SCUOLA NORMALE SUPERIORE
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A method to selectively degrade proteins of the secretory pathway

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Candidate:
Lara Vecchi

Supervisor:
Dr. Oscar Burrone

Co-supervisor:
Dr. Marco Bestagno

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ABSTRACT

Proteins that enter the secretory pathway, either soluble or membrane bound, are subjected to ER quality control mechanisms: if protein folding is aberrant or delayed, proteins are subjected to additional folding cycles or selected for ER-associated degradation (ERAD). In this process, proteins that fail to reach their terminal folding, and thus selected for ERAD, are recognised and retro-translocated to the cytosol for ubiquitinylation and subsequent degradation by the proteasome.

The most characterised mammalian ubiquitin ligase is HRD1. For its activity HRD1 needs the interaction with SEL1L an ER resident type I transmembrane glycoprotein. SEL1L has been defined as an adaptor protein because, beside its interaction with the ubiquitin ligase HRD1, it interacts either with proteins involved in substrate recruitment or in the retro-translocation process.

To induce degradation of specific targets we designed and constructed a new class of fusion molecules, termed *degradins,* where the NH_2-terminal part consists of a target-specific binding domain, such as an antibody-derived scFv or a ligand of a receptor, fused to the COOH-terminal 372 aminoacids of human SEL1L. The SEL1L moiety lacks the first NH_2-terminal 401 amino acids probably involved in identification and recognition of ERAD substrates, but retains the luminal portion, homologous to the yeast protein Hrd3p and involved in the interaction with the ubiquitin ligase HRD1, and the transmembrane and cytosolic domains.

By testing the system on two different models (FcεRI-α chain and TGEV spike protein S) we found that degradins were able to induce retro-translocation and proteasome-mediated degradation of the target.

To study the retro-translocation induced by degradins, we developed a system based on the specific *in vivo* biotinylation of a reporter engineered by the addition of a biotin acceptor peptide (BAP). The BAP peptide is recognised by the cyt-BirA enzyme that catalyses the addition of a biotin molecule in the cytosol. Proteins normally residing in the ER lumen can get biotinylated only after their dislocation to the cytosol. As a model, the MHC-Iα was chosen because target of retro-translocation induced by US2 or US11 immunoevasins of HCMV; the system demonstrated to be very specific and efficient in detecting all types of

intermediates of retro-translocation or completely retro-translocated molecules. Altogheter, my results strongly suggest that the degradin system can be efficiently used to degrade specific target proteins depending on the target recognition moiety, moreover our *in vivo* biotinylation represents a novel and unique method for the study of retro-translocation process.

List of abbreviations

Ab: antibody
AD: Alzheimer disease
APC: antigen presenting cell
APN: aminopeptidase N
Apob: apoliprotein B
APP: amyloid precursor protein
ATP: adenosine triphosphate
BAP: biotin acceptor peptide
Bip: binding protein
C-terminal: Carboxy terminal
C_H: immunoglobulin constant region of heavy chain
C_L: immunoglobulin constant region of light chain
CNX: calnexin
CRT: calreticulin
CTL: cytotoxic T lymphocyte
Da: Dalton
DC: dendritic cell
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethylsulfoxide
DUB: deubiquitinylating enzyme
EDEM: ER degradation-enhancing α mannosidase-like protein
Elf2: elongation factor 2
EndoH: endoglycosidase H
ER: endoplasmic reticulum
ERAD: ER associated degradation
ERAP: ER amino peptidase
Erdj: ER protein containg a j domain
ERQC: ER quality control
Fc: fragment crystalizable
FcεRI: high affinity IgE receptor
FCS: Fetal calf serum
FITC: fluorescein isothiocyanate
Fv: variable fragment
GPI: glycosylphosphatidylinositol
HCMV: human cytomegalo virus
HCV: Hepatitis C virus
HIV: Human immunodeficiency virus
HLA: human leukocyte antigen
HRP: Horse Radish Peroxidase
Hsp: heat shock protein
Ig: immunoglobulin
ITAM: immunoreceptor tyrosine-based activation motif
mAb: monoclonal antibody
mdα: membrane dimeric FcεRI-α chain
Met: methionine
MHC: major hystocompatibility complex
min: minutes
MoI.: moltiplicity of infection
MRH: mannose receptor homology
N-terminal: Amino-terminal
NLS: nuclear localisation signal
O.D: optical density
o/n: over night
PAGE: polyacrylamide gel electrophoresis
PBS: Phosphate buffered saline
PDI: protein disulphide isomerase
PLC: peptide loading complex

PNGase: peptide N-glycanase
RNC: ribosome nascent chain complex
rpm: revolution per minute
RT: room temperature
^{35}S: solfour
scFv: single chain fragment variable
sdα: soluble dimeric FcεRI-α chain
SEC: secretion signal peptide
SIP: Small Immuno Protein
SiRNA: small interfering RNA
SIT: suicide intrabody technology
SR: SRP receptor
SRP: Signal Recognition Particle
SVIP: small p97/VCP interacting protein
TAP: Transporter associated with Antigen Processing
Tc: T cell
TCR: T cell receptor
TGEV: *Transmissible gastroenteritis virus*
TM: transmembrane domain
TNF: tumor necrosis factor
TRAM: Translocating chain Associating Membrane protein
TPR: tetratricopeptide repeats
TSE: transmissible spongiform encephalopaty
UBA: Ubiquitin Associated Domain
Ubc: ubiquitin conjugating enzyme
UBL: Ubiquitin Like modifiers
UGGT: UDP-glucose Glycoprotein Glucosyltransferase
UPR: Unfolded Protein Response
UPS: ubiquitin proteasome system
V_H: immunoglobulin variable region of heavy chain
V_L: immunoglobulin variable region of light chain

INTRODUCTION

1 ER quality control: from protein biogenesis to protein degradation

1.1 Protein folding: general aspects

Protein biogenesis is an error prone process due to the high rate of translation, cellular stress or nutrient deprivation that can cause incorrect aminoacids incorporation.

Folding efficiency and rate can be compromised by spontaneous errors during transcription and translation or by genetic mutations, toxic compounds and cellular stress [1,2].

The resulting aberrant proteins are not able to undergo correct post-translational modifications and attain the correct conformation.

Aberrant proteins represent a threat for the cell because of their possible aggregation and precipitation with toxic effects.

Indeed, many neurodegenerative disorders seem to be linked with defects in clearing protein aggregates [3-5]. Furthermore there are other human genetic diseases, such as autosomal dominant neuroypophyseal diabetes mellitus and polyglutammine diseases (spinobulbar muscular atrophy SBMA), that seem to be linked with the accumulation of misfolded proteins aggregates, caused by deficiency in degradation machinery activity [6].

These findings have helped to understand the pivotal role of having a functional control of protein folding for cell viability.

To avoid protein aggregation cells have evolved several pathways that target defective proteins for proteolysis.

This process is very important and is underlined by the finding that perhaps 30% of newly synthesised polypeptides are destroyed during or soon after translation [7].

A great number of proteins are sorted for the secretory pathway. These proteins can be soluble or membrane anchored and, in both cases, they need to be transported through the ER membrane to reach the lumen of ER, in the first case, or in order to be inserted in ER membrane, in the second case [8]. The vast majority of secreted and membrane proteins carry at the N-terminus a cleavable hydrophobic domain, the signal sequence that ensures the insertion of the protein

in the ER. Translation of secreted or membrane proteins starts in the cytosol since the signal sequence emerges from the ribosome and is recognised by signal recognition particles (SRP) [9]. The complex formed is called Ribosome Nascent chain Complex (RNC) and is delivered to the membrane through the interaction between SRP and SRP receptor (SR). Afterwards RNC is released to the Sec61 translocon, SRP and SR dissociate and translation continues pushing the protein in the ER lumen through the proteinaceous pore formed by Sec61.

If the protein contains hydrophobic domains they are inserted into the ER membrane from the nascent polypeptide, process ensured by Sec61 [10,11].

The transmembrane domain must be recognised once it arrives inside the channel and afterwards it must be released in the lipid phase. The Sec61 channel must open in order to allow polypeptide segments to cross the lipid bilayer but it also must let hydrophobic segments to pass through its walls to enter into the lipid bilayer [12].

A candidate for being a chaperone that assists membrane protein to be inserted in the ER membrane is the Translocating-chain-Associating Membrane Protein (TRAM) that spans the ER membrane six times [13] and is located at the front of Sec61 channel [12,14].

To be transported through the secretory pathway, membrane and secretory proteins must acquire their proper folding state; acquisition of correct folding is possible though the ER Quality Control mechanisms (ERQC) [15].

ERQC comprises pathways that ensure a protein to acquire the proper folding and pathways that make the cell able to detect proteins not properly folded that therefore must be degraded.

The primary mediators of the ER quality control are called chaperones; they evaluate substrate conformation and help protein to fold. Chaperones belong to different families: the heat shock family includes BiP (HSP70), Erdj (HSP40) and GRp90 [16], the lectin family includes calnexin, calreticulin and EDEM [17], in addition there are chaperones that are substrate specific, like HSP47 [18].

A number of different ER-resident proteins, such as enzymes involved in co- and post-translational modifications, were also shown to assist protein folding.

These enzymes act preventing the formation of aberrant conformations. Some of these are enzymes acting on signal sequence cleavage and oligosaccharide addition, or they are enzymes that trigger aminoacid isomerisation and oxidation

(peptidylprolyl cis-trans isomerase and protein disulphide isomerase, PDI, respectively) [19-21].

Chaperones have two main ways of action: they can stabilise nascent polypeptide chains on ribosomes initiating their folding, or they can act downstream translation to complete the folding process [22,23].

Chaperones monitor protein folding through detection of exposed hydrophobic patches or through excessive protein dynamics associated with the non compact structure characteristic of partially folded proteins [24].

Hydrophobic domains are commonly buried in folded proteins because of their tendency to form insoluble aggregates that can have toxic effects.

Chaperones such as the heat shock protein Hsp70 (BiP) and chaperonins (Hsp60) primarily recognise hydrophobic aminoacid side-chains exposed by non-native proteins and promote their folding through ATP-regulated cycles of binding-release [25,26].

BiP is a very abundant ER resident chaperone that acts in an early phase binding the polypeptide during its traslocation across ER membrane. Its N-terminal portion harbors ATPase activity whereas the peptide binding domain is localised at the C-terminal portion [27,28].

BiP originally found associated with immunoglobulins [29] is an important player during the folding of numerous proteins within the ER.

BiP is able to bind the substrate after ATP hydrolysis; as different number of folding cycles can occur in order to ensure the protein to fold properly, different cycles of ATP hydrolysis are essential for substrate binding-release to BiP [30,31].

Due to the weak ATPase activity of BiP ATP hydrolysis occurs by the help of Hsp40 co-chaperone that contain a J domain (ERdj); this domain consists of four helices and is essential for the interaction with the ATPase domain of BiP. The second helix contains charged and basic residues involved in binding to BiP [31].

ERdj family comprises five proteins identified, so far, and among them ERdj5 displays oxidoreductase activity.

ERdj5 binds Bip via its DnaJ domain and contains four PDI-like domains that explain its activity in reducing disulphide bonds of misfolded proteins and its activity in preventing protein aggregation [32].

Through its binding to BiP Erdj5 probably creates a link between the two different activities of BiP in promoting the proper folding of proteins and in removing misfolded proteins.

Intramolecular and intermolecular disulphide bonds are important for the stabilisation of tertiary and quaternary structure of proteins. The oxidising environment supplied by the ER compartment favours the formation of disulphide bonds; this process is catalysed by members of protein disulphide isomerase (PDI) family, which possess thiol oxidoreductase activity.

Oxidative folding occurs through two phases, one co-translational, during which intra-molecular disulphide bonds are created between cysteines of the nascent chains, and one post-translational, during which disulphide bonds are rearranged into the native state.

The oxidative folding during BiP pathway is displayed by PDI, which is a redox-driven chaperone participating in the ERAD of several substrates during their translation [33].

PDI catalyses the formation of a disulphide bond in the nascent polypeptide; its action is favoured by BiP that keeps the substrate in a conformation that ensure PDI to have access to the cysteines involved in a disulphide bond [33].

If protein folding is delayed, proteins are subjected to additional folding cycles or, whether the protein has become irreversibly unfolded, it is addressed to proteasomal degradation through ER-associated degradation (ERAD) [34].

1.1.1 Folding of glycoproteins

Several covalent modifications take place in the ER and allow the protein to acquire the proper conformation; these modifications include disulphide bond formation, N-glycosylation and GPI addition [35]. The N-glycosylation is particularly important because it allows the interaction with molecular chaperones that assist the glycoprotein folding. In fact, the vast majority of proteins that does not acquire the correct glycosylation remain in a misfolded state and is degraded.

Moreover N-linked oligosaccharides addition increases protein solubility, avoiding the formation of protein aggregates, and is essential for glycoprotein transport to the Golgi apparatus and in general for protein sorting [36].

The N-glycosylation provokes binding of a preformed oligosaccaride ($Glc_3Man_9GlcNAc_2$) to asparagine side chains [37].

The addition of this preformed oligosaccharide occurs as the nascent polypetide is inserted in the ER through the Sec61 channel, and is performed by the enzyme oligosaccharide transferase [38].

This synthesis is rapidly followed by the removal of the two outermost glucose residues by the enzymes glucosidase I and II [39].

The removal of terminal glucose residues by glucosidases I and II results in a glycoprotein that contains a $Glc_1Man_9GlcNAc_2$ moiety specifically recognised by the lectin-like chaperones calnexin and calreticulin that promote its folding.

Calnexin and calreticulin are the most extensively studied chaperones with lectin activity [40,41]. Calnexin is a type I ER membrane protein, while calreticulin is its soluble analogue that localises in the ER by a C-terminal KDEL sequence [42,43] KDEL sequence is responsible of the binding to a membrane bound receptor that shuttles from the ER to the Golgi, causing the KDEL containing protein to relocalise in the ER compartment [44].

Following calnexin-calreticulin folding cycle, glucosidase II removes the remaining glucose residue; if the resulting protein has been able to reach its proper folding can exit from the ER through COPII vesicles that bud from the ER [45,46]. If instead, the protein is not completely folded, it is recognised because it exposes hydrophobic patches or because it harbors an insufficiently compact structure.

For those proteins that have not been able to reach the correct folding, cycles of deglucosylation-reglucosylation take place in the ER mediated by the enzyme UDP-glucose glycoprotein glucosyltransferase (UGGT), that adds back a glucose residue to the N-linked glycan [47]. The resulting monoglucosylated proteins can re-enter the calnexin-calreticulin cycle because they are specifically recognised by these two chaperones [48]. Thus, cycles of deglucosylation-reglucosylation make possible to restart the protein folding process giving to glycoproteins more chances to reach their native conformation.

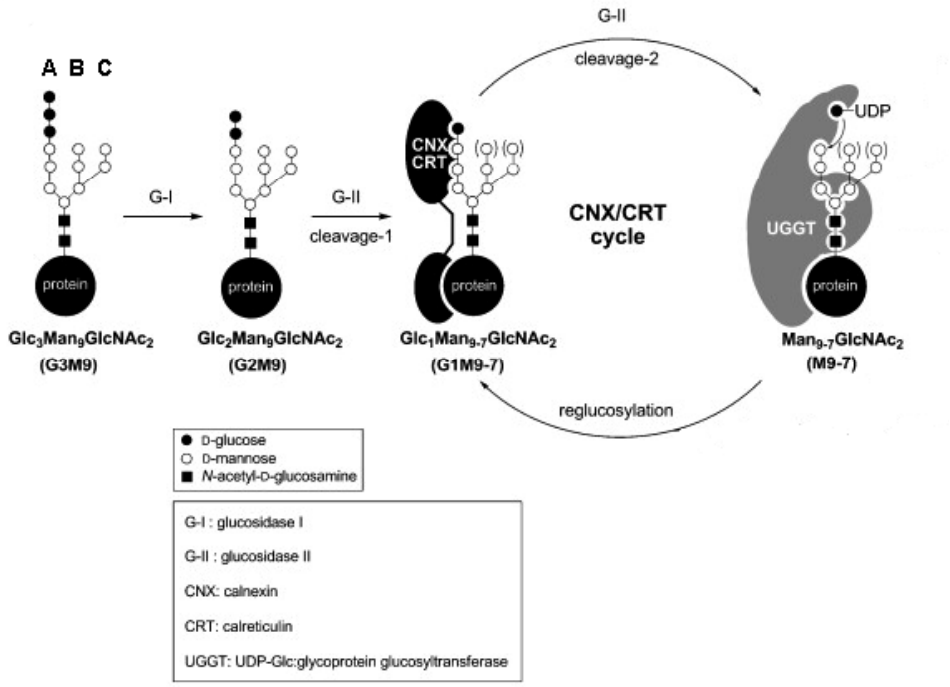

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Figure 1: A preformed oligosaccaride ($Glc_3Man_9GlcNAc_2$) is added to asparagine side chains and is organised in three branches (A, B and C). Glucosidase I and II remove the two outermost glucose residues leading to a monoglucosylated protein, specifically recognised by calnexin and calreticulin that promote protein folding. If the protein is not able to reach its proper conformation it can re-enter the calnexin-calreticulin cycle, by the action of glucosidase II that removes the remaining glucose residue, and UGGT that readd a glucose moiety, allowing the protein to be recognised, once again, by calnexin and calreticulin.

Oxidative folding during the post-translational phase requires the activity of ERp57, a member of the PDI superfamily, that associates with calnexin and calreticulin and is involved in glycoprotein quality control [49]. ERp57 carries out the reduction, isomerisation and oxidation of non-native disulphide bonds similarly to PDI but much more slowly [50].

Calnexin and calreticulin also promote oxidative folding because responsible of the retention of misfolded proteins; this activity facilitates the interaction between the substrate and ERp57 leading to the inhibition of protein aggregates formation [41,51,52].

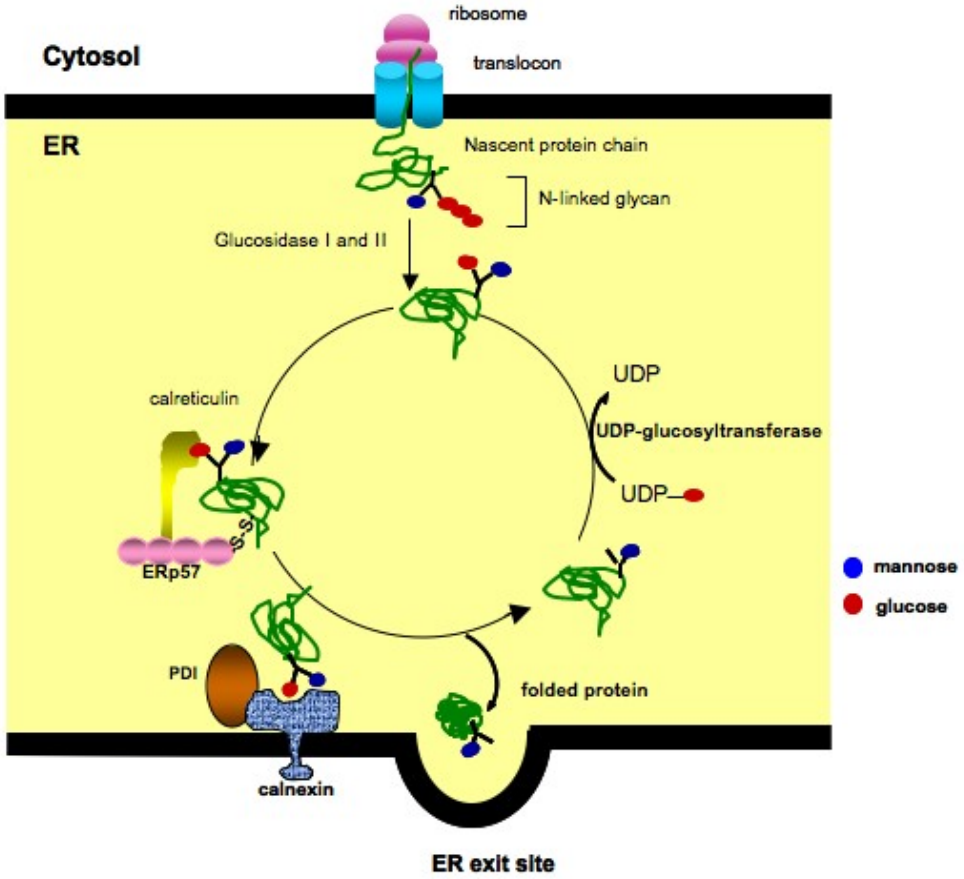

Figure 2: Cooperation between calnexin, calreticulin and ERp57 in glycoprotein folding. Glycoproteins are modified by N-glycosylation, during or soon after translation. Glucosidase I and II remove the two outermost glucose residues, the resulting monoglucosylated form is specifically recognised by calnexin and calreticulin and enter the folding cycle. ERp57 interact specifically with calreticulin promoting protein oxidative folding. Finally glucosidase II removes the remaining glucose residues and the protein modified in this way is able to exit ER through COP-II vesicles or if not properly folded can re-enter the calnexin-calreticulin cycle.

1.2 Disposing of ER proteins

Soluble and membrane proteins that fail in the acquisition of the correct folding must be degraded in a process called ER associated degradation (ERAD) [34].

ERAD takes place after the recognition of the substrate, using as mediators ER lectins and molecular chaperones such as BiP. Chaperones and lectins that promote protein folding can, on the other side, decide which proteins must be targeted to degradation.

In this process, terminally unfolded or misfolded proteins, upon recognition, are retro-translocated to the cytosol, a process also known as dislocation, for degradation by the 26S proteasome complex, a proteolytic particle enriched at ER membrane [53].

For the disposal of misfolded proteins from the ER, removal of α1,2-bonded mannose residues from the N-glycan is required as it allows the generation of a code deciphered by ERAD machinery [54].

Mammalian ER α1-2 mannosidase I is a member of GH47 family that contains also EDEM 1, 2 and EDEM 3 proteins [17,55,56]. EDEM (ER degradation-enhancing α mannosidase-like proteins) subfamily has been described as player in substrate de-mannosylation [57].

ER α1-2 mannosidase I and the EDEM family members function sequentially leading the protein to expose a terminal α(1,6)-bonded mannose on the glycan which is recognised by proteins containing a mannose 6-phosphate receptor homology-domain (MRH-domain), Yos9 in yeast and OS-9 and XTP3-B in mammals. These proteins, due to the presence of the MRH domain functional in oligosaccharide binding [58], function as lectin and promote the recognition of misfolded proteins [59].

EDEM1, through its interaction with calnexin, avoid futile calnexin calreticulin folding cycles when the protein is already irreversibly unfolded. In addition EDEM1 accelerates ERAD by preventing the formation of disulphide-bonded dimers or covalent aggregation containing misfolded proteins [57]. This function is carried out by EDEM1 through its specific association with an ER resident member of the ERdj family named ERdj5, which catalyses the reduction of disulphide bonds and, in turn, recruits BiP preventing aggregation of misfolded proteins and in this way promoting ERAD process.

ERdj5 accelerates ERAD and the model proposed is that once misfolded proteins are transferred from calnexin to EDEM, ERdj5 bound to EDEM reduces the disulphide bonds of misfolded proteins disaggregating multimeric complexes. At the same time ERdj5 keeps the substrate in an unfolded dislocation competent state [32,60] and catalyses the conversion of ATP form of BiP into ADP-BiP causing the release of BiP which is now able to strongly bind the substrate.

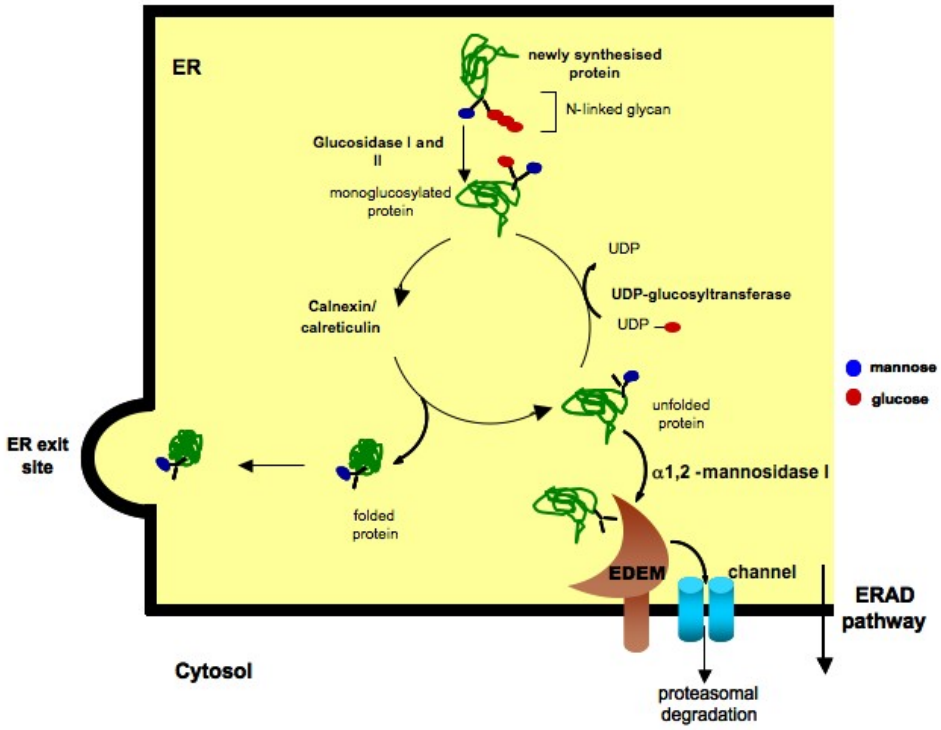

Figure 3: ER protein disposal. When a protein becomes irreversibly unfolded the action of ER mannosidase I and EDEM generates a signal for ERAD represented by a terminal α(1-6)-bonded mannose avoiding, in this way, the protein to re-enter another futile calnexin calreticulin folding cycle. The unfolded protein, by the action od EDEM is maintained in an unfolded dislocation competent state and therefore can be retro-translocated from the ER to the cytosol.

1.3 The unfolded protein response

Accumulation of aberrant proteins results in the induction of the unfolded protein response (UPR), an ER-to-nucleus and ER-to-cytosol signalling pathway. The ER-to-nucleus signalling gives rise to a transcriptional programme which increases expression of chaperones and enzymes required for protein modification, ER membrane expansion, protein trafficking from the ER and the disposal of misfolded proteins, while the ER-to-cytosol signalling results in the attenuation of protein synthesis [61].

Accumulation of misfolded proteins results in BiP dissociation from three ER transmembrane proteins that function as UPR transducers: IRE1, PERK and ATF6.

Following dissociation from BiP, ATF6 is transported to the Golgi where it gets cleaved by two proteases S1P and S2P [62,63]. The cytosolic N-terminal 377 aminoacids fragment, composed of a basic leucine zipper, becomes an active

transcriptional factor that translocates to the nucleus [64-67]. The outcome is the induction of ER stress response genes [64].

The UPR transducer IRE1 is a protein kinase and ribonuclease associated to the ER membrane that during stress conditions binds to misfolded protein. This binding activates IRE1 oligomerisation keeping IRE1 away from Bip and enabling its activation by trans-autophosphorylation [68]. Activated IRE1 causes, through its ribonuclease activity, the splicing of XBP1 mRNA independently from the spliceosome. The spliced form of XBP1 mRNA produces a protein that activates the transcription of its target genes [69].

UPR induced genes encode for proteins involved in different steps of ERAD; proteins acting in the recognition and in the ER-to-cytosol dislocation of misfolded proteins. The spliced form of XBP1, in particular, targets genes expressing proteins involved in ER protein disposal [69].

The ER-to-cytosol signalling of UPR decreases the rate of protein translation by the activity of the transmembrane protein kinase PERK that contains a cytosolic kinase activity. PERK, under ER stress conditions and upon dissociation from BiP, catalyses its autophosphorylation and activation [70], once activated, PERK is able to block protein synthesis, catalysing the phosphorylation of the translation initiation factor eIF2α [71].

A prolonged UPR signalling can lead to cell death, through induction of apoptosis. In particular, IRE1 dependent unfolded protein response seems to be anti-apoptotic while PERK dependent signalling might trigger apoptosis [72].

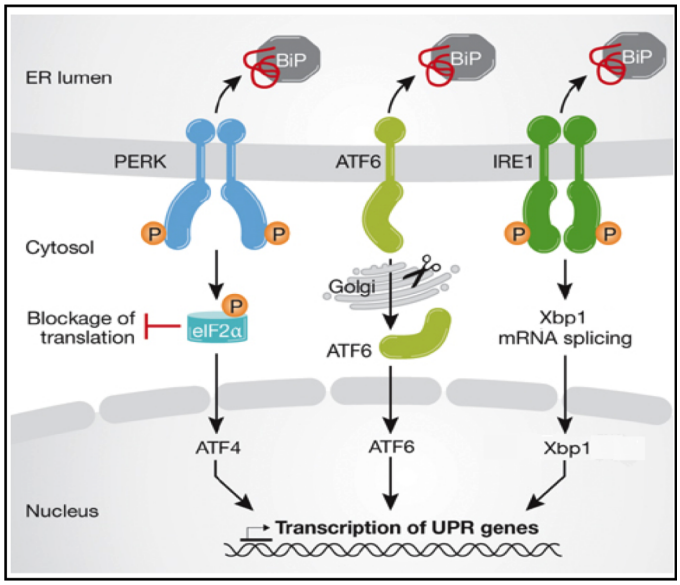

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Figure 4: Unfolded protein response pathway. Unfolded protein accumulation causes BiP to disassociate from IRE1, PERK and ATF6 UPR transducers. Following the release of BiP IRE1 can catalyse it autophosphorylation and get activated. Activated IRE1 promotes alternative splicing of XBP1 mRNA leading to XBP1 protein product that can translocate to the nucleus and act as a transcription factor
Also PERK catalyses its autophosphorylation, upon release of BiP, and subsequently it catalyses eIF2α phosphorylation
ATF6 upon release of BiP is processed leading to release of its cytosolic portion that is now able to translocate in the nucleus and activate expression of UPR genes.

1.4 Ubiquitin proteasome system

The main protein degradation activity in the cell is carried out by the ubiquitin-proteasome system [73-75].

For proteasomal degradation proteins need to be ubiquitinylated through the participation of different ubiquitin ligases whose catalytic domain is always localised in the cytosol even if the ligase is anchored to the ER membrane [76].

Once recognised, proteins that must be degraded are targeted to the proteasome, which is a multisubunit with proteolytic activitiy shielded from cytoplams and nucleoplasm, in order to highly regulate proteolysis and to avoid non-selective protein degradation [77]. Protein degradation is a highly specific process, proteins have different half-lifes that range from minutes to years. This diversity in protein half-life ensures the right concentration of proteins within the cell; a balance

between synthesis and degradation rates is essential because the concentration of a protein determines its biological activity.

Proteasomal degradation requires energy in part for protein unfolding which confers to the protein the capability to access the proteolytic activity of the proteasome. The specificity of the degradation process is vastly provided by protein polyubiquitination that enables proteasomal recognition.

Ubiquitin proteasome system (UPS) controls cell cycle progression, signal transduction, cell death, immune responses, metabolism, protein quality control and development through degradation of short lived regulatory or structurally aberrant proteins [78,79]. Ubiquitin dependent proteolysis is fundamental in the adaptive immune response, because it leads to the production of peptides that can be presented on the major hystocompatibility complex (MHC) class I antigen presentation pathway. Peptides derived from proteasomal degradation of non-self antigens can be loaded on MHC class I molecules and presented to CD8+ cytotoxic T-lymphocytes (CTL).

1.4.1 Ubiquitinylation

Ubiquitin is a 76 aminoacids long protein that plays its main role in misfolded protein degradation, but also in many different cellular processes, by its covalent conjugation to cellular proteins [80-82].

Ubiquitinylation proceeds through the formation of a covalent amide bond between the C-terminal glycine residue of ubiquitin and the ε amino group of a lysine residue of the substrate [74,83]. Upon attachment on the lysine of the substrate, the ubiquitin becomes substrate for the attachment of another ubiquitin molecule.

In spite of the presence in the ubiquitin polypeptide sequence of seven lysines, all potentially involved in chain formation, mainly Lys^{48} and Lys^{63} are well-characterised residues involved in ubiquitinylation in vivo [83].

Proteins can be modified with polymers of ubiquitin that can display both proteolytic and non-proteolytic functions, depending on the conformation of the polyubiquitin chains, or with ubiquitin monomers, which is always connected to non-proteolytic processes [84].

Monomers of ubiquitin can bind to the target as single moiety (monoubiquitinylation) or as multiple single moieties (multiple monoubiquitinylation) [85].

Monoubiquitinylation can alter protein activity and localisation; it is mainly associated with sorting of proteins, viral budding and membrane trafficking due to its role in endocytosis [86,87].

Polyubiquitinylation that involves Lys^{48} is associated with a degradative fate, whereas Lys^{63} (although it has been recently described as a target for 26S proteasome [88]) is generally associated with non-degradative fate.

Lys^{63} polyubiquitinylation acts during DNA repair, kinase signalling [89], intracellular protein trafficking [90] and transcription [91].

Through the targeting of proteins for proteasomal degradation, Lys^{48} polyubiquitinylation plays an essential role in the regulation of cell cycle, immune signalling, DNA repair and apoptosis by controlling the abundance of cell cycle regulatory proteins, apoptotic and anti apoptotic proteins [86].

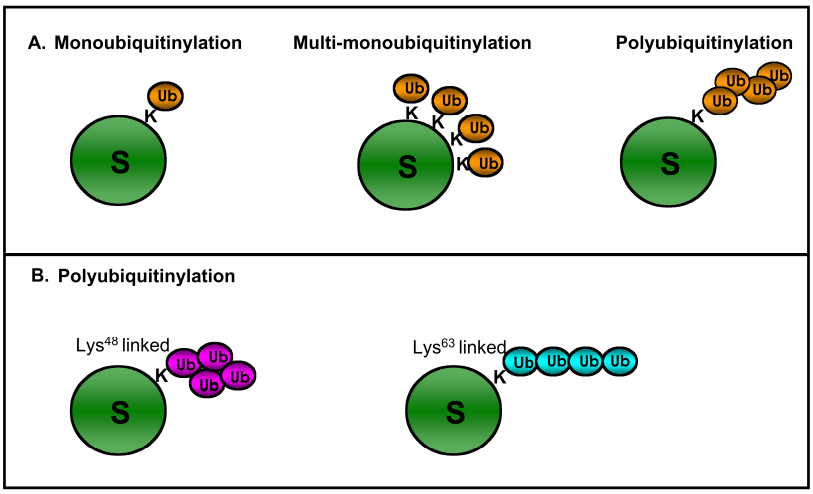

Figure 5: Schematic representation of different types of ubiquitinylation. A) The substrate can be modified with a monomer of ubiquitin (monoubiquitylation), with multiple monomers of ubiquitin (multi-monoubiquitinylation) or with ubiquitin chains (polyubiquitinylation). B) The most common lysine residues involved in the formation of polyubiquitin chains are Lys^{48}, associated with degradative fate, and Lys^{63}.

Basically the ubiquitinylation mediated degradation requires the activity of three enzymes: E1, E2 and E3. The process occurs through the following steps:

1. The E1 enzyme activates ubiquitin by hydrolysing a molecule of ATP establishing a thioester bridge between the cysteine in the active centre of E1 and the glycine 76 of the ubiquitin carboxy terminus. Different E1 enzymes are present in the cytosol and in the nucleus [92].
2. The activated ubiquitin is transferred to a member of ubiquitin conjugating enzyme family E2 (or Ubc) with the same thioester bond. E2 enzymes can be divided in groups; every group has the specificity for a particular set of E3 enzymes.
3. E3 enzymes recognise target proteins that must be degraded and are able to interact with E2 enzymes. E3-target complexes recognise E2-ubiquitin complexes and this interaction allows the transfer of ubiquitin from the E2 enzyme to the target [93].
4. The E3 enzyme releases the ubiquitinylated protein
5. These steps are repeated until a chain of at least four ubiquitin subunits is attached to the target. In some cases polyubiquitinylation is obtained by the activity of ubiquitin elongating factors termed E4 enzymes [94].

