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V247M α-sarcoglycan mutant: uncovering the ERAD pathway of a type I membrane protein

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RIASSUNTO

Le proteine non correttamente ripiegate e/o mutate sono riconosciute dal sistema di controllo qualità del reticolo endoplasmatico (ER) (ER-QC) ed eliminate attraverso una specifica via, chiamata degradazione associata al reticolo (ERAD). La selezione avviene attraverso l'identificazione dei difetti strutturali da parte di specifiche proteine coinvolte nel controllo qualità del reticolo. La degradazione conseguente può essere suddivisa in tre vie: ERAD-L per proteine con difetti luminali, ERAD-M per difetti nella porzione di membrana ed ERAD-C in caso di difetti citosolici. Le E3 ubiquitin-ligasi risiedenti nel reticolo sono elementi chiave di ERAD e ciascuna sembra agire prevalentemente in una particolare via piuttosto che in un'altra, assicurando quindi lo specifico riconoscimento e smaltimento di proteine aventi difetti particolari, anche se non è ancora chiaro nei dettagli come ciò avvenga.

Nel muscolo, mutazioni a carico di proteine associate in un complesso tetramerico alla distrofina, chiamate sarcoglicani (αβγδ), causano nell'uomo distrofie muscolari chiamate distrofie dei cingoli. E' stato precedentemente dimostrato che il mutante di α-sarcoglicano V247M, una proteina di tipo I con un difetto luminale, viene ubiquitinato e degradato dal proteasoma, tappa finale di ERAD. Scopo di questo studio è indagare i componenti del sistema degradativo atti a riconoscere questa proteina come mutante e le E3 ligasi coinvolte nella sua ubiquitinazione, processo fondamentale per l'indirizzamento al proteasoma. Il fine ultimo è chiaramente quello di individuare possibili bersagli farmacologici per lo sviluppo di una possibile terapia, non presente attualmente. Numerose patologie dovute a problemi di ripiegamento/mutazioni di proteine stanno emergendo, e con esse anche possibili trattamenti farmacologici che agiscono sui pathways di ER-QC ed ERAD.

Nel reticolo le proteine malripiegate e/o mutate sono ritenute in soluzione da chaperoni e lectine, che giocano un ruolo indispensabile in ER-QC e nel delivery dei substrati ai complessi formati dalle E3 ligasi. Tra questi ho dimostrato che GRP94 è coinvolta nel riconoscimento di V247M α-SG, mentre BiP e OS9 non sembrano implicati, anche se ulteriori studi a questo riguardo risultano necessari. In letteratura i substrati modello di proteine di tipo I con difetti luminali vengono degradati grazie all'azione di due E3 ligasi, chiamate gp78 e HRD1. Attraverso l'uso di E3 ligasi mutate nel sito catalitico e di RNAi ho dimostrato che V247M α-SG è degradato grazie all'azione di HRD1 ma non di gp78. Mediante saggi di immunoprecipitazione, ho anche dimostrato che HRD1 interagisce strettamente con il sarcoglicano mutato così come UBC6e, l'ubiquitin-coniugasi partner di HRD1, e SEL1L, il recettore associato alla ligasi. Un'altra ligasi, RFP2, si è dimostrata in grado sia di interagire con il sarcoglicano mutato, sia di bloccarne la degradazione se deleta del sito catalitico coinvolto nell'ubiquitinazione. Per permettere a molte delle proteine di membrana di essere estratte dal reticolo e dirette al citosol, dove risiede il proteasoma, la AAA-ATPasi p97 sembra avere un ruolo fondamentale, grazie alla forza motrice fornita dall'idrolisi dell'ATP e il supporto dato da Derlina-1. Ho dimostrato, grazie all'uso di un dominante negativo, che l'azione di p97 è necessaria per la

retrotraslocazione di V247M α-SG e, grazie ad esperimenti di co-immunoprecipitazione, che il mutante interagisce strettamente sia con p97, sia con Derlina-1.

Questi risultati descrivono per la prima volta il pathway degradativo di α-sarcoglicano V247M, che, in qualità di proteina di membrana di tipo I con difetti nella porzione luminale, segue la via classica ERAD-L guidata dall'E3 ubiquitin-ligasi HRD1. Nel processo di riconoscimento e indirizzamento al proteasoma questo mutante è inoltre assistito dalla E3 ubiquitin-ligasi RFP2.

In un progetto in collaborazione con la Dr. R. Sacchetto (Dip. Scienze Sperimentali Veterinarie), mi sono occupata di un'altra proteina, SERCA1a. La proteina mutata è causa nell'uomo della miopatia di Brody e recentemente un fenotipo muscolare simile, chiamato Pseudomiotonia, è stato riscontrato anche in alcune vacche Italiane di razza Chianina che presentano la mutazione R164H a carico di SERCA1a. Sia nell'uomo che negli animali, la riduzione della attività della calcio ATPasi SERCA1a è correlata alla ridotta quantità di proteina presente nel muscolo.

L'espressione eterotopica di SERCA1a mi ha permesso di dimostrare che la proteina è degradata attraverso il proteasoma: l'inibizione dell'attività degradativa porta, infatti, a un aumento della quantità di proteina. Oltre ciò, studi funzionali mi hanno permesso di dimostrare che la proteina così recuperata è anche enzimaticamente attiva, indice del fatto che un suo recupero farmacologico potrebbe risultare efficace non solo nei casi di Pseudomiotonia ma anche in quelli di miopatia di Brody.

SUMMARY

Misfolded/mutated proteins are identified by the endoplasmic reticulum (ER) quality control system (ER-QC) and eliminated through the ER-associated protein degradation (ERAD) pathway. The presence of structural defects identifies these proteins as ERAD-L, -M, or -C substrates and results in selection of distinct degradation pathways. ER-associated E3 ubiquitin ligases are key components of the ERAD machinery, and distinct E3 ligases seem to control specific ERAD pathways.

In human muscles, mutations on sarcoglycans, proteins that form a tetramer complex (αβγδ) associated to dystrophin, lead to a Limb Girdle Muscolar Dystrophy (LGMD). It has been demonstrated that the V247M α-sarcoglycan mutant, a type I membrane protein with a luminal defect, is ubiquitinated and degraded by the proteasome, the last ERAD component. Aim of this project is to investigate ERAD components involved in recognition, ubiquitination and retrotraslocation of the mutated protein. The attention has been focused particullary on the E3 ligases that seem to be crucial factors assuring both selectivity and specificity to ERAD pathways. Emerging literature regarding several diseases involving mutated/misfolded proteins augurs well for treatments that involved common or peculiar ER-QC and ERAD steps. Due to the fact that until now, there is no known therapy for muscular dystrophies, I believe this study is relevant both to disclose an important biological process but also to identify possible molecular targets to treat sarcoglycanopathies.

A set of ER chaperones and lectins recognize and transport misfolded proteins to the site of dislocation. Among these, I have demonstrated that the chaperone GRP94 is probably involved in V247M mutant recognition, while BiP and OS9 do not seem to be implicated, however, their role is still under investigation. Usually gp78 and HRD1 are the E3 ligases involved in the ERAD-L pathway of model substrates. By using mutated variants of, or siRNAs for these E3 candidates, my results show that HRD1, but not gp78, is specifically involved in the disposal of the α-sarcoglycan mutant. In addition, the E2 ubiquitin-conjugase enzyme UBC6e and the cargo receptor SEL1L, well-known HRD1 partners, cooperate in the disposal of V247M α-SG. Immunoprecipitation experiments validated the interaction of HRD1, UBC6e and SEL1L with the α-SG mutant. Moreover another E3 ligase, RFP2, is involved in the degradation of V247M α-SG being able both to co-immunoprecipitate with the protein and to block its disposal, if a mutated variant is expressed. The driving force to eradicate misfolded membrane proteins from the ER is usually provided by the AAA-ATPase p97. My experiments demonstrated that p97, together with the ER-associated Derlin-1, form the so-called "dislocon", the proteinaceous environment for the hydrophilic luminal domain of V247M mutant to cross the ER membrane.

These results describe for the first time the ERAD pathway for the disposal of the type I membrane protein V247M α-sarcoglycan, a pathway leaded by the E3 ligase HRD1.

Moerover, recognition and delivery to proteasome of this mutant is also assisted by the E3 ubiquitin ligase RFP2.

In collaboration with Dr. R. Sacchetto (Dept. of Veterinary), I also carried out a study aimed to check whether the R164H mutant of the polytopic membrane protein SERCA1a is also an ERAD client. Mutations in SERCA1a are responsible for Brody Disease, a human inherited congenital disorder that affects skeletal muscles, because of SERCA1a loss of function. A similar muscular disorder, named congenital Pseudomyotonia, has been described in the Italian Chianina cattle. In affected animals and Brody's patients, decreased calcium ATPase activity perfectly correlates with reduced expression of SERCA1a protein. I demonstrate that inhibition of proteasome not only rescued the expression of R164H SERCA1a mutant transfected in HEK-293 cells, but also restored the enzymatic activity.

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INTRODUCTION

Dystrophin and dystrophin glycoprotein complex (DGC)

Dystrophin is coded by the longest gene in human genome: it is 2.4 million base pairs in size and takes over 16 hours to be transcribed and co-transcriptionally spliced. The result is a protein of about 427 kDa in size that represents about 0.002% of total striated muscle proteins (Hoffman et al., 1987; 1988). Dystrophin is, in fact, a major component of the plasma membrane cytoskeleton in muscle fibers that connects the intracellular side to the surrounding extracellular matrix. Dystrophin is composed by four distinct functional domains: the N- terminal actin binding domain; a central linear rod domain containing 24 spectrin-like repeats; a cysteine-rich domain involved in binding with the C-terminal of dystroglycan (DG) and a C-terminal domain that interacts with syntrophin (α1 and β1) and α-dystrobrevin. DG consists of a highly glycosylated extracellular alpha subunit (α-DG) and a transmembrane beta subunit (β-DG), on which the dystrophin binding occurs. Both are encoded by the gene Dag1 and generated by post-translational cleavage and processing. α-DG completes the link from the cytoskeleton to the basal lamina by binding a major component of the extracellular matrix, laminin α2, but also other proteins such as agrin and perlecan (Sandonà & Betto 2009; Michele & Campbell, 2003). Besides these proteins also a complex of four glycoproteins can be co-purify with dystrophin from muscle membranes, the sarcoglycans: γ-sarcoglycan (SG), δ-SG, β-SG, and α-SG (Nigro et al., 1996; Campbell & Kahl, 1989; Ervasti & Campbell, 1991; Ohlendieck et al., 1991; Ibraghimov-Beskrovnaya et al., 1992; Worton, 1994).

All these proteins form a sub-complex that associates to the previously described proteins in the complex related to dystrophin called dystrophin glycoprotein complex (DGC) (Figure 1). Therefore the DGC is a multimeric group of soluble, integral, membrane-associated proteins located in the sarcolemma of both cardiac and skeletal muscle fibers.

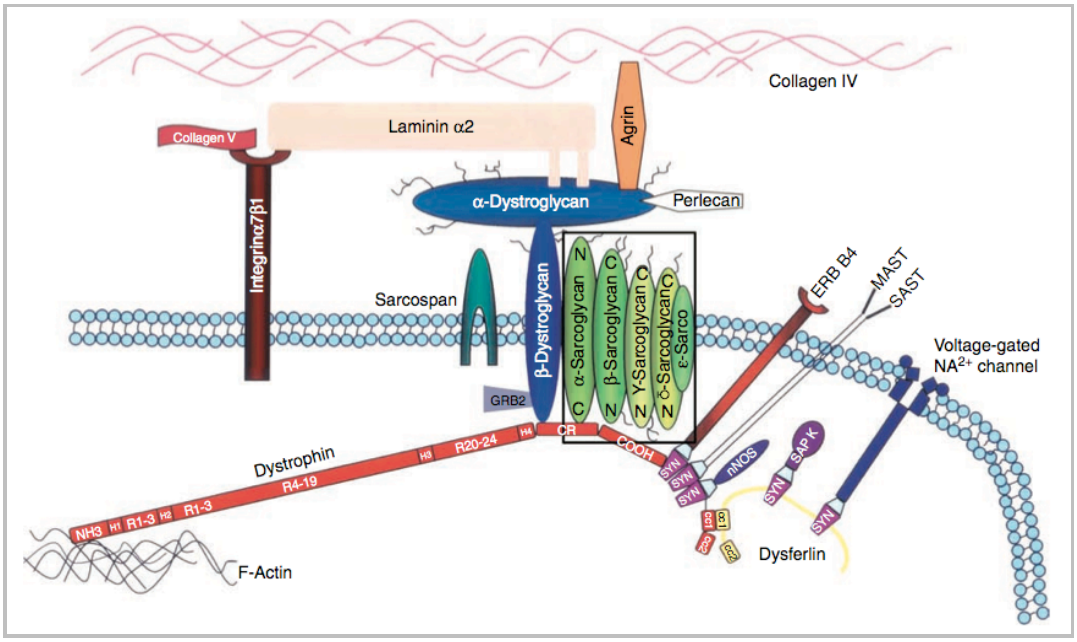


Figure 1. The dystrophin glycoprotein complex (Draviam et al., 2006).

The DGC is thought to provide structural support to the plasma membrane and protect it from the mechanical stress of contractile activity by transmitting the lateral tension generated by muscle contraction to the extracellular matrix (Sandonà & Betto, 2009). In fact, lengthening contractions cause an increase in membrane tension on the sarcolemma, which can lead to small tears in the membrane. The membrane repair mechanism subsequently reseals these membrane tears thus restoring the membrane integrity of myofibers. If the association of the sarcolemma to the basal lamina is disrupted or non present for any reasons, a small membrane tears caused by lengthening contractions expand, can lead to the loss of a large segment of membrane, and eventually to muscle-cell necrosis (Han et al., 2009). Thus, the presence of a whole DGC allows the basal lamina to prevent the sarcolemma from rupturing: muscle contraction will not lead to breakage if the yield strength of the adhesive (DGC) is high enough. A role of the DGC in signalling has also emerged from the identification of nNOS (neuronal nitric oxide synthase) associated via α-dystrobrevin (Bredt, 1999); in 2007, Chen et al. identified the 16 kDa subunit of vacuolar H^+-ATPase as a novel sarcoglycan-interacting protein; moreover, dystroglycan has direct as well as indirect roles in NMJ formation thanks to its binding with rapsyn, a protein with a crucial role in anchoring/stabilizing acetylcholine receptors at synaptic sites (Winder, 2001).

Perturbation of the DGC causes muscular dystrophy

Gene defects in almost all the components of the DGC are responsible for muscular dystrophy, thus reinforcing the fundamental role of the complex in striated muscle physiology. Muscular dystrophies (MD) are a group of degenerative muscle disorders characterized by progressive skeletal muscle weakness. They are due to genetic defects in muscle proteins that lead to progressive wasting. A group of MD refer to defects in the sarcoglycans, so they are collectively named sarcoglycanopathies.

Sarcoglycanopathies

These dystrophies are included for clinical characteristics to the large group of Limb-Girdle Muscular Dystrophies (LGMD). LGMD can be genetically subdivided into type 1, characterized by autosomal dominant inheritance, and type 2 due to heterogeneous autosomal recessive mutations. These dystrophies have a high variable age of onset and progression but the unifying theme is the initial involvement of shoulder and pelvic girdle muscles (in black in the side picture, www.mda.org) that may or may not be associated with cardiomyopathy (Durbeej & Campbell, 2002). The LGMD have been estimated to account for about 5% of dystrophies in patients with normal dystrophin staining. Patients have in fact a clinical presentation with symptoms including progressive proximal muscle weakness, calf hypertrophy, elevated serum creatine kinase (10-120 times the upper limit of normal) that could be due to defects in plasma membrane stability (Duggan & Hoffman, 1996). Sarcoglycanopathies are defined as LGMD type 2D, 2E, 2C and 2F with reference to mutations in α, β, γ- and δ-sarcoglycans, respectively. Importantly, a primary

mutation in any one of sarcoglycans leads often to a secondary deficiency of the entire sarcoglycan complex with variable consequences for the stability of the other remaining components at the cell membrane (Sandonà & Betto, 2009).

