Sérgio Tiago Freitas Ribeiro

# Quality Control of E-cadherin and Hereditary Diffuse Gastric Cancer

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra, para prestação de provas de Mestrado em Investigação Biomédica, no ramo de Oncobiologia.

Dissertation presented to the Faculty of Medicine of the University of Coimbra for the fulfillment of the requirements for a Master degree in Biomedical Research, branch of Oncobiology

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This work was done in the Center of Ophthalmology and Vision Sciences, at Institute of Biomedical Research of Light and Image at Faculty of Medicine at University of Coimbra and at Unity of Proteomics of Center of Neurosciences and Cellular Biology at technology park of Cantanhede – Biocant with the supervision of Doctor Joana Simões-Correia and Doctor Paulo de Carvalho Pereira.

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Part of this work was presented as poster at the following scientific meetings: 6th National Meeting on Cell Signaling and XXI Porto Cancer Meeting, with the title: E-cadherin is a substrate of Autophagy, with the participation of: Sérgio Ribeiro, Henrique Girão, Raquel Seruca, Paulo Pereira, Joana Simões-Correia

Este trabalho foi realizado no Centro de Oftalmologia e Ciências da Visão, no Instituto Biomédico de Investigação da Luz e Imagem (IBILI) da Faculdade de Medicina da Universidade de Coimbra e na Unidade de Proteómica do Centro de Neurociências e Biologia Celular da Universidade de Coimbra no Parque Tecnológico de Cantanhede – Biocant, sob a orientação da Doutora Joana Simões-Correia e do Doutor Paulo de Carvalho Pereira.

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Se há um adjetivo possa escolher para caracterizar este magnífico ano, no qual tive a oportunidade de realizar este trabalho, é evolução. Evolução, quer a nível científico, na forma de encarar os problemas/resultados na maneira de pensar e levantar novas questões quer a nível pessoal, no qual poderia descrever uma lista infindável de assuntos. Assim é às pessoas que estiveram envolvidas direta ou indiretamente neste processo quero deixar o meu sincero obrigado!

Paulo, Henrique, Joana, Hugo, João, Carla, Steve e Gladys, não esquecendo obviamente a família.

# ACRONYMS AND ABBREVIATIONS LIST

3-MA – 3-methyladenine

ACN – acetonitrile

ATG – autophagy-related gene

ATP – adenosine triphosphate

BSA – bovine serum albumin

CAMs – cell-cell adhesion molecules

CFTR – cystic fibrosis transmembrane conductance regulator

CHO – Chinese hamster ovarian

CMA – chaperone-mediated autophagy

DGC – diffuse gastric cancer

DMSO – dimethyl sulphoxide

DSP – Dithiobis[succinimidyl propionate]

DTME – Dithio-bismaleimidoethane

ECL – enhanced chemiluminescence

EMT – epithelial-mesenchymal transition

EOGC – early onset gastric cancer

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum associated degradation

FA – formic acid

FBS – fetal bovine serum

GC – Gastric cancer

GFP – green fluorescent protein

HDGC – hereditary diffuse gastric cancer

HRP – horseradish peroxidase

Hsc – heat shock cognate

Hsp – heat shock proteins

IDA – information dependent acquisition

IGC – intestinal-like gastric cancer

IGCLC – International Gastric Cancer Linkage Consortium

IP – immunoprecipitation

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

LC-MS/MS – liquid chromatography coupled to tandem mass spectrometry

LC3 – microtubule-associated protein 1 light chain 3

MS – mass spectrometry

MTOC – microtubule organizing center

PBS – phosphate buffered saline

PE – phosphatidylethanolamine

PFA – paraformaldehyde

PM – plasma membrane

PQC – protein quality control

PVDF – Polyvinylidene difluoride

QC – quality control

SDS–PAGE – sodium dodecylsulphate–polyacrylamide gel electrophoresis

UPR – unfolded protein response

UPS – ubiquitin-proteasome system

WT – wild type

# THESIS ORGANIZATION

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

The first chapter consists of an introduction to the subject. There is a general introduction to Hereditary Diffuse Gastric Cancer and the implications of mutations in E-cadherin as a major cause of this disease. The chaper also briefly describes the principal mechanisms involved in protein quality control, including protein degradation by the ubiquitin-proteasome system (UPS) and the lysosome-dependent degradation. At the end of the chapter the major objectives of the work are presented.

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

In third chapter, the results obtained in this study are shown in figures and tables and are described in the text. This chapter presents data showing that autophagy involved in the degradation of both WT and mutant (R749W and E757K) E-cadherin. In the same chapter the molecular partners binding to WT and mutant E757K E-cadherin are identified.

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

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

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# ABSTRACT

E-cadherin is a transmembrane protein that mediates cell-cell adhesion and is a well-known tumor suppressor. Germline mutations of CDH1 (gene encoding E-cadherin) are the only known genetic cause of Hereditary Diffuse Gastric Cancer (HDGC).

The aims of this work are to establish the role of autophagy in the regulation of WT and HDGC-associated mutant E-cadherin and to identify the putative molecular players that are likely to be implicated in the quality control and stabilization of E-cadherin at plasma membrane.

The autophagy was induced in stable CHO cell lines expressing WT or HDGC-associated CDH1 missense mutations. Protein expression (total amount or membrane fraction) was analyzed by Western Blot and, where appropriate, by biotinylation of membrane proteins. Subcellular distribution was assessed by immunofluorescence confocal microscopy. The interactome of WT and mutant form of E-cadherin was identified by liquid chromatography coupled to tandem mass spectrometry.

Data show that E-cadherin is a substrate of autophagy. Induction of autophagy by serum deprivation leads to a dramatic decrease in the total levels of WT E-cadherin. In addition, the mutant forms of E-cadherin that accumulates as intracellular aggregates are also cleared following serum deprivation. Consistently, immunofluorescence data shows that intracellular aggregates of mutant E-cadherin colocalize with autophagic vesicles that is revealed by GFP-LC3. Consistent with a role for autophagy in regulation of E-cadherin, data show that both lysosome inhibitors and impairment of autophagy by knocking down Atg7, result in a stabilization of WT and mutant E-cadherin. In this study we also identified, for the fist time, some binding partners that selectively associate with mutant E-cadherin. These include, Hsp90beta, lamin-A/C and microtubules associated proteins. Conversely the E757K mutant E-cadherin is unable to associate with proteins such as p120-catenin, Hsc70 and FAM that bind to WT E-cadherin. Other proteins, such as alpha- and beta-catenin, associate to a different extent with WT and mutant forms of E-cadherin.

# RESUMO

A caderina-E é uma proteína transmembranar e um importante supressor tumoral, responsável em larga medida pela manutenção da adesão entre células adjacentes. A importância desta proteína como supressor tumoral é bem ilustrada pela observação de que as mutações no gene CDH1 (que codifica a cadherina-E) em células germinativas são a única causa genética conhecida para o cancro gástrico difuso hereditário (HDGC).

Os principais objectivos deste trabalho consistem em avaliar o papel da autofagia na regulação da quantidade e distribuição subcelular da caderina-E. Procuraram ainda identificar-se os interactores moleculares especificamente associados às formas mutantes da caderina-E comparando-os com os que interagem com a forma WT da proteína. Procuraram interpretar-se estes resultados no contexto do controlo de qualidade proteico e em particular nos mecanismos responsáveis pela estabilização da caderina-E na membrana plasmática.

O efeito da autofagia na regulação da caderina-E foi estudado em células CHO que expressam quer a forma WT quer as variantes mutantes (mutações "*missense")* da caderina-E associadas ao HDGC. Os níveis totais de proteína foram determinados por western blot. Os níveis de caderina-E membranares foram determinados na sequência da biotinilação de proteínas membranares seguida por western blot e confirmados, sempre que apropriado, por microscopia confocal de imunofluorescência. O interactoma da caderina-E (WT e mutante) foi determinado por espectrometria de massa associada a cromatografia líquida.

Os resultados mostram que a caderina-E é um substrato para a autofagia. De facto, a indução de autofagia por privação de soro, conduz a um decréscimo dramático nos níveis totais de caderina-E WT. Por outro lado, a caderina-E mutante que se acumula na forma de agregados intracelulares desaparece rapidamente em situações de privação de soro. A hipótese de que a autofagia participa na degradação destas formas de caderina-E é corroborada pelos resultados de imunofluorescência que mostram claramente que os agregados intracelulares de caderina-E mutantes se co-localizam com vesículas autofágicas que integram o marcador quimérico GFP-LC3. Por outro lado, quer os

inibodores do lisossoma quer a inibição da autofagia (por silenciamento da Atg7) resultam numa estabilização das formas mutantes e WT da caderina-E.

Neste trabalho, identificaram-se, pela primeira vez, algumas das proteínas que interatuam seletivamente com a forma mutante da caderina-E associada ao HDGC. Estas proteínas incluem Hsp90beta, lamina A/C e proteínas associadas aos microtúbulos. Por outro lado a forma mutante E757K da caderina-E não se associa a algumas proteínas que geralmente se encontram associadas à forma WT da caderina-E, tais como catenina p-120, Hsc70 e FAM. Foi ainda identificado um outro grupo de proteínas, onde se incluem a alfa e beta-catenina, que se associam em quantidade/extensão diferente à forma mutante e WT da caderina-E.

# Chapter 1: Introduction

## Introduction to Cancer

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries [1]. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets [2].

Causes for cancer development are to a large extent multifactorial and most likely involve genetic predispositions as well as environmental risk factors that drive the normal cell into highly malignant derivatives by a progressive transformation process.

A single cell can acquire a set of advantageous alterations that allow autonomous proliferation, invasion of adjacent tissues and metastization. Most types of human tumor cells are thought to share common alterations in cell physiology that collectively dictate malignant growth. These set of alterations are known as the six hallmarks of cancer and are the following: (i) the self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory (antigrowth) signals, (iii) evasion of programmed cell death (apoptosis), (iv) limitless replicative potential, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis [3].

During the development of most types of human cancer, some cells escape the primary tumor and invade adjacent tissues, colonizing in new sites in the body. Formation of metastasis are thought to be responsible to about 90 % of human cancer deaths [4]. Tissue invasion and metastization are very complex processes and the genetic and biochemical determinants involved remain to be completely elucidated. A number of studies over the years have attempted to identify proteins and protein modifications that are associated with various forms of cancer, particularly by contributing to increased invasive potential and increased metastatic potential. These proteins include cell-cell adhesion molecules (CAMs), like E-cadherin that mediates cell-to-cell interaction, and integrins, which link cells to extracellular matrix substrates. The loss of E-cadherin function characterizes the progression of the majority of epithelial cancers (including gastric cancer that will be

described hereafter) [3]. Although no fully understood, this loss is commonly a result of the acquisition of mutations in E-cadherin encoding gene (CDH1), which include splice site and truncate mutations, caused by insertions, deletions, and nonsense mutations, as well as missense mutations [5].

## Gastric Cancer

Gastric cancer (GC) is the fourth most common form of cancer worldwide and is the 10$^{th}$ leading cause of mortality [6]. The incidence rates of gastric cancer is about twice as high in males as in females and the prevalence varies widely between different regions of the world, with high prevalence in South America, Eastern Europe and Central Asia [2]. Due to the late diagnosis of gastric cancer, the survival rates remain low, and even after curative treatments or after adjuvant therapy approximately 60 % of the affected patients die [7].

GC can be divided into two main histological subtypes: intestinal-like gastric cancer (IGC) characterized by glandular structures morphology; and diffuse gastric cancer (DGC) characterized by poorly differentiated cells that exhibit infiltrative growth. The vast majority of GCs are sporadic, which can be associated with environmental factors, such as *Helicobacter pylori* infection, high-fat diet, or smoking, and is often associated with somatic mutations acquired in the gastric tissue [8]. However, there is a small percentage (1-3 %) of GC cases that were clearly shown to arise as a result of an inherited genetic-predisposition component and are referred as hereditary diffuse gastric cancer (HDGC) cases.

### Hereditary diffuse gastric cancer

HDGC is an autosomal dominant cancer predisposition syndrome caused by heterozygous germline mutation in the E-cadherin gene (CDH1), on chromosome 16q22. CDH1 germline mutations can also be identified in sporadic early onset gastric cancer (EOGC), which is defined by the presence of de novo mutations in patients with less then 40 years of age at the time of diagnosis [9]. Different patterns of CDH1 germline mutations have been described as truncating, deletion, insertion, splice site, non-sense, silent, and missense alterations. Currently, there are 122 CDH1 germline mutations described, 72 % of these are non-missense and 28 % missense mutations [10].

The definition of the HDGC syndrome was established by the International Gastric Cancer Linkage Consortium (IGCLC) that defined the following clinical criteria for diagnosis of HDGC: (i) Two or more documented cases of GC in first or second degree relatives, with at least one diagnosed before the age of 50; (ii) three or more cases of documented DGC in first/second degree relative, independent of age of onset; (iii) individuals with DGC before the age of 40 years without a family history and (iv) individuals and families with diagnoses of both DGC (including one case below the age of 50 years) and lobular breast cancer [10,11]. The prophylactic total gastrectomy is the single valid approach for management of asymptomatic carriers of CDH1 germline mutations in HDGC families [11].