E3 enzymes possess the ability to ligate activated ubiquitin molecules to the substrate; there are several E3 enzymes and the way of action can be very different.

Based on their structure E3 enzymes can be assigned in two groups: the HECT (homology to E6AP C-terminus) E3s and the RING finger E3s, which are in turn subdivided in two families, the U-box [95,96] and the classic RING fingers.

The RING finger is characterised by the presence of cysteines and histidines that bind to zinc ions allowing the structure to be stabilised, the U-box, instead, share the same pattern of aminoacid residues but do not contains the series of cysteines and histidines and the structure is probably stabilized by salt-bridges and hydrogen bonds.

The RING finger E3s bind both the substrate protein and the E2 enzyme bound to ubiquitin and catalyses the direct transfer of ubiquitin to the substrate, whereas, for HECT E3s pathway is different; after E3 binding to both substrate and E2 enzyme,

ubiquitin is transferred first form E2 enzyme to an internal active cysteine residue of HECT E3 enzyme and then to the substrate's lysine [97].

RING finger E3s are susceptible to auto-ubiquitinylation and this causes their short half-life. HRD1, which is the best known E3 enzyme, requires the interaction with an adaptor protein, the ER resident protein SEL1L, for its stabilisation, otherwise it catalyses its auto-degradation [98].

In order to be target for 26S proteasomal degradation proteins must be polyubiquitinylated with at least four molecules of ubiquitin [99].

The ubiquitin chain is recognised by 26S proteasome complex, then it is inserted into the catalytic side where a series of proteases rapidly degrade the protein into small peptides [100].

Ubiquitinylation is also essential for the process of dislocation of proteins from the ER to the cytoplams. Disruption of ubiquitinylation machinery causes an inhibition of protein dislocation [101-103].

In addition to ubiquitin there are other members of the family of ubiquitin-like modifiers (UBL), such as SUMO 1, 2 and 3, Nedd8 and ISG15. Several UBLs have been described to modify proteins following an enzymatic pathway similar of that of ubiquitin [104,105].

Ubiquitin like modifiers intervene in the regulation of several cellular processing, such as transcription, DNA repair, autophagy and cell cycle control.

Among a long list of poorly studied UBLs Fat10 is the only one that seems to be involved in substrate degradation in an ubiquitin independent manner [106].

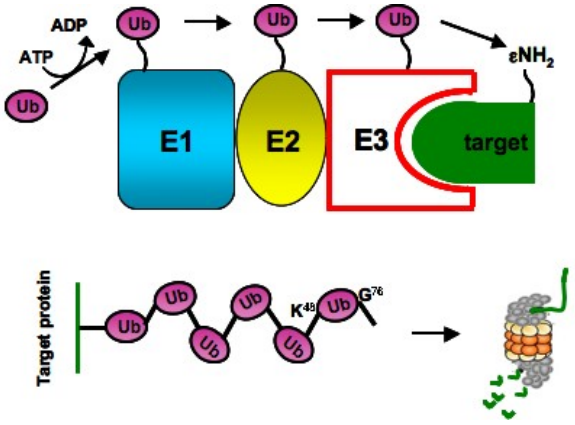

Figure 6: Through the hydrolysis of ATP E1 enzyme activates the ubiquitin and establishes a thioester bond with glycine 76 residue of ubiquitin C-terminus. Once activated, ubiquitin is transferred to E2 enzyme . E3 enzyme binds to the E2 enzyme and catalyses the tranfer of ubiquitin from E2 to the substrate that it recognises specifically. Polyubiquitinylated proteins are targets of proteasomal degradation.

1.4.2 The proteasome

The 26S proteasome is a large protein complex responsible for intracellular protein degradation, a process that requires energy. The proteasome contains in fact an ATP-dependent protease that collaborates with the ubiquitin system. Proteasome complex is composed by the catalytic subunit 20S of about 750 kDa and the 19S regulatory subunit of about 700 kDa [107-109]. This regulatory subunit can bind to one or both sides of 20S subunit in order to form an enzymatically active proteasome that could be the so-called 26S or 30S complexes. In the case of the 26S, only one 19S subunit is bound, while in the case of 30S proteasome, both sides of 20S subunit are bound by the 19S subunit [110].

The role of the 19S subunit is to recognise ubiquitinylated proteins and probably it also plays a role in substrate unfolding and transfer to the 20S catalytic subunit.

The 20S proteasome is a barrel shaped structure build up of four rings; two β rings form the inner proteolytic chamber while two α rings constitute the outer part of the proteasome.

Each ring of both yeast and mammalian 20S proteasome contains seven different α subunits α1-7 or seven different β subunits β1-7 giving rise to hetero-heptameric rings.

Proteolytic activity is carried out by the innermost part of proteasome, which constitute the main chamber, whereas α rings regulate the access of substrate within the catalytic core.

Only subunits β1, β2 and β5 of the main chamber show proteolytic activity, in particular, caspase like activity, trypsin-like activity and chimotrypsin-like activity respectively [111].

The release of substrate within the proteasome is regulated by different regulator proteins, such as the 11S regulator particle and the ATP dependent 19S regulator particle or PA700 [112].

PA700 is composed of about 20 subunits with a size of 25-110 kDa; six of these subunits are members of a large protein family characterised by domains containing ATP-binding motifs, named the AAA family ATPases, that together with two large regulatory components, Rpn1 and Rpn2, form the so-called “base” [113].

The base presumably functions as ubiquitin receptor [114] whereas the other sub-complex of PA700 is called the “lid” and does not present ATPase activity.
ATPases seem to have the role of continuously supply of energy for protein unfolding, to allow the penetration of proteins into the channel of 20S proteasome and the subsequent degradation [115,116].
The base complex of PA700 is bound to the outermost α ring of 20S proteasome and is able to open a narrow channel following ATP hydrolysis [117].
Ubiquitinylated proteins show a steric impediment for the entry into the proteasome structure; this implies the necessity of a deubiquitinylation activity coordinated with proteasome function [118-120]. Deubiquitinylation activity physically linked with protease activity of the proteasome is accomplished by lid components of PA700 [121].

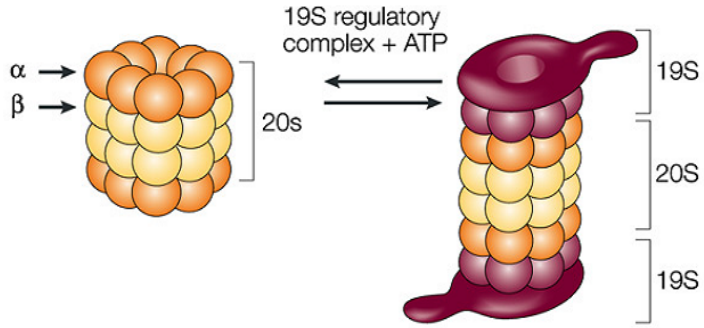

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Figure 7: Proteasome structure. The 20S catalytic core of the proteasome is build up of four rings; the two outermost are α rings whereas the inner part is formed by two β rings that display the proteolytic activity. Each α ring contains seven different α subunits and each β ring contains seven different β subunits. In order to get activated the proteasome can bind one or two 19S subunits giving rise to 26S or 30S proteasome.

1.4.3 Deubiquitinylation

Deubiquitinylating enzymes (DUBs) play an essential role in regulating proteasomal degradation. Deubiquitinylases (DUBs) are specific proteases that act on polymeric ubiquitin by cleavage of the C-terminal glycine of ubiquitin.

DUBs are grouped in a large family subdivided in five classes: ubiquitin C-terminal hydrolases (UCHs; [122]) ubiquitin specific processing proteases (USPs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMM/MPN+; [123]), ovarian tumour proteases (OTUs; [124]) and JAMM motif (zinc metallo) proteases. The UCH, USP, OTU and Josephin families are cysteine proteases whereas JAMM/MPN+ family members are zinc metalloproteases [125].

The main activity of DUBs is the removal of polyubiquitin chains to allow the substrate to entry within the proteasome and get degraded. However several other functions have been linked to DUBs.

DUBs can act to recycle ubiquitin contributing to ubiquitin homeostasis, to allow the escape of proteins from degradation either proteasomal or lysosomal or to control protein trafficking [126,127]. DUBs act removing non-degradative signals, reversing ubiquitin signalling or in editing of ubiquitin modifications by trimming ubiquitin chains [128].

Substrates that are targeted for proteasomal degradation are recognised by the regulator particle of the proteasome. Proteasome associated deubiquitinating enzymes can start to shorten the polyubiquitin chain after substrate recognition.

Lid components have been associated with deubiquitinylating and ubiquitin receptor activity.

In yeast lid complex of PA700 contains Rpn10 and Rpn13 subunits are thought to serve as ubiquitin receptors [129-132]. Among the other components (Rpn3, Rpn5-9, Rpn11-13 and Rpn15) Rpn11 has been described to display metalloprotease activity that cleaves peptide bond between the proximal ubiquitin and polyubiquitin chain [118,119].

In mammals yeast homologue of Rpn13 has been discovered and named hRpn13/Admr1. hRnp13 recruits UCH37 a previously described deubiquitinylating enzyme (DUB) associated with the proteasome [133]. Another deubiquitinylating enzyme associated with the proteasome is Ubp6 in yeast and Usp14 in mammals. Both DUBs are not a constituent component of proteasome structure but they associate with Rpn1/Rpn2 subunits of PA700 [114].

1.5 ERAD pathway

The ER associated degradation pathway comprises recognition of misfolded proteins, retro-translocation from the ER lumen to the cytosol, polyubiquitinylation and proteasomal degradation [134-136].

Misfolded proteins and non-assembled proteins are retained in the ER bound to chaperons such as Hsp70 family, calnexin and calreticulin or by lectins. After recognition the misfolded protein initiates the retro-translocation process.

The retro-translocation of the substrate is an essential process that cause the dislocation of the misfolded protein from the ER lumen, where it is recognised to the cytoplasm where proteasome is present and degradation can occur.

The spatial separation of the site of protein folding and the site of degradation, confers to the ERAD system an activity specific only for terminally misfolded proteins and not for folding intermediates.

The ER to cytosol dislocation process could occur through a channel that would lead misfolded proteins to cross the ER membrane and arrive to the cytosol. A putative retro-translocation channel is sec61p in yeast [137] and Sec61 in mammals [138].

In mammals Derlin-1, an ER membrane proteins with four transmembrane domains, has been described to participate in the retro-translocation process [139]. In yeast the homologue of Derlin-1, Der1p shows the same topology and although it was demonstrated to be essential in misfolded protein degradation a clear evidence of its involvement in retro-translocation is missing [140]. A further hypothesis, that will be described in more details later on, is that proteins would be exported from the ER within lipid droplets [141].

Another open question concerns the organisation of the retro-translocation and ubiquitinylation processes. One of the most probable hypothesis is that the protein is ubiquitinylated during retro-translocation, and after ubiquitinylation the ATPase p97 (cdc48 in yeast) is recruited to the ER membrane to supply the energy through ATP hydrolysis, for dislocation or for the release of substrates that have been already retro-translocated [142].

Following release of the misfolded protein the polyubiquitin chain is trimmed by the activity of deubiquitinylating enzymes (DUBs), step required to enter inside the proteasome. The activity of peptide *N*-glycanase, that displays deglycosylation

activity in the cytosol, could also be required for protein unfolding that is required for the efficient release of proteins within the proteasome.

Substrates are released in the 20S proteasome by the help of an ATP-dependent activator known as PA700 or regulatory complex 19S which binds 20S proteasome in order to form a complex known as 26S [100,108].

Once within the 20S catalytic core of the proteasome the substrate is broken down into peptides.

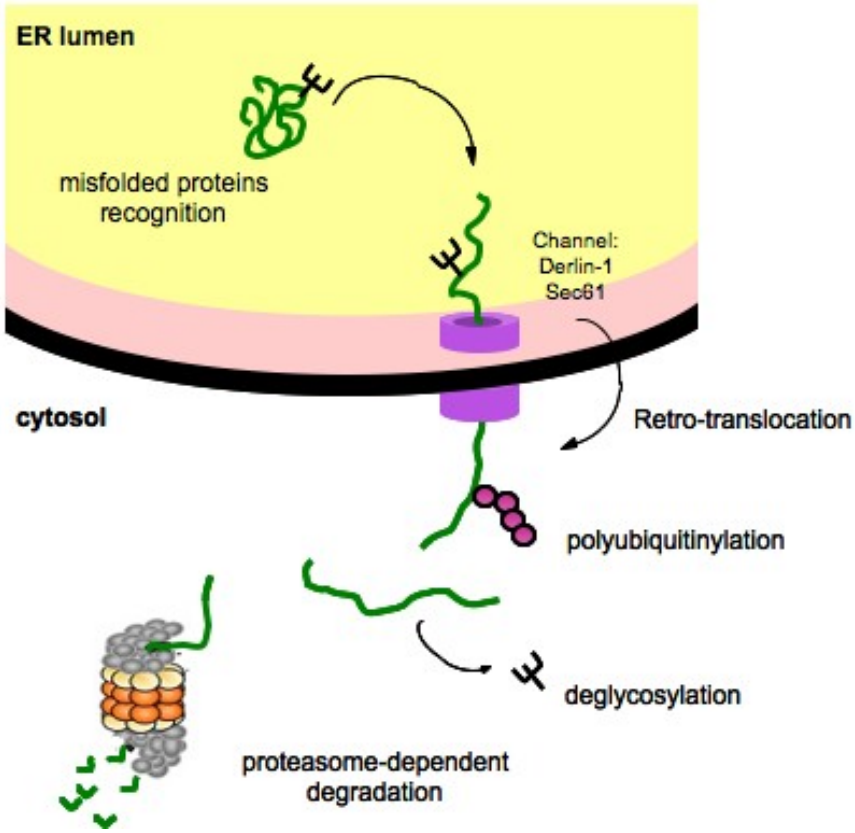

Figure 8: ERAD pathway. Misfolded proteins are recognised within the ER by chaperones and lectins, once recognised they are committed for degradation. To get degraded by the proteasome misfolded proteins must dislocate from the ER to the cytosol probably through a channel or within lipid droplets. During retro-translocation the protein is polyubiquitinylated, a step required for the recognition by the proteasome. In order to entry within the proteasome steps deglycosylation and deubiquitinylation are critical. Finally the protein is broken down into small peptides.

1.5.1 ERAD in yeast

Several ERAD pathways have been described in yeast and can occur depending on the type of the protein and on the localisation of the misfolded moiety.

Ì) The ERAD-L pathway is activated when soluble, secretory or membrane proteins have a defect in their luminal portion; ÌÌ) the ERAD-C pathway is involved

when a protein contains defects in the cytoplasmic region; ÌÌÌ) the ERAD-M pathway occur when a protein contains a lesion located into the transmembrane domain [143,144].

ERAD –L exploits the activity of Hrd1p ubiquitin ligase, which is a RING finger E3 enzyme that forms a complex with the adaptor protein Hrd3p, responsible of substrate recruitment [145,146].
Hrd1p interacts with the putative retro-translocation channel Der1p through Usa1p protein [147]. Usa1p is a double-spanning membrane protein that was found to be upregulated by the UPR [148] and that mediating the interaction between Hrd1p and Der1p seems to create a link between the ubiquitinylation process and dislocation to the cytosol [147].

ERAD C requires the activity of the Doa10 ubiquitin ligase, whose activity also depends on its RING finger. Doa10 localises at the ER/nuclear envelope, it contains 14 transmembrane domains and both its N- and C-termini are facing to cytosol, where most of the molecule resides.
It forms a complex with E2 enzymes Ubc6 and Ubc7, Cue1, a protein that stimulates Ubc7 and with Ubx2p, a membrane protein that, in turn, binds the ATPase Cdc48 [147,149].
Doa10p targets membrane proteins containing misfolded cytosolic domain toward proteasomal degradation [144].

Much less is known about ERAD-M pathway: it seems to depend on Hrd1 complex, but, differently from ERAD-L, it is independent of Usa1p and Der1p [144,150,151].
All these pathways converge to the ATPase cdc48 in order to ensure protein dislocation into the cytosol and ubiquitinylation.

1.5.1.1 Hrd1p ubiquitin ligase complex in yeast

In yeast ERAD M and ERAD L are carried out by the activity of Hrd1p ubiquitin ligase complex. Hrd1p is a multispan ER membrane protein characterised by the

presence of two distinct domains, one hydrophobic localised at the N-terminus containing the six transmembrane segments, and one hydrophilic localised at the C-terminus. The C–terminal portion contains the RING-H2 motif, required for the degradative function [145,152]. As mentioned before the E2 enzymes responsible of soluble and transmembrane protein degradation are Ubc6p and Ubc7p [153].

Ubc6p is an integral membrane protein while, Ubc7p is recruited to the membrane by Cue1p [146,154].

The activity of the E3 enzyme Hrd1p can be carried out thanks to its interaction with the protein Hrd3p [155]. Hrd3p is an ER glycoprotein that contains a single membrane span localised near the C-terminus and a large N-terminal luminal domain with different sets of repeated regions [156,157].

An essential role of Hrd3p during ERAD is to maintain the stability of Hrd1p [157].

A number of studies have been performed on Hrd1p and Hrd3p interaction and functions in which it was demonstrated that the Hrd1p N-terminal transmembrane domain is essential and sufficient for the binding to Hrd3p. Moreover this transmembrane region mediates the Hrd3p stabilisation effect on Hrd1p [155]. Furthermore the Hrd3p domain involved in Hrd1p stabilisation was investigated. Hrd3p contains two luminal domains; the first one starts from residue 1 and ends with residue 390 and the second one (residues 391-767) shows a high homology with the *C. elegans* protein SEL-1 and murine SEL1L [152,158,159]. The C-terminus contains the transmembrane region and a short cytosolic tail.

The main function of Hrd3p is the substrate recognition and the formation of complexes with chaperones.

Both Hrd1 and Hrd3 encoding genes are induced by the IRE1 yeast homologue Ire1p UPR transducer [148].

The Hrd1p complex also contains Yos9p, a lectin that specifically binds to misfolded glycoproteins and accelerates ERAD [58,160]. Yos9 requires protein demannosylation, a signal for protein disposal, in order to bind the substrate [161,162].

Another protein, Usa1p, was found to be part of the complex and probably it assists the clearing of luminally misfolded glycoproteins from the ER, allowing the interaction between Der1p and Hrd1p ligase and, therefore, probably linking retro-translocation and ubiquitinylation processes [58,160,163]. Moreover, Usa1p has been demonstrated to induce Hrd1p oligomerisation a step that seems to be required for protein degradation [164].

Another participant of the Hrd1p complex is Kar2p/BiP, which is a component of the HSP70 family whose action is keeping misfolded proteins in soluble state [165,166].

The Hrd1 complex consists of Hrd3p, Der1p, the ER lectins Yos and BiP; all these proteins together form a luminal surveillance complex that is able to recognise misfolded proteins and to promote their degradation [150].

Once recognised by the ER lectins the protein is bound to the ubiquitin ligase, subsequently the protein starts to be retro-translocated into the cytosol and the process of ubiquitinylation seems to happen concomitantly with the process of retro-translocation.

Extraction of the ubiquitin-modified protein occurs with the assistance of Cdc48 ATPase and its cofactors Ufd1p and Npl4p; this complex most likely provides the energy for pulling proteins out of the ER [167]. Alternatively the Cdc48 complex could allow the release of already retro-traslocated substrate [168].

1.5.2 ERAD in mammals

ERAD pathways are not described in such a deep detail as in yeast, however some homologous proteins have been identified between mammals and yeast. For example mammalian homologue of Doa10p, involved in degradation of protein bearing cytosolic misfolded domains, has been discovered and named TEB4-MARCHVI. This protein catalyses its own ubiquitinylation and for this reason it has been postulated to act as E3 ubiquitin ligase, although substrates of this predicted ubiquitin ligase have not been described so far [169].

Hrd1p mammalian homologue was also discovered and named HRD1/Synoviolin and is, so far, the best E3 enzyme characterised in mammals acting during both ERAD-L and ERAD-M pathways [152,170].

Another well characterised ubiquitin ligase is gp78, which is the first identified ER-membrane spanning ubiquitin ligase of mammalian cells; it has been shown to ubiquitinylate a variety of substrates and interestingly gp78 appears to target all substrates of the different pathways ERAD L, M and C [171].

In addition to the membrane ubiquitin ligases there are also E3 enzymes [172] involved in ERAD that are located in the cytosol. For example Parkin, a ubiquitin ligase with two RING fingers, was identified to mediate ubiquitinylation of Pael-R

but it also recognises non ER substrates possibly due to its interaction with cytosolic Hsp70 [173].

Another cytosolic E3 implicated in degradation of CFTRΔF508 a mutant of the cystic-fibrosis channel, which is retained within the ER and subjected to ERAD, is CHIP. CHIP is a U box ubiquitin ligase that function together with cytosolic chaperones in protein quality control [174].

1.5.2.1 HRD1/SEL1L ubiquitin ligase complex in mammals

Among the different identified mammalian E3 ligases, the best characterised one is HRD1/synoviolin, a 671 amino acid long ER transmembrane resident protein with six transmembrane segments and a RING finger motif localised to the cytosolic side. It is also characterised by the presence of a proline-rich cluster not found in yeast [175].

HRD1 displays an ubiquitinylation activity by means of its catalytic RING finger, which *in vitro* catalyses the transfer of ubiquitin from E2 enzymes to substrates or its self-ubiquitinylation [176].

HRD1 interacts specifically with the E2 enzyme UBC7 and its activity is restricted to Lys^{48} polyubiquitinylation [175].

A protein found to interact with HRD1 is HERP, the mammalin homologue of the yeast protein Usa1p. It contains a cytoplasmic ubiquitin like domain and is required for the degradation of some substrates [177].

HERP is recruited to the dislocation machinery by its binding to the putative dislocation channel Derlin-1 and although its activity seems to be dispensable for glycoprotein breakdown, it seems to be essential for breakdown of non-glycosylated proteins [178].

The mammalian homologue of yeast Hrd1p interacting protein, Hrd3p, was identified with the protein SEL1L, which interacts with mammalian HRD1 and allows the recognition of misfolded substrates that, subsequently, come in contact with the ubiquitin ligase HRD1. SEL1L is a component of an ER multiprotein complex implicated in the recognition and dislocation of misfolded proteins [98,179].

SEL1L is a type I transmembrane glycoprotein with five N-linked glycans; the bulk of the protein composed of eleven copies of the short tricopeptide-like Sel1

repeats exposed to the ER lumen [179,180] and a type II fibronectin domain present at the N-terminus of its large luminal part [181]. This domain is a small compact two-disulphide-bond module, which is missing in SEL1L invertebrate homologues suggesting the acquisition of an ulterior function in higher organisms.

The central portion of the protein is divided in three large clusters of Sel-1 like repeats, a subtype of the tetratricopeptide repeat (TPR) from residues 183 to 326, 373 to 554 and from 627 to 699 residues. TPRs are present in multiple copies in several proteins and are modules for protein protein interaction.

These domains consist of degenerate 34 aminoacids repeats oftenly organized in tandems with conserved aminoacid residues, hydrophobicity and spacing patterns. In particular a TPR is arranged in antiparallel amphipatic α-helices that help proteins interaction and multiprotein complexes assembly. These repeats seem to bind transcriptional regulators and component of signal transduction cascade [182].

The fibronectin domain probably can bind to chaperones or directly to misfolded proteins; alternatively it can recruit substrate recognition proteins such as EDEM or OS-9 the mammalian homologue of the yeast Yos9.

The Hrd3-like motif originally discovered in yeast Hrd3p consists of twelve aminoacids residues from 664 to 675 and is important for the interaction with the ubiquitin ligase HRD1.

The proline rich motif is located at the C-terminus of SEL1L (from 770 to 793 residues) and mediates protein-protein interactions [181].

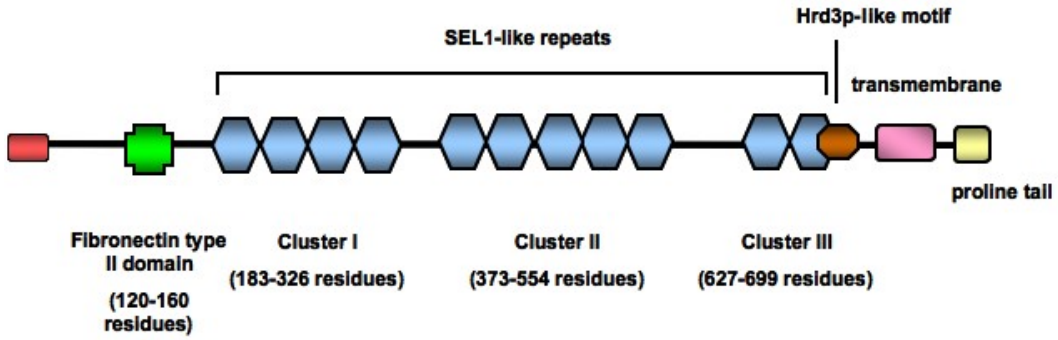

Figure 9: Organisation of SEL1L domains. SEL1L contains a signal sequence for its insertion in the ER lumen, a fibronectin type II domain, three clusters of SEL1-like repeats, an Hrd3p-like motif, a transmembrane domain that anchors the protein to the ER membrane and a proline rich tail.

SEL1L has a short half-life of about 180 minutes and its degradation seems to be proteasome dependent. Moreover, SEL1L remains completely susceptible to Endoglycosidase H treatment, suggesting that it resides exclusively in the ER compartment and does not traffic through the Golgi and secretory pathway [98].

SEL1-L interacts with the transmembrane ERAD component HRD1 and with the ATPase VCP/p97. It also interacts with Derlin-1 and Derlin-2, two multispanning membrane proteins that seem to have a role during ER-to-cytosol dislocation of proteins [139,179].

These interactions are abolished when the C-terminal portion of SEL1L, containing seven luminal SEL1 repeats, the transmembrane and cytosolic regions, are deleted ($SEL1L_{1-372}$); this suggests the requirement of an intact luminal portion of SEL1L for the formation of the complex [183].

During UPR the expression of HRD1 is induced by IRE-XBP1 pathway whereas expression of SEL1L is induced by ATF6 [184].

SEL1L is also associated with cell fate determination, cell differentiation, cell transformation and cancer progression [181].

Two ER-resident lectins OS-9 and XTP3-B were found to bind to SEL1L and the binding requires a domain that shows a conserved mannose 6-phosphate homology (MRH), which doesn't interact with the substrate.

OS-9 and XTP3-B bind to glycans of proteins and probably recognise ERAD substrates by the presence of a lower mannose structure deriving from α-mannosidase and EDEM demannosylation [183].

SEL1L coprecipitation with XTP3-B, OS-9 and HRD1 ubiquitin ligase suggested that in mammalian cells the homologue of the yeast complex Hrd1p-Hrd3p-Yos9 is conserved.

This complex would form a scaffold for ERAD of both glycosylated proteins and non-glycosylated misfolded proteins [185].

SEL1L and HRD1 are involved in degradation of many substrates: among them unassembled μ_s chains are well-defined substrates of ERAD whose degradation has been described to depend on both SEL1L and HRD1 expression [186].

Recruitment of proteins to HRD1 occurs either directly or indirectly through transient association of SEL1L with OS-9, XTP3-B, EDEM1, BiP or GRP94 a member of the Hsp90 family [60,183].

Others SEL1L interactors are PDI and calnexin and two proteins UBXD8 and UBXD2 that seem to be associated with the dislocation machinery and likely involved in recruitment of p97 to the dislocation complex [187].

It has recently been suggested different requirements for ERAD of soluble or membrane proteins that bare defects in the luminal domain (ERAD-L), by using the same ERAD substrate in two different versions: membrane tethered and soluble. The finding was that only soluble substrates of ERAD-L strictly require the participation of proteins of the HRD1 pathway. ERAD of soluble substrates, named ERAD-L_S, depends on the activity of the HRD1 ubiquitin ligase, SEL1L and the ER lectins OS-9 and XTP3-B. In contrast ERAD of membrane tethered proteins, that carry defects in luminal domains, seems to not be affected by inactivation of those HRD1 pathway proteins and is named ERAD-L_M [188].

Participation of OS-9 and XTP3-B is dispensable when proteins are membrane anchored thus, in contrast to yeast, demannosylation in mammals is not only linked to the binding to lectins. So up to now the need to unravel how ERAD membrane proteins are selected for degradation persists.

OS-9 and XTP3-B seem to be interchangeable during shuttling of soluble misfolded proteins to the dislocon and in this work it is hypothesised that membrane tethered proteins can laterally diffuse within the ER membrane and reach the dislocon.

Both SEL1L and HRD1 belong to the family of unfolded protein response genes that are induced during ER stress due to the accumulation of misfolded proteins [189].

Induction of SEL1L and HRD1 encoding genes ensure a more efficient recognition of misfolded substrate and degradation.

Recently it has been discovered the existence of other two splicing isoforms of SEL1L: with SEL1L-A it is indicated the transmembrane glycoprotein resident in the ER while the other two are secreted isoforms (SEL1L-B and –C) both lacking the SEL1L membrane –spanning region. SEL1L-B retains the SEL-1-like repeats and is able to participate in the formation of multi-protein complexes while SEL1L-C that lacks the repeats does not maintain this ability, suggesting that SEL-1 like

repeats are functionally responsible of multi-protein complex formation and signal transduction [190].

Peroxisomal localisation of SEL1L-C suggested a role in peroxisomal quality control that targets protein for degradation through an ubiquitinylation pathway similar to ERAD [191].

Beside the role in protein degradation SEL1L is a crucial participant in cell cycle regulation, tumour progression and tumour invasiveness through modulation of genes related to cell-matrix interactions [192]. In addition a series of human primary breast carcinomas show reduced levels or complete absence of SEL1L [193]. Both in breast cancer and human pancreatic carcinomas SEL1L levels affect expression of genes involved in cellular growth likely acting on the TGFβ- signalling pathway [192,193].

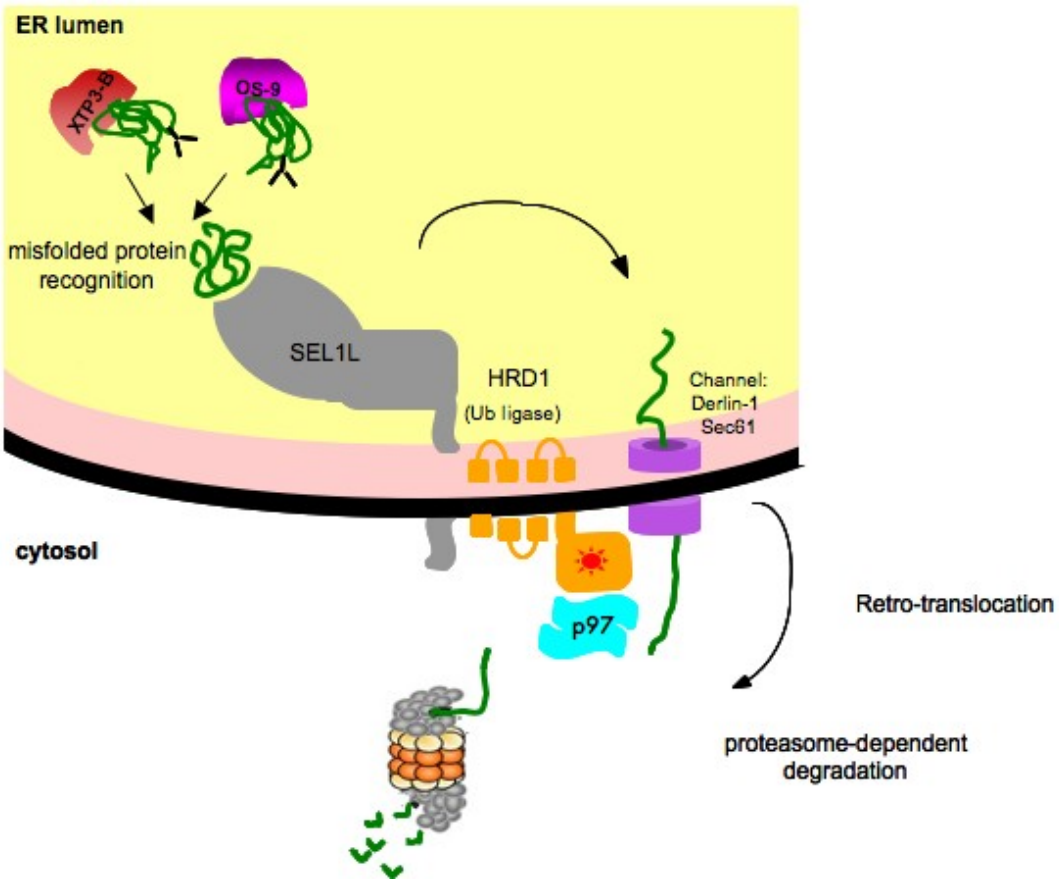

Figure 10: HRD1/SEL1L complex during ERAD pathway. SEL1L, OS-9 and XTP-3B cooperate in substrate recognition. After recognition the substrate can start to dislocate from ER lumen to the cytosol, probably crossing the lipid bilayer through a channel, or by ER extraction within lipid droplets. Retro-translocation requires ATP hydrolysis carried out by the p97 ATPase, which probably drives the dislocation process, or allows the substrate, already dislocated, to be released in the cytosol.

1.5.3 Retro-translocation machinery

Membrane and secreted proteins must be retro-translocated into the cytosol to be degraded by the proteasome.
There are basically two theories about how protein retro-translocation into the cytosol can occur: the first hypothesis bases the retro-translocation process on the activity of a channel called retro-translocon whereas the second one is the lipid droplets hypothesis that suggests that these particles have a direct role in dislocation [141].

1.5.3.1 The retro-translocation channel

Regarding the first hypothesis there are evidences of the involvement of Sec61 in the retro-translocation process. Sec61 constitutes a proteinaceous pore for the delivery in the ER of secreted, ER resident, or membrane anchored proteins.
Subunits of Sec61 were found to bind various ERAD substrates during degradation. MHC class I molecules have been found to bind Sec61 before their delivery to the proteasome when coexpressed with two small transmembrane proteins of human cytomegalovirus (HCMV) termed US2 and US11 immunoevasins that down-regulate MHC-I expression with the aim of escape the immune system [194]. Moreover, Cholera toxin once endocytosed within the cell and transported to the ER needs to be retro-translocated to the cytosol through its binding to Sec61 [138].
One hypothesis is that the differential binding partners induces modifications of the Sec61 channel in order to function in the opposite ways of translocation into the ER and retro-translocation into the cytosol [195].
However the involvement of Sec61 in retro-translocation process remains controversial and still under investigation. In some cases it has been demonstrated the exit of proteins from the ER fully glycosylated; this suggests that the channel involved in retro-translocation should be larger than the import channel that has to accommodate only the polypeptide chain [194,196].

In addition the yeast Der1p and the family of Derlin proteins in mammals have been described as candidates for being putative retro-translocation channels. The yeast Derp is a component of the Hrd1 ERAD-L complex through its interaction to Usa1.

Yeast Der1p is predicted to span the ER membrane four times with both N-terminus and C-terminus facing the cytosol [140]. In yeast it was proposed that the retro-translocation channel for misfolded luminal domains consists of Hrd1 ubiquitin ligase complex, Der1p and Usa1p. Usa1p would be the mediator for the association between Hrd1p and Der1p in order to link together ubiquitinylation and retro-translocation [147].

Derlin-1 is the mammalian homologue of the yeast Der1p that shares the same topology [98,139] and is probably implicated in retro-translocation [140,197].

There are evidences that Derlin-1 binds to misfolded and ubiquitinylated proteins. Down-regulation by RNA interference of Derlin-1 of *C. Elegans* provokes ER stress, supporting the hypothesis of its essential activity during ERAD process [98].