Sarcoglycans

Data from patients and related animal models demonstrate that sarcoglycans have a fundamental role at the muscle plasma membrane, but only when they form a complex. In fact they are organized as a tetrameric complex (α-β-γ-δ-SG), with a strict equimolar stoichiometry, present only in striated muscle (Jung et al., 1996). All sarcoglycans are single-pass transmembrane protein with a short intracellular tail and a large extracellular glycosylated portion rich in cysteines.

Six sarcoglycans have been cloned so far: α-, β-, γ-, δ-, ε- and ζ-sarcoglycan (table 1). α- and γ-sarcoglycan are expressed exclusively in skeletal and cardiac muscle, whereas ε-, β-, δ- and ζ-SG are more widely distributed. So, in striated muscles the principal tetrameric sarcoglycan complex is formed by α-β-γ-δ-SG, whereas in other tissues the tetramer is exclusively composed by ε-β-δ-ζ-SG (Sandonà & Betto, 2009).

Protein	Gene	Chromosome	Disease	AA	Mass[a]	N	C
α-Sarcoglycan	*SGCA*	17q21.33	LGMD-2D	387	50	2	5
β-Sarcoglycan	*SGCB*	4q11	LGMD-2E	318	43	3	5
γ-Sarcoglycan	*SGCG*	13q12	LGMD-2C	291	35	1	4
δ-Sarcoglycan	*SGCD*	5q33.3	LGMD-2F	290	35	3	4
ε-Sarcoglycan	*SGCE*	7q21.3	MDS	405	45	1	4
ζ-Sarcoglycan	*SGCZ*	8p22	Unknown	299	35	1	4

[a]Observed molecular mass (kDa) based on results from SDS-PAGE.
Abbreviations: LGMD, limb-girdle muscular dystrophy; MDS, Myoclonus–dystonia syndrome; AA, number of amino acid residues; N, number of putative N-linked glycosylation sites in the extracellular portion of the protein, according to http://www.uniprot.org; C, number of cysteine residues.

Table 1. Human sarcoglycans (Sandonà & Betto, 2009).

α, β, γ and δ-SG possess putative phosphorylation sites in the intracellular domain, indicating a possible post-translational modulation of proteins or complex. The cysteine cluster present in the extracellular domain of all sarcoglycans is predicted to be important for their tertiary structure and for the assembly of the whole complex (Chan et al., 1998). Cysteines in β, γ and δ-SG are in a fixed position similar to that of the epidermal growth factor EGF, suggesting a possible receptor-like function (Chen, 2006) and it has been demonstrated that β, γ and δ-SG contain intramolecular disulfide bonds (Chan et al., 1998). Despite the presence of the mRNAs of all SGs in myoblasts, neither the complex nor the single protein products accumulated. SG polypeptides can be detected only in trace in differentiating C2C12 cells on D4 (day 4 of differentiation) while on D8 all SGs are detected at the same intensity, consistent with the claim of a stoichiometric assembly of the complex (Noguchi et al., 1999; Hack et al., 2000). The following sequential events for sarcoglycan association have been proposed: β-SG seems to play a pivotal role by

interacting with δ-SG in the first step; thereafter, γ-SG is added to the β/δ-SG core and α-SG is recruited in the final step. The tetrameric complex is then targeted to the plasma membrane, in a process not yet completely understood (Chan et al., 1998; Shi et al., 2004). α-SG is the only sarcoglycan not requiring specific partners to translocate to the plasma membrane when expressed alone although not in a very stably way: it co-localizes, in fact, with clathrin and recycling endosomes. Its stability, instead, increases when associated with the other SGs (Draviam et al., 2006). Following, a short description of the four muscle SGs is provided.

α-SG, also called adhalin from the Arabic word for muscle "adhal", is a type I integral membrane protein of 50 kDa molecular weight, discovered by Roberds et al. in 1993. It is composed (Figure 2) by a N-terminal signal sequence, cleaved during co-translational translocation into endoplasmic reticulum, a large extracellular domain, one transmembrane domain from Leu-291 to Val-311 and a short C-terminal intracellular domain from Met-312 to His-387. It contains two consensus sites for N-linked glycosylation, Asn-174 and Asn-246, and two consensus sites for phosphorylation at Thr-336 and at Ser-377. It is an ecto-ATPase with an ATP binding sequence, conserved in all species, from Ala-220 to Asp-234 (Betto et al., 1999; Sandonà et al., 2004). The ecto-ATPase activity of α-SG requires both Ca^{2+} and Mg^{2+} and the requirement of Ca^{2+} is supported by the presence of a putative Ca^{2+}-binding site in the extracellular portion of the protein. In fact, sequence analysis identified the presence of a cadherin-like domain from Leu-28 to Leu-133 (Dickens et al., 2002; Sandonà et al., 2004). Moreover, recently it has been demonstrated that α-SG is required for FGF-dependent myogenic progenitor cells proliferation and differentiation (Cassano et al., 2011).

MAETLFWTPLLVVLLAGLGDTEAQQTTLHPLVGRVFVHTLDHETFLSLPEHVAVPPAVHITYHAHLQGHP
DLPRWLRYTQRSPHHPGFLYGSATPEDRGLQVIEVTAYNRDSFDTTRQRLVLEIGDPEGPLLPYQAEFLV
RSHDAEEVLPSTPASRFLSALGGLWEPGELQLLNVTSALDRGGRVPLPIEGRKEGVYIKVGSASPFSTCL
KMVASPDSHARCAQGQPPLLSCYDTLAPHFRVDWCNVTLVDKSVPEPADEVPTPGDGILEHDPFFCPPTE
APDRDFLVDALVTLLVPLLVALLLTLLLAYVMCCRREGRLKRDLATSDIQMVHHCTIHGNTEELRQMAAS
REVPRPLSTLPMFNVHTGERLPPRVDSAQVPLILDQH

Figure 2. Amino acid sequence of α-SG: in orange the signal peptide, in blue the cadherin-like domain (putative Ca^{2+} binding site), in light violet the two putative N-glycosylation sites, in red the ATP binding site, underlined in green the transmembrane sequence.

β-SG is a type II glycoprotein first reported by Ervasti et al. in 1990 as a 43 kDa protein associated to dystrophin. The short intracellular N-terminal domain is 65 amino acids long, the transmembrane region goes from I-66 to I-86 while the extracellular C-terminal comprehend 232 amino acids.

γ-SG is a type II glycoprotein that was also first reported by Ervasti et al. in 1990 as a 35 kDa protein that associate to dystrophin. The intracellular N-terminal tail is very short, only 36 amino acids long, its transmembrane region goes from Leu-38 to Leu-58 and other 233 amino acids form the extracellular C-terminal.

δ-SG is a type II glycoprotein that weights as γ-SG: when Ervasti discovered γ-SG, did not noticed that the band consisted out of two overlapping proteins, so δ-SG has been

identified lately by Nigro et al. in 1996. The very short intracellular N-terminal tail is 35 amino acids long, its transmembrane region goes from Phe-36 to Leu-56 and other 233 amino acids form the extracellular C-terminal. γ and δ-SG are closely related with 55% of identity and 70% of similarity (Ozawa et al., 2005).

Pathogenetic mechanisms of sarcoglycanopathies

The majority of sarcoglycanopathies is associated to missense mutations resulting in a codon that codes for a different amino acid, with a striking prevalence of mutations involving α-SG (Roberds et al., 1994; Fanin et al., 1997; Vainzof et al., 1999; www.dmd.nl). Besides missense mutations, also deletions, non-senses, insertions have been observed (Bönnemann et al., 1995; Piccolo et al., 1995; Carrié et al., 1997; Mendell et al., 2010; www.dmd.nl). It should be notices that all but one (M321R) missense mutation causing LGMD-2D, map in the extracellular domain of the protein. Among these, the most frequently reported, R77C, and five others map in the cadherin-like domain, the putative Ca^{2+}-binding site of the protein; other five mutations are located in the highly conserved putative ATP-binding domain. It has been demonstrated that the reduced expression of α-SG mutants is a post-translational event, because the mRNA level is unchanged (Carrié et al., 1997). This implies that all SG mutants (either those potentially affecting the enzymatic activity or those that apparently should not have functional implication) are recognized as incorrect/misfolded by the quality control of the cell and are prematurely disposed. This means that these altered SGs can be considered as processing mutants, and the associated disorders can be included in the group of Unfolded Protein Diseases (UPD).

Therapies to treat sarcoglycanopathies

There is no known therapy for any muscular dystrophies. The unique goal of treatments is to control symptoms, to maximize the patient's quality of life and to help patients to walk as long as possible. Steroids are often used to reduce inflammation and therefore slow down muscle wasting; physical activity is very recommended, while creatine supplementation gives some benefits (Tarnopolsky et al., 2004; Passaquin et al., 2002) however, until now, none of such treatments has been effective in cure dystrophies. Gene therapy, exon skipping and new chemical compounds could be potential therapeutic strategies as shown by emerging literature on the field (Bowles et al., 2011; Mendell et al. 2010; Sermet-Gaudelus et al., 2010; Cirak et al., 2011a; Tedesco et al., 2011; Cirak et al., 2011b; De Moor et al., 2011; Carre-Pierrat et al., 2011; Goyenvalle et al., 2011). Both gene and cell therapy strategies, devoted to express the wild type form of proteins, have been developed in rodent. However, significant challenges have to be overcome, in particular the delivery of the gene into such a large amount of muscles, and more work is needed before the use of such techniques in a clinical trial can be envisaged.

Pharmacological strategies aimed at “cure” the gene instead of replacing it should avoid the hurdles presented by gene- and cell-based therapy. Exon skipping and stop codon read through approaches, for example, are very promising in Duchenne Muscular

Dystrophy where a large part of defects are deletions or nonsense mutations (Hoffman et al, 2011). However, exon skipping is not appropriate for the cure of sarcoglycanopathies as the mutated proteins are short and don't have dispensable sequence that could be skipped away, like instead in dystrophin. Moreover the use of molecules able to promote stop codon read through is promising in dystrophies, however in sarcoglycanopathies it must take into account that the number of nonsense mutations is considerable small, compared to missense mutations (83% of LGMD-2D patients present amino acid substitution; www.dmd.nl).

Missense mutations or deletions may produce misfolded proteins that can provoke a peculiar 'loss of function': the protein is missing from its proper cellular site because removed by the cell's quality control system (QCS) and subsequently degraded principally by the ubiquitin-proteasome system. Unfortunately, the legitimate action of QCS may results in the disposal of proteins retaining, at least in part, their function. In fact, recently several independent groups demonstrate the therapeutic potential of proteasome inhibition in Duchenne Muscular Dystrophy and Becher Muscular Dystrophy (Gazzerro et al., 2010; Assereto S et al., 2005). Other groups have provided the evidence that is possible to rescue certain disease-causing SG mutants, either by inhibiting proteasome or by interfering with the QCS (Gastaldello et al., 2008; Bartoli et al., 2008; Soheili et al., 2011). These results suggest that the pharmacological retargeting of a "processing mutant", acting either on the substrate itself, on specific cellular factors or on the global folding environment of the cell (discuss in the next paragraph), may represent a new therapeutic way to treat sarcoglycanopathies.

Unfortunately, so far, nothing is known about how native or misfolded SGs are processed in the cell.

Processing of proteins in the endoplasmic reticulum (ER)

The capacity of cells to discriminate among functional and correctly folded protein and the misfolded ones, that should be recognized and destroyed, is of fundamental importance for the maintenance of cell homeostasis. Up to one third of newly synthesized proteins are secretory or membrane protein that are co-translationally translocated into the ER. Assisted by chaperones and modifying enzymes, folding of the polypeptide starts as soon as the chain emerges in the ER. Folding is however an error-prone and complex process in which genetic mutations, viral infections and cellular alteration (such as heat shock and oxidative stress), frequently cause the misfolding of newly synthesized or pre-existing proteins. Accumulation of such molecules is harmful for the cell because they can aggregate, impairing functionality of the ER, finally leading to amyloid fibril deposition, such as in Parkinson, Alzheimer or Huntington disease (Hoseki et al., 2010; Mehnert et al., 2010).

The success of the folding process in the ER is carefully monitored by an ER quality control mechanism that allows only correctly folded proteins to be transported to their final destination, whereas misfolded or unassembled proteins are retained in the ER and subsequently targeted to degradation: the decision-making process is termed "ER-quality

control" (ER-QC), the following process is known as "ER-associated degradation" (ERAD) (Ellgaard et al., 1999).

ER-QC and ERAD require the perfect coordination of three subcellular compartments: 1) the ER lumen, where ERAD substrates are recognized and delivered to retrotranslocation, 2) the ER lipid bilayer, where substrate retrotranslocation from the ER to the cytoplasm and tagging for degradation take place, and 3) the cytoplasm, where the final removal of the misfolded protein occurs. To all these events participate and cooperate a large number of proteins, almost all described and discovered in yeast and mammals in the last 15 years.

An alternative degradation route known to extract aggregates of misfolded proteins, requires the targeting of part of ER to the autophagy-lysosome system. Through the generation of double membrane vesicles that engulf these species with a portion of the surrounding environment, cells can deliver to degradation the entire content of these vesicles thanks to the fusion with lysosomes.

The ER has four major physiological functions: 1) it is the site of membrane and secretory protein synthesis; 2) it is the site where the majority of secretory and transmembrane proteins fold into their native conformation (they undergo post-translational modification and formation of disulfide bonds); 3) it is the place where the Ca^{2+} is stored; 4) the ER membrane is involved in the biosynthesis of lipids and sterols (Schröder, 2008). Perturbation of any of these functions might lead to ER stress and the Unfolded Protein Response (UPR), a finely regulated cascade of processes that slows down protein translation and increases the ability of the ER to handle misfolded proteins, but eventually, if unsuccessful, might lead to apoptosis.

Entering the ER and the folding process

Ribosomes are the ribonucleoprotein particles of the microsome fraction that synthetize polypeptide chains. Ribosomes are classified as being either free or membrane-bound. Secretory, membrane proteins but also proteins that need the formation of disulfide bonds for their folding, are co-translationally inserted into the ER, in which chaperones assist their folding and exclusive enzymes catalyze co- and post-translational modifications (Brodsky & Wojcikiewicz, 2009; Vembar & Brodsky, 2008). Usually, proteins to be inserted into the ER have a specific signal sequence, which is recognize in the cytoplasm by signal recognition particles (SRP). The SRP targets the ribosome-nascent polypeptide chain complex to the cytosolic face of the ER where the alignment between the ribosome exit tunnel and an aqueous channel, in ER membrane, occurs. Protein translocation is in fact mediated by an evolutionarily conserved hetero-trimeric protein-conducting aqueous channel called Sec61 (Park & Rapoport, 2011).

The first modification of newly synthetized type I proteins is the removal of the N-terminal signal sequence (by a signal peptidase) followed by the attachment of a $Glc_3Man_9GlcNAc_2$ unit (Glc: glucose; Man: mannose; GlcNAc: *N*-acetylglucosamine, Figure 3) on proteins having an Asn in the Asn-X-Ser/Thr glycosilation motif (X: any amino acid).

The ER is an oxidative compartment in which the disulphide bond formation, operated by protein disulphide isomerases (PDI), is facilitated; moreover it contains chaperones and enzymes that catalyze the proper folding of proteins (Schröder, 2008).

The oligosaccharide unit, attached to a newly-synthesized protein, is extensively modified by removal and addition of sugar residues in the ER and Golgi compartments (Helenius & Aebi, 2004).