## E-cadherin

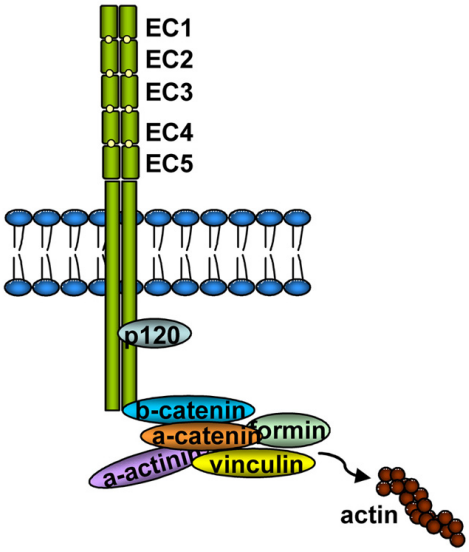
E-cadherin, the major component of the adherens junctions, is a protein that mediates cell-cell adhesion, in a calcium-dependent manner, through homophilic bindings between molecules on adjacent epithelial cells. This 120 kDa transmembrane glycoprotein is coded by CDH1 gene and expressed in epithelial cells [12].

The E-cadherin extracellular domain composed of five repetitive subdomains, called cadherin repeats or EC domains, and each subdomain contains calcium-binding sequences (Figure 1). The interaction of calcium ions with these sequences controls the conformation of the extracellular domain.

The adherens junction complex is stabilized by binding of beta-catenin to alpha-catenin [13]. Impairment of the adherens junction components, in particular E-cadherin and beta-catenin, has been demonstrated to play an important role in induction of epithelial-mesenchymal transition (EMT) in development and tumorigenesis [14].

E-cadherin is involved in many biological processes including cell adhesion, morphogenesis, cytoskeletal organization and cell sorting/migration, as well as in pathological conditions such as cancer [15]. This well-known tumor suppressor protein is involved in suppression of invasion and metastasis formation of epithelial cells. The bridges formed by E-cadherin between cells result in the transmission of antigrowth and other signals via cytoplasmic interactions with intracellular signaling circuits namely through beta-catenin. The loss of function of E-cadherin is present in the majority of

epithelial cancers. Experimentally, the forced expression of E-cadherin in cultured cancer cells and in a transgenic mouse model of carcinogenesis impairs invasive and metastatic phenotypes, whereas interference with E-cadherin function enhances both capacities [3].




Loss of E-cadherin expression is frequently associated to genetic events such as splice site and truncation mutations caused by insertions, deletions, and nonsense mutations, in

**Figure 1 - E-cadherin complex**
The extracellular region of E-cadherin consists of five cadherin-type repeats (EC domains; extracellular cadherin domains) that are bound together by $Ca^{2+}$ ions. The core-universal catenin complex consists of p120 catenin and beta-catenin, which in turns binds alpha-catenin. In a less understood way, alpha-catenin binds to actin and actin-binding proteins, such as vinculin, alpha-actinin and formin-1.
Adapted from N. Rivard, Frontiers in Bioscience, 2009

addition to missense mutations [5]. These genomic alterations of the CDH1 gene causing loss-of function of E-cadherin, have been identified in a variety of tumors. Somatic E-cadherin mutations were identified in sporadic lobular subtype of breast cancer, endometrial and ovarial carcinomas. In addition, these genetic alterations of E-cadherin (mutations, deletions and methylations) are the only recognized genetic cause of HDGC [14,16–18].

The E-cadherin missense mutations found in cancer frequently lead to native-state destabilization, and lead to the development of DGC earlier in life [19]. E-cadherin folding is surveyed by mechanisms of protein quality control (PQC) and HDGC-associated

mutations can be prematurely degraded by the endoplasmic reticulum associated degradation (ERAD), a mechanism responsible for the clearance of misfolded and unstable proteins, dependent on the proteasome [20].

## Protein Quality Control

Eukaryotic protein homeostasis, or proteostasis, enables healthy cell and organismal development as well as ensures a better adaptation to aging by protecting cells against acute and chronic insults. Proteostasis is a complex and integrated biological network within cells, comprising various pathways that control the biogenesis, folding, trafficking and turnover of proteins present within and outside the cell. Deficiencies in this PQC system lead to many metabolic, oncological, neurodegenerative, and cardiovascular disorders [21].

The maintenance of the protein folding is essential to proteostasis and many diseases appear to be caused by deregulation of protein folding including loss-of-function diseases (cystic fibrosis) and gain-of-function diseases (like Alzheimer's, Parkinson's and Huntington's disease). The accumulation of misfolded, aggregation prone and potentially cytotoxic polypeptides can be generated by mutations, transcriptional and translational errors or cellular and environmental stresses [22]. To maintain the cellular function and viability, it is important that molecular mechanisms that recognize and eliminate misfolded or otherwise damaged or obsolete proteins function properly. During evolution several systems evolved to ensure these critical functions. These systems include, but are not limited to, the ubiquitin-proteasome system (UPS) and the lysosome-dependent degradation.

### The ubiquitin-proteasome system

The UPS is the main proteolytic system involved in the selective degradation of soluble proteins. The UPS is a complex system involved in numerous cellular events where protein degradation is required either to dispose obsolete proteins or to regulate various biological processes. The UPS can be, very broadly, divided into two different parts: recognition and targeting of proteins destined for degradation and proteolytic degradation of targeted proteins. Recognition and targeting of proteins is mediated by the attachment of a small peptide called ubiquitin to the protein. The following degradation involves recognition of the ubiquitin chain attached to the protein substrate and subsequent

unfolding of the substrate and degradation by a large proteolytic particle called the proteasome [23].

### The proteasome

The proteasome is a, 26S, multicatalytic protease that degrades polyubiquitinated proteins to produce small peptides. It is composed of two subcomplexes: the 20S core particle that carries the catalytic activity, and a 19S regulatory particle schematically represented in Figure 2. The 20S core particle is a barrel-shaped structure arranged as a stack of four rings, two α and two β, each of them composed of seven subunits. The general structure is αββα, being that the catalytic sites are located in the β-rings. In fact, among the seven subunits that compose the β-rings three have catalytic properties, β1, β2 and β5, which mediate caspase-like, trypsin-like or chemiotrypsin-like activity, respectively. One or both ends of the 20S barrel can be capped by a 19S regulatory particle that is composed of 17 distinct subunits, nine in a "base" subcomplex and eight in a "lid" subcomplex. One important function of the 19S regulatory particle is to recognize polyubiquitinated proteins. A second function is to open an orifice in the α-ring, which allows the substrate to enter the proteolytic chamber. Six different ATPase subunits are present in the base of 19S regulatory particle indispensable for functions that require metabolic energy like channel-opening and the substrate-unfolding. Following substrate degradation, short peptides that have been derived from the substrate are released [24–26].

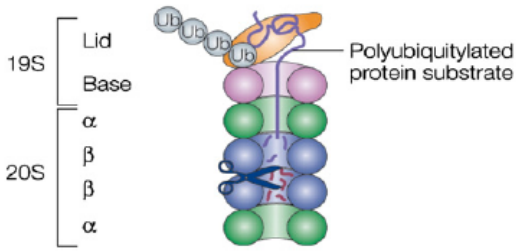



**Figure 2 – Schematic representation of the structure and function of the 26S proteasome**
Adapted from A. Ciechanover, Nature reviews. Molecular cell biology, 2005

### Ubiquitin conjugating cascade

Ubiquitin is a small (76 residue), heat-stable and highly evolutionarily conserved protein ubiquitously expressed in all eukaryotic cells.

Most of the 26S substrates have to be polyubiquitinated so the prime tag for proteasomal degradation is a chain of four or more ubiquitin moieties covalently linked to lysine residue(s) of the subtract [25]. Ubiquitin has seven internal lysines (K6, K11, K27, K29, K33, K48, and K63) that can be linked through the amino terminal to the carboxyl terminal of another ubiquitin molecule, forming polyubiquitin chains [27]. K48-linked polyubiquitin chains represent the canonical proteasomal degradation tag, but more recently other chains were also identified as being able to target substrates to degradation, although often by unconventional mechanisms, these include K11 and- K63-linked polyubiquitin chains [28].

Ubiquitination of protein substrates is a very complex process involving a large number of enzymes and other ancillary proteins. In the most simplified configuration an enzymatic cascade involving three major classes of enzymes E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase) is responsible for targeting substrates by attachment of an ubiquitin chain. The human repertoire consists of two ubiquitin-specific E1 activation enzymes, about 30 E2 conjugation enzymes, and more than 1000 E3 ligases providing a great versatility in substrate recognition and enabling diversity in ubiquitin chain linkages added to substrates [23,29,30]

## Autophagy (Lysosomal-dependent degradation)

Autophagy was initially thought to be a form of cell response and adaptation to lack of nutrients, it is now realized that autophagy is a highly regulated and multipurpose system. The best-characterized form of activation of autophagy remains nutrient deprivation. When nutrients are scarce, autophagy allows a cell to break down its own components, including proteins, organelles and even pathogens that reach the cytosol after cell invasion, and recycle important molecules. It is, in this context, a very important PQC system that represents the adaptation of a single cell to starvation/ nutrient deprivation. The cell is forced to break down part of its own reserves to stay alive until there is food available in the surroundings [31,32]. In unicellular organisms such as yeasts, starvation response is one of the primary functions of autophagy, but in fact this function extends up to humans. For example, even on a day-to-day basis, autophagy is activated between meals in organs such as the liver to maintain its metabolic functions, supplying amino

acids and energy through catabolism [33]. The field of autophagy has undergone rapid expansion in recent years, due for the most part, to a better molecular characterization of the different steps of this process, which include cargo-recognition, sequestration from the cytosol, delivery to lysosomes, degradation and recycling of the essential components of the macromolecules degraded. This has also revealed new biological functions for autophagy and an unanticipated cross talk between various proteolytic pathways and between other components of PQC such as chaperones.

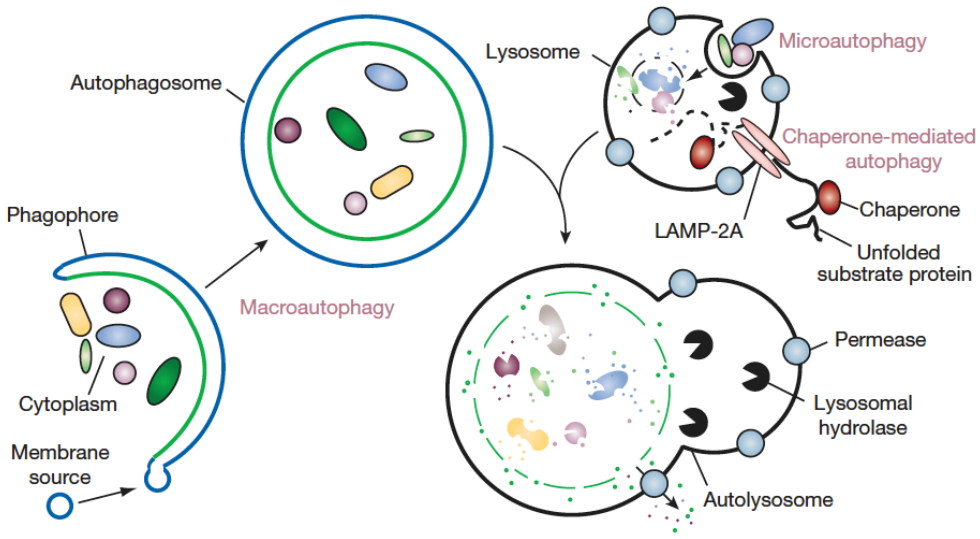



**Figure 3 - Different types of autophagy**
In macroautophagy, the cargoes are sequestered within a unique doublemembrane cytosolic vesicle, an autophagosome. Lysis of the autophagosome inner membrane and breakdown of the contents occurs in the autolysosome after the fusion with lysosome. Microautophagy refers to the sequestration of cytosolic components directly by lysosomes through invaginations in their limiting membrane. CMA involves direct translocation of unfolded substrate proteins across the lysosome membrane through the action of a cytosolic and lysosomal chaperone hsc70, and the integral membrane receptor LAMP-2A (lysosome-associated membrane protein type 2A).
Adapted from N. Mizushima, Nature, 2008

Cytosolic cargo is recognized and targeted for autophagy through different mechanisms that set the basis for the distinction of several types of autophagic pathways. The three best-characterized forms of autophagy, shown in Figure 3, include macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [31]. Variants of each of this type of autophagy have been described and named to indicate the cargo preferentially degraded: mitophagy (autophagy of mitochondria), pexophagy (autophagy of

peroxisomes), lipophagy (autophagy of lipid droplets), and aggregophagy (autophagy of aggregates) [28].

The essential component of these proteolytic systems is the lysosome, single membrane vesicles that contain in their lumen the larger variety of cellular hydrolases including proteases, lipases, glycosidases, and nucleotidases [34].

### Macroautophagy

The macroautophagy (also referred to as autophagy) is the only process that can mediate the degradation of larger substrates such as organelles, microbes, and protein aggregates.

