43 is its unusual high turnover, with a half-life of 1-3 hours in both cell culture and whole tissues. This high turnover of Cx43 implicates that, more than synthesis, mediation of the degradation mechanisms play a vital role in the regulation of GJIC, in response to both physiological stimuli and environmental alterations [1, 3]. Typically, regulation of GJIC can be achieved at two levels: i) short term regulation, through alterations on the channel conductance and permeability, mainly as a result of phosphorylation/dephosphorylation of specific residues of Cx43 [3], and ii) slow regulation, which consists on alterations on the number of channels present in the PM, due to changes on the rate of Cx43 biosynthesis, GJ assembly and turnover (Figure 2). In any case, Cx43-interacting partners have a preponderant role, either by the interaction itself, or through subsequent post-translational modifications (PTM) in Cx43 protein. Although Cx43 can undergo different PTM, including hydroxylation, acetylation, disulfide binding, nitrosylation, and palmitoylation, several studies have ascribed to phosphorylation and ubiquitination of Cx43 a main role on regulation of its turnover and on the GJ remodeling.

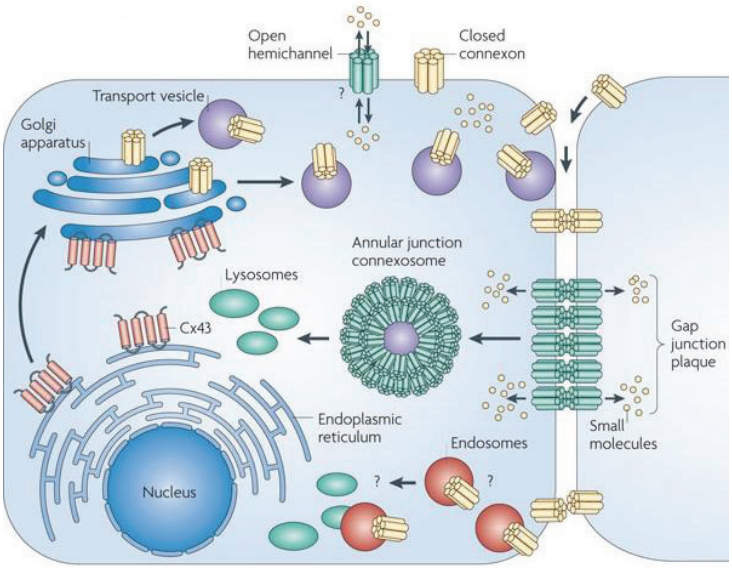

Figure 2 – Cxs life-cycle. Cxs are synthesized and co-translationally inserted into the ER, before its oligomerization in connexons and migration to the PM, via the secretory pathway. GJIC is accomplished by exchange of small molecules and ions between adjacent cells. Removal of GJ from the PM is achieved through the endocytic pathway, and Cxs targeted for degradation end up in the proteasome or in the lysosome. (Adapted from Laird, et al. 2010)

1.2.1. Transcriptional regulation of human Cx43

At the transcriptional level, several transcription factors have been involved in the regulation of Cx43 expression, in a cell-independent manner, such as specificity protein 1 (Sp1) or adaptor protein 1 (AP-1), and cell-dependent ones, such as cardiac-specific Nkk2.5, T-box 5 (Tbx5) or GATA4 [10].

More than one binding site for AP-1 can be found in the Cx43 promoter. It has been reported that transcription of the human Cx43 gene is induced through protein kinase C (PKC) activation in uterine smooth muscle cells, which can be of a great physiological impact to the muscle contraction of the uterus, namely during labor [11]. Consistently, some hormones and growth factors have been pointed to have effects upon Cx43 expression through AP-1–dependent mechanisms. Examples of these are the downregulation of Cx43 upon progesterone stimulation in the uterus or, conversely, its upregulation by insulin growth factor 1 (IGF-1) and angiotensin II, upon stenosis of the saphenous vein [12, 13]. In cardiac myocytes, activation of the Wnt-signaling pathway and dibutyryl-cAMP were shown to induce Cx43 protein and RNA expression [10].

Besides, in the past few years it become apparent that epigenetic alterations are involved in the regulation of Cx43 expression. Thus, the role of histone modifications and DNA methylation were established as key regulators, and more recently, several microRNAs have been related to control of Cx43 expression [14].

1.2.2 Channel gating and trafficking of Cx43

Channel gating can be achieved through structural rearrangement of the hexamers, as a consequence of Cx43 conformational changes induced by PTM and/or intra- and intermolecular interactions. Several models have been proposed to explain channel gating based on conformational changes of Cx43. For instance, the Cx43NT is responsible for regulating channel gating and permeability. Indeed, mutations in the NT are causal with various pathologies, such as oculodentodigital dysplasia (ODDD), a developmental disease that affects eyes, teeth, limbs and causes cardiac arrhythmia [15, 16]. Also, the interaction between CT and CL domains of Cx43 can account for pH-dependent channel closure. Interestingly, based on this mechanism, a novel hemichannel inhibitor, Gap19, was recently described. Gap19 mimics the Cx43CL and competes for the interaction with the CT, thus impeding the CT-CL interaction, ultimately reducing hemichannel opening. Since this effect is specific upon inhibition of hemichannels, GJ remain fully functional, which can constitute a valuable tool in some pathologies associated with hemichannel impairment [17].

Trafficking of Cx43 to the PM can be modulated through interaction of several proteins with Cx43, mostly through Cx43CT. Altered trafficking may affect the number of channels present at the PM and, consequently, the level of GJIC. Thus, interaction of Cx43 with cytoskeleton elements, such as microtubules and actin filaments, as well as junctional proteins, namely cadherins and occludins, and with scaffolding proteins, such as zonula occludens-1 (ZO-1), is of utmost importance for regulation of Cx43 stability at the PM [2, 3]. Many of the Cx43-binding partners are recruited to GJ and interact with Cx43 for regulation of GJ function. Therefore, binding can only occur at certain stages of GJ life cycle, and consequently a precise regulation of this process is crucial. PTM of Cx43 assume a preponderant role upon this tight regulation [3].

1.2.2.1 Phosphorylation of Cx43

In general, phosphorylation results in alterations of protein conformation, hydrophobicity or charge that may ultimately modify its normal function. Cxs have multiple phosphorylation sites, either in serines (Ser, S) or tyrosines (Tyr, Y), being its life cycle affected by covalent ligation of phosphate groups. Indeed, phosphorylation of Cx43 has been implicated in several cellular processes, such as regulation of its intracellular traffic, assembly, turnover and interactome [18-20]. Although several kinases have been described to phosphorylate Cx43, depending on the kinase involved and the phosphorylated residue, the consequences regarding Cx43 distribution, turnover, channel conductivity and GJIC may vary.

Regarding channel gating, the kinases involved are cyclin-dependent kinase 2 (CDC2), mitogen-activated protein kinases (MAPKs) and PKC. All of them correlate with inhibited GJIC, and indeed, phosphorylation of S368 by PKC leads to channel closure and reduced half-life of Cx43. Conversely, protein kinase cAMP-dependent (PKA) and casein kinase 1 (CK1) regulate GJIC by allowing the assembly and

docking of new GJ, whereas protein kinase B (Akt) seems to be responsible for the regulation of the traffic and stability of gap junctional-Cx43 [18]. Phosphorylation of S373 by Akt is responsible for mediating the interaction between Cx43 and ZO-1, stabilizing Cx43 at the PM.

Additionally, phosphorylation of Cx43 can induce its further ubiquitination. Indeed, it is well established that epidermal growth factor (EGF) induces hyperphosphorylation of Cx43, which increases its ubiquitination and subsequent internalization and degradation [21]. Recently, it was also shown that activation of PKC, with concomitant phosphorylation of Cx43 on S368, leads to subsequent ubiquitination and degradation of the protein by the proteasome [22].

1.2.2.2 Ubiquitination of Cx43

The attachment of ubiquitin (Ub) moieties to a certain protein/substrate (ubiquitination) is a PTM that was initially described as a tag for protein degradation and thus, a modulator of protein longevity. However, more recent studies have provided evidence that besides degradation, ubiquitination is involved in many other cellular processes, namely in the modulation of protein activity and regulation of protein localization. Indeed, more than a signal for protein degradation, ubiquitination should also be seen as a modulator of protein-protein interaction [23].

Ultimately, ubiquitination consists in the attachment of a polyUb chain to the ε-NH_2 group of a lysine (Lys, K) residue on the target protein. The monomeric unit of such chain is Ub, a 76 amino-acid protein, ubiquitously distributed in all tissues of eukaryotic organisms. The cascade of Ub-conjugation comprises a three-step reaction, which is catalyzed by three key enzyme groups – Ub-activating enzymes (E1), which activate Ub, allowing its transfer to one of the Ub-conjugating enzymes (E2). Subsequently, E2 associates to an Ub-protein ligase (E3) to perform the ultimate covalent bonding between Ub and the substrate. The interaction E2-E3 is also required during the polyUb chain assembly, when it is needed as a specific degradation signal [24]. Different types of polyUb chains can be formed depending on the Lys residues involved in Ub-Ub binding required for chain assembly. Lysine 48 (K48) polyUb chains are the canonical signals recognized by the proteasome, however it is known that the proteasome can also recognize linear ubiquitinated substrates, as well as heterologous chains of Ub/SUMO and monoubiquitinated proteins [25, 26]. K63 linked chains are otherwise signals that can lead to endocytosis of its target proteins. Editing of Ub chains can be regulated not only by the attachment of Ub moieties, but also by its cleavage, catalyzed by deubiquitinating enzymes (DUBs). Recent reports suggest that ubiquitinating and DUB activities compete along the endocytic pathway and that different DUBs may have distinct roles, either promoting protein degradation or allowing recycling [27]. Therefore, the Ub chain attached to protein substrates depends upon the balance and orchestrated action of both E3 ligases and DUBs.

A pioneer study published by Laing, almost 20 years ago, provided the first evidences that Ub has a major role in controlling the life cycle of Cx43. In this study, the authors used a CHO-ts20 cell line, with depleted E1 function to show that, in these conditions, an accumulation of Cx43 occurs, as well as an enlargement of Cx43-containing GJ plaques. This data suggests that Ub is a needed signal for degradation of Cx43 [28]. Later, some other groups contributed to elucidate the pathways and molecular partners involved in Cx43 ubiquitination, and phosphorylation events arise as important regulators. For example, EGF and tumor-promoting phoebe ester 12-O-tetradecanoylphorbol-13-acetate (TPA), both known as activators of MAPK signaling, have been shown to contribute to increased Cx43 ubiquitination, internalization, and degradation [23, 29].

Neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) was the first E3 ligase described to be responsible for Cx43 ubiquitination and additionally, it was shown that Nedd4 is required for internalization and degradation of Cx43 [30, 31]. After this discovery, other two Ub-ligases were pointed to interact and ubiquitinating Cx43 - tripartite motif-containing protein 21 (TRIM21) and WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) [32]. More recently, it was demonstrated that sad ubiquitination regulatory factor 2 (Smurf2) is recruited to Cx43 GJ in response to TPA exposure, thus contributing to GJIC downregulation through enhanced endocytosis of Cx43 [32].

To perform its signaling function, Ub moieties attached to their substrates have to be recognized by Ub adaptors that “decode” the signal and trigger a subsequent downstream response. Regarding Ub chain topology, as for other proteins, it is likely that the attachment of different polyUb chains to Cx43 directs it towards different pathways, which can be determined by interaction with different adaptor proteins. It was previously shown that ubiquitinated Cx43 can be recognized by the endocytic adaptor epidermal growth factor substrate 15 (Eps15), which contains Ub-binding domains (UBD), and drives the internalization of Cx43 localized at the PM [33] (Figure 3). Besides, interaction of ubiquitinated Cx43 with the autophagy adaptor sequestosome-1 (p62/SQSTM1) has also been demonstrated as an important part of macroautophagy degradation of internalized Cx43 [34].

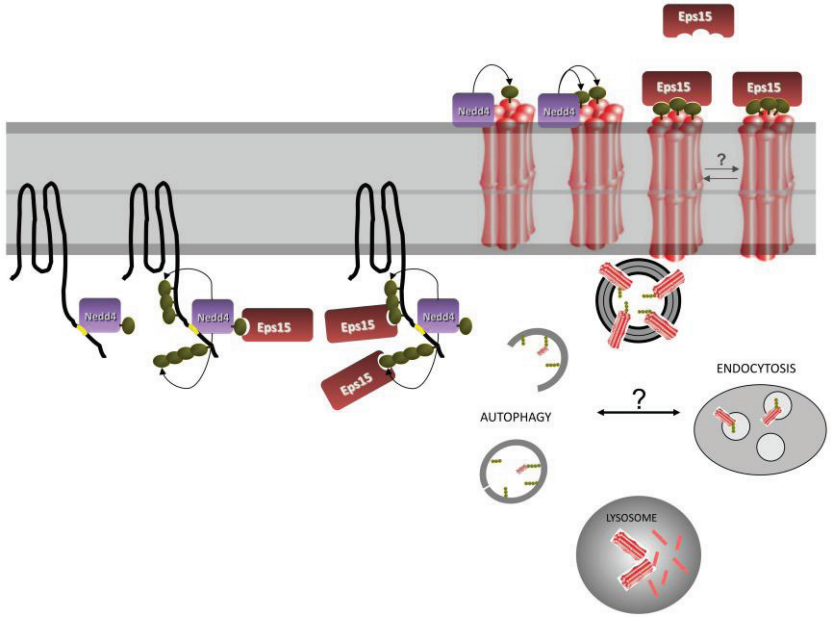

Figure 3 – Internalization of gap junctional Cx43 involves interaction with Nedd4 and Eps15. Ubiquitination of Cx43 is mediated by Nedd4 and ubiquitinated Cx43 is recognized by Eps15 that drives the internalization of the protein. Degradation of endocytosis Cx43 occurs in the lysosome, delivered either by the endocytosis or autophagy machinery.

1.2.3 GJ degradation

Although channel closure can regulate GJIC in a transient and reversible manner, a more accurate and definitive level of control involves degradation of Cx43. Indeed, the amount of Cx43 at the PM can have direct effect on the level of GJIC and in the activity of hemichannels. Thus, and given the high turnover of Cx43, its degradation rate has a preponderant impact at the level of GJIC. Along the years, several studies support the definition of three degradation pathways for Cxs, namely the proteasomal, endosomal and autophagosomal pathways.

1.2.3.1 Proteasomal degradation

Degradation by the proteasome is often associated with the protein quality control performed in the ER, being called ER-associated protein degradation (ERAD). The ER is the cell compartment responsible for the proper folding of newly synthesized proteins. Proteins that acquire their native state are retrotranslocated to the Golgi or the cytosol, being further addressed to their proper localization, whereas proteins unsuccessfully folded are labeled for degradation through ERAD [35]. In general, this process involves a complex network of ER-associated proteins that recognize the abnormal proteins and retrotranslocate them into the cytosol, where the ubiquitination cascade and proteasomal degradation take place [36]. This constitutes a protein quality control mechanism, ensuring that only proteins that acquire the correct folding are delivered to the cell surface through the secretory pathway.

In accordance, it was shown that proteasome inhibition results in the accumulation of Cx43, suggesting that ERAD is involved in its degradation [37]. Consistently, experiments where proper folding of

Cx43 is prevented chemically, with dithiothreitol (DTT), showed an enhanced Cx43 translocation from the ER to the cytosol and increased proteasomal degradation as a consequence [38, 39]. Although the interaction between Cx43 and the Ub-binding Cx43-interacting protein of 75kDa (CIP75) is required for the ERAD degradation of Cx43, in contrast with the other ERAD substrates, this process seems to be Ub-independent, since the interaction CIP75/Cx43 still occurs in Cx43 mutants with no Lys residues, thus not susceptible to be ubiquitinated [38] (Figure 4).

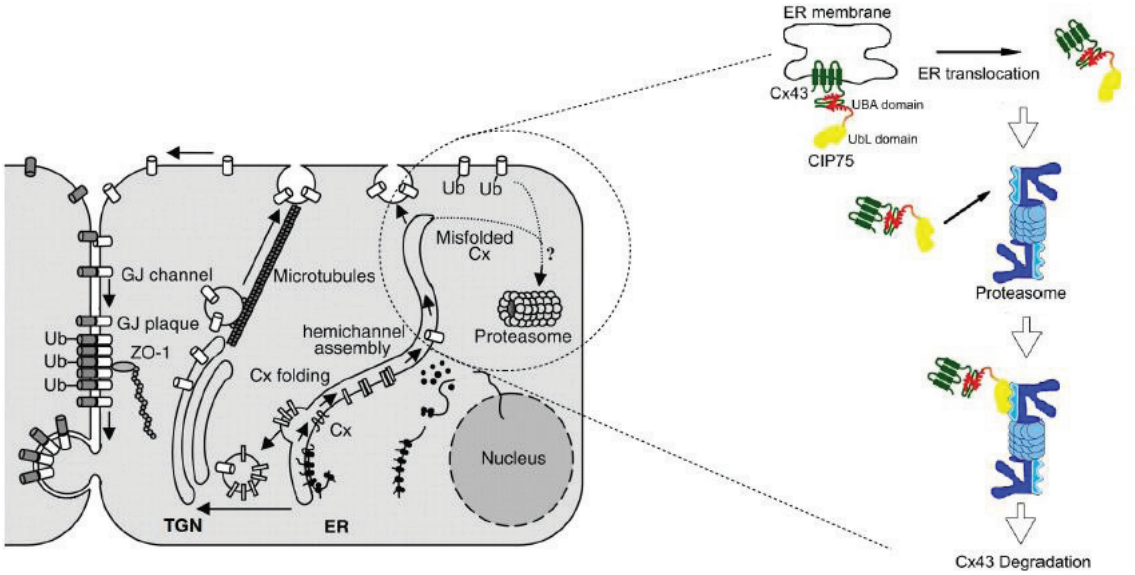

Figure 4 – Proteasomal degradation of Cx43 - a CIP75 dependent mechanism. Either ubiquitinated or non-ubiquitinated Cx43 can be targeted for proteasomal degradation, in the context of ERAD. CIP75 acts as a shuttle to transport misfolded and non-ubiquitinated Cx43 from the ER to the proteasome for degradation. CIP75 binds to the C-terminal tail of ER-localized Cx43 via the UBA domain, helping its translocation from the ER to the cytosol, and then transporting Cx43 to the proteasome. (Adapted from Sáez JC, et al. 2003 and from Su V, et al. 2010)

1.2.3.2 Degradation of endocytosis GJ – endo-lysosomal and autophagosomal pathways

The secretory pathway is responsible for the delivery of GJ to the PM whereas GJ removal is achieved by its endocytosis. However, the process whereby GJ are internalized is significantly different from those involved in the canonical internalization of PM proteins. Indeed, internalization of GJ, shared by two connected cells, comprises the engulfment of the two associated membranes from both cells into cytoplasmic double-membrane vesicles, termed annular GJ or connexosomes. Endocytosis of GJ involves the recruitment of clathrin, by the clathrin adaptor protein (AP-2) and disabled-2, and includes also the GTPase dynamin-2. Additionally, intact actin filaments and the actin retrograde motor myosin VI are required for the endocytosis of Cx43 [40, 41]. However, the inhibition of Cx43 internalization by filipin and methyl-β-cyclodextrin (MBC), as well as its interaction with caveolin suggests that endocytosis of GJ can also occur through caveolae [42].

Following internalization, the final destination of endocytosis gap junctional-Cx43 is the lysosome, a membrane-enclosed organelle containing acidic hydrolases and low internal pH, maintained by its

membrane proton pumps, which guarantee efficient degradation of the targeted proteins. It has been shown that, in cells treated with several lysosomal inhibitors, such as ammonium chloride, chloroquine (CQ) or Bafilomycin A1 (Baf), total levels of Cx43 increase and a significant part of this Cx is accumulated in lysosomes [43]. Still, there is evidence that internalized Cx43 can be recycled back to the PM, once it was verified that Cx43 co-localizes and interacts with Rab11 [44].

As described above, ubiquitination of Cx43 localized at the PM is required to recruit the endocytic protein Eps15 that promotes the removal of GJ from the PM. Accordingly, results from our lab show that either the depletion of Eps15 or the overexpression of a mutated form of Eps15 that does not bind Ub, result in an accumulation of Cx43 at the PM [34]. Recent studies carried out by Leithe et al. demonstrated that UBD-containing proteins of the endocytic machinery, such as the hepatocyte growth-factor regulated tyrosine kinase substrate (Hrs) and the tumor susceptibility gene 101 (Tsg101), that mediate the endosomal sorting complex required for transport (ESCRT)–dependent trafficking of proteins, are responsible for directing Cx43 towards lysosomal degradation, preventing its recycling [33, 45]. Accordingly, siRNA-depletion of Hrs and Tsg101 results in accumulation of a phosphorylated and ubiquitinated subpopulation of Cx43 in early endosomes, supporting the identification of Hrs and Tsg101 as crucial regulators of trafficking of Cx43 to lysosome [23].

Besides endocytosis, macroautophagy is another degradation pathway which ultimately leads to lysosomal degradation of its substrates. Autophagy is a catabolic process whereby damaged/obsolete long-lived proteins and organelles are degraded engulfed by a double-membrane vesicle, the autophagosome, that expands and fuses with the lysosome where degradation of the sequestered material takes place [46]. The core macroautophagy (thereafter referred as autophagy) machinery, composed mainly by autophagy related proteins (Atg), can be divided into four functional subgroups, according to the different stages of the autophagic process: i) the induction of autophagosome formation, mediated by the mammalian homolog of the yeast autophagy-related gene 1 (Atg1)/UNC-51-like kinase 1 (ULK1) complex (Atg1, Atg11, Atg13, Atg17, Atg29 and Atg31), ii) the membrane delivery to the expanding phagophore, by Atg9 and its cycling system (Atg2, Atg9 and Atg18), iii) the vesicle nucleation, mediated by class III phosphatidylinositol 3-kinase (PI3K-III) complex (Vps34, Vps15, Vps30/Atg6/Beclin 1, and Atg14) and finally iv) the vesicle expansion, by the concerted action of two Ub-like conjugation systems: the Atg12 (Atg5, Atg7, Atg10, Atg12 and Atg16) and Atg8 (Atg3, Atg4, Atg7 and Atg8) [46, 47].

Autophagy can be activated by multiple stressors, namely nutrient deprivation, oxidative stress or hypoxia. In this context, autophagy constitutes a survival mechanism, by the replenishment of the intracellular pool of amino acids, ensuring energy demand when nutrients are scarce. Additionally, autophagy can also be part of a process of protein quality control, by selectively degrading unwanted damaged or obsolete cellular components [48, 49].