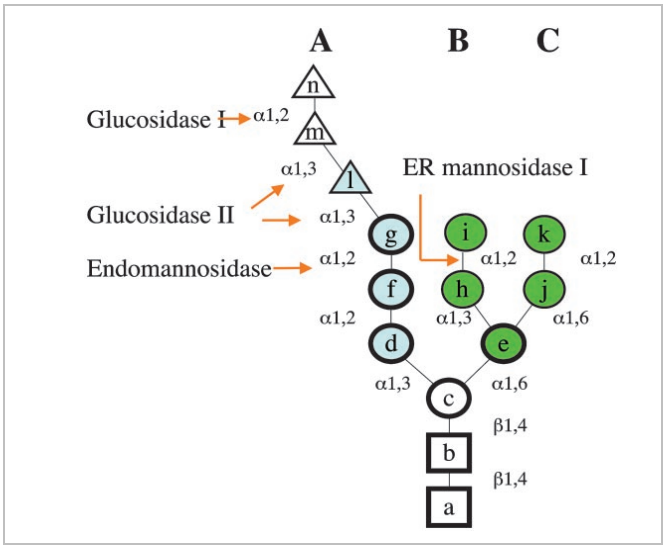


Figure 3. $Glc_3Man_9GlcNAc_2$ (G3M9), the *N*-linked core oligosaccharide added to a nascent polypeptide. A, B and C indicate the three branches of the oligosaccharide tree. Arrows indicate the cleavage sites of some glycosidases known to be involved in oligosaccharide trimming (Helenius & Aebi, 2004).

The attachment of G3M9 *N*-glycan is made by oligosaccharyltransferase (OST) and is followed by the subsequent removal of the two outer glucoses by glucosidase I and II. The resulting monoglucosylated *N*-glycans are recognized by ER-resident calnexin (CNX) and calreticulin (CRT), which promote folding of the glycoprotein recruiting a PDI (i.e., ERp57). It is important to point out that CNX is a membrane protein that binds substrates co-translationally, while CRT is a soluble protein that interacts primarily with secreted proteins, so, the processing of soluble and membrane-bound proteins differ from the very beginning. After removal of the innermost glucose, proteins are competent for ER exit and transit through Golgi where further modifications might be operated. At the end of this process, proteins can reach their final destination. However, if the native form is not achieved, after the first round of folding, re-glucosylation by the folding sensor UDP-glucose glycosil-transferase (UGGT) can occur. This mono-glucosylated form of proteins can be recognized and reinserted again into the CNX/CRT cycle, where they attempt additional rounds of folding. However, proteins that fail to attain their correct conformations must be eliminated. This is facilitated by mannosidases with the help of EDEMs (ER degradation enhancing alpha-mannosidase-like protein). These ER factors trim mannose residues from the N-glycan B branch and escort misfolded glycoproteins to ERAD, thus preventing their permanent trapping into the CNX/CRT folding cycle (Hosokawa et al., 2010) (Figure 4).

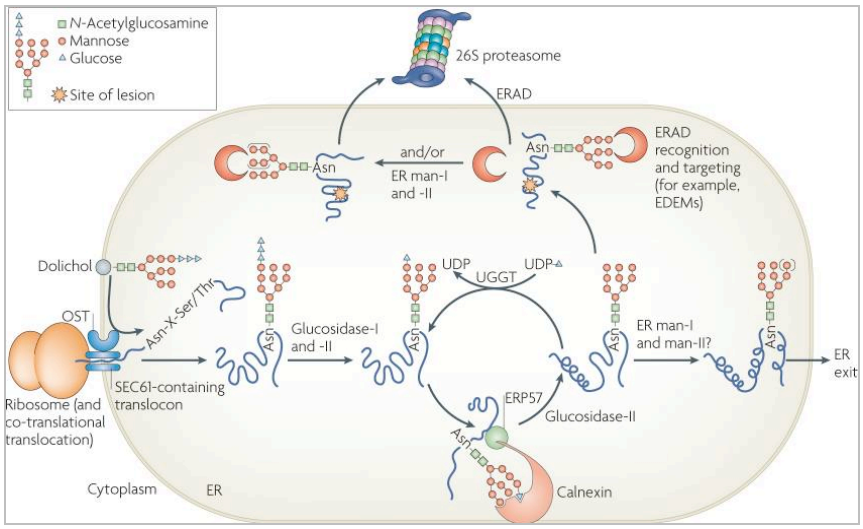


Figure 4. Early events for newly synthetized proteins. After translocation in the ER the polypeptide chain is recognized by glucosidase I and II and subsequently by CNX/CRT and the folding occur. If the native conformation is not achieved, UGGT folding sensor and mannosidases intervene, initially by reintroducing the protein in the folding cycle. Mannose trimming, however, assures terminally misfolded proteins and unused complex partners being delivered to ERAD (Vembar & Brodsky, 2008).

How and when the "folding timer" operates in recognition of the misfolded protein is still a challenging issue. A generally accepted feature is that a key for the recognition of misfolded or partially folded polypeptides or of unassembled proteins is the presence of exposed hydrophobic amino acid residues (Määttänen et al., 2010). However, no sequence has been shown to be necessary and sufficient to target proteins to degradation (except for the Cox2 cassette reported by Mbonye et al., 2006) and until now it seems to be clear that no widespread "degron" signal is involved. Studies with glycoproteins suggest that a "glycan code" is critical for their recognition. In fact mannose trimming and modifications, in particular those imposed by the slow acting ER mannosidase I (ManI), have been proposed to create a time window for folding before surveillance by ERAD components occurs (Smith et al., 2011) (Figure 5). ERAD surveillance passes through glycans, utilized as specific tags or signals, and a spectrum of carbohydrate-binding proteins (lectins) that can recognize misfolded polypeptides by different glycan chain exposition on their surface (Helenius & Aebi, 2004). Thus the disposal of misfolded glycosylated proteins requires both a wrong conformation and a correct glycan signal.

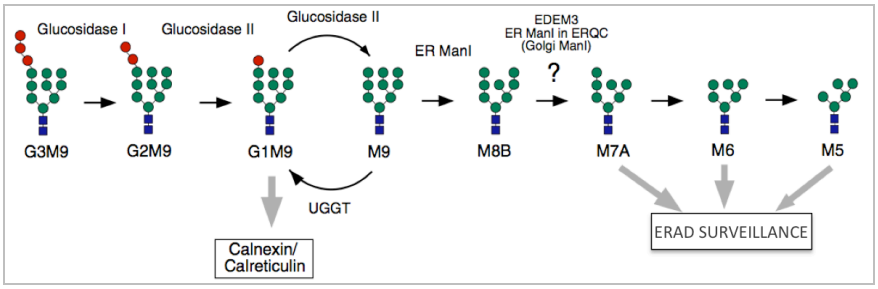


Figure 5. Mannose trimming. After formation of the M8B by ManI, misfolded species can be recognized by surveillance ERAD component (adapted from Hosokawa et al., 2010).

ER-QC: surveillance, recognition and dislocation

During ER-QC, three main recognition steps have been identified, each requiring distinct proteins. First of all, newly synthetized proteins are bound to ER chaperones that attend them during the CNX/CRT cycle. Then, if the protein does not reach the required conformation or is an unwanted protein, the second recognition step intervenes. This involves principally lectins that recognize and deliver these species to adapters and co-factors that, in the third step, provide the environment to escort selected polypeptides from ER-QC to ERAD.

Very important for the folding process but also, if any, for an efficient retrotranslocation to the cytosol, is that folding intermediates and misfolded proteins remain associated to ER chaperones (Nakatsukasa K & Brodsky, 2008). Two ER-resident proteins are known to deal with this process, GRP94 and BiP. **GRP94**, or "Glucose-Regulated Protein", is an abundant, soluble, obligate dimeric ER chaperone, member of the Hsp90 family. GRP94 is involved in the early recognition of misfolded proteins, both glycoproteins and non-glycoproteins (Christianson et al., 2008). The basis for GRP94 clients selectivity, how it binds its clients and the impact it has in the folding process is still unclear, though its ATPase activity appears to be essential for its chaperoning activity (Marzec et al., 2011). GRP94 is also a calcium-binding protein that, together with calreticulin, provides a Ca^{2+} ER storage capacity up to 30 µM (Eletto et al., 2010), however it is not known if this property affects its chaperoning activity.

BiP, or "immunoglobulin heavy chain Binding Protein" is an ER chaperone member of the Hsp70 family and it is the first chaperone that binds each nascent chain. It is also known to escort non-glycosylated misfolded proteins to the ERAD machinery (Hosokawa et al., 2008), in fact the prolonged association of Bip with misfolded mutants seems to prevent their export (Määttänen et al., 2010).

The molecular link between the ER-QC and ERAD adaptors has been elusive until Gauss and colleagues reported in yeast a relationship between the ER-QC lectin Yos9p and Hrd3p, a protein involved in protein dislocation from ER (Gauss et al. in 2006). Lately this association was confirmed also in mammals by demonstrating that the mammalian variants of Yos9p, OS-9 and XTP3-B, can decode the trimmed mannose of terminally misfolded proteins and escort them to the ER membrane-associated protein SEL1L (described below) for retrotranslocation (Bernasconi et al., 2008; Christianson et al., 2008). **OS-9** is a soluble lectin that contains one MRH domain (mannose 6-phosphate receptor homology), indicative of its role in mannose recognition, but no ER retention motifs, suggesting that it is retained by other ER factors. **XTP3-B** is an additional ER lectin that shares several features of OS9, but contains two MRH domains. It has been proposed that the MRH domains of OS-9 and XTP3-B, instead of mediating a direct interaction with substrates (Alcock & Swanton, 2009), may interact with glycans on other ER-resident components, such as SEL1L (Hosokawa et al., 2008). OS-9 is proposed to facilitate the transfer of ERAD substrates from GRP94 to SEL1L, as the binding of OS-9 to GRP94 or SEL1L was demonstrated to be mutually exclusive (Christianson et al., 2008). As OS9, also

XTP3-B seems to play a relevant role in delivering ERAD substrates from chaperones, like BIP, to SEL1L (Hosokawa et al., 2008; Christianson et al., 2011).

It is important to mention that ER lectins and chaperones involved in the second recognition step, are able to recognize not only misfolded proteins but also unnecessary native species, in a still elusive manner. An example is the recognition of unassembled subunits of multimeric protein complexes that, although properly folded, must be retrotranslocated and degraded in order to not overload ER (Vembar & Brodsky, 2008). The determinants for ER disposal are still to be identified and the literature in this field is growing and seething.

The last ER-QC step involves membrane components that are recognized by lectins and chaperones and in turn engages ERAD machineries.

Herp is a receptor for non-glycosylated BiP substrates and contains an ubiquitin like binding domain. It is an ER membrane protein, up-regulated during ER stress, with both the N- and C- terminal facing the cytoplasm (Kokame et al., 2000). Herp interacts with cytosolic proteins involved in ERAD like ubiquilin, a shuttle factor that delivers ubiquitinated substrates to the proteasome (Kim et al., 2008).

SEL1L is the mammalian orthologue of yeast Hrd3p. It is a highly glycosylated type I membrane protein that strictly associate, in ER membrane, with HRD1 (an E3 ligase described in the next paragraph) in a 1:1 complex. SEL1L acts as a molecular adaptor for lectins transporting ERAD substrates and facilitates the subsequent retrotranslocation of misfolded species (Christianson et al., 2008). It contains a large soluble region in the ER lumen containing several TPR (tetratricopeptide repeat)-type domains, known to mediate protein-protein interactions, however their function in SEL1L remains to be elucidated (Bagola et al., 2011).

Derlins are three ER membrane proteins crossing the membrane four times, with the N- e C-terminal both in the cytoplasmic side. Derlins are involved in the turnover of both soluble and polytopic substrates and have been shown to bind the substrates both before and after their extraction from ER membrane (Bagola et al., 2011). Derlins have been observed to form homo-oligomers and heteromultimeric complexes (Lilley & Ploegh, 2005; Carvalho et al., 2010) including SEL1L and HRD1. They were thought to take part in the formation of the ER export channel through which misfolded proteins can cross the ER membrane to reach the cytoplasm (Lilley & Ploegh, 2004), however it has been recently suggested that they just facilitate the release of ERAD substrates following their transfer across the membrane (Greenblatt et al. 2011). Derlins are in fact rhomboid pseudoproteases that lack the catalytic active site, which functions are poorly understood, but are predicted to retain the overall rhomboid architecture, proposed to compress and deform the bilayer in order to facilitate the exposure of substrates.

One of the last processes taking place in the ER and mandatory for the retrotranslocation of ERAD substrates into cytosol is the reduction of disulphide bonds of damaged or misassembled proteins. The release of these covalent bonds helps the crossing through the membrane. In charge for this function could be an EDEM-binding ER resident protein, called **ERdj5**, highly conserved in mammals, that has been discovered

recently (Ushioda et al. 2008). It has reductase activity, cleaves the disulfide bonds of misfolded proteins and accelerates ERAD through its physical and functional associations with EDEM and the ER-resident chaperone BiP.

To be degraded, ERAD substrates must be tagged with a polyubiquitin chain just before entering the proteasome. Ubiquitination of ERAD substrates occurs contemporarily with retrotranslocation from the ER. To clarify how ERAD substrates are prepared for degradation, a short description of the ubiquitination process is provided below.

The ubiquitin-proteasome system

Attachment of ubiquitin (Ub), a 76 amino acids long regulatory protein highly conserved and expressed in all tissue, modulates proteins' function in various cellular processes such as cycle progression, DNA repair, endocytosis and cell signaling. Among these functions the ubiquitin labeling of misfolded/unwanted protein is a point of no return for dislocation from the ER and destruction.

Ubiquitin is one of the most versatile molecular signals in the cell because of its ability to modify substrate proteins in its monomeric form (mono-ubiquitination) or polymeric form (poly-ubiquitination) as it can be conjugated to preceding Ub moieties building, consequently, many types of Ub chain. Ubiquitin contains 7 Lys residues through which the poly-Ub chain formation occurs, resulting in the assembly of various kinds of chains (Ikeda & Dikic, 2008). Polyubiquitin chains linked through Lys48 of ubiquitin are characteristically associated with proteasomal degradation. However, linkages through several of the other seven Lys residues in ubiquitin have recently been also shown to target protein to proteasomal degradation (Xu et al., 2009). Most substrates are ubiquitinated through a stable isopeptide bond formed between ubiquitin and the ε-NH_2 group of an internal Lys residue in the substrate, then, additional ubiquitin moieties are added to generate the poly-ubiquitin chain. On the other hand, in some cases, ubiquitin can be conjugated to the α-NH_2 group (Bloom et al., 2003; Ciechanover, 2005) of the first amino acid of a protein and there are few examples of Ub conjugation to Thr, Ser or Cys residues (Ishikura et al., 2010; Shimizu et al., 2010).

Ubiquitin ligases are the critical determinants of substrate selection and are also considered to be important components in controlling poly-Ub chain formation (Ikeda & Dikic, 2008; Xu et al., 2009). Ubiquitin is conjugated to target misfolded protein in a highly regulated process with specificity conferred by over 1,000 different ubiquitin ligases (E3) working together with a subset of ubiquitin conjugating (E2) enzymes (Figure 6). Protein ubiquitination requires first the intervention of an E1 Ub-activating enzyme that activates Ub in an ATP-dependent manner. After that, Ub is transferred to E2 conjugating enzymes.