Supporting this evidence experiments of RNA interference of Derlin-1 have been performed showing a marked increase of CFTRΔF508 [198].

Other evidences supporting the view of Derlin-1 as a key component of retro-translocation channels come from experiments in which MHC class I molecules are retro-translocated into the cytosol of HCMV infected cells by the interaction with the p97 ATPase and Derlin-1. In particular Derlin-1 is able to bind prevalently glycosylated but also deglycosylated MHC class I molecules [98,139]. Moreover, it associates with peptide-N-glycanase at the ER membrane, suggesting the possible role in recruiting cytosolic deglycosylating enzymes, consistently with its interaction with both MHC class I molecules glycosylated and deglycosylated [199].

More recent studies showed that Derlin-1 interacts with the ATPase p97 and through this binding it can associate with the ubiquitin ligases HRD1 and gp78 [179,200].

Derlin-1 has been associated with the retro-translocation of Cholera toxin although it is not excluded its transport to the cytosol through Sec61p channel. The hypothesis is that, once recruited to Derlin 1, cholera toxin is transferred to Sec61 [201].

Further evidences that Derlin-1 could be involved in retro-translocation came from an *in vitro* retro-translocation approach. In this study retro-translocation of fluorescent pro-alpha factor from ER microsomes was measured as decrease in fluorescence due to the presence in the cytosolic fraction of a quenching agent. Antibody against Derlin-1 blocked the decrease in fluorescence while antibody against Sec61 allowed a decrease in fluorescence to occur due to capability of pro-alpha factor to retro-translocate and loose its fluorescence by the activity of the quenching agent [202].

In mammals there are other two Der1p-homologues, Derlin-2 and Derlin-3 that similarly to Derlin-1 contain four hydrophobic domains with both N-terminus and C-terminus facing the cytosol [203].
Derlin-2 and 3 demonstrated also to be required for degradation of misfolded glycoproteins; they associate with the substrate, p97 and EDEM.
Derlin-2 helps the formation of p97-EDEM complex mediating the association of p97 with the substrate.

To complicate a poorly understood scenario there is the discovery of another protein, TRAM1, probably involved in disposal of ER degradation substrates. TRAM1 was previously characterised for its function during translocation of nascent polypeptides in the ER [204].
Lack of TRAM1 limits the extraction of proteins from the ER and leads to an increased UPR [205]. In this work it is also demonstrated that the role of TRAM1 is important for the disposal of membrane substrates independently of their glycosylation status but it doesn't act on soluble substrates.

More recently BAP31, a three membrane spanning ER protein, previously demonstrated to be involved in protein sorting [206], has been described to be possibly implicated in retro-translocation process. In fact, BAP31 has been found to interact with the Sec61 translocon and with a factor acting during the insertion into ER membrane of membrane anchored protein, TRAM [207].
Studies on ERAD of CFTRΔF508 evidenced BAP31 important activity during ERAD process.

BAP31 knock down experiments have shown an increase in the amount of CFTRΔF508 but the fraction of CFTRΔF508 coimmunoprecipitated with Derlin-1 was lower. This result suggested that Bap31 has an important role in promoting the interaction of Derlin-1 with the substrate [207].

In yeast another putative retro-translocation channel, build up by Hrd1p oligomerisation following Usa1p activity, has been proposed. In particular the multiplication of transmembrane segments of Hrd1p has been demonstrated to be required for protein degradation probably due to its activity in dislocation of proteins from ER to cytosol [208,209].

1.5.3.2. Lipid droplets in ER to cytosol dislocation

Lipid droplets are organelles for the storage of lipids in mammalian cells that can form soon after an increase in fatty acids levels [210]; they bud from the ER surrounded by a single layer of phospholipids and are believed to be formed by lipid ester deposition between the two leaflets of ER membrane [211,212].
Protein composition of lipid droplets differs form the ER protein content due to the presence of proteins such as adipophilins and perilipin, that are specifically inserted in lipid droplets [213]. Recognition of these lipid droplets specific proteins could help in the identification of these structures.
A possible involvement of lipid droplets in the escape of proteins from the ER in order to allow their proteasome-dependent degradation has been recently proposed for type I membrane proteins. The protein taken into consideration was the MHC class I molecule targeted for retro-translocation and degradation by the two immunoevasins of the human cytomegalovirus, US2 and US11 [194]. These proteins are expressed by HCMV in order to avoid the recognition by the immune system of viral peptides loaded on MHC-I molecules of infected cells.
The hypothesis is that MHC-I molecules can be discarded from the ER either as part of lipid droplets or as a structure composed solely of MHCI molecules and phospholipids. US2 and US11 are supposed to be able to create a suitable environment for the formation of bicellar structures through the recruitment of proteins that could promote lipid rearrangements [141]. To support this hypothesis there are works on apoliprotein B (ApoB) in which it has been demonstrated that in

cells that actively degrade ApoB there is an association between lipid droplets and ApoB, ApoB is ubiquitinylated and recruits the proteasome [214].

1.5.4 ATP dependent extraction of ER proteins to the cytosol

In order to reach the cytosol, proteins with a degradative fate must follow a series of steps that are still a matter of intensive study.

What is already known is that after retro-translocation proteins that are substrates for degradation are recognised by a series of ubiquitin binding proteins that escort proteins from the ER membrane to the proteasome [215,216].

An essential protein involved in these steps is, in yeast, the ATPase cdc48 and in mammals ATPase p97.

The p97 and cdc48 couple ATP hydrolysis with different cellular activities, among which there is the activity in releasing proteins with degradative fate from the ER [217]. The p97 consists of six subunits each containing two ATPase domains (D1 and D2) that are arranged in a homo-hexameric ring configuration. The bottom of the hexameric ring forms a central pore that seems to accommodate the protein targeted for degradation [218]; this hexameric structure seems to undergo large dynamic conformational changes during ATP hydrolysis that are likely linked to the process of unfolding of the protein [219,220].

The p97 and cdc48 are abundant in the cytosol and in the nucleus but a considerable fraction is tightly associated to the ER membrane on the cytosolic side [142,221].

The cdc48 seems to be recruited to the membrane through UBX-domain-containing membrane protein Ubx2 [149,222], while in mammals p97 can be associated to the membrane by the interaction with the valosin-containing protein VIMP [98].

Beyond their pivotal role in ubiquitin-dependent degradation [223] these ATPases are involved in many cellular processes such as apoptosis, post-mitotic membrane fusion of ER and Golgi membranes, spindle assembly after mitosis, nucleic acid repair and replication [224,225].

They can bind to different partners and the ability to form different complexes seems to determine their different functions.

In yeast cdc48 has two associated factors, Ufd1 and Npl4, which contain poly-ubiquitin binding domains. These two cofactors are conserved in mammals and are called UFD1 and NPL4. The binding of the ATPase with these two proteins directs the activity towards ER degradation [226].

The complex cdc48-Ufd1-Npl4 has been described to be implicated in the release of polypeptides from the ER membrane [227].

Also p97 has been associated with protein extraction from the ER [223] and also in mammals there are evidences that for this activity it requires the binding to two cofactors present in a heterodimeric complex: UFD1 and NPL4 [223,228].

The UFD1 and NPL4 bind ubiquitinylated substrates through ubiquitin binding domains whereas previous studies indicate that p97 transiently associates with proteasome. This suggests a role of p97 in facilitating the interaction between ubiquitinylated proteins associated with NFD1 and NPL4 and the proteasome [229,230].

Noteworthy, is the finding that p97 is always detected in association with SEL1L and Derlin-1 [179].

The ATPase activity of p97 can act on retro-translocation by providing the energy for dislocation or allowing the release of substrates that have been already retro-translocated [136].

A recent work has collected a number of observations that suggest the function of p97 as unfoldase. The p97 would lead the substrate to acquire a poorly folded state that would allow proteasomal degradation [231].

Regarding p97 interaction with substrates an endogenous inhibitor of ERAD has recently been characterised and named SVIP (small p97/VCP interacting protein) [232].

SVIP was previously described to be localised in the cytosol and to be anchored to the ER membrane through myristoylation. In this work it was described that SVIP overexpression causes cellular vacuolation due to the dilatation of ER [233] and more recently it has been described to form a complex with Derlin-1 and p97 [232].

In particular it was postulated that SVIP reduces the association of ERAD substrates with the gp78 ubiquitin ligase and p97, avoiding protein ubiquitinylation and degradation [232]. These findings confirm either the essential role of p97 during ERAD and also the need of mechanism to block an uncontrolled degradation processed.

1.5.5 Deglycosylation and ubiquitin escort factors

The link between proteasome machinery and degradation of substrates retro-translocated to the cytosol seems to be determined by deubiquitinylating enzymes, deglycosylating enzymes and ubiquitin binding factors [216].

Peptide N-glycanase (PNGase) a deglycosylating enzyme has been proposed as a possible candidate for protein-proteasome connection. PNGase was discovered in yeast and is responsible for the deglycosylation of N-linked glycoproteins. Despite of its broad range of substrates it acts only on misfolded glycoproteins that have been dislocated from the ER to the cytosol [234,235].

Analysing possible interactions Rad23 protein was identified as a PNGase interactor [236].

There are evidences that the 26S proteasome is bound to a fraction of Rad23 (Rad23p) through its N-terminus ubiquitin like domain [237]. Therefore, it is possible that PNGase interacts with the proteasome through its interactor Rad23, suggesting an involvement of PNGase in the ERAD process [238,239].

Rad23 is part of a class of ubiquitin-like domain (UBL) proteins that include also Dsk2. UBL domain confers to these proteins the ability to bind 26S proteasome [237].

Besides their UBL domain Rad23 and Dsk2 contain an ubiquitin associated domain (UBA), which is a sequence of about 45 aminoacid residues specific for ubiquitin chains [240]. Due to the presence of UBL and UBA domains that recognise polyubiquitinylated proteins Rad23 and Dsk2 have been proposed as shuttling factors able to target ubiquitinylated substrates toward the proteasome by binding simultaneously both the proteasome (through UBL domain) and the substrate (through UBA domain) and for this reason they are collectively named ubiquitin receptors [132,241,242].

Rpn1 and Rpn10 subunits of the proteasome are able to interact with UBA-UBL proteins Rad23 and Dsk2 allowing the release of substrate to the proteasome [215].

2. HCMV dependent dislocation/degradation of MHC class I molecules

2.1 MHC class I structure and function

MHC class I molecules are glycoprotein expressed on the cell surface involved in immunodetection and elimination of virally infected cells or transformed cells.
They can bind a broad array of peptides that are generated intracellularly, in order to present them at cell surface to cytotoxic T cells [243].
Dendritic cells, which are very potent antigen presenting cells (APC), are also able to present on MHC-I peptides that derive from exogenous antigen in a peculiar pathway called "cross-presentation" [244].
An MHC class I molecule contains a large α chain associated non-covalently with a β2 microglobulin. The α chain is a type I transmembrane glycoprotein of about 45 kDa and is encoded by genes in A, B and C regions of the human HLA complex and contains hydrophobic transmembrane segment and hydrophilic cytoplasmic tail.
The β2 microglobulin is an invariant protein with a weight of about 12 kDa. Association between α chain and β2 microglobulin is essential for the membrane expression of MHC-I molecules.
Both α chain and β2 microglobulin are synthesised by polysomes in the ER. MHC-I assembly involves the activity of ER resident chaperones, in particular calnexin.
Moreover, for the stability of class I molecules the binding to an 8-9 aminoacids long peptide is required [245,246].
Cell specialysed in the recognition of peptides loaded on MHC-I molecules are the Cytotoxic T lymphocytes (CTLs). CTLs are CD8+ cells restricted to the recognition of peptides presented by MHC-I molecules by the cell surface receptor termed TCR. Since every kind of nucleated cell expresses MHC-I, CTLs can recognise all types of transformed or infected cell.
CTLs immune response takes place through two different steps: the first phase serves to activate and differentiate naive T_C cells into functional CTLs.
Upon interaction of TCR expressed on T_C cell with peptide presented on MHC-I molecules by antigen presenting cell, in particular dendritic cells (DCs), T_C cells start to proliferate and differentiate into CTLs.

The second phase involves the recognition by antigen activated CTLs of MHC-I molecules complexed with a specific peptide on target cells. Following recognition CTLs initiate the effector phase that leads, by the secretion of lytic granules to the killing of the target cell.

CTLs immune response is directed to allogenic cells, transformed cells, virus infected cells and chemically conjugated cells. The capability of CTLs to recognise and destroy viral infected cells forced different viruses to develop strategies to evade the immune system. Several viruses have been found to produce proteins interfering with specific or non-specific host defences. For instance a viral defence against non-specific host defence is represented by the capability to overcome interferon α and β activities, which are expressed by the cell in response to viral infection. Other viruses continuously mutate their proteins in order to avoid recognition while other viruses interfere with the immune response suppressing peptide presentation by MHC-I molecules [247].

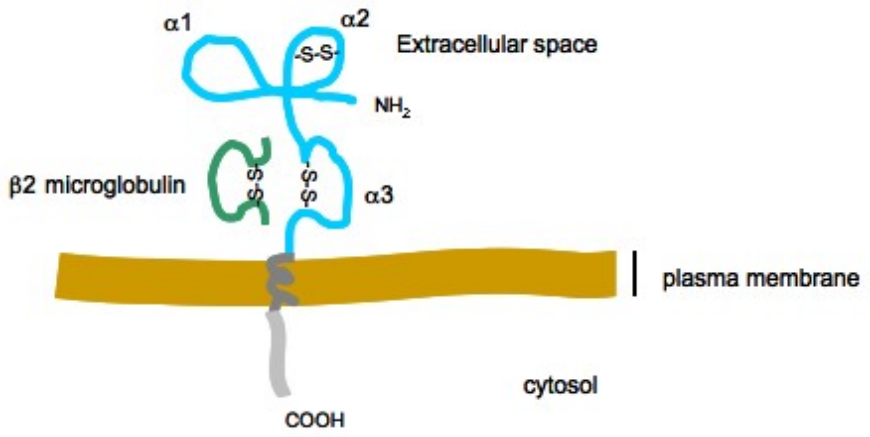

Figure 11: Structure of MHC class I molecule constituted by the α chain with three domains (α1, α2 and α3) and β2 microglobulin. The α chain creates a groove for the binding to peptides.

2.1.1 Folding of MHC-I molecules

During translation MHC-Iα binds rapidly the transmembrane chaperone calnexin and the associated ERp57, a member of protein disulphide isomerase (PDI), which enables the formation of proper disulphide bonds in glycoproteins that have been just inserted into the ER membrane [248,249]. Association with calnexin ensures inhibition of α chain degradation and allows the formation of disulphide bonds in the α2 and α3 domains by ERp57.

Afterwards MHC-Iα chain associates with β2 microglobulin in a highly unstable heterodimer which is stabilised by calreticulin that replaces calnexin [245,246,250].
This binding cause the change in MHC-Iα conformation which is now able to bind peptides and to enter the peptide loading complex (PLC) whose components are: MHC-Iα-β2 microglobulin heterodimer, calreticulin, ERp57, tapasin, the transporter associated with antigen processing (TAP) and Bap31.
Tapasin plays an important role during MHC-Iα folding acting as a bridge between TAP and the other components of the PLC. Tapasin allows the stabilisation of either TAP or the entire PLC [251,252] and is linked to ERp57 through a disulphide bond [253].
Peptides presented on MHC class I are produced mainly in the cytosol and can come from various sources including endogenous or viral cytosolic proteins, proteins retro-translocated to the cytosol from the ER or proteins internalised.
All of these proteins generate peptides following proteasomal degradation that are then transported into the ER by the transporter associated with antigen processing (TAP). Peptides that match with the binding groove of MHC-I are loaded on class I molecules with the assistance of the PLC.
However, peptides trasported through TAP are frequently longer than the 8-10 residues length suitable for MHC-I loading and therefore, the activity of ER associated peptidases is required. In humans there are two ER aminopeptidases named ERAP1 [254,255] and ERAP2 [256] that seem to complement their function; ERAP1 cleaves peptides with hydrophobic residues whereas ERAP2 cleaves peptide with basic residues [256,257].
Thanks to the physically association to TAP, MHC-Iα-β2 microglobulin complex can easily associate with peptides.
Following peptide association MHC-I acquires more stability, dissociates from calreticulin and TAP and its vesicular export from the ER can start through a process that involves Bap31 and possibly other export receptors [258].

2.2 HCMV immunevasion: affecting MHC class I-peptide complex recognition

Human cytomegalovirus (HCMV) can cause serious diseases in immunosuppressed or immunodeficient patients and in children; it infects many

cell types including epithelial, glial fibroblast endothelial cells and monocytes/macrophages. HCMV replicate slowly in most cells and it establishes a latent state in monocyte/macrophages: its life cycle is actually characterised by periodic reactivation and replication. HCMV can survive thanks to its ability to compromise the immunological mechanisms that ensure the recognition of infected cells by T lymphocytes and natural killer cells [259,260].

The ability to inhibit this recognition is characteristic of many viruses that encode proteins that affect cell surface expression of MHC-Iα: down-regulation of MHC-Iα molecules is a mechanism that the virus uses to escape the cellular immune response and is known as immunoevasion [261].

In particular HCMV contains in its genome a unique short region S (US) that encodes for eight membrane glycoproteins involved in immunoevasion and thus called immunoevasins [262].

These proteins are differentially expressed during the viral life cycle and with different ways of action they affect cell surface expression of MHC-Iα. Among these proteins, US3 is the earliest expressed after infection and prevents trafficking of newly synthesised MHC-Iα molecules causing their retention in the ER compartment [263]. Afterwards two proteins that induce specific proteasomal degradation of MHC-Iα are expressed: these proteins called US2 and US11 are independently sufficient for the dislocation of MHC-Iα molecules newly synthesised in the ER [235,262].

MHC-Iα molecules upon interaction with US2 and US11 start the dislocation to the cytosol and, as soon as the exposition to the cytosolic face has occured, the enzyme N-glycanase removes the N-glycan from its sequence allowing the protein to entry into the proteasome [264-266].

MHC-Iα molecules degradation pathway induced by US2 and US11 follows a series of steps very similar to the ERAD of misfolded proteins [136,264].

A protein that works both at early and late times post-infection is US6, that blocks TAP, thus preventing peptide loading on MHC class I molecules [267].

2.2.1 US2 and US11 activity

US11 is a type I transmembrane glycoprotein with an N-terminal ER luminal portion, a transmembrane domain and a short cytoplasmic tail.
The specificity of US11 activity is directed to α1 and α2 luminal domains of only some MHC-I molecules. Depending on the aminoacid composition of these two domains US11 association and affinity with MHC-Iα can be different. In fact, US11 causes downregulation of MHC-I molecules, in particular it is specific for HLA-A2 and for members of the region C (HLA-C) [268,269].
Further elucidations on how US11 mediates MHC-Iα retro-translocation and degradation come from the findings that US11 seems to work independently of the tertiary structure of MHC-I molecules, thus working both on folded or misfolded molecules and it catalyses the polyubiquitinylation of MHC-I [270,271].
The US11 pathway exploits the activity of the ERAD proteins Derlin-1 and SEL1L. SEL1L is involved in the retro-translocation of MHC-I molecules and is essential for US11 mediated MHC-I molecules dislocation into the cytosol [98,180].
The proposed model is that US11 has the specific function of delivering MHC-Iα molecules to HRD1/SEL1L complex, promoting their rapid dislocation and degradation [187].
US11 transmembrane domain is essential for its ability to induce retro-translocation of MHC-Iα molecules, it contains a glutamic residue which could interact with host proteins; this suggestion is supported by the finding that a US11 mutant in which glutamic residue 192 is mutated to leucine (US11Q192L) is no more able to induce dislocation of MHC molecules because Derlin-1 binding site is disrupted [271].
Noteworthy, once expressed in the cell, US11 induces the UPR response activating XBP-1 splicing, while the US11Q192L does not.
This indicates that UPR is not induced merely by US11 expression, but is dependent on the interaction between US11 and Derlin-1 within the ER lipid bilayer [272].

US2 shares the same topology of US11 and, in spite of the presence in the sequence of a site of N-glycosylation, US2 can be found both in glycosylated and

non-glycosylated form. The non-glycosylated form is supposed to derive from an inefficient insertion in the ER, caused by the presence of a non-cleavable signal peptide, which could diminish the efficiency of ER insertion. Inefficient insertion would therefore lead a fraction of protein to be localised in the cytosol in a non-glycosylated form [273].

US2 recognises MHC-Iα molecules through an immunoglobulin–like fold, which targets the luminal part of MHC-Iα soon after synthesis. Following interaction with US2, MHC-Iα molecules are transported back to the cytosol, where steps of deglycosylation and proteasome-dependent degradation take place [270].

US2 interacts with HLA α2 and α3 regions and is able to down modulate HLA-A2, HLA-B27 and HLA-G [269].

Compared to US11 activity, the US2 pathway is less understood, but it seems to be dependent on the activity of signal peptide peptidase, a protease that cleaves polypeptide within the membrane [274]. Furthermore it has been demonstrated the critical requirement of a functional ubiquitin system [275] and very recently it has been characterised the TRC8 E3 ligase acting in the US2 pathway [276].

Differently from US11, US2 seems to display its activity preferentially on properly folded MHC-Iα molecules, which upon US2 contact and dislocation are modified with 3-5 ubiquitins [271].

In order to promote MHC-Iα molecules dislocation, US2 needs its cytoplasmic tail, where some important residues reside: cysteine 187, serine 190, tryptophan 193 and phenylalanine 196 are likely involved in the interaction with the retro-translocation machinery. Upon tight interaction between US2 and MHC-Iα has occured, the cytoplasmic tail of US2 would engage the retro-translocation machinery [277].

To further confirm these suggestions the cytoplasmic tail of US2 was fused to US3, thus conferring to US3 degradative activity on MHC-Iα [278].

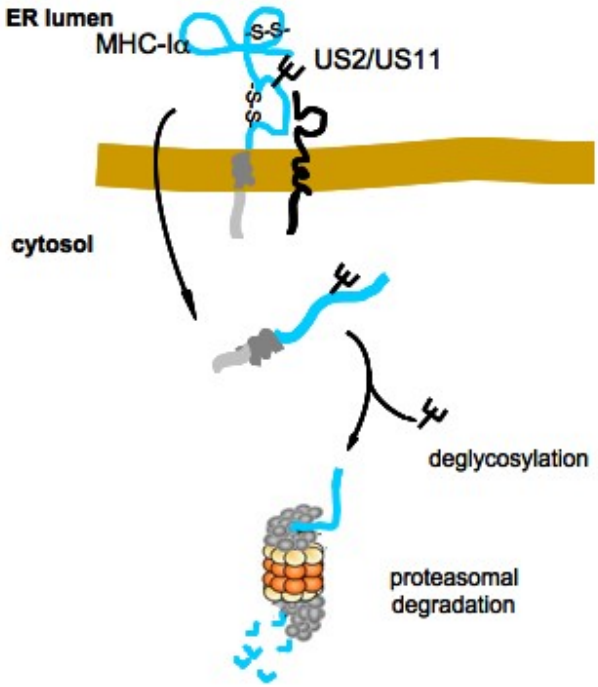

Figure 12: Illustration of US2 and US11 degradative activity against MHC-Iα molecules. Once engaged by US2 and US11, MHC-Iα molecules start to dislocate from the lumen of ER to the cytosol, probably crossing the lipid bilayer through a channel. After retro-translocation MHC-Iα molecules are subjected to a deglycosylation step by enzymes localised in the cytosol and finally they are transferred to the proteolytic chamber of the proteasome in order to get degraded.

3 Immunoglobulins

Antibodies or immunoglobulins are glycoproteins expressed by B lymphocytes, as membrane bound or secreted molecules; antibodies are the central effectors of the humoral immune response due to their ability in recognising and binding foreign molecules with high specificity [279].

All antibodies are made up of four polypeptide chains; two light chains of 23 kDa and two heavy chains of 50 to 75 kDa. Every light chain is bound to a heavy chain by an interchain disulphide bond and by a combination of ionic bonds, hydrogen bonds and hydrophobic interactions.

Similar interactions are responsible for the binding of the two identical heavy chains.

A peculiar characteristic of immunoglobulins is the presence of homologous repeated sequences either in the heavy chain and in the light chain; these domains are about 110 aminoacids long that give rise, once folded, to a compact globular structure. These immunoglobulin domains are formed by two series of β sheets with antiparallel orientation. The resulting immunoglobulin domain is stabilised by hydrophobic interactions and by conserved disulphide bonds that link together the two series of β sheets [280].

Both heavy and light chains contain constant and variable regions; variable regions are responsible for antigen binding, while constant regions are responsible for effector functions.

There are five different isotypes in the constant region of heavy chain that generate five classes of antibodies: IgA, IgE, IgG, IgD and IgM. Depending on which type of constant region the antibody contains, different effector functions can be carried out.

IgA, IgD and IgG contain three constant domains and a hinge region whereas IgM and IgE contain four constant domains and no hinge region [281].

IgG: is the most represented class of immunoglobulins, made up by two γ heavy chains. IgGs can activate the complement and bind with high affinity the FC receptors expressed on phagocyte membrane mediating the opsonization.

IgM: are expressed as monomers on B cell surface or are secreted by plasmacells as pentamers or hexamers [282].

IgA: are the predominant immunoglobulins at the level of external secretion such as oral surface where they can be found as dimers or trimers [283].
IgD: are present in the serum at very low levels, but together with IgM are the main immunoglobulins expressed on the surface of mature B lymphocytes [282].
IgE: is the less abundant immunoglubulin class in the serum, they are involved in allergies and in particular they mediate sudden hypersensitive reactions that give raise to asthma, orticaria and anaphylactic shock [281].

3.1 IgE-FcεRI interaction: effector mechanisms

IgE immunoglobulins are able to induce a strong immune response involved in allergic reactions and immunity against parasites; recent studies also suggested an important role of IgE in cancer immune-surveillance [284,285].
IgE heavy chains contain four constant domains responsible for effector functions (εCH1, εCH2, εCH3 and εCH4) displaying six N-linked glycosylation sites and one variable domain (VH) [286].
In order to exert the effector functions IgE must bind to the high affinity IgE receptor (FCεRI) mainly expressed in mastcells. For binding to the receptor the IgE εCH3 region is essential [287].
IgE high affinity receptor is a membrane glycoprotein that plays a fundamental role in hypersensitive reactions mediated by IgE. In humans it exists in two different forms: one tetrameric and the other one trimeric [288].
The first one is constituted by a tetrameric complex that comprises one α subunit, one β subunit and a γ subunit homodimer ($\alpha\beta\gamma_2$) and is expressed on IgE associated effector cells, mastcells and basophils. The second form is constituted by a trimeric complex ($\alpha\gamma_2$) which lacks the β subunit and is expressed on Langerhans cells [289,290], monocytes, eosinophils [291], peripherical dendritic cells [292] and platelets [293] and is involved in the internalisation of IgE/Antigen complexes. The β chain is known to act as an amplifier of IgE-mediated functions and seems to enhance expression of FcεRI molecules [294,295].
Subunit α is responsible of binding to IgE; in particular the CH3 domain of IgE is recognised by FCεRI-α chain. The α subunit is in turn constituted by two

extracellular immunoglobular domains (D1 and D2): the D2 domain is the main responsible of IgE interaction and the D1 domain seems to have structural relevance in the maintaining of high affinity for IgE and a correct folding of D2 domain [296,297].

The α subunit is highly glycosylated because it contains seven sites of glycosylation [298]. The β subunit is essential for α subunit expression on the cell surface and, together with β subunits, for the regulation of several genes transcription and for signal transduction cascade that ends up with secretion of mediators and the synthesis of proinflammatory metabolites.

The two γ subunits form a heterodimer through disulfide bonds and in the γ subunit cytosolic tails conserved motifs, known as ITAM, are located. ITAM motifs are able to interact with tyrosine-kinase proteins during signal transduction cascade. The binding of the antigen with the complex IgE-FcεRI, causes aggregation of FcεRI molecules, a rapid phosphorylation of tyrosine residues and degranulation of mastocytes [299].

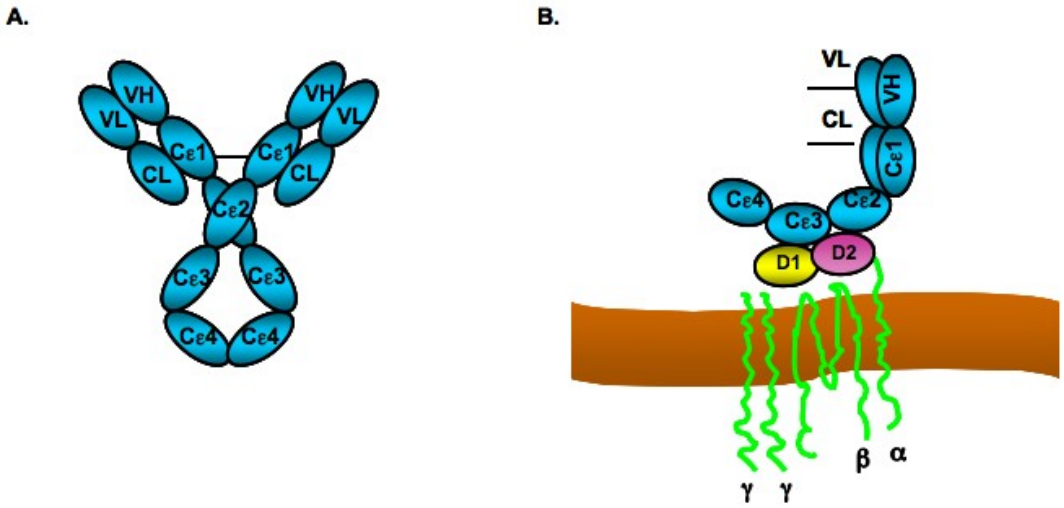

Figure 13: A) IgE structure characterised by the presence of four constant domains (Cε 1-4) B) Illustration of FcεRI constituted by one β chain, two γ chains and one α chain. FcεRI-α chain contains two domains: D1, responsible for IgE binding and D2 domain. IgE binds the receptor through its Cε3 domain.

3.2 Antibodies as biotechnological tool

Monoclonal antibodies derive from a single clone of B lymphocytes, they display a single VH-VL combination and are specific for a single epitope [300].
Monoclonal antibodies (mAbs) have proved very useful not only as biotechnological tools (for example in antigen detection and purification), but also as therapeutic and diagnostic tools.
However, since murine mAbs were responsible of several side effects, chimeric antibodies containing only the mouse-variable regions of the mAb and human constant regions have been produced showing reduced antigenicity [301]. Another limitation in therapeutic usage of antibodies is represented by the immunoglobulins low capacity in penetrating poorly perfused tissues, including tumours; moreover the Fc fragment can influence the availability of the antibody to bind its antigen, because it can be captured by specific receptors on the membranes of monocytes, macrophages and natural killer cells.
These limitations have favoured the design and production of small antibody derived molecules that maintain the specific binding to the antigen, but do not show the disadvantages of antibody molecules.

Among all the different antibody fragments produced, the major breakthrough in this field is represented by the “single chain Fv” (scFv) antibody molecules. The scFvs are monovalent antibody molecules expressed as single polypeptide chain (single chain Fv) by the insertion of a flexible peptidic linker between the C-terminus of a VH and the amino terminus of a VL (or *vice versa*) [302]. The flexible linker ensures the correct interaction between VL and VH [303].
Due to their small size, scFvs present a series of advantages over complete immunoglobulins, including a better penetrability into tissues and reduced toxicity [304-306].
In order to increase the avidity of scFv molecules another class of dimeric single chain antibody molecules has been developed: the small immunoproteins (SIPs). SIPs have been obtained by the addition to VL and VH domains of the CH3 dimerizing domain from human IgG1 (γ1CH3), resulting in a bivalent scFv [307]. SIPs overcome a series of limitations of scFvs: first SIPs maintain the bivalency of the original antibody, in addition, when administered to patients, their stability is higher

compared to scFv and, moreover, SIPs can penetrate tissues with the same efficiency, but with the advantage of having a slower clearance and therefore a longer permanence within tissues and tumours as well [308].

Since many proteins are expressed intracellularly, the idea was to use mAbs directly *in vivo* to interact with the antigen and block its function.

For this purpose, the production of antibody fragments allowed an easier selection of the antibodies able to fold properly also in the cytosol [309,310], giving the possibility to obtain a new class of antibodies named intrabodies [311].

Intrabodies are any kind of antibody, including antibody fragments that remain confined within the cell [312].

Intrabodies could be very useful to block protein-protein interactions, or protein-nucleic acid interaction, to inhibit directly the function of an enzyme but also to redirect the target protein toward a different cellular compartment [313].

As a matter of fact, intrabodies can be directed into different cellular compartments depending on the specific signal sequence used.

Intrabodies can be directed into cytosol just through deletion of the leader peptide, to the nucleus through the use of NLS (nuclear localisation signal), to peroxisomes using SKL sequence for peroxisome retention or to the ER through the addition at the C-terminus of the protein of a KDEL sequence or other sequences described to be ER retention signals, such as transmembrane domains of ER resident proteins [312,314].

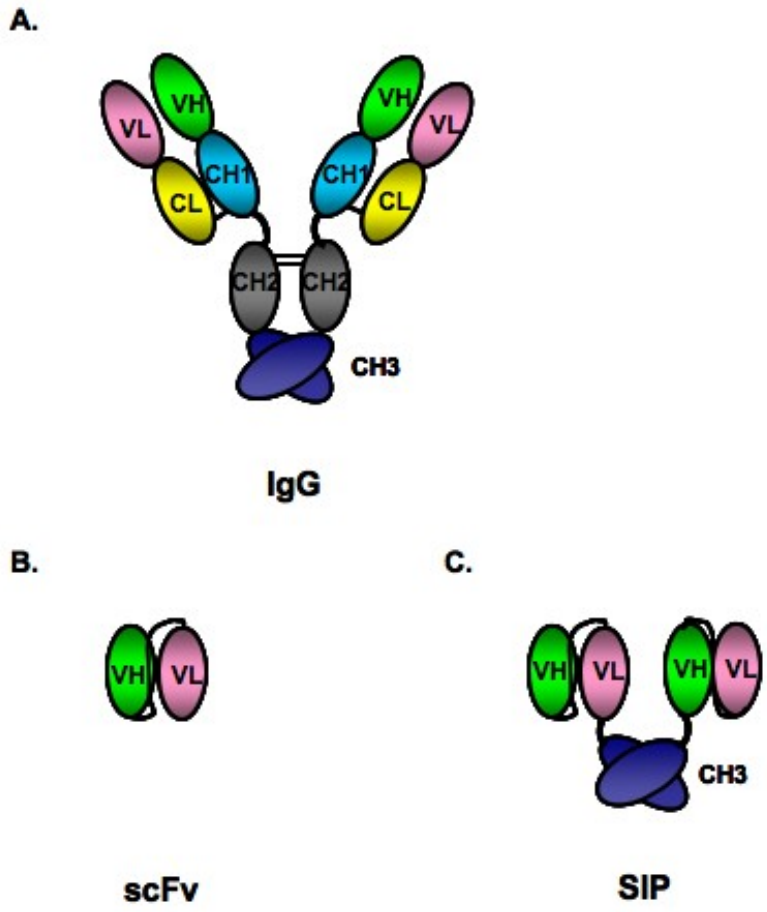

Figure 14: schematic representation of a complete IgG (A) scFv composed by VH and VL domains (B) and SIP that contains, in addition, the CH3 dimerizing domain which results in a divalent scFV (C).

In this work we exploited the specificity of IgE for the FcεRI-α chain and the specificity of antibody molecules in scFv format (in particular $scFv^{9E1}$) to induce specific degradation of the target, in this case the FcεRI-α chain.