Cellular energy levels are strikingly related with the autophagy machinery by the means of three major energy-sensing pathways – Akt, adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) [50]. Oxygen or nutrient deprivation can activate AMPK through ADP:AMP accumulation, which negatively regulates mTOR, either by AMPK-mediated phosphorylation of mTOR, or by activation of its upstream repressor tuberous sclerosis complex (TSC). Also, limited oxygen upregulates hypoxia-responsive genes, which are capable of suppressing mTOR signaling. Similarly, amino acid withdrawal results in the inactivation of mTOR. Conversely, Akt mediates autophagy inhibition under conditions of abundance of nutrients and growth factors [51].

Either AMPK and mTOR can lead to phosphorylation of the ULK1 complex, and these three proteins have been described to take part of the same feedback loop [46]. The activity of the ULK1 kinase is therefore needed for the recruitment of Vps34 to the phagophore. Hence, Vps34 complex, containing Vps15, Beclin 1, and Atg14, is specifically recruited to the phagophore to phosphorylate phosphatidylinositol (PI), producing PI(3)P. Differential interaction of the core proteins of PI3K-III, mainly the Beclin 1 interactome, can either upregulate or downregulate autophagy, a dynamic strictly dependent of the nutrient signaling [50].

At the stage of phagophore elongation, the lipidation of the cytosolic microtubule-associated protein 1 light chain 3 (Atg8/LC3) – LC3-I occurs with subsequent formation of LC3-II that translocates to the autophagosome membrane. The recruitment of LC3-II to the autophagosome constitutes a reliable indicator of autophagic activity, however, it can only be used as a complementary tool to measure autophagic flux. Figure 5 illustrates the autophagic process and the molecular players involved in the key steps [52].

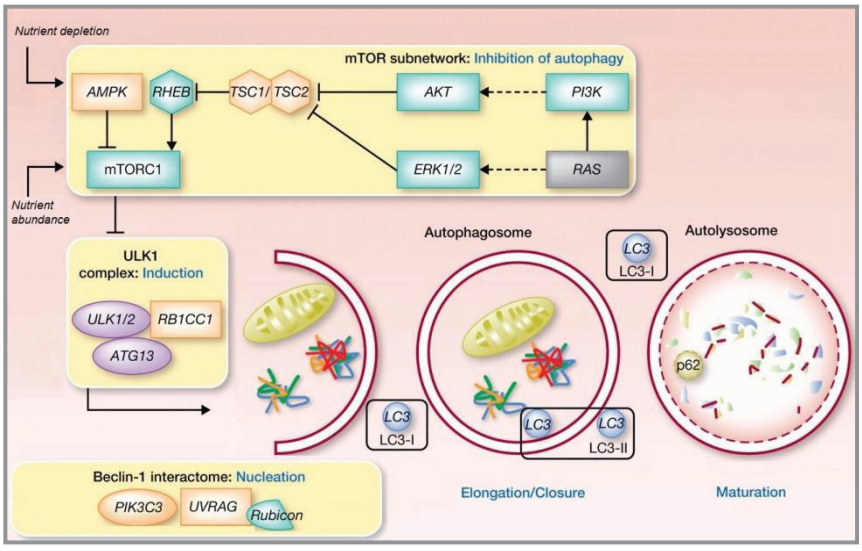

Figure 5 – The autophagy pathway and machinery. Nutrient limitation, cellular stress and reactive oxygen species (ROS) are some of the activating factors of autophagy. AMPK and mTOR signaling regulate autophagy triggering, and subsequent downstream effects of induction, nucleation and elongation of autophagic vesicles, driven by the autophagy core machinery. Lipidation of cytosolic LC3-I into LC3-II with its concomitant recruitment to the autophagosome is one of the hallmarks of autophagic flux. Autophagosomes sequester target substrates for degradation, upon its fusion with the lysosomes. Solid arrows: direct interaction, dashed arrow: indirect interaction,

orange fill: positive autophagy regulators, teal fill: negative regulators, gray fill: both positive and negative regulators. (Adapted from Gorski, et al. 2012)

Furthermore, one of the designed functions of autophagy is the clearance of polyubiquitinated substrates via interaction with p62, an Ub-binding protein that is simultaneously a substrate of autophagy. Besides the UBD that recognizes the Ub attached to the substrate, p62 contains a LC3-interacting region (LIR) which allows its binding to LC3, thus acting as a bridge between ubiquitinated substrates and autophagy machinery, driving ubiquitinated cargos to degradation through autophagy [53, 54]. Other autophagy adaptors have also been described, such as neighbor of BRCA1 (NBR1), which is structurally very similar to p62. Consistently, NBR1 contains an UBD and a LIR domain, and it has been described to participate in the selective autophagic degradation of peroxisomes [55]. In this context, as in many others, it plays a co-operative role along with p62 in the degradation of autophagy substrates [56].

Additionally, ubiquitination plays an essential role on the regulation of autophagy, through the control of activity, recruitment, and turnover of autophagy components [56, 57]. Consistently, several E3 ligases have been associated with autophagy regulation, and besides their activity-dependent role upon ubiquitination and regulation of autophagy machinery stability, E3 ligases can also have activity-independent roles, by facilitating the recruitment of autophagy adaptors, namely p62 and NBR1 [58]. Indeed, sad ubiquitination regulatory factor 1 (Smurf1) was recently identified to take part of selective virophagy and mitophagy, in a tissue-specific manner and independently of its E3 ligase activity [59, 60]. Also, Nedd4 has recognized functions upon ubiquitination of Beclin 1, which can regulate both its proteasomal degradation and interaction with the PI3K-III catalytic subunit Vps34 [61]. Regulation of autophagy by the ubiquitination machinery involves also the concerted activity of DUBs. For instance, it was shown that Ub protease Ubp3p/Bre5p participates in the selective autophagic degradation of ribosomes in yeast [62]. However, to the date, little is known about the role of DUBs in autophagy.

Recently, studies carried out in our lab demonstrated that internalized GJ may also be degraded through autophagy. Enclosure of Cx43 in vesicles containing the autophagy-related protein LC3, co-localization with p62 and accumulation of Cx43 upon Atg5 and Atg7 knockdown were the first evidences to that assumption [34, 63]. Strikingly, we demonstrate that Nedd4-mediated ubiquitination of Cx43 is required for targeting of GJ towards autophagy degradation, and that the endocytic adaptor Eps15 is recruited to the PM upon autophagy induction and is needed for further autophagic degradation of Cx43. Interaction between Cx43 and p62 is also seen, but apparently, Eps15 and p62 did not form part of the same complex, being interaction with p62 instead a post-internalization event [34].

1.3. Cx43 remodeling in the ischemic heart

Normal heart function relies on correct electrical and metabolic coupling between cardiomyocytes, which are accomplished by a proper GJIC. Several members of the Cx family are expressed in the human heart, namely Cx40, Cx43 and Cx45, which show a differential distribution along the heart tissue. While Cx43 is expressed in the atria, the ventricles, His bundle, and in the distal Purkinje system, Cx45 expression is restricted to the sino-atrial node (SAN), atrio-ventricular node (AVN), and bundle branches [64]. A schematic of Cx40, Cx43 and Cx45 expression in the heart is present on Figure 6.

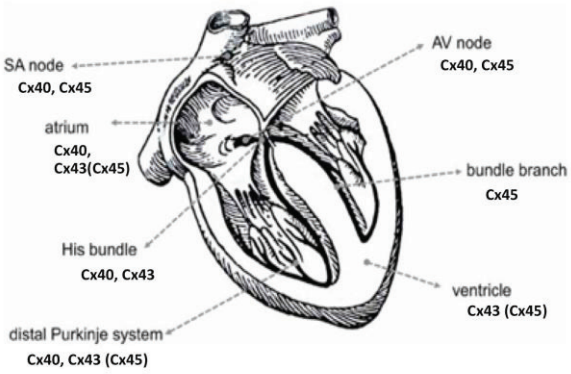

Figure 6 - Distribution of Cxs in the human heart. Distribution of Cx40, Cx43 and Cx45 in the adult human heart. Different regions of the heart show specific profiles of Cx expression, which reflects its differential requirements upon the degree of electrical coupling. (Cx) indicates relatively small amounts. (Adapted from Verheule S, et al. 2013)

Cx43 is the major GJ protein found in the ventricular muscle of the heart, being localized mainly at the longitudinal termini of the cardiomyocytes – the intercalated discs (IDs) – where it ensures the correct anisotropic impulse propagation, required for a synchronized and coordinated heart beating [65]. In cardiomyocytes, Cx43 can also be present in mitochondria, where it has been implicated in enhanced ischemic preconditioning response. Accordingly, some authors support that during stress conditions, as occurs in myocardial ischemia (MI), the levels of mitochondrial Cx43 raise, which could contribute to keep the mitochondrial permeability transition pore in a closed state, delaying the release of apoptotic proteins and cytochrome c, thus reducing ischemia/reperfusion (I/R) injury [66-68].

The role of Cx43 in electric conduction in the heart, either in physiological or pathological conditions, has been studied for years, through the use of different models [69]. $Cx43^{+/-}$ mice show less expression of the total Cx43 levels (about 50%) resulting in a comparable reduction of Cx43-based GJ, which was associated with decreased conduction velocity [70]. However, in Cx43 conditional knockout mice, total ablation of cardiac Cx43 expression besides deceleration of ventricular conduction velocity, results in develop of ventricular arrhythmias. Altogether, these studies suggest that GJ coupling has to be reduced to very low levels to impair impulse conduction and lead to arrhythmogenesis [71, 72].

Unsurprisingly, dysfunction of GJ regulation and impairment of GJIC have been associated with several cardiomyopathies, such as heart failure and MI. Indeed, it has been consistently shown that Cx43 remodeling associated with cardiac malfunction involves protein dephosphorylation, that likely contributes to channel closure, and subcellular redistribution, in which Cx43 is removed from the IDs and is further degraded or lateralized, thus contributing to the impairment of impulse propagation [70, 73].

MI, a condition characterized by the reduction of blood supply to the heart muscle, mainly as a result of a coronary artery dysfunction, is the most common type of heart disease and one of the major causes of death worldwide. Deprivation of oxygen and nutrients associated with MI induces pronounced metabolic changes in the heart, which include an abrupt interruption of oxidative phosphorylation and creatine phosphate and ATP-depletion. In addition to an energetic imbalance, MI also leads to decrease of intracellular pH, as well as accumulation of lactic acid, reactive oxygen species (ROS) and other damaging metabolites, alterations that compromise cell integrity and function [74, 75]. Reperfusion, the restoration of blood supply to the myocardium after ischemia, can either limit the extent of the injury, or be even more harmful, depending on its timing. Whereas after mild ischemia, reperfusion tends to be a period of cell recovery and re-adaptation after injury, reperfusion following severe ischemia tends to exacerbate the process, accentuating its harmful effects.

Hemichannels, the biogenetic precursors of GJ, which in physiological conditions control cardiomyocyte volume, can also undergo pathological remodeling during MI. There is extensive evidence that during ischemia, occurs hemichannel opening and, as a consequence, ATP release, which exacerbates the ATP-depleted state of ischemic cardiomyocytes [76, 77]. Therefore, it has been suggested that inhibition of hemichannels can be protective against ischemic-induced injury. Indeed, administration of Gap19, a selective hemichannel inhibitor, prevented hemichannel opening, which led to cardiomyocyte protection against volume overload and cell death following I/R, both *in vitro* and *in vivo* [17].

Oxygen and nutrient-deprivation, characteristics of ischemia, lead to overall loss of kinases activity, which result in severe alterations on the phosphorylation profile of cardiac proteins. Regarding Cx43, while it is mainly phosphorylated in physiological conditions, during MI Cx43 undergoes dephosphorylation, with its concomitant translocation to the lateral sarcolemma. Hence, regulation of the phosphorylation state of Cx43 is particularly important in the heart, where intercellular communication has to be strictly controlled. Although in several pathological conditions, namely during MI, defects in electrical coupling and impulse propagation can lead to cardiac malfunction, a reduction in metabolic coupling can be considered beneficial to prevent propagation of harmful signals, and to restrict the damage to a small area of the heart. Consistently, dephosphorylation of the S325, S328, and S330 is promptly induced after ischemia, and is observed on the population of Cx43 that undergoes ischemia-induced lateralization [70, 78]. Ischemic preconditioning, characterized by brief episodes of ischemia and reperfusion preceding a period of sustained reperfusion, promotes PKC activation, which results in increased phospho-S262- and phospho-

S368-Cx43. Phosphorylation of both residues has been correlated with the ability of cardiomyocytes to resist to ischemic injury, and with prevention of Cx43 lateralization [45]. Additionally, ischemia also leads to a rapid dephosphorylation of S365, a PKA target. Since the dephosphorylation of S365 is required for subsequent S368 phosphorylation, this residue has been referred as the GJIC ‘‘gatekeeper” [79].

It is conceivable that ischemia-induced phosphorylation/dephosphorylation events that lead to a redistribution of Cx43 and disruption of electrical and chemical GJIC have a protective purpose upon injury, preventing lesion spreading. However, while the loss of electrical GJIC occurs after a few minutes of ischemia, the metabolic coupling is sustained for a substantial period of time, possibly allowing the propagation of ischemic injury. It has been suggested that dephosphorylation of Cx43 is the initial step in GJ internalization in the ischemic heart, however little is known about the fate of internalized GJ (Figure 7) [70, 80]. Accordingly, it was recently published that phosphorylation of S373 by Akt activates a 14-3-3 mode-1 binding motif on Cx43, which can drive the protein through internalization during acute cardiac ischemia [81]. Consistently, enhanced proteolysis of Cx43 has been implicated in ischemia-induced cardiac dysfunction, and several studies, both with different animal models and patients with cardiovascular disease, were essential to the understanding of such GJ remodeling and degradation. Indeed, when normal, hypertrophic and ischemic hearts were compared, total amount of Cx43, in the left ventricle, was reduced by 40% in diseased hearts [82]. Also, a 30% to 40% reduction of GJ area per ID is observed in ischemic ventricles, being this reduction limited to a few cell layers around the affected area [83].

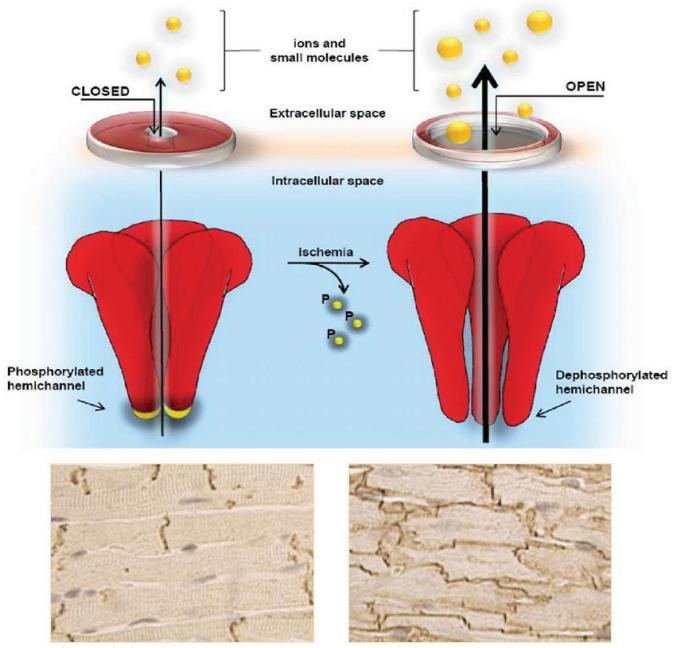

Figure 7 – GJ remodeling and Cx43 dephosphorylation are features of the ischemic heart. Cx43 is mainly phosphorylated in physiological conditions, in the heart, and localizes at the IDs (left panel). In the ischemic heart, Cx43 is dephosphorylated and further lateralyzed. Additionally, hemichannel opening occurs during ischemia (right panel). (Adapted from Lampe PD et al. 2006)

Despite all the efforts of the scientific community devoted to this field, the molecular mechanisms and machinery involved in ischemia-associated degradation of Cx43 remain elusive. As reviewed above, degradation of Cx43 can occur in the proteasome, mainly in the context of ERAD, or in the lysosome, as part of autophagy and/or endocytic pathways [34, 41]. Studies carried out in our lab demonstrated that Nedd4-mediated ubiquitination of Cx43 acts as a triggering signal for the internalization and degradation of GJ [30, 33]. Furthermore, we showed that ubiquitination of Cx43 is required for starvation-induced degradation of GJ by autophagy, through a mechanism that involves the recruitment of the autophagy adaptor p62. Even though autophagic degradation of Cx43 has already been established, the pathophysiological impact of this finding, namely in the context of MI, is still unknown.

Although autophagy occurs constitutively in the normal myocardium, it is upregulated in response to stress stimuli, such as ischemia. While in basal conditions it is well established that autophagy is vital to the maintenance of protein quality control, the consequences of its upregulation in ischemia have been a matter of intense debate. Indeed, studies with mice showed that 30 minutes of ischemia lead to activation of autophagy, and its inhibition upon treatment with 3-methyladenine (3-MA) or Baf increases cardiomyocyte death, showing that ischemia-induced autophagy may have a protective role [84]. It is thought that autophagy-mediated protection can be due to the consequent downregulation of apoptosis. In these conditions, autophagosomes might help the clearance of damaged mitochondria, preventing the release of cytochrome c and inhibiting the formation of apoptotic bodies. Conversely, Matsui et al. demonstrated that inhibition of autophagy, by heart-specific knockdown of Beclin 1, results in smaller infarct sizes and less apoptotic cell death in mice subject to cardiac I/R, suggesting that autophagy plays a detrimental role under these circumstances [85].

Regardless, the role of Beclin 1 in cardiac autophagy, namely during I/R, constitutes another important controversial axis, mainly because Beclin 1 interactome can be extensive and modulated by different stimuli [86]. Whereas some authors, demonstrated that, upon I/R, knockdown of Beclin 1 results in increased apoptosis [84], others showed that cardiac-specific heterozygotic Beclin $1^{+/-}$ mice are more resistant to I/R injury [87]. Nevertheless, it is known that Beclin 1 besides interaction with Vps34 interacts also with Rubicon, which slows down autophagosome-lysosome fusion. For that reason, Beclin $1^{+/-}$ mice may be more resistant to I/R injury because they clear autophagosomes more efficiently through enhanced lysosomal fusion, instead of less formation of vesicles, as can be initially assumed [85, 87]. In fact, it is important to notice that impaired clearance of autophagosomes, which is called frustrated autophagy, can constitute one of the worst-case scenarios. Indeed, stopping the autophagic flux may lead to exosome secretion, accompanied by release of metalloproteinases and activation of inflammation, which could promote cardiac rupture [88].

These observations highlight the importance of unraveling the molecular partners of the main autophagy mediators all the way through ischemia and I/R. The initiators mTOR and AMPK, and Beclin 1, as

the main trigger of downstream events, are the well-studied members of the autophagy machinery under I/R. However, more recently, it was also reported that alterations on the phosphorylation state of glycogen synthase kinase 3β (GSK-3β) modulate autophagy in a phase-dependent manner, either in ischemia and I/R. Dephosphorylation of GSK-3β at S9 leads to activation of autophagy, however protective roles of GSK-3β also include increased glucose availability for glycolysis during acute ischemia. Additionally, during I/R, phosphorylation of GSK-3β can also have impact upon inhibition of apoptosis and mitochondrial permeability transition pore.

In order to conciliate all these apparently contradictory observations, it has been proposed that the dichotomous role of autophagy likely depends on the nature, extent and severity of the stimuli, as well as on the autophagy players involved. Thus, a cardioprotective role of ischemia-induced autophagy has been attributed to AMPK, a ubiquitous sensor of cellular energy status that promotes autophagy upon ATP depletion, whereas detrimental autophagy has been associated with up-regulation of Beclin 1 [86, 88].

2.Objectives

GJIC is essential for the maintenance of a normal heart function. Exchange of second messengers, ions and small molecules between adjacent cardiomyocytes ensures efficient electric activation and action potential propagation, resulting in coordinated contraction. Therefore, dysfunction of GJ regulation has been associated with conduction block and arrhythmogenesis under several pathological conditions, such as MI and cardiac hypertrophy. The regulation of GJIC is rather complex and is likely to involve events such as channel closure, changes in intercellular trafficking and degradation of Cx43. Remodeling of cardiomyocyte GJ at the onset of MI has been extensively reported, and includes Cx43 dephosphorylation, removal from the IDs and redistribution to the lateral sarcolemmal membranes. Albeit its importance and all the efforts to elucidate the mechanisms associated with this GJ remodeling, the molecular players and pathways involved are still obscure. Therefore, a main goal of this work is to elucidate the molecular mechanisms underlying the ischemia-induced degradation of Cx43.

Studies carried out in our group have demonstrated that Nedd4-mediated ubiquitination of Cx43 downregulates GJIC, by promoting the Eps15-mediated internalization of Cx43-containing channels. Furthermore, we showed that activation of autophagy, by starvation, leads to degradation of Cx43, through a mechanism that requires its ubiquitination and the recruitment of the autophagy adaptor p62. Since autophagy is known to be promptly induced in the ischemic heart, in response to oxygen and nutrient-deprivation, we hypothesized that degradation of Cx43 under these circumstances occurs, at least in part, by autophagy. With this study we intend to establish the molecular players that assist in autophagic degradation of Cx43 in ischemic cardiomyocytes.

Although autophagy is required to maintain protein quality control under basal conditions, mediating the degradation of abnormal or obsolete proteins and organelles, the consequences of its upregulation in response to stimuli, such as ischemia, have been a matter of intense debate in the scientific community. Indeed, if for one hand, activation of autophagy can constitute a pro-survival response, ensuring energy replenishment and removal of damaged proteins and organelles, it can also be harmful leading to cell death. In an attempt to conciliate these apparent contradictory effects, it has been suggested that the dual role of autophagy in ischemia relies on the nature, extent and severity of the stimuli, as well as on the autophagy players involved. The prevalent theory stands for a cardioprotective role of ischemia-induced autophagy via AMPK, being up-regulation of Beclin 1 the proposed molecular hallmark of exacerbated autophagy and cell death. Therefore, another main objective of this study is to unravel the players and signaling pathways underlying Cx43 degradation. In particular, we will evaluate the involvement of AMPK and Beclin 1 in the degradation of Cx43 in cardiomyocytes subjected to either ischemia or reperfusion.

Altogether, this study aims to unravel the molecular mechanisms responsible for the ischemia-induced degradation of Cx43, which can contribute to the identification of direct targets for heart protection. By understanding the molecular players involved, it might be possible to envision new pharmacological strategies that can revert pathological GJ remodeling, in order to preserve GJIC in MI.

3. Material and Methods

3.1 Chemicals

All chemicals not otherwise stated were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dorsomorphin dihydrochloride (DH) was from Tocris Bioscience, Bafilomycin A1 (Baf) from Bioaustralis and PYR-41 and MG-132 from Calbiochem.