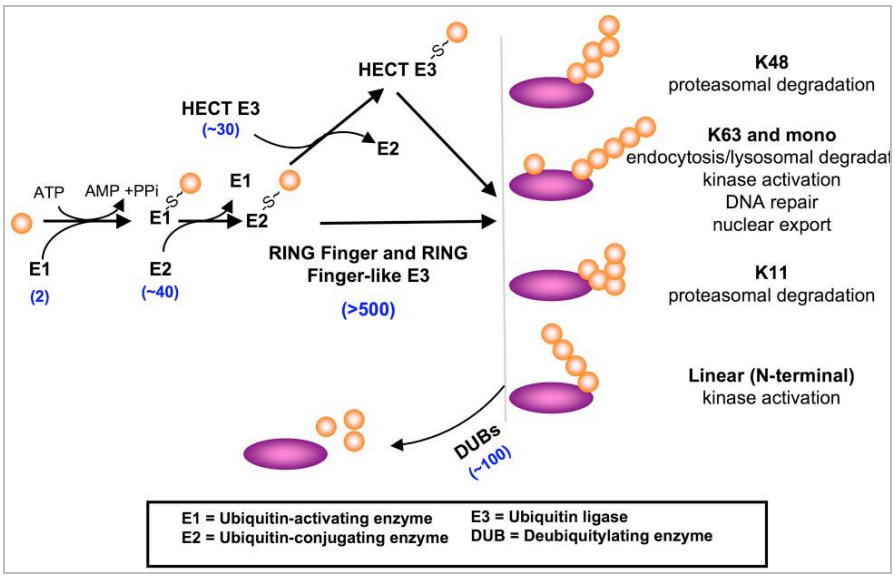


Figure 6. Conjugation of Ub and role of the Ub chain, linked to the Lys (K) through which the chain is built up (Tsai & Weissman, 2011).

E2 enzymes might act either by transferring Ub to a HECT-type E3 ligase (Homologous to the E6AP Carboxyl Terminus) or catalyzing substrate ubiquitination in combination with RING (Really Interesting New Gene) or RING finger-like E3 or U-box E3 ligases (Ikeda & Dikic, 2008; Kostova et al., 2007; Deshaies & Joazeiro, 2009). Unlike the RING finger E3s, that recognized E2s thanks to the coordination of two Zn^{2+} ions, the U-box E3s do it independently. In selected cases, an E4 ubiquitin chain extension enzyme intervenes in the process and has been shown to facilitate ERAD (Tsai & Weissman, 2011; Vembar & Brodsky, 2008). The size of the Ub-chain seems in fact to be a key determinant for example to complete ER dislocation of ERAD substrates (Jarosch et al., 2002; Weissman et al., 2011).

Diverse E3 ligases lead distinct ERAD pathways

Fundamental linkers between the E2 conjugase enzymes and the right ERAD substrate to be tagged with Ub are the E3 ubiquitin ligases. However, E3 ligases are assisted by a dynamic and organized number of proteins organized in functional modules (Mayor, 2001; Christianson et al., 2011). It is important to point out that ERAD pathways have been almost defined in yeast: this helped in the uncovering of well-conserved ERAD pathway in mammals, where the topic is still very open. Clearly, in higher organisms these pathways are greatly expanded to adapt to the increasing cellular complexities. To accommodate the topological diversity of protein defects in fact, distinct pathways work side by side to monitor misfolding (Ismail & Ng, 2006). It is emerging that specialized ERAD pathways are characterized by distinct E3 ligase competent for ERAD substrates with distinct lesions and selection starts since the recognition phases. Recognition of ERAD substrates depends on the site of the lesion. ERAD membrane substrates can be distinguished on the base of defects residing in their luminal, cytosolic and membrane domains, so that the competent ERAD pathway is named, respectively, ERAD-L, ERAD-C or ERAD-M (Figure 7). Despite the classification some proteins might escape the theorized

pathway and the criteria that drive them is still missing. Moreover, the activity of individual ligases or their co-factors may be controlled in a spatially, temporally or tissue-specific manner (Mehnert et al., 2010) and thereby can vary in cells in different developmental or physiological situations.

In the case of membrane ERAD substrates, independently of the site of lesion, ubiquitination proceeds or can occur concomitantly with retrotranslocation. Diversely, to be degraded, soluble ERAD substrates must be first retrotranslocated to the cytoplasm because the enzymes required for their ubiquitination resides in this compartment (Vembar & Brodsky, 2008).

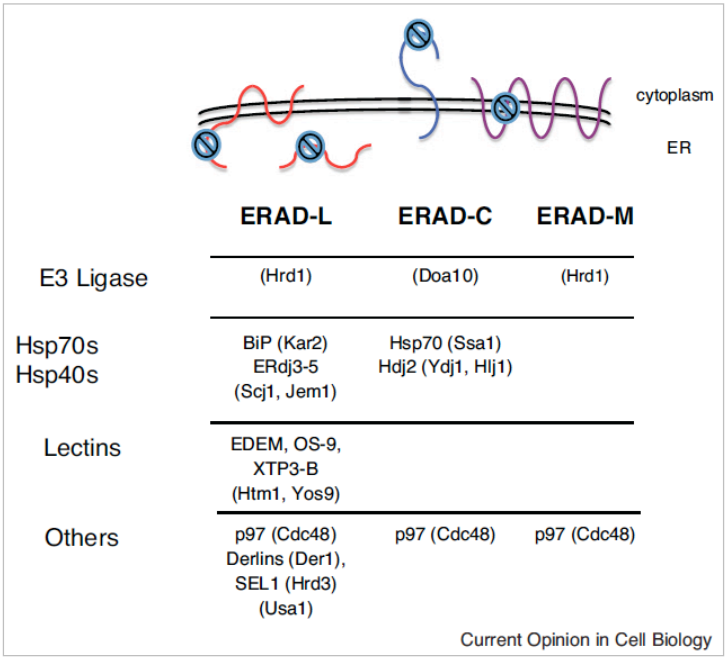


Figure 7. Classification of ERAD pathways acting on topological different substrates with misfolding in the luminal domain (ERAD-L), in the cytosolic domain (ERAD-C) or in the transmembrane domain (ERAD-M). In parenthesis is indicated the name of the related yeast factor (Brodsky & Skach, 2011).

Among the over 1,000 E3 ubiquitin ligases reported so far, only few of them seem implicated in ERAD (table 2) (Bagola et al., 2011; Mehnert et al., 2010), although their number is increasing. Most of them are poorly described and only few targets have been identified; in mammals, just two E3 ligases, **HRD1** and **gp78**, were investigated in detail and numerous substrates are now identified.

Yeast	Mammals	Localization
Hrd1	Hrd1/synoviolin	integral ER membrane
	gp78/AMFR	integral ER membrane
Doa10	Teb4/MARCH6	integral ER membrane
	TRC8	integral ER membrane
	RFN5/RMA1	integral ER membrane
	RFP2	integral ER membrane
	CHIP	cytosolic
	Parkin	cytosolic

Table 2. A list of established yeast and mammal E3 ligases involved in ERAD and their cyto-localization (adapted from Bagola et al., 2011).

E3 ligases, when not needed, can down-regulate themselves by self-ubiquitination, heterologous ubiquitination and also by a hierarchical cascade of ubiquitination, all mechanisms leading to proteasomal degradation (Weissman et al., 2011) (Figure 8).

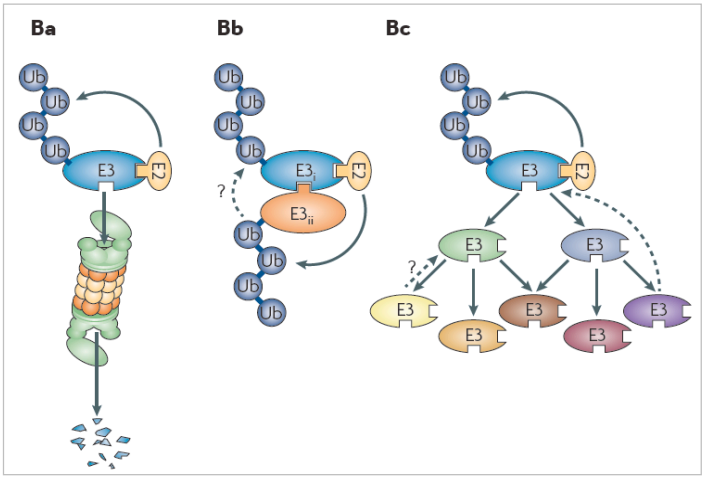


Figure 8. Degradation of E3-ligases. The degradation can be mediated by self-ubiquitination (Ba), by a heterologous ligase (Bb) or by a hierarchical cascade of ligases (Bc) (Weissman et al., 2011).

As for the E3 ligases also ERAD components must be controlled in their relative abundance. A process, recently discovered and called "ERAD tuning" can modulate the degradative capacity of ER, selectively clearing EDEM1 and OS-9, and also other ERAD factors from the ER in the so-called EDEMosomes (Figure 9), small LC3-I-coated vesicles that can subsequently fuse with lysosomes (Calì et al., 2008).

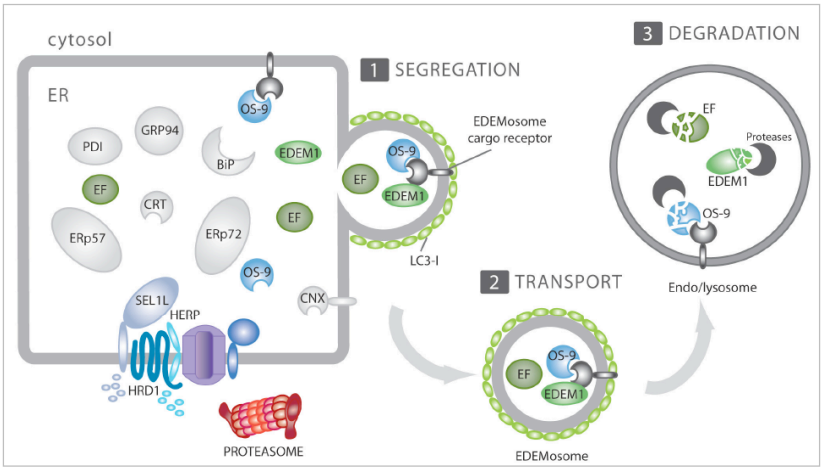


Figure 9. Degradation through EDEMosomes of selected ERAD components (Reggiori et al., 2011).

Retrotranslocation of ERAD substrates

The heart of all ERAD pathways are multiprotein transmembrane complexes formed around the E3 ligases that coordinating the events on both sides and within the ER membrane, catalyze substrate ubiquitination and retrotranslocation into the cytoplasm (Smith et al., 2011) (Figure 10).

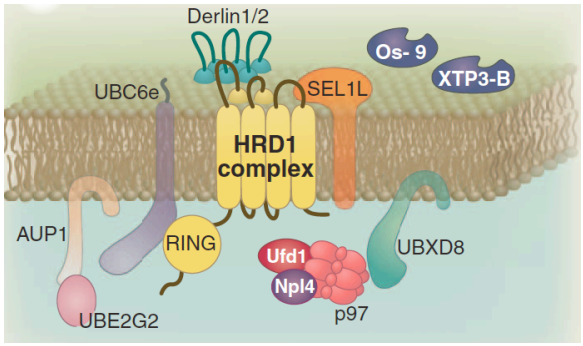


Figure 10: the HRD1 complex, one of the most characterized in mammal ERAD. As shown here, HRD1 is surrounded by a number of proteins helping in substrate selection, delivery, ubiquitination and extraction (Smith et al., 2011).

The poly-Ub chain added to a misfolded protein acts probably as a handle for the displacement of ERAD substrates through the retrotraslocation complex. The AAA-ATPase **p97** and other proteins, like Derlins and E3 complexes, probably compose the complex, but this topic is still controversial. A role for Sec61 has also been hypothesized, but crystallographic structure suggests that the pore size is too small (only 3 Å), making it difficult to imagine how this translocon could supports the dislocation of ERAD substrates that might display oligosaccharides moieties extending for about 30 Å from the polypeptide chain (Hebert et al., 2010). Recent data indicates a close, perhaps direct interaction of E3 ligases and proteasome. It has been recently shown that both the main E3 ligases, gp78 and HRD1, are able to capture the 20S subunit of the proteasome, directly linking retrotranslocation to degradation (Christianson et al. 2011). This raises the possibility that E3 ligases/proteasome connection may be independent of the 19S particle, however further studies are needed to confirm this hypothesis.

What is known about retrotranslocation is that p97 with two cofactors, **Npl4** (nuclear protein localization 4) and **Ufd1** (ubiquitin fusion degradation 1), is able to recognize ubiquitinated substrates and extract them, in an ATP-dependent manner, from the ER to the cytosol, where the 26S proteasome is located (Ye et al., 2001; Bebeacua et al., 2012). p97 is an AAA-ATPase (known as Cdc48 in yeast) that assembles in hexameric rings and forms a two level ring-shaped structure with a pore in the middle. p97, as other members of its family, acts either by translocating or remodeling the substrate, thanks to the ATP driving force. p97 has a broad spectrum of functions including fusion of homotypic membranes, protein degradation, and activation of membrane-bound transcription factors that most likely are possible as it is diverted into different cellular pathways via its binding to specific cofactors (Meyer et al., 2000; Madsen et al., 2009; Bagola et al., 2011; Wolf & Stolz, 2012). The two most characterized p97 adaptors are p47 and the Npl4-Ufd1 complex: p47 directs p97 to fusion events and protein degradation other than ERAD (Pye et al., 2007), while Npl4 and Ufd1 in a 1:1 complex, direct p97 to the essential extraction role in ERAD. Ufd1 contains an N-terminal domain, similar to that of p97, through which they both recognize synergistically long polyubiquitin chains: the lack of this domain lead to a dominant negative mutant that retains proteins in the ER (Ye et al., 2003). Its C-terminal domain is instead involved in interaction with both p97 and Npl4. Npl4 has a C-terminal zinc finger domain that interacts with ubiquitin and a N-terminal Ub-like domain that interacts with p97 (Pye et al., 2007). So, via its N-terminal, p97 interacts with

multiple regulatory cofactors and protein like, for example, the E3 ligase gp78. This site of binding overlaps with the one for UBX (ubiquitin regulatory X) containing proteins and with the binding site of Npl4, meaning that competitive binding is a crucial determinant for p97 actions (Hänzelmann & Schindelin, 2011). Therefore p97 acts like a bridge between the E3 complex, that ubiquitinates the substrates, and the proteasome, the last step during proteins' route to degradation.

Degradation of ERAD substrates

Just after substrate retrotraslocation, but before degradation, a very important process is the removal of glycan moieties, operated by a cytosolic *N*-glycanase (PNGase). Trimming of the oligosaccharide tree is necessary before entering the narrow proteolytic chamber of proteasome, so the protein should be nude and unfolded to be able to pass through (Gallastegui & Groll, 2010).

For the same reason, the removal of polyubiquitin moieties from substrates is also essential. Members of a growing family of enzyme named DUBs (deubiquitinating enzymes) can catalyze the *en-bloc* removal or the trimming of the just attached polyubiquitin chain. This process is of fundamental importance to maintain the free ubiquitin pool in cells. Moreover it has to be very regulated: the removal of ubiquitin too early can antagonize substrate degradation due to its dissociation from the proteasome (Lee, 2011). Inhibition or perturbation of the proteasomal activity results in fact in accumulation of polyubiquitinated species in the cytosol.

The degradation after retrotraslocation requires, as mentioned, the proteasome, a very large complex located in the cytoplasm. The proteasome is composed by two 19S caps, each containing six AAA-ATPase proteins to form a ring that help to direct the flow of degradation to the 20S core. The 19S subunit is involved in the recognition of the polyubiquitin chain, in the ATP-dependent substrate unfolding necessary for entry in the 20S chamber and in ubiquitin recycling. Polyubiquitinated model substrates bound to the proteasome enhance their own degradation by facilitating gate opening and allosterically activating the peptidase activities (Bech-Otschir et al., 2009). The gate to the 20S proteasome is in fact usually closed and only Ub-bound substrates can get in (Sorokin et al., 2009). The 20S core accommodates proteases with chymotryptic, tryptic and peptidyl-glutamyl-like activity that destroy proteins in peptides of about eight amino acids long, which can then be further degraded, by peptidases, in single amino acids that can be recycled in synthesizing new proteins (Gallastegui & Groll, 2010; Navon & Ciechanover, 2009; Sorokin et al., 2009).