4. TGEV spike protein S

The porcine *Transmissible gastroenteritis virus* (TGEV) is a member of the *Coronaviridae* family of the *Nidovirales* order [315,316]. It is an enveloped positive strand RNA virus that replicates both in enteric and respiratory tissues of newborn piglets, causing a mortality near to 100% [317]. The TGEV structure is build up by four structural proteins: the spike glycoprotein S which conditions with its structure the enteric and respiratory tropism, the membrane protein (M) and the envelope protein (E), that are embedded in the virus envelope together with S protein and finally the nucleoprotein (N), that is bound to RNA + genome in the nucleocapsid.
At the structural level, TGEV virus contains three different compartments: the envelope essential for virus infectivity, the internal core made up by the carboxy-terminus of M protein and the nucleocapsid consisting of the RNA genome and the nucleoprotein N [318-320].
The cellular receptor for TGEV infection is the porcine aminopeptidase N (pAPN), which is recognised by the viral protein S. S protein is a large type I transmembrane glycoprotein, which is also responsible for membrane fusion [321-323].

Against TGEV spike protein a mAb with neutralizing activity was produced and named 6A.C3 [324]. Oral administration into piglets of either mAb 6A.C3 or its derived SIP version has demonstrated to have a protective function against TGEV infection. 6A.C3 thanks to its neutralizing activity is able to inhibit TGEV
In this work we have used the TGEV S protein as second model for the validation of our system to induce specific protein-*knock out*. In this case the specificity to act against S protein is conferred by the $scFv^{6A.C3}$ molecule [324,325].

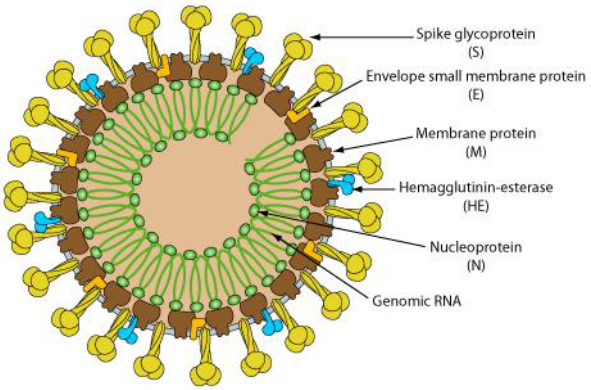

Image downloaded from http://expasy.org/viralzone/all_by_species/30.htm

Figure 15: TGEV structure.
The S protein is present in the envelope and is essential for virus infectivity, interaction with the host cell receptor. The M glycoprotein is essential for envelope formation whereas N protein constitutes the nucleocapsid and is probably involved in viral RNA synthesis

5 Protein biotinylation

Beside the involvement of biotin in several metabolic pathways, biotin has a very interesting feature that resides in the high affinity for avidin, a glycoprotein present in eggs and for streptavidin, the avidin homologous produced in *Streptomyces avidinii.*

Avidin and streptavidin can bind to biotin through a non-covalent interaction that is the strongest known, so far. Moreover (strept)avidin is a homotetramer in which every monomer can bind to biotin resulting in a tetravalent protein [326].

Therefore, the strong binding between biotin and avidin could be exploited for a number of diagnostic and research purposes and for purifications of proteins. For these reasons, with the aim of mimic the physiological biotinylation of a system has been developed (*in vivo* biotinylation; [327]).

This system is based on the activity of *E. coli* enzyme BirA, which is the best biotin ligase characterised.

BirA enzyme catalyses the biotinylation reaction through two ATP-dependent steps that allow the formation of a covalent linkage between the aminic group of a lysine residue, present within protein sequence, and the carboxylic group of biotin. During biotinylation reaction, ATP hydrolysis causes the formation of the intermediate biotinyl-AMP which is hydrolysed, in turn, in order to guarantee the transfer of biotin to lysine residue located within the BirA domain called biotin carboxyl carrier protein or BCCP [328].

In order to identify the BirA consensus sequence a combinatorial study of protein biotinylated sequences was performed and a series of biotinylated peptides were found out [329]. Analysis on N-terminal and C-terminal truncation variants of one of these peptides allowed the identification of a 14 aminoacid residues peptide able to mimic biotin acceptor domain of BirA; the sequence found GLNDIFEAQKIEWH was called Biotin Acceptor Peptide (BAP) [327].

Since the BAP peptide constitutes an efficient target for the BirA enzyme, the addition of a BAP peptide in the sequence of heterologous proteins confers to those proteins the possibility to get biotinylated once in contact with BirA enzyme. Proteins bearing the BAP peptide and BirA can be coexpressed in mammalian cells in order to obtain biotinylated proteins *in vivo* (*In vivo* biotinylation). *In vivo*

biotinylation can be performed for secretory or membrane proteins through the expression of a BirA modified by the addition of a SEC leader peptide [330].

In this work the in vivo biotinylation has been exploited for the study of molecules that, once committed for degradation, dislocate from the ER to the cytosol in the so called retro-translocation process.

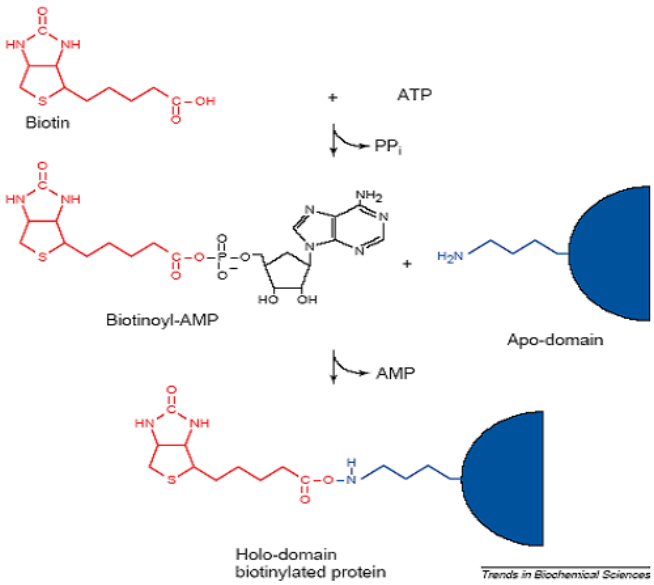

Figure 16: Biotinylation reaction steps.
BirA enzyme catalyses the biotinylation reaction through two ATP-dependent steps; the first step catalyses the formation of the intermediate biotinyl-AMP, which is afterwards hydrolysed, in order to transfer the biotin to lysine residue present within protein sequence.

AIMs of the study

The main objective of the present work was to exploit the ubiquitin-proteasome machinery to obtain specific degradation of defined proteins within the secretory pathway.

Up to now, different strategies have been used to obtain intracellular specific protein inactivation: siRNA have been used to inhibit protein expression, while intrabody technology was used to inactivate proteins by inhibiting protein function or by promoting target protein relocalisation and subsequent inactivation.

A great advantage of the use of intrabodies is given by the high specificity for the target. As a consequence of this high specificity, the possibility to have off target effects is extremely low.

In order to bring together the possibility to inhibit target protein expression and to maintain the specificity conferred by antibodies, the idea was to create a fusion protein between a recognition moiety, represented by a scFv molecule or any kind of molecule able to specifically recognise the target such as a ligands, peptides, receptors or whatever antibody fragment and a moiety displaying a degradative activity.

A candidate for being the degradative moiety was SEL1L, an ER transmembrane protein functioning during ERAD. SEL1L is involved in the recruitment of misfolded substrate in the lumen of ER, that after recognition are delivered to proteasome machinery for degradation.

With the aim of specifically recognise proteins within the secretory pathway and in order to induce their degradation, we designed a new class of molecules termed *degradins.* Degradins result from the fusion of the COOH-terminal 372 aminoacids of human SEL1L with an antibody-derived scFv or a ligand of a receptor (*scFv-degradin* or *ligand-degradin*). The COOH-terminal portion of the human SEL1L were previously demonstrated to be essential during ERAD [183].

Degradins would therefore engage target proteins within the ER, promoting their dislocation to the cytosol and their proteasome-dependent degradation.

ER-to-cytosol dislocation of aberrant proteins is a complex process poorly characterised, so far, in part due to the absence of proper approaches for its study.

Up to now, there are just a few techniques that enable the detection of proteins that, once engaged within the ER lumen, are dislocated to the cytosol, where they are degraded by the proteasome.

Those experiments are mainly focused on the study of very well known substrates of ERAD such as the MHC class I molecule.

MHC-I molecules are targeted for dislocation and degradation by two viral proteins of Human cytomegalovirus named US2 and US11.

So far, in order to detect retro-translocated MHC-I molecules most experiments were based on the separation of the cytosolic fraction or on the detection of deglycosylated molecules that accumulate in the cytosol, because retro-translocated, upon proteasome inhibition [235,275].

These techniques share the same weak points, they have a low efficiency due to the inability to detect retro-translocated molecules without the use of proteasome inhibitors and are not very specific.

To overcome the limitations of these techniques and in order to enhance the specificity and the efficiency of retro-translocated molecules detection, we decided to exploit a compartmentalised reaction that can occur only on those molecules that have been dislocated to the cytosol where the enzyme displays its function. The reaction is the *in vivo* biotinylation carried out by the enzyme BirA [331].

To assess whether biotinylation could be useful to study retro-translocation, we chose a membrane target protein already described to be substrate of the retro-translocation process. This protein was the MHC-I α chain that we engineered through the addition of a BAP peptide at the N-terminus to be recognised by the BirA enzyme once dislocated to the cytosol.

Membrane or secreted proteins bearing the BAP peptide are directed towards ER lumen, during their synthesis, therefore the BAP peptide would become accessible to BirA enzyme only when the BAP-protein was retro-translocated to the cytosol in order to get degraded by the proteasome.

The engineered BAP-MHC-Iα was used to validate the method, using two HCMV derived proteins, US2 and US11 that are well known to induce MHC-Iα downregulation by inducing its retro-translocation to the cytosol and proteasome-dependent degradation.

MATERIALS AND METHODS

Construction of degradins

By homology with the yeast Hrd3p mutant lacking the NH_2-terminal luminal portion and the ability to interact with the misfolded protein recognition system, but still able to bind the ubiquitin ligase Hrd1p and the other elements of the translocon, we created a number of recombinant proteins containing the COOH-terminal 372 aminoacids of the human SEL1L fused to different recognition domains. The recognition domain can be represented by a scFv from an antibody specific for a defined antigen or a ligand for a determined receptor. This class of molecules has been named as *degradins*, because they are expected to specifically recognise their target proteins within the ER and retain the ability to induce their degradation after retro-translocation to the cytosol.

Preparation of scFv degradin specific for FCεRI-α chain

Total RNA was extracted from the human cell line HEK 293 using the RNeasy Mini Kit (Qiagen). After cDNA synthesis with oligo-dT and MuMLV Reverse Transcriptase (Invitrogen) using 2 µg of total RNA as substrate, a fragment of 1138 bp, containing codons 423-794 of human SEL1L (corresponding to positions 402-773 of the mature protein) was amplified by PCR using primers SEL1L-NheI (AGTAGCTAGCGGAAGTGACATTGTACCTCA) and SEL1L-EcoRI (TCAGAATTCTTACTGTGGTGGCTGCTGCTCT) and Kod High-Fidelity DNA polymerase (Novagen).

The amplification product was digested NheI/EcoRI and inserted in a pcDNA3 vector expressing the anti-FcεRI $scFv^{9E1\ 332}$ modified by insertion in the NheI site of a linker (GGCAAACCAATCCCAAACCCACTGCTGGGCCTGGAT) encoding the SV5 tag (GKPIPNPLLGLD).

The final construct pcDNA3-$scFv^{9E1}$-SV5-SEL1L was named $scFv^{9E1}$-degradin.

A further truncated version of degradin, completely lacking the SEL1L luminal portion, was obtained by amplifying the cDNA with primers SEL1L-NheI (TCAGCTAGCCTGGTCCATATCAAGTTGGGTGA) and SEL1L-EcoRI.

The amplification product was also in this case inserted in pcDNA3-scFv^{9E1} vector using NheI-EcoRI digestions.
The construct used as a control for ER retention operated by the KDEL retention sequence [44,314] was obtained by inserting in the BspEI/XhoI sites of the said pcDNA3-scFv^{9E1} a linker encoding the SV5 tag followed by the KDEL sequence (tccggaGGCAAACCAATCCCAAACCCACTGCTGGGCCTGGATAGTACTAAAGA TGAGCTGtagctcgag).
The control constructs containing an irrelevant scFv was obtained by substituting the HindIII/BspEI cassette encoding the scFv^{9E1} with the corresponding cassettes encoding different scFvs.
The cassette encoding the anti-FcεRI scFv^{9E1}-degradin was in addition subcloned into the pcDNA3-Hygro vector (Invitrogen) for the generation of double transfected stable clones.
scFv^{9E1}-degradin construct was modified by substitution of SV5 tag with another tag obtained in the lab.

Preparation of FCεRI α chain specific ligand degradin

The ligand-degradin expressing vector has been obtained from the previously described plasmid pCDNA3-sec-SV5-CH3/CH4, encoding a secretion signal (sec), the SV5 tag and human IgE domains CH3-CH4 [333].
This vector was modified by the addition of a linker to introduce a NheI site downstream the CH4 domain gene
Linker sequence: GATCCGGTGGCGCTAGCG
A HindIII/NheI fragment was excised from the resulting plasmid and transferred to the plasmid pcDNA3-scFv^{9E1}-degradin, yielding the construct pcDNA3-sec-SV5-CH3/CH4-SEL1L that was named Ligand$^{Fc_\varepsilon}$-degradin.
We further produced this construct using a different tag identified in the lab amplifying the CH3-CH4-SEL1L fragment using the following plasmids:
Primer CH3 for with BglII site TCTGAGATCTTGTGCAGATTCGAACCCGAGA
SEL1L Primer reverse with EcoRI site:
TCAGAATTCTTACTGTGGTGGCTGCTGCTCT

The PCR product was digested with BglII-EcoRI enzymes and ligated in a plasmid present in the lab expressing the SEC leader peptide followed by the sequence expressing the tag with a BamHI site at the 3' end compatible with BglII.
The resulting plasmid is the sec-tag-CH3-CH4-SEL1L (Ligand$^{Fc_\varepsilon}$-degradin).
A truncated form of this construct was generated, lacking the sequence encoding the CH3 domain, which is the domain responsible for interaction with FcεRI [287], to be used as a non-binding degradin control.
Amplification of the fragment CH4-SEL1L lacking CH3 domain was performed using the SEL1L EcoRI primer and the following forward primer:
CH4 BglII TGTAAGATCTGGCCCGCGTGCTGCCCCGGAA
CH4-SEL1L PCR product was ligated in a plasmid pcDNA3-sec-tag using BglII-ECORI sites obtaining the construct sec-tag-CH4-SEL1L (Ligand$^{Fc_{\varepsilon\Delta}CH3}$-degradin).
In order to obtain also for the Ligand$^{Fc_\varepsilon}$-degradin the control in which SEL1L lacks the domain essential for the induction of ERAD pathway the sequence encoding for SEL1L in plasmid sec-tag-CH3-CH4-SEL1L was substituted with the sequence encoding for SEL1L TM cyto (SEL1LΔ) obtaining the Ligand$^{Fc_\varepsilon}$-SEL1LΔ.

Preparation of SVIP expressing plasmid

The plasmid expressing SVIP protein was obtained by amplifying the SVIP coding sequence from human cDNA using the following plasmids:
Primer forward: TATTCTAGATTCCGGAAACTGTCCACCTAAGTCCACCT
Primer reverse: TATTCTAGATTATGAAACTGTCCACCTAAGTCCA
PCR fragment was cloned using HindIII-BspEI digestion in a plasmid that contains H6 tag [334].

Preparation of constructs for TGEV inhibition assay

With the aim of inhibit TGEV infective particles formation a specific anti-S protein scFv was fused with SEL1L.
The scFv^{9E1}-degradin construct was digested using a HindIII-BspEI in order to substitute the scFv^{9E1} with scFv^{6AC3} obtained from pcDNA3-6AC3 swine αSIP [325].
The resulting construct is the specific S-degradin pcDNA3-scFv^{6AC3}-degradin.

As control of ER retention scFv^{6AC3}-KDEL plasmid was constructed by substitution of scFv^{9E1} from pcDNA3-scFv^{9E1}-KDEL with scFv^{6AC3} using HindIII-BspEI digestion.
The constructs scFv^{6AC3}-degradin, scFv^{9E1}-degradin, 6AC3 swine αSIP and scFv^{6AC3}-KDEL were subcloned in pcDNA3- Hygro(+) vector using HindIII-ApaI digestions.

Preparation of BAP-HLA-A2

The plasmid pcDNA3-sec-SV5-BAP-CysEMI (Predonzani et al) was modified by the addition of a linker encoding the BAP peptide (GLNDIFEAQKIEWH) using BamHI/NheI digestion.
Linker sequence:
GGATCCGCCGGAGGCTCTGGAGGCCTGAACGATATTTTCGAAGCTCAGAAAA
TCGAATGGCACGAAGCTAGC
The intermediate obtained was sec-SV5-BamHI-BAP-NheI.
RNA was prepared from a pool of lymphocytes derived from different donors using Rneasy kit (Qiagen) in order to ensure the presence of the specific HLA sequence.
Upon standard RT-PCR, HLA-A2 was amplified using the following plasmids:
oligo A2 EcoRI: taggaattcTCACACTTTACAAGCTGTGAGA
oligo A2 Nhe: ataggctagcGGCTCTCACTCCATGAGGT
Both HLA-A2 PCR and the vector pcDNA3-sec-SV5-BamHI-BAP-NheI were digested with NheI/EcoRI restriction enzymes and ligated in order to obtain the final construct named pcDNA3-sec-SV5-BAP-MHC-Iα.
pcDNA3.1 plasmids encoding for the HCMV proteins US2 and US11 were kindly provided by Domenico Tortorella [102,270,271].

Mutagenesis of US11 expressing plasmid: preparation of US11QL

Starting from plasmid pcDNA3.1-US11 (Domenico Tortorella) mutagenesis was performed using Stratagene mutagenesis kit following manufacturer directions with the following oligos:
US11 Q192L forward:
GTGGCAGTGATTCTAGTGTTTTGGGGGCT

US11 Q192L reverse:
CCCCCAAAACACTAGAATCACTGCCACCA

Cell culture and virus

HEK 293 were used for stable transfections and (expressing SV40 large antigen and suitable for transient transfection,[335]) grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Gibco BRL) supplemented with 10% of fetal calf serum (FCS, Life Technologies Gibco BRL). HEK 293 T cells were grown in the same conditions as above and were used for transient transfections.
Baby hamster kidney (BHK21) stably transformed with gene coding for porcine aminopeptidase N (BHK pAPN, [336]) were grown in DMEM 5% FCS with addition of 1.5 mg/ml of G418 (Gibco 50 mg/ml) as selective agent.
HEK 293 cells stably expressing mdα were grown in DMEM 10% FCS using as selective agent G418 at 400 μg/ml.
HEK 293 mdα-scFv^{9E1}-degradin double stable clone (clone G6) was maintained in DMEM 10% FCS supplemented with 400 μg/ml of both G418 and hygromicine used as selective agents to ensure continuous expression of mdα and scFv^{9E1}-degradin respectively.

Cells transfection

BHK pAPN stable transfections were performed using 10 μg of pcDNA3 constructs previously linearised with a BglII digestion. DNAs were transfected using lipofectamine 2000 (Invitrogen) following manufacturer directions.
BHK pAPN stable clones were maintained in medium containing 400 μg/ml of Hygromicine (Invitrogen 50 mg/ml) as selective agent.
HEK 293 T cells were transiently transfected with calcium phosphate technique whereas HEK 293 were used to obtain stable transfected clones.
Cells were plated in 6-well Petri dishes. Four hours before transfection fresh medium was added. 2.5-5 μg of plasmidic DNAs were resuspended in 50 μl of TE 0.1X (10 mM Tris, 1 mM EDTA) and added to a solution of $CaCl_2$ 57.5 mM. Further 26 μl of $CaCl_2$ 2 M were added and the resulting solution was applied

under constant agitation to 250 μl of HBS 2X (280 mM NaCl, 10 mM KCl, 1.5 mM NaH_2PO_4, 12 mM dextrose and 50 mM Hepes).
16 hours after transfection medium was discarded and serum free medium was added for 24 hours.

Cellular lysis

HEK 293 transfected cells (corresponding to about $5x10^5$ cells) were lysed with 100 μl of SDS buffer (100 mM TRIS HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and subsequently sonicated to disrupt DNA.
BHK pAPN stable clones were lysed with 100 μl of TNN lysis buffer (100 mM Tris-Hcl pH 8; 250 mM NaCl; 0.5% NP40) supplemented with Protease inhibitory cocktail (PIC, Sigma) according to manufacturer instructions, and were centrifugated at 15,800x g for 5 minutes in order to clear the lysates of insoluble material.

Cycloheximide treatment

A cycloheximide treatment was performed 16 hours after transfection on cells cotransfected with mdα and degradins. Cells were treated up to 4 hours with 50 μg/ml of cycloheximide to analyse mdα stability.

Proteasome inhibitor treatment

HEK 293 stable clones expressing mdα and clone expressing both mdα and $scFv^{9E1}$-degradin (G6 clone) were treated with 20 μM of MG132 proteasome inhibitor (50 mM in DMSO, Sigma) or with the same volume of DMSO for 6 hours.
HEK 293 T cells transiently transfected with plasmids expressing degradins were treated 16 hours after transfection as described above.

TGEV infection

In this study TGEV strain PUR46-MAD [337,] was used.
BHK pAPN stable clones were plated in duplicate in 35 mm dishes in order to obtain about 10^6 cells. Before performing TGEV infection one series of plates were used to count the cells in order to ensure the normalization of the results.
TGEV infection was performed using 1 MOI of TGEV diluted in 500 μl DMEM 2% FCS.
One hour of TGEV internalisation was performed gently shaking every 15 minutes. Following internalisation virus infection was maintained for 16 hours.

TGEV titration

BHK pAPN cells were seeded in 24-well tissue culture plates. Serial dilution of supernatants collected from BHK pAPN stable clones infection were made in DMEM 2% FCS with 40 μg of DEAE-dextran per ml (Pharmacia). Dilutions containing viruses were applied to the cells. After 1 hour of virus adsorption the inoculum was replaced with medium containing 2% FCS, 40 g of DEAE dextran per ml and 1% agarose. The cells are incubated for 2-3 days at 37° and finally fixed with 10% formaldehyde and stained with 0.1% crystal violet. The final step is the count of the plaques formed ([338] in collaboration with I. Sola and L. Enjuanes-Centro Nacional de Biotecnologia-Madrid [324,325]).

EndoH and PNGase F samples deglycosylation

In order to visualise protein localisation we used two different deglycosylating enzymes: EndO H_f and PNGaseF (NEB, New England Biolabs, Beverly, MA). EndoO H_f cuts N-glycosylation ER dependent and the sensitivity to this enzyme reflects an ER localisation whereas PNGase act on all types of N-glycosylation.
Samples containing the glycoprotein are heat denatured with 10% of denaturing buffer at 100°C for 10 minutes. 10% of specific buffer (G5 0.5 M sodium citrate pH 5.5 for ENDO Hf and G7 sodium citrate PH7.5 for PNGase F) was added and for the reaction of PNGase F 10% of NP40 must be added. Samples were incubated with a proper amount of enzyme for 1 hour at 37°C.

After another denaturation with SDS containing sample buffer the samples were loaded on SDS-PAGE.

Western blot analysis

Cell extracts and supernatants were loaded on a SDS-Page and transferred on PVDF membrane (Millipore) previously activated in methanol. Transfer on PVDF membrane was done in Tris-Glycine buffer (Tris 25 mM, glycine 192 mM and 20% methanol).

To detect degradin expression both in transient and stable transfections primary antibody used was mAb anti SV5 tag antibody (Invitrogen) and mAb anti tag (produced in our lab).

FCεRI-α chain expression was detected using mAb 9E1, expression of BAP-MHC-Iα and sα-SV5-BAP was detected using anti-SV5 antibody. .

TGEV S protein expression was detected with murine mAb 1D.G3, TGEV M and N protein expression were detected with murine mAbs 25.22 and 3D.C10 respectively [318,338,339,340].

In all cases secondary antibody used was anti mouse Fcγ peroxidase labeled (Jackson).

To normalize the amount of extract loaded on the gels, blots were in addition incubated with anti-actin antibody (Sigma) and with a goat anti rabbit HRP-conjugated (Thermo Scientific).

Upon deglycosylation treatment with EndoHf and PNGaseF enzymes, both sdα and mdα were detected using a rabbit polyclonal antibody against Fcγ (DAKO) and by the use of a goat anti rabbit antibody HRP-conjugated (Thermo Scientific).

ELISA screening of swine a SIP 6AC3 expressing clone

BHK-pAPN cells stably transfected with a SIP version of 6AC3 antibody were selected analyzing supernatants.

96 multi well Maxisorp ELISA plates (Nunc) were coated with goat anti porcine IgA 1 μg/ml (Serotec) for 2 hours at 37°. After 3 washes with PBS containing tween 0.05% (PBS-tween), plates were blocked with and BSA 1% in PBS-tween.

Supernatants of a 96 multiwell plates of BHKpAPN transfected with 6AC3αSIP were collected and incubated on plate for 1h
Plates were washed and incubated with a goat anti porcine IgA peroxidase labeled 1 μg/ml (Serotec). Reaction was performed using tetramethylbenzidine (TMB) peroxidase substrate (Sigma).

In vivo biotinylation to detect retro-translocated molecules

HEK 293 T cells were transfected with 0.5 μg of BirA plasmid, 0.5 μg of plasmid expressing SV5-BAP-MHC-Iα and 1 μg of US2 or US11 expressing plasmid. 16 hours after transfection medium was discarded and cells were incubated for 24 hours with biotin 100 μM (Sigma) diluted in serum free medium.
To study retro-translocation induced by degradins HEK 293T cells were transfected with 0.5 μg of BirA plasmid, 2.5 μg of sα-SV5-BAP and 2.5 μg of degradin expressing plasmid. Also in this case serum free medium was applied 16 hours post tranfection and biotin serum free medium was added for 24 hours.
Experiments of detection of biotinylated molecules during proteasome inhibitor treatment were performed as follows: cells were transfected with the same quantity of plasmids just described. 16 hours after transfection medium was discarded and serum free medium containing biotin was added with 20 μM of MG132 proteasome inhibitor (Sigma) or the same volume of DMSO. Proteasome inhibitor treatment was carried on for 6 hours in presence of biotin. Afterwards cells were lysed with Sample buffer and supernatants were collected.

Gel retardation assay

Lysates of HEK 293T cells transfected with SV5-BAP-MHCα or sαBAP were incubated or not for 1hour at RT with 1 μg of purified streptavidin (Invitrogen). After streptavidin incubation samples were loaded on SDS-Page under denaturing conditions and blotted on PVDF membrane (Millipore).
Detection of protein was performed using anti SV5 mAb; biotinylated molecules can be discriminated from the non-biotinylated ones because they are bound very strongly by streptavidin. Binding to streptavidin changes migration of biotinylated molecules that migrates as higher molecular weight protein.

Samples incubated and not incubated with streptavidin were loaded in parallel to determine aproximatively the percentage of molecules shifted because of their biotinylated status.

ELISA detection of biotinylated retro-translocated molecules

96 multi well Maxisorp ELISA plates (Nunc) were coated with mAb anti-SV5 in carbonate buffer. Lysates of 293 cells were applied and incubated for 1 h. Biotinylated molecules were detected with Streptavidin HRP-conjugated (Jackson).

Flow cytometry assay

HEK 293-mdα clone was analysed by flow cytometry using a FACS-CaliburTM (Becton Dickinson). The expression of the receptor was detected using as primary antibody mAb 9E1 and anti mouse IgG fluorescein conjugated (KPL, Kirkegaard and Perry Laboratories, Gaithersburg, MD) as secondary antibody.
In order to verify the intracellular localisation of degradins a FACS analysis was performed. HEK 293T cells transiently transfected with plasmids expressing degradins were analysed using as primary antibody mAb anti-SV5 and as secondary antibody a goat anti mouse IgG fluorescein conjugated (KPL).

IgE binding assay on mdα expressing clones

FACS analysis was performed to study mdα binding of IgE in mdα expressing clone and mdα-degradin double stable transfected clone (G6 clone). The analysis was done using purified human IgE (Calbiochem, Canada) and as secondary antibody a FITC-conjugated goat anti human IgE (DAKO) was used.

S^{35} pulse-chase experiment

293 cells were transfected using calcium phosphate technique; 16 hours after transfection medium was discarded and cells were starved for 30 minutes with DMEM Methionine/Cysteine free with 10% of dialyzed FCS and biotin.

Following starvation cells were labeled for 30 minutes with ^{35}S Methionine/Cysteine 200 μCi/ml and afterwards medium was discarded and complete DMEM was added to chase the radioactive label. Chase period was performed for different times (from 30 minutes to 4 hours) in presence or absence of proteasome inhibitor MG132 (Sigma).

Immunoprecipitation of S^{35} labeled extracts

Cell lysates obtained using SDS electrophoretic sample buffer were diluted with 400 μl of TNN lysis buffer and digested with DNaseI for 1h to disrupt DNA and ensure the immunoprecipitation to work properly. Samples were immunoprecipitated using anti SV5 (1:500) or with 9E1 mAb (1:250) and 25 μl of Protein A-sepharose for 1 h. This suspension was applied to micro Bio-spin chromatography columnns (Biorad); columns were washed three times with TNN and twice with PBS. Bound proteins were eluted from the columns using 50 μl of reducing SDS electrophoretic sample buffer.

RESULTS

Design and construction of degradins

With the aim of specifically targeting proteins within the secretory pathway for degradation, a new class of fusion molecules was designed and constructed, which we termed *degradins*. Degradins contain a target recognition moiety specific for a particular target and a degradative moiety able to induce target degradation (Figure 1A).

The recognition moieties can be derived from many different sources (monoclonal antibodies, antibody fragments derived from libraries, antibody heavy chains V regions, specific binding peptides, ligands or receptors) characterised by a high specificity for the target.

In my construct the degradative moiety is represented by the C-terminal 372 residues of SEL1L (positions 402-773 of the mature protein), which is an adaptor protein in the ERAD pathway that binds the substrate and both HRD1 and Derlin-1 proteins. SEL1L is therefore able to link substrate recognition with substrate retro-translocation.

This portion of SEL1L was chosen as degradation moiety after sequence alignment with yeast and *C. elegans* homologues, Hrd3p and SEL1 respectively. Hrd3p contains two regions in its luminal domain; the first one, localised at the N-terminus (residues 1-390), is probably involved in substrate recruitment, while the second one (residues 390-767) mediates the stabilisation of Hrd1p ubiquitin ligase avoiding its autoubiquitinylation and degradation [155]. In addition indications about the right choice of SEL1L domain essential for the induction of protein degradation came out from studies in which the human SEL1L C-terminal portion (residues 373-end) was assessed to be essential for interactions with HRD1, OS-9 and XTP-3B [183]. Those interactions seem to be crucial for dislocation, ubiquitinylation and the subsequent proteasome-dependent degradation.

The resulting degradin is an ER-resident type I transmembrane protein; its binding moiety is localised at the N-terminus on the luminal side and allows the recognition of ER localised target proteins (proteins in transit through the ER or ER resident).

A target molecule would be engaged within the ER by the recognition-specific domain of a degradin and would be committed for the degradative pathway by the SEL1L moiety which promotes the ER to cytosol dislocation process.
The possibility to use different target recognition moieties provides the system a high degree of flexibility. Therefore, whichever secretory or membrane-bound protein can theoretically be directed towards proteasome-mediated degradation, once a specific ligand is identified.

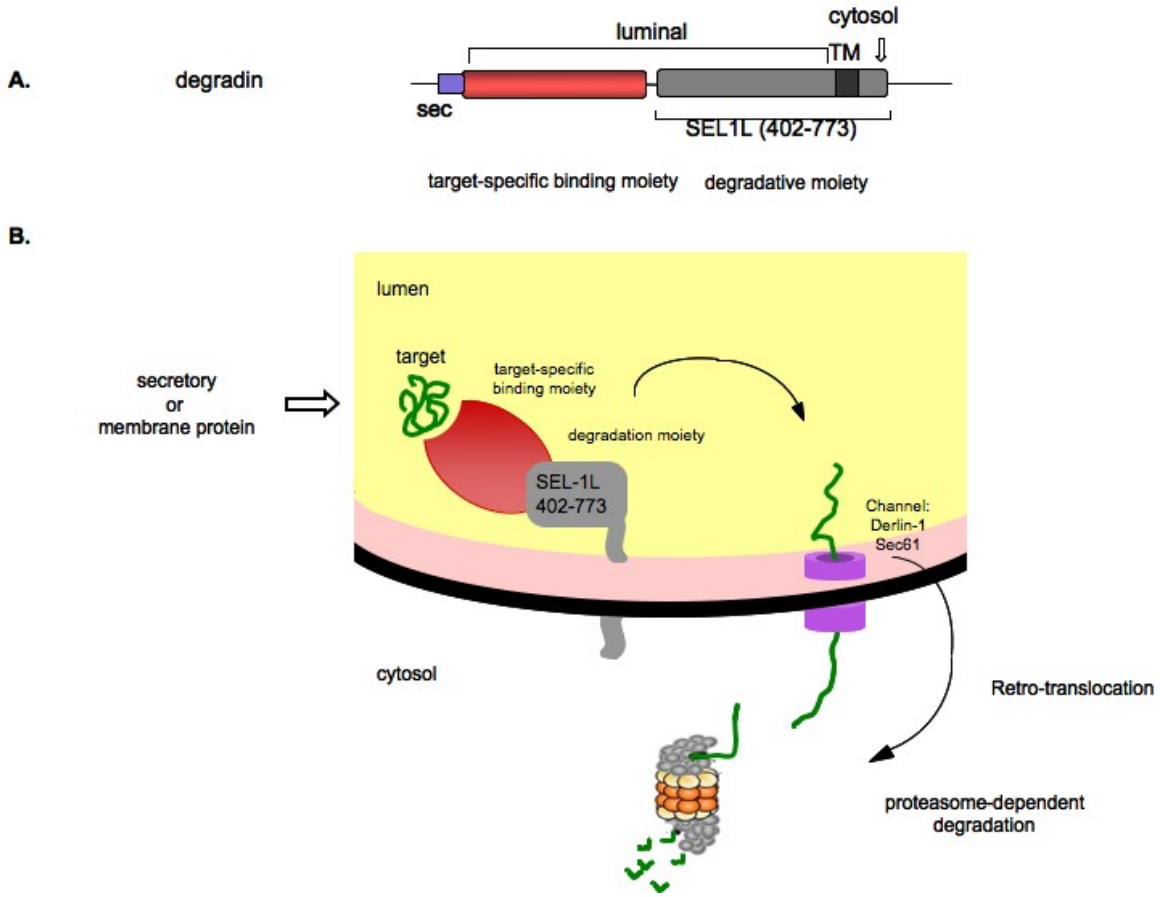

Figure 1: A) schematic representation of degradin obtained by the fusion of a specific target recognition moiety with SEL1L (402-773) portion
B) Illustration of presumed degradin activity. Degradin is an ER membrane anchored protein with both recognition and degradative moieties facing the lumen of the ER. Thus, the target recognition moiety is able to recognise either secretory or membrane tethered target proteins within the ER. Once engaged by degradin the target would start to dislocate to the cytosol to be degraded by the proteasome.

As a model protein to be targeted for degradation the FcεRI-α chain was chosen (Figure 2A); this model gave me the opportunity to test the degradin system on two forms of FcεRI-α chain previously obtained in our lab, one soluble and the other one membrane anchored. Both versions contain the FcεRI-α chain

extracellular domains D1 and D2 and are fused through a five aminoacids linker to the CH3 dimerization domain derived from human IgG1 heavy chain (γ1). The resulting constructs encode a soluble receptor protein (sdα ^{332}) or membrane bound receptor protein (mdα; Vangelista, Cesco Gaspere unpublished work) depending whether the CH3 was derived from the secretory or membrane bound γ1 heavy chain respectively. Both proteins are expressed as dimers as shown in figure 2B and C.