3.2 Cell culture and treatments

The atrial cardiomyocyte cell line, HL-1, [89] was cultured in gelatin/fibronectin (0.02% gelatin/0.1% fibronectin) coated culture vessels and maintained in Claycomb medium (Sigma-Aldrich, St. Louis, MO) supplemented with 0.1 mM of Norepinephrine, 2 mM L-Glutamine, 10% fetal bovine serum (FBS) and Penicillin/Streptomycin (100 U/mL:100 μg/mL), at 37ºC under 5% CO_2.

The mouse embryo fibroblast cell line, NIH3T3, was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA), supplemented with 10% FBS, 1% Penicillin/Streptomycin (100 U/mL:100 μg/mL) and 1% GlutaMAX (Life Technologies, Carlsbad, CA), at 37ºC under 5% CO_2.

When indicated, chemical ischemia was induced by a buffer exchange to an ischemia-mimetic solution (in mM: 137 NaCl, 5.4 KCl, 1.0 $MgSO_4$, 1.0 $CaCl_2$, 5 Na-HEPES, 20 taurine, 5 creatine, 5 sodium pyruvate, 1 KCN, 20 2-deoxy-D-glucose, pH 7.4). Metabolic ischemia was induced by a buffer exchange to an ischemia-mimetic solution (in mM: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 5 calcium lactate, 20 2-deoxy-D-glucose, 20 Na-HEPES, pH 6.6) and by placing the dishes in hypoxic pouches (GasPakTM EZ, BD Biosciences), equilibrated with 95% N_2/5% CO_2. After the indicated times of ischemia, reperfusion was induced by a buffer-exchange to complete Claycomb Medium and normoxia conditions.

Lysosome-dependent degradation was inhibited by the addition of 50 nM Baf and macroautophagy-dependent degradation by the addition of 5 mM 3-MA [90], or 50 nM Okadaic Acid (OA) [91]. 80 μM DH was added to inhibit AMPK-dependent processes [92], and 10 μM PYR-41 to inhibit activity of E1 enzymes [93]. Proteasome inhibition was achieved by the use of 10 μM MG-132. Protein synthesis was inhibited by the addition of 50 μg/mL cycloheximide (CHX) [94].

3.3 Animal models

Wistar rats were obtained from our local breeding colony (Faculty of Medicine of University of Coimbra, Coimbra, Portugal). All animals received care in accordance with the Portuguese Law on Experimentation with Laboratory Animals (last amendment, 2004), which is based on the principles of laboratory animal care as adopted by the EC Directive 86/609/EEC for animal experiments.

3.3.1 Neonatal primary cultures of rat cardiomyocytes

Primary cell cultures were obtained from 3-day-old (P3) Wistar rats of both sexes, euthanized by decapitation. Hearts were removed and cardiac cells were isolated from the ventricles by enzymatic digestion with Trypsin/DNase, as previously described [95]. Briefly, after removing the hearts from P3 decapitated pups, the ventricles were separated from the atria, rinsed with phosphate-buffered saline (PBS) solution, and minced into 1-mm-wide pieces with scalpel blades. Sedimented tissues were then resuspended in a solution of Trypsin/DNase (0.08% Trypsin/13 µg/ml DNase I). After shaking the tissues for 10 minutes (min) at 37°C, dislodged cells were recovered by centrifugation, and the procedure was repeated, with 0.09% Trypsin/7 µg/ml DNase I, until all the tissue was fully digested. The products of all digestions were pooled and resuspended in DMEM, containing 10% FBS, and transferred through a screen (70 µm) into a non-coated flask. Incubation for 90 min, in a humidified incubator at 37ºC with 5% CO_2, was performed in order to eliminate non-cardiomyocyte cells. After that, the remaining non-attached cells, enriched with cardiomyocytes, were resuspended in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL) and 1% GlutaMAX, and plated at a density of $3{,}3x10^5$ cells/cm^2 on gelatin/fibronectin-coated (0.02% gelatin/0.1% fibronectin) plates and/or coverslips, resulting in a confluent, uniformly aligned cardiac cell monolayer. When indicated, chemical ischemia was induced by a buffer exchange to an ischemia-mimetic solution, as described before. After indicated times of ischemia, reperfusion was induced by buffer-exchange to complete DMEM medium.

3.3.2 Organotypic heart slice cultures

Organotypic heart slice cultures were obtained from postnatal day 4 (P4) – 7 (P7) Wistar rat pups of both sexes, as described previously [96]. Briefly, to establish organotypic heart cultures, rats were euthanized by decapitation, hearts were excised, the atria were removed, and the ventricles sagitally sliced (1mm thickness) by the use of a rodent heart matrix (Harvard Apparatus, UK). Heart slices were transferred into a semiporous membrane (Millicell-CM 0.4 µm membrane, Millipore, France), and inserts were placed in 6-well plates with 1 ml of DMEM, supplemented with 2 mM GlutaMAX, 10% FBS and Penicillin/Streptomycin (100 U/mL:100 µg/mL), at 37ºC and 5% CO_2. Cultures were maintained for one week prior to the performance of experiments. When indicated, ischemia was induced by a buffer exchange to an ischemia-mimetic solution, and/or by placing the dishes in hypoxic pouches equilibrated with 95% N_2/5% CO_2, as described before. After indicated times of ischemia, reperfusion was induced by a buffer-exchange to complete DMEM medium and/or normoxia conditions. Where indicated, 100 nM Baf and 10 mM 3-MA were used. Pre-treatment with the drugs were performed 2 hours (h) before ischemia induction. After the experiments, slices were either embedded in OCT (Tissue-Tek, Histolab, Sweden) for cryosectioning, or snap-frozen in liquid nitrogen for biochemical studies, before storage at -80°C.

3.3.3 Langendorff heart perfusion model

Hearts were isolated from 10- to 12-week-old Wistar rats (450 ± 25 g body weight, n=2-4 per condition). Animals were anesthetized with ketamine chloride (75 mg/kg, intraperitoneally (i.p.), Parke-Davis, Ann Arbor, MI, USA), and injected with heparin (50 IU i.p.). When indicated, CQ was administered to the animals (60 mg/kg of body weight, i.p.), 4 h before experiments. After cervical dislocation, a thoracotomy was performed and hearts were excised into ice-cold PBS. The aorta was cannulated and the coronary circulation maintained at 70-80 mmHg, at a constant flow rate of 15 ml/min, with a modified Krebs-Henseleit (KH) buffer (containing in mM: 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2P0_4, 10 HEPES, 1.25 $CaCl_2$ and 10 glucose, pH 7.4). Buffer was equilibrated with 95% O_2/5% CO_2 at 37ºC. Perfusion medium was passed through water-jacketed tubing and cylinders, and the temperature was maintained at 37°C with a temperature-controlled circulating water bath. Normal perfusion was maintained for 10 min, and hearts were then either perfused for further 20, 40, 100, 120 or 220 min, for control conditions, or exposed to no-flow ischemia for 20, 100, 120 or 220 min. After 20 or 100 min of no-flow ischemia, a period of 20, 100 or 200 min of further reperfusion was performed at the same flow rate used before ischemia. Electrocardiography (ECG) monitoring of the hearts was performed during perfusion protocols. Immediately after the Langendorff procedure, apical region of the heart was embedded in OCT, for cryosectioning, and the remaining tissue of the heart was snap-frozen in liquid nitrogen for biochemical studies, before storage at -80°C.0

3.4 Genetic manipulation of cell cultures (cell transfection, siRNA-mediated knockdown and transduction)

One day before transfection, HL-1 cells were seeded in gelatin/fibronectin coated 35 x 10mm plates so that the cells were 70% confluent at the time of transfection. For each transfection sample, 3.5 μg plasmid DNA were diluted in 50 μl Opti-MEM I Reduced Serum Media (Life Technologies, Carlsbad, CA). Subsequently, 7 μl Lipofectamine 2000 (Life Technologies, Carlsbad, CA) transfection reagent were diluted in 50 μl Opti-MEM medium and incubated for 5 min at room temperature. After 5 min of incubation, diluted DNA and Lipofectamine were combined and incubated together for an additional 20 min. After incubation time was completed, DNA/Lipofectamine complexes were added to the normal medium of the cells. The cells were incubated at 37 ºC, under 5% CO_2, for 48 h prior to the experiments performance. The cell culture medium was replaced after 24 h of DNA/Lipofectamine incubation.

Plasmids expressing mCherry-Cx43-Ub(AA) were generated by subcloning Cx43 and Ub (AA) into a pcDNAENTRBPmCherry vector, and plasmids expressing Cx43-Ub(AA) were generated by subcloning Cx43 and Ub (AA) into a pCMV7myc vector [30].

siRNA-mediated knockdown was achieved by transfecting twice at intervals of 24 h. One day before transfection, cells were seeded in 35 x 10mm plates so that the cells were 50% confluent at the time of

transfection. For each transfection sample, 45 pmol siRNA were diluted in 50 ml Opti-MEM medium, and 2 ml Lipofectamine 2000 transfection reagent were diluted in 50 μl Opti-MEM medium. Incubation proceeded as described above, and DNA/Lipofectamine complexes were added to the normal medium of the cells, incubated at 37ºC, under 5% CO_2, for 48 h prior to the experiments performance. The experiments were performed 24 h after the second transfection. siRNA targeting Beclin 1 (s80166 (GGUACCGACUUGUUCCCUAtt) or s80167 (ACCAUGCAAUGGUAGCUUUtt)), p62 (s71143 CCAAUGUCAAUUUCCUGAAtt) or s7144 (GGAACUCGCUAUAAGUGCAtt)), and Nedd4 (s9417 (GGCGAUUUGUAAACCGAAUtt)) and a non-targeting control sequence were obtained from Ambion, Life Technologies (Silencer Select Pre-designed siRNA).

Cell transduction of NIH3T3 was performed with lentivirus carrying a short hairpin RNA construct against Atg7 (5'-GACTGCAGTGCAGATGA-3' and 5'-AAGCACCATCATGCTGGATAT-3'), packed in 293T cells transfected by the calcium phosphate protocol. Supernatants containing viral particles were harvested after 72 h, and 1 ml of the supernatant was used for cell transduction after incubation in the presence of polybrene as described [34, 97].

3.5 Western blot (WB) analysis

After appropriate treatments, HL-1 cells or primary cardiomyocytes cultured in gelatin/fibronectin coated 35 x 10 mm plates were washed twice in ice-cold PBS, scraped off the dishes and lysed in 150 μl RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40 and 0.1% SDS, pH 7.5), containing protease inhibitors and phosphatase inhibitors (protease inhibitor cocktail (Roche, Basel, Switzerland), 2 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM iodoacetamide and 2 mM sodium ortovanadate). Cell lysates were incubated 15 min on ice, and the solubilized fraction was recovered in the supernatant after centrifugation at 3200 rpm for 5 min. Protein concentration was determined by the DC Protein Assay (BioRad, Hercules, CA, USA), after which 30 μg of the supernatants were denatured with 2x Laemmli buffer (total extract/input), and boiled at 95ºC for 5 min.

Tissue homogenates were prepared by homogenization in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 5mM EDTA, 1% NP-40 and 0.2% SDS, pH 7.5) supplemented with protease inhibitors, as described above. Homogenization was performed in a tissue grinder (Potter-Elvehjem PTFE Tissue Grinder, Corning Life Sciences, NY, USA), followed by brief sonication (3 pulses of 2'', 180 watts). Homogenates were incubated 30 min on ice, and then centrifuged at 3200 rpm for 5 min. After, supernatants were briefly sonicated, as previously, and subsequently centrifuged at 13200 rpm for 20 min. Determination of total protein was performed by the DC Protein Assay, after which 50 μg of the supernatants were denatured with 3x Laemmli buffer (total extract/input), boiled at 95ºC for 5 min, and sonicated.

Total cell lysates and tissue homogenates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T) (20mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 7.6), probed with appropriate primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. All antibodies used in this work are listed in Table 1. The proteins of interest were visualized by chemiluminescence using a VersaDoc system (BioRad). Densitometric quantification was performed in unsaturated images using ImageJ (National Institutes of Health, Bethesda, MD).

3.6 Immunoprecipitation (IP)

After appropriate treatments, HL-1 cells or primary cardiomyocytes cultured in gelatin/fibronectin coated 60 x 15 mm plates were washed twice in ice-cold PBS, scraped off the dishes and lysed in 400 µl RIPA buffer, as described above. The samples were centrifuged at 3200 rpm for 5 min, and 5% of the total amount of protein in the lysates was denatured (inputs), being the remaining lysates used for IP. Briefly, supernatants were incubated with 10 µg of goat polyclonal antibodies directed against Cx43. Non-specific antibodies (anti-green fluorescent protein (GFP), 10 µg) were used for negative controls. Incubation proceeded overnight, at 4ºC, with gentle agitation. Thereafter, 40 µg of protein G-Sepharose (GE Healhcare Biosciences, Uppsala, Sweden) were added to the samples and incubation proceeded for 1.5 h, at 4ºC. The samples were then centrifuged and the protein G-Sepharose sediments washed 3 times in RIPA buffer, eluted with 2x Laemmli buffer and denatured at 95ºC, for 5 min. WB analysis of the eluates was performed as already described.

IP of Cx43 from tissue lysates, either from organotypic cultures or Langendorff hearts, was performed as described for cell cultures. Briefly, 1.3 mg of total protein were incubated with 10 µg of goat polyclonal antibodies directed against Cx43, whereas non-specific antibodies (anti-GFP, 10 µg) were used for negative controls. Incubation with protein G-sepharose proceeded as previous and WB analysis of the eluates was performed.

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/-	SICGEN (Cantanhede, Portugal)
anti-Cx43	rabbit polyclonal	710700	WB/IF	1:500/1:50	Life Technologies (Carlsbad, CA)
anti-Cx43	rabbit polyclonal	Sc9059	WB/IF	1:500/1:25	Santa Cruz Biotechnology (Heidelberg, Germany)
anti-Cx43	mouse monoclonal	610062	IF	1:25	BD Transduction Laboratories (CA, USA)
anti-NP-Cx43	mouse monoclonal	13-8300	WB/IF	1:500/1:50	Life Technologies (Carlsbad, CA)

anti-ZO-1	rabbit polyclonal	61-7300	IF	1:100	Life Technologies (Carlsbad, CA)
anti-Troponin T	mouse monoclonal	Ab33589	IF	1:50	Abcam (Cambridge, UK)
anti-LC3	rabbit polyclonal	pa116931	WB	1:500	Thermo Fisher Scientific (Waltham, MA, USA)
anti-LC3 B	rabbit polyclonal	3868	IF	1:100	Cell Signalling Technology (MA, USA)
anti-p62	rabbit polyclonal	5114S	WB	1:1000	Cell Signalling Technology (MA, USA)
anti-ACC	rabbit polyclonal	3662	WB	1:500	Cell Signalling Technology (MA, USA)
Anti-pACC (Ser79)	rabbit polyclonal	3661	WB	1:500	Cell Signalling Technology (MA, USA)
anti-AMPKα1	rabbit polyclonal	07-350	WB	1:500	Merck Millipore (Darmstadt, Germany)
Anti-pAMPKα1 (Thr172)	rabbit polyclonal	07-681	WB	1:500	Merck Millipore (Darmstadt, Germany)
anti-Beclin 1	rabbit polyclonal	B6186	WB	1:500	Sigma-Aldrich (St. Louis, MO, USA)
anti-Nedd4	rabbit polyclonal	ab14592	WB	1:2000	Abcam (Cambridge, UK)
anti-Eps15	rabbit polyclonal	sc-1840	WB	1:1000	Santa Cruz Biotechnology (Heidelberg, Germany)
anti-Ubiquitin	mouse monoclonal	P4D1	WB	1:1000	Covance (Princeton, NJ, USA)
anti-NBR1	mouse monoclonal	H00004077-M01	WB	1:500	Abnova (Taipei, Taiwan)
anti-E Cadherin	mouse monoclonal	ab1416	WB	1:1000	Abcam (Cambridge, UK)
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	IP	-	SICGEN (Cantanhede, Portugal)
anti-Tubulin	mouse monoclonal	T6199	WB	1:2000	Sigma-Aldrich (St. Louis, MO, USA)
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 488 anti-rabbit	goat	A-11034	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)
alexa 568 anti-rabbit	goat	A-11011	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)
alexa 488 anti-mouse	goat	A-11001	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)
alexa 568 anti-mouse	goat	A-11004	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)

3.7 Biotinylation of cell surface proteins

HL-1 cells grown on 60 x 15 mm gelatin/fibronectin coated culture dishes were rinsed twice with 2 mL of ice-cold PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, followed by the addition of 1 mL of the same

ice-cold solution containing 1 mg/mL of freshly added Sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA). After 30 min at 4ºC, to stop subcellular trafficking, the medium was discarded and the plates were washed three times with PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 mM glycine. The cells were scraped in 400 µl RIPA buffer supplemented with protease inhibitors. After 15 min on ice, the cells were centrifuged at 3200 rpm, for 5 min. 5% of the total amount of protein in the supernatants was denatured (inputs), as already described, being the remaining lysates used for precipitation with neutravidin. Briefly, samples were incubated with 30 mL of Neutravidin beads (Pierce, Rockford, IL, USA), for 2 h, at 4ºC under agitation. Beads were washed three times with RIPA buffer, and the final pellets resuspended in 2x Laemmli buffer and denatured at 95ºC, for 5 min. The beads were pelleted and the solubilised proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies directed against Cx43.

3.8 Triton X-100 fractionation assay

The detergent solubility assay with 1% Triton X-100 was performed essentially as described previously by others [98]. 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 inhibitors (protease inhibitor cocktail, 2 mM PMSF, 10 mM iodoacetamide and 2 mM sodium ortovanadate). Samples were then ultracentrifuged at 100,000g for 50 min 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. 2x Laemmli buffer was then added to the Triton X-100 soluble and insoluble fractions and denatured at 95ºC for 5 min before SDS-PAGE analysis.

3.9 Immunofluorescence staining

HL-1 cells or primary cardiomyocytes grown on gelatin/fibronectin coated glass coverslips were fixed with 4% paraformaldehyde (PFA) in PBS, for 10 min. The samples were then washed three times with PBS, permeabilised with 0.2% v/v Triton X-100 in PBS, for 10 min, and blocked with 2.5% BSA for 20 min. Incubation with primary antibodies against Cx43, LC3B, or Troponin T proceeded for 1 hour at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for an additional hour 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 0.25% w/v BSA containing 0.02% sodium azide in PBS. For controls, primary antibodies were omitted. The images were collected by confocal microscopy using a Zeiss LSM 710 (Carl Zeiss AG, Jena, Germany).

Tissue samples (5 µm cryosections) were fixed with acetone, at -20ºC for 10 min. The samples were then washed three times with PBS, and blocked with 5% BSA for 1 hour prior to incubation with primary antibodies. Incubation with primary antibodies against Cx43, NP-Cx43, ZO-1 or LC3B 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. Nuclei were stained with DAPI. All antibodies were diluted in 5% BSA. For controls, primary antibodies were omitted. The images were collected by confocal microscopy using a Zeiss LSM 710.

3.10 Transmission electron microscopy (TEM)

HL-1 cells grown on 60 x 15 mm gelatin/fibronectin coated culture dishes were scraped and fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) supplemented with 1 mM/L calcium chloride. After rinsing, the samples were post-fixed in 1% buffered osmium tetroxide for 1.5 h, rinsed again and post-fixed in 1% aqueous uranyl acetate for 1 hour. After rinsing in distilled water, the samples were dehydrated in a graded acetone series (30–100%), impregnated and embedded using an Epoxy embedding kit (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). Ultrathin sections were cut on an Ultramicrotome (EM UC6+EM FC6) (Leica, Solms, Germany). Sections were mounted on copper grids and stained with lead citrate 0.2% for 8 min. Observation of the samples was carried out on a FEI-Tecnai G2 Spirit Bio Twin (FEI, Hillsboro, OR, USA) at 80kV.

3.11 Statistical analysis

Data presented in this work is representative of at least 3 independent experiments. Data was analyzed by standard computer programs (GraphPad Prism 6 for Windows, version 6.01, GraphPad Software, Inc.) and is expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $P<0.05$.

4. Results

4.1 Ischemia induces degradation of Cx43 in cardiomyocytes

Myocardial ischemic injury results from severe impairment of the coronary blood supply, usually produced by partial or complete blockage of the coronary arteries. The loss of oxygen causes mitochondrial oxidative phosphorylation to stop, resulting in a rapid ATP depletion, and an upregulation of anaerobic glycolysis, as a compensatory mechanism for ATP production. This metabolic shift leads to the accumulation of hydrogen ions and lactate, which results in intracellular acidosis and inhibition of all forms of energy metabolism. Additionally, energy loss leads to functional impairment of cardiac ion pumps that ensure the proper electrolytic balance needed for muscle contraction and, as a result, ventricular arrhythmias may arise, as well as cellular and mitochondrial swelling [99]. When blood flow is restored to the myocardium, in a process that is called reperfusion, the extent of damage can be either controlled or exacerbated, which can be defined by the extent or severity of the initial ischemia stimulus [100].

A variety of techniques have been used to simulate ischemia and I/R in cell cultures. Severe hypoxia, using nitrogen concentrations between 95% and 100% has been consistently used, in combination with substrate deprivation through the use of a glucose-free medium, or by using a non-metabolizable analogue, 2-deoxy-D-glucose. Alternatively, other groups use potassium cyanide, an inhibitor of the oxidative metabolism, in combination with nutrient depletion, which results in a blockage of energy production. Hence, use of cyanide results in cell injury comparable with simulated ischemia, even in the presence of oxygen [101].

It has been widely shown that MI induces GJ remodeling, which include channel closure and Cx43 dephosphorylation, subcellular redistribution and degradation [70, 80, 102]. However, the molecular mechanisms underlying Cx43 degradation in cardiomyocytes subject to ischemia remain elusive. Therefore, to address what is the best method to simulate ischemia and I/R *in vitro*, and how Cx43 levels are affected in each case, we subjected neonatal primary cultures of rat cardiomyocytes to chemical ischemia, by the use of potassium cyanide. Results presented in Figure 8A show that chemical ischemia leads to a decrease in the total levels of Cx43, in a time-dependent manner. We also tested the effect of further reperfusion in these cultures, after 30 minutes of ischemia, by replacing the “ischemic medium” with complete cell growth medium, for 120 minutes, after which we evaluated the total levels of Cx43 by WB, and its subcellular distribution by confocal microscopy. Our results show that reperfusion induces further degradation of Cx43 (lane 6 of Figure 8A and lower panel of Figure 8B).

It has been established that ubiquitination constitutes a signal for internalization and degradation of Cx43 upon a variety of stimuli. To evaluate the levels of Cx43 ubiquitination under ischemia, we performed IP assays. Results presented in Figure 8A show that the amount of Ub moieties bound to Cx43 increases upon chemical ischemia.