So, briefly summarizing, substrates are recognized by chaperones and undergo ER-QC pathway: if the correct folding is achieved proteins can continue their route to Golgi to be further modified or delivered to their proper localization. Otherwise, if the folding is not reached, proteins are escorted to ERAD. Through a highly specific process, still not completely understood, substrates are recognized and then delivered to an E3 complex that ubiquitinates and probably partially translocates them from the ER to the cytosol.

Proteins are finally extracted in a not well-comprised process that seems to involve both E3 complex and p97 complex. To complete the route to destruction, in the cytosol chaperones and shuttle proteins assure substrates delivery to the proteasome, responsible of their degradation.

This is the current view of ERAD, but this topic is very debated, and last year a provocative scenario has been proposed where even two rounds of Ub attachment and removal are necessary for the degradation of ERAD substrates (Ernst et al., 2011) The substrate ubiquitination, performed by the E3 ligases embedded in ER membrane, is immediately followed by a round of de-ubiquitination, operated by DUBs associated to p97. The trimming off the polyubiquitin chain is supposed to be necessary to allow the substrate to enter the central channel of the p97 complex during retrotranslocation. As proteasome recognition is mediated by Ub, a second round of ubiquitination is then necessarily operated by either ER-resident or cytosolic E3 ligases (Tsai & Weissman, 2011). Despite very interesting, because the fundamental role for DABs, the model leaves many open questions, like in particular the identity of the E3 ligases involved in the second round of ubiquitination.

We are clearly just beginning to scratch the surface of these fascinating and peculiar routes to destruction, which have begin to be clarified only recently.

Rescuing of processing mutants by acting on ERAD

Nowadays, more than 100 disorders ranging from cystic fibrosis, to liver disease, epilepsy, blindness but also Alzheimer's disease, Parkinson and Huntington share conserved cellular pathology in which misfolded ER substrates are prematurely degraded or accumulate as toxic aggregates (Brodsky & Skach, 2011; Schwartz & Ciechanover, 2009; Mehnert et al., 2010). Deciphering ER-QC and ERAD is therefore of fundamental importance not only to shed new light in the pathogenetic mechanisms of Unfolded Protein Diseases but also to identify potential targets for pharmacological therapies.

The strategies for a drug therapy are directed either at the substrate itself, at specific cellular factors, or at the global folding environment as they strictly depend on the protein defect or on the effects that the misfolded protein can cause. In spite of the discovery of the ubiquitin-proteasome system less than 30 years ago, a therapeutic approach, surprisingly effective, concerns the inhibition of the last step of this pathway, the proteasome, through inhibitors of the proteolytic activity. Founder of this category of compounds is Bortezomib (Velcade) approved in 2003 to treat multiple myeloma and in 2005 to treat mantle cell lymphoma and now in diverse clinical trials to treat diverse forms of cancer (www.clinicaltrial.gov). Despite the pleiotropic role within the cell of proteasome, its inhibition by the drug might lead to a huge accumulation of misfolded proteins, the induction of UPS followed by cell death. This strategy can be used not only to eliminate cells in which the accumulation of misfolded proteins can cause impairments due to aggregation in the ER but can also be used to avoid the destruction of mutants that despite the defect still retain their biological function. Proteasomal inhibition reduces in fact the degradation of such mutants but also of unassembled proteins,

facilitating their maturation and targeting (Gastaldello et al., 2008; Altier et al., 2011). However, in these cases, the terminal effect of cell apoptosis due to proteasomal inhibition can nullify the treatments. Fortunately, in this field, new categories of compounds have been recently discovered and named “potentiators”, “correctors”, “blocking” and “trafficking” drugs. These very promising drugs derive from the high-throughput screening of huge libraries of compounds (Pedemonte et al., 2011). They can, as the name of each category explains, help the processing of mutants however in a not well-understood way. Some of these compounds are now in clinical trials: the potentiator called ivacaftor (VX-770), for example, has been demonstrate to improve lung function and patient-reported respiratory symptoms (ongoing clinical trial, Ramsey et al., 2011) in cystic fibrosis patients with G551D CFTR mutant.

Clearly stabilization of the misfolded protein may not always be the appropriate clinical strategy, in particular cases, stimulation and promotion of the residual activity of ERAD factors might be an effective approach. This is the case, for example, of Parkin E3 ligase: mutations in parkin gene are reported in 50% patients with juvenile Parkinson’s disease and parkin gene therapy has been shown to rescue the phenotype of a rat model of such disorder (Yamada et al., 2005; Nalepa et al., 2006). A reduced amount of HRD1 has been reported in neuronal cells of Alzheimer’s disease patients and this down regulation is accompanied by an increase of APP (the mutant amyloid precursor) and Aβ peptide (the processed form of APP, which forms aggregates in patients) (Kaneko et al., 2010). Other targets could be chaperones, lectins, DUB enzymes, which specificity for different polyubiquitin chain linkages varies considerably, and generally all the proteins involved in recognition and delivery of the mutant protein from the ER to the proteasome. Possibly the up/down-regulation of a precise interactor during the pathway can lead to rescue or improvement of the quantity/quality of the substrate.

Undoubtedly, the “holy grail” in the drug treatment of UPS would be the development of inhibitors, or more generally chemicals, that can modulate the activity of E3 ubiquitin ligases. As E3 ligases seem to be the carriers of ERAD specificity, these drugs can provide the selectivity that Velcade lacks (Eldridge & O'Brien, 2010).

A side project

Last year, I had the opportunity to start a collaboration with Dr. R. Sacchetto (Veterinary Dept.) to study the pathogenetic mechanism of a disease affecting a Chianina cattle, called Pseudomyotonia (Drögemüller et al., 2008). The cow suffers of exercise-induced muscle contracture due to the reduced activity of the skeletal muscle protein SERCA1a. R. Sacchetto identified the mutation present in SERCA1 protein (R164H amino acid substitution), and noticed that the activity of the enzyme was closely related to the protein amount present in muscle sample. Moreover, the reduced amount of SERCA1a was due to post-translational events, as the level of SERCA transcription is comparable to that found in the wild type animal. Therefore Pseudomyotonia (PMT) presents the characteristic of an Unfolded Protein Disease. Hence, I decided to investigate PMT to determine its pathogenetic mechanism and possibly to identify new molecular targets for the treatment of this veterinary disorders that unfortunately has a human counterpart in Brody's disease. To this end, herein follows a brief description of SERCA1a and of related diseases.

Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA)

In all eukaryotes, the ER, or its specialized sub compartment in striated muscle cells, the sarcoplasmic reticulum (SR), is a fundamental intracellular calcium store. In muscle resting condition, a free calcium concentration of 1-2 mM is present in the SR in contrast to a cytosolic concentration of 0.1 μM (Schröder, 2008). However, upon electrical stimulation during the excitation-contraction coupling, calcium is rapidly released from SR in order to activate the contraction mechanism. The excitation-contraction coupling is the mechanism through which the action potential leads to muscle contraction. When the motor neuron releases the acetylcholine neurotransmitter at the neuromuscular junction, the depolarization propagates from the membrane of the muscle cell to the T tubule where voltage dependent calcium channel, the dihydropyridine receptor (DHPR) is located. T tubule are plasma membrane invagination that insert deeply inside muscular cell, bringing the depolarization signal close to the site of calcium release, the terminal cisternae of SR. Depolarization causes subsequently the opening of the mechanically coupled Ryanodine receptor (RyR) present on the terminal cisternae with a consequent strong release of calcium that rises from 10^{-9}M to 10^{-5}M a concentration able to activate the contraction mechanism. Contraction persists as long as the cytosolic calcium concentration is high, which means that removal of Ca^{2+} ions is necessary for relaxation. Calcium handling is therefore of foundamental importance for muscle work and is achieved through the concerted action of three major classes of SR calcium-regulatory proteins: luminal calcium binding proteins (calsequestrin, histidine-rich calcium-binding protein, junctate and sarcalumenin) for calcium storage; SR calcium release channels (type 1 ryanodine receptor or RyR1 and IP_3 receptors) for calcium release; and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps for calcium re-uptake. Proper calcium storage, release, and re-uptake are essential for normal skeletal muscle function (Rossi & Dirksen, 2006).

SERCA proteins are coded by three genes (ATP2A1-3) and are differently expressed tissues. SERCA1 is expressed in fast-twitch skeletal muscles where its last two exons are alternatively spliced to generate both adult (SERCA1a) and neonatal (SERCA1b) isoforms. SERCA2 similarly has alternative splicing that results in SERCA2a-d production: the two main expressed isoforms are SERCA2a, the principal isoform present in cardiac, slow-twitch skeletal muscle and neonatal skeletal muscle, and 2b, found in both non-muscle tissues and smooth muscle. SERCA3 is broadly expressed (Rossi & Dirksen, 2006).
SERCA 1 is responsible for most Ca^{2+} influx into SR. This occurs with a stoichiometry of two Ca^{2+} ions per ATP hydrolyzed, as SERCA pumps calcium against gradient. SERCA is a polytopic membrane protein with ten transmembrane helices called M1-M10 and both C- and N-terminus facing the cytosol (Figure 11).

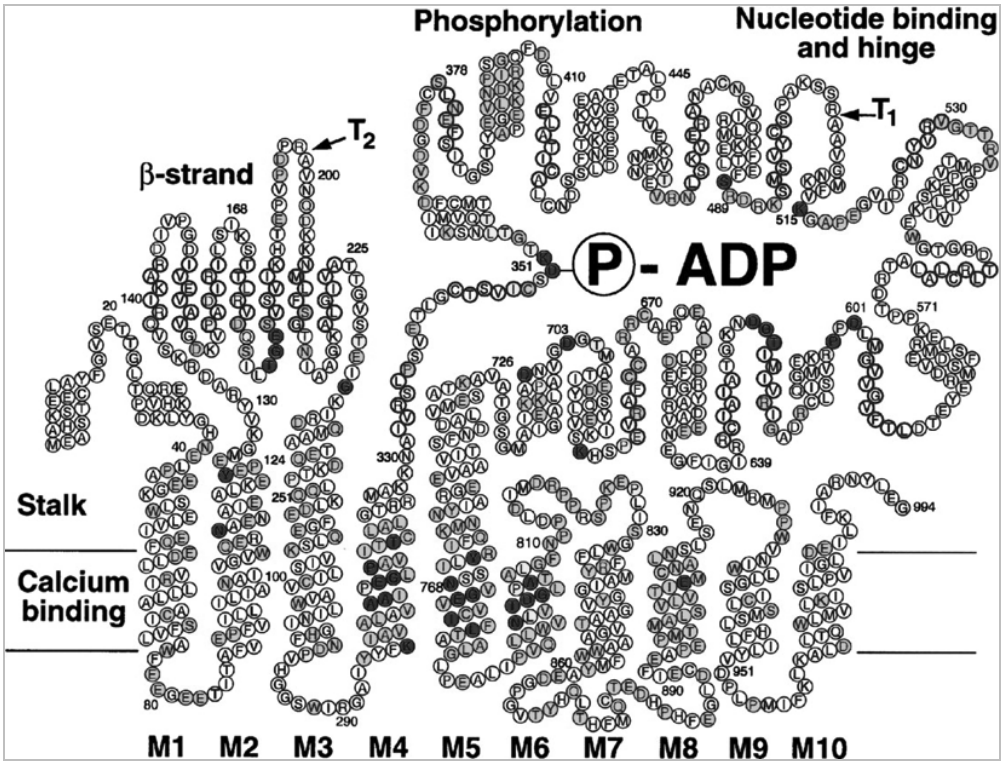


Figure 11. Structure of SERCA1a (MacLennan et al., 1997).

The structure of SERCA protein identifies four different domains, named P, N, A and M. P-domain is the phosphorylation domain, the catalytic part of SERCA. It contains the aspartic acid residue transiently phosphorylated during the reaction cycle and is responsible for the conformational changes. N-domain, or nucleotide binding domain, is inserted in the P-domain and contains residues that bind ATP and phosphorylate the P-domain acting as protein kinase. The A-domain, or actuator, located at the N-terminal, is responsible of P-domain de-phosphorylation during the reaction cycle. M-domain is the membrane domain and the calcium binding sites are located in M4, M5, M6 and M8 (Kühlbrandt, 2004).

The regulation of SERCA activity is done by phospholamban. It binds to the Ca^{2+}-pump preventing Ca^{2+} from being pumped back into the SR. During muscle relaxation,

phospholamban is phosphorylated by Protein Kinase A (PKA) or Ca^{2+}-calmodulin-dependent protein kinase and this event inhibits its activity.

Brody Disease and Congenital Pseudomiotonia (PMT)

Brody disease is a rare inherited skeletal muscle disorder, due to mutations in ATP2A1 gene encoding SERCA1 (Odermatt et al., 1996). It is characterized by an increasing impairment of muscle relaxation during exercise, with consequent muscle contracture, due to markedly reduced Ca^{2+} re-uptake into SR. SERCA1 deficiency has been reported in all cases of human BD examined so far. The time required to reach resting intracellular Ca^{2+} levels during relaxation is increased several fold in Brody's muscle cells. Almost normal resting cytosolic calcium concentration might be, however reached, through compensatory mechanisms, that can be hypothesized in Ca^{2+} removal by plasma membrane Ca^{2+} ATPases (PMCAs), by Na^{+}/Ca^{2+} exchangers in the plasma membrane, by mitochondrial Ca^{2+} uptake, or by the proliferation of sarcoplasmic or endoplasmic reticulum containing compensating levels of SERCA2 or SERCA3 (MacLennan et al., 1997).

Recently, a muscular disorder defined as congenital pseudomyotonia has been described in Chianina cattle in which a missense mutation in the ATP2A1 gene yields an R164H substitution in a highly conserved region of SERCA1, essential for the cycle of ATP-dependent Ca^{2+} transport. This missense mutation is associated with a drastic protein reduction (Drögemüller et al., 2008; MacLennan & Green, 2000).

Both BD and PMT are characterized by exercise-induced impairment of muscle relaxation and consequent muscle contracture. Moreover, sarcoplasmic reticulum enriched microsomal fractions showed a markedly decreased Ca^{2+}-ATPase activity in Brody's patients, in close agreement with measurements of Ca^{2+}-ATPase activity in microsomal sarcoplasmic reticulum fractions from PMT affected Chianina muscles (Grünberg et al., 2010).

Clinical symptoms, genetic and biochemical results indicate that pseudomyotonia can be considered the counterpart of human BD and Chianina cattle represents therefore a suitable non-conventional animal model for the investigation of the pathogenesis of this rare human disease.

RESULTS AND DISCUSSION

The choice of HEK-293 cells

The widespread use of the heterologous cell line HEK-293, is due to the fact that these cells are extremely easy to cultivate and to transfect, so they can be used to express and subsequently analyze and study protein(s) of interest in a human genetic background.

It has been previously demonstrated that in HEK-293 cells constitutively expressing β-γ-δ-sarcoglycan, the transfection with wild type (WT) α-SG can lead to the formation of the sarcoglycans tetramer on the cell membrane. Conversely, the transfection of LGMD-2D associated α-SG missense mutants defective in their extracellular domain, leads to their poly-ubiquitination and degradation by the proteasome (Gastaldello et al., 2008). Hence, the quality control system ER-QC, is able to recognize and intercept these mutant proteins and deliver them to ERAD degradation. Moreover it has been shown that, upon proteasomal inhibition with Velcade, MG132 or lactacystin, it is possible to rescue protein expression of selected mutants, i.e. R77C, D97G, R98H, P228Q and V247M (Figure 12).