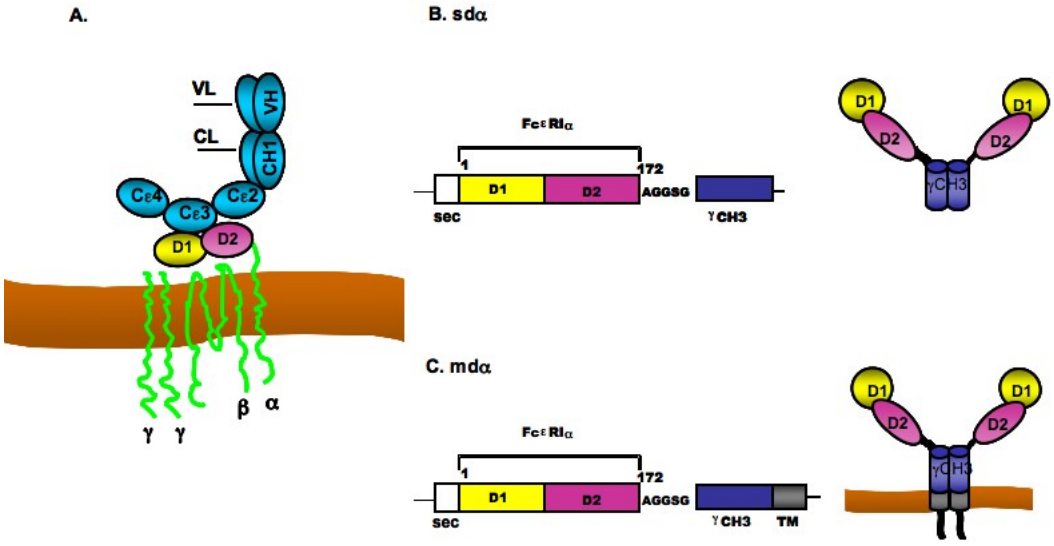

Figure 2: Figure: A) FcεRI is constituted by one β chain, two γ chains and one α chain. FcεRI-α chain contains two extracellular domains: D1, and D2 responsible for IgE binding. IgE binds the receptor through its Cε3 domain.
B) Schematic representation of the soluble dimeric version of FcεRI-α chain obtained by fusing domains D1 and D2 of α chain with the secretory γ_1CH3 dimerization domain, sdα
C) Schematic representation of membrane version of FcεRI-α chain that contains, domains D1 and D2 fused to the membrane γ_1 CH3 domain, mdα.

Besides the possibility to test the degradin system on either soluble or membrane anchored proteins, the choice of FcεRI-α chain allowed me to test two different target recognition moiety, one represented by a scFv (scFv-degradin) and the other by the FcεRI-α chain ligand, the IgE molecule (Ligand-degradin).

A monoclonal antibody against domain D1 of FcεRI-α chain, 9E1, was previously produced in our lab (Vangelista, Cesco Gaspere unpublished work).

From mAb 9E1 a single chain antibody molecule was obtained through amplification of the VL and VH domains and assembly in the VL-VH orientation with a linker to maintain the association between the two domains ($scFv^{9E1}$).

The degradin containing the target recognition moiety $scFv^{9E1}$ was obtained by fusing it to the SEL1L 402-end portion degradative moiety. The resulting $scFv^{9E1}$-degradin is specific for FcεRI-α chain (Figure 3A).

As a control of degradin specificity, a scFv-degradin with an irrelevant recognition moiety was produced (irrelevant-degradin; Figure 3B).

An intrabody with the ER retention signal KDEL was also constructed using $scFv^{9E1}$; the obtained construct $scFv^{9E1}$-KDEL would be able to bind to FcεRI-α chain, but lacking the degradative moiety it should only cause a retention in the ER compartment (Figure 3D). I used this intrabody to compare the effect of simple retention with the possible effect of degradation induced by degradins.

To facilitate the detection of degradins, the 12 aminoacid-long SV5 tag was inserted between the recognition and the SEL1L moieties (Figure 3A and 3B) or between the recognition moiety and the KDEL sequence in the case of $scFv^{9E1}$-KDEL (Figure 3D).

The FcεRI-α specific Ligand-degradin was constructed using as recognition moiety the CH3 and CH4 domains of the human ε heavy chain that are known to bind efficiently the FcεRI-α [287]. This FcεRI-α specific Ligand degradin was named $Ligand^{Fc_\varepsilon}$-degradin (L^{Fc_ε}-degradin). In this case the SV5 tag was inserted at the N-terminus of the sequence immediately downstream the secretion signal (Figure 3C).

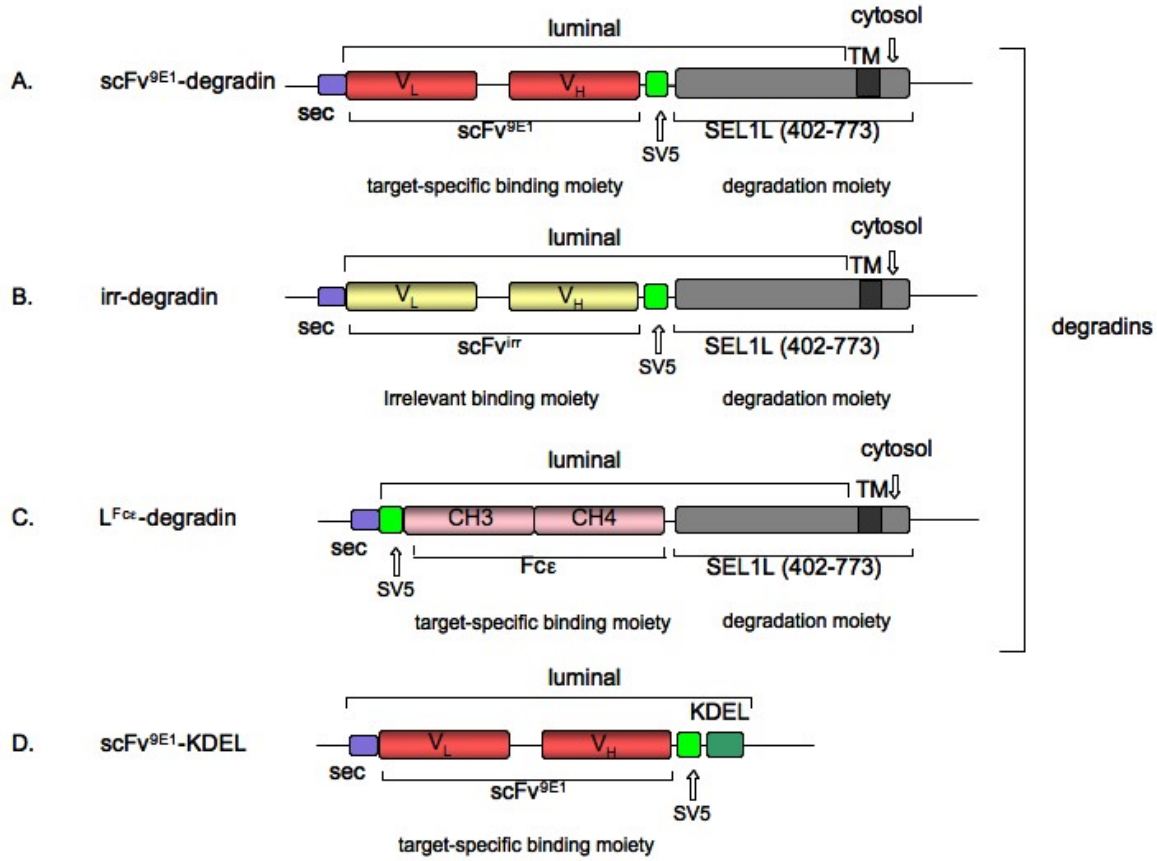

Figure 3: Schematic representation of constructs. A) degradin specific for FcεRI-α chain where the recognition moiety is the mAb 9E1-derived scFv^{9E1}. B) degradin with irrelevant specificity obtained by substitution of scFv^{9E1} with scFv^{irr}. C) The Ligand-degradin where the FcεRI-α chain specific recognition moiety is represented by CH3 and CH4 domains of IgE. D) Control construct for ER retention; constituted by the scFv^{9E1} fused to the C-terminus to a KDEL sequence.

Degradin expression and localisation

The plasmids encoding the constructs described above were transiently transfected into HEK 293 T cells and the expression of degradins was tested on extracts and supernatants.

Extracts and supernatants were loaded on SDS-PAGE and Western blotting was performed using an anti-SV5 antibody to check their expression.

As shown in figure 4A, degradins were well expressed and were found only at the intracellular level, whereas scFv^{9E1}-KDEL, in spite of the presence of the KDEL signal for ER retention, was detected in large amounts also in supernatants (Figure 4B).

Since the mAb 9E1 VH domain contains a glycosylation site, scFv^{9E1}-KDEL can be visualised in Western blotting as two separate bands corresponding to the glycosylated and the non-glycosylated forms (Figure 4A and B). The glycosylation

pattern was confirmed treating supernatants of HEK 293 T cells expressing scFv^{9E1}-KDEL with PNGaseF, which is able to remove all glucidic side chains from glycoproteins (Figure 5C). PNGaseF treatment caused the disappereance of the upper band of scFv^{9E1}-KDEL, but did not change the mobility of the lower band, which should therefore correspond to the non-glycosylated form (Figure 5C). It appears from this experiment that scFv^{9E1}-KDEL is only partially glycosylated due to an inefficient site of glycosylation.

All degradins are visualised as two separate bands, corresponding to the glycosylated and partially glycosylated forms due to the presence in the SEL1L sequence of two sites of glycosylation (position 411 and 587 of the entire SEL1L) maintained in the SEL1L (402-end) fragment of degradins (Figure 4A, see also EndoH treatment in Figure 5B).

In addition scFv^{9E1}-degradin also contains the 9E1 VH glycosylation site, whereas L$^{Fc_\varepsilon}$-degradin contains three additional glycosylation sites derived from the εCH3 domain.

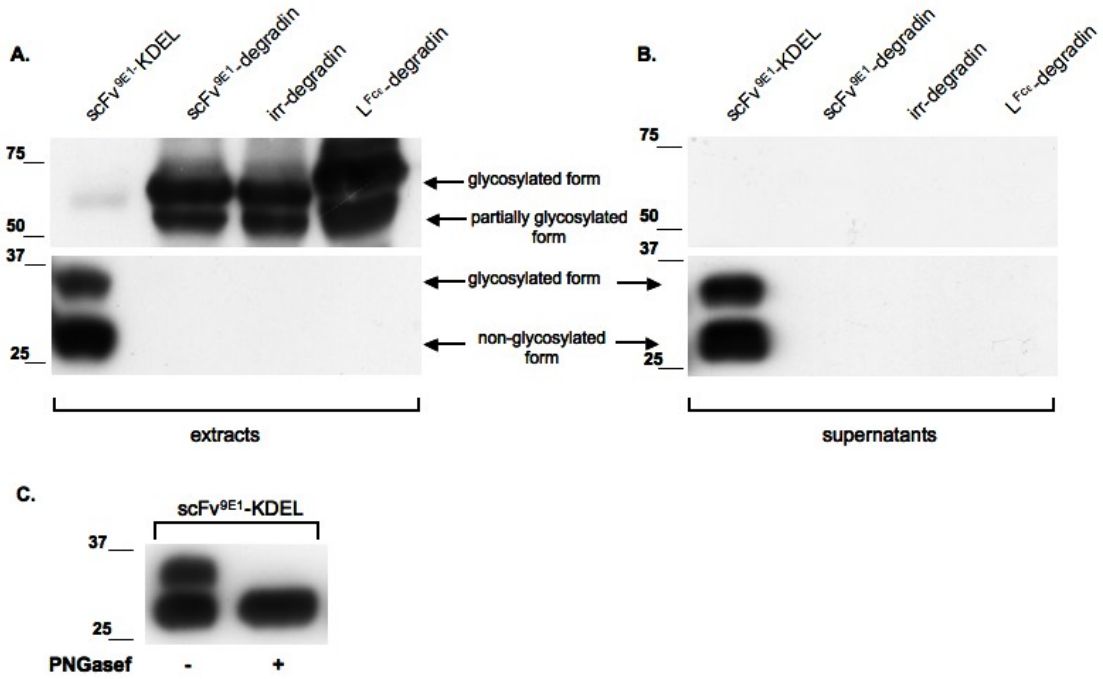

Figure 4: Western blotting on extracts (A) and supernatants (B) of HEK 293 T cell transiently transfected with plasmids encoding degradins or the intrabody scFv^{9E1}-KDEL. Expression of transfected proteins was detected by mAb anti-SV5.
C) The low efficiency of glycosylation of 9E1 VH was confirmed by treating supernatants of cells transfected with scFv^{9E1}-KDEL with PNGaseF.

The different degradins were expected to be confined within the cell and in particular because of the activity of SEL1L transmembrane domain in establishing ER retention [183], they were expected to localise completely in the ER.

In order to confirm degradin localisation a cytofluorimetric analysis was done on HEK 293 T cells transiently transfected with degradins or with an SV5 tagged membrane version of IgE (tm_LIgE, [330]) as a positive control of both, transfection and anti-SV5 antibody detection.

As shown in figure 5A, all types of degradins, either irrelevant, $scFv^{9E1}$ or L^{FC_ε}, were not detected on the cell surface while the SV5 tagged membrane IgE was.

To further assess degradin intracellular localisation we performed a deglycosylation treatment with the endoglycosidase enzyme EndoH. EndoH deglycosylates only molecules that bear a high mannose N-glycan, characteristic of ER localised proteins, whereas Golgi-dependent glycosylated proteins are resistant to EndoH.

As shown in figure 5B all degradin types demonstrated to be fully sensitive to EndoH deglycosylation demonstrating their total ER localisation.

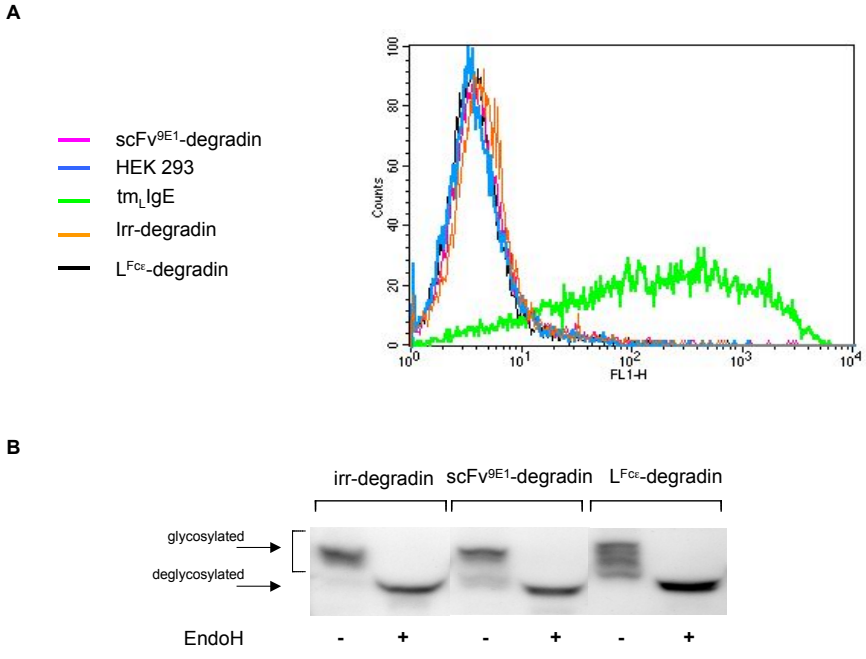

Figure 5: Intracellular degradin localisation was confirmed by cytofluorimetric analysis using mAb anti-SV5 (A). ER localisation was assessed by deglycosylation treatment with EndoH enzyme followed by Western blotting analysis and detection with anti-SV5 mAb (B).

scFv^{9E1}-degradin causes a strong decrease of the soluble form of FcεRI-α chain (sdα)

In order to analyse degradin activity on the soluble version of the FcεRI-α chain (sdα), HEK 293 T cells were transiently cotransfected with plasmids encoding sdα and each one of the different degradins.

The effect of degradins on sdα secretion and eventual retention in the ER, due to the ER localisation of degradins, was analysed by Western blotting in cellular extracts and supernatants.

To compare the effect in decreasing target protein accumulation induced by degradins with simple ER retention, such as that mediated by the KDEL retention signal, scFv^{9E1}-KDEL was also cotransfected with sdα.

As shown in figure 6A lane 1, scFv^{9E1}-degradin blocked completely sdα secretion as no molecules can be detected in supernatants.

In contrast the irrelevant-degradin, in which the scFv moiety does not recognise the target, allows sdα secretion (lane 3), confirming the specificity of scFv^{9E1}-degradin.

The scFv^{9E1}-KDEL allowed a small amount of sdα to be secreted as visualised in supernatants of figure 6A, lane 2: this is most likely due to the incomplete intracellular retention of scFv^{9E1}-KDEL, which can be detected in considerable amounts in the supernatant.

The detection of sdα in the supernatant when coexpressed with scFv^{9E1}-KDEL led us conclude that scFv^{9E1}-degradin is more efficient in preventing sdα secretion.

When looking at the intracellular content (Figure 6B lane 2), it is possible to see that scFv^{9E1}-KDEL caused a large accumulation of sdα in the ER, and prevented its transit through the Golgi, as can be deduced by the absence of the higher molecular weight glycosylated forms. On the contrary, very little target protein was detected in cellular extracts of scFv^{9E1}-degradin transfected cells (Figure 6B lane 1). Thus, together with sdα absence in supernatants, this result suggests that the degradin induced active degradation of the target, sdα.

The ER localisation of the sdα lower molecular weight form was confirmed by treating sdα accumulated protein when coexpressed with scFv^{9E1}-KDEL with the endoglycosydase EndoH that, as described previously, is not able to deglycosylate proteins with Golgi-dependent glycosylation.

Since mAb 9E1 demonstrated to be much less sensitive in the detection of deglycosylated FcεRI-α chain molecules, in this case an anti human Fcγ antibody (DAKO) was used. As shown in figure 6C when coexpressed with scFv^{9E1}-KDEL sdα is fully deglycosylated by EndoH confirming its ER localisation.

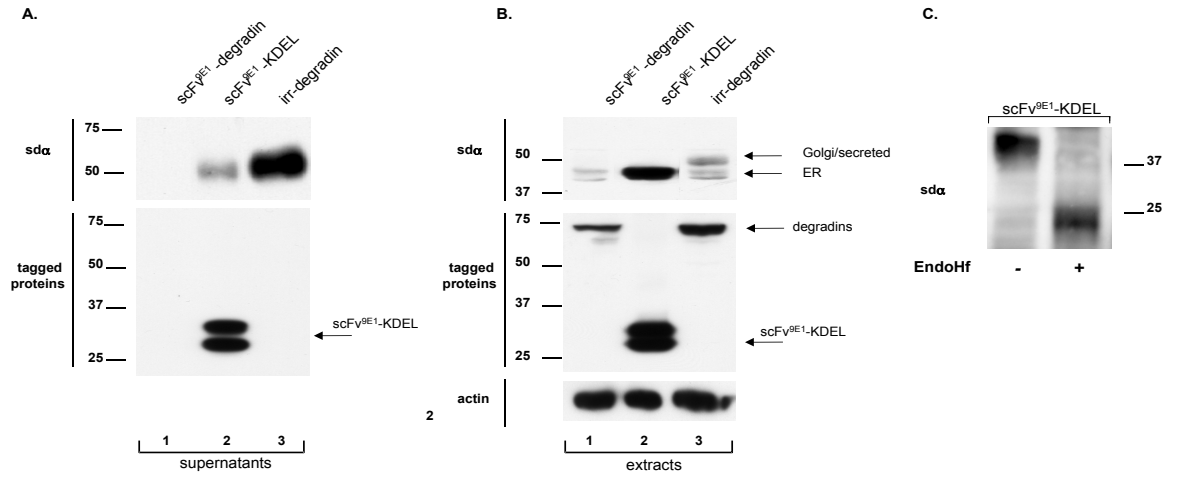

Figure 6: Analysis of scFv^{9E1}-degradin activity on sdα. Detection of sdα using 9E1 mAb on extracts (A) and supernatants (B) of HEK 293 T cells cotransfected with sdα and the indicated proteins. Detection of degradins was performed using anti-SV5 antibody. Anti-actin antibody was used as loading control.
C) Extracts of cell expressing sdα and scFv^{9E1}-KDEL were treated or not treated with EndoH. sdα was detected using a rabbit anti human Fcγ (DAKO) and a goat anti rabbit Ab peroxidase conjugated (Thermo Scientific).

scFv^{9E1}-degradin causes a strong decrease on membrane version of FcεRI-α chain (mdα)

In order to obtain further evidences on the applicability of degradins, the system was tested on the membrane bound version of the same FcεRI-α chain (mdα).
Analysis of mdα levels was done by Western blotting of total extracts of HEK 293 T cells cotransfected with a plasmid encoding mdα and different types of degradin plasmids as reported in figure 7A. As an additional control mdα was also cotransfected with a vector without any insert (pcDNA3 mock).
As shown in figure 7A, scFv^{9E1}-degradin caused a strong decrease in the amount of mdα, compared to pcDNA3 mock and to irrelevant-degradin (compare lane 4 with lanes 1 and 2 respectively). When coexpressed with scFv^{9E1}-degradin, mdα could be detected in a partially glycosylated form corresponding to the protein

localised in the ER, that has not yet undergone Golgi-dependent glycosylation (see also Figure 7B).

The expression of the irrelevant-degradin did not block mdα trafficking to the Golgi apparatus. In fact most of mdα acquired a complete glycosylation state and migrated as a higher molecular weight protein.

When compared to the cotransfection with a pcDNA3 mock, irrelevant-degradin caused a partial impairment in mdα expression.

This effect could be the consequence of the residual activity of the SEL1L fragment of degradins in recognising misfolded proteins, therefore increasing their degradation. Alternatively, degradins could alter the ER environment and induce an UPR response.

However, a very large difference between irrelevant-degradin and scFv^{9E1}-degradin activities was clearly visualised, confirming the essential role of the target recognition moiety.

When coexpressed with scFv^{9E1}-KDEL mdα was completely retained in the ER but with a small decrease in the protein amount indicating a compromised degradative activity. This result is visualised in figure 7A, lane 3, where only the incomplete glycosylation form of mdα was detected. Interestingly, scFv^{9E1}-KDEL displayed a retention activity that was more efficient on mdα than on sdα (compare figure 7A with figure 6A in which sdα is partially secreted). It is likely that scFv^{9E1}-KDEL forms a complex with sdα that can be secreted, while the complex formed by scFv^{9E1}-KDEL and mdα would not be able to proceed to the Golgi, due to the membrane anchoring effect exerted by mdα transmembrane domain.

Also for membrane bound proteins, therefore, the impairment of target protein expression seems to be specifically induced by scFv^{9E1}-degradin upon the recruitment of the target through the recognition moiety.

The ER localisation of mdα lower molecular weight form was confirmed by treating mdα coexpressed with scFv^{9E1}-KDEL with the endoglycosydase EndoH. As shown in figure 7B when coexpressed with scFv^{9E1}-KDEL, mdα was fully deglycosylated by EndoH, confirming its ER localisation.

The degradation of mdα induced by scFv^{9E1}-degradin was measured by blocking protein synthesis 16 hours after transfection with cycloheximide at 50 μg/ml and by anlysing cell extracts at different time points (up to 4 hours) by western blotting.
When coexpressed with scFv^{9E1}-degradin mdα was almost completely disappeared after 30 minutes, whereas when coexpressed with irr-degradin it took at least 4 hours to reach a similar level of reduction (Figure 7C).
A similar result was obtained when cells were pulsed for 30 minutes with $^{[35]}$S-Methionine and chased up to 4 hours.
The amount of mdα at the starting point was already decreased in the presence of scFv^{9E1}-degradin, suggesting a very fast degradation induced by degradins.
A decrease in the amount of mdα was visible after 4 hours when expressed with the irr-degradin, whereas an almost complete absence of mdα was visualised after only 2 hours when expressed with scFv^{9E1}-degradin (Figure 7D).
These results further suggest that scFv^{9E1}-degradin actively induces degradation of the target protein.

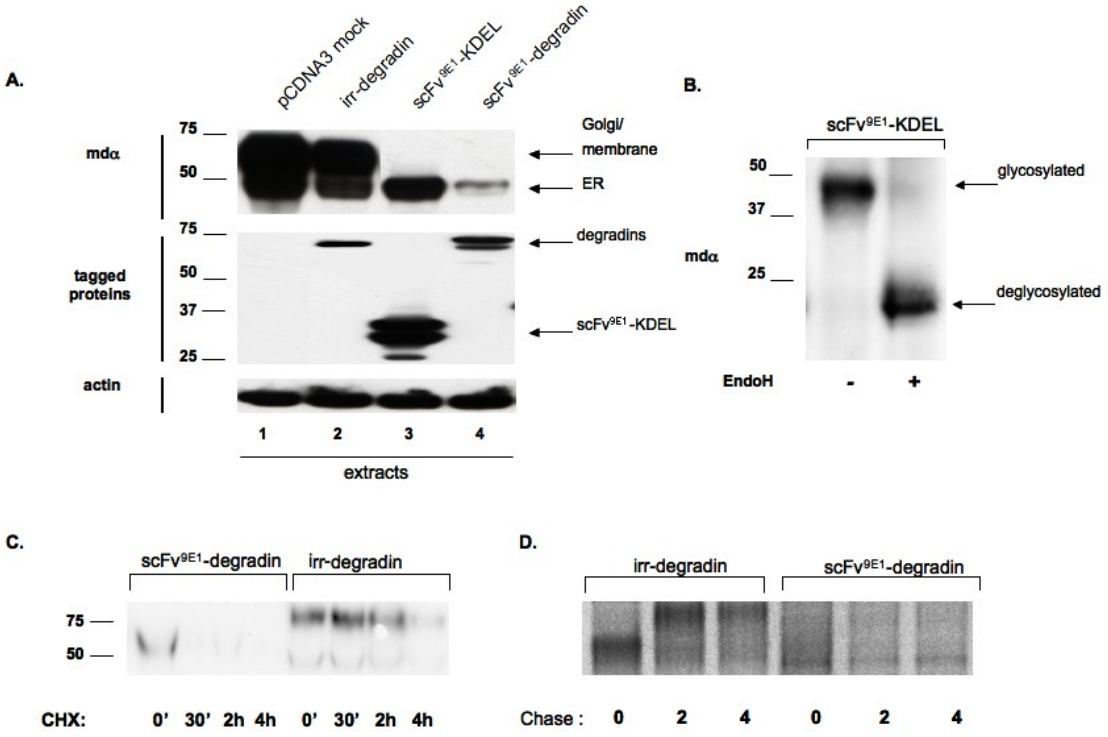

Figure 7: A) scFv^{9E1}-degradin effect on mdα. Extracts of HEK 293 T cells cotransfected with plasmids encoding mdα and the indicated proteins were analysed by Western blotting using 9E1 mAb to check mdα expression. Anti-SV5 antibody was used to detect degradins and scFv^{9E1}-KDEL. Anti-actin antibody was used as loading control B) Extracts of cell expressing mdα and scFv^{9E1}-KDEL were treated or not treated with EndoH. mdα was detected using a rabbit anti human Fcγ (DAKO) and a goat anti rabbit Ab peroxidase conjugated (Thermo Scientific).
C) Measurement of mdα half-life when coexpressed with scFv^{9E1}-degradin or with irr-degradin. 16 hours after transfection cells were treated with cycloheximide 50 μg/ml for the indicated times.
D) Pulse-chase experiment with [^{35}S]-Methionine. After a pulse of 30 minutes cells were chased for the indicated times.

Activity of the Ligand-degradin (L$^{Fc_\varepsilon}$-degradin)

To further validate the method, I investigated the possibility to induce degradation of the FcεRI-α receptor protein exploiting the specificity of its ligand.

A Ligand-degradin (L$^{Fc_\varepsilon}$-degradin), containing the human ε-CH3 and ε-CH4 domains, specific for the interaction with the FcεRI-α chain, was tested by cotransfection with plasmids encoding both mdα and sdα followed by western blotting analysis.

As shown in figure 8A and B, L$^{Fc_\varepsilon}$-degradin caused a strong decrease in sdα levels even if, when compared to scFv^{9E1}-degradin, seemed to have lower effect in blocking sdα secretion (Figure 8A compare lane 2 and 3).

As shown in figure 8B, lane 2, analysis of the cellular extracts showed that L^{Fc_ε}-degradin did not cause sdα ER accumulation.

The same effect of large target protein decrease caused by L^{Fc_ε}-degradin was observed also for mdα; consistently with the partial secretion of sdα, the residual mdα can be visualised in two different glycosylation forms that could reflect mdα capability to reach the Golgi apparatus (Figure 8C, lane 2).

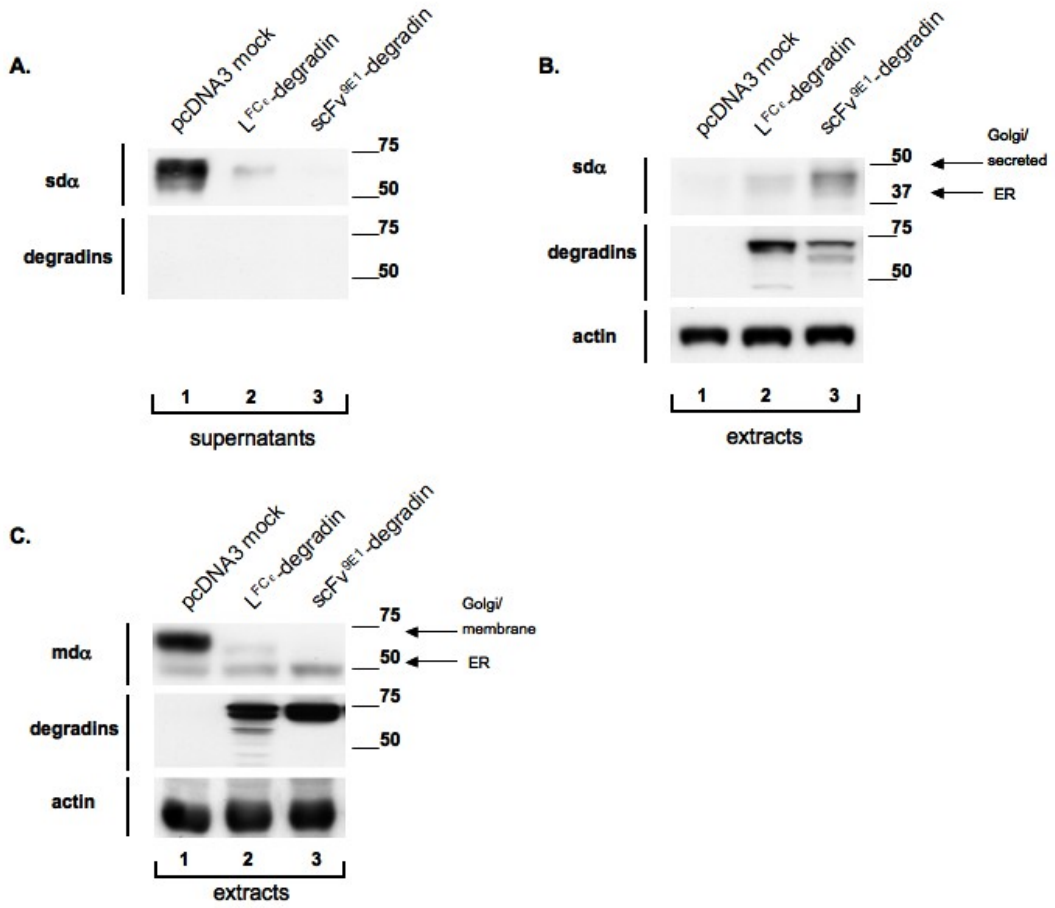

Figure 8: L^{Fc_ε}-degradin efficiency was compared with $scFv^{9E1}$-degradin efficiency by Western blotting analysis on supernatants (A) and extracts (B) of HEK 293 T cells transiently transfected with sdα encoding plasmid or on extracts of cells transfected with mdα encoding plasmid (C). 9E1 mAb was used to check sdα and mdα expression, anti-SV5 antibody was used to check degradin expression and anti-actin antibody was used as loading control.

Degradins do not affect the accumulation of an irrelevant protein

The specific effect of degradins was further investigated by testing their activity on an exogenous irrelevant protein; for this purpose we cotransfected a secretory tagged version of a scFv ($scFv^{1E10\ 330,341}$) with degradins.

Degradins did not affect neither the expression nor the secretion of the irrelevant protein. As shown in figure 9, coexpression with a pcDNA3 mock or with each one of the degradins resulted in comparable expression levels of the irrelevant protein.

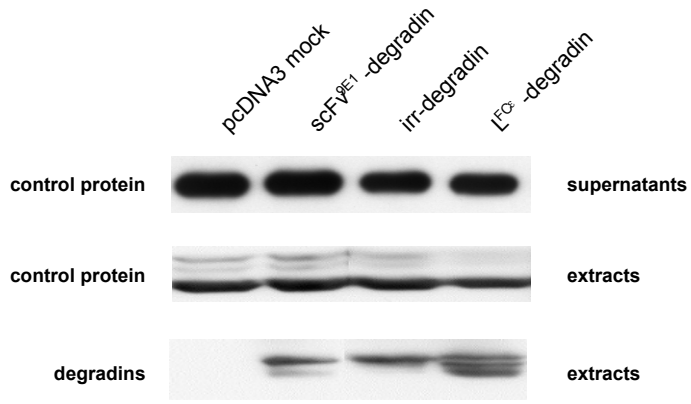

Figure 9: Effect of degradins expression on an irrelevant protein. Western blotting analysis was performed on HEK 293 T cell trasfected with plasmid encoding the irrelevant secretory control protein scFv^{1E10} with each one of the plasmids encoding degradins or with pcDNA3 mock. Detection of degradins and scFv^{1E10} were carried out using anti-SV5 antibody.

The relevant role of the SEL1L moiety

In order to further investigate the requirement of the SEL1L luminal region for the degradin activity, a control construct containing a deleted version of the SEL1L luminal domain and maintaining only the transmembrane and cytosolic portions of SEL1L (SEL1LΔ) was produced (scFv^{9E1}-SEL1LΔ Figure 10B). The SEL1L ER luminal region has been described to interact with components of the ERAD machinery (in particular with the ubiquitin ligase HRD1) and to be essential for the induction of misfolded protein degradation. Therefore constructs containing SEL1LΔ should not induce target degradation but instead only intracellular target retention.

The same control was also produced for L^{Fc_4}-degradin obtaining the L^{Fc_4}-SEL1LΔ (Figure 10D).

As a further control of L^{Fc_ε}-degradin specificity, a deleted version of the ligand was produced ($L^{Fc_{\varepsilon\Delta}}$-degradin Figure 10E). This construct maintains only the human εCH4 domain but lacks the human εCH3, essential for FcεRI-α chain binding [287].
The $scFv^{9E1}$-degradin, $scFv^{9E1}$-SEL1LΔ and L^{Fc_ε}-degradin constructs were produced either using the SV5 tag or using a Tag obtained in our lab whereas L^{Fc_ε}-SEL1LΔ $L^{Fc_{\varepsilon\Delta}}$-degradin were produced only as Tagged version.