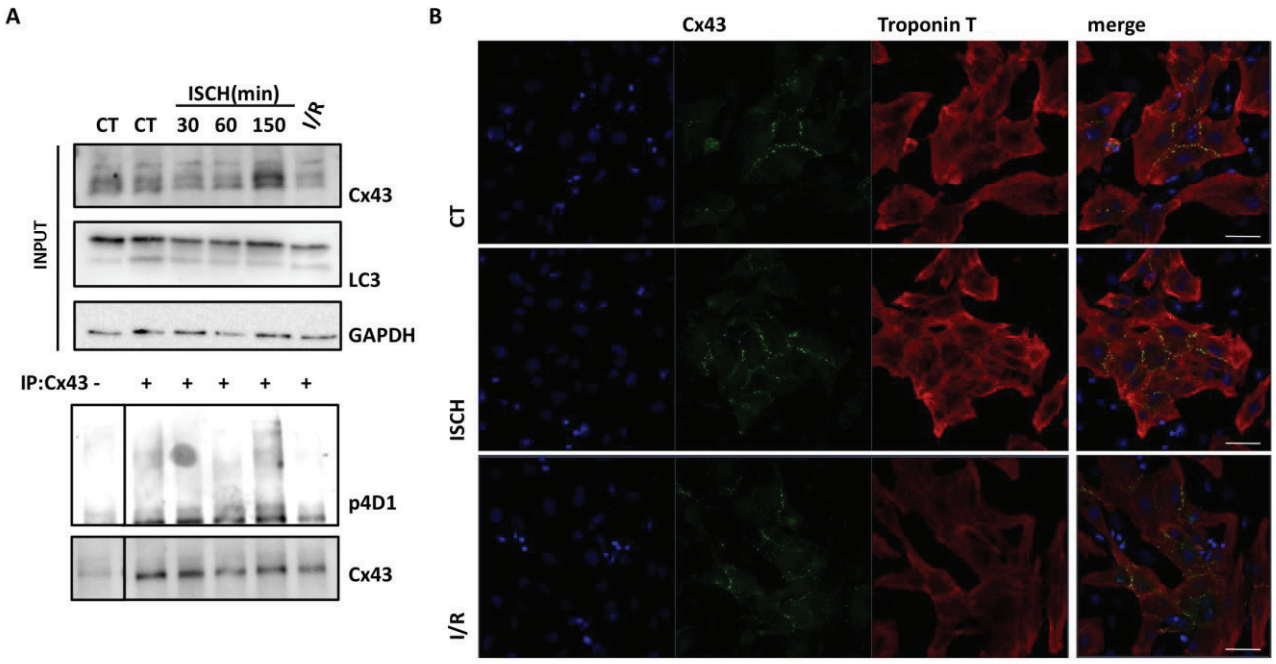

Figure 8 – Chemical ischemia and I/R induce degradation and subcellular redistribution of Cx43 in cardiomyocytes. A. Neonatal primary cultures of rat cardiomyocytes were incubated either in normal (CT), or ischemic conditions (ISCH), with a cyanide-based ischemia-mimetic solution, for 30, 60 or 150 min. Reperfusion was induced, after an initial period of 30 min of ischemia, by 120 min of incubation in normal conditions, with complete cell growth medium (I/R). Total amount of Cx43 and the levels of the autophagy marker LC3 were evaluated by WB. Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) was used as loading control. Inputs represent about 5% of the total protein of cell lysates used for IP. Cx43 was further immunoprecipitated and the immunoprecipitates were probed against Ub (P4D1) and Cx43. **B.** Primary cultures grown in gelatin/fibronectin coated coverslips were subjected to normal (CT), 30 min of chemical ischemia (ISCH) or 30 min of chemical ischemia, followed by 120 min of reperfusion (I/R). Cells were fixed with 4% PFA for 10 min and used for immunocytochemistry using specific antibodies directed against Cx43 and Troponin T. Nuclei were stained with DAPI. Scale bars, 40 µm

Although our protocol for establishing the primary cultures of cardiomyocytes is optimized in order to obtain a culture as pure as possible, without “contamination” of other cell types co-existent in the heart, we cannot ensure a 100% pure cardiomyocyte culture. To evaluate the purity of our primary cultures, we labeled the cells with cardiac Troponin T, a specific marker of cardiomyocytes (Figure 8B). Although our cultures are relatively enriched in cardiomyocytes (55% Troponin T positive cells: 45% Troponin T negative cells), the significant presence of cells other than cardiomyocytes leads us to choose a more homogenous model. The atrial cardiomyocyte cell line, HL-1, the most common and widely used cell model of cardiomyocytes, was previously shown to express Cx43 endogenously and respond to ischemia [84]. Therefore, all the studies presented hereafter were carried out with this cell line.

First, to evaluate whether the effects of ischemia are comparable to those obtained with the primary cultures, we subjected HL-1 cells to chemical ischemia, after which we evaluated the total levels of Cx43 and its ubiquitination. The results presented in Figure 9A show that chemical ischemia results in a decrease in the total amount of Cx43 and increased ubiquitination of the protein, as with primary cultures. In parallel, in an attempt to define the best model of simulated ischemia, we also subjected HL-1 cells to metabolic ischemia, by incubating the cells with a medium with 2-deoxy-D-glucose and in hypoxic conditions, for different periods of time. Also, in this case, the total amount of Cx43 gradually decreases, reaching about 40% of basal levels after 240 minutes (Figure 9B). Additionally, the ratio of ubiquitinated/non ubiquitinated Cx43 increases in response to metabolic ischemia (Figure 9C).

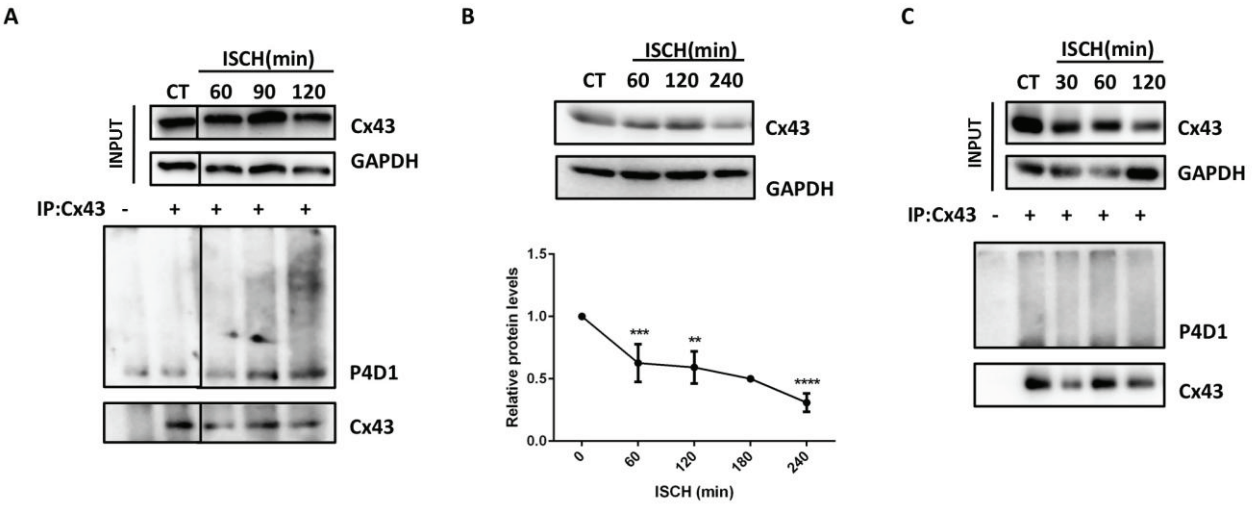

Figure 9 – Ischemia induces degradation of Cx43 in cardiomyocytes. A. HL-1 cells were either subjected to chemical ischemia (ISCH), with a cyanide-based ischemia-mimetic solution, or maintained in normal conditions (CT), for 60, 90 or 120 min. The total amount of Cx43 was evaluated by WB. GAPDH was used as loading control. Inputs represent about 5% of the total protein of cell lysates used for IP. Cx43 was further immunoprecipitated and the immunoprecipitates were probed against Ub (P4D1) and Cx43. **B.** HL-1 cells were either subjected to metabolic ischemia (ISCH), by placing the cells in hypoxic pouches with a deoxyglucose-based medium, or maintained in normal conditions (CT), for 60, 120 or 240 min. The total amount of Cx43 in each condition was evaluated by WB and after densitometric analysis; values were plotted on a graph. Values are mean ± SD (n=4). **p < 0.01, ***p < 0.001, **** p < 0.0001. **C.** HL-1 cells were either subjected to metabolic ischemia (ISCH), by placing the cells in hypoxic pouches with a deoxyglucose-based medium, or maintained in normal conditions (CT), for 30, 60 or 120 min. GAPDH was used as loading control. Inputs represent about 5% of the total protein of cell lysates used for IP. Total levels of Cx43 were evaluated by WB, and Cx43 was further immunoprecipitated and the immunoprecipitates were probed against Ub (P4D1) and Cx43.

Taking this into account, and given that the use of any irreversible form of metabolic inhibition, such as cyanide, can complicate interpretation of cell mortality upon further simulated reperfusion, we decided to proceed the studies using the model of metabolic ischemia (hereafter referred as ischemia) [101]. With this model, we mimic the extracellular environment and the reduced intracellular energy state that occur in the ischemic heart, *in vivo*. Consistently, composition of the medium mimics the extracellular hyperkalemia,

through a high concentration of potassium, an acidic pH, and 2-deoxy-D-glucose which ultimately inhibits cellular metabolism.

Having established that ischemia constitutes a stimulus for degradation of Cx43 in HL-1 cells, and since, in the heart, Cx43 is mainly localized at PM domains, we wanted to elucidate which population of cellular Cx43 is being degraded upon ischemia. For this purpose, we determined the effect of ischemia upon membrane-localized Cx43, by the use of two different approaches, cell surface protein biotinylation and Triton X-100 insolubility assays, which allow the discrimination between HC and GJ plaques, respectively. Through biotinylation assays, we show that the levels of Cx43 at the PM decrease almost 20%, after 2 hours of ischemia, and 65% after 4 hours (Figure 10A). Since Cx43 localized at GJ plaques is not accessible to biotin, we performed Triton X-100 subcellular fractioning assays, and isolated gap junctional Cx43 in the Triton X-100 insoluble fraction [98]. Using this approach, we demonstrate that the amount of Cx43 in the insoluble fraction decreases about 80%, while in the soluble fraction the levels of Cx43 diminish 60% (Figure 10B). Altogether, these results demonstrate that ischemia-induced protein degradation mainly targets Cx43 localized at the PM.

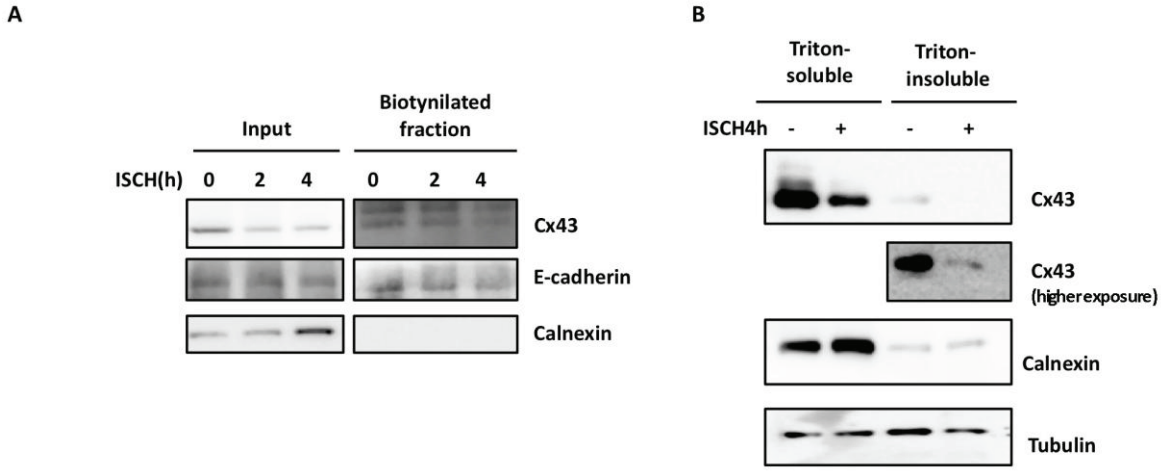

Figure 10 – Cx43 localized at the PM is the main target of ischemia-induced degradation. A. HL-1 cells were either incubated in ischemic conditions, by placing the cells in hypoxic pouches with an ischemia-mimetic solution, or maintained in normal conditions (CT), for 2 or 4 h, and then subjected to cell surface protein biotinylation. The biotinylated fraction of cell lysates was precipitated with NeutrAvidin beads, and further analyzed by WB using goat polyclonal antibodies against Cx43. E-Cadherin was used as a positive control for the biotinylated fraction, corresponding to the PM, and Calnexin was used as a negative control. **B.** Cells were either incubated in normal (CT), or ischemic conditions (ISCH), 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 goat polyclonal antibodies against Cx43. Calnexin was used to differentiate the Triton X-100-soluble from the –insoluble fraction. Tubulin was used as a loading control.

4.2 Autophagy is responsible for degradation of Cx43 in HL-1 cells subject to ischemia

A downregulation of Cx43 at the onset of MI has been reported in several studies, some of them suggesting a role for both the proteasome and the lysosome, however the exact molecular pathways

enrolled on the regulation of Cx43 turnover remain elusive [80]. It is well established that degradation of Cx43 can occur either in the proteasome, through ERAD, or in the lysosome, as consequence of endocytosis or autophagy. To test the involvement of the two proteolytic systems in ischemia-induced degradation of Cx43, we subjected HL-1 cells to ischemia, either in the presence of a lysosomal inhibitor, Baf, or a proteasomal inhibitor, MG-132 [103]. Baf is a commonly used lysosomal inhibitor, since it specific inhibits vacuolar-type H^+-ATPases, thus preventing acidification of organelles containing this enzyme, such as lysosomes and endosomes. It is also reported that Baf blocks the process of fusion of the autophagosome with the lysosome, ultimately preventing lysosomal degradation of targeted cargo [52]. While Baf is able to revert partially the ischemia-induced degradation of Cx43, no protective effect was observed after treatment with MG-132 (Figure 11), suggesting that degradation of Cx43 occurs mainly in the lysosome.

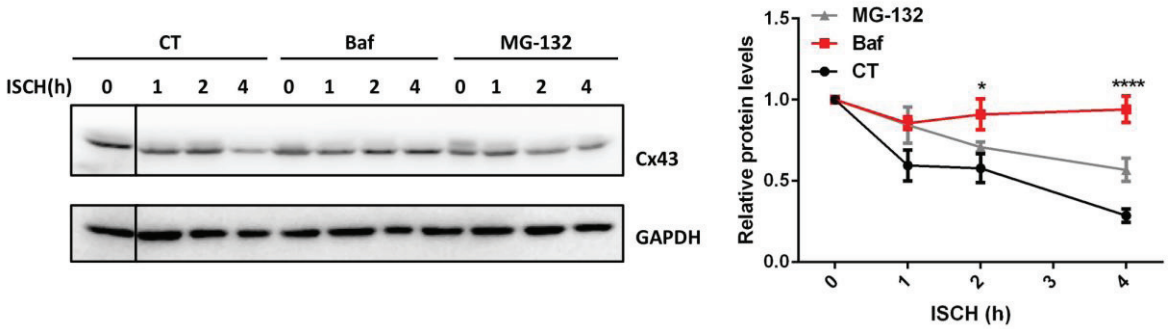

Figure 11 – Ischemia-induced degradation of Cx43 occurs mainly in the lysosome. HL-1 cells were either incubated in ischemic conditions (ISCH), by placing the cells in hypoxic pouches with an ischemia-mimetic solution, for 1, 2 or 4 h, in the presence of 50 nM Baf or 10 μM MG-132, for the indicated periods. Cells incubated in the absence of the inhibitor, or with vehicle, were used as controls (CT). Total levels of Cx43 were addressed by WB analysis and calculated from densitometric analysis. GAPDH was used as loading control. Values are mean ± SD (n=3). *p = 0.05, ****p < 0.0001 vs CT.

Previous studies carried out in our group have demonstrated that starvation leads to degradation of Cx43 through autophagy. Since the absence of oxygen and nutrients in ischemia is known to activate autophagy, we hypothesized that degradation of Cx43 in ischemic conditions occurs, at least in part, through autophagy [34]. To address this question, we determined the levels of Cx43 in HL-1 cells after 4 hours of ischemia, either in the presence or absence of chemical inhibitors of macroautophagy, 3-MA and OA, besides inhibition of the lysosome [52]. 3-MA inhibits autophagy by blocking autophagosome formation through the inhibition of PI3KIII, while OA is an inhibitor of protein phosphatase 2A (PP2A). PP2A is a serine/threonine phosphatase which negatively regulates Akt, thus activating autophagy [46]. The data presented in Figure 12 shows that treatment with drugs that inhibit autophagic degradation partially reverts the ischemia-induced degradation of Cx43 (at least 50% recovery), strongly supporting that autophagy plays an important role in the turnover of Cx43 during ischemia. Consistently, p62 and Beclin 1 levels also decrease, indicating that ischemia is inducing protein degradation by autophagy (Figure 12). Moreover, the

decrease of LC3-II in ischemia and its stabilization in the presence of Baf and OA with no cumulative effect, suggests that, under these circumstances, ischemia is promoting autophagic degradation by inducing the fusion of constitutively formed autophagosomes with the lysosome, rather than increasing autophagic flux.

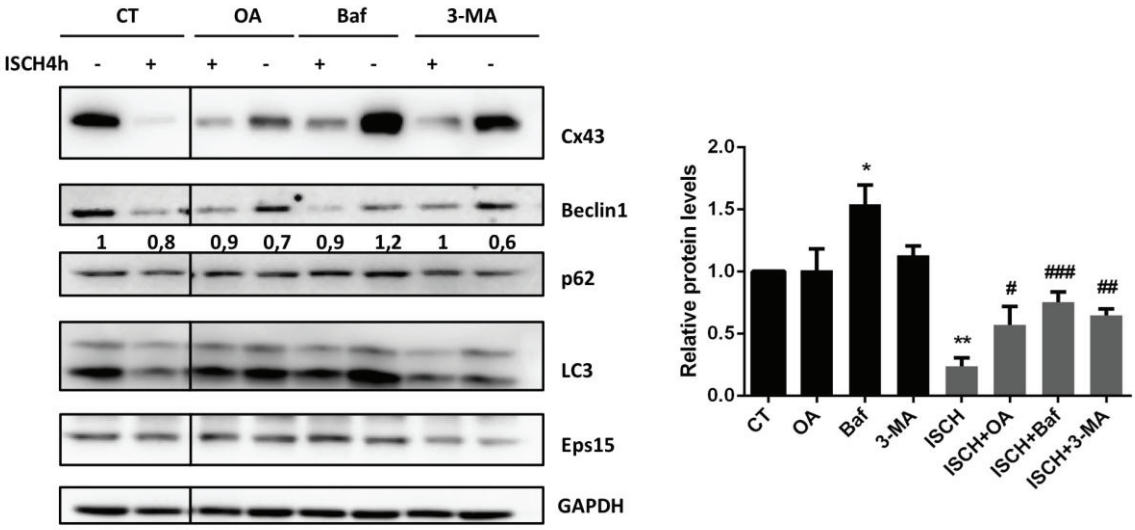

Figure 12 - Autophagy is responsible for degradation of Cx43 in ischemic cardiomyocytes. A. HL-1 cells incubated in normal or ischemic (ISCH) conditions, for 4 h, were treated with autophagy inhibitors (50 nM OA, 50 nM Baf, or 5 mM 3-MA). Cells incubated in the absence of the inhibitor, or with vehicle, were used as controls (CT). Total levels of Cx43 and autophagy players Beclin 1, p62 and LC3 were evaluated by WB and calculated from densitometric analysis. GAPDH was used as loading control. Values are mean ± SD (n=3). *p = 0.05, **p < 0.01 vs CT. #p = 0.05, ##p < 0.01, ###p < 0.001 vs ISCH.

Additionally, we performed TEM to investigate ultrastructural features usually ascribed to autophagy and to assess autophagic flux under our experimental conditions. Our results show that in control cells, initial autophagic vacuoles (structures with double membrane and containing morphologically intact cytoplasmic contents) are mainly present, while in ischemic cells, it was rather observed the presence of degradative autophagic vacuoles, whose contents start to look partially digested, reflected by an increase in its electrodensity [104]. Inhibition of the autophagosome-lysosome fusion in control conditions, using Baf, results in the accumulation of vacuoles containing numerous small vesicles that likely correspond to amphisomes and multivesicular bodies. Importantly, when we inhibit the lysosome during ischemia, we observed a reduced electrodensity of the degradative autophagic vacuole contents, suggesting that Baf is impeding the ischemia-induced degradation of autophagy cargo (Figure 13).

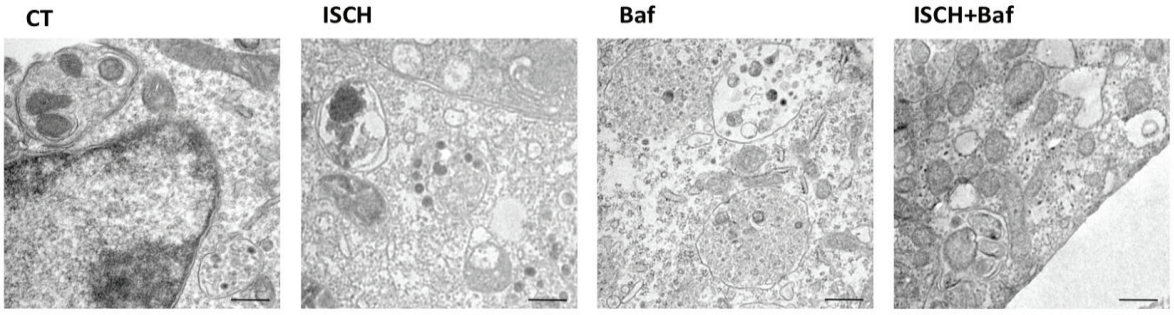

Figure 13 - Autophagy is enhanced in ischemic cardiomyocytes. HL-1 cells incubated in normal (CT) or ischemic (ISCH) conditions, for 4 h, either in the presence or absence of 50 nM Baf. Cells were fixed in 2.5% glutaraldehyde and used for TEM. Representative images of each experimental condition, with focus on autophagy-related structures. Scale bars, 0.5 µm.