Autophagy is initiated by the formation of a double-membrane structure, the phagophore (Figure 3). The elongation of this membrane depends on two ubiquitin-like conjugation reactions. First, autophagy-related gene 12 (ATG12) is conjugated to ATG5 resulting in the formation of an oligomeric ATG5-ATG12-ATG16L complex. This complex is then needed for the conjugation of ATG8 homologues (termed microtubule-associated protein 1 light chain 3-I – LC3-I – in mammalian) to phosphatidylethanolamine (PE) on the phagophore membrane, resulting in LC3-II (conjugated form). This process is catalyzed by an ubiquitination-like reaction performed by an E1-like enzyme (Atg7) and an E2-like enzyme (Atg3) [30]. LC3-II proteins are also involved in membrane biogenesis of autophagosomes via their membrane fusion activity [35]. Autophagosomes are formed by closure of the phagophore into a double-membrane vesicle [30].

The autophagosomes often form at the cell periphery are moved linearly toward the perinuclear region at the microtubule organizing center (MTOC) area where lysosomes are localized. The mechanism involved in this transport remains to be elucidated, however the involvement of LC3 in this regulation is an interesting possibility, as it was originally identified as microtubule-associated protein light chain 3 [36].

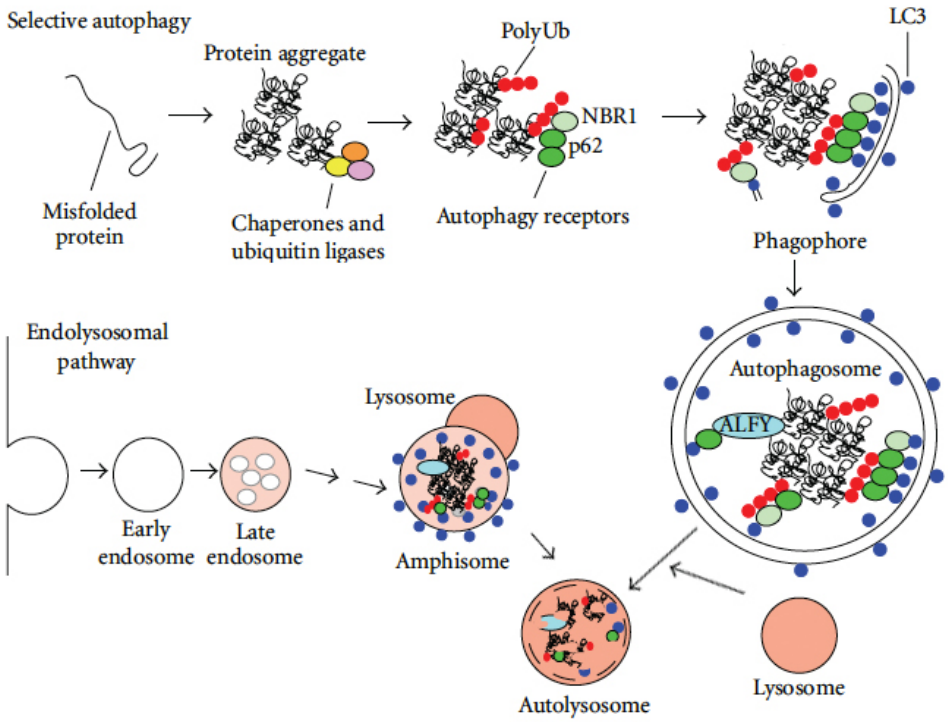
The fusion of autophagosomes with late endosomes or lysosomes is the final step in this process that results in degradation of their contents (Figure 3, last step) [30].

### Selective macroautophagy

Macroautophagy was initially described as a form of indiscriminate degradation by which cytosolic substrates are degraded ‘‘in bulk’’. However, although this may still be true for

soluble cytosolic proteins that are trapped along with other cargo as the autophagosomes form, the selective autophagy (Figure 4) determines the selective degradation of organelles, bacteria, ribosomes, specific proteins, and protein aggregates by autophagy. In this PQC, proteins acting as autophagy receptors such as p62 and NBR1 that bind directly to LC3 (that are themselves degraded later by autophagy) play an important role on identification and selection of proteins that might be degraded [30,31].

The formation of larger protein aggregates is regarded as a cellular defense mechanism because the large aggregates or inclusions are less toxic to the cell than the presence of smaller microaggregates dispersed throughout the cell [37]. Selective autophagy is an important quality control (QC) system and is part of a basal constitutive autophagy that can also be induced or boosted by various stressors including oxidative stress, infections, protein aggregation, and proteasomal inhibition [30].



### Microautophagy

This is probably the less characterized and less understood form of autophagy in mammals. In yeast, microautophagy (Figure 3) occurs at the vacuole (equivalent to the lysosome) and makes use of some macroautophagy genes and some microautophagy-specific genes [31,38,39].

In microautophagy the sequestration of cargo occurs directly at the surface of the lysosomes by invaginations of the lysosomal membrane, where they are rapidly degraded. Microautophagy activity can be detected under basal conditions in many cell types, but there is currently no information as to whether this pathway can be further upregulated under specific cellular conditions [31].

### Chaperone-mediated autophagy

As opposed to the macroautophagy and microautophagy, CMA, as so far, only been

**Figure 4 - Selective macroautophagy**
Ubiquitination of misfolded-protein aggregates induces the recruitment of p62 and NBR1. These cargo receptors also bind to LC3-II (blue dots) of the inner surface of the phagophore. The autophagosome fuses with a late endosome or with a lysosome, but the end point is in both cases the formation of an autolysosome where the contents are degraded.
Adapted from T. Lamark, Journal of Cell Biology, 2012

observed in mammals [39].

All CMA substrates described until now are, soluble proteins [31], that are selectively targeted, one-by-one, to the lysosomes and are then translocated across the lysosomal membrane into the lumen where they are degraded by resident proteases (Figure 3) [31]. Substrates for this pathway are cytoplasmic proteins that contain in their amino acid sequence a pentapeptide motif biochemically related to the sequence KFERQ. When exposed (i.e. during protein misfolding or disassembly of protein complexes) this sequence is recognized by the cytoplasmic form of the chaperone hsc70 in a process that can be modulated by the associated co-chaperones, leading to the delivery of the substrate protein to a receptor at the lysosomal membrane. This receptor was indentified as the lysosome-membrane protein type 2A (LAMP-2A), and binds to CMA substrates facilitating, in conjunction with the lysosomal form of hsc70, their translocation into the lysosomal lumen where substrates are degraded [40].

Basal CMA activity occurs in all cells, but it can be further upregulated in response to several stressors including prolonged starvation and conditions leading to protein damage, such as oxidative stress. About 30% of cytosolic proteins bear the CMA-targeting motif in their sequence, which makes them putative CMA substrates. However, degradation through this pathway depends on the accessibility of this motif to hsc70, suggesting that conformational changes in the substrate protein, posttranslational modifications, or changes in interacting proteins that usually mask the motif, could be triggers for CMA degradation [31]. These might explain the relatively small number of CMA substrates that have been identified so far.

## Chaperones in Protein Quality Control

The proteostasis network consists of various biological pathways. The macromolecular components of these pathways comprise over 1000 general and specialized chaperones, folding enzymes, and degradation components as well as trafficking components, that determine compartment localization [22].

Protein folding, unfolding, and refolding are constantly occurring throughout the lifetime of nearly all proteins. Chaperones promote folding and maintenance of conformation within the cell largely by minimizing misfolding and aggregation, but the chaperones also escort terminally misfolded proteins or irreversibly damaged proteins to the proteolytic pathways for degradation [41].

The chaperones associated with the endoplasmic reticulum (ER), e.g. calnexin and calreticulin, BiP and ERp 57 are able to recognize misfolded proteins and help their retention in the ER, allowing only correctly folded proteins to reach the cytosol, where a complex consisting of heat shock proteins (Hsp): Hsp70/Hsp40 and Hsp90 chaperone systems mediate the PQC by facilitate folding by encapsulation of newly synthesized proteins [30].

In inherited diseases associated with a folding deficiency, it is well established that a change in the amino acid sequence of a protein can significantly alter folding energetics and, therefore, the normal function of the protein. In this process the chaperones participate in protein folding/unfolding, repairing misfolded proteins, and preventing the aggregation of unfolded/misfolded proteins. One example are the chaperones like the heat

shock proteins BiP (also termed Grp78, an ER homologue of Hsp70) and its co-chaperone Hsp40 turned out to prevent aggregate-formations of attached proteins [42].

## Quality control at the plasma membrane

The selective recognition and elimination of conformationally defective membrane proteins from post-ER compartments has been postulated more than a decade ago. Direct perturbations of transmembrane domains by insertion of charge residues or depletion of lipid rafts enriched in sphingolipid can also sensitize plasma membrane (PM) proteins for conformational destabilization and subsequent recognition by the peripheral QC.

Some documented structural defects in the PM proteins are related with their rapidly elimination from cell surface. The mutant variant of cystic fibrosis transmembrane conductance regulator (CFTR) is one example of this process. However the structural perturbation that is necessary and sufficient to target a PM proteins for degradation remains to be determined [43]. Interestingly, it has been shown that autophagy might also function as a mechanism of QC at the PM, resulting in degradation of PM proteins (such as connexins), in an ubiquitination dependent manner [44]. Ubiquitin-binding clathrin adaptors (e.g. epsin1 and eps15/eps15R) mediate the rapid endocytosis of aberrant PM proteins. These clathrin adaptors can recognize both K63-linked and K48-linked poly-Ubiquitin chain. In addition, BAG-1, an Ubiquitin-like domain containing Hsc70 co-chaperone, may link the chaperone-PM protein complex to Ubiquitin-binding adaptors to the internalization and lysosomal sorting machinery through its Ubiquitin-like domain [43].

# Chapter 2: Methods

## Cell culture

Chinese Hamster Ovarian (CHO) cells (ATCC number: CCL-61) were the cellular model used in this work. Stable CHO cells were generated by transduction of wild type (WT) human E-cadherin, mutant E-cadherin (R749W or E757K) or empty vector (Mock), as described by Simões-Correia [20]. Cells were grown in completed medium: alpha-MEM medium (Gibco, Invitrogen) supplemented with 10 % fetal bovine serum (FBS; Gibco, Invitrogen), 1 % penicillin-streptomycin (Gibco, Invitrogen) and 5 mg/ml blasticidin (Gibco, Invitrogen), in a humidified incubator with 5 % $CO_2$ at 37 ºC.

When applicable, cells were transient transfected with 500 ng/ml of plasmids encoding green fluorescent protein (GFP)–LC3 using Lipofectamine 2000 (Invitrogen), following the manufacturer instructions.

Atg7 -/- cells were generated by with transduction with lentivirus containing the ATG7 shRNA, using 8 μg/ml of polybrene (Sigma).

### Reagents and Cell treatments

**Starvation**

Cells were plated in six-well plates and grown to about 70–80 % confluence. Then the growth medium was replaced, after rinsed with phosphate buffered saline (PBS), with completed medium for control conditions or with alpha-MEM medium (without FBS) for nutrient deprivation conditions.

**Chloroquine**

Cells were plated in six-well plates and grown to about 70–80 % of confluence. Then the growth medium was replaced, after rinsed with PBS, with completed medium or medium without FBS containing 50 μM chloroquine.

## Immunofluorescence and microscopy

The cells were seeded on glass coverslips and grown to at least 80-90 % confluence. The cells were kept on ice and were washed with PBS/$Ca^{2+}$ (0.05 mg/ml) three times, fixed

with 4 % paraformaldehyde (PFA) in PBS for 10 minutes, washed with PBS, blocked with 5 % bovine serum albumin (BSA) in PBS and incubated with primary antibody diluted in PBS containing 5% BSA for 1 hour at room temperature. The primary antibody used was the mouse monoclonal anti E-cadherin (clone HECD-1; 1:300; Invitrogen Corporation). Secondary antibodies were Alexa 488 goat anti-mouse or Alexa 594 goat anti-mouse (Invitrogen). The coverslips were mounted on slides using Vectashield with DAPI (Vector Laboratories).

Images were collected using an Axiovert 200 fluorescence microscope (Carl Zeiss) equipped with a 63× objective and 1.4 numerical aperture and subjected to deconvolution with the manufacturer's software.

## Biotinylation of cell surface proteins

The precipitation of biotinylated proteins was used to analyze the amount of protein at the plasma membrane.

A confluent cell monolayer was incubated with 0.5 mg/ml of Sulpho-NHS-SS-biotin (Thermo Scientific) in PBS/$Ca^{2+}$ (0.05 mg/ml) for 30 min on ice. Whole cell lysates were prepared according to the protocol described above. An aliquot of 400 μg of protein (with the same volume) was incubated overnight at 4 ºC under shaking conditions with 50 μl of Streptavidin agarose beads (Amersham Biosciences). To separate the surface fraction from the cytoplasmic fraction, the samples were spin down, and the supernatant (cytoplasmic fraction) was recovered, quantified and eluted in Laemmli buffer. The pellet (surface fraction) was washed with PBS/$Ca^{2+}$ (0.05 mg/ml) and also eluted in Laemmli buffer. The samples were loaded on 7.5 % sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblot analysis.

## SDS–PAGE and western blotting

Cells were lysed in cold catenin lysis buffer (1 % Triton X-100, 1 % Nonidet P-40 in PBS) enriched with a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma).