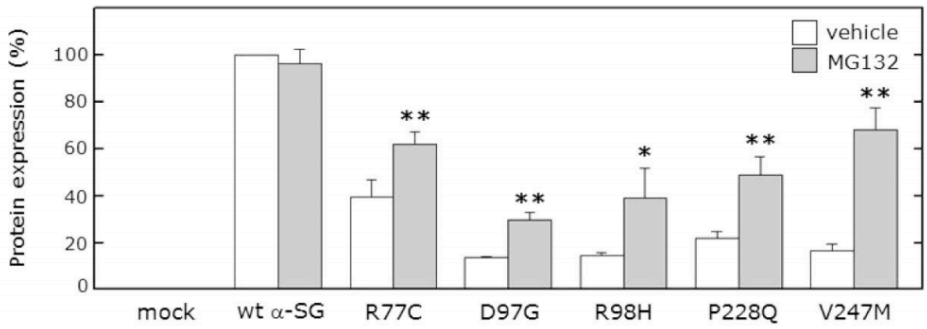


Figure 12. Protein rescue of α-SG missense mutants upon 8 hours of proteasomal inhibition with 10 μM MG132. Densitometric analysis of mutants' expression before treatment, white bars; after proteasomal inhibition, grey bars (Gastaldello et al., 2008). WT α-SG expression of untreated cells was set to 100%.*p<0.05; ** p<0.01.

Upon proteasomal inhibition the level of all mutant proteins increased. In all but R77C case, it has been also demonstrated the correct tetramer formation and its plasma membrane localization. Tetramers, containing an α-SG mutant subunit, have been proved to interact with endogenous β-distroglycan. As in skeletal muscle such interaction is considered indispensable to guarantee the structural function of sarcoglycan complex, it is probable that, in HEK cells, besides quantitative, the mutant rescue is also functional. On the other hand, R77C mutant, despite the high level of protein expression, was not found at the cell surface, except in traces, even after proteasomal inhibition. This mutant is probably trapped in the ER as it can be detected as large spots in intracellular compartments. Moreover, co-immunoprecipitation experiments show that only a minimal amount of the mutant can assemble with the β-γ-δ-SG subunits (Gastaldello et al., 2008). Conversely, rescue of R77C mutant protein expression and localization was obtained by inhibiting α-mannosidase I by using kifunensine or deoxymannojirimycine,

both in human cells and in a mouse knock-in model (Bartoli et al., 2008). At the same time, Kobuke et al., in 2008, showed that the, independently generated, α-sarcoglycan$^{H77M/H77M}$ knock-in mouse (in mouse the aminoacid at position 77 is a His) presented no pathological signs and the H77M mutant protein is normally maturated, assembled and localized at cell membrane. This suggests that, differences in the processing of mutant proteins between human and mouse are possible, probably because of the different genetic background of the two species (Kobuke et al., 2008). Since the murine model does not seem to be the best choice to study the processing and trafficking of sarcoglycans, the heterologous cells HEK-293 become the better and easiest model to work with. HEK-293 cells transfected with wild type and mutant forms of sarcoglycans, in fact, recapitulate the phenotype observed in muscle cells, moreover the HEK-293 genetic background, that seems to be important in this field, is the human one.

The choice of V247M α-SG mutant

Among analyzed α-SG mutants, the "weirdest" mutation is the V247M substitution. This mutation, in fact, should not impair either the functionality neither the glycosylation of the sarcoglycan because substitution occurs on the amino acid designed as "X", in the putative glycosylation site, after Asn246 where glycan attachment occurs, so that any amino acid is allowed in this position. Beside the theory, practically, as previously reported, this missense mutation causes LGMD-2D in human.

Therefore, I decided to focus my attention on V247M mutant processing and trafficking for several reasons: 1) this mutation is not expected to have dramatic consequences on protein functionality, 2) recovery of V247M mutant expression and localization is good upon proteasomal inhibition, and 3) the V247M mutant is not prone to aggregate, as, on the contrary, happens to R77C mutant. In Figure 13, compare V247M protein distribution inside the cells (a) with that of R77C (b).

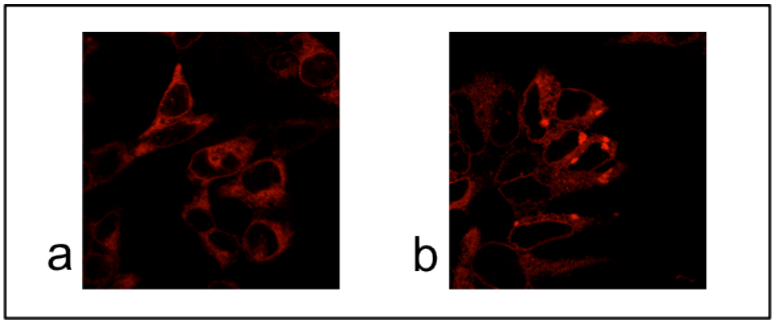


Figure 13. Immunofluorescence performed on permeabilized cells transfected with V247M α-SG (a) or R77C α-SG (b). Cells were stained with α-SG antibody and a secondary antibody TRITC-conjugated. V247M is diffusely distributed thoroughly cells and no dots are visible (a), while numerous intensely stained dots are visible in R77C transfected cells (b).

Co-localization experiments lead us to demonstrate that V247M is confined, as previously hypothesized, in the ER as shown by the yellow signal obtained in double staining experiments with α-SG and either calnexin or BIP, two ER-resident proteins (Figure 14).

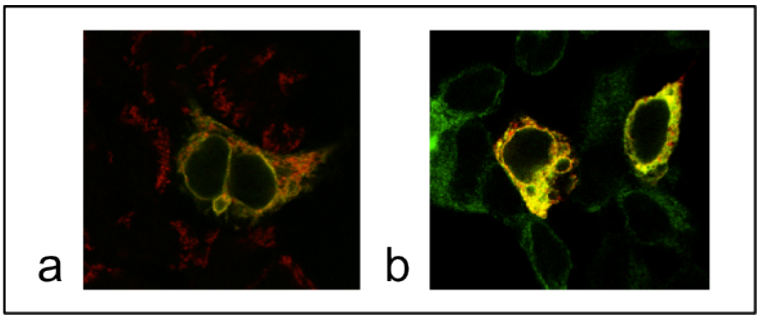


Figure 14. Immunofluorescence performed on permeabilized cells transiently transfected with V247M α-SG. (a): cells were incubated with primary antibodies against calnexin and α-SG then with secondary antibody conjugated with TRITC (red) to recognized calnexin and FITC (green) to localize α-SG. (b): cells were incubated with primary antibodies against BiP and α-SG then with secondary antibody conjugated with TRITC (red) to label α-SG and FITC (green) to mark BiP. A yellow signal characterized the overlap of green (FITC) and red (TRITC) signals meaning proteins co-localization.

α-sarcoglycan staining perfectly matched that of BiP, a luminal ER protein, whereas the co-localization of α-SG and calnexin is less good, in fact, there are several zones of calnexin staining (red) where α-SG is excluded. It's known that calnexin, an ER membrane-bound protein, localizes also on the MAM (mitochondria-associated membrane) (Lynes EM et al., 2011) where the ER is in very close contact with mitochondria, and it is possible that folding proteins are excluded from these specialized ER regions.

Taken together these data strongly suggest that V247M α-SG is a missense mutant recognized by the ER-QC resident proteins and hence destined to ERAD degradation unless forced toward another way, as in the case of proteasomal inhibition.

Ubiquitination of V247M α-SG

A prerequisite to be a true ERAD substrate is that a protein must be ubiquitinated, a process that usually occurs on Lys (K) residues. Gastaldello et al. in 2008 have demonstrated that the V247M mutant is ubiquitinated, however no information on the residues involved in such process was provided. α-SG has five lysines, four in the extracellular domain and one in the intracellular domain, as depicted in Figure 15.

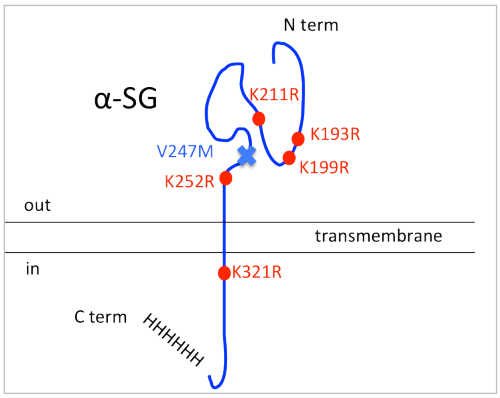


Figure 15. α-SG is a type I membrane protein containing five lysines. The lysines are positioned as specified by red dots. V247M missense mutation is indicated as a light blue cross. A polyHIS signal (6xHis) at C-terminal was previously inserted.

In-silico experiments permit to predict the lysine(s) involved in the ubiquitination of a protein. By using bioinformatics software like BDM-PUB, Ub-Pred and the more recent

CKSAAP_UbSite, it is possible to obtain a ranking score of the propensity of a particular Lys in a polypeptide to be site of ubiquitination. These tools, based on proteome of *S. cerevisiae*, have a very good success in ubiquitination prediction of yeast proteins, whereas are thought to be less precise when utilized in testing human proteins, even if the ER-QC and ERAD pathways are pretty well-conserved among these species. The outputs of the three different software, using human α-SG as query sequence, have been unable to indicate a specific Lys as the preferred site of ubiquitination. The BDM-PUB software predicted K211, CKSAAP_UbSite indicated K252 and Ub-Pred, K211 and K252, however in all cases the ranking score was low. With these unsatisfactory results from the bioinformatic analyses, and because the Lys residues in α-SG are only five, I decided to perform a site-directed mutagenesis study to uncover the Lys residue(s) that regulate proteasomal degradation of V247M mutant. In order to preserve the amino acid charge, Lys residues were changed in Arg. As ubiquitination can occur on a single residue, but may involve also ancillary sites, mutagenesis of V247M α-SG was performed changing a single Lys at time but also generating mutants with combination of two, three, four and five Lys>Arg substitutions (Figure 16). The expected result was that without the proper Lys residues for ubiquitination, the V247M mutant could skip the subsequent proteasomal degradation, as reported for other proteins (Weissman et al., 2011; Feng et al., 2007).

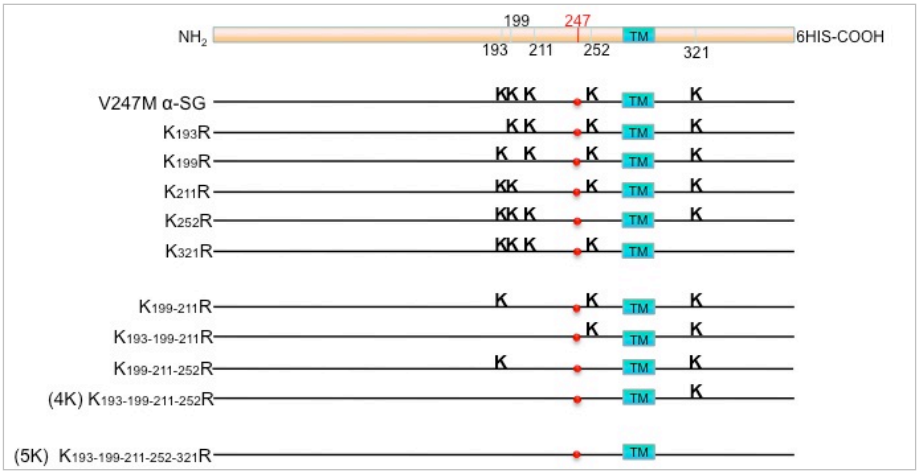


Figure 16.
Lys (K) > Arg (R) mutants obtained with site directed mutagenesis. The red dot indicates the position of V247M in α-SG, the K indicates the presence of a lysine, TM is for transmembrane.

HEK-293 cells were transfected with the different K>R constructs and, after 48 hours, total cell lysate was prepared and analyzed by western blot. α-SG protein expression was always normalized with reference to the amount of β-actin, used as loading control. The expression of each K>R mutant was compared to that of WT α-SG in order to evaluate the amount of protein increase, if any, due to the escape from degradation. As shown in the bar chart in Figure 17, V247M α-SG expression, as expected, is reduced compare to WT α-SG indicating its active degradation. On the other hand, unexpectedly, neither single nor multiple K>R substitutions preserved V247M α-SG from disposal.

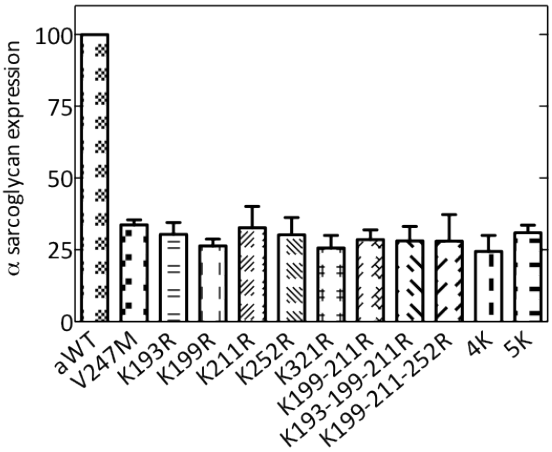


Figure 17. WT α-SG, V247M and K>R mutants expressions 48 hours after transfection in HEK-293 cells. The expression of V247M α-SG is about 30% of that of WT α-SG (as previously reported). Importantly, no significative difference is evident among V247M and the K>R-V247M mutants, WT α-SG protein was set to 100 %.

The expression of V247M mutants bearing the K>R substitutions does not statistically differ from that of the sole V247M missense mutant, meaning that lysines availability is not required to direct V247M to the proteasome. In fact, not only the single K>R substitution had no effect on V247M protein degradation, but also changing all five lysine residues, so that theoretically no sites for ubiquitination were available, was able to prevent protein disposal. Since lysines are the most common ubiquitination sites and the effects on protein expression were measured at steady state (48 hours after transfection), I asked whether K>R substitution(s) could slow down, instead of block, the degradation rate of the protein. Thus, cycloheximmide (CHX) chase experiments were performed to inhibit protein neosynthesis analyzing in this way the true rate of degradation. After CHX addition, a time course experiment of four hours, a time interval sufficient to degrade half of V247M protein, was performed; WT α-SG was used as a control. This experiment was carried out with all K>R mutants (data not shown) but here the results obtained with the 5K-V247M mutant are reported, as it nicely recapitulates the entire outcome (Figure 18).

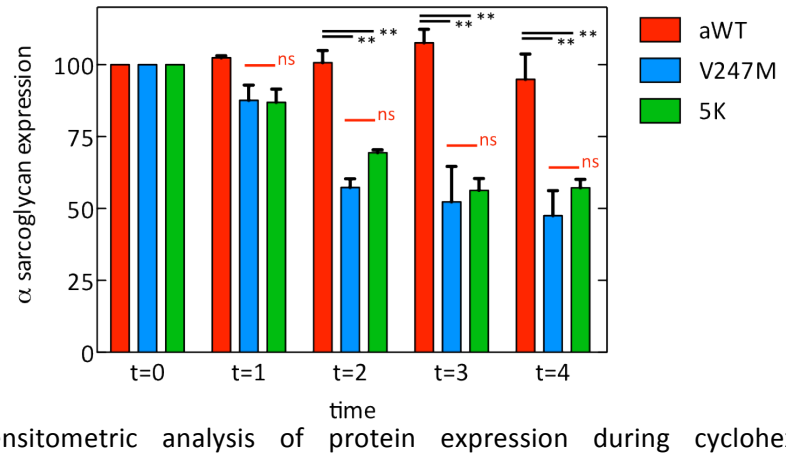


Figure 18. Densitometric analysis of protein expression during cycloheximmide chase experiments. To assess the degradation rate of WT α-SG, V247M and 5K-V247M, the amount of each protein at t=0 is set to 100 % and during the chase expression is compared to that of each sample at time zero. WT α-SG protein expression, red bars; V247M, blue bars; 5K-V247M mutant, green bars. The degradation rate of 5K-V247M did not significantly differ from that of V247M, both are rapidly removed compare to wild type α-SG whose level remain almost invariant during the entire experiment. ns: non significative; ** $p<0.01$.