We also evaluated Cx43 levels in response to ischemia in a fibroblast cell line that constitutively expresses Cx43, with a stable knockdown (KD) for the essential autophagy gene Atg7 – NIH3T3 cells [34]. In these cells, macroautophagy is compromised, which is proved by the decreased levels of LC3 protein. The stabilization of Cx43 in Atg7 KD cells subjected to ischemia constitutes an additional proof to autophagic involvement in the ischemia-induced degradation of the protein (Figure 14).

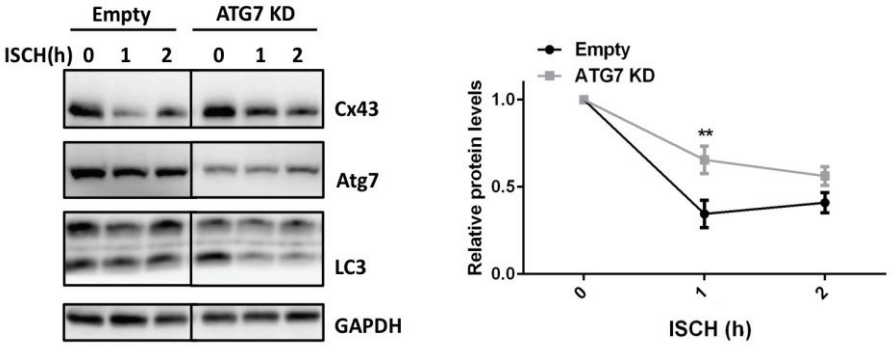

Figure 14 - Cx43 is protected from ischemia-induced degradation in cells knocked down for Atg7. NIH3T3 cells, with a stable KD for Atg7, were subjected to either normal (CT) or ischemic (ISCH) conditions, for 1 or 2 h. Total levels of Cx43 and LC3 were evaluated by WB and calculated from densitometric analysis. GAPDH was used as loading control. Values are mean ± SD (n=2). **p < 0.01 vs CT

To further evaluate the effect of ischemia upon the subcellular distribution of Cx43, in HL-1 cardiomyocytes, we performed immunofluorescence confocal microscopy experiments. In agreement with data presented above, we show that staining for Cx43 decreases upon 4 hours of ischemia. Importantly, the levels of co-localization between Cx43 and LC3 increase in ischemia, which is more visible when lysosomal degradation is inhibited (Figure 15), strongly suggesting that Cx43 is being degraded within autophagic vesicles.

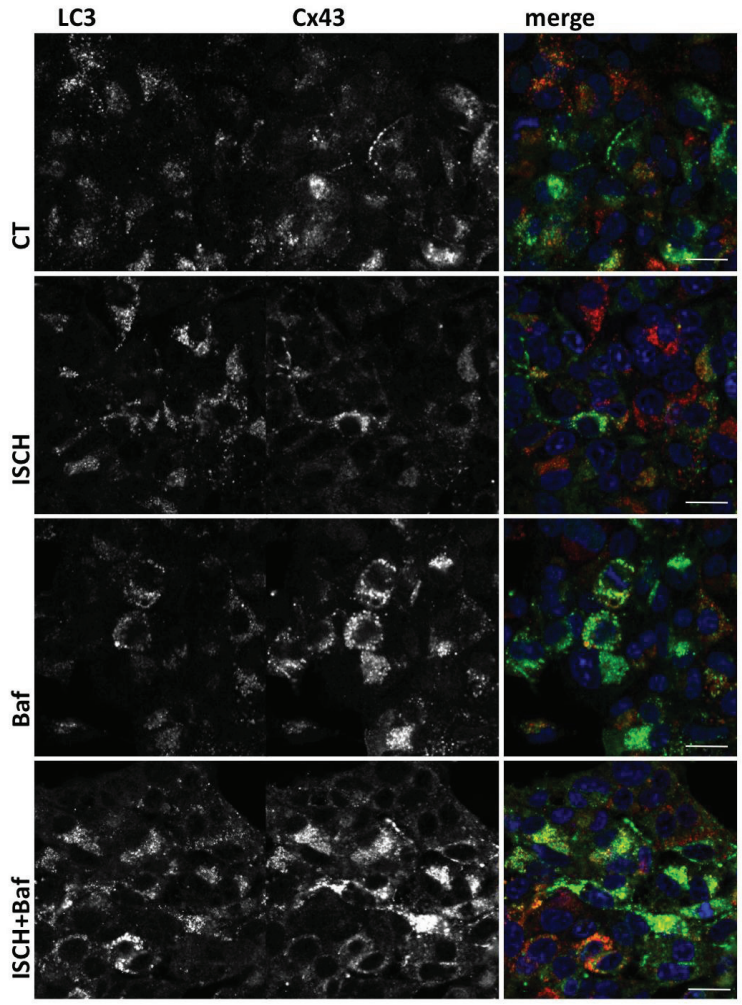

Figure 15 – Ischemia induces co-localization of Cx43 with LC3-positive autophagosomes. HL-1 cells were grown on gelatin/fibronectin coverslips, either in normal (CT) or ischemic conditions (ISCH) for 4 h, either in the presence or absence of Baf. Cells were fixed with 4% PFA for 10 min and used for immunocytochemistry using monoclonal antibodies directed against Cx43 and polyclonal antibodies directed against LC3. Subcellular distribution of Cx43 and co-localization of both proteins was further evaluated by confocal microscopy. Nuclei were stained with DAPI. Scale bars, 20 µm

We have previously demonstrated that Cx43 degradation by starvation-induced autophagy involves the interaction with Eps15, p62 and LC3 [34]. To evaluate if these autophagic players are also involved in ischemia-induced degradation of Cx43, we performed IP assays. The results presented in Figure 16 show that the relative amount of p62, LC3 and Eps15 that co-IP with Cx43 is similar in controls and ischemia, suggesting that these proteins form a complex that is degraded when autophagy is promoted. Furthermore, the amount of LC3 and p62 that is co-IP with Cx43, in the presence of Baf, both in control and ischemia, increases above its respective control levels, while interaction with Eps15 is not significantly affected. Not surprisingly, inhibition of early phases of autophagy, either with 3-MA or OA, does not result in a increased interaction between Cx43 and the autophagy machinery once, in this case, we are inhibiting upstream events of the autophagy process, thus preventing the formation of the complex between the three proteins.

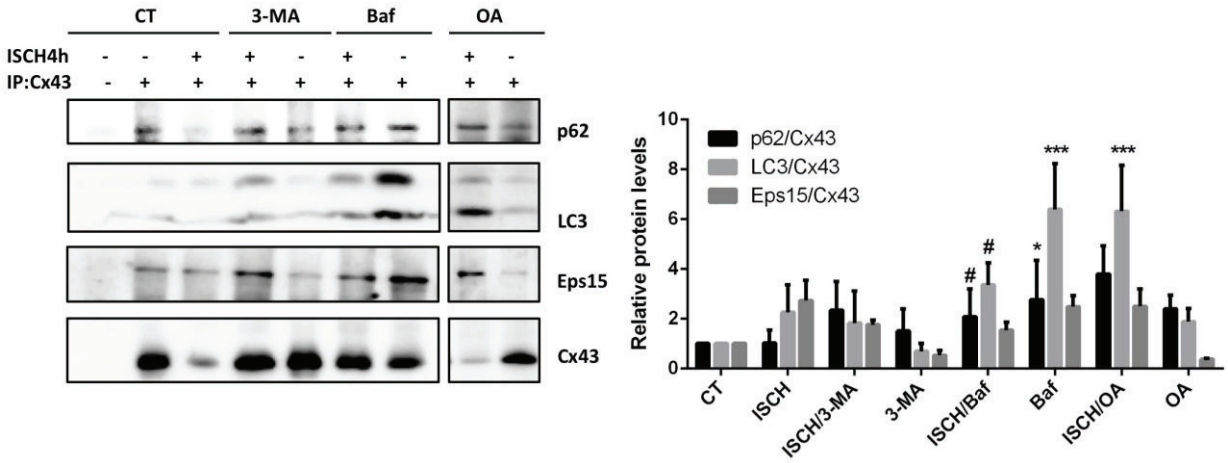

Figure 16 – Cx43 interacts with the autophagy machinery in ischemic cardiomyocytes. HL-1 cells incubated in normal or ischemic (ISCH) conditions, for 4 h, were treated with autophagy inhibitors (50 nM OA, 50 nM Baf, or 5 mM 3-MA). Cells incubated in the absence of the inhibitor, or with vehicle, were used as controls (CT). Cell lysates were used for IP of Cx43, and then the interaction with the autophagy machinery evaluated by WB, with antibodies against Cx43, p62, LC3 and Eps15. Representative graph shows the ratio p62/Cx43, LC3/Cx43 and Eps15/Cx43 calculated from densitometric analysis of WB. Values are mean ± SD (n=3). *p = 0.05, ***p < 0.001 vs CT. #p = 0.05 vs ISCH.

Since p62 acts as an autophagy adaptor that mediates the interaction of ubiquitinated substrates with LC3-containing autophagosomes, we evaluated the effect of p62 silencing upon Cx43 degradation and its interaction with LC3. The results presented in Figure 17A show that knockdown of p62 not only protects Cx43 from ischemia-induced degradation, but also leads to a decrease of its interaction with LC3 (Figure 17B). Strikingly, in the absence of p62, we observe an increased interaction with NBR1, another autophagy receptor involved in the degradation of ubiquitinated substrates (Figure 17B).

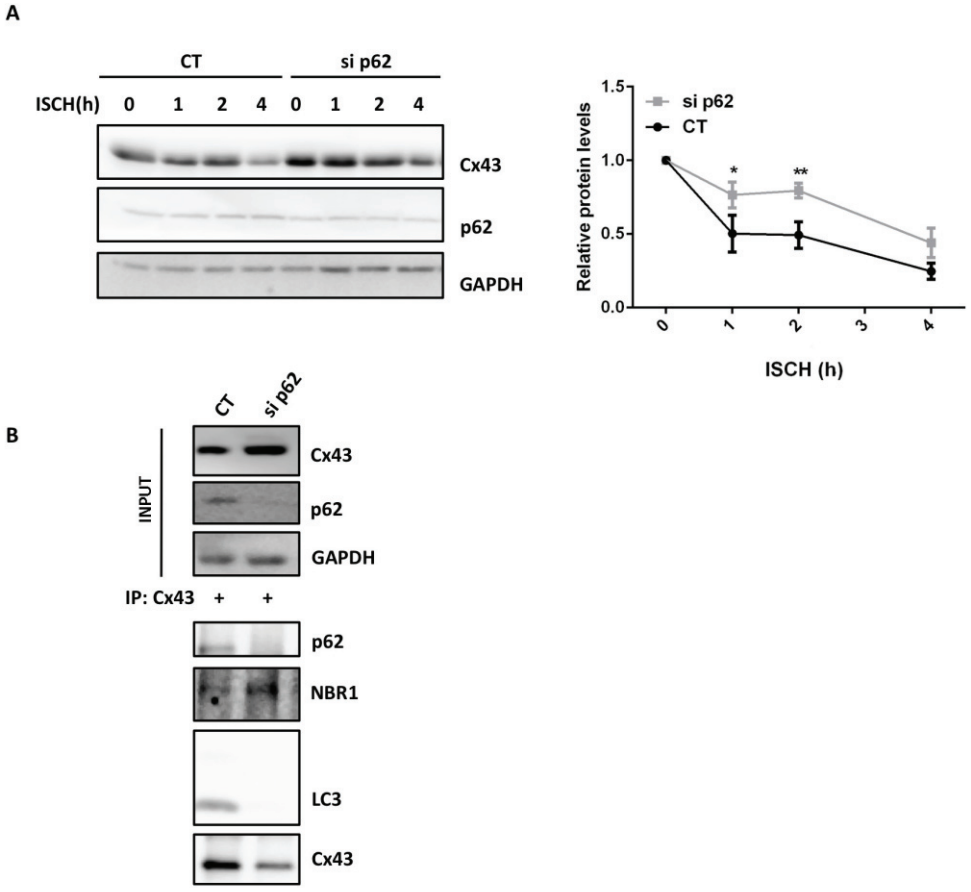

Figure 17 – Ischemia-induced degradation of Cx43 occurs through a p62-dependent mechanism. A. HL-1 cells transfected either with scramble siRNA (CT) or siRNA against p62 were maintained in control medium or subjected to 1, 2 or 4 h of ischemia. Total levels of Cx43 and p62 were analyzed by WB and calculated from densitometric analysis. GAPDH was used as loading control. Values are mean ± SD (n=3). *p = 0.05, **p < 0.01 vs CT. **B.** Cells incubated in control conditions were transfected either with scramble siRNA (CT) or siRNA against p62, after which Cx43 was immunoprecipitated and the interaction with NBR1 and LC3 evaluated by WB. Inputs represent about 5% of the total protein of cell lysates used for IP. GAPDH was used as loading control.

Altogether the data presented up to this point suggests that ischemia-induced autophagy promotes degradation of Cx43 through a mechanism that depends on p62, most likely to recruit ubiquitinated Cx43 to the autophagosome and thus enabling its degradation.

4.3 Ubiquitination of Cx43 is a signal for its ischemia-induced degradation in HL-1 cells

Since one of the established functions of p62 is to mediate the autophagic degradation of ubiquitinated substrates, we hypothesized that ubiquitination of Cx43 acts as a signal for GJ degradation by autophagy. To address this question, we evaluated the levels of ubiquitinated Cx43 and the interaction with the autophagy players p62 and LC3, following 4 hours of ischemia. Data presented in Figure 18 shows that the amount of ubiquitinated Cx43 is maintained, though total levels of Cx43 decrease. Moreover, in basal conditions, in the presence of Baf, the levels of ubiquitinated Cx43 are increased, suggesting that Ub targets

Cx43 for lysosomal degradation in cardiomyocytes. Moreover, as described above, the ratio Cx43/LC3 and Cx43/p62 is not significantly altered with ischemia, while in the presence of Baf the interaction of Cx43 with p62 and LC3 increases, both under basal conditions and ischemia.

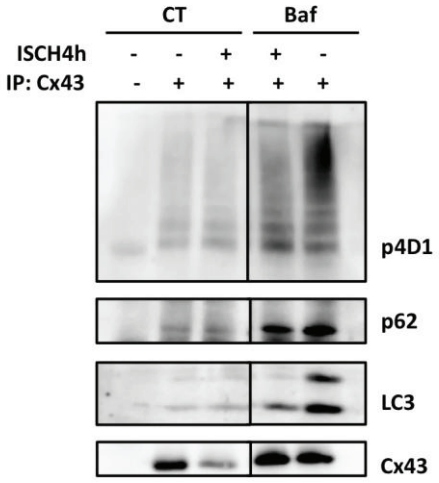

Figure 18 - Ischemia induces the degradation of ubiquitinated Cx43 by autophagy. HL-1 cells were maintained in control conditions (CT), or subjected to 4 h of ischemia (ISCH), either in the presence or absence of 50 nM Baf. Cell lysates were used to immunoprecipitate Cx43. Immunoprecipitates were analyzed by WB and probed with antibodies against Ub (P4D1), p62 and LC3, and Cx43.

In an attempt to prove the role of Ub in ischemia-induced degradation of Cx43, we determined the levels of Cx43 in HL-1 cells subjected to ischemia, either in the presence or absence of an E1 Ub-activating enzyme inhibitor, PYR-41. Ischemia-induced degradation of Cx43 was partially prevented in cells treated with PYR-41 (Figure 19A), further suggesting that ubiquitination of Cx43 enhances the degradation of the protein.

Moreover, to address the signaling role of Cx43 ubiquitination in the turnover of the protein, we overexpressed a chimeric Cx43 protein in which an Ub molecule was fused in-frame to the C-terminus of Cx43, in HL-1 cells. The half-life of endogenous Cx43, either in the presence or absence of Cx43–Ub, was determined by a CHX-chase assay, in which protein synthesis was inhibited with 50 µg/mL of CHX for different periods of time. The results presented in Figure 19B show that the overexpression of Cx43–Ub results in a decrease of the half-life of endogenous Cx43 from 4 hours to less than 2 hours. Additionally, to demonstrate that ubiquitination of Cx43 accelerates the ischemia-induced degradation of the protein, we investigated the levels of endogenous Cx43 in HL-1 cells transfected with a chimeric protein (mCherry–Cx43–Ub), subjected or not to ischemia. Consistent with a role of Ub in Cx43 degradation, the overexpression of mCherry–Cx43–Ub decreases the stability of the endogenous protein, under basal conditions (a 60% reduction) and, more importantly, increases its degradation during ischemia (about 20% more degradation) (Figure 19C).

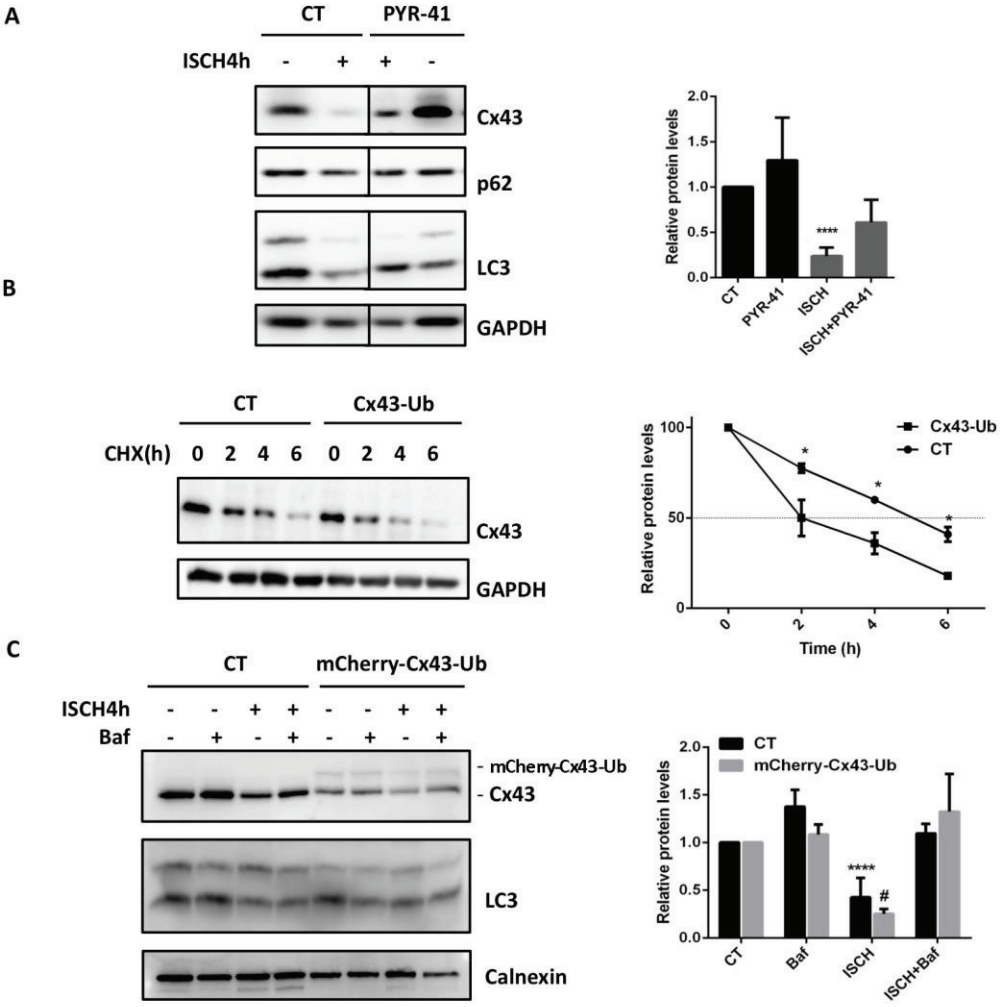

Figure 19 - Ubiquitination is a required signal for ischemia-induced degradation of Cx43. A. HL-1 cells were incubated in normal (CT) or ischemic (ISCH) conditions for 4 h, either in the presence or absence of 10 µM PYR-41. Total levels of Cx43, p62 and LC3 were evaluated by WB and calculated from densitometric analysis. GAPDH was used as loading control. Values are mean ± SD (n=3). ****p < 0.0001 vs CT. **B.** Levels of endogenous Cx43 in HL-1 cells transfected either with Cx43-Ub, or mock (CT). Half-life of the endogenous protein was assessed by incubation in the presence of 50 µg/mL cycloheximide (CHX), for 2, 4 or 6 h, followed by WB analysis using antibodies against Cx43. GAPDH was used as loading control. **C.** Levels of endogenous Cx43 in HL-1 cells transfected either with mCherry-Cx43-Ub, or mCherry (CT), were maintained in normal or ischemic conditions (ISCH), in the presence or absence of 50 nM Baf. Total levels of Cx43 and LC3 were analyzed by WB. Calnexin was used as loading control.

Since Nedd4 was previously associated with Cx43 ubiquitination and subsequent degradation in conditions of nutrient deprivation [2], we evaluated whether Nedd4 is required for ischemia-induced degradation of Cx43. Unexpectedly, in cells knocked down of Nedd4, degradation of Cx43 is not prevented and its levels of ubiquitination increase upon ischemia. However, in basal conditions, the silencing of Nedd4 result in a increase of 1.2 fold in the amount of Cx43 together with a decrease in ubiquitination levels, thus suggesting that Nedd4-mediated ubiquitination of Cx43 is required for degradation of the protein in basal conditions, but not in ischemia (Figure 20).

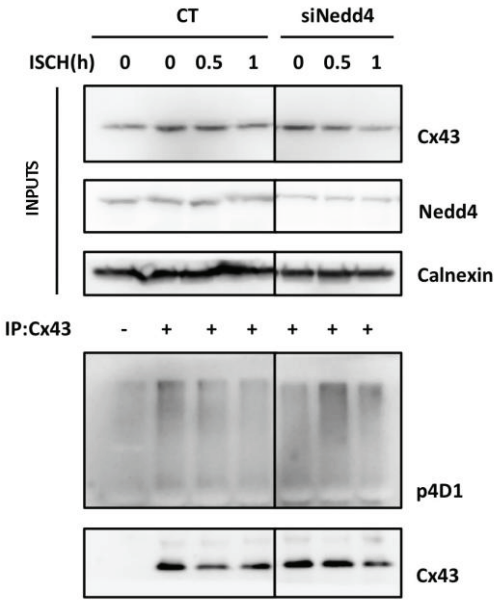

Figure 20 – Ischemia-induced degradation of Cx43 is mechanism independent of Nedd4. Cells transfected with scramble siRNA (CT) or siRNA against Nedd4, were maintained in control medium or subjected to 0.5 or 1 hour of ischemia (ISCH). Total levels of Cx43, Nedd4 and LC3 were evaluated by WB. Cx43 was further immunoprecipitated and the levels of ubiquitinated Cx43 were evaluated. Inputs represent about 5% of the total protein of cell lysates used for IP. GAPDH was used as loading control.