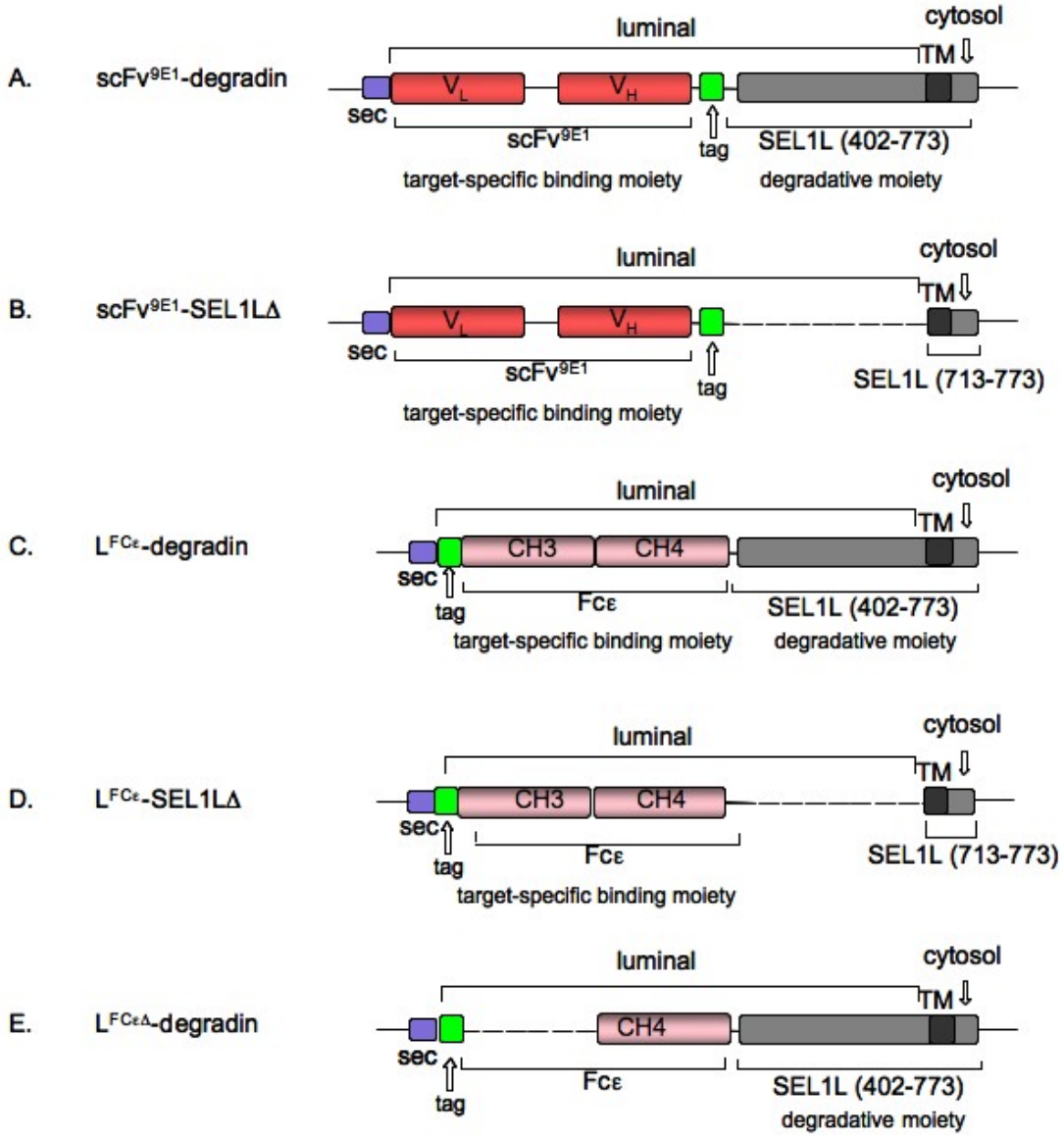

Figure 10: Schematic representation of: A) $scFv^{9E1}$-degradin; B) the control $scFv^{9E1}$-SEL1LΔ degradin in which the target recognition moiety is maintained whereas the degradation moiety was deleted (dotted line) but maintains the transmembrane (TM) and cytosolic portions.
C) L^{Fc_ε}-degradin; D) mutated Ligand-degradin with the luminal part of SEL1L deleted (dotted line) as in B (L^{Fc_ε}-SEL1LΔ); E) $L^{Fc_{\varepsilon\Delta}}$–degradin (deleted Ligand-degradin) that lacks the εCH3 (dotted line) responsible of binding to FcεRI-α chain,

In order to compare scFv^{9E1}-degradin and scFv^{9E1}-SEL1LΔ activities each one of these two proteins or the irrelevant-degradin were coexpressed with sdα. As shown in figure 11A while irrelevant-degradin allowed sdα to be secreted in the supernatant (lane 2), both scFv^{9E1}-SEL1LΔ and scFv^{9E1}-degradin strongly inhibited sdα secretion (lane 3 and 4 respectively).

At the intracellular level however, a difference between scFv^{9E1}-SEL1LΔ and full length degradin activities could be appreciated. As visualised by Western blotting on cellular extracts (Figure 11B) while scFv^{9E1}-SEL1LΔ produced a large intracellular accumulation of sdα in the ER, comparable to that caused by scFv^{9E1}-KDEL, scFv^{9E1}-degradin did not (compare lanes 4, 5 and 3 respectively).

Taken together these results strongly suggest that the SEL1L luminal domain is essential to induce an active degradation of the target, since the presence of the transmembrane and cytosolic domains alone only caused intracellular accumulation.

The deleted Ligand-degradin (L$^{Fc_{\varepsilon\Delta}}$-degradin) and SEL1L deleted version of Ligand-degradin (L$^{Fc_{\varepsilon}}$-SEL1LΔ) were also analysed for their effect on sdα expression and compared to the activity of the full length L$^{Fc_{\varepsilon}}$-degradin.

As shown in figure 11C, sdα secretion as expected was not impaired by Ligand$^{Fc_{\varepsilon\Delta}}$-degradin (lane 2), due to its inability to bind FcεRI-α chain whereas sdα secretion was completely prevented by both L$^{Fc_{\varepsilon}}$-degradin and L$^{Fc_{\varepsilon}}$-SEL1LΔ (lanes 3 and 4).

However, while only traces of sdα can be detected intracellularly when coexpressed with L$^{Fc_{\varepsilon}}$-degradin, L$^{Fc_{\varepsilon}}$-SEL1LΔ produced a large ER retention of sdα (Figure 11D compare lanes 2 and 4).

This result is consistent with an important role of the SEL1L luminal region for the degradative activity of degradins

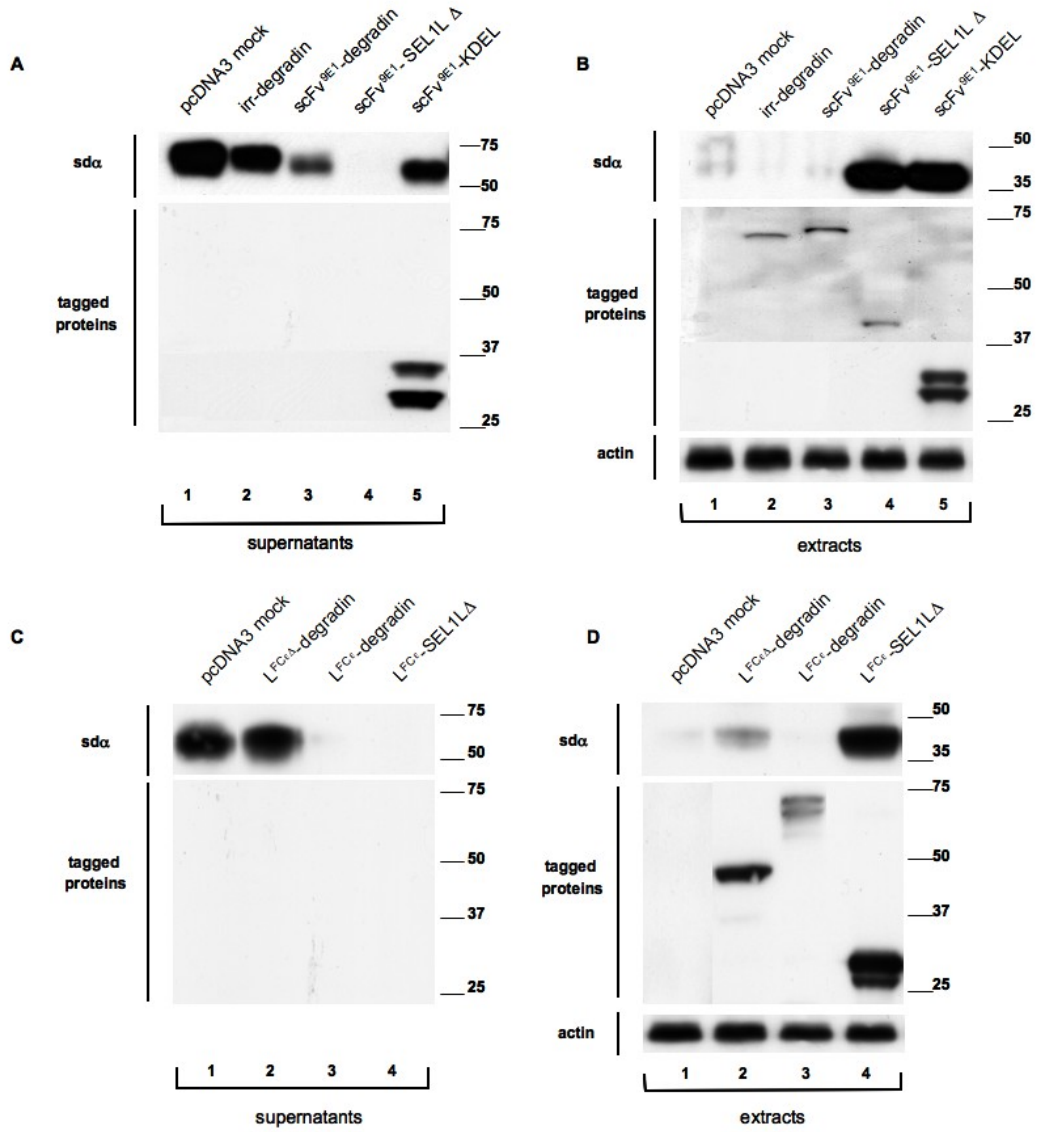

Figure 11: Western blotting of extracts and supernatants of HEK 293 T cells cotransfected with sdα encoding plasmid and plasmids encoding the proteins indicated on the figure. A-B) Comparison between scFv^{9E1}-degradin and scFv^{9E1}-SEL1LΔ activities on sdα expression
C-D) Ligand$^{Fc_{\varepsilon}}$-degradin activity was compared with Ligand$^{Fc_{\varepsilon\Delta}}$-degradin and Ligand$^{Fc_{\varepsilon}}$-SEL1LΔ controls. For sdα detection mAb 9E1 was used and anti-tag mAb was used for degradin detection. Anti-actin antibody was used as loading control.

Similar effects of the deleted degradin were also obtained with the membrane version of FcεRI-α chain (mdα).

As shown in figure 12A lane 4, scFv^{9E1}-SEL1LΔ did not impair mdα expression, but instead displayed a retention activity as visualised by the presence of the ER glycosylated form of mdα, similar to scFv^{9E1}-KDEL (compare lanes 4 and 5).

As shown in figure 12B lane 4, also the $L^{Fc_{\varepsilon}}$-SEL1LΔ produced ER retention although not complete, and partial activity in decreasing mdα accumulation probably correlated to ER stress. The $L^{Fc_{\varepsilon\Delta}}$-degradin instead, did not have any effect on mdα expression and localisation, consistently with its inability to bind the target (Figure 12B, lane 2).

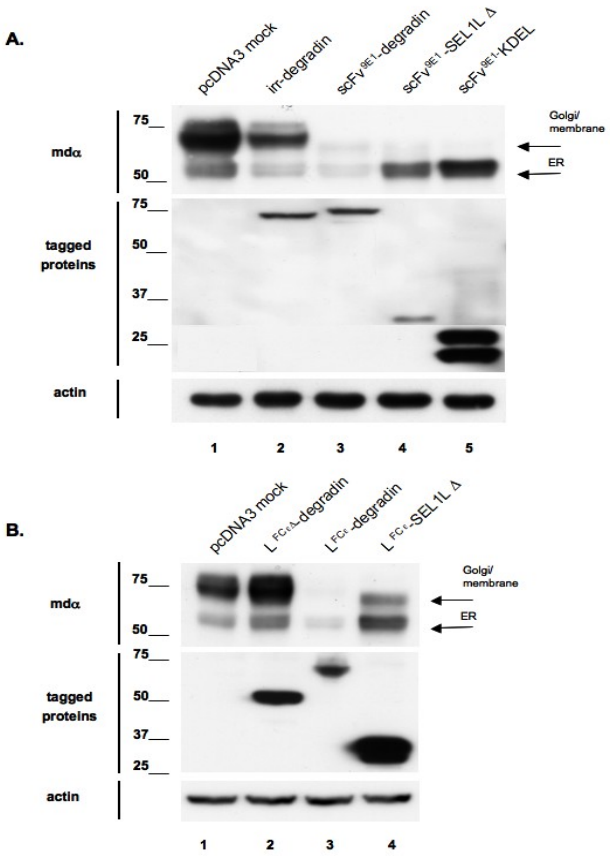

Figure 12: Western blotting of extracts of HEK 293 T cells cotransfected with mdα and plasmids encoding the indicated proteins. A) Comparison between $scFv^{9E1}$-degradin and the deleted degradin $scFv^{9E1}$-SEL1LΔ
B) The $Ligand^{Fc_{\varepsilon}}$-degradin was compared with the deleted Ligand ($L^{Fc_{\varepsilon\Delta}}$-degradin) and with the deleted degradin ($L^{Fc_{\varepsilon}}$-SEL1LΔ) controls. For mdα detection mAb 9E1 was used, anti-tag mAb was used for degradin detection and anti-actin antibody was used as loading control.

Degradins-induced degradation and the proteasome machinery

The design of degradins was based on the assumption that the activity of SEL1L in inducing misfolded protein degradation through the ubiquitin proteasome pathway would be maintained. Therefore target degradation induced by degradins would take place by recruiting ubiquitin ligases and the proteasomal machinery.

To confirm this assumption I used the proteasome inhibitor MG132 in cells co-expressing degradins and the target, in order to block proteasome dependent degradation.

For this purpose HEK 293 T cells were transiently transfected with mdα and degradins encoding plasmids and, 16 hours after transfection, treated with MG132 for 6 hours. The outcome of this experiment was that MG132 addition caused an increased intracellular accumulation of target protein, an effect that was more evident with scFv^{9E1}-degradin rather than with L$^{Fc_\alpha}$-degradin (Figure 13A).

In particular, due to the ER localisation of scFv^{9E1}-degradin, the increased amount of mdα was almost completely retained in the ER and only a small portion was detected in the terminal glycosylated form. The accumulation of glycosylated mdα molecules during MG132 treatment resulted in the ability of a certain amount of mdα to reach the Golgi where the its N-glycosylation is modified.

The inhibition of degradation caused by the proteasome inhibitor MG132 confirmed that degradin-induced degradation was at least in part dependent on the proteasome activity. The incomplete rescue in protein expression could be due to an involvement of autophagy or by an induction of autophagy, caused by MG132 treatment. It has been reported that autophagy induction seems to protect the cell form death in situations in which the proteasome is inhibited (reviewed in [342]).

This could be the main reason why MG132 treatment did not result in a complete inhibition of degradation since substrates of ERAD (in this case mdα) would be re-routed to autophagy during proteasome inhibition.

This possibility could be investigated by treating cells with proteasome and autophagy inhibitors.

When the same experiment was performed on sdα no increase was visible upon MG132 treatment (data not shown).

Since deglycosylation is an important step during proteasome-dependent glycoprotein degradation we decided to use sα-SV5-BAP, a soluble SV5 tagged version of α chain (αD1D2-SV5-BAP [330]; see also figure 26A) that can be detected using anti-SV5 antibody. Anti-SV5 antibody should detect all sα-SV5-BAP molecules, either glycosylated and deglycosylated, while mAb 9E1 displayed a much reduced affinity for deglycosylated FcεRI-α chain molecules.

As expected, degradins were still functional against sα-SV5-BAP since the inhibition of sα-SV5-BAP secretion was evident with both scFv^{9E1}-degradin and L$^{Fc_\alpha}$-degradin (Figure 13B).

Although MG132 treatment did not produce an increase in sα-SV5-BAP secretion in the presence of degradins, I found that, interestingly, the non-degraded accumulated sα-SV5-BAP molecules migrate as lower weight band (Figure 13B) corresponding to the deglycosylated molecules (data not shown).

In the absence of proteasome inhibitors these deglycosylated molecules were very poorly represented, whereas once accumulated upon treatment with MG132, their detection was enabled (Figure 13C).

As a further analysis the different stability of degradin in the presence or absence of its target (mdα) was also assessed. As visualised in figure 13D scFv^{9E1}-degradin stability seemed to be somehow reduced by the coexpression with the target mdα, while no differences were visible with the irr-degradin. This indicates that only a small fraction of degradin was degraded, while most of it could exhibit a longer persistence than its susbstrate.

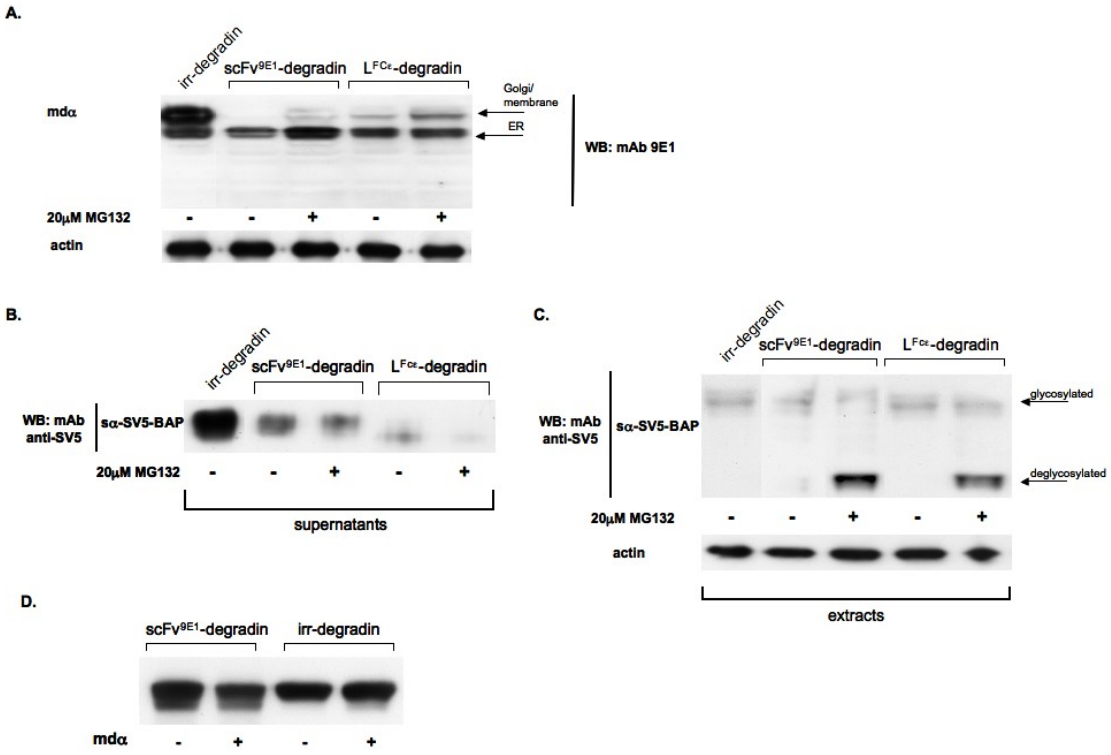

Figure 13: A) mdα was coexpressed in HEK 293 T cells with one of the indicated proteins: 16 hours after transfection cells were treated for 6 hours with 20 μM MG132. mdα was detected using mAb 9E1 and as loading control anti-actin antibody was used.
B-C) The same experiment was performed coexpressing sdα with the indicated proteins; supernatants (B) and extracts (C) were analysed by Western blotting using mAb anti-SV5 and as loading control anti-actin antibody.
Degradin expression was analysed in the presence or absence of its target mdα (D)

Use of the endogenous ERAD inhibitor: SVIP

An endogenous ERAD inhibitor known as SVIP has been described. SVIP is a cytosolic protein, anchored to the ER membrane through myristoylation [233]. It has been described to form a complex with Derlin-1 and p97 and to reduce association of ERAD substrates with p97, thus avoiding protein ubiquitinylation and degradation [232].

To test the ability of SVIP to inhibit degradin-mediated target degradation (what would constitute a further confirmation of proteasome involvement in this process) HEK 293 T cells were transfected with plasmids encoding mdα, degradins (scFv^{9E1}-degradin, irrelevant and L$^{Fc\varepsilon}$-degradin) and SVIP. An irrelevant protein was transfected in the samples in which SVIP is not present, in order to have the same amount of exogenous mRNA.

Figure 14 shows that SVIP expression led to an increase in the intracellular level of mdα either when coexpressed with scFv^{9E1}-degradin or when coexpressed with L$^{Fc\varepsilon}$-degradin, although increase in mdα expression was more evident in the case of the L$^{Fc\varepsilon}$-degradin, showing accumulation of both completely glycosylated and incompletely glycosylated bands in agreement with the result obtained with MG132 (compare Figure 14 and 13A). This result suggests that SVIP through its activity in inhibiting retro-translocation is able to partially block target degradation further suggesting the proteasome-dependent pathway of degradins.

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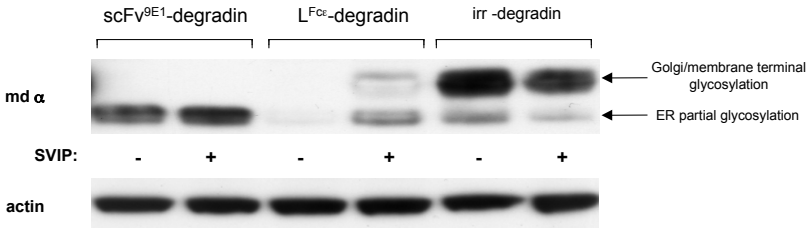

Figure 14: Analysis of SVIP activity on degradin-induced degradation. mdα was coexpressed in HEK 293 T cells with degradins and SVIP or with a control protein. mAb 9E1 was used to check mdα expression on cellular extracts of transfected cells. Anti-actin antibody was used as loading control.

Degradin can induce degradation of a constitutively expressed target

To demonstrate the ability of degradins to constitutively knock-down a specific target, scFv^{9E1}-degradin was stably transfected into a HEK 293 cell line constitutively expressing mdα, membrane expression levels of mdα were analysed by cytofluorimetry and Western blotting.

As shown in figure 15A, Western blotting analysis revealed a significantly lower amount of mdα in the clone stably expressing degradin (clone G6), when compared to the non-transfected cell line.

An ulterior confirmation of this result came from cytofluorimetric analysis using either the anti FcεRI-α chain mAb 9E1 (Figure 15B) or the ligand, a purified human IgE (Figure 15C).

As shown in figure 15B G6 clone showed a very strong decrease in mdα expression in comparison to the clone expressing only mdα; consistently also the ability to bind IgE resulted much reduced (Figure 15C).

The G6 cell line was maintained in culture for long periods without changes in the profiles of mdα expression, demonstrating that degradins can constitutively induce a strong and sustained target degradation.

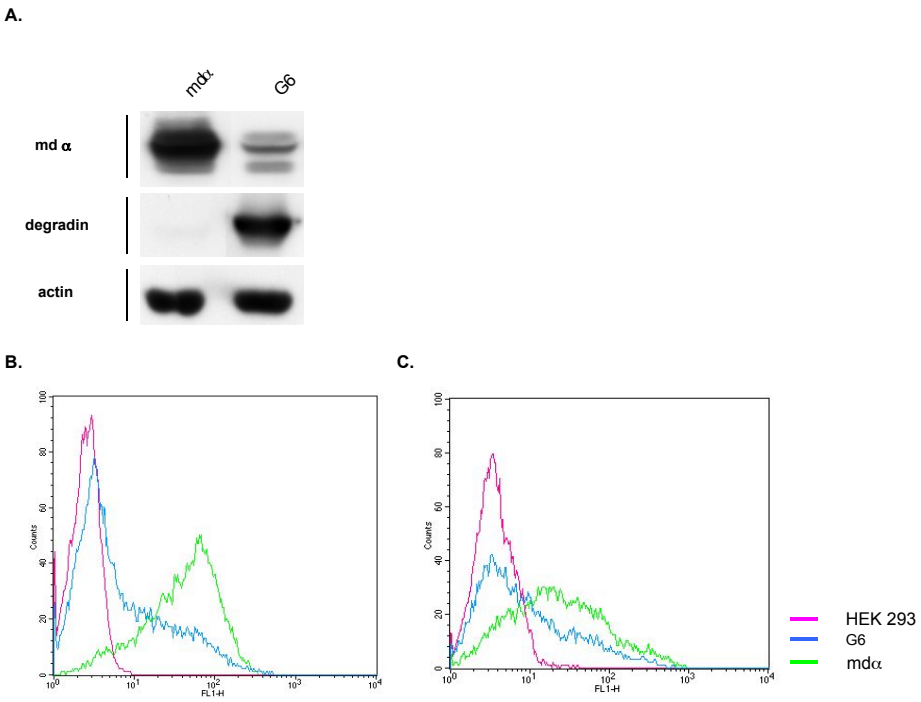

Figure 15: mdα levels on HEK 293 cells constitutively expressing mdα and scFv^{9E1}-degradin
Western blotting analysis using mAb 9E1 to detect mdα and anti-SV5 to detect degradin. Anti-actin was used as loading control.
B and C) Cytofluorimetry on HEK 293-mdα/degradin (clone G6) stained with mAb 9E1 to detect mdα expression on the cellular membrane (B) or with human IgE to evaluate binding to mdα (C).

To confirm that the reduced expression of mdα was the consequence of the degradin activity cells expressing mdα and G6 cells were treated for 6 hours with the proteasome inhibitor MG132. As shown in figure 16, an increased amount of mdα was observed in the presence of the proteasome inhibitor, thus confirming that the degradin-induced-degradation takes place at least in part through the proteolytic activity of the proteasome.

These results indicate that the reduced expression of mdα is due a continuos degradation induced by scFv^{9E1}-degradin.

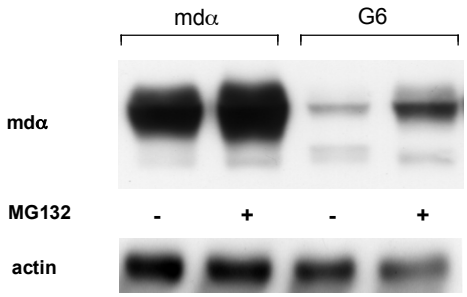

Figure 16: Proteasome inhibitor treatment on mdα clone and G6 clone. 20 μM MG132 treatment was performed for 6 hours. Anti-actin was used as loading control.

The TGEV spike protein S, a second model for degradin

A second model of degradins was also developed and tested. The target protein in this case is represented by the spike protein S of the coronavirus TGEV, while the S-specific degradin was obtained using as recognition moiety a scFv derived from mAb 6A.C3 (scFv$^{6A.C3}$ [324,325]).

Also in this case the control scFv$^{6A.C3}$-KDEL was produced (Figure 17).

It should be noted that S-protein is absolutely essential for the viral particle to be infective, yet it is not required for the assembly of the viral particle. Thus the scFv$^{6A.C3}$-degradin/S protein model represents an ideal case in which the degradin directly targets a relevant biological effect.

The BHK pAPN cell line, stably expresses the aminopeptidase N which is the receptor for the porcine TGEV [336] and is therefore highly permissive for viral infection and replication. I used this cell line to generate four different stable transfectants expressing the anti S protein scFv$^{6A.C3}$-degradin (S-degradin), scFv$^{6A.C3}$-KDEL (S-KDEL), irrelevant-degradin or the secretory anti-S protein αSIP-6A.C3 (S-SIP; Figure 18). The latter one was previously demonstrated to be actively secreted in the extracellular medium with a strong neutralization activity on virus infection (Figure 18).

The scFv$^{6A.C3}$-degradin, irrelevant-degradin and scFv$^{6A.C3}$-KDEL localise in the ER, the first two as transmembrane proteins, while the last one is retained in the ER through the KDEL sequence.

A scheme of the constructs and the corresponding stable transfectants are shown in figures 17 and 18.

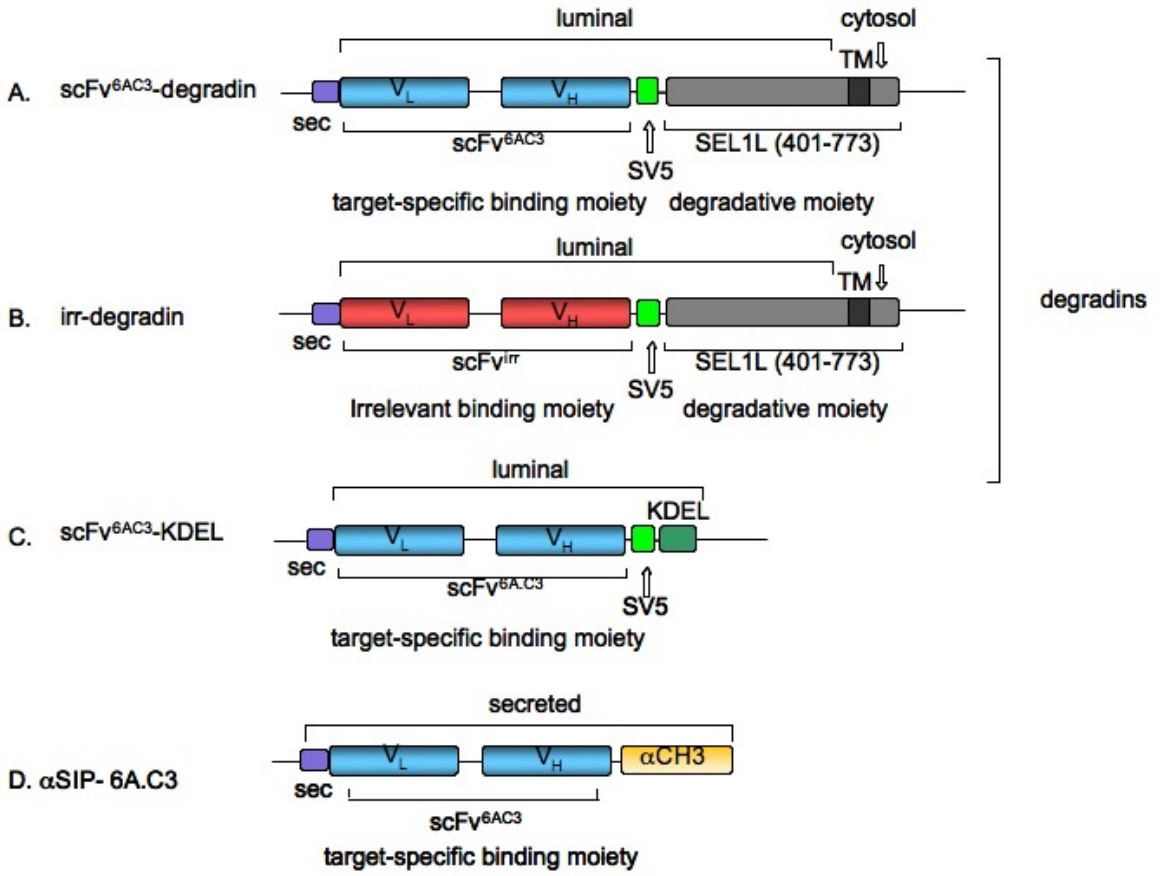

Figure 17: Schematic representation of constructs. A) TGEV S protein specific scFv$^{6A.C3}$-degradin B) with irrelevant specificity C) scFv$^{6A.C3}$-KDEL D) and SIP version of mAb 6A.C3.

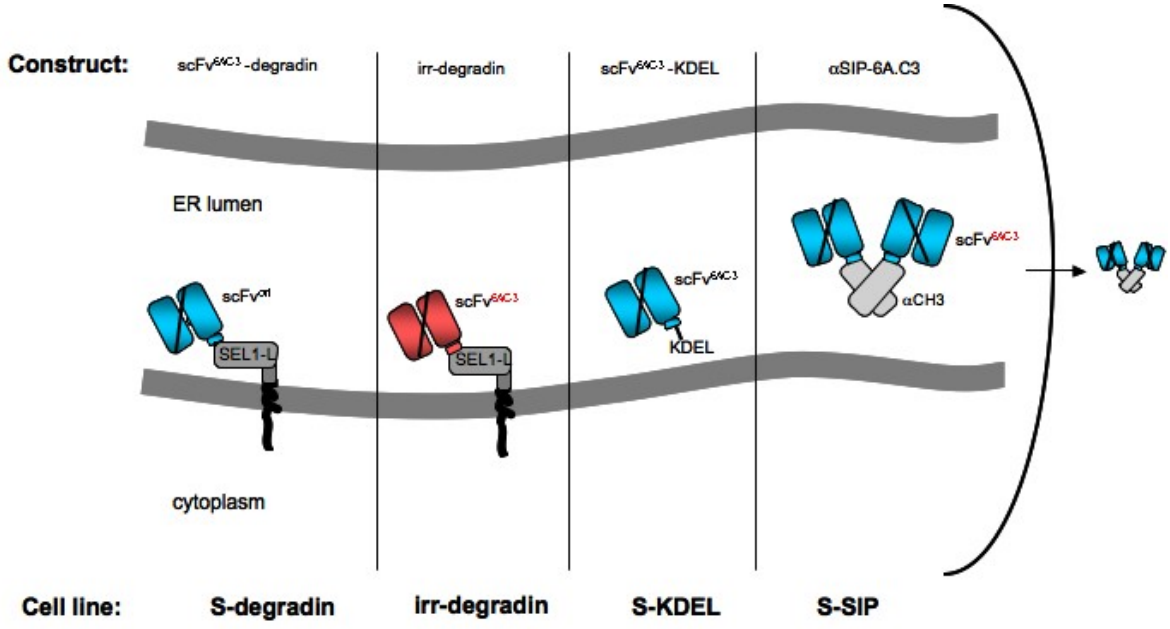

Figure 18: Intracellular localisation of the constructs transfected into BHK pAPN cell line. scFv$^{6A.C3}$ was expressed in three different version: as degradin anchored at ER membrane (S-degradin), as KDEL tagged localised in ER lumen (S-KDEL) and as αSIP-6A.C3 which is secreted in the extracellular medium (S-SIP). As a control of specificity, an irrelevant-degradin was also expressed, tethered to the ER membrane.

The four different cell lines and the original untransfected one (BHK pAPN) were then infected with TGEV. If the S-specific degradin was functional we expected a specific decrease in the accumulation of protein S with no alteration of the other viral proteins.

As visualised by Western blotting on lysates of TGEV infected cells, scFv$^{6A.C3}$-degradin heavily impaired S protein expression, whereas N and M proteins were normally expressed demonstrating, once more, the specificity of the degradin. Thus, while the virus could infect cells leading to a normal expression of N and M proteins expression of S protein was heavily compromised because of degradation concomitantly with S-degradin expression (Figure 19), on the contrary, the irrelevant degradin did not affect neither S protein expression nor M and N protein expression.

The scFv$^{6A.C3}$-KDEL also caused a decrease in S protein expression, most likely through intracellular retention.

As previously demonstrated, αSIP-6A.C3, neutralizes the virus extracellularly, thus inhibiting virus infection [325], clearly reflected in the reduced accumulation of all the three viral proteins S, N and M (Figure 19).