During CHX chase interval, WT protein (red bars) was not significantly degraded, meaning that this protein is quite stable in its native conformation. This is not the case for V247M α-SG (blue bars) and its 5K mutant (green bars). During the CHX chase experiments, in fact, their level decreased with the same rate (no significative differences) even if the 5K mutant lacks all the lysines and therefore, ubiquitination should not be possible.

To verify that V247M and 5K-V247M mutants are similarly processed by the cells and do not have distinct mechanisms of removal, I performed experiments with inhibitors of diverse degradative pathways. To exclude the involvement of autophagy, bafilomycin A1 (inhibitor of autophagosome/lysosome fusion) and leupeptin (protease inhibitor) were used. To block proteasomal activity, the medium was supplemented with the well-known irreversible inhibitor MG132, as described previously.

As reported in the bar chart (Figure 19), MG132 treatment slowed down the degradation rate of V247M and 5K-V247M mutants by comparable levels. The amount of the two proteins raised from 30% (untreated cells white bars) to about 60% (MG trated red bars) compared to WT α-SG (first bar in the bar chart). On the contrary, treatment with both leupeptin (yellow bars) and bafilomycin A1 (green bars) had no effect on the disposal of the two mutants. When both proteasomal and autophagy pathways were inhibited by contemporarily treating cells with MG132 and bafilomycin A1 (blue bars) the rescue is comparable to the one obtained with the MG132 treatment only. These data clearly indicate that both proteins are degraded not only at the same rate but also through the same pathway, the ubiquitin-proteasome system.

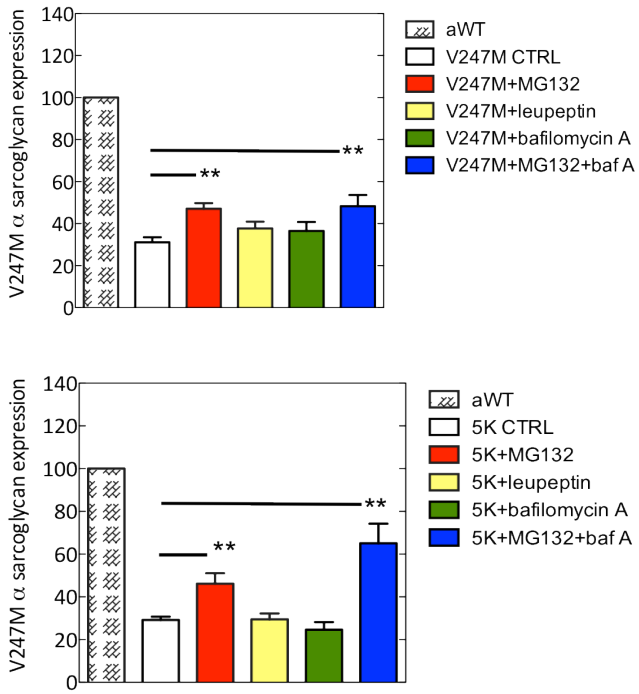


Figure 19. V247M and 5K-V247M mutants' expressions upon treatments with inhibitors of the ubiquitin-proteasome and of autophagy pathways. WT α-SG protein expression always set to 100 %). Cells after transfection were treated with vehicle (control) (white bar), 10 µM MG132 (red bars) to block proteasome, 20 µg/ml leupeptin (yellow bars) a protease inhibitor, 100 nM bafilomycin A (green bars) a blocker of the autophagosome/lysosome fusion, or 10 µM MG132 plus 500 nM bafilomycin A. Only treatments blocking proteasome lead to a significative rescue in protein expression for both V247M and 5K-V247M mutants. ** $p<0.01$.

To definitely verify that 5K-V247M mutant is effectively delivered to proteasome degradation, a proof that ubiquitination occurs on this protein was required. To this end, HEK-293 cells were transiently transfected with WT α-SG, V247M and 5K-V247M

constructs. Eight hours before lysis, both proteasome and deubiquitinase enzymes (DUBs) were blocked by using MG132 and N-ethylmaleimide (NEM), respectively. α-SG proteins (both WT and mutants) were immunoprecipitated (IP) with a specific antibody. After SDS PAGE and blotting, the level of ubiquitination was assessed by probing blot with an antibody able to recognize both mono- and poly-ubiquitin moieties. In Figure 20 are shown the western blots of total cell lysates (on the left) and of the IP proteins (on the right). Both blots were cut; the upper part was stained with anti-ubiquitin antibody, while the lower part with anti α-SG and β-actin antibodies. The band just under that of α-SG is an indication of the efficacy of MG132 treatment. This band, recognized by the α-SG specific antibody, corresponds, in fact, to the non-glycosylated form of α-SG, which can be also observed after PNGase treatment (not shown). The "nude" α-SG form is most likely the result of N-glycanase action and accumulates in the cytosol upon proteasomal inhibition. The antibody against mono- and poly-ubiquitin chain labeled both V247M mutant and 5K-V247M, as well as a part of the IP WT α-SG. These data demonstrated that, despite lacking the conventional sites of ubiquitination (the five Lys residues), the 5K-V247M mutant is anyway ubiquitinated.

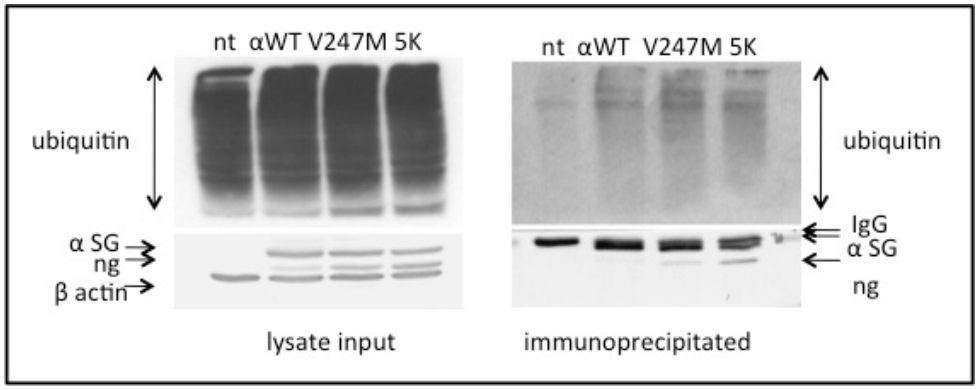


Figure 20. Left: 40 µg of cell lysates (input). Right: immunoprecipitated proteins with α-SG antibody (nt: empty vector; αWT: WT α-SG; 5K: 5K-V247M mutant). The blot was stained with the Ub antibody (up) and with α-SG and β-actin antibodies (down). ng, non glycosilated form of α-SG; IgG, heavy chain of the antibody used in the IP assay.

In the literature it has been recently demonstrated that, even if not as preferred sites, also N-terminal (Bloom et al., 2003), serine (Ishikura et al., 2010), cysteine (Williams et al., 2007) and threonine (Wang et al., 2007) residues could be alternative ubiquitination sites.

α-SG possesses 20 threonine and 20 serine and three free cysteine residues, highlighted respectively in yellow, green and light blue in Figure 21.

MAETLFWTPLLVVLLAGLGDTEAQQTTLHPLVGRVFVHTLDHETFLSLPEHVAVPPAVHITYHAHLQGHPDLPRWLRY
TQRSPHHPGFLYGSATPEDRGLQVIEVTAYNRDSFDTTRQRLVLEIGDPEGPLLPYQAEFLVRSHDAEEVLPSTPASR
FLSALGGLWEPGELQLLNVTSALDRGGRVPLPIEGRKEGVYIKVGSASPFSTCLKMVASPDSHARCAQGQPPLLSCYD
TLAPHFRVDWCNVTLVDKSVPEPADEVPTPGDGILEHDPFFCPPTEAPDRDFLVDALVTLLVPLLVALLLTLLLAYVM
CCRREGRLKRDLATSDIQMVHHCTIHGNTEELRQMAASREVPRPLSTLPMFNVHTGERLPPRVDSAQVPLILDQH

Figure 21. Amino acid sequence of α-SG: in orange the signal peptide, in blue the cadherin-like domain, in light violet the two putative glycosylation syte, in red the ATP binding site, underlined in green the transmembrane sequence. Highlighted in green serines, in yellow threonines, in light blue free cysteins.

Ishikura et al. previously demonstrated that another type I membrane protein, TCRα, one of the eight transmembrane protein that make up the TCR complex, does not possess a lysine on which the ubiquitination can occur to deliver the protein to degradation, when unassembled. They reported that the substitution of two conserved serine residues to alanine in the protein tail prevented the ubiquitination to take place (Ishikura et al., 2010).

A new site directed mutagenesis analysis of the α-SG cytosolic tail was thus planned in order to assess if any of these reported and alternative ubiquitination residues are involved in α-SG ubiquitination. Among these, the most conserved ones in α-SG tail of seven mammalian species were chosen (Figure 22) and substituted with alanine.

```
Human____MCCRREGRLKRDLATSDIQMVHHCTIHGNTEELRQMAASREVPRPLSTLPMFNVHTGERLPPRVDSAQVPLILDQH
Mouse____MCFRREGRLKRDMATSDIQMFHHCSIHGNTEELRQMAASREVPRPLSTLPMFNVRTGERLPPRVDSAQMPLILDQH
Rat______MCCRREGRLKRDMATSDIQMFHHCSIHGNTEELRQMAASREVPRPLSTLPMFNVRTGERLPPRVDSAQMPLILDQH
Hamster__MCCRREGQLKRDMATSDIQMVHHCTIHGNTEELRQMAARREVPRPLSTLPMFNVRTGERLPPRVDSAQVPLILDQH
Rabbit___MCCRREGRLKRDLATSDIQMVHHCTIHENTEELRQMAASREVPRPLFPLPMFNVRTGERMPPRVDSAQVPLILDQH
Pig______MCCRREGRLKRDLATSDIQMVHHCTIRGNTEELRQMAASREVPRPLSTLPMFNVRTGERLPPQVDNAQVPLILDQH
Bos______MCCRREGRLKRDLATSDIQMVHHRTIRGNTEELRQMASSREVPRPLSTLPMFNVHTGERLPPQVDSAQVPLILDQH
identity_MC RREG LKRD ATSDIQM HH  I  NTEELRQMA  REVPRPL  LPMFNV TGER PP VD AQ PLILDQH
```

Figure 22. Results of BLAST performed on the sequence of α-SG's tail of seven mammalian species. Bolded at the bottom the identity of amino acids, in red only threonines, serine and cysteine conserved.

So far, preliminary results indicate that none of C313A, T326A, S327A, or T367A single point mutations and T326A-S327A double mutations seem to be involved in the ubiquitination, as none leads to a rescue in protein expression.

Therefore, more experiments are undoubtedly needed to confirm these results but also to finally uncover α-SG's cryptic ubiquitination residue(s).

V247M in its long way to destruction

To better study the degradation pathway of V247M α-SG missense mutant I decided to generate a population of cells that constitutively express only this protein, herein named V247M cells. It is important to underline that trafficking of WT α-SG can be studied in HEK-293 cells even when expressed alone as it localizes in the cell membrane even without the other SGs (Draviam et al., 2006). So, I utilized this property of α-SG to dissect the ERAD pathway involved in the disposal of its mutants.

As previously said, E3 ligases play a central role in ERAD as they give selectivity and specificity to substrates recognition. Among the most studied E3 ligases, I focused my attention on gp78 and HRD1, both orthologues of the Hrd1 E3 ligase descripted in yeast. This E3 ligase in yeast is required for degradation of ERAD substrates with membrane or luminal lesions (like V247M α-SG), while membrane proteins with cytoplasmic lesions are dealt by Doa10 the orthologue of gp78 (Kostova et al., 2007; Carvalho et al., 2006). In mammals these two ligases are described in the ubiquitination of various ERAD substrates with different lesions (Christianson et al., 2008; Morito et al., 2008; Bernasconi et al., 2010; Burr et al., 2011; Ballar et al., 2011). It is important to underline that the requirement for a specific E3 ligase by different ERAD substrates is still cryptic, even if it

has been reported for example a stringent requirement for HRD1 for a soluble substrate with luminal lesions (ERAD-L_S), that became dispensable for the same substrate when membrane tethered (ERAD-L_M) (Bernasconi et al., 2010). The decision-making process of partition of substrates is still unknown, making it impossible to determine, merely looking at the protein and its lesions, the followed pathway and the necessary interactors during ERAD. Moreover more than one E3 ligase can be involved in the degradation of a mutant protein, as demonstrated for CFTR where three E3 ligases seem to intervene (Younger et al., 2006; Morito et al., 2008; Ballar et al., 2010).

Christianson and colleagues have recently proposed a new definition of human ERAD networks using different approaches, such as proteomics, genomics and responses to ER stress (Christianson et al., 2011). They identified 71 previously uncharacterized HCIPs (High-confidence Candidates Interacting Proteins) for a role in ERAD, meaning that, despite most of the work on ERAD refers to only a small number of ERAD proteins, there is a large amount of them that still has to be explored.

V247M has a HRD1-dependent, gp78-independent degradation pathway

To dissect the E3(s) involved in V247M mutant ubiquitination and disposal, by RNA interfering, I initially screened two candidate ligases, gp78 and HRD1. 72 hours after shRNA transfections, cells were lysed and the lysate was analyzed by western blotting. The shRNA sequences used to down regulate the ligases were from the literature (Christianson et al., 2008 for HRD1 and Zhong et al., 2004 for gp78). Effectiveness of gp78 silencing (about 50% of knock down) was verified on the recombinant wt gp78 co-transfected with the shRNA, whereas that of HRD1 (almost complete knock down) on the endogenus protein. In Figure 23 are reported the blots.

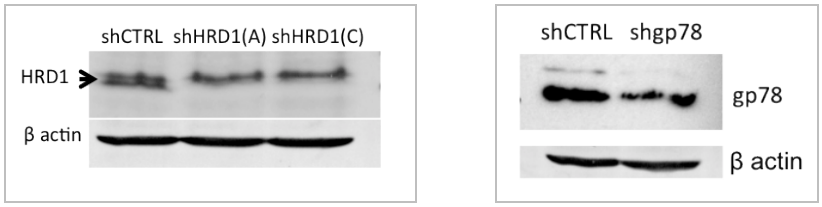


Figure 23. Left: 40 µg of cell total cell lysates obtained from V247M cells transfected with shRNA ctrl in lane 1 or with two different shRNA against HRD1 called (A) in lane 2, or (C) in lane 3. Right: 40 µg of cell total cell lysates obtained from V247M cells co-transfected with 0.5 µg of WT gp78 together with 1.5 µg shRNA ctrl (lane 1) or 0.5 µg of WT gp78 together with 1.5 µg shRNA gp78 (lane 2) (1:3 ratio). 72 hours after transfection cells were lysed and the lysate loaded on a gel to assess the effectiveness of silencing probing each blot with specific antibody and β-actin, used as loading control. In HRD1 blot an unspecific band is recognized by HRD1 antibody immediately upstream the band of the endogenous protein.

Down regulation of a candidate E3 ubiquitin-ligase possibly involved in the recognition of a mutant protein (V247M) is expected to cause the trapping of the mutant into the ER, as without the competent E3 ligase, the mutant is no longer retrotranslocated, ubiquitinated and delivered to degradation by the proteasome. Therefore, when down

regulation of an E3 ligase causes the recovery of V247M mutant, this suggests the involvement of the E3 ligase in the degradative pathway of the mutant.