4.4 AMPK and Beclin 1 protect Cx43 from ischemia-induced degradation in HL-1 cells

It has been established that the molecular machinery involved in autophagic degradation varies with the different stages of MI/reperfusion. Indeed, it was shown that AMPK is activated right upon autophagy induction in ischemia, while in later stages and during reperfusion, degradation by autophagy mainly depends on Beclin 1 [87]. To address the role of AMPK-mediated autophagy upon Cx43 degradation, we subjected cells to different periods of ischemia, either in the presence or absence of the AMPK inhibitor Dorsomorphin Hydrochloride (DH), after which we evaluated the total levels of Cx43, as well as AMPK activation. The results presented in Figure 21 show that in the first 60 minutes of ischemia, degradation of Cx43 is accompanied by a robust activation of AMPK, demonstrated by the presence of phosphorylated-AMPK (pAMPK) and phosphorylated-ACC (pACC), a known downstream target of AMPK. Strikingly, during this period of ischemia, inhibition of AMPK leads to impaired autophagic flux and prevents degradation of Cx43. Nevertheless, for longer periods of ischemia (4 hours), the activity of AMPK diminishes (as demonstrated by decreased levels of pAMPK and pACC) and inhibition of AMPK does not present any protective effect regarding Cx43 degradation, suggesting that the autophagic process is being sustained by different molecular players. Since Beclin 1 up-regulation has been previously associated with autophagy in later periods of ischemia or reperfusion [3], we investigated the role of Beclin 1 in autophagy-mediated degradation of Cx43, through siRNA silencing. The data presented on Figure 21 shows that knockdown of Beclin 1 protects Cx43 from degradation, being this effect more robust in prolonged periods of ischemia.

Accordingly, both AMPK inhibition and Beclin 1 silencing result in the stabilization of LC3-II, suggesting that ischemia-triggered autophagic flux is impaired.

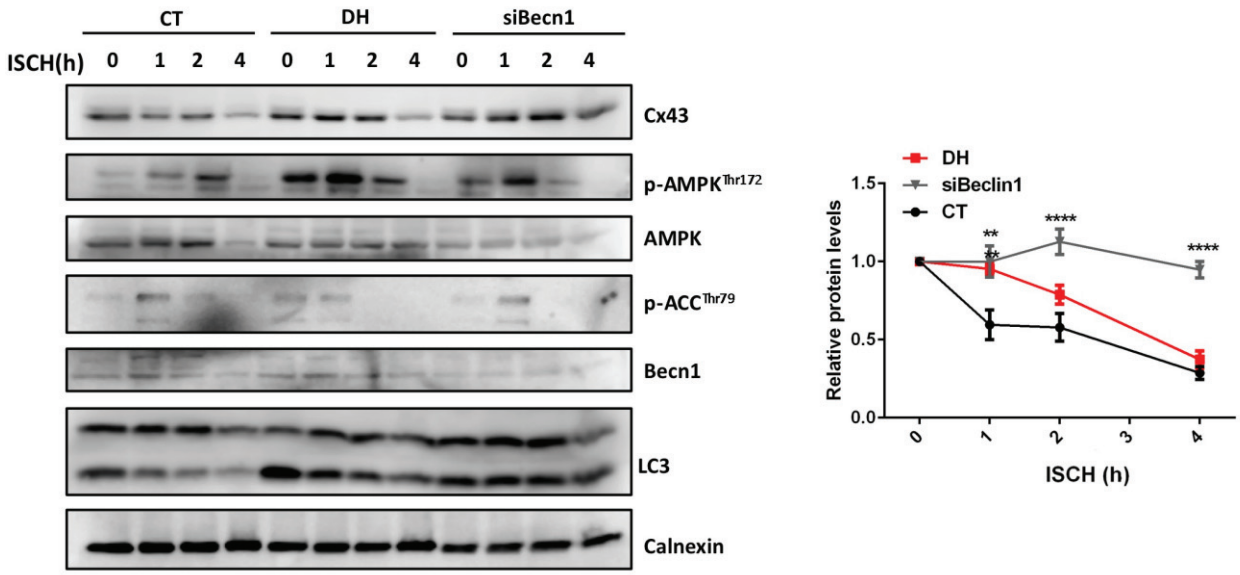

Figure 21 - AMPK and Beclin 1 protect Cx43 from ischemia-induced degradation. HL-1 cells were incubated in normal or ischemic (ISCH) conditions for 1, 2 or 4 h. Where indicated, cells were either transfected with scramble siRNA (CT), siRNA against Beclin 1, or treated with 80 µM DH. Activity of AMPK, by the levels of p-AMPK and p-ACC, autophagy activation, by the levels of p-AMPK and LC3, and levels of Cx43 were analyzed by WB. Total levels of Cx43 were calculated by densitometric analysis and plotted on a graph. Values are mean ± SD (n=3). **p < 0.01, ****p < 0.0001 vs CT.

4.5 I/R induces further degradation of Cx43 in HL-1 cells

Reperfusion, the restoration of blood supply to the myocardium after ischemia, can either limit the extent of the ischemic injury or be even more harmful, depending on its timing [100, 105]. Thus, we investigated the role of reperfusion in the autophagic degradation of Cx43. After 60 minutes of ischemia, ischemic medium was replaced by complete cell growth medium and cells were returned to normoxia conditions. For equivalent periods of ischemia, reperfusion following ischemia induces higher levels of degradation of Cx43 (Figure 22A). We then evaluated the effect of the inhibitors Baf and 3-MA, and our results show that both inhibitors stabilize Cx43, at least partially, in cells exposed to 30 or 120 minutes of reperfusion (compare lanes 6-7 with lanes 8-9 and 10-11 of Figure 22B), strongly suggesting that, during reperfusion, degradation of Cx43 relies on autophagy. However, when cells were treated with Baf or 3-MA only during the reperfusion phase, only the lysosomal inhibitor Baf protects Cx43 from degradation, while 3-MA presents no effect (Figure 22C). These results suggest that degradation of Cx43 in I/R is triggered in ischemia, during which autophagic vesicles are formed, being subsequently degraded in reperfusion.

Additionally, we evaluated the interaction of Cx43 with p62 and LC3 during reperfusion, by IP assays. Results presented in Figure 22D show that the relative amount of p62 and LC3 that co-IP with Cx43 increases in reperfusion, in comparison to ischemia.

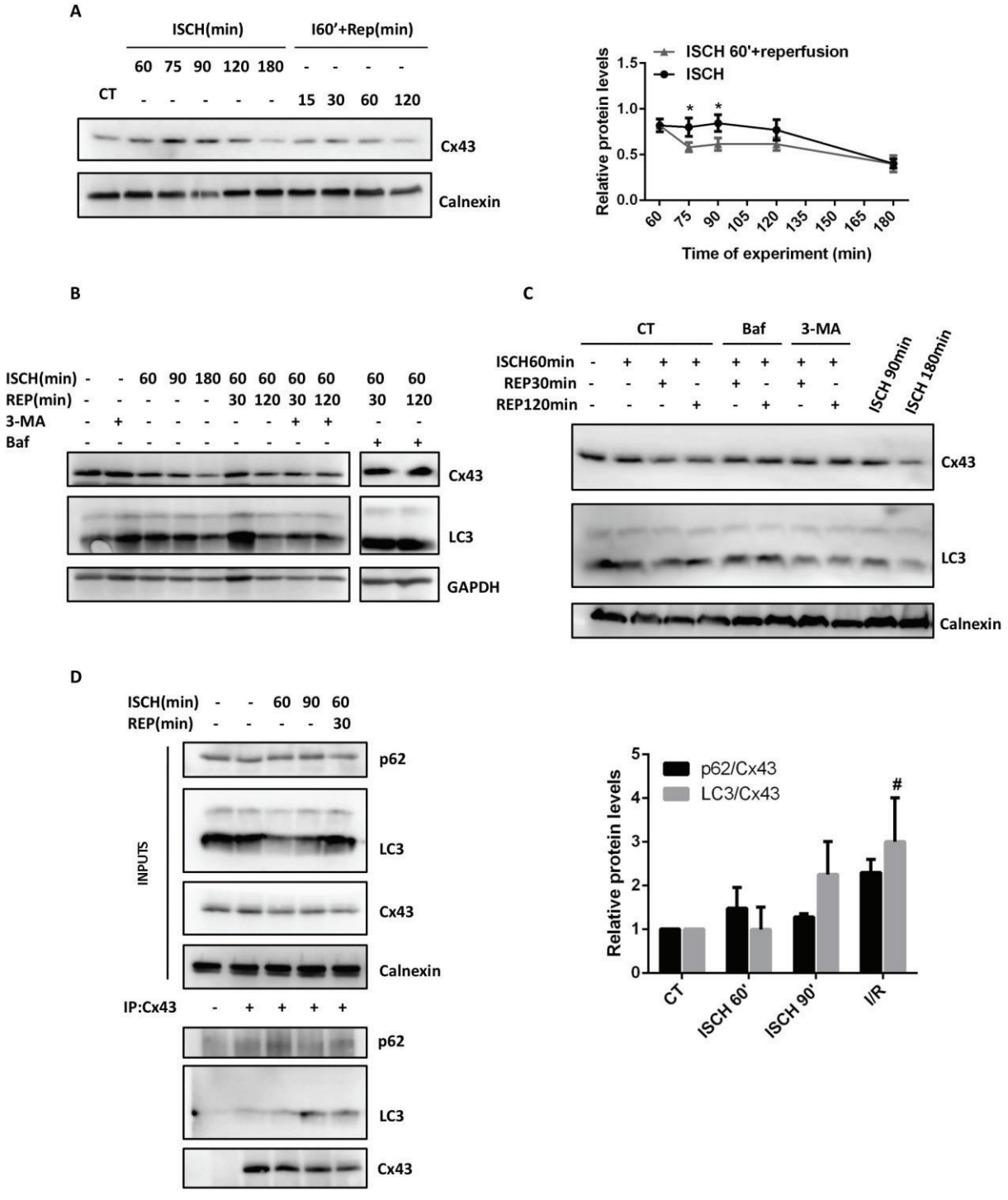

Figure 22 - I/R induces degradation of Cx43. A. HL-1 cells were incubated in ischemic conditions for 60 min, followed by further ischemia or reperfusion for 15, 30, 60 or 120 min. Cx43 and LC3 levels were evaluated by WB, calculated from densitometric analysis and plotted on a graph. **B.** HL-1 cells were incubated in ischemic conditions for 60 min, followed by further ischemia or reperfusion for 30 or 120 min. 50 nM Baf or 5 mM 3-MA were added to the culture medium, both during ischemia and reperfusion. Levels of Cx43 and LC3 were assessed by WB. Calnexin was used as loading control. **C.** HL-1 cells were incubated in ischemic conditions for 60 min, followed by further ischemia or

reperfusion for 30 or 120 min. 50 nM Baf or 5 mM 3-MA were added to the culture medium only during the reperfusion phase. Levels of Cx43 and LC3 were assessed by WB. Calnexin was used as loading control. **D.** Cx43 from HL-1 cells was immunoprecipitated and interaction with p62 and LC3 were further analyzed. Representative graph shows the p62/Cx43 or LC3/Cx43 calculated from densitometric analysis of WB. Values are mean ± SD (n=3). #p=0.05 vs ISCH 60 min. Inputs represent about 5% of the total protein of cell lysates used for IP. Calnexin was used as loading control.

To further elucidate the machinery involved in reperfusion-induced degradation of Cx43, we evaluated the roles of AMPK and Beclin 1. Results shown in Figure 23 demonstrate that Cx43 degradation is prevented mainly in cells knocked down for Beclin 1, while only a modest effect is observed with AMPK inhibition, consistent with a Beclin 1-mediated role of autophagy during reperfusion.

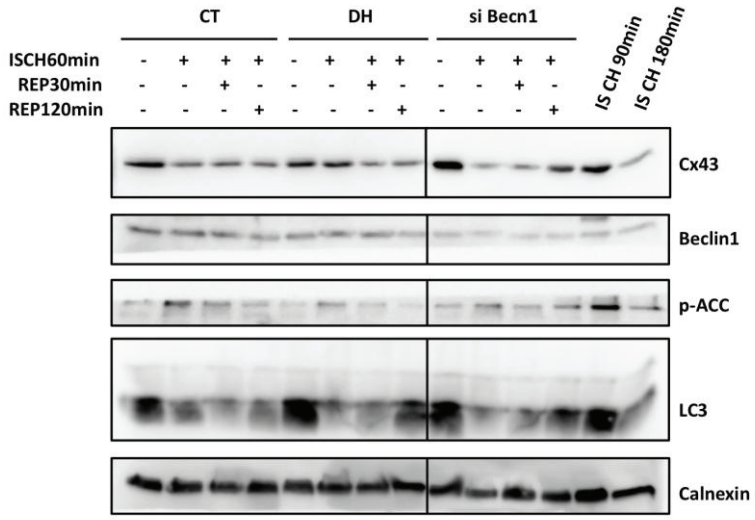

Figure 23 - I/R-induced degradation of Cx43 relies upon Beclin 1-mediated autophagy. A. HL-1 cells were either incubated in normal or ischemic conditions (ISCH), either in the presence or absence of 80 µM DH or siRNA against Beclin 1. Cells transfected with scramble siRNA were used as controls (CT). Total levels of LC3 and Cx43 were assessed by WB. pACC was used as a measure of DH-mediated AMPK inhibition. Calnexin was used as loading control.

4.6 Cx43 is degraded through ischemia-induced autophagy in organotypic heart slices

Once we have established the role of autophagy in ischemia-induced degradation of Cx43 in the cardiac cell line HL-1, we further investigated this mechanism in a more complex biological system, closer to what happens in the heart. For this purpose, we used short term culturing of heart slices, which has been reported to be a powerful and suitable tool to study cardiac physiology in a 3-dimensional *in vitro* model. Some recent studies provided evidence that support the viability of these cultures up to 8 days, while they retain multiple properties of fresh isolated heart tissue, namely protein expression patterns and electrophysiological properties [96]. Therefore, to validate the results obtained in our cell-based studies in a more physiological approach, we reproduce some of the previous experiments in organotypic heart cultures. First, we subjected organotypic heart slices to chemical ischemia for 30 or 120 minutes, or 30 minutes of chemical ischemia followed by 60 minutes of reperfusion. Then, we evaluated the levels of Cx43

in response to ischemia and I/R, by WB, and further immunoprecipitate Cx43 to assess its levels of ubiquitination. Our results show that, as occurs in the cell-based models, Cx43 is degraded in response to chemical ischemia, and more so during I/R, which is accompanied by an increased ratio of ubiquitinated/non-ubiquitinated Cx43 (Figure 24).

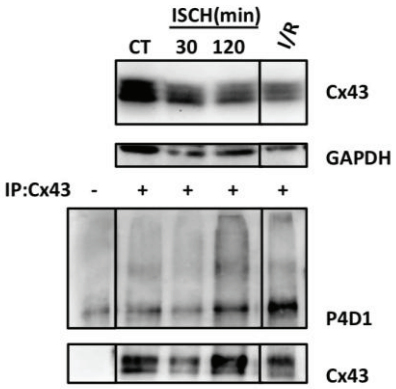

Figure 24 – Chemical ischemia leads to degradation of Cx43 in organotypic heart cultures. Organotypic heart slices were either maintained in control conditions (CT), or subjected to 30 or 120 min of chemical ischemia (ISCH), by the use of a cyanide-based ischemia-mimetic medium. I/R was induced for 60 min, after an initial period of 30 min of ischemia. Cx43 levels were evaluated by WB. GAPDH was used as loading control. IP of Cx43 was further performed, and immunoprecipitates analyzed by WB and probed with antibodies against Ub (P4D1) and Cx43. Inputs represent about 5% of the total protein of cell lysates used for IP.

Since we have previously validated the metabolic ischemia as the best model to study the impact upon Cx43 degradation, we also use this model in further studies with organotypic heart slices. To determine whether some of the features of the ischemic heart are preserved in these cultures, we subjected the organotypic heart slices to 120 minutes of ischemia, by the use of an ischemia-mimetic medium combined with hypoxia. In agreement with what happens in the ischemic heart, results presented in Figure 25A show that, in organotypic heart slices, Cx43 is dephosphorylated upon ischemia induction, which is represented by the presence of a fast migrating band of Cx43. Furthermore, we subjected the cultures to different periods of ischemia, 30, 60 and 120 minutes, and evaluated the total levels of Cx43 and its distribution along the tissue by confocal microscopy. Consistently, results presented in Figure 25B show that ischemia leads to a gradual decrease in the total levels of Cx43.

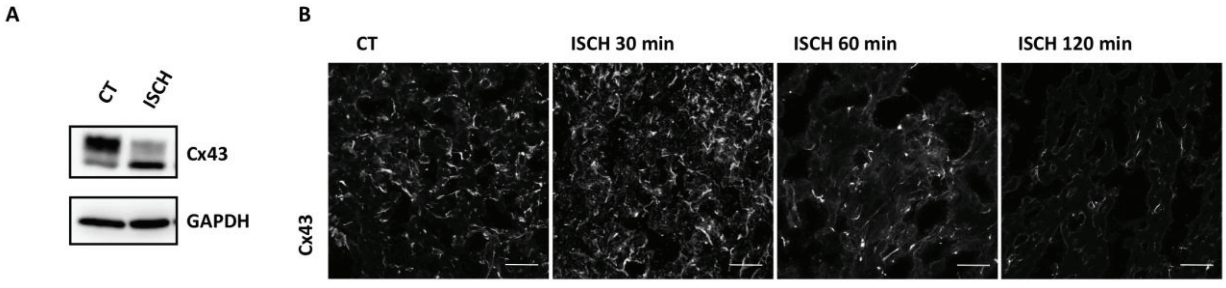

Figure 25 - Ischemia leads to degradation of Cx43 in organotypic heart cultures. A. Organotypic heart slices were either subjected to ischemia (ISCH), by placing the cells in hypoxic pouches with a deoxyglucose-based medium, or

maintained in normal conditions (CT), for 120 min, after which Cx43 levels were evaluated by WB. GAPDH was used as loading control. **B.** Organotypic heart slices, subjected to 30, 60 or 120 min of ischemia, were fixed with acetone for 20 min, and further stained with monoclonal antibodies directed against Cx43. Total levels of the protein were evaluated by confocal microscopy. Scale bars, 40 µm

To address whether in this model Cx43 is likewise degraded by autophagy, organotypic heart slices were subjected to 4 hours of ischemia, either in the presence or absence of 3-MA or Baf, and total levels of Cx43 were assessed by WB and confocal microscopy. WB results, presented in Figure 26A, shown that similarly to the cell model, autophagy and lysosome inhibition partially revert ischemia-induced degradation of Cx43. Consistently, results of confocal microscopy (Figure 26B) support the involvement of autophagy in this process.

To evaluate the role of Cx43 ubiquitination, we performed Cx43 IP experiments and evaluated the levels of ubiquitinated Cx43. In agreement with the results obtained in HL-1 cells, ischemia leads to an increased ratio of ubiquitinated/non ubiquitinated Cx43 (Figure 26C).

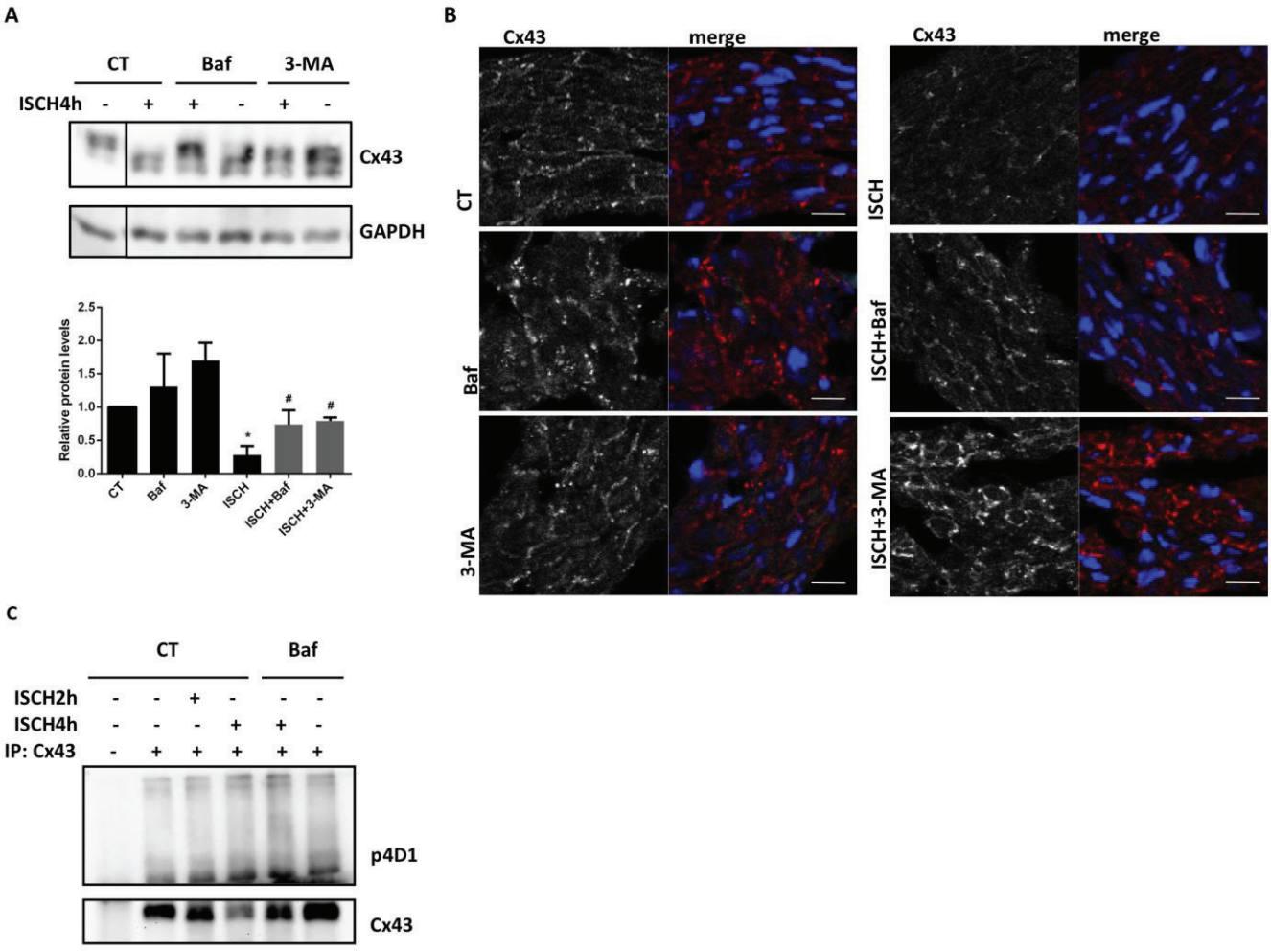

Figure 26 - Ischemia leads to autophagic degradation of Cx43 in organotypic heart cultures. A. Organotypic heart slices were either maintained in control conditions (CT), or subjected to 4 h of ischemia (ISCH), either in the presence or absence of 10 mM 3-MA or 100 nM Baf. Total levels of Cx43 were evaluated by WB, calculated from densitometric analysis and plotted on a graph. Values are mean ± SD (n=3). *p = 0.05 CT. **B.** Organotypic heart slices subjected to 4 h

of ischemia, either in the presence or absence of 10 mM 3-MA or 100 nM Baf, were fixed in acetone for 20 min, and further stained with monoclonal antibodies directed against Cx43. Nuclei were stained with DAPI. Scale bars, 25 µm **C.** Organotypic heart slices subjected to control or ischemia conditions, either in the presence or absence of 100 nM Baf, were lysed and used for IP of Cx43. Ubiquitination of Cx43 was further evaluated, by WB, using antibodies against Ub (P4D1).