The proteins were quantified using a modified Bradford assay (DC Protein Assay, Bio-Rad) or BCA Protein Assay (Pierce) in case of immunoprecipitated proteins, following

the manufacture instructions. For following analysis, 25 µg of total proteins or immunoprecipitates of E-cadherin from 1 mg of total proteins were denatured in Laemmli buffer, boiled for 5 minutes, and loaded in a 7.5 or 12 % SDS–PAGE. After electrophoretic separation, the proteins were transferred to nitrocellulose blotting paper (Amersham Biosciences) or to a polyvinylidene difluoride (PVDF) membrane with a sandwich transfer system (Bio-Rad).

The membranes were blocked with 5 % non-fat milk and 0.5 % Tween-20 in PBS and probed with the appropriate primary antibodies: anti E-cadherin (1:1000, BD Biosciences or 1:2500, Invitrogen Corporation), beta-catenin (1:1000; Sigma), GAPDH (1:5000; Abcam), LC3 (1:1000; Thermo Scientific), Atg7 (1:1000; Sigma). Specific bound antibodies were detected by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized after enhanced chemiluminescence (ECL) detection (BioRad) by chemiluminescence system (BioRad).

The blotted strips were quantified with the ImageJ software. All results are representative of at least three experiments. Data are expressed as a sample mean ± standard deviation (SD). The different samples were compared using the Student's T test and two-tailed probability.

## Liquid Chromatography coupled to tandem Mass Spectrometry

### Immunoprecipitation

To immunoprecipitate the proteins of interest for mass spectrometry (MS), we used the dynabeads co-immunoprecipitation (IP) kit (Invitrogen). In this protocol the antibody was firstly bound covalently to dynabeads and then the beads-bound antibody was used for the IP experiments.

Each 1 mg of cells was lysed in 9 µl cold extraction buffer (IP buffer 1× supplied in the kit, 0.5 % tritonX-100, 100 mM NaCl, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (Sigma)). 5 mg of total protein isolated for each condition was quantified using the BCA protein assay (Pierce). Each 1 mg of dynabeads was previous incubated with 5 µg of antibody of E-cadherin or goat IgG (Santa Cruz Biotechnology) for negative control), and then total proteins were incubated with beads-antiboby for 3

hours with shaking conditions at 4 ºC. The following procedure was performed as indicated by the manufacturer.

The elutes were dried by rotary evaporation under vacuum using a vacufuge plus vacuum concentrator (Eppendorf). All samples were denatured in Laemmli buffer during 5 minutes and then resolved in an "Any kD Resolving Gel" (BioRad) followed by staining with silver or coomassie blue.

### Silver staining

A modified silver staining method according to O'Connell [45] was implemented for the detection of low level proteins separated by electrophoresis. After electrophoresis the gel was rinsed with distilled water and then immersed in the fixation solution (25 % (v/v) methanol and 5 % (v/v) acetic acid) during 30 minutes. Then, the gel was washed by consecutive immersion for 10 minutes in alcoholic solutions of gradually decreasing concentrations (50 % (v/v) ethanol followed by 30 % (v/v) ethanol). The gel was immersed in a sodium thiosulfate solution (0.2 mg/ml) for one minute and for 20 minutes in a silver nitrate solution (2 mg/ml). Finally the developing solution (0.7 ml/l of a solution of 37 % of formaldehyde, 30 mg/ml sodium carbonate anhydrous and 10 mg/l of sodium thiosulfate) was added. The developing solution was changed by stop solution (50 mg/ml Tri and 2.5 % (v/v) acetic acid) when the desired staining was achieved.

### Colloidal coomassie staining

Despite the lower sensitivity compared with silver staining, coomassie blue staining protocol, described by Candiano [46], was performed because it is fully compatible with subsequent MS. This staining was performed as previously described with some slight modifications. Briefly, after electrophoresis, the gel was rinsed in distilled water following by immersion in the staining solution (10 % (v/v) of 85 % solution of phosphoric acid, 10 % (w/v) ammonium sulfate, 20 % (v/v) methanol and 0.2 % Commassie Brilliant Blue G (Pierce)). The commassie powder was added to the solution with a filter to prevent clotting of the dye and allows development of colloidal particles.

Gel was allowed to stain overnight under agitation and then the gel was washed with abundant water and maintained in water with sodium azide until further analysis.

### Sample processing and peptide extraction

After of Coomassie blue staining, the entire gel lanes were sliced into 37 bands of equal size using an "OneTouch GridCutter" (Gel Company). Then, each band was sliced in small pieces and was transferred to a tube with 1 ml of water. The bands were distained with 1 ml of distaining solution (50 mM ammonium bicarbonate and 30 % acetonitrile). After mix at 850 rpm for 15 minutes the distained solution was removed. The gel bands were washed with water for 10 minutes at 850 rpm and then the water was removed and the pieces were dehydrated on vacufuge plus vacuum concentrator (Eppendorf) during 1 hour.

To digest the proteins 30 µl of trypsin were added (15 ng/µl in solution of 10 mM ammonium bicarbonate) and left for 10 minutes on ice. Then, 15 µl of 10 mM ammonium bicarbonate were added (at final concentration of trypsin 10 ng/µl) and incubated overnight at room temperature in the dark. The peptides were collected to low binding microcentrifuge tubes (Eppendorf) and the gel pieces were removed by sequential addition of 50 µl of following solutions: acetonitrile (ACN) in 1% formic acid (FA; 30, 50, and 98 % of ACN, respectively).

The peptides were dried by rotary evaporation under vacuum (Vacufuge plus Vacuum Concentrator) and ressuspended in 22 µl of a solution of 2 % CAN and 0.1 % FA followed by vortex and sonication in a water bath (during 1 minute with pulses of 1 second). The peptide mixtures were centrifuged for 15 minutes at maximum speed and transferred into a new tube.

Before performing MS/MS analysis the peptide mixtures were cleaned/desalted using stop-and-go-extraction tips (stage tips) with C18 stationary phase (Thermo Fisher Scientific). Briefly, the peptide mixtures were equilibrated with 80 µl of the solution of 5 % FA and tip columns were pre-conditioned with 180 µl of 90 % ACN and 5 % FA and equilibrated with 30 µl of 5 % FA. Peptide samples were loaded on the column for 3 times followed by washing step with 5 % FA solution and elution to new tubes with 40 µl

of 70 % ACN and 5 % of FA. Elutes were dried by rotary evaporation and the samples were adjusted to 22 µl and transferred into vials for posterior MS/MS analysis.

### Protein identification

Protein identification experiments were carried out on a hybrid quadruple/linear ion-trap mass spectrometer (4000 QTrap; Applied Biosystems Sciex) using a nano-electrospray ionization source (Nanospray II, Applied Biosystems Sciex) and a dual gradient pump (Ultimate 3000 LC, LC packing Dionex). The mass spectrometer was programmed for information dependent acquisition (IDA) scanning full spectra, followed by an enhanced resolution scans to determine the ion charge states, and set the appropriate collision energy for fragmentation. The IDA cycle was programmed to perform 6 MS/MS on multiple charged ions (+1 to +4) and performed two repeats before adding ions to the exclusion list for 60 seconds.

Samples were loaded onto a trap column (PepMap C18), at 6 µl/minute during 5 minutes. The peptides separation was carried out by nano-LC on a PepMac C18 reverse phase nano column (75 µm × 15 cm, 3 µm, 100 Å, Dionex) at 250 nl/minute or on a Monolithic C18 reverse phase nano column (150 mm × 0.1 mm, 130 Å, Onyx) at 1 µl/minute. Peptides were eluted into the mass spectrometer with an acetonitrile gradient in 0.1 % FA (2 to 98 % ACN, in a multiple step gradient for 50 minutes) using a nano-electrospray source.

# Chapter 3: Results

## The role of autophagy in E-cadherin quality control

In normal epithelial cells, E-cadherin is synthesized, binds to beta-catenin and is transported to the lateral membrane to constitute the adherent junctions [47]. However, hereditary diffuse gastric cancer (HDGC)-associated CDH1 missense mutation (e.g. R749W and E757K) are less expressed at the cell surface [20]. It is already established by our group that a number of HDGC-associated E-cadherin mutations can be degraded by the ubiquitin-proteasome pathway [20]. Additionally, lysosome-dependent degradation has been shown to regulate the levels of wild type (WT) E-cadherin, namely in epithelial mesenchymal transition [48]. Autophagy is a lysosome-dependent proteolytic pathway involved in protein quality control of aberrant proteins. We hypothesized that the lysosome-dependent autophagy pathway could be involved in the degradation of E-cadherin. To gain further insight into the mechanisms and to address if autophagy is involved in degradation of WT and/or mutant E-cadherin a number of experiments were conducted in Chinese hamster ovarian (CHO) cell lines. A well established stimulus to induce autophagy is starvation or the cell culture equivalent of nutrient (or serum) deprivation [49]. In this work, CHO cells stably transduced with an empty vector (Mock) or with the WT, R749W or E757K human E-cadherin were serum-deprived and the levels of E-cadherin were determined by immunoblotting after resolution on sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

### Mutants forms of E-cadherin (R749W or E757K) present low expression levels of E-cadherin and different distribution in the cell

We assessed the total protein content in cells expressing WT or mutant forms of E-cadherin. As already described by our group [20], mutant E-cadherin R749W and E757K express lower levels of total protein (presented in Figure 5) that correspond to 60 % and 25 %, respectively, of the WT protein expression. It has been shown that mRNA levels of mutant and WT E-cadherin are approximately the same [20], suggesting that mutant forms of E-cadherin are pos-transcriptionally regulated, possibly by increased degradation.

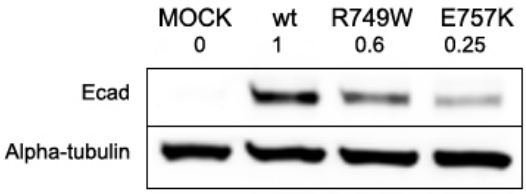



**Figure 5 - Mutants (E749W and E757K) and WT E-cadherin expression pattern in CHO cells**
Immunoblot detection of E-cadherin (Ecad) and Alpha-tubulin (as a control). The cells with an empty vector (MOCK) and cells expressing WT or mutant (R749W or E757K) human E-cadherin were resolved into a SDS-PAGE. The same amount of total protein (25 μg) was used. This image shows the representative results repeated at least tree times.

We analyzed the subcellular distribution pattern of WT and mutant E-cadherin by immunofluorescence (Figure 6). As described before [20] the WT human E-cadherin localizes at the plasma membrane (PM), whereas mutants R749W and E757K are predominantly found accumulated in perinuclear regions, with lower levels at the PM. The different levels of surface expression of WT and mutant forms (R749W and E757K) of E-cadherin were also verified by immunoblot after biotinylation assay (Figure 7). This results show that cells overexpressing mutant E-cadherin (R749W and E757K) have lower levels of this protein at the PM compared to the cells with WT E-cadherin.

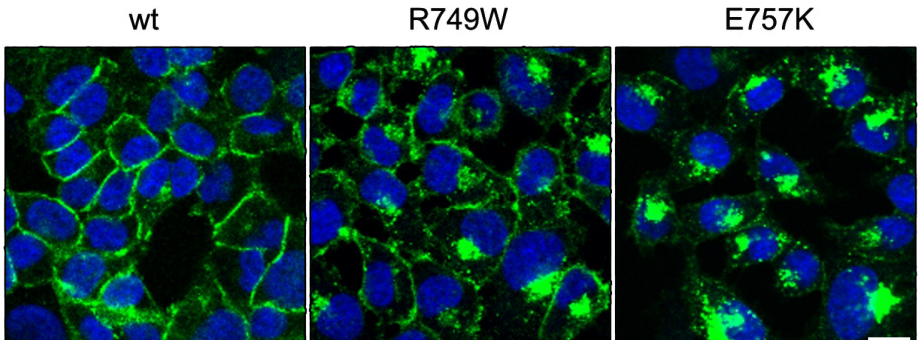



**Figure 6 - Subcellular distribution pattern of WT and mutant (R749W and E757K) E-cadherin**
CHO cells with stable transduction with WT or mutant R749W or E757K human E-cadherin. Cells were fixed, permeabilized and co-immunostained with anti-human E-cadherin. E-cadherin was visualized with FITC-conjugated antibody. Images were acquired by confocal microscopy. Scale bar represents 10 μm.

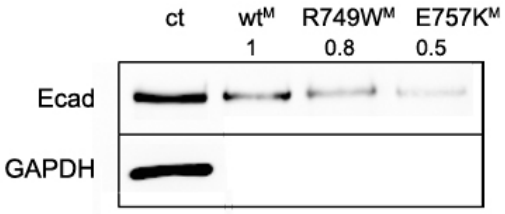



**Figure 7 – Plasma membrane expression of WT and mutant (E749W and E757K) E-cadherin in CHO cells**

Immunoblot detection of membrane fraction ($^{M}$) of E-cadherin (Ecad) obtained by biotinylation assay from CHO cells with stable expression of WT or mutant forms (R749W and E757K) of human E-cadherin. 25 μg of total protein lysate of WT cells was used as control reference (ct). Anti-GAPDH antibody was to evaluate the efficiency of the membrane fraction isolation. The values represent the relative amounts of proteins and were obtained by normalization with the WT.