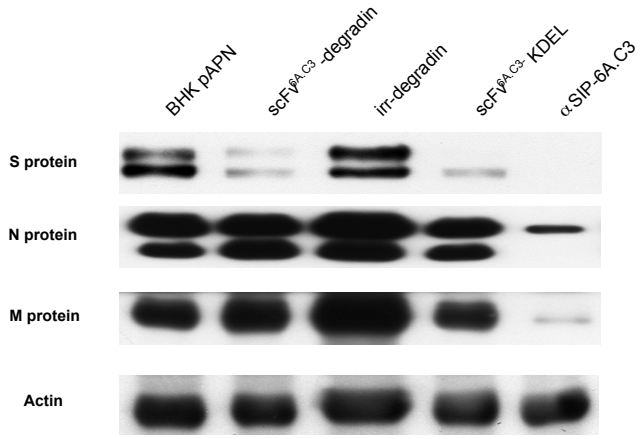

Figure 19: Untransfected BHKpAPN cell line and BHKpAPN cell lines expressing the proteins indicated on the figure were infected with 1 M.O.I. of TGEV. Lysates were analysed by Western blotting. TGEV S protein expression was detected with murine mAb 1D.G3, TGEV M and N protein expression were detected with murine mAbs 25.22 and 3D.C10 respectively. Anti-actin antibody was used as loading control.

scFv$^{6A.C3}$-degradin inhibits TGEV infective particles production at intracellular level

The yield of infective viral particles produced from the different cell lines was then tested in a plaque forming assay [338] by I. Sola (L. Enjuanes lab-Centro Nacional de Biotecnologia-Madrid [324,325]) and the result obtained is shown in figure 20.
The scFv$^{6A.C3}$-degradin as well as scFv$^{6A.C3}$-KDEL caused a strong reduction in viral titres (1-3%) compared to the irrelevant-degradin. It can be deduced that, because of the reduction in the production of S protein, inhibition of the formation of infective viral particles at the intracellular level was the consequence of the activity of scFv$^{6A.C3}$-degradin and scFv$^{6A.C3}$-KDEL.
As expected, and as previously described, 6A.C3 soluble SIP version completely inhibited the formation of infective TGEV particles. Its neutralization activity however, takes place at extracellular level directly inhibiting the infection.

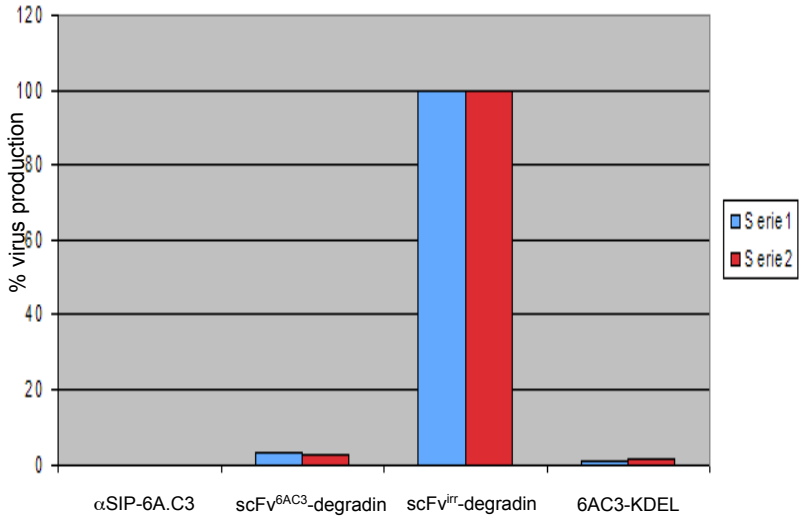

Figure 20: Histogram of TGEV titration of supernatants of BHKpAPN stable clones. The number of TGEV infective particles obtained after TGEV infection of irrelevant-degradin cell line represents 100%. The results of TGEV titration of the other cell lines were compared to the irr-degradin.

Degradin and retro-translocation

Since the proteolytic activity of the protesome was demonstrated to be essential during degradin-induced degradation the idea was to further characterise the pathway of degradation focusing on the retro-translocation process.

Retro-translocation is an essential step during ERAD that allows the release of misfolded proteins from the ER to the cytosol in order to ensure their proteasome-dependent degradation.

Due to the absence of a specific and efficient system to detect retro-translocated molecules we therefore developed a method that allows direct identification of proteins retro-translocated from the ER to the cytosol. The system is based on the specific *in vivo* biotinylation of the protein of interest tagged with the 15 aminoacids long biotin-acceptor-peptide (BAP) by the *E. Coli* biotin ligase BirA expressed in the cytosol. We reasoned that if a BAP-tagged protein localised in the lumen of the ER is dislocated to the cytosol, it should become selectively biotinylated by the cytosolic BirA while, the non retro-translocated ones should not.

In order to test the method we used as a model the human A2 allele of the MHC class I α chain (MHC-Iα), which has been extensively studied and described to be targeted by two HCMV proteins (US2 and US11) for dislocation to the cytosol and for proteasomal degradation. US2 and US11 carry out a degradative activity against MHC-Iα molecules with the aim of escape the host immune system [98,235].

In order to confer to MHC-Iα the possibility to become biotinylated an engineered version of MHC-Iα fused with a BAP peptide at the amino-terminal side was designed. Since MHC-Iα molecule is a type I transmembrane protein with an ER luminal N-terminal portion, when the BAP peptide is inserted at the N-terminus it should be accessible to the cytosolic BirA only when dislocated from the ER to the cytosol, by means of US2 and US11 activities.

It has been already demonstrated that secreted and membrane proteins modified with the addition of the BAP peptide at the N-terminus, due to their insertion in the ER during translation, cannot get biotinylated by cytosolic BirA (cyt-BirA) but they can get fully biotinylated by a version of BirA redirected to the secretory pathway (sec-BirA) [330].

The BAP-MHC-Iα chain was therefore engineered by fusing at the N-terminus of the sequence the 12 aminoacids long SV5 tag to allow recognition by an anti-SV5 antibody followed by the 15 aminoacids long BAP peptide (Figure 21A).

The BAP peptide of the resulting BAP-MHC-Iα would be completely inserted in the ER lumen and would be recognised by cyt-BirA only upon retro-translocation to the cytosol.

Once in the cytosol, the BAP peptide should be rapidly recognised by cyt-BirA resulting in the covalent addition of a biotin molecule to a lysine within the BAP sequence.

Figure 21B illustrates dislocation and biotinylation steps expected for MHC-Iα molecules.

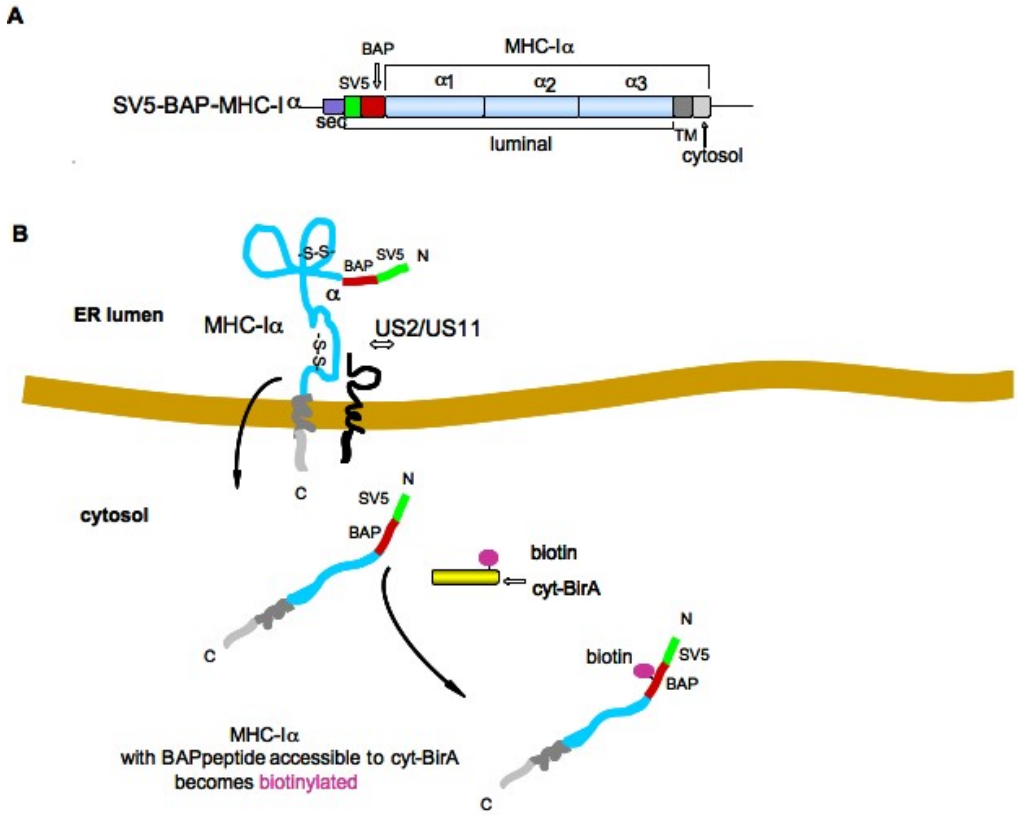

Figure 21: A) schematic representation of the engineered version of MHC-Iα chain. BAP-MHC-Iα contains a secretion signal (sec) upstream the SV5 and BAP peptide encoding sequences
B) Schematic representation of retro-translocation induced by US2 or US11. MHC-Iα molecules dislocated from the ER to the cytosol could be biotinylated by cyt-BirA enzyme present in the cytosol.

Gel retardation assay to monitor biotinylation

In order to discriminate biotinylated molecules from the non-biotinylated ones, a gel retardation assay, illustrated in figure 22, was used.
The assay is based on a Western blotting retardation of the biotinylated molecules upon incubation with streptavidin. Non-biotinylated molecules do not bind to streptavidin, whereas biotinylated ones bind strongly streptavidin, giving rise to a complex with higher molecular weight, which is resistant to SDS-PAGE conditions and can therefore be visualised in Western blotting as a slower migrating band.

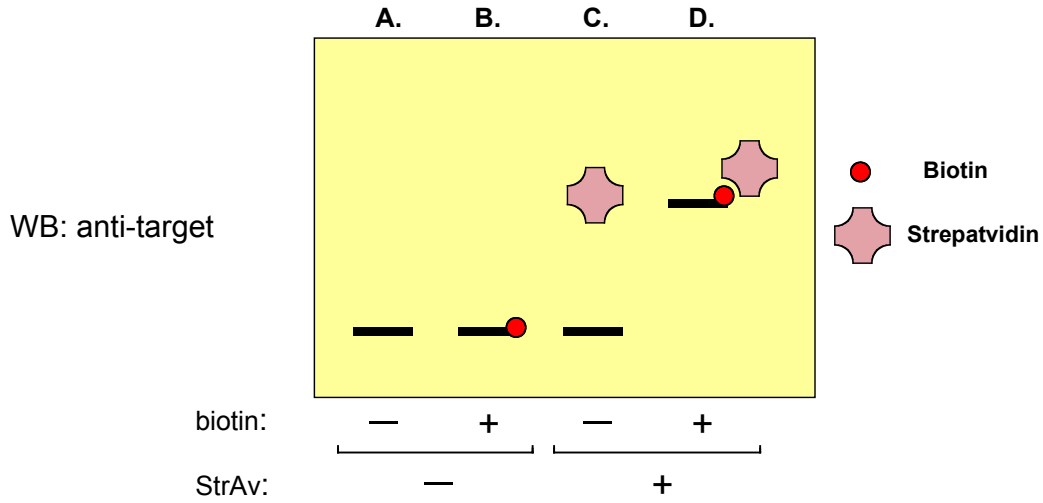

Figure 22: Gel retardation assay scheme. Efficiency of biotinylation can be detected by Western blotting analysis using the specific antibody for the protein whose biotinylation status is under investigation.
The scheme represents the four possible outcomes of biotinylation and subsequent incubation of the sample with streptavidin.
A. protein non-biotinylated and not incubated with streptavidin
B. protein biotinylated but not incubated with streptavidin
C. protein non-biotinylated and incubated with streptavidin
D. protein biotinylated and incubated with streptavidin

Retro-translocation of BAP-MHC-Iα

A three-plasmids transient transfection was performed in HEK 293 T cells with plasmids encoding BAP-MHC-Iα, cyt-BirA, and one of the two immunoevasins US2 or US11 or control pcDNA3 in a medium containing biotin.

Total lysates of the transfected cells were then incubated in the presence or absence of streptavidin and loaded on SDS-PAGE in order to perform the SDS-PAGE retardation assay using anti-SV5 (tag) antibody in Western blotting.
As shown in figure 23A, BAP-MHC-Iα expression is decreased, as expected when cotransfected with either US2 or US11. The decrement with US2 is less pronounced than with US11. Interestingly, a significant portion of BAP-MHC-Iα still present in cells expressing US2 and US11 was biotinylated, as visualised by the presence of the retarded band, indicating exposure to the cytosol.
In contrast, in the absence of immunoevasins, only a slight amount of BAP-MHC-Iα was biotinylated possibly representing misfolded molecules that become substrates of ERAD.
To further validate the *in vivo* biotinylation system in the study of retro-translocation, an experiment was performed using a US11 mutant, US11Q192L, which is still able to bind MHC-Iα molecules but is not able to induce their degradation because of the disruption of its interaction with Derlin-1 [98,343]. For these reasons US11 Q192L represents an excellent control of the specificity of the in vivo biotinylation system when compared to the US11 activity.
As shown in figure 23B, US11Q192L was unable to induce BAP-MHC-Iα retro-translocation, as demonstrated by the almost complete absence of BAP-MHC-Iα biotinylated molecules. On the other hand, also in this case US11 expression allowed the biotinylation of a high rate of BAP-MHC-Iα molecules.
This result strongly suggests that *in vivo* biotinylation of luminal BAP-tagged proteins is a valid method to monitor retro-translocation from the ER to the cytosol.

The same result was obtained following immunoprecipitation with the anti-SV5 antibody of extracts from cells labelled with [^{35}S]-Methionine in pulse-chase experiments. The advantage of this experiment resides in the fact that molecules produced during the pulse period can be followed during the chase period in which, the [^{35}S] labelling is stopped, by the incorporation of cold methionine.
As shown in figure 23C, after 30 minutes of [^{35}S]-Methionine pulse, BAP-MHC-Iα amounts were decreased when cotransfected with US2 encoding plasmid with a fraction of molecules becoming biotinylated.

Following the chase period of two hours, a decrease in the amount of BAP-MHC-Iα when coexpressed with US2 became more evident, due to the proteasome-dependent degradation of the molecules produced during the pulse period.

Although the amount of retro-translocated/biotinylated molecules was much reduced, a significant relative increase with respect to the non-biotinylated ones was observed in the presence of US2. Moreover, the biotinylated amount of protein was much increased compared to the non biotinylated ones (Figure 23C) indicating that during the chase period dislocation and degradation of the molecules previously produced continue to take place.

The decrease of BAP-MHC-Iα observed after the chase period in control transfection without US2 (pcDNA3 mock) must be ascribed to protein turnover and spontaneous degradation of misfolded proteins. Indeed, the majority of the intracellular BAP-MHC-Iα present after the chase in cells expressing US2 are already delocalised to the cytosol, as evidenced by the relative intensity of the retarded and non-retarded bands.

It is important to note that our method allows to detect retro-translocated molecules which is otherwise impossible. Indeed, the other systems used to discriminate luminal from dislocated molecules needs the addition of proteasome inhibitors. In those conditions the accumulation of retro-translocated molecules can be visualised because cytosolically localised proteins become deglycosylated by the enzyme PNGase producing a lower molecular weight band. However, we have obtained that even in the presence of MG132, the deglycosylated molecules represent only a fraction of all the cytosolically delocalised ones.

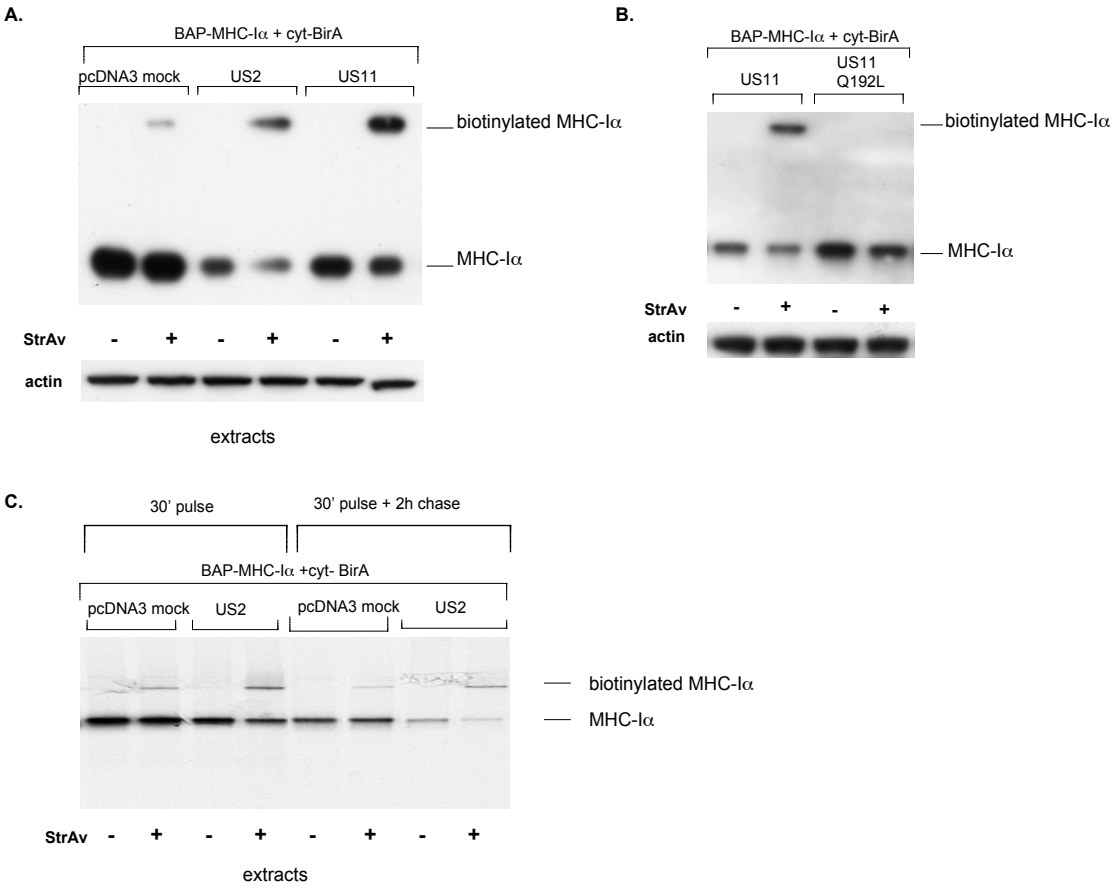

Figure 23: A) Gel retardation assay was performed on extracts of HEK 293 T cells transfected with plasmids encoding BAP-MHC-Iα, cyt-BirA and one of the following proteins: US2, US11 or an irrelevant protein (pcDNA3 mock). Cellular lysates were incubated or not with streptavidin and loaded on SDS-PAGE. Western blotting was performed using anti-SV5 mAb.
B) BAP-MHC-Iα biotinylation was compared between cotransfection with US11 or with the US11 mutant, US11Q 192 L
C) S^{35} pulse-chase experiment. HEK 293 T cells well labeled with [^{35}S]-Methionine and immunoprecipitated with mAb anti-SV5. Immunoprecipitated samples were incubated or not with streptavidin and loaded on SDS-PAGE.

As a consequence of retro-translocation of BAP-MHC-Iα induced by US2 or US11, dislocated molecules become substrate of the proteasome.

The proteasome inhibitor MG132 was used to further confirm that biotinylation takes place only on dislocated molecules. Up to now retro-translocation of BAP-MHC-Iα molecules has been studied through the detection of deglycosylated molecules in the cytosol when the proteasome is inhibited [235].

As shown in figures 24, when coexpressed with either US2 (24A) or US11 (24B) BAP-MHC-Iα degradation was reduced and the amount of biotinylated (retro-translocated) molecules clearly increased.

In our cells, very few deglycosylated molecules accumulated after proteasome inhibition compared to previous works [235], in particular when coexpressed with US11 (Figure 24A), while these molecules are more represented in the presence of US2 (Figure 24B).

As expected for cytosolic localised proteins, practically all deglycosylated MHC-Iα molecules get biotinylated, but also a significant amount of non-deglycosylated ones was also biotinylated.

After proteasome inhibitor treatment the biotinylation of BAP-MHC-Iα molecules increased in all cases, when expressed with US2 or US11 but also when coexpressed with an irrelevant protein.

This suggests that proteasome inhibition caused an increase in spontaneous retro-translocation rate, possibly through the activation of the UPR, which increases the expression of proteins involved in the retro-translocation and therefore would increase the efficiency of the process.

As concluding remark the analysis of retro-translocated molecules by *in vivo* biotinylation allowed us to detect also biotinylated molecules that bear a normal glycosylation, confirming its higher efficiency and the possibility to detect retro-translocation in the absence of proteasome inhibitor.

This result also indicates that deglycosylation takes place after dislocation and is the rate limiting step. In other words, as soon as deglycosylated, dislocated molecules are rapidly degraded by the proteasome and can accumulate only upon inhibition of the proteasome.

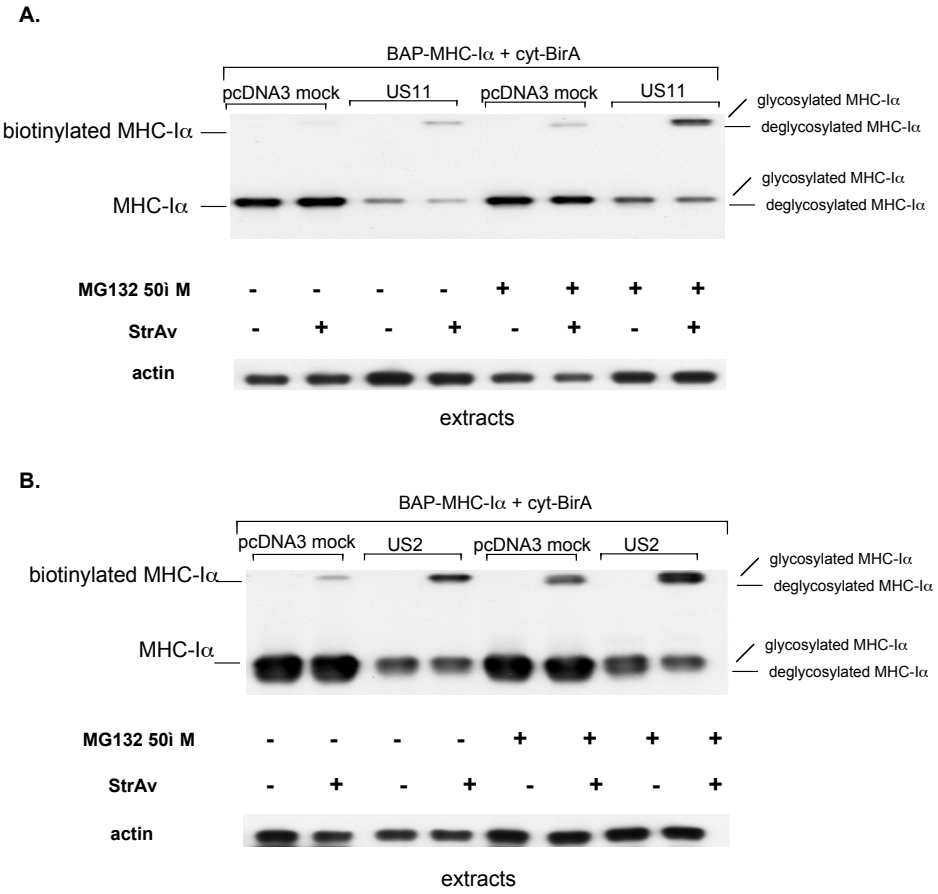

Figure 24: HEK 293 T cells were transfected with plasmids encoding BAP-MHC-Iα, cyt-BirA either with US2 (A) or with US11 (B) or with pcDNA3 mock. Cellular lysates were incubated or not with streptavidin and loaded on SDS-PAGE. MHC-Iα detection was performed using anti-SV5 mAb. As loading control anti-actin antibody was used.

Quantification of BAP-MHC-Iα retro-translocated molecules

One important aspect of our new method to detect retro-translocated molecules is that it allows the quantification in a precise and easy way.

Quantification was performed by ELISA as shown in figure 25A; BAP-MHC-Iα molecules were captured with mAb anti-SV5, and biotinylated molecules were detected using HRP-conjugated streptavidin.

As shown in histogram of figure 25B the amount of BAP-MHC-Iα biotinylated molecules was much higher in the presence of US2 or US11 compared to pcDNA3 mock transfection.

Proteasome inhibitor treatment caused an increase of biotinylated BAP-MHC-Iα molecules amount, either when coexpressed with US2 or US11, or with an irrelevant protein (pcDNA3 mock).

This suggests, in agreement with Western blotting results, an increased rate of retro-translocation due to UPR activation caused by proteasome inhibition, which leads a higher amount of BAP-MHC-Iα to reach the cytosol where it gets biotinylated.

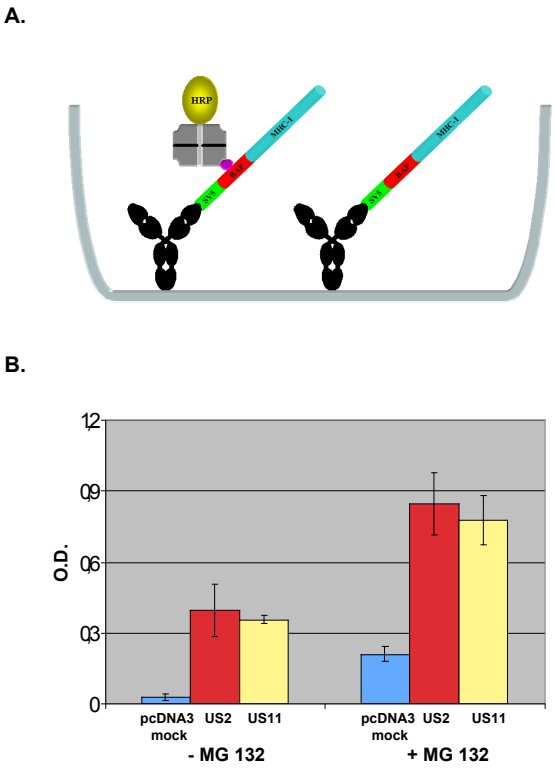

Figure 25: A) Schematic illustration of ELISA assay to detect biotinylated BAP-MHC-Iα molecules B) ELISA assay performed in triplicate and O.D: measurements were plotted in a histogram.

Degradins induce retro-translocation of target substrates

Since the *in vivo* biotinylation system demonstrated to be an important tool to monitor retro-translocation we wanted to exploit the method to study the mechanism of degradation induced by degradins.

For this purpose we used sα-SV5-BAP, a soluble version of FcεRI-α chain in which an SV5 tag and a BAP peptide were added at the C-terminus (αD1D2-SV5-BAP [330]; Figure 26A).

As shown in figure 26B, and similarly to what observed for sdα, when coexpressed with scFv^{9E1}-degradin only a small portion of sα-SV5-BAP could reach the extracellular medium and when coexpressed with scFv^{9E1}-SEL1LΔ the inhibition of secretion was almost complete.

Interestingly, as shown in figure 26C, all the intracellular sα-SV5-BAP present in cells expressing the irrelevant-degradin or scFv^{9E1}-degradin was biotinylated, indicating their cytosolic localisation. However, the intracellular material found with the irr-degradin represents only a small fraction since most of it was secreted suggesting an active role of retro-translocation and degradation.

On the other hand, the scFv^{9E1}-SEL1LΔ, massively retained sα-SV5-BAP in the ER, with only a small fraction getting biotinylated, thus confirming the important role of 402-773 SEL1L residues for the retro-translocation process. Of note, biotinylation was total for the deglycosylated sα-SV5-BAP and only partial (about 20%) for the glycosylated molecules.

While more than 90% of sα-SV5-BAP molecules are already deglycosylated when coexpressed with scFv^{9E1}-degradin only a small fraction of these molecules was shuttled to the cytosol and deglycosylated when coexpressed with scFv^{9E1}-SEL1LΔ and, as expected all deglycosylated molecules get biotinylated.

In addition is noteworthy the inability of the secreted sα-SV5-BAP molecules to get biotinylated by cyt-BirA.

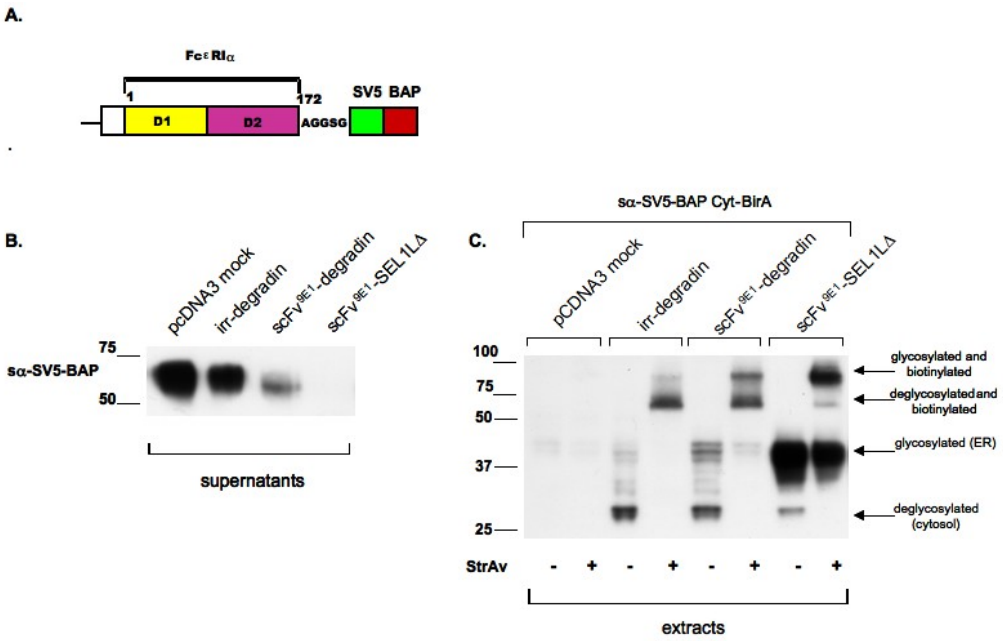

Figure 26: A) Schematic representation of sα-SV5-BAP, the FcεRI-α chain soluble version with an SV5 tag and a BAP at the C-terminus.
B-C) *In vivo* biotinylation was performed on an SV5-BAP version of sdα: sα-SV5-BAP to study retro-translocation induced by scFv^{9E1}-degradin.
sα-SV5-BAP was expressed in HEK 293 T cells with cyt-BirA and the proteins reported on the figure. Secretion of sα-SV5-BAP was analysed on supernatants (B) and the biotinylation on cellular lysates (C). Lysates were therefore loaded on SDS-PAGE upon incubation in the presence or absence of streptavidin. sα-SV5-BAP detection was performed using anti-SV5 mAb (C).

In vivo biotinylation of sα-SV5-BAP was also performed with the L$^{Fc_\varepsilon}$-degradin.

As shown in figure 27A also sα-SV5-BAP secretion was almost completely blocked by L$^{Fc_\varepsilon}$-degradin and slightly impaired by L$^{Fc_\varepsilon}$-SEL1LΔ whereas, the deleted ligand degradin (L$^{Fc_{\varepsilon\Delta}}$-degradin) did not affect the secretion.

Intracellular biotinylation of sα-SV5-BAP takes place in a considerable manner when coexpressed with L$^{Fc_\varepsilon}$-degradin either on glycosylated or on deglycosylated molecules. When coexpressed with SEL1LΔ only a fraction of the total sα-SV5-BAP accumulated gets biotinylated.

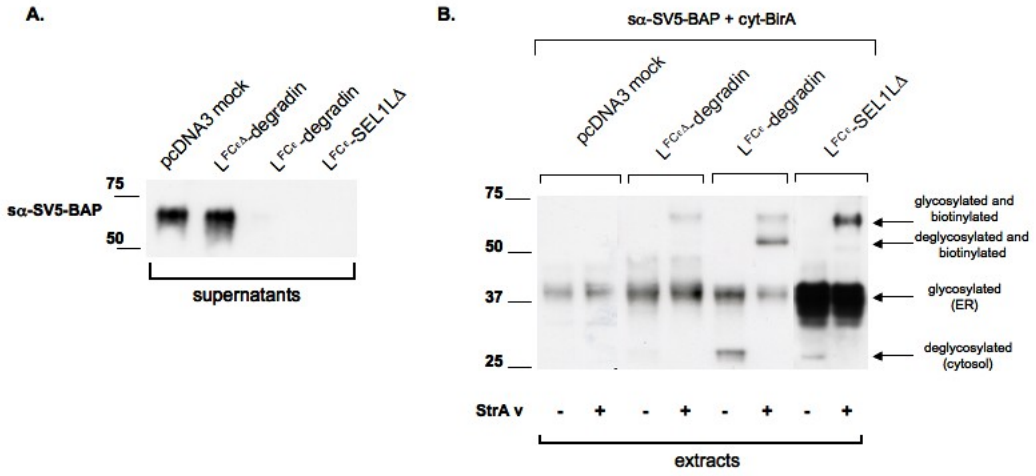

Figure 27: *In vivo* biotinylation was performed on sα-SV5-BAP to study retro-translocation induced by Ligand$^{Fc\varepsilon}$-degradin.
sα-SV5-BAP was expressed in HEK 293 T cells cyt-BirA and the proteins reported on the figure. sα-SV5-BAP secretion was analysed in the supernatants (A) whereas biotinylation was detected on cellular lysates (B). Lysates were loaded on SDS-PAGE upon incubation in the presence or absence of streptavidin. sα-SV5-BAP detection was performed using anti-SV5 mAb (B).

We then studied the effect of proteasome inhibition during ER-to-cytosol dislocation of sα-SV5-BAP induced by degradins.

For this purpose a coexpression of sα-SV5-BAP, cyt-BirA and degradins (irrelevant, scFv^{9E1}-degradin and L$^{Fc\varepsilon}$-degradin) was performed in HEK 293T cells, treated 16 hours after transfection cells were with 20 μM MG132 for 6 hours.

As shown in figure 28A the secretion of sα-SV5-BAP was strongly affected by both scFv^{9E1}-degradin and L$^{Fc\varepsilon}$-degradin.

Proteasome inhibitor treatment did not cause accumulation of sα-SV5-BAP in the extracellular medium neither when coexpressed with the irrelevant-degradin nor when coexpressed with the specific L$^{Fc\varepsilon}$-degradin.

This is consistent with the fact that, in the particular case of the soluble version of FcεRI-α chain, MG132 blocks degradation of already retro-translocated molecules not affecting dislocation itself.

Indeed upon MG132 treatment, accumulation of sα-SV5-BAP was observed intracellularly mainly in the deglycosylated form. As expected these deglycosylated molecules were completely biotinylated regardless whether cell were treated or not with MG132.

In contrast following MG132 treatment also the non-deglycosylated molecules present, get biotinylated while in the absence of MG132 only a fraction of them (Figure 28B).

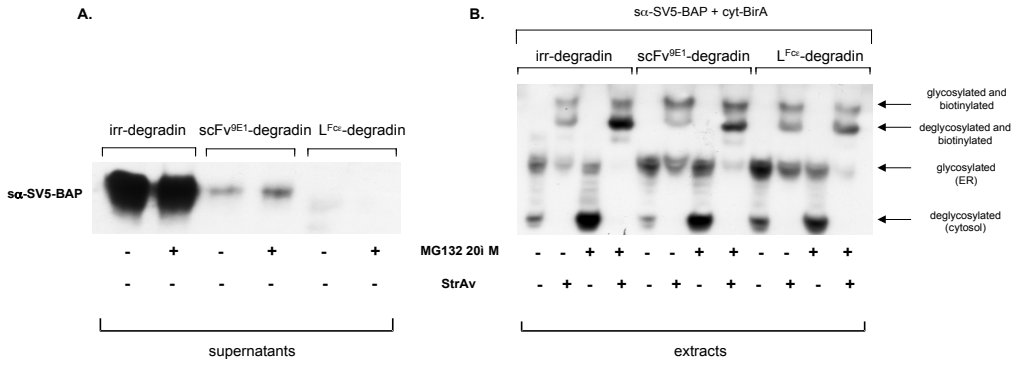

Figure 28: *In vivo* biotinylation was applied to study retro-translocation of target molecules induced by degradins, on an SV5-BAP version of sdα: sα-SV5-BAP during proteasome inhibitor treatment. sα-SV5-BAP secretion was tested by Western blotting on supernatants (A). Biotinylation was analysed on extracts of HEK 293 T cells treated and not treated with MG132 and incubated or not with streptavidin and analysed by Western blotting (B).