4.7 I/R induces degradation of Cx43 in the Langendorff heart perfusion model

The results presented up to this point constitute an important contribution to establish the molecular mechanisms underlying the autophagic degradation of Cx43 in cardiac cells subject to ischemia and I/R. Therefore, in the final stage of this work, we investigated the pathophysiolgical impact of this mechanism in the heart. For that purpose, we used the *ex vivo* Langendorff perfusion system to evaluate the effect of ischemia and I/R in the rat heart. Langendorff perfusion system is a relatively easy and reliable model to study the physiology of the ischemic heart, since it enables the examination of cardiac contractile strength and heart rate without the complications of an intact animal, mainly the influence of neuronal and endocrine systems. In the Langendorff model, the heart is excised from the animal, mounted in the system, and then perfused with a nutrient-rich oxygenated solution, in a retrograde manner through the aorta. In this model, no-flow ischemia can be induced simply by the interruption of the coronary flow.

It is well established that during MI, Cx43 undergoes dephosphorylation and further lateralization, therefore, we started to subject the rat hearts to 30 minutes of no-flow ischemia, after which we evaluated the Cx43 migration profile, by WB, and its subcellular distribution, by confocal microscopy. Our results show that upon 30 minutes of ischemia, Cx43 is dephosphorylated, which is represented by the presence of a fast migrating band of Cx43, not visible in the control (Figure 27A). Moreover, this lower band is specifically recognized by antibodies raised against the non-phosphorylated form of Cx43 (NP-Cx43). Confocal images show that in the control hearts, Cx43 is mainly localized at the IDs, whereas upon ischemia, Cx43 localizes at the lateral membranes of the cardiomyocytes (Figure 27B). In addition, using the antibodies against NP-Cx43, we demonstrate that the NP-Cx43 is present not only at the IDs, but also at the lateral membranes (Figure 27C).

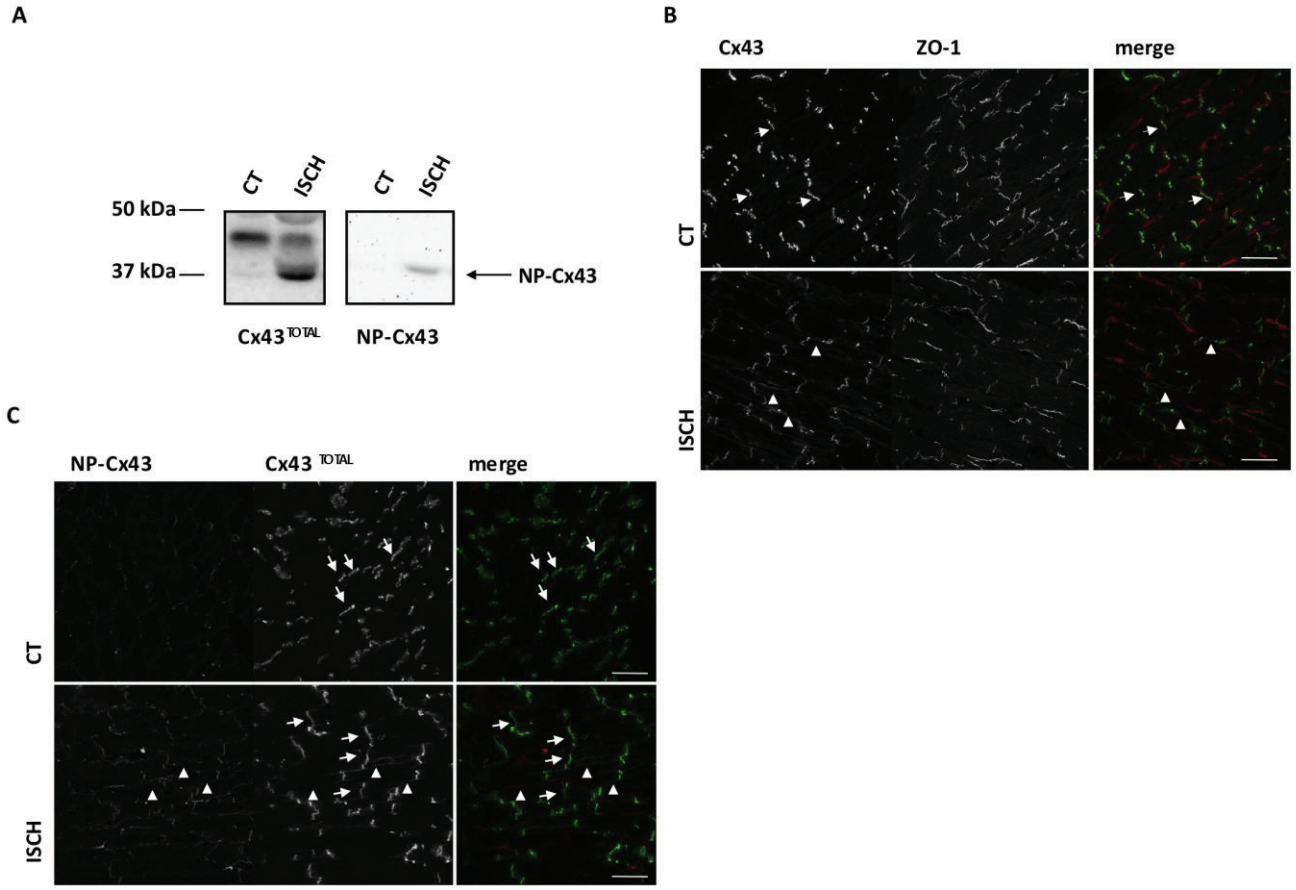

Figure 27 – Ischemia-induced remodeling of Cx43 is reproduced in the Langendorff heart perfusion model. Hearts from 10-week-old rats were maintained using a Langendorff apparatus for 10 min, with modified KH buffer, followed either by 30 min of normal perfusion (CT) or no-flow ischemia (ISCH). **A.** Lysates of control or ischemic hearts were analyzed by WB, using antibodies against total Cx43 and NP-Cx43. **B.** Cryosections of control or ischemic hearts were fixed with acetone and used for immunocytochemistry using monoclonal antibodies directed against total Cx43 and polyclonal antibodies directed against ZO-1. In control hearts Cx43 is mostly localized at the IDs (arrows) while in ischemic hearts there is an accumulation of Cx43 at the lateral membranes (arrow heads). Scale bars, 25 µm **C.** Cryosections of control or ischemic hearts were fixed with acetone and used for immunocytochemistry using monoclonal antibodies directed against NP-Cx43 and polyclonal antibodies directed against total Cx43. In control hearts Cx43 is mostly phosphorylated and localized at the IDs (arrows) while in ischemic hearts there is an accumulation of dephosphorylated Cx43 at the IDs and lateral membranes (arrow heads). Scale bars, 25 µm

Next, we evaluated the ischemia-induced degradation of Cx43 over time. For this purpose, we subjected the rat hearts to 20, 100, 120 or 220 minutes of no-flow ischemia. For controls, equivalent periods of perfusion were tested. Results presented on Figure 28 show that ischemia induces a rapid and consistent dephosphorylation of Cx43. However, surprisingly, total levels of Cx43 do not significantly vary along the time of ischemia. Only after 120 minutes of no-flow ischemia, Cx43 levels slightly decrease. To further investigate the effect of reperfusion, we subjected the hearts to two different periods of ischemia (20 and 100 minutes), followed by various periods of reperfusion, by restoring the coronary flow to the levels applied before ischemia induction. The results obtained show that after a short initial ischemia stimulus (20 minutes), reperfusion is either harmless, if applied during a short period of time (20 minutes), or leads to degradation of Cx43, if it was sustained during long periods of time (100 minutes). On the other

hand, when the initial stimulus of ischemia is longer (100 minutes), reperfusion leads to further degradation of Cx43, in a time-dependent manner (compare lane 12 with 13 of Figure 28). Importantly, the levels of LC3, which does not vary during ischemia, are decreased during reperfusion, suggesting that autophagy can be also the mechanism responsible for the I/R-induced degradation of Cx43 in the *ex vivo* model.

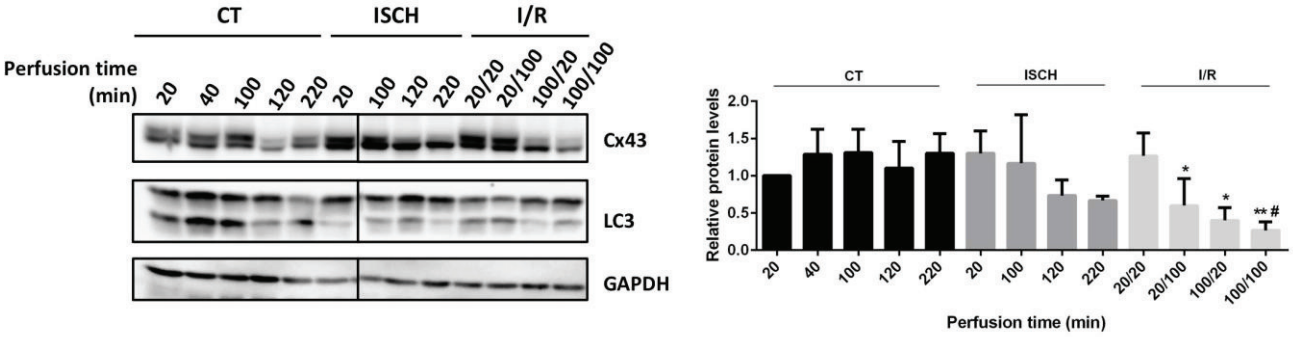

Figure 28 – I/R induces degradation of Cx43 in the ischemic heart. Hearts from 10-week-old rats were maintained using a Langendorff apparatus for 10 min, with modified KH buffer, followed either by 20, 40, 100, 120 and 220 min of normal perfusion (CT) or 20, 100, 120 and 220 of no-flow ischemia (ISCH). Reperfusion (I/R) was induced by restoring normal perfusion for 20 or 100 min, after 20 or 100 min of no-flow ischemia. Lysates of control or ischemic hearts were analyzed by WB, using antibodies against Cx43 or LC3. GAPDH was used as loading control. The total amount of Cx43 in each condition was evaluated by WB and after densitometric analysis; values were plotted on a graph. Values are mean ± SD (n=3). *p=0.05, **p < 0.01 vs CT (equivalent perfusion time), #p=0.05 vs ISCH 100 min.

Furthermore, to evaluate the cardiac electric activity and the heart rates under our various experimental conditions, we performed monitoring through ECG. Our results show that right after ischemia induction, occurs a rapid decrease in the heart rate (about 50%), and we also observe some of the widely established indicators of acute ischemia, such as the ST-segment elevation (Figure 29). The heart rate decreases in a time dependent manner, and during sustained ischemia, the heart goes into asystole, i.e. its electrical activity ceases. We also observe that after an initial ischemia stimulus of 20 minutes, reperfusion enables the recovery of the electric activity of the heart, whereas when the initial ischemia is prolonged (100 minutes), despite reperfusion reestablish the electric activity of the heart, it remains completely disorganized.

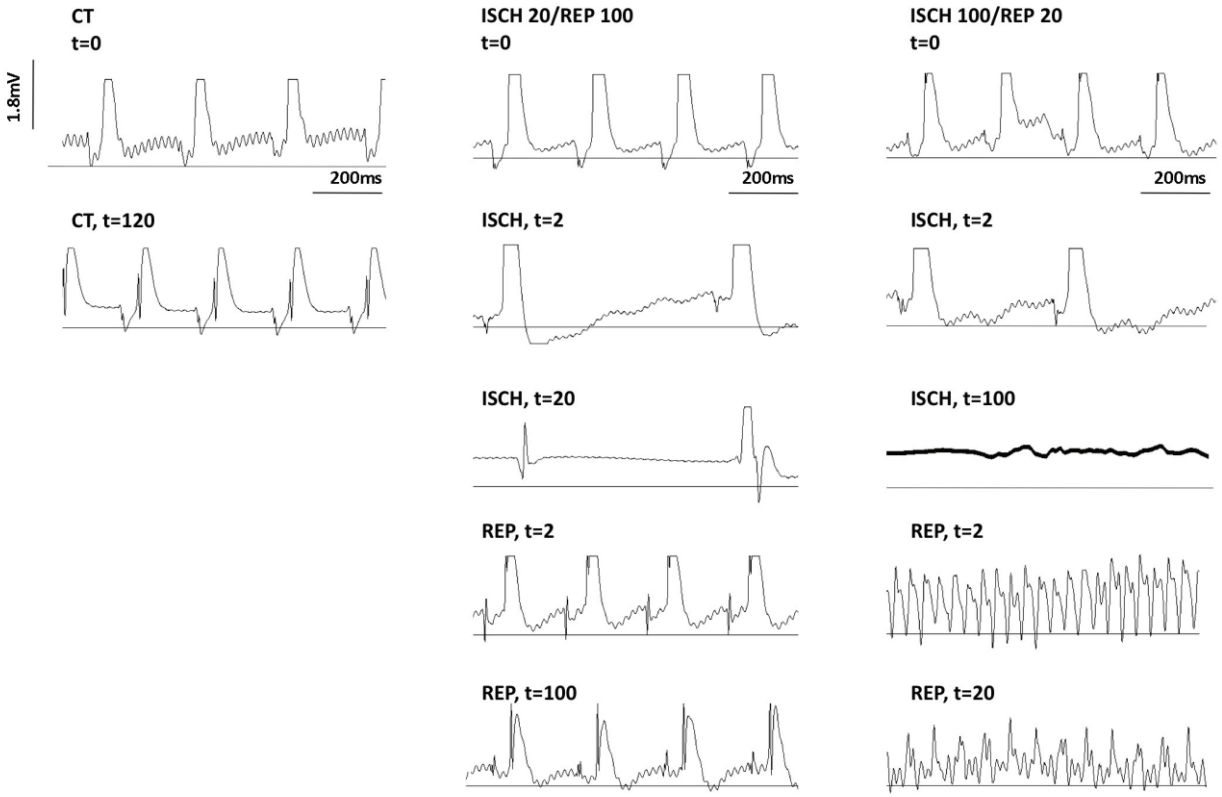

Figure 29 – ECG monitoring of the Langendorff hearts. Hearts from 10-week-old rats were maintained using a Langendorff apparatus for 10 min, with modified KH buffer, followed either by 120 min of normal perfusion (CT), 20 or 100 min of no-flow ischemia (ISCH), followed by 100 or 20 min of reperfusion (I/R), respectively. ECG monitoring of the hearts were performed.

Moreover, in order to address the involvement of autophagy in Cx43 degradation, we evaluated the subcellular distribution of Cx43 and its co-localization with LC3, by confocal microscopy, in hearts subjected to 220 minutes of ischemia. Results presented in Figure 30 show that, although the total levels of the protein are slightly diminished, Cx43 undergoes a significant redistribution. Indeed, after 220 minutes of ischemia, a small population of Cx43 remains at the IDs, being the protein mainly localized in the cytosol. Strikingly, the cytosolic population of Cx43 presents large co-localization with LC3, supporting the hypothesis that Cx43 is being targeted for autophagic degradation in ischemia.

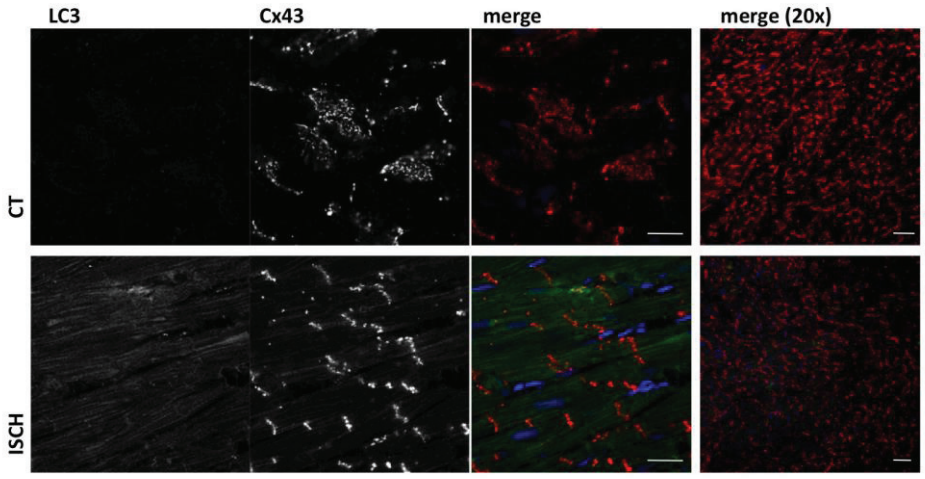

Figure 30 – Cx43 co-localizes with LC3-positive vesicles in the ischemic heart. Hearts from 10-week-old rats were maintained using a Langendorff apparatus for 10 min, with modified KH buffer, followed either by 220 min of normal perfusion (CT) or no-flow ischemia (ISCH). Cryosections of control or ischemic hearts were fixed with acetone and used for immunocytochemistry using monoclonal antibodies directed against Cx43 and polyclonal antibodies directed against LC3. Scale bars, 25 µm

In order to confirm the role of autophagy machinery in the degradation of Cx43 upon ischemia and I/R, we also performed IP experiments, and further evaluated the interaction between Cx43 and Eps15 and p62, two adaptor proteins that have already been associated with autophagy-mediated degradation of Cx43. Additionally, to understand if ubiquitination plays a role upon degradation of Cx43 in the ischemic heart, we determined the levels of ubiquitinated Cx43, and its interaction with the E3 Ub ligase Nedd4. Results presented in Figure 31 show that, during ischemia, there is an increased interaction of Cx43 with Nedd4, which is accompanied by an increased trend in the ratio ubiquitinated/non-ubiquitinated Cx43. Interaction with Eps15 and p62 presents a tendency to increase during ischemia, while during reperfusion, a robust increase in the interaction of Cx43 with both proteins is observed.

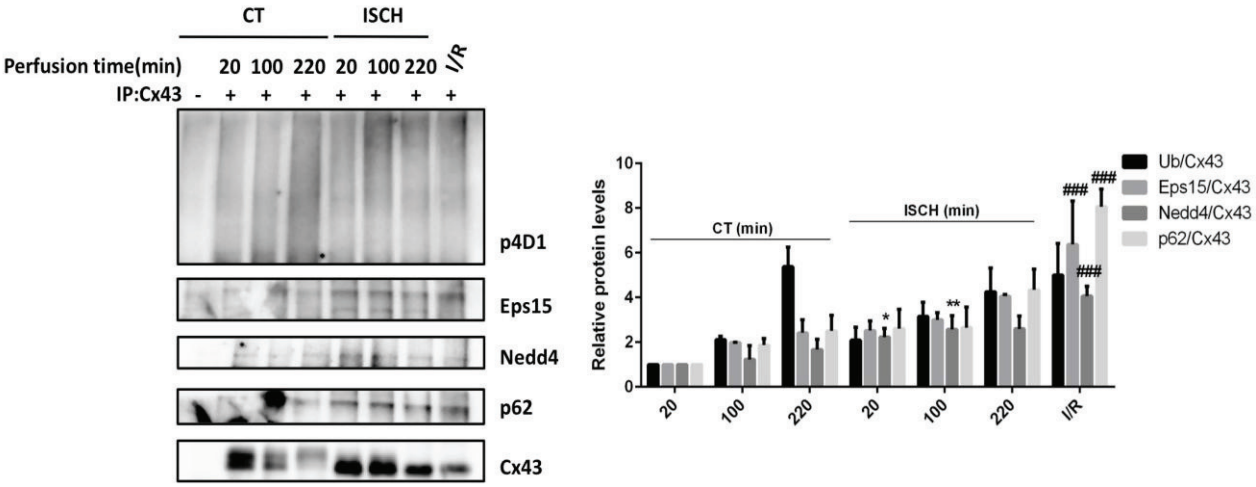

Figure 31 – Cx43 is ubiquitinated and interacts with the autophagy machinery in the ischemic heart. Hearts from 10-week-old rats were maintained using a Langendorff apparatus for 10 min, with modified KH buffer, followed either by 20, 100 or 220 min of normal perfusion (CT) or 20, 100 or 220 min of no-flow ischemia (ISCH). Reperfusion (I/R) was

induced by restoring normal perfusion for 100 min, after 20 of no-flow ischemia. Lysates of control or ischemic hearts were used for IP of Cx43, and immunoprecipitates further analyzed by WB, using antibodies against Ub (P4D1), Eps15, Nedd4, p62 and Cx43. The ratio Ub/Cx43, Eps15/Cx43, Nedd4/Cx43 and p62/Cx43 in each condition was calculated after densitometric analysis of WB, and values plotted on a graph. Values are mean ± SD (n=3). *p=0.05, **p < 0.01 vs CT (equivalent perfusion time), ###p<0.001 vs ISCH 20 min.

To confirm the involvement of autophagy in this ischemia-induced degradation of Cx43, CQ was administered to the animals, prior to the induction of ischemia in the Langendorff system. Consistently with the cell-based approaches, the results obtained showed that in animals treated with CQ, Cx43 is stabilized after 220 minutes of no-flow ischemia (Figure 32).

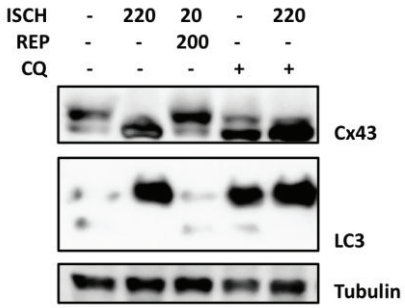

Figure 32 – Degradation of Cx43 is reverted by CQ in the ischemic heart. Hearts from 10-week-old rats, either prior injected with 60 mg/kg CQ or not, were maintained using a Langendorff apparatus for 10 min, with modified KH buffer, followed either by 220 min of normal perfusion (CT) or no-flow ischemia (ISCH). Reperfusion (I/R) was induced by restoring normal perfusion for 200 min, after 20 of no-flow ischemia. Lysates of control or ischemic hearts were analyzed by WB, using antibodies against Cx43 and LC3. Tubulin was used as loading control.