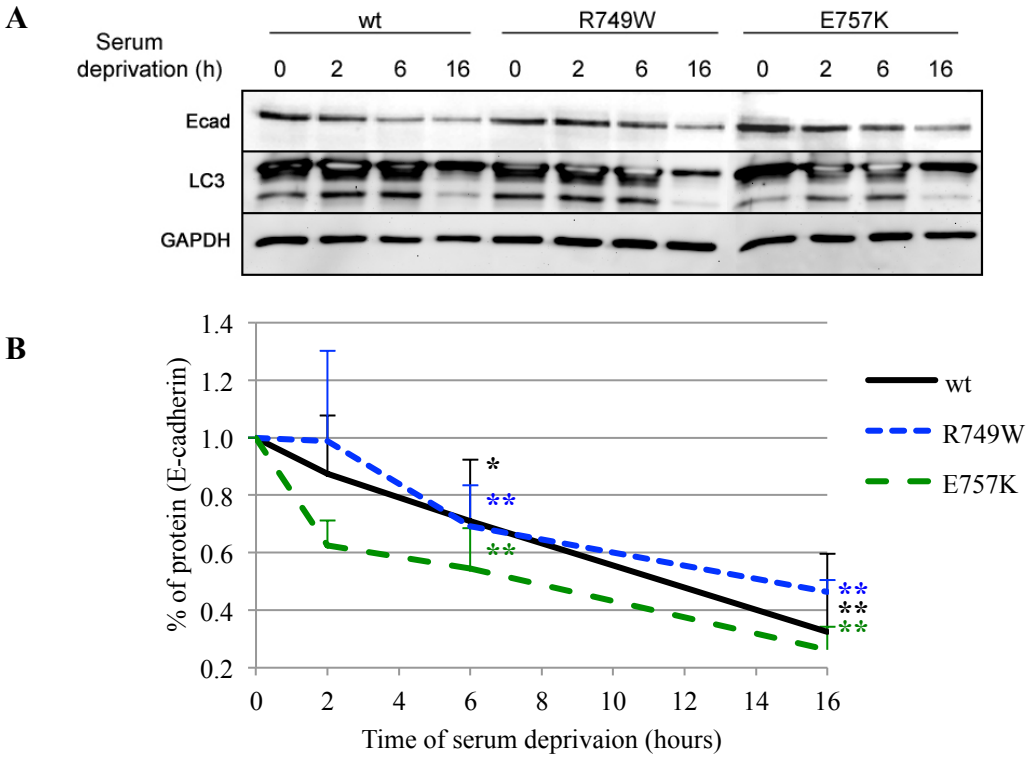



**Figure 8 - E-cadherin is downregulated upon nutrient deprivation**

Levels of E-cadherin (Ecad) in CHO cells stabling expressing WT or mutant forms (R749W and E757K) of human E-cadherin. The cells were incubated with serum-deprived medium for 2, 6 or 16 hours or complete medium (time zero).

(A) Immunobloting showing the time-dependent decrease in WT and mutant forms of E-cadherin. To monitor the activation of autophagy the anti-LC3 antibody was used after transient transfection of cells with GFP-LC3 construct. The top band refers to the LC3-I and the lower band is the LC3-II. In all conditions, 25 μg of protein cell lysates was loaded. Anti-GAPDH antibody was used as loading control. (B) Levels of E-cadherin upon serum deprivation expressed as a percentage of the levels detected in control cells (time zero) for each cell line (WT, R749W or E757K)

(p value was calculated by student's T-test in relation to time zero for each condition, *=$p<0.005$ and **=$p<0.001$; error bars represent standard deviations of at least three independent experiments).

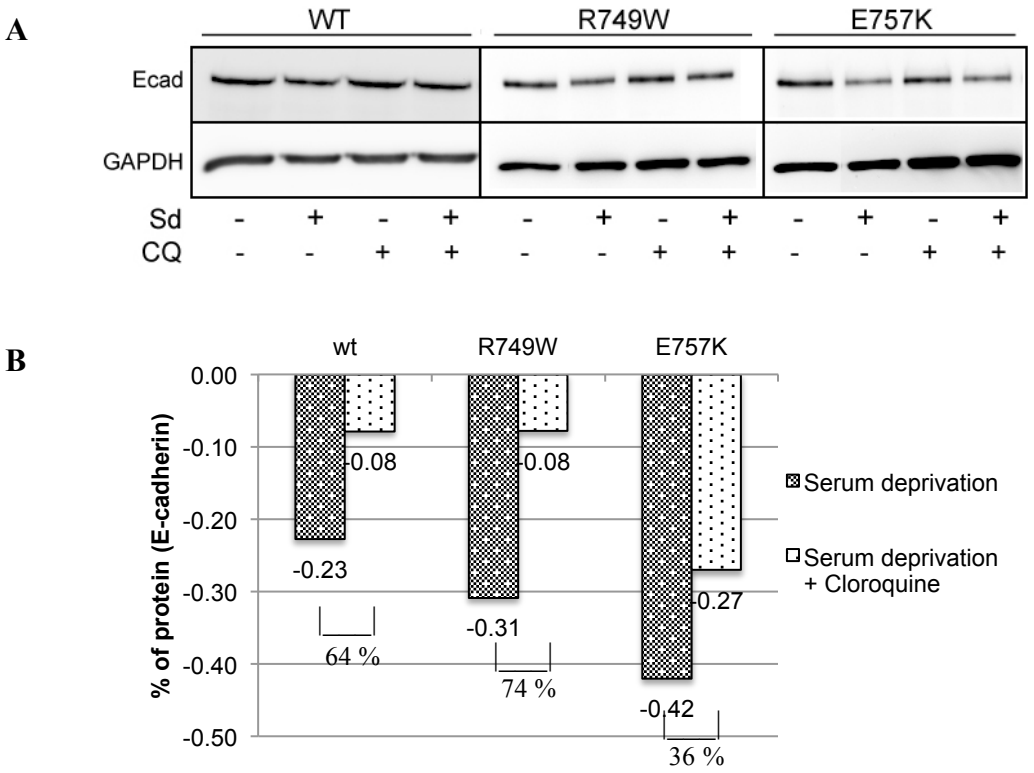
### Nutrient deprivation decreases the levels of WT and mutant (R749W and E757K) E-cadherin

To analyze the role of autophagy in protein quality control of E-cadherin and evaluate if it differently influences WT and mutant forms, CHO cells stably expressing WT and mutant E-cadherin (R749W or E757K) were serum starved and analyzed over time, using time-points of 2, 6 or 16 hours. The levels of E-cadherin were subsequently determined by immunoblotting. Figure 8A shows a representative immunoblot that confirms the time-dependent decrease in the levels of E-cadherin under nutrient deprivation. It is also possible to observe the typical increase in the levels of LC3-II during nutrient deprivation, indicating that autophagy is activated. Figure 8B is a graphical representation of the percentage of WT and mutant E-cadherin expressed over time, upon nutrient deprivation, normalized to the amount expressed in time zero (non-nutrient deprivation conditions). Both WT and mutant forms of E-cadherin show decreased expression. Data shows that both WT and R749W mutant E-cadherin have a 30 % decrease, whereas E757K mutant E-cadherin decreased to 50 % after 6 hours of serum deprivation.

### The effects of serum deprivation on WT and mutant E-cadherin can be selectively reverted by inhibiting autophagy

The lysosomes are critical organelles in autophagy and inhibition of the lysosome is often used as a control to verify the involvement of autophagy in protein degradation. To verify if autophagy participates in the degradation of E-cadherin, cells were incubated with the lysosomal inhibitor (chloroquine) under serum deprivation. The results in Figure 9B show that the blockage of autophagy by inhibition of the lysosome with chloroquine leads to an accumulation of E-cadherin indicating that the lysosome is involved in the degradation of E-cadherin in these conditions. The E-cadherin is stabilized upon incubation with chloroquine when the cells are incubated with serum-deprived medium (Figure 9). The WT and mutant forms (R749W and E747K) of E-cadherin present different percentages of protein stabilization, 64 % 74 % and 36 % respectively. It is also possible to observe an increase of the total amount of WT and mutant E-cadherin after 6 hours when the cells are incubated with chloroquine in relation to controls (Figure 9A)

implying that the lysosome is also involved in E-cadherin degradation in basal




conditions.

## E-cadherin colocalize with autophagosomal marker (LC3) and the aggregates of mutants E-cadherin are degraded upon nutrient deprivation

To verify if autophagy is implicated in the degradation of the aggregated forms of E-cadherin, CHO cells stably transduced with WT or mutant forms (R749W or E757K) of human E-cadherin were transient transfected with green fluorescent protein- microtubule-associated protein 1 light chain 3 (GFP-LC3), and treated during 6 hours without serum and/or chloroquine. The immunofluorescence images (Figure 10) show an increase of autophagosomal structures labeled with GFP-LC3 after serum deprivation. The inhibition of the lysosomal proteases, by chloroquine, leads to an increase in the number and size of

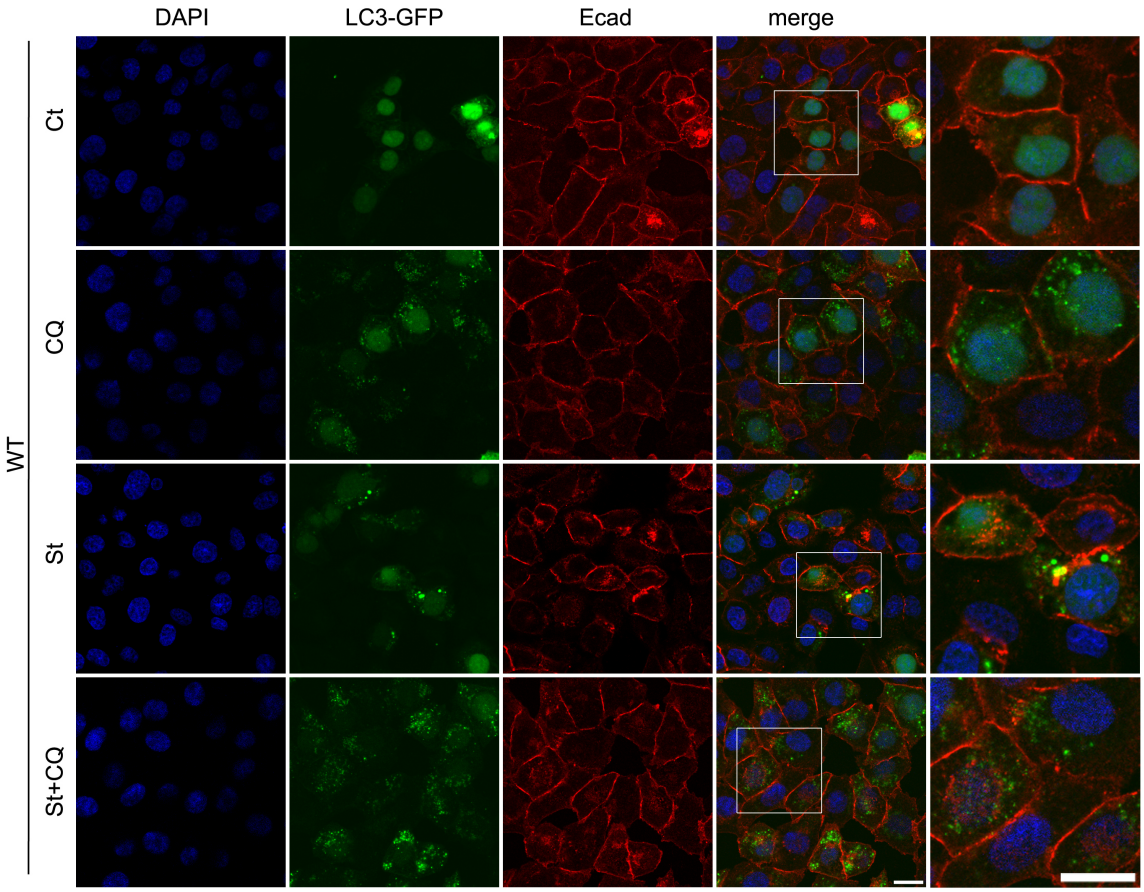
**Figure 9 - Stabilization of E-cadherin with chloroquine**

CHO cells (expressing WT, R749W or E757K E-cadherin) were serum-deprived (Sd), treated with chloroquine (50 μM; CQ) or serum-deprived and treated with chloroquine for 6 hours. Cells with no treatment were used as a control of tretments. (A) Representative immunoblot detection of E-cadherin (Ecad) and GAPDH as a loading control. Aliquots with the same amount of total protein (25 μg) were resolved on SDS-PAGE. (B) E-cadherin levels represented as a percentage of control without treatments corresponding to zero percent of each cell line (WT, R749W or E757K). The stabilization of protein with chloroquine is present in percentage for each condition. The amount of protein was determined by densitometric quantification of the bands corresponding to E-cadherin immunoblot.

GFP-LC3-labeled vesicles compared with untreated cells. Following six hours of nutrient deprivation the characteristic aggregates of mutant forms of E-cadherin disappears or decrease in R749W or E757K E-cadherin respectively.