To conclude, the degradin system turned out to be a very specific and efficient method to induce degradation of particular targets. In order to induce specific degradation of a protein either antibody fragments or ligands can be used, but also the specificity of peptides for particular targets could be exploited.

As expected target degradation induced by degradins occurs through the retro-translocation of the target from the ER to the cytosol and through the ubiquitin-proteasome machinery.

DISCUSSION

The degradin system

In the present thesis a new system to induce knock-out of proteins within the secretory pathway is presented. It allows to specifically induce degradation of particular targets within the ER.

This protein knock-out system is based on the activity of molecules, named degradins, that result from the fusion between two different protein moieties, one with target recognition activity and the other one with degradative activity. The target recognition moiety could be represented by whichever target specific binding peptide, antibody fragment, ligands or receptors but could be also a partner protein that specifically interacts with the target.

To induce degradation of the target through the proteasome machinery I wanted to exploit the activity of proteins involved in ERAD [344] and in particular those proteins that create a link between substrate recognition within the lumen of the ER and retro-translocation.

The protein selected was SEL1L, involved in substrate recruitment to the ubiquitin ligase HRD1 [179].

SEL1L has been defined as an adaptor protein that is able to recruit the substrate and to bind both the ubiquitin ligase HRD1 and the putative retro-translocation channel Derlin-1. Thus, SEL1L creates a link between recognition of the substrate and its retro-translocation.

By multiple alignment of SEL1L sequence with those of its homologues from yeast and *C. elegans*, Hrd3p and SEL1, we identified a C-terminal luminal portion of SEL1L sharing similarities with the Hrd3p and SEL1 luminal regions described to interact with the ERAD machinery; the region 402-end portion of SEL1L was therefore chosen as the degradative moiety.

The choice of this SEL1L fragment was afterwards supported by the demonstration of the essential role of 373-end SEL1L fragment during ERAD [183]. A fine mapping of the SEL1L region essential for protein degradation could lead to the definition of a smaller degradative moiety of degradins.

SEL1L contains several domains: among them, the proline rich and the Hrd3p-like motifs, whose essential role during ERAD has been already demonstrated [183], are

maintained in degradins. The fibronectin type II domain on the contrary is missing in degradins and it has been already demonstrated to be dispensable for SEL1L activity during ERAD. It has been proposed to bind to chaperones or misfolded proteins or alternatively it is involved in the recruitment of other target recognition proteins such as EDEM or OS-9 [181].

SEL1L contains in addition several domains: the SEL1-like repeats (TPR repeats). The TPR domain is arranged in antiparallel amphipatic α-helices that help proteins interaction and multiprotein complexes assembly [182], in fact it has been associated with protein-protein interaction and in the recruitment of proteins in several processes. Degradins contain an almost complete SEL1-like repeats (TPR) cluster II and a complete TPR cluster III and, through the addition of a target recognition moiety, they are able to induce both dislocation and proteasome-dependent degradation of the target.

Approach for testing the activity of degradins

To validate the degradin system we chose two different target models; the first one was represented by the FcεRI-α chain and gave us the opportunity to test the system either on a soluble or on a membrane version of the same molecule; in addition it could be targeted by two types of binding moiety, the ligand (IgE) or a specific scFv. The second model was the TGEV spike protein S, that allowed a direct analysis of degradin activity on a biological property, in this case viral infectivity.

The choice of FcεRI-α chain gave me the possibility of testing degradins on two minimal receptors constituted by the extracellular D1 and D2 domains of FcεRI-α chain in a secreted (sdα [332]) or membrane form (mdα, Vangelista, Cesco Gaspere unpublished work) and was facilitated by the availability in the lab of mAb 9E1 specific for FcεRI-α chain and its derived scFv^{9E1}, as well as different versions of the ligand, IgE.

The possibility to test degradin activity on the same molecule with or without a membrane anchor, allowed me to validate the system for any kind of molecule in transit through the ER, whether secreted or membrane bound, and to analyse if

there were differences between the behaviours of soluble and membrane proteins during degradin-induced degradation or during the ERAD pathway in general.
By exploiting the well documented activity of the αSIP-6A.C3 (derived from the neutralizing anti-S protein mAb 6A.C3) in inhibiting TGEV infective particles formation [325], the TGEV S protein was also a good candidate to validate the degradin system in blocking at the intracellular level the formation of infective viral particles.
Using as recognition moieties the scFv^{9E1} and the truncated Fcε version (domains εCH3 and εCH4) it was possible to obtain two different degradins specific for FcεRI-α chain (scFv^{9E1}-degradin and L$^{Fc_\varepsilon}$-degradin), whereas for the anti-S degradin the construct was based on the scFv$^{6A.C3}$ (scFv$^{6A.C3}$-degradin).
Both scFv^{9E1}-degradin and L$^{Fc_\varepsilon}$-degradin were well expressed and, as verified by cytofluorimetric analysis, localised intracellularly.
The presence in the SEL1L sequence of two sites of N-glycosylation at positions 411 and 587 maintained in the SEL1L 402-end fragment of degradins allowed to perform an endoglycosydase treatment to study their localisation. The sensitivity to endoglycosydase H treatment indicated that degradins were primarily localised in the ER compartment, without reaching the Golgi. This complete ER localisation of degradins is likely due to signals localised in the transmembrane and cytosolic portions (residues 717 to 773) of SEL1L (http://www.uniprot.org/uniprot/Q9UBV2.html). This signal is also conserved in the degradin versions completely lacking the luminal region of SEL1L (SEL1LΔ), consistently with the effect of target retention at the ER level obtained by both scFv^{9E1}-SEL1LΔ and L$^{Fc_\varepsilon}$-SEL1LΔ.

The activity of degradins is target specific

By testing the activity scFv^{9E1} and L$^{Fc_\varepsilon}$-degradins on sdα and mdα expression, I could show that both degradins induced efficiently a decrease of either soluble or membrane proteins in transit through the ER, blocking almost completely their secretion or membrane expression with no intracellular retention.
The degradin activity was dependent on the specific interaction with the target and the recognition moiety; both irrelevant-degradin and L$^{Fc_{\varepsilon\Delta}}$-degradin did not block

either sdα secretion or mdα expression at the cell surface confirming the specificity of the system.
In the case of the irrelevant-degradin only a slight decrease in both mdα and sdα expression was observed. It is possible that this was the consequence of the residual activity of the SEL1L 402-end fragment in recognising misfolded proteins In this context the decrease observed could be due to the enhanced degradation of the fraction of molecules that were present in a misfolded state and that were not completely recognised by the ERAD machinery in conditions of proteins overexpression. In fact a clear difference between irrelevant and specific degradins was always visualised.
Alternatively, the SEL1L 402-end fragment could induce, by sequestering SEL1L ER partners, an UPR response that would increase misfolded protein recognition and inhibit protein translation.

The experiments with cycloheximide and [^{35}]S-Methionine pulse-chase experiments suggest a decreased stability of mdα when expressed with the specific scFv^{9E1}-degradin. Therefore it is likely that degradins are able to actively induce specific target degradation upon substrate recruitment by their recognition moiety.

In addition the stability of degradins in the presence or absence of the target was investigated as well.
Degradins expression was only slightly affected by the presence of the target, indicating that degradin upon recognition of the target is in a small fraction degraded it-self. Nevertheless, degradins persistence seems to be higher than its target suggesting the possibility to act on several target molecules before getting degraded.

Degradation versus retention induced by degradins

When compared to the intrabody bearing a KDEL signal (scFv^{9E1}-KDEL) degradins demonstrated higher efficiency in inhibiting sdα secretion. The effect of incomplete retention of sdα by scFv^{9E1}-KDEL could be explained by the presence

of scFv^{9E1}-KDEL also in the supernatants; this could reflect an oversaturation of the KDEL receptor. Therefore, the excess scFv^{9E1}-KDEL expressed would be free to proceed to the secretory pathway and to reach the extracellular medium.
The scFv^{9E1}-KDEL as well as the two constructs that produce retention (scFv^{9E1}-SEL1LΔ and L$^{Fc_\varepsilon}$-SEL1LΔ), displayed a modest degradative activity on both sdα and mdα when compared to the specific degradins (scFv^{9E1}-degradin and L$^{Fc_\varepsilon}$-degradin). This modest degradative activity could be the consequence of the induction of ER stress due to the overload of proteins in the ER (in this case the target proteins) which cannot be secreted or expressed on the cell surface but, instead, remain largely accumulated in the ER compartment.
Using SEL1LΔ degradins the essential role of the SEL1L moiety in the induction of degradation was further confirmed.
Therefore, degradins act, as expected, through the specific recognition of the target molecule and by inducing its degradation through the SEL1L moiety.
Although there are some cases in which a KDEL intrabody could be sufficient to efficiently inhibit the target protein expression (as obtained for TGEV S protein, see ahead), in other cases they produce ER retention, while degradins do not.
By producing a stable transfectant of scFv^{9E1}-degradin in a cell line already expressing mdα, it was possible to obtain a continuous protein knock-out system, stable in time.
This result was important because it confirmed that the effect in decreasing target expression was neither correlated to cell viability, nor to the altered expression of one of the two transiently cotransfected plasmids.

Degradation induced by degradins is achieved through the proteasome machinery

The involvement of the proteasome in the degradin-induced degradation was confirmed by the experiments performed in the presence of proteasome inhibitors. During MG132 treatment a good but not complete rescue of target protein expression was observed. This result indicated the involvement of the proteasome, at least in part, in the degradation of the target, but did not exclude the possible involvement of autophagy. In particular autophagy can be activated

during conditions of ER stress caused by proteasome inhibition. Autophagy induction could therefore allow a partial degradation of ERAD substrates with a compensatory effect that the cell exploits to avoid death [342].

The proteasome inhibition led to an increase of both partially and Golgi-dependent glycosylated mdα molecules. The completely glycosylated mdα molecules possibly originate following accumulation caused by MG132 treatment. Once accumulated, a fraction of mdα molecules not involved in any binding, would be released to the secretory pathway and therefore free to acquire a Golgi dependent glycosylation. On the contrary, only deglycosylated FcεRI-α chain soluble molecules that have been likely already retro-translocated to the cytosol accumulated during MG132 treatment. Deglycosylated FcεRI-α chain soluble molecules are very poorly represented in the absence of proteasome inhibitors, most likely because they are immediately degraded.

This notion was confirmed using the SV5 tagged soluble version of FcεRI-α chain that could be very efficiently detected using the anti-SV5 antibody. On the contrary, using sdα, the detection of these molecules could not be achieved because of the low sensitivity of mAb 9E1 in recognising FcεRI-α deglycosylated molecules.

Cytosolic deglycosylation is a step that takes place before the entry of the substrate within the proteolytic chamber of the proteasome and is required for an efficient degradation upon substrate dislocation from the ER to the cytosol.

Soluble proteins, probably because free from any kind of membrane anchor or constraint, seem to be dislocated to the cytosol very fast and, once in the cytosol, immediately deglycosylated and degraded, thus explaining why they were the only kind of molecule that accumulate during proteasome inhibition. The retro-translocation process is still functional during proteasome inhibition and leads to the dislocation of the soluble SV5 tagged FcεRI-α chain molecules in the cytosol. Since no proteasomal degradation takes place, the dislocated deglycosylated molecules accumulate.

Consistent with previous reports the rapid degradation of deglycosylated molecules explains why their visualisation was only possible upon inhibition of the proteasome [194,270].

The finding that glycosylated mdα molecules (but not sdα) increased during proteasome inhibition may suggest that in the particular case of FcεRI-α, the membrane version (mdα) requires a functional proteasome for its extraction from the ER membrane whereas the soluble one (sdα) does not. This result also suggests different mechanisms operating for membrane and secretory proteins.. This hypothesis is supported by previous works in which it has been demonstrated that membrane proteins, for example MHC class II molecules, require an intact proteasome for their dislocation to the cytosol [345,346].

Degradins and their ability in inhibiting TGEV infective particles formation

Very promising results were obtained with the experiments on the TGEV virus, which represents the second model used to test our degradin knock-out technology.

Since the absolute requirement of the TGEV spike protein S for virus infection was already demonstrated, we focused on a degradin containing a target recognition moiety specific for the TGEV S protein derived from a well characterised mAb (6A.C3).

I observed that in the TGEV S protein model the scFv$^{6A.C3}$-degradin and the intrabody scFv$^{6A.C3}$-KDEL were equally efficient in inhibiting S protein expression and infective viral particles formation.

This inhibition, in contrast to what previously described [325], did not imply extracellular neutralization of infection but impaired the intracellular assembly of infective particles.

The use of TGEV spike protein S as a model highlights the possibility to use degradins to directly influence a biological property such as viral infectivity. Moreover, these experiments suggested a wider applicability of degradins confirming their ability to work in cells constitutively expressing them and suggesting their possible use *in vivo*.

The intracellular inhibition of infective viral particles formation could represent an important application of the degradin system opening the possibility to induce degradation of proteins essential for viral infectivity in other systems. For instance,

degradins could prove useful to block virus spreading in chronic infections such as HIV or HCV. HIV infected patients are characterised by very long lasting infections in which the morbidity is caused by the immunodeficiency arising from $CD4^+$ T cells depletion. Thus degradins could avoid infection of the residual fraction of $CD4^+$ T cells blocking the onset of the immunodeficiency.

Comparison between the degradin technology and other knock-out systems

From all these evidences we can therefore draw the conclusion that the degradin protein knock-out technology can be applied on targets in transit through the ER or ER resident, against which a target recognition moiety is available. The pathway of degradation induced by degradins exploits at least in part the activity of the proteasome, although the participation of autophagy was not investigated.
However, independently of the degradative pathway, the degradin system represents a novel tool for the induction of specific target proteins degradation with some interesting features.
It acts at the protein level; it makes possible to use antibodies that confer very high specificity but also ligands, peptides whichever antibody fragment or specific interacting partner able to specifically recognise the target, theoretically making possible degradin application to a wide number of targets.
The interesting aspect of degradins reside in their easy manipulation; it could be possible to construct degradins with multiple copies of the same target recognition moiety or with different target recognition moieties against the same protein, in order to increase even more the efficiency of the system.
At the same time, recognition moieties with different specificities could be used in order to degrade proteins acting in different steps of the same pathway and therefore efficiently inhibiting that pathway. Using different recognition moieties, the degradation of a multiprotein complex could be achieved.
In this context degradins with two different scFv moieties fused through a linker (bispecific scFv) could be used.
Through a careful choice of the immunogenic peptide, the production of antibodies either highly specific for the target or directed against a domain conserved between different proteins could be obtained. Depending on the purpose the knock-out of several proteins of a particular family could be required, for example

with the aim of inhibiting a process carried out by proteins that share a redundant function.

Up to now, several methods have been developed to induce specific protein knock-out: among others, they include intracellular antibodies and RNA interference.

Antibody usage at intracellular level has been favoured by the development of recombinant proteins based on the use of antibody fragments such as the scFv [302].

They have been particularly useful in the development of techniques to isolate those antibodies that are able to fold also in cytosolic reducing conditions [310,311,347,348].

Since intrabodies can be further modified in order induce a particular cellular localisation, the inhibition of the target can be achieved either by directly neutralizing the target activity or by diverting the target into a different cellular compartment, blocking its secretion or membrane expression [349,350]. In some cases, however target relocalisation using intrabodies was not achieved; for instance coexpression of the target and the intrabody, harboring a signal for a particular intracellular localisation, can cause a relocalisation of the intrabody at the site of target expression and not *vice versa* [351].

Intrabodies could be very useful for the recognition of proteins with aberrant conformations since it is possible to obtain antibodies specific for a particular conformation.

For example, they have been successfully used against the amyloid precursor protein (APP) involved in Alzheimer disease avoiding its appearance on the cell surface [352], against PrP^{c} protein, by avoiding its conversion to the PrP^{sc} aberrant conformation [353] and against the mutated Huntingtin protein in Huntington disease, by reducing aggregates formation [354].

The degradin system offers the great advantage of actively degrade the target, no intracellular accumulation or target relocalisation are induced thus avoiding the possible formation of toxic aggregates within the cell.

In the latest years much interest has been raised by the RNA interference technology. This technology efficiently induces protein knock out acting at the

mRNA level through a 19-21 base pair long double strand RNA (dsRNA), which leads, through its association with the RISC complex, to target mRNA degradation [355].

Nevertheless, the presence of off-target effects could be considerably high due to the possible redundancy of the target mRNA sequence. Additional limits of the RNA interference technology reside in the inability in discriminating between different conformations of the same protein and the inability to act on proteins already expressed or, on proteins that come in contact with the cell from the external environment, such as bacterial proteins and toxins. Another disadvantage emerges in situations where the target protein originates from a single nucleotide mutation; in this case the RNA interference should act at the site of the mutation which could be not accessible by the RISC complex and in addition it could not efficiently discriminate between the wild type and the mutated mRNA. In many of these cases degradins could instead be functional.

It is interesting to mention some protein knock-out systems that have been previously described. One of them was based on the construct of a fusion protein between a chemokine and the cytosolic fragment derived from the HIV protein Vpu. In this way specific degradation of the chemokine receptor was reported [356].

A different approach for degradation of cytosolic proteins has been described: the suicide (or silencing) intrabody technology (SIT). This approach exploits the TNF-dependent-degradation of the inhibitory protein IkBα, which has been fused to an intrabody specific for a particular target. The resulting suicide intrabody is degraded by the proteasome together with the degradation of its target [357].

A different strategy to obtain knock-out of cytosolic proteins was based on the activity of subunits of the cytosolic SCF ubiquitin ligase complex, named Fbox-containing proteins. These proteins act as adaptors between the substrate and the cytosolic ubiquitin ligase Skip1. The F-box containing proteins modified by the addition of a moiety specific for the target were able to induce degradation of the chosen target protein [358].

As mentioned above for intracellular antibodies an aspect that makes our system a very promising way to obtain specific protein knock-out is represented by the possibility to act on a particular protein conformation. A number of human diseases are caused by single point mutations that could be hardly discriminated

by specific siRNAs molecules, but that can deeply change protein conformation, which could be, instead specifically recognised by a monoclonal antibody.

The possibility to use conformational antibodies could confer to degradins the unique possibility to discriminate between aberrant and proper conformations, inducing degradation of only the aberrant one. Moreover, there are even cases where the aberrant conformation originates without the presence of point mutations such as in the Creutzfeld-Jacob disease caused by prions.

Proteins bearing an aberrant conformation can produce aggregates with toxic effects for the cells; neurodegenerative disorders are likely the most attracting class of protein aggregation-related diseases such as Alzheimer disease (AD), one of the most frequent disorders in aging population [359].

AD is accompanied by the formation of neuritic amyloid plaques, neurofibrillary tangles and neuropil threads. It involves the excessive formation of a product of the amyloid precursor protein (APP) metabolism: the amyloid β peptide 1-42 ($A_{\beta 1-42}$). This results in $A_{\beta 1-42}$ accumulation in the neuronal ER and at extracellular level [359,360]. Pathogenesis of AD seems to be indeed correlated with the conversion of natural monomer to toxic oligomers [361,362].

The Transmissible Spongiform Encephalopaties (TSE) class of diseases is another example of how conformational changes can subvert a normal phenotype to a pathological phenotype. TSEs are caused by a very peculiar infective agent, the prion, which is an autocatalitically replicating proteins [363] responsible of BSE, Creutzfeld-Jacob disease and Scrapie [364].

The protein involved is Prp^{c} that undergoes conformational modification by mechanisms so far poorly understood, giving rise to an insoluble version of the protein named Prp^{SC} .

There are reports that show the activity of the proteasome in clearing aggregated proteins, even inclusions, when the production of the abnormal protein is stopped [365]. Therefore, degradin technology could be used not only to avoid protein aggregation of newly synthesised proteins, but also in clearing pre-existing aggregates. Intrabodies, which have demonstrated to reduce protein aggregates, due to their lower efficiency in inhibiting protein expression could be less efficient than degradins on pre-existing aggregates. Thus theoretically, degradins could be used to obtain an improvement of the disease and not only to avoid its progression.

Other possible applications of the degradin system could include the inactivation of bacterial antigens in transit through the ER. Bacterial pathogens, such as "*Salmonella typhimurium"* and "*Chlamidia trachomatis*", are able to persist within the cell in intracellular vacuoles that protect the bacteria from the innate immune response and within these elements bacterial replication can occur [366,367]. Although vacuoles isolate the bacteria from cellular structures, there are proteins released by the pathogens that get into the cellular secretory pathway. Examples are the "*S. typhimurium*" Slrp protein, possibly involved in the interference with MHC-I peptide presentation, due to the induction of ER stress [368] and "*Chlamydia trachomatis"* proteins and lipids (MOMP, IncA and LPS) whose trafficking through the ER could induce ER stress as well [369].
In these cases the siRNA approach would not be useful, because the bacterial RNA does not enter the cell, while degradins act at the protein level and can therefore be efficient in contrasting the effects of bacterial infections.
Degradins could also be exploited to study particular phenotypes arising from the knock-out of specific proteins in cell cultures but also *in vivo* through the delivery of degradins encoded by viral vectors. In addition, they could be used to target proteins involved in metastatic behaviour of cancer cells, such as metalloproteinases or cathepsins, with the aim of blocking the metastatic transformation of tumours.

A further possible application of degradins could be the selective redirection of antigens to proteasome degradation in antigen presenting cells (APC), including dendritic cells, to increase presentation of antigen-derived peptides in order to activate antigen-specific cytotoxic CD8+ T-lymphocytes (CTL). CTLs are in fact activated by the interaction of the T cell receptor with the antigen-derived-peptides bound to MHC class I molecules on the surface of specialised antigen-presenting cells. Peptide loading on MHC-I requires antigen to be previously processed by the proteasome, that executes the initial antigen proteolytic degradation. The generated peptides are in turn transferred to the ER and loaded to class I MHC molecules.
The degradin model could be exploited to efficiently generate a cytotoxic CD8+ response against an antigen when a specific monoclonal antibody is available: an antigen-specific degradin would efficiently direct the antigen towards proteasome

degradation and generate peptides that can be loaded to MHC I molecules and activate a CD8+ response.

An antigen could also be artificially generated, even if a specific antibody is not available: A recombinant target for a degradin could be created by fusing the antigen to a different protein, against which is possible to create a specific degradin. Coexpression of the two constructs (target antigen and degradin) in the same APC cell, such as DCs, will lead to target degradation and efficient peptide presentation in association with MHC I molecules, thus inducing lymphocyte activation. The genetic constructs encoding the target and degradin could be administered to a patient by DNA vaccination, for example by biolistic gene transfer, which efficiently transfects dermal antigen presenting cells, such as dendritic cells and macrophages; a further level of control that these constructs are expressed specifically in antigen presenting cells could be achieved by having their expression controlled by a dendritic cell-specific promoter, such as that of CD11c.

In vivo biotinylation as a tool for the study of retro-translocation

Proteasome mediated degradation requires a step of retro-translocation from the ER to the cytosol: to get a better insight into the events, that following target recognition by degradins lead to proteasome degradation, a new method that allows identification of the retro-translocated molecules was developed.

The system is based on the specific in vivo biotinylation of the protein of interest, which is modified by the addition of a 15 aminoacids long biotin-acceptor-peptide (BAP peptide).

This peptide is specifically biotinylated by the *E. coli* biotin ligase BirA, whose expression could be directed to the cytosol of eukariotic cells (cyt-BirA). Moreover, as the specificity of cyt-BirA enzyme in biotinylating BAP containing molecules only in the cytosolic compartment was previously assessed ([330], Arnoldi, Predonzani, Burrone Unpublished work), we expected that as soon as an ERAD substrate would reach the cytosol, it would immediately become substrate of the biotin ligase cyt-BirA resulting in the covalent addition of a biotin to the unique lysine residue present in the BAP peptide.

This system was tested to detect retro-translocation of the human MHC-Iα which is recognised as a well established and widely used target of retro-translocation induced by two HCMV immunoevasins US2 and US11.

The in vivo biotinylation approach demonstrated to be a very efficient and specific system for the detection of retro-translocated molecules. With this system it was possible to detect a large amount of MHC-Iα molecules that get biotinylated, because of their dislocation to the cytosol, only in the presence of US2 and US11 and without proteasome inhibitors.

In previous studies retro-translocated MHC-Iα molecules were identified as deglycosylated cytosolic proteins [270]. In addition our *in vivo* biotinylation method was able to detect a large amount of MHC-Iα molecules that get biotinylated in the cytosol, and therefore have undergone retro-translocation, but that are still glycosylated. As expected, the few deglycosylated molecules detected in the presence of proteasome inhibitors were completely biotinylated due to their cytosolic localisation.

Thus, the *in vivo* biotinylation was not only able to detect all the deglycosylated molecules but also a large amount of glycosylated ones. We hypothesised that MHC-Iα glycosylated and biotinylated molecules could represent an intermediate state of retro-translocation; these molecules are probably still anchored to the ER membrane, but have already started to dislocate to the cytosol from their N-terminus with the N-glycans localised inside the ER lumen. According to this hypothesis, only the MHC-Iα completely retro-translocated to the cytosol were found fully deglycosylated (Petris, Vecchi, Burrone. Unpublished work).

The glycosylated and retro-translocated MHC-Iα molecules were undetectable using previous methods and this could have lead to an underestimation of the rate of MHC-Iα retro-translocated proteins.

Since deglycosylated molecules are immediately transferred to the proteasome for degradation, *in vivo* biotinylation enables the visualisation of the proportion of molecules in each step of the retro-translocation and degradation processes. This prompted us to propose that, on the other side, the extraction of proteins from the ER membrane is a rather slow process.

An interesting finding was that treatments with proteasome inhibitors led to the accumulation of retro-translocated/biotinylated molecules even in the absence of immunoevasins, as shown by Western blotting and ELISA assays.
The increase of retro-translocation/biotinylation during proteasome inhibition is likely caused by the activation of ER stress following the accumulation of misfolded proteins that, in turn, cause the UPR induction. The UPR, which has been already described to be activated during proteasome inhibition, is responsible of the expression of proteins involved either in protein folding, such as chaperones, and in ERAD, such as SEL1L and Derlin-1, in order to retro-translocate and degrade more efficiently misfolded proteins [184]. Also a more efficient retro-translocation could be the cause of the increased proportion of biotinylated and deglycosylated molecules accumulated in the cytosol.

All the results obtained with the *in vivo* biotinylation together with the finding that in the cytosolic fraction MHC-Iα molecules can be detected only in a deglycosylated status (Petris, Vecchi, Bestagno, Burrone. Unpublished work) the finding that also glycosylated MHC-Iα molecules were ubiquitinylated let us to confirm a previously proposed model for MHC-Iα retro-translocation, which could be possibly expanded to all single membrane span proteins [370].
As illustrated in figure 29, the idea is that once engaged by US2 or US11, MHC-Iα molecules start the retro-translocation process from their N-terminus, leading this portion, containing the BAP peptide to face the cytosol. The portion of the molecule containing the N-glycan would still be protected from the activity of the cytosolic PNGase because localised within the lumen of the ER.
As soon as the BAP peptide becomes accessible to the cyt-BirA enzyme it gets biotinylated. The resulting biotinylated molecules still bound to the ER are glycosylated, whereas after extraction from the ER membrane a complete deglycosylation can occur in the cytosol. Since deglycosylated molecules are rapidly degraded, their visualisation can be achieved only in the presence of proteasome inhibitors that block their rapid disappearance.

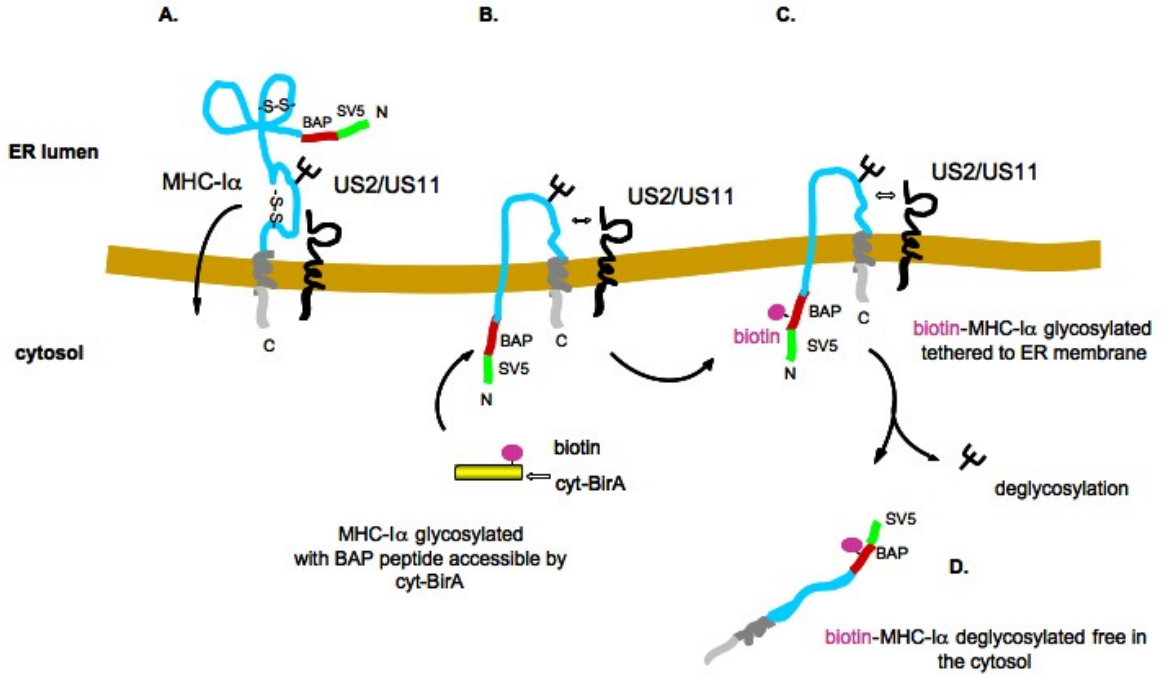

Figure 29: A) MHC-Iα molecules are engaged within the ER by US2/US11 immunoevasins B) Once engaged by US2/US11 SV5-BAP-MHC-Iα starts the retro-translocation process from the N-terminus C) BAP peptide can come in contact with cyt-BirA in the cytosol leading to the formation of partially dislocated MHC-Iα biotinylated molecules D) MHC-Iα can finally reach the cytosol (limiting step) where they are deglycosylated

Since the in vivo biotinylation was validated also on other ERAD models (Petris, Vecchi, Burrone unpublished work) we have used it in the study of retro-translocation induced by degradins.

For this purpose we used an engineered version of FcεRI-α chain containing a BAP peptide, sα-SV5-BAP [330].

When expressed with scFv^{9E1}-degradin and L$^{Fc_\varepsilon}$-degradin intracellular sα-SV5-BAP was biotinylated as visualised by the gel retardation assay (see figures 26 and 27 of results chapter).

On the other hand, when coexpressed without degradins sα-SV5-BAP was rapidly secreted and no retro-translocated/biotinylated molecules were visualised.

The irrelevant-degradin however, allowed sα-SV5-BAP to be secreted but, it also caused a small fraction of intracellular molecules to get biotinylated. The two specific scFv^{9E1} and L$^{Fc_\varepsilon}$ degradins induced very efficiently retro-translocation/biotinylation since the total amount of sα-SV5-BAP was much reduced.

The target specific degradins most likely induce retro-translocation/biotinylation of the target independently on its folding state, mediated by the activity of the target recognition moiety. Some residual activity of the SEL1L (402-end) fragment in the recognition of misfolded proteins could also be present as shown by the effect of the irrelevant-degradin. While the intracellular amount of sα-SV5-BAP coexpressed with the irrelevant-degradin probably reflects the percentage of misfolded molecules because almost completely retro-translocated/biotinylated, the secreted material instead represents the correctly folded fraction suggesting that the ERAD machinery is not sufficient in the recognition of misfolded protein during overexpression conditions.

This non complete efficiency of the ERAD machinery is supported by a previous work in which a fraction of the misfolded "null Hong Kong" truncated version of the secretory antitrypsin protein, normally non secreted and degraded by ERAD, can escape the ER quality control system and be secreted. However its secretion was completely blocked by overexpression of OS-9, a protein that in cooperation with SEL1L is involved in the recognition of misfolded protein [69].

Therefore, it is possible that the 402-end SEL1L fragment can supplement the endogenous ERAD machinery leading to a more efficient recognition of the misfolded proteins. To confirm this role of the 402-end SEL1L portion, overexpression of this fragment should be compared with that of the wt SEL1L protein. To support the hypothesis SEL1L overexpression should therefore cause a slight decrease in mdα and sdα expression, through a more efficient degradation of the misfolded fraction.

Alternatively, the effect visualised by the overexpression of the irrelevant degradin could be due to the induction of a UPR response as already decribed for the immunoevasin US11 [272].

Taken together all these data further confirm that degradins induce retro-translocation of the target molecule to the cytosol where it gets deglycosylated and degraded.

So far, just a few techniques have been described for the detection of retro-translocated molecules.

One is based on the subcellular fractionation that allows the detection of those proteins that after retro-translocation form the ER are localised in the cytosol. This technique has been very useful in the identification of deglycosylation as a step first demonstrated for MHC-Iα degradation, taking place in the cytosol before degradation [275].

Another method is based on the detection of retro-translocated molecules, by cytosolic fluorescent enrichment following ER to cytosol dislocation of a GFP-tagged protein into the cytosol [371].

Retro-translocation was also studied by looking at the decrease of fluorescence-labelled ERAD substrate that, when dislocated, can come in contact with a quencher agent and dislocation to the cytosol is measured as a decrease in fluorescence [202].

All these techniques however, were able to detect retro-translocated molecules only in the presence of proteasome inhibitors, which leads to protein accumulation, favouring the detection of retro-translocated molecules.

In vivo biotinylation offers a great advantage consisting in the possibility to easily detect retro-translocated molecules, independently of whether they are glycosylated or not, and without the use of proteasome inhibitor.

Through the possibility to discriminate and separate biotinylated proteins from the non-biotinylated ones, studies about the characteristics of retro-translocated molecules could be carried out.

The *in vivo* biotinylation turned out to be a very efficient and specific system; it can be exploited to study the events that bring a protein to the cytosol allowing the detection of all types of retro-translocated molecules. It is a simple and reproducible method that can be used to study the retro-translocation pathway but also to study the intrinsic tendency of a protein to misfold or the different tendency of the same protein to misfold in different tissues. In addition, since the BAP peptide is rather short it should not alter protein functions.

An interesting and unresolved question that *in vivo* biotinylation could help to understand regards the temporal organisation of retro-translocation and ubiquitinylation processes.

Up to now ubiquitinylation was seldom described to take place during retro-translocation event, even before extraction from the ER membrane. Although with the *in vivo* biotinylation we were able to detect a large fraction of MHC-Iα

molecules facing the cytosol during the retro-translocation process, but still attached to the ER membrane, no evidence of ubiquitinylation was found. The detection of ubiquitinylated molecules was not achieved probably because of the tight sequentiality of ubiquitinylation and deubiquitinylation processes. However, performing radioactive pulse-chase experiments it should be possible to detect the ubiquitinylated MHC-Iα molecules, still attached to the ER membrane that should get fully biotinylated.

With the *in vivo* biotinylation system it could be possible to study whether the presence of a transmembrane domain can influence both retro-translocation and ubiquitinylation processes.

Finally, since biotinylation allows the purification of retro-translocated molecules it can be exploited to study the modifications that take place in the cytosol and to identify partners associated with the retro-translocated molecules.

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