5. Discussion

GJIC is essential for the maintenance of a proper electrical and metabolic cellular coupling, which assumes particular importance in excitable tissues, such as the heart. Efficient communication between heart cells is crucial for the rapid anisotropic impulse propagation required for a synchronous and coordinated heart beating. Therefore, the mechanisms involved in the maintenance of GJIC have to be strictly regulated, as the impairment of intercellular communication may contribute to the development of disease. Accordingly, several cardiomyopathies, such as heart failure and MI, have been related to dysfunctional GJIC, as a consequence of GJ deregulation. Indeed, remodeling of cardiomyocyte GJ in the ischemic heart has been extensively reported, however the exact molecular mechanisms behind this effect remain unclear. Alterations on Cx43, the main component of cardiac GJ, have been identified, namely its dephosphorylation, subcellular redistribution and enhanced turnover at the onset of MI. Nevertheless, the underlying signaling, molecular mechanisms and partners involved remain largely undefined.

The results presented in this thesis show that activation of autophagy in the ischemic heart is responsible for degradation of Cx43. Using cell-based models, such as neonatal primary cultures of cardiomyocytes and the cardiac cell line HL-1, we demonstrate that total levels of Cx43 decrease in response to ischemia in a time-dependent manner. Furthermore, we show that ischemia-induced degradation of Cx43 can be partially reverted either by macroautophagy inhibitors (3-MA and OA), or by lysosomal inhibitors (Baf), as well as by the knockdown of Atg7 and p62. Importantly, proteasome inhibition, with MG-132, presented no protective effect, suggesting that degradation of Cx43 in ischemic cardiomyocytes cannot be ascribed to the proteasome. Moreover, we provide evidence showing that reperfusion following ischemia leads to enhanced degradation of the protein, when compared to similar periods of ischemia. Strikingly, we establish that degradation of Cx43 in response to ischemia or I/R relies on different molecular players and signaling pathways. Indeed, we demonstrate that AMPK-mediated autophagy is responsible for the degradation of Cx43 during early periods of ischemia, while Beclin 1-mediated autophagy constitutes the major pathway for degradation of Cx43 in late periods of ischemia and during reperfusion, in HL-1 cells. These findings were corroborated in more complex models, such as organotypic heart slices, where ischemia and I/R also lead to an autophagy-mediated degradation of Cx43. In the *ex vivo* model, the Langendorff heart, only I/R was able to induce a robust degradation of Cx43, while no-flow ischemia results in a slight degradation of the protein, only after prolonged times.

The establishment of the best model of simulated ischemia and I/R was the first objective of this study. We tested a cyanide-based model of chemical ischemia, and a model of metabolic ischemia based on hypoxia combined with a deoxyglucose-based medium. Results from both approaches were similar, either in the cell-based models, or in the organotypic heart cultures. However, once there is some controversy about the use of irreversible forms of metabolic inhibition, such as this cyanide-based model that interfere with

mitochondrial activity and can complicate interpretation of cell mortality upon further reperfusion, we found more appropriate in a pathophysiological context to proceed the studies using the hypoxia/deoxyglucose-based model. With this approach, the cells are subjected to an environment that mimics what happens in ischemia *in vivo*, which include absence of oxygen (hypoxia), and deprivation of nutrients (glucose) and acidosis. Additionally, the establishment of a cell-based model that could better reproduce the effects of ischemia upon cardiomyocytes was also a priority in this study. Given that in our hands the primary cultures of cardiomyocytes were not 100% pure, which could lead to a misinterpretation of the results obtained, we decided to choose a more homogenous, reliable and reproducible model – the HL-1 cardiomyocyte cell line. Since this cell line is established as a privileged model for studying many aspects of cardiac cell physiology, expresses high levels of endogenous Cx43, and is relatively easy to manipulate genetically, in opposition with the primary cultures, it constituted the best compromise for our further studies.

The subcellular fractioning performed in HL-1 cells, based on the biotinylation assays and the partitioning of Cx43 into the Triton insoluble fraction, suggest that Cx43 localized at the PM is the preferential target for ischemia-induced degradation. However, the confocal microscopy data shows that a significant proportion of Cx43 localizes intracellularly. These apparently conflicting results can be explained by the fact that Cx43 recovered in the Triton X-100–insoluble fraction may also arise from lysosomal membranes [106]. Since these organelles take part of the autophagy pathway, the presence of Cx43 in the Triton-insoluble fraction upon ischemia reinforces the hypothesis of autophagic involvement in Cx43 degradation.

When we inhibit the proteasome with MG-132, ischemia-induced degradation of Cx43 is not prevented. However, a slight protection conferred by MG-132 is observed. This protection may be explained by inhibition of the degradation of a small population of Cx43 in the context of ERAD, as a mechanism of protein quality control.

To demonstrate the involvement of the autophagy machinery in ischemia-induced degradation of Cx43, in HL-1 cells, we used chemical and genetic inhibition of autophagy, and in both cases the degradation of Cx43 was only partially reverted. This can be explained on the behalf of two possible scenarios – either by only partial inhibition of the process by our strategies (knockdown of p62 and Atg7 are not complete, neither 3-MA nor OA block all forms of autophagy), and/or by the involvement of other degradation pathways, such as endocytosis. Consistent with this hypothesis, inhibition of the lysosome, a common organelle to both endocytosis and autophagy, results in a more robust accumulation of Cx43. To clarify this question, additional studies need to be performed in order to discriminate the involvement of endocytosis and/or autophagy in degradation of Cx43 in the ischemic heart.

Interestingly, the results presented in this study show that in basal conditions, in HL-1 cells, Cx43 interacts with both LC3-I and -II, while in ischemia Cx43 mainly interacts with LC3-II, which is particularly

evident when we use OA to inhibit autophagy or Baf to inhibit autophagosome/lysosome fusion. Based on this data, it is conceivable to propose a model in which, under basal conditions, Cx43 binds to non-phagosomal LC3-I, after which it is directed to the autophagosome, with the concomitant maturation of LC3-I into LC3-II. On the other hand, in ischemia, due to PTM and/or conformational changes, the interaction of Cx43 with cytosolic LC3-I may be impaired, being Cx43 recruited directly to the autophagosome to be rapidly degraded. Indeed, recent *in silico* studies from our lab have identified the presence of a putative LIR motif in the Cx43NT, that might mediate interaction with LC3, which could explain these discrepancies [107]. Based on this putative motif, we hypothesized that under basal conditions, the binding of Cx43 to LC3 occurs though p62, thus requiring prior ubiquitination of Cx43, whereas during ischemia, the interaction of Cx43 with LC3 could be direct, mediated by the LIR motif. This could ascribe a role to Ub in the context of a quality control mechanism under basal conditions, while in ischemia, the autophagic degradation of the protein would depend on the direct interaction Cx43-LC3. However, additional studies are required to address this hypothesis.

Previous reports have shown that degradation of Cx43 by starvation-induced autophagy depends on prior Nedd4-mediated ubiquitination of the protein, and subsequent recruitment of Eps15 that acts as an autophagy adaptor, linking Cx43 and the autophagy machinery [34]. However, in our experimental conditions, the silencing of Nedd4 in HL-1 cells, did not present any protective effect against ischemia-induced Cx43 degradation. This can suggest that either the remaining levels of Nedd4, after silencing, are enough to catalyze Cx43 ubiquitination, or that another E3 ligase can be involved in this process, upon ischemic induction. Indeed, other E3 ligases have been associated with Cx43 ubiquitination and/or regulation of GJIC, such as TRIM21 and Smurf2 [108, 109]. Furthermore it is conceivable to suggest that Nedd4 plays an indirect role in the process, namely by regulating the autophagy machinery itself [58]. Indeed, Platta and colleagues demonstrated that Nedd4 catalyzes the ubiquitination and further proteasomal degradation of Beclin 1 [61]. In line with this study, it is likely that in the absence of Nedd4, accumulation of Beclin 1 contributes to enhance autophagy and consequently promote Cx43 degradation, instead of preventing it. Concerning Eps15, whereas its binding to Cx43 tends to increase with ischemia, it does not accumulate under autophagy or lysosomal inhibition, suggesting that interaction Eps15-Cx43 is only transitory and most likely involved in the internalization step of GJ degradation.

Previous studies showed that Ub signals starvation-induced degradation of Cx43 [34]. The results obtained either with HL-1 cells, or organotypic heart slices, suggest that ubiquitination is required for autophagic degradation of Cx43 under basal conditions, as demonstrated by the accumulation of ubiquitinated Cx43 in the presence of Baf. Although during ischemia we observe an increased ratio of ubiquitinated/non-ubiquitinated Cx43, the concomitant inhibition of the lysosome does not result in a

further increase of that ratio. These results can suggest that ischemia is just accelerating the degradation of ubiquitinated Cx43, instead of promoting the attachment of additional Ub moieties.

It is well established that p62 has a preponderant role on bridging ubiquitinated targeted substrates and the autophagosome [54]. In agreement, the results obtained in HL-1 cells show that in the absence of p62, Cx43 is resistant to ischemia-induced degradation, suggesting that recognition of ubiquitinated Cx43 by p62 is necessary for its concomitant recruitment and direction of the complex to the autophagosomes. For that reason, it would be expected to observe an increased interaction of Cx43 with p62 in ischemic conditions, however, the results obtained show that the interaction of Cx43 with p62 upon ischemia induction is not significantly altered, neither in the presence of Baf. Importantly, we demonstrate that the amount of p62 that is co-IP with Cx43, under basal conditions and in the presence of Baf, accompanies the accumulation of Cx43. This reinforces the idea that autophagy degradation of ubiquitinated Cx43, through a p62-dependent mechanism, occurs constitutively, as a mechanism of maintenance of protein quality control, and that ischemia only promotes the fusion of autophagic vesicles with the lysosome, rather than increasing the autophagic flux. Additionally, we show that knockdown of p62 leads to an increased interaction of Cx43 with NBR1 that is known to play a co-operative role along with p62 in the degradation of several autophagy substrates [56]. Although this data can suggest some level of redundancy regarding their role as adaptors for autophagic degradation of Cx43, the stabilization of Cx43 in the absence of p62 suggests that either NBR1 cannot direct Cx43 for degradation, or that both proteins are required to mediate the degradation of Cx43 by autophagy.

Various reports have shown that reperfusion is more detrimental than ischemia for cardiac cells, in part due to activation of autophagy [110]. Moreover, the severity of the ischemia that precedes reperfusion, significantly determines the extent of damage caused by autophagy activation when blood flow is restored [99]. Indeed, reperfusion after mild ischemia tends to be a period of cell recovery and re-adaptation after injury, whereas the activation of autophagy during reperfusion after severe ischemia is usually irreversible and deleterious, often leading to cell death. Consistent with this model, in this study we show that degradation of Cx43 is accentuated in reperfusion, in comparison with ischemia alone. Additionally, we demonstrate that during I/R, Cx43 levels are stabilized upon lysosomal inhibition. However, when autophagy is inhibited in its early phases, at the PI3KIII level, with 3-MA, no protective effect was observed. This suggests that the differential effect of autophagy inhibition on reperfusion-induced Cx43 degradation depends on the phase in which the degradation process is impaired. We speculate that, during reperfusion, the use of inhibitors of early phases of autophagy is irrelevant, since Cx43 has already reached a “point of no return” in the degradation process. According to this model, activation of autophagy during ischemia commits/signals Cx43 to lysosomal degradation, with reperfusion enhancing the degradation of Cx43-containing autophagic vesicles formed during ischemia. In agreement with this hypothesis, the stabilization

of Cx43 during reperfusion is only achieved when we used inhibitors of autophagosome-lysosome fusion, such as Baf.

The impact of autophagy activation in the heart, being either protective or harmful, is a controversial subject that has been a matter of intense debate in the scientific community. It is reasonably consensual that the dichotomous role of autophagy in the ischemic heart is largely associated with the severity of the stimuli, and, consequently, with the autophagy players involved [87]. The most widely accepted model, that attempts to conciliate the observations reported in the literature, states that AMPK is activated upon ischemia induction, by ATP depletion, while in later periods of ischemia or reperfusion, Beclin 1 plays a preponderant role [87, 111]. Consistent with this hypothesis, in this study we show that inhibition of AMPK protects Cx43 from degradation only in the first hours of ischemia, while silencing of Beclin 1 induces a protective effect against Cx43 degradation in longer periods of ischemia or during reperfusion. Taken together, this data supports a model in which degradation of Cx43 during early periods of ischemia depends on AMPK, whereas in late periods of ischemia and in reperfusion the process relies on Beclin 1 activation.

Additionally, given that GJIC is of extreme importance in the heart, the establishment of the functional consequences of autophagic degradation of Cx43, namely upon GJIC, would be of utmost importance. We can speculate that in the ischemic heart, a significant reduction in the Cx43 levels could reduce electric and metabolic coupling between heart cells, however further studies are required to proper address these questions. Indeed, we have performed a scrape-loading assay, in HL-1 cells, which allows visualization of the diffusion of a low–molecular weight dye permeable (lucifer yellow) through Cx channels, providing a good assessment of GJIC [112]. However, the hemichannel opening resultant of ischemia induction hinder our assay, since in cells subjected to ischemia, the diffusion of the probe was not restricted to GJ channels, making the results unreliable. Therefore, additional and more accurate studies are required to assess GJIC in our experimental conditions. Electrophysiology studies and a targeted dye-transfer, through microinjection, can constitute good approaches.

A direct extrapolation of these cell-based results to the heart should be considered with caution given the different subcellular distribution of Cx43 in HL-1 cells, when compared with the heart-resident cardiomyocytes. Indeed, in HL-1 cells, a significant part of Cx43 is localized in intracellular vesicles, being only a small part of Cx43 population localized at the PM. On the other hand, in the heart, Cx43 localizes mainly at the PM, more precisely at the IDs. Therefore, it is possible to argue that the autophagic degradation of Cx43 in HL-1 cells may be facilitated by the presence of the protein intracellularly, whereas in the heart, degradation of Cx43 requires its prior removal from the PM, which likely involves different machinery and signaling pathways. However, the results obtained with organotypic heart slices, a multicellular model of the myocardium that preserves some of the cardinal features of the heart, seem to

corroborate the results obtained with HL-1 cells. Nevertheless, organotypic slices were obtained from P4-P7 rat pups, and it was previously demonstrated that in rats, spatiotemporal distribution of some cardiac membrane proteins is a time-dependent process along the development of the heart. Indeed, Cx43 and N-Cadherin were not fully accumulated at the IDs until postnatal day 90 [113]. This makes the organotypic model similar to HL-1 cells, and for this reason, poorly representative of the heart physiology. Therefore, to evaluate the effect of ischemia in a more complex biological model that better resembles what happens at a mature organ level, we used the Langendorff system. When we use this *ex vivo* approach, we observed large differences comparing with both *in vitro* models. While in HL-1 cells and organotypic slices ischemia is a sufficient stimulus for the degradation of Cx43, in the heart, only I/R leads to such an extent of damage.

Previous studies, based on the use of cell-based models, demonstrated that internalization and degradation of Cx43 require phosphorylation and ubiquitination events, that depend on the presence of ATP [3]. Therefore, it is conceivable to anticipate that the same occurs in the heart. However, the large ATP depletion that characterizes ischemic events, occurs after a few minutes in the no-flow ischemic model that we use in the Langendorff, and may compromise this process. The autophagic process, by definition, is a mechanism of cell defense that ultimately leads to ATP production by increasing catabolism. However, being a mechanism that requires formation of vesicles and numerous processes of fusion and progression of these vesicles, residual levels of ATP are needed to maintain a continuous flux. Accordingly, we can suggest that, in the Langendorff heart, since ATP levels are scarce, the autophagic flux may somehow stop during ischemia, and only when reperfusion restores ATP levels, degradation of Cx43 can finally take place. Additionally, and in accordance with the results obtained in the HL-1 cardiomyocyte cell line, we can speculate that ATP depletion during no-flow ischemia can lead to activation of AMPK-mediated autophagy. Given the dichotomous role of autophagy in the ischemic heart, and the establishment of AMPK as a mediator of protective autophagy, AMPK triggering could account for the relatively modest levels of Cx43 degradation during early stages of ischemia. On the other hand, detrimental autophagy activation, likely mediated by Beclin 1 during late periods of ischemia and reperfusion, could explain the increased degradation of Cx43 during reperfusion in the *ex vivo* model. However, additional studies would be required to support our hypothesis.

We have previously demonstrated that internalization of Cx43 localized at the PM requires both the Nedd4-mediated ubiquitination of Cx43 and subsequent interaction with Eps15 [34]. Also, we have established that interaction of Cx43 with p62 is required for autophagic degradation of Cx43, likely as a post-internalization event [34]. To address whether these events are also involved in the internalization and degradation of ID-localized Cx43, we looked for interaction of Nedd4, Eps15 and p62 with Cx43 during ischemia in the Langendorff heart. Our results show that interaction of Cx43 with the three partners tends to increase during ischemia. Interaction with Nedd4 is mainly increased in the initial periods of ischemia, which is accompanied by an increased trend in the ratio ubiquitinated/non-ubiquitinated Cx43. This is

consistent with our model, in which ischemia induces the targeting of Cx43 for degradation, through Nedd4-mediated ubiquitination of the protein. During reperfusion, an increased interaction of Cx43 with Eps15 would enable internalization of Cx43 and further recruitment of p62 would direct Cx43 towards autophagic degradation. A proposed model for ischemia-induced degradation of Cx43 is illustrated in Figure 33.

Additional studies in more complex systems, such as *in vivo* models, are needed to establish the real pathophysiological significance of Cx43 autophagic degradation in the ischemic heart, namely its impact on GJIC and, consequently, in the electrophysiological properties of the heart. For example, to investigate the involvement of the autophagy pathway in the degradation of Cx43 in the ischemic heart, we can perform occlusion of the lateral ascending aorta *in vivo*, after which we can determine the interaction of Cx43 with the autophagy machinery. This might be particularly important in the context of ischemia-induced arrhythmias, where an impairment of GJIC has been consistently implicated. Understanding the molecular mechanisms involved in the degradation of Cx43 and its consequences would be a powerful tool to setting up direct targets for heart protection. Based on the data presented in this study, it is tempting to suggest that a special attention should be given to the pharmacological strategies used to preserve GJIC and correct electrical impulse propagation during ischemia and/or reperfusion. The data gathered in the present work may open new avenues for the development of targeted strategies, directed against AMPK or Beclin 1, that better accomplish the maintenance of heart function.

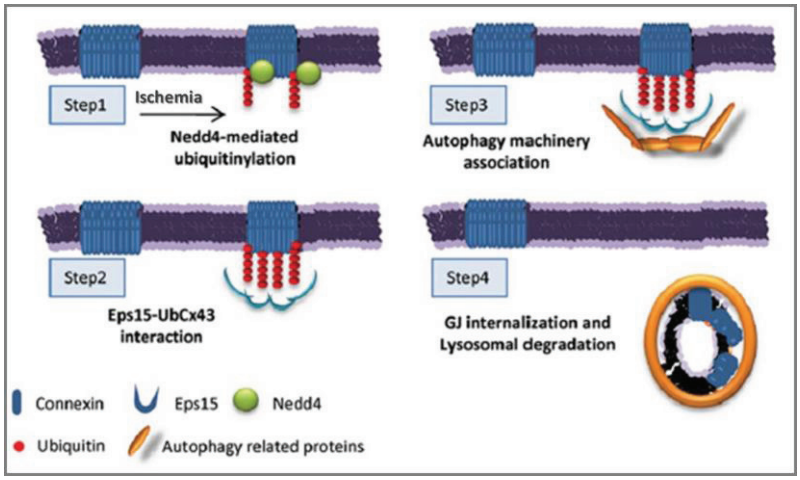

Figure 33- Proposed model for ischemia-induced degradation of Cx43. Under ischemic conditions, Cx43 is ubiquitinated by Nedd4, a recognized signal for interaction with Eps15 and proteins of the autophagy machinery. Consequently, Cx43 is internalized and degraded through autophagy, after fusion with the lysosomes. (Adapted from Bejarano E, et al. 2012).

6. Conclusions

Normal heart function relies on correct electrical and metabolic coupling between cardiomyocytes, which are accomplished by a proper GJIC. Exchange of second messengers, ions and small molecules between adjacent cardiomyocytes ensure efficient electric activation and action potential propagation, resulting in coordinated contraction. Therefore, dysfunction of GJ regulation has been associated with conduction block and arrhythmogenesis under several pathological conditions, such as MI. Remodeling of cardiomyocyte GJ at the onset of MI has been extensively reported, and includes Cx43 dephosphorylation, removal from the IDs and redistribution to the lateral sarcolemmal membranes. Albeit its importance and all the efforts to elucidate the mechanisms associated with this GJ remodeling, the molecular players and pathways involved remain obscure.

The results presented in this thesis show that degradation of Cx43 in cardiomyocytes, in response to ischemia, occurs through autophagy. Using cardiac cell lines, we show that during ischemia, ubiquitination of Cx43 enables the recruitment of Eps15 and subsequently p62 that is responsible for further directing Cx43 to LC3-positive autophagosomes. Moreover, we provide evidence showing that reperfusion following ischemia leads to enhanced degradation of the protein, when compared to similar periods of ischemia. Strikingly, we establish that degradation of Cx43 in response to ischemia or I/R relies on different molecular players and signaling pathways. Indeed, we demonstrate that AMPK-mediated autophagy is responsible for the degradation of Cx43 during early periods of ischemia, while Beclin 1-mediated autophagy constitutes the major pathway for degradation of Cx43 in late periods of ischemia and during reperfusion, in HL-1 cells. These findings were corroborated in more complex models, such as organotypic heart slices, where ischemia and I/R also lead to an autophagy-mediated degradation of Cx43.

In the *ex vivo* model, the Langendorff heart, only I/R was able to induce a robust degradation of Cx43, while no-flow ischemia results in a slight degradation of the protein, only after prolonged times. The molecular mechanism likely involves prior ubiquitination of ID-localized Cx43, mediated by Nedd4, and subsequent internalization through interaction with Eps15. p62 bridges internalized Cx43 and the autophagosome, ensuring the delivery of Cx43 to autophagic degradation.

Altogether, the data gathered in the present work may help on the understanding of the molecular mechanisms involved in Cx43 degradation in the ischemic heart. The establishment of the exact molecular partners during each phase of either ischemia or reperfusion could be a powerful tool to setting up direct targets for heart protection. Based on the data presented in this study, it is tempting to suggest that a special attention should be given to the pharmacological strategies used to preserve GJIC and correct electrical impulse propagation during ischemia and/or reperfusion. The data present in this work may open new avenues for the development of targeted strategies, directed against AMPK or Beclin 1, that better accomplish the maintenance of heart function.

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