## Destabilization of E-cadherin at the plasma membrane by inhibition of Autophagy

Autophagy is important in homeostasis in the cell and our results indicate that it might have a role in post-transcriptional regulation of E-cadherin. To demonstrate that autophagy is involved in the regulation of the amount of E-cadherin protein in the cell, we established autophagy-null cells lines, by stable transduction of a short-hairpin RNA (shRNA) against a fundamental autophagy player, Atg7. We observe an increase of total amount of E-cadherin by inhibiting of autophagy (Figure 11B), reveling that autophagy regulates E-cadherin levels. Interestingly, using biotinylation of PM E-cadherin, we also demonstrate that, despite the increase in total levels, WT and mutant E-cadherin are destabilized at the PM in the cells with stable inhibition of autophagy (Figure 11A).



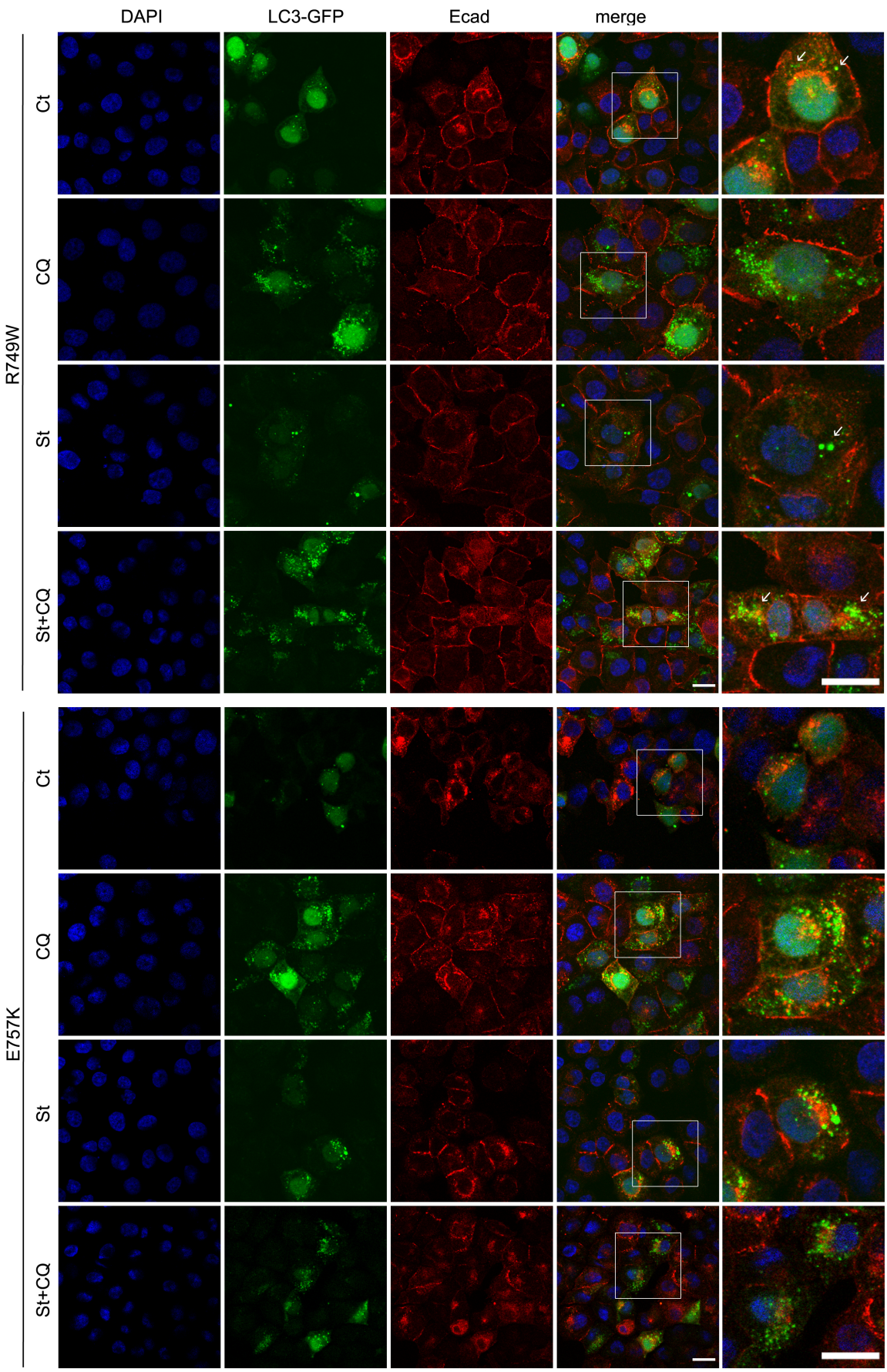

DAPI
LC3-GFP
Ecad
merge
R749W
Ct
CQ
St
St+CQ
E757K
Ct
CQ
St
St+CQ

**Figure 10 - E-cadherin partially localizes in autophagosomal structures**

Immunofluorescent confocal microscopy shows the immunolocalization of E-cadherin and LC3. CHO cells stably transduced with WT, R749W or E757K human E-cadherin (red) were transiently transfected with GFP-LC3 (green). Cells were incubated under control conditions (Ct), serum deprivation (Sd), lysosome inhibitor (chloroquine, CQ,; 50 μM) or serum deprivation and lysosome inhibition during 6 hours (Sd+CQ). The cells were then fixed, permeabilized and immunostained with anti-human E-cadherin antibody. E-cadherin was visualized with Texas Red-conjugated secondary antibody (red) and the nuclei were stained with DAPI (blue). The fourth column shows the merged images and the boxes regions are shown at hither amplification in last column. The arrows indicate some of the LC3-positive autophagosomal structures. Scale bar represents 10 μm.

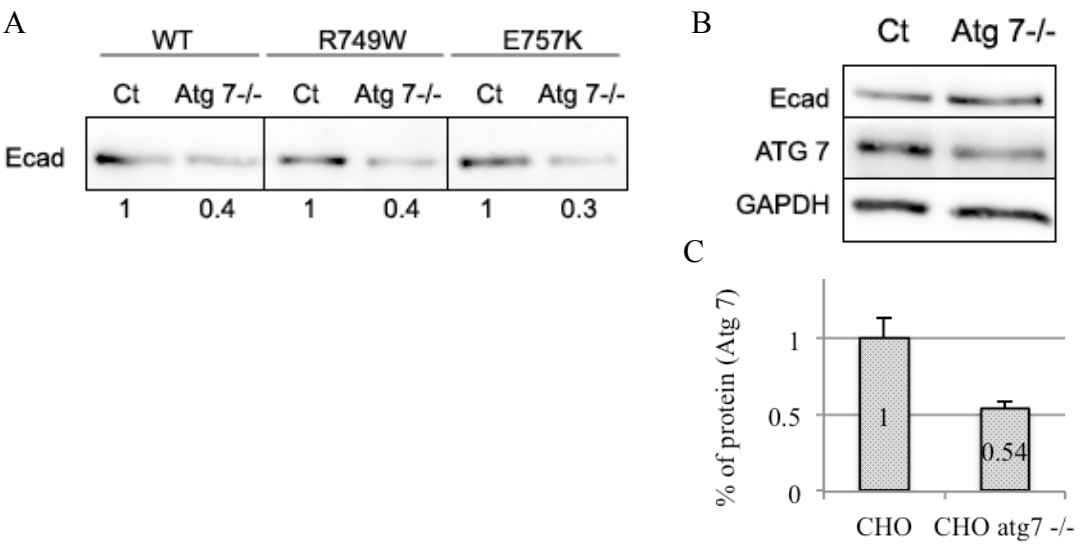



**Figure 11 – Membrane E-cadherin decreases by inhibiting the autophagy**

(A) Immunoblot detection of membrane fraction of E-cadherin (Ecad) obtained by biotinylation assay from CHO cells with stable expression of both WT or mutant forms (R749W and E757K) of human E-cadherin and shRNA of ATG7 (Atg 7-/-) or the empty vector (Ct). The values represent the relative amount of E-cadherin in relation to each control. (B) Immunoblot detection of E-cadherin, Atg7 or GAPDH from CHO cells Atg7-/- and control cells (with empty vector). 25 μg of total protein lysate was used. The amount of Atg7 protein was determined by densitometric quantification and normalized to the control GAPDH. (C) Atg7 protein levels from cells overexpressing shRNA ATG7 (Atg7 -/-) or empty vector (Ct). Experiments were performed in triplicates and the graph shows the means ± SD.

## Interactome of E-cadherin

To identify the mechanisms that underlie the protein quality control (PQC) of WT and HDGC-associated mutant E-cadherin, the binding partners of these proteins were identified by mass spectrometry (MS) after immunoprecipitation (IP) of human E-cadherin from stable CHO cell lines.

### Validation of immunoprecipitation method

The experimental procedure included IP of WT and mutant E-cadherin followed by identification of binding partners by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

This experimental design requires the generation of appropriate controls in order to assess the non-specific interactions (proteins that bind nonspecifically to the beads or the constant regions of the antibody), which consist of a negative control for the IP using goat IgG.

Data in Figure 12 allows for the comparison between the bands revealed by silver staining following IP of both WT E-cad and a control with IgG. It is possible to see different bands, at different molecular weighs, between the two conditions. The bands

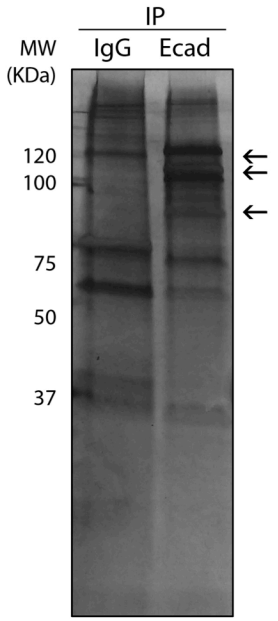



**Figure 12 - Silver Staining of WT E-cadherin binding partners.**
Separation pattern of the proteins from IgG and E-cadherin (Ecad) immunoprecipitations. For each condition were used 1 mg of total protein lysate. The purified proteins were resolved by SDS-PAGE followed by gel staining with silver. The arrows indicate bands only present in E-cadherin IP condition (not in the negative control) that may correspond to specific E-cadherin interactors.

that are observed only in samples that were incubated with antibodies against E-cadherin represent its interactome, and confirm that our strategy purifies proteins associated with the E-cadherin.

The IP procedure was validated by IP of E-cadherin protein followed by SDS-PAGE and western blot in Polyvinylidene Difluoride (PVDF) membrane probed with E-caderin (Figure 13A). It is possible to identify a 120 KDa band that corresponds to mature E-cadherin protein.

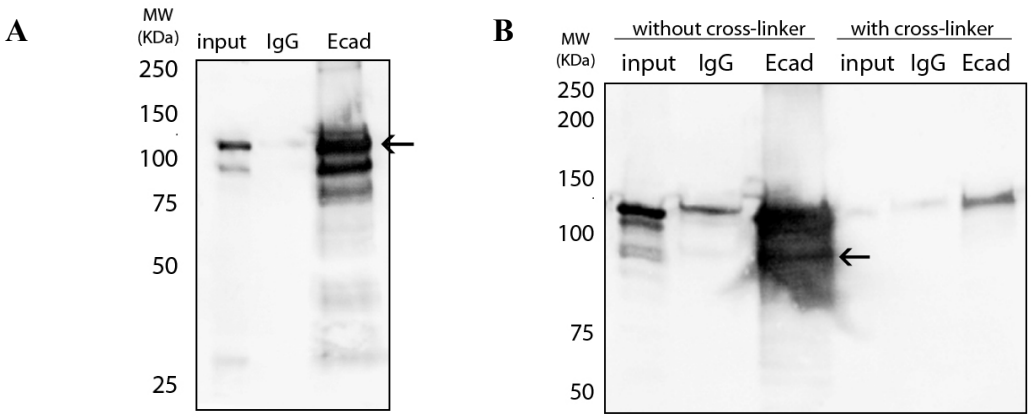



**Figure 13 - Validation of the IP experimental procedure**

CHO cells with stable expression of WT E-cadherin (Ecad) were lysed and the lysates were quantified and immunoprecipitated. (A) Immunoblot of E-cadherin immunoprecipitated fraction or control fraction (IgG immuneprecipitate). 25 µg of total cell lysate was loaded in the input. (B) The same cells were treated with/without cross-linkers (Dithiobis[succinimidyl propionate] (DSP) plus Dithio-bismaleimidoethane (DTME)) before cell lysis. Beta-catenin immunobot in control conditions and in cross-linker condition. The arrows indicate the expected bands that correspond to mature E-cadherin (120 KDa) in A and beta-catenin protein (94 KDa) in B.

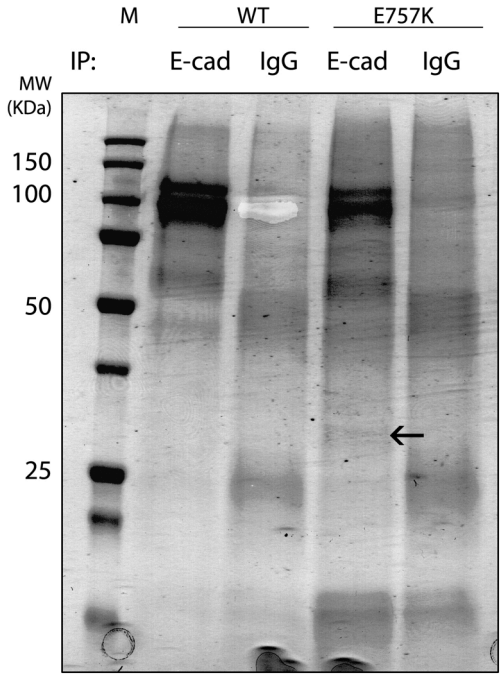
Immunoblot of beta-catenin was used as positive control of IP because this protein is a well-known interactor of E-cadherin (Figure 13B). Chemical crosslinkers facilitate co-IP of weakly interacting binding partners, so the cocktail with Dithiobis[succinimidyl propionate] (DSP) and Dithio-bismaleimidoethane (DTME) was tested as stabilizer of labile E-cadherin interaction. We were not successful on with IP of cross-linked samples

(Figure 9B), as confirmed from the absence of beta-catenin protein under crosslinking conditions. This method was not used for the following experiments.

### Identification of new binding partners: different interactome of WT and mutant E-cadherin

After validation of E-cadherin IP with western blot (Figure 13A), it was possible to progress with identification of the interactome by MS.

We used lysates from CHO cells stably expressing WT and E757K mutant E-cadherin for IP, and then the purified proteins were electrophoretically separated in an "any kD resolving gel". After SDS-PAGE, the gel was stained with coomassie brilliant blue G in order to obtain a visual perception of protein separation. This is fully compatibile with subsequent proteomic analysis and enables the identification and definition of the bands to be analyzed by MS.



From the results obtained (Figure 14) it is possible to perceive some differences between the band pattern of proteins in WT and mutant conditions. Such differences provide a clue that WT and mutant form of E-cadherin protein have a different interactome.

Following SDS-PAGE and coomassie blue staining, entire gel lanes were sliced into 37 bands of equal size and digested with trysin. The peptides extracted from different bands were analyzed by LC-MS/MS. For protein identification, the peptide sequence information was searched in two protein databases SwissProt and NCBInr. Two different search engines were used (Protein Pilot software, AB SCIEX and MASCOT, Matrix Science) witch use different probabilistic algorithms to score the peptides analyzed. The identified proteins are listed in Table 1.

**Table 1 - Interactome of WT and mutant (E757K) E-cadherin identified by LC-MS/MS**

| | | | | | Number peptide matches (>95%) | |
|---|---|---|---|---|---|---|
| | | | | | WT Ecad | E757K Ecad |
| Cadherin-1 | E-cadherin | Cell adhesion molecule binding | P12830 | Human | 8 | 6 |
| Catenin alpha-1 | Alpha E-catenin | Actin filament binding | P26231 | Mouse | 21 | 11 |
| Catenin beta-1 | Beta-catenin | Molecule binding | Q02248 | Mouse | 11 | 5 |
| Metal transporter CNNM4 | Ancient conserved domain-containing protein 4 (mACDP4) | Transmembrane Ion transport | Q69ZF7 | Mouse | 1 | |
| Protein FAM40A | FAM40A | Protein binding (regulation of cell morphogenesis) | Q8C079 | Mouse | 1 | |
| 14-3-3 protein beta/alpha | Protein kinase C inhibitor protein 1 (PCIP-1) | Protein domain specific binding | Q9CQV8 | Mouse | 1 | |
| Catenin delta-1 | p120 catenin | Protein domain specific binding | P30999 | Mouse | 5 | |
| Tubulin alpha-1A chain | Alpha-tubulin 2 | Structural molecule activity | P68362 | Chinese hamster | 1 | |
| Heat shock cognate 71 | HSC70 | Chaperone | P19378 | Mouse | 1 | |
| Heat shock protein HSP 90-beta | HSP84 | Chaperone | P11499 | Mouse | | 3 |
| Lamin-A/C | Lamin-A/C | Structural molecule | G3HG95 | Chinese | | 1 |

**Figure 14 - WT and mutant (E757K) E-cadherin and co-immunoprecipitated proteins**
Pattern band of the polyacrylamide gel stained with coomassie blue of E-cadherin or IgG immunoprecipitate from CHO cell overexpressing WT or mutant (E575K) E-cadherin. 7.5 mg of total protein for each condition was used for IP. The arrow indicates a band that may disclose specific binding partner of mutant E-cadherin. M: molecular weight marker.

| | | | | | |
|---|---|---|---|---|---|
| | | activity | | hamster | |
| Tubulin alpha-1B chain | Alpha-tubulin 1 | Structural molecule activity | P68361 | Chinese hamster | 3* |
| Tubulin beta-5 chain | Class I beta-tubulin | Structural molecule activity | P69893 | Chinese hamster | 3* |

*Two peptides were identified in negative control (IgG) of E757K E-cadherin

The identified proteins listed in Table 1 can be considered as specific interactors of E-cadherin because they were identified only in E-cadherin immunoprecipitates and absent in negative control (IgG). Some binding partners such as p120 catenin, alpha and beta-catenin, that were previously described, are well known interactors of E-cadherin and their identification validates our strategy, with a high confidence level (based in the high number of peptides matches). These results reveal a number of proteins that interact only with WT E-cadherin (p120 catenin, FAM40A and the chaperone heat shock cognate 70 (Hsc)). The specific interactors of mutant form (E575K) of E-cadherin include the chaperone heat shock proteins (Hsp) 84, proteins that belongs to the intermediate filaments (Lamin-A/C), and proteins that constitute microtubes (alpha and beta-tubulin). Table 1 also shows that some proteins interact both with WT and mutant forms of E-cadherin proteins such as alpha and beta-catenins, although less significantly with the mutant form, as expected.

# Chapter 4: Discussion and conclusions

E-cadherin is a well-known tumor suppressor protein that acts by preventing the invasion and metastasis of epithelial cells. When the function of this protein is compromised, frequently by hypermethylation or mutation of the gene that encodes the protein (CDH1), tumors often develop. In fact, inactivation of E-cadherin is present in the majority of invasive epithelial cancers.

A good example that illustrates the carcinogenic potential of mutation in human E-cadherin is Hereditary Diffuse Gastric Cancer (HDGC), a syndrome directly associated to alterations in E-cadherin gene including mutations, deletions and methylation, leading to loss-of-function of the protein. A significant proportion (28 %) of germline mutation in CDH1 gene gives rise to single amino acid substitutions, resulting in a codon that codes for a different amino acid [10]. It was recently shown that different missense mutations in the E-cadherin gene that leads to loss-of-function have different pathogenicity in vivo, manifested by the age of onset of the disease [19].

The reasons why such mutations lead to non-functional protein are still not completely clear. It appears that in many instances the amount of protein that reaches and/or is stabilized at the plasma membrane is insufficient to ensure proper function[13,20]. The reasons that underlie the loss of protein stability at plasma membrane (PM) are not yet fully understood and are most likely different for different mutations. For example, some mutations (as the E-cadherin missense mutations used in this work) were described to result in aberrant proteins that are rapidly degraded by the endoplasmic reticulum associated degradation (ERAD) that is an important part of the protein quality control (PQC) system. It was shown before that by using specific chemical chaperones it is possible to promote the escape from ERAD, thus restoring the E-cadherin trafficking to the cell membrane and rescuing its function [20].

In this study we used cell lines that express different mutant forms of E-cadherin (R749W and E757K). The results show that both cell lines express considerably lower levels of total protein (60 % and 25 %, for R749W and E757K, respectively, as compared to the expression levels of wild type (WT) protein (presented in Figure 5). Since it has been shown that mRNA levels of mutant and WT E-cadherin are comparable, it is tempting to

suggest that mutant forms of E-cadherin are pos-translationally regulated, possibly by increased degradation. In addition, immunofluorescence images show different subcellular distributions patterns between WT and mutant forms of E-cadherin (presented in Figure 6). The WT human E-cadherin localizes at the PM, whereas mutants R749W and E757K are predominantly found in perinuclear regions in aggregates and at lower levels at the PM. To further confirm the cell imaging studies, the amount of E-cadherin was quantified after precipitation of biotinylated-membrane proteins followed by immunoblot. Data in Figure 7 indicates that the missense mutations of E-cadherin destabilize the protein at the PM as compared to the WT protein, corroboring the immunofluorescence data.

One of the major goals of this study was to identify additional mechanisms and key molecular players that are involved in regulating PQC of pathogenic mutant forms of E-cadherin. Some preliminary observations suggest that proteolytic pathways (including the ubiquitin-proteasome system (UPS) and autophagy) are likely to play a role in regulation of the levels of mutant E-cadherin at PM. This role is, apparently not limited to proteasomal ERAD but likely involves mechanisms and/ or molecules that regulate the stability E-cadherin at PM.

The participation of proteolytic events in regulation of protein stability at the PM is recent but not novel. In fact an increasing number of membrane proteins are shown to be pos-transcriptionaly regulated either by UPS or by autophagy. One well-established example is the role of both UPS and macroautophagy in degradation of mutant forms of cystic fibrosis transmembrane conductance regulator (CFTR), namely the deletion mutant DeltaF508. It was shown that macroutophagy was involved in degradation of protein aggregates and of unfolded membrane forms of this mutant channel [30,50]. In addition to cystic fibrosis, the errors in protein folding occur in many protein-misfolding disorders like alpha1-antitrypsin deficiency, neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease [51]. Recent work form our group has also revealed a role for ubiquitin and macroautophagy in regulation of stability of the GAP-junction protein connexin-43 at PM. It was demonstrated that ubiquitination of the

connexin molecule by Nedd4 is required to trigger its autophagy-dependent internalization and degradation [44].

Autophagy was initially described in the 1970s as a form of cell response and survival under nutrient deprivation and starvation was, indeed, the first described stimulus to activate autophagy. Likewise, removal of serum from the medium in cultured cells or starvation in animals are also the best characterized stimuli for autophagy [31]. Early work, done for the most part in rodents, demonstrated that the availability of nutrients and the hormonal changes associated to food intake regulate autophagic activity in liver [52].
The activation of autophagy would be beneficial for the energetic balance in the cell, because the recycling of amino acids resulting from protein breakdown in lysosomes serves to sustain protein synthesis even in the absence of amino acids coming from the extracellular environment through the diet. These amino acids could also be utilized directly to obtain adenosine triphosphate (ATP) through their entry at different steps of the Krebs cycle. In addition to sustaining cell metabolism during starvation, it was found in more recent years that this lysosome-mediated autophagic process has an important role in maintenance of proteins and organelles quality control (QC) by eliminating damaged proteins and organelles that accumulate during stress and aging [31,53].

As it became clear that autophagy had functions other than providing nutrients by degrading proteins, the molecular mechanisms involved in autophagy began to be characterized in more detail and new biochemical and cell biology tools evolved to better characterize and manipulate autophagy. One of the most frequently used biochemical tool to assess autophagy is the conversion of microtubule-associated protein 1 light chain 3-I (LC3-I) to LC3-II [54].
LC3 is a ubiquitin-like protein initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking amino acids from the C terminus (LC3-I) and is finally modified into the phosphatidylethanolamine (PE)-conjugated form, LC3-II.
Thus, an increase in the level of LC3-II reflects the induction of autophagy and/or inhibition of autophagosome formation and is the only protein marker that is reliably

associated with completed autophagosomes. Furthermore, the proteolytic capacity of a cell, which likely varies with cell type, age, transformation and/or disease, may have an important impact on autophagy. As a consequence, levels of LC3-II are also tissue- and cell context-dependent. As a general rule, however, the induction of autophagy results in an increase of LC3-II over time of nutrient deprivation (as presented in Figure 8A, lower band of LC3). The three cell lines used in this study (with WT, R749W or E757K E-cadherin) present different basal levels of LC3-II (Figure 8A, time zero). This is probably due to the fact that expression of each mutant E-cadherin affects differently the ability of the cells to activate and respond to autophagy. For example the increased stress of the ER (due to excessive production of misfolded protein) can induce stress via the unfolded protein response (UPR), which, in turn, was shown to activate autophagy [55].

Results in Figure 8 show that the mutant R749W shows the highest levels of LC3-II at basal conditions as compared to both WT and the mutant E757K form of E-cadherin. It is well established in the literature [54] that prolonged starvation or nutrient deprivation for long periods leads to sustained autophagy and to a rapid degradation of LC3-II via lysosomal turnover (Figure 8A, time 16). Thus, the alterations on the levels of LC3-II should be interpreted with caution. Although levels of LC3-II might be useful in comparing different cell types (or cells expressing different mutations) for the same periods of nutrient deprivations and similar conditions, it does not allow for a direct comparison in prolonged starvation. In addition to the ratio LC3-II/ LC3-I, it has been proposed that LC3 flux is a more reliable marker for activation of macroautophagy [54].

The results shown in Figure 8B show that there is a time-dependent decrease in the levels of both WT and mutant forms of E-cadherin following nutrient deprivation.

Significantly, data in Figure 9B show that the lysosome inhibitor, chloroquine, stabilizes these proteins.

In cultured mammalian cells, tens or hundreds of autophagosomes are formed in the cytoplasm within a few hours of nutrient deprivation; these autophagosomes are subsequently delivered to the perinuclear region where they fuse with lysosomes. The contents of the autophagosome are then degraded by lysosomal hydrolases [36].

In further support of a role for autophagy in degradation of E-cadherin, immunofluorescence data show that cytoplasmic aggregates of E-cadherin co-localize with LC3 suggesting that E-cadherin is entrapped by isolation membrane, of the autophagosomes or related vesicles (Figure 10). Then, these autophagosomes fuse with lysosomes resulting in clearance of the aggregated forms of mutant E-cadherin under nutrient deprivation (Figure 10).

The levels of autophagy attained under normal nutrient conditions must be insufficient to degrade and clear from the cytoplasm the aggregated forms of the mutant R749W and E757K E-cadherin. However, prolonged activation of autophagy by 16 hours of nutrient deprivation leads to a decrease in intracellular aggregates in cells expressing the mutant R749W E-cadherin. The aggregates of mutant E757K E-cadherin show a more modest decrease in the intracellular levels of aggregated E-cadherin.

Membrane proteins are typically thought to be internalized by endocytosis, ultimately resulting in the fusion of the endosomes with the lysosomes. Endocytosis occurs by one of two different pathways: clathrin-dependent and clathrin-independent. In clathrin-dependent endocytosis the cytoplasmic domains of plasma membrane proteins are specifically recognized by adaptor proteins and packaged into clathrin-coated vesicles that are brought into the cell.

Clathrin-independent endocytosis, by contrast, may come in many forms, and it has been less studied. However, there are also specialized clathrin-independent endocytosis pathways, such as macropinocytosis and phagocytosis, that are actin driven and may, in fact, represent stimulated forms of this pathway. This mechanism occurs due to the presence of many transmembrane proteins, in cell surface, that lack cytoplasmic sequences for the recruitment and internalization into clathrin-coated vesicles [56].

Although these two pathways remain the major classical mechanisms involved in endocytosis and degradation of membrane proteins, increasing evidences, in recent years, indicate that both the UPS and autophagy are involved in selective degradation of membrane proteins. The requirement of ubiquitination for the internalization of membrane proteins was recognized some years ago and was shown to involve endocytic

adaptors for lysosomal targeting that can recognize K63-, K11- K29 and K48-linked poly-Ubiquitin chains [43].

The precise mechanism for E-cadherin endocytosis is, however, incompletely understood. Interestingly, recent studies have presented evidence that E-cadherin might be internalized by either clathrin-dependent [57,58] or clathrin-independent mechanisms [59,60], suggesting that multiple pathways may also exist for E-cadherin internalization.

In fact there are proteins that can be internalized by mechanisms that involve ubiquitination and autophagy and, more importantly proteins that are degraded by mechanisms that involve a crosstalk between the endocytic machinery and autophagy. For example, work in our group showed that the membrane protein connexin 43 is ubiquitinated and that, ubiquitination result in decreased half-life of proteins at the PM. The ubiquitination is an important signal that recruits membrane proteins (such as connexin) to degradation in the lysosome by a process that involves the participation of macroautophagy [44]. For many substrates, specific features or components on the surface of the organelle or particle serve as docking points for what are known as cargo recognition molecules, which bring along the autophagic machinery leading to the formation of the autophagosome membrane around the specific cargo. Molecules such as p62 or NBR1 have been recently described as cargo recognition proteins that mediate the binding of ubiquitination proteins to the autophagosome-associated protein LC3 critical for autophagosome maturation, thereby controlling their packing into autophagosomes [31]. Ubiquitination of different proteins depends on many factors and is regulated at numerous levels. Ultimately, the state of ubiquitination of a certain protein is regulated by the balance of the rates of ubiquitination and deubiquitination. Ubiquitination, itself, is regulated by various enzymes and ancillary proteins. E-cadherin present in its structure the “PEST” sequence motives that are often recognized by specific ubiquitin ligases. However, when beta-catenin binds, at the juxtamembrane region, to E-cadherin it masks the “PEST” motives so that E-cadherin cannot be ubiquitinated as it is inaccessible to the ligases. This prevents ubiquitination and internalization of E-cadherin at PM ensuring proper function for the protein. Hakai is an E3 ubiquitin-ligase that mediates ubiquitination that enhances endocytosis

of E-cadherin [61]. It is however conceivable those mutant forms of E-cadherin do not bind properly to beta-catenin and thus the protein is rapidly ubiquitinated and degraded thus accounting for the tumorigenic phenotype associated with expression of mutant forms of E-cadherin [62].

These and other examples illustrate new mechanisms for regulation of membrane protein stability and recycling. These mechanisms involving ubiquitination (mediated by specific ubiquitin ligases) account for an important crosstalk between autophagy and endocytosis.

The loss of function of mutant forms of E-cadherin is largely associated with the loss of protein at PM and this is determined by the amount of protein that traffics to and reaches the PM as well as the stability of the protein. Both processes are influenced by a complex interplay between molecular chaperones and proteolytic systems, including macroautophagy and UPS.

The importance of PQC in stability of membrane proteins is well illustrated by mutations in disease-causing mutations of membrane proteins such as in cystic fibrosis. This disease results primarily from a mutation (Delta F508) in the chloride channel, CFTR, which is a large transmembrane protein of 170 KDa that in the WT conformation is misfolded to a large extent being rapidly degraded by the ERAD, a process mediated by the UPS. However the deletion of the amino acid F508 leads to a conformational change that is sufficient to recruit the ERAD machinery leading to almost total degradation of the protein. A number of tissue culture and in vivo experiments have shown that by preventing degradation of mutant CFTR it is possible to increase the amount of protein present at the PM and therefore rescue the phenotype associated with the disease [50].

Similar to CFTR, our group has shown that mutant forms of E-cadherin lead to conformational changes in the protein, resulting in its rapid degradation through ERAD, mediated by the UPS. Additionally, the pathogenicity of the mutations is dependent on the nature of the mutation that is present, an on the structural impact that it induces [19]. Moreover, specific chemical chaperones, as dimethyl sulphoxide (DMSO), were shown to be able to promote trafficking of E-cadherin to the PM, rescuing E-cadherin function in one of the mutants [20]. Interestingly, the rescue of expression by treatment with chemical chaperones depends on the manipulation of the trafficking machinery, namely by downregulating ARF6-dependent endocytosis of E-cadherin [63].

Data presented here also show that different mutations affect differently the trafficking of E-cadherin to the PM, resulting in decreased PM expression of the mutants (R749W and E757K) to 80 and 50 % respectively (Figure 7), when compared to the WT form. In addition to decreased levels at PM, there is an accumulation of intracellular aggregates of mutant E-cadherin, as revealed by immunofluorescence microscopy (Figure 6). These intracellular aggregates (Figure 6) are putative substrates for autophagy. The data in support of this hypothesis is very robust, since the clearance of intracellular aggregates induced by nutrient deprivation is blocked by inhibiting the lysosome with chloroquine (Figure 10, right column).

The biotynilation experiments further suggest that activation of macroautophagy affects not only the levels of E-cadherin that accumulate intracellularly but also the stability of E-cadherin at the PM.

To confirm the effects of activation of autophagy in E-cadherin by nutrient deprivation we have also used an alternative strategy by using cells that are autophagy-incompetent (or limited), by silencing of the critical autophagy protein Atg7. The Atg7 is required for LC3-I and phosphatidylethanolamine (PE) conjugation resulting in LC3-II production, essential for autophagosome formation. The Atg7 knockdown (about 50 % of protein reduction) causes a 60-70 % E-cadherin reduction at the plasma membrane (Figure 11A), despite the increased expression of E-cadherin in the total lysate (Figure 11B). Although these results are not easy to interpret, it is tempting to speculate that an active autophagy is required to maintain E-cadherin at the PM. This could, for example reflect the need for autophagy in the clearance of intracellular misfolded forms of E-cadherin, ensuring that properly folded E-cadherin is not trapped in aggregates of misfolded protein, and reaches the PM. This hypothesis would require further experimental support.

In fact there is a decrease in the protein levels of both WT and the mutant E-cadherin at PM following six hours of nutrient deprivation. This suggests that, by mechanisms that still remain to be identified, the proteolytic systems are involved not only in the degradation of intracellular E-cadherin but also in the E-cadherin present at PM.

These mechanisms are probably very complex and may involve selective recruitment of ancillary proteins such as chaperones to the E-cadherin present at PM.

Altogether, these observations are consistent with a model of emerging complexity in regulating stability of proteins at PM. This model, that has been described as peripheral PQC involve, in many instances, ubiquitination of the membrane substrate, recruitment of ubiquitinating enzymes, and various adaptors (including endocytic adaptors), ultimately resulting in degradation of membrane proteins in the lysosome [43]. The identity of these different adaptors and molecular players involved in the crosstalk between the ubiquitination/edocytic machinery and the autophagy/lysosomal compartments is still not fully understood although it is an area of fast growing progress.

Interestingly, the data obtained herein with MS revealed that WT and mutant E-cadherin bind selectively to specific chaperones. The data does not allow to distinguish between interactors that selectively associate with E-cadherin at the PM versus interactors that associate with intracellular E-cadherin, like the aggregates in mutant E-cadherin. Nevertheless, the cell biology data suggests that regulation of stability of E-cadherin at PM is closely associated with proteolysis, and that autophagy selectively affect subcellular distribution of E-cadherin.

Altogether, both MS and cell biology data, suggest that regulation and stability of E-cadherin at PM is not only dependent on the amount of protein that reaches the PM but also results from the intricate regulation by other factors that affect the total levels of E-cadherin at PM. The recent concept of peripheral PQC [43,50] is likely to affect the stability of mutant proteins at PM by selective interaction with key players that influence quality control at PM. In this study, different molecular players interact differently with different E-cadherin forms (WT and E757K) and this may account to explain the specific pathogenicity of the different mutant forms of E-cadherin. This is also likely to open new avenues in the strategies for therapy of forms of cancer that are associated with loss of function of E-cadherin. This is particularly important when the loss of function is directly related with the decreased stability of the protein at the PM.

The MS data shows the different interactome with WT or mutant form of E-cadherin. Data from MS revealed that most proteins that interact with WT E-cadherin are well-established molecular partners that interact with WT human E-cadherin as described in previous studies [13,14,62,64]. The catenin (p120, alpha and beta) are the best-characterized

binding partners of E-cadherin. Only the p120 catenin, that stabilizes E-cadherin at the PM, was not found associated with the mutant form of E-cadherin (E757K), suggesting that E757K is not stable in the PM. The alpha and beta-catenin were found to associate with mutant E-cadherin, although to a lower extent (Table 1), demonstrating that the cadherin-catenins complex is not properly established in the mutant context, as previously demonstrated by other experimental approaches [20].

Another interesting binding partner of E-cadherin that was shown in this study is the deubiquitinating enzyme FAM, that was suggested in previous studies to interact with E-cadherin [64]. Significantly, our results show that only WT E-cadherin binds to FAM. FAM associates with E-cadherin and beta-catenin during trafficking to the PM. FAM deubiquitinating enzyme prevent endocytosis and lysosomal trafficking of many PM proteins like E-cadherin by reversing their ubiquitination [64]. This results further supports a model in which increased ubiquitination of mutant E-cadherin at PM, presumably by absence of deubiquitination by FAM may further account for the ubiquitin-dependent internalization of mutant E-cadherin. The final destination of this mutant E-cadherin is likely to be the lysosome, as also suggested by our results (Figure 9A).

Another example of a protein that may have an important role in PQC is the kinase C inhibitor protein that only binds to WT E-cadherin.

This is the first study where the binding partners of E757K mutant E-cadherin were identified. The results show that different molecular chaperones bind selectively to WT or mutant (E757K) form of E-cadherin. While Hsc70 binds to WT E-cadherin, Hsp84 (Hsp90beta) binds only to mutant E-cadherin. Based on these results it is possible to speculate that a selective association may exist between these molecular chaperones and the trafficking of WT and mutant E-cadherin to the PM, ultimately determining the amount of E-cadherin that reached, and is stabilized at, the PM. For example, there is a considerable crosstalk between the Hsp70 and Hsp90 chaperone complexes, but in general Hsp90 protects proteins from unfolding and aggregating, whereas Hsp70 is responsible for their degradation in cases when unfolding or aggregation cannot be prevented [30].

Other molecular partners identified in E757K mutant E-cadherin belong to constituents of microtubules, alpha- and beta-tubulin. All too frequently, attempts to express

hydrophobic integral membrane proteins in mammalian cells, results in mistargeting of the heterologous protein to cytoplasmic aggregates. Heterologous expression of CFTR, for example, leads to the formation of aggregates that accumulate in a single juxtanuclear region [65]. This microtubule-dependent movement is a possible mechanism that explains the interaction between mutant E-cadherin and microtubules. Although these two proteins identified (alpha- and beta-tubulin) were found in negative control (IgG) of mutant E-cadherin only. These are shown in Table 1 but should be interpreted with caution.

Ultimately the results of the study suggest that mutant forms of E-cadherin may interact differently with different key players involved broadly in the concept of peripheral PQC. This may provide new clues that are likely to be useful in devising new strategies to increase stability of E-cadherin at PM and rescue the invasive and metastatic potential associated with loss of E-cadherin in HDGC.

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