As shown by western blot (on the left) and densitometric analysis (on the right) in Figure 24, down regulation of gp78 does not lead to a rescue in V247M protein expression, compared to the protein expression produced by a control shRNA. Diversely, down-regulation of the E3 ligase HRD1 with shRNA (C) produced a significant rescue of V247M expression, indicating the involvement of HRD1 E3 ligase in V247M mutant disposal.

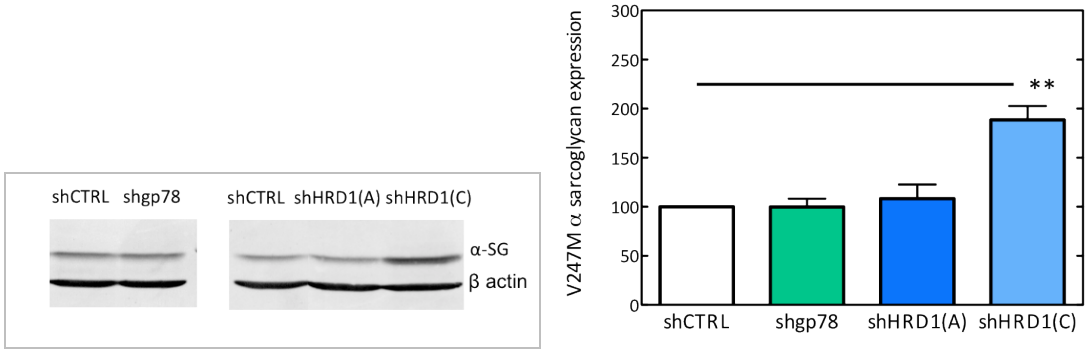


Figure 24. Left: example of western blot of 40 µg of total proteins from V247M cells transfected with indicated shRNAs. On the left in the bar chart, densitometric analysis of western blots demonstrates a significative rescue in V247M expression due to down regulation of E3 ligase HRD1 with shRNA (C); ** p<0.01.

To further investigate the role of HRD1, I took advantage of the dominant negative mutant of the protein. I utilized the well-characterized HRD1 mutant in which Cys 291 in the RING finger domain was mutated to alanine (called HRD1-C1A). Utilization of this inactive HRD1 prevented the degradation of a variety of misfolded substrates (Kikkert et al., 2004; Bernardi et al., 2010). Importantly, overexpression of native HRD1 did not stimulate ER stress (Bernardi et al., 2010). As a control for gp78 role, I utilized the so-called Ring Finger Mutant (RFM) with C337S and C374S substitutions: it has been shown that, as for HRD1 C1A mutant, also RFM-gp78 was able to block the degradation of specific ERAD substrates (Morito et al., 2008).

Figure 25 shows the effects of inactive HRD1 and gp78 ligases on V247M mutant processing. The expression of inactive HRD1-C1A E3 ligase significantly delayed the degradation of V247M mutant protein, confirming its involvement in the ERAD pathway for V247M α-SG. Consistent with the shRNA data, inactive RFM-gp78 was without effects on mutant rescuing.

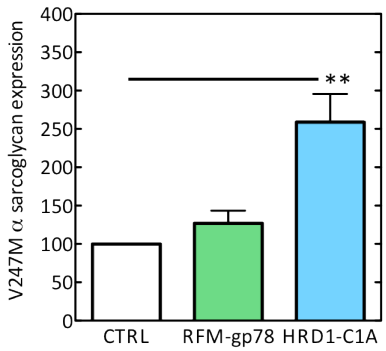


Figure 25. Results of the densitometric analysis of western blots performed on protein from transfected cells with empty vector (ctrl), RFM-gp78 mutant in light green and HRD1-C1A mutant in light blue. As shown, the expression of inactive HRD1-C1A E3 ligase significantly reduced the degradation rate of V247M mutant protein; ** p<0.01.

To confirm the interaction between V247M α-SG and HRD1, I performed an immunoprecipitation assay as previously described. To ensure a sufficient amount of misfolded substrate the proteasome inhibitor MG132 was added to cells for 8 hours before lysis except to cells transfected with the mutant form of the HRD1 because in this case the misfolded protein is anyway preserved to degradation. As shown in Figure 26, both WT HRD1 and HRD1-C1A mutant co-immunoprecipitated with the misfolded substrate, suggesting an intimate and specific interaction between the ligase and V247M α-SG.

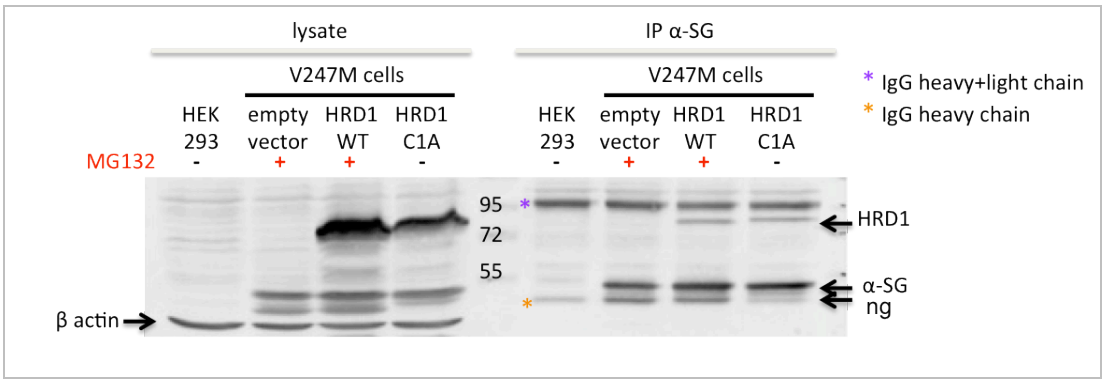


Figure 26. On the left, 40 µg of the lysates obtained from HEK-293 cells and V247M cells before immunoprecipitation. Numbers indicate molecular weights of pre-stained marker. On the right, samples resulting from IP with α-SG monoclonal antibody. V247M cells were transfected as indicated with empty vector, wild type HRD1 or HRD1-C1A (inactive form) and treated when specified (+) with the proteasome inhibitor MG132, 10 µM. The blot was stained with specific antibodies for HRD1, α-SG and β-actin, used as loading control and to verify the goodness of IP. Heavy and light chains of the monoclonal antibody used for the IP were recognized by the murine secondary antibody and are indicated by purple and orange stars, respectively.

SEL1L is an established HRD1 co-factor and critical component of ERAD pathways (Christianson et al., 2011). It associated stoichiometrically with HRD1, but not with gp78, even though the two ligases are homologous. The importance of SEL1L and HRD1 interaction is indicated by the fact that SEL1L critically determines the stability of the HRD1-SEL1L complex (Iida et al., 2011) moreover SEL1L has a documentate scaffolding role during ERAD (Christianson et al., 2008; Hosokawa N et al., 2008; Mueller B et al., 2008; Bernasconi et al., 2010). Thus, considering that the involvement of HRD1 has been established (Figure 26), it is predictable that also SEL1L might have a role in the disposal of V247M mutant. To verify this possibility, RNAi experiments were performed using shRNA (Christianson et al., 2008) against SEL1L. 72 hours after transfection, cells were lysed and proteins analyzed by western blotting (Figure 27). Under my experimental conditions, even if RNAi caused only a moderate reduction of SEL1L (40%), a significant rescue in V247M protein expression was observed. SEL1L is important for the interaction with XTP3-B and OS-9 lectins as well as with HRD1 (Christianson et al., 2008), constituting a sort of docking platform for the delivery of client proteins to ERAD. SEL1L down-regulation produces the rescue of V247M mutant because of the reduced availability of

the nexus between proteins that recognize the misfolded protein and the E3 ligase HRD1 that secures its disposal.

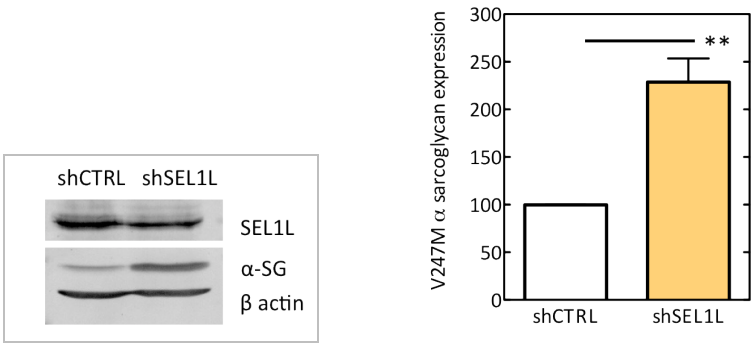
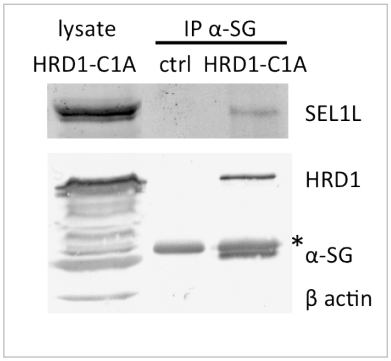


Figure 27. Left: example of western blot of 40 µg of total proteins from V247M cells transfected with indicated shRNAs. On the left in the bar chart, densitometric analysis demonstrates a significative rescue in V247M expression due to down regulation of SEL1L; ** $p<0.01$.

To confirm the physical interaction between the mutant protein, SEL1L and HRD1, another IP experiment was performed in V247M cells transfected with the dominant negative HRD1-C1A protein. As shown in Figure 28, IP with the α-SG antibody sedimented the V247M mutant together with SEL1L and HRD1, as postulated. These data confirm the essential role of SEL1L in scaffolding ERAD substrates to the HRD1 transmembrane complex.

Figure 28. In the first lane 40 µg of proteins lysate of V247M cells transfected with HRD1-C1A. Second and third lanes are sample proteins obtained after IP with α-SG antibody. In the second lane proteins were IP from HEK-293 lysate, used as control, in the third lane proteins were IP from V247M expressing cells transfected with HRD1-C1A. The blot was stained with specific antibodies for SEL1L, HRD1, α-SG and β-actin, used as loading control and to verify the goodness of IP. Asterisk indicates the heavy chains of the monoclonal antibody used for the IP, recognized by the used murine secondary antibody.



After the identification of the HRD1-SEL1L complex, we asked whether UBC6e, also called UBE2J1, the E2 conjugase demonstrated in other conditions to be the cognate of HRD1 (Christianson et al., 2011) and reported to be required for the exit of a type I membrane protein (Mueller et al., 2008), is also involved in the route to degradation of V247M α-SG. This ubiquitin-conjugase is able, in concert with HRD1, to deliver the Ub to the ERAD substrate. To verify this hypothesis, we took advantage of an UBC6e mutant in its catalytic site, C91S, which has been demonstrated to delay ERAD substrates degradation (Lenk et al., 2002; Mueller et al., 2008). V247M cells were transfected with wild type UBC6e and C91S-UBC6e and after 48 hours cells were lysed and proteins subjected to SDS page. As shown in Figure 29, overexpression of wild type UBC6e does not influence the rate of degradation of V247M mutant, whereas C91S-UBC6e significantly delays V247M degradation.

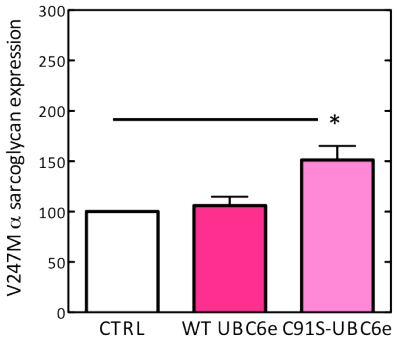


Figure 29. Results of the densitometric analysis of western blots of proteins from cells transfected with empty vector (ctrl), WT UBC6e (cyclamen bar), and C91S-UBC6e mutant (pink bar). As it can be appreciated the transfection with the mutant E2 conjugase, delaying V247M mutant degradation, results in its accumulation; *p<0.05.

To confirm the role of UBC6e, the interaction of the E2 conjugase with V247M mutant was analyzed with IP experiments. V247M cells were transfected alternatively with empty vector, wild type UBC6e or inactive C91S-UBC6e; HEK-293 were used as control. 48 hours after transfection cells were lysed and proteins subjected to immunoprecipitation with the anti α-SG antibody. To ensure an appropriate amount of V247M to be immunoprecipitated, cells that were transfected with the empty vector and with WT UBC6e, were treated with the proteasome inhibitor MG132. As shown in Figure 30, the expression of endogenous UBC6e in HEK-293 cells is robust and the transfected WT protein has the same molecular weight of the endogenous one. C91S-UBC6e instead has a higher molecular weight, due to the insertion of an HA tag. The IP with the α-SG monoclonal antibody (shown on the right panel) demonstrated the interaction between V247M α-SG and both the wild type and the inactive E2 conjugase, confirming that also this enzyme is a component of the degradation pathway for the V247M α-sarcoglycan mutant.

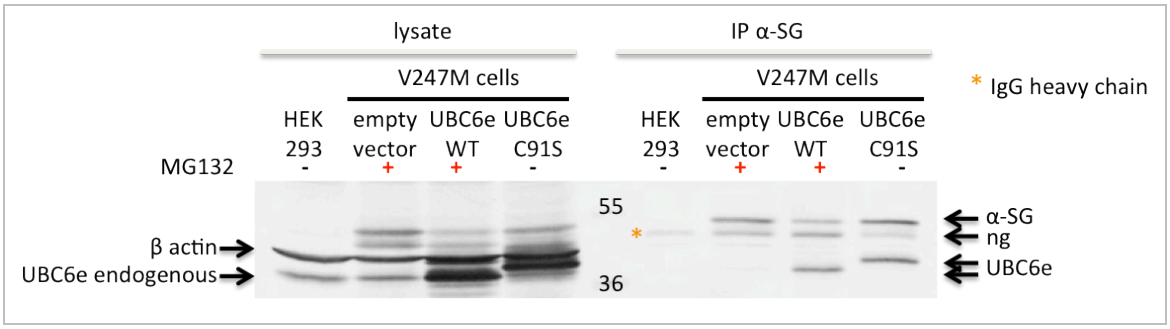


Figure 30. On the left, 40 µg of the lysates obtained from HEK-293 cells and V247M cells before immunoprecipitation. In the middle, numbers indicate molecular weights of pre-stained marker. On the right, samples resulting from IP with α-SG monoclonal antibody. V247M cells were transfected as indicated alternatively with empty vector, with WT UBC6e or C91S-UBC6e (inactive form) and treated when indicated (+) with the proteasome inhibitor MG132, 10 µM. The blot was stained with specific antibodies for UBC6e, α-SG and β-actin, used as loading control and to verify the goodness of IP. The heavy chains of the monoclonal antibody used for the IP were recognized by the murine secondary antibody and are indicated with an orange star. ng: non glycosilated form of α-SG.

The E3 ligase RFP2 cooperates with HRD1 in the disposal of V247M

RFP2, also known as TRIM13, is a RING finger protein (Skoblov et al., 2006) recently identified as an E3 ligase involved in ERAD (Lerner et al., 2007). It is expressed in skeletal muscle as well as in HEK-293 (Baranova et al., 2003; Lerner et al., 2007). RFP2 is an unstable protein with self poly-ubiquitination activity, similarly to other E3 ligases; as expected, the removal of RING domain determines the lack of its Ub-ligase activity (Altier et al., 2010). The role of RFP2 in the disposal of ERAD substrates is still poorly characterized and CD3-δ and CaV 1.2 channels (Lerner et al., 2007; Altier et al., 2010) are the only substrates known to be removed by this ligase.

In the quest for additional E3 ligases possibly involved in the disposal of V247M α-SG, even though the leading role of of HRD1 was established, experiments were performed to evaluate whether RFP2 might also be involved. In these experiments, cells were transfected either with WT RFP2-GFP (E3 ligase conjugated with a GFP tag) or its dominant negative form that lack the RING domain (Δ131-RFP2), known to inhibit the ubiquitination activity of RFP2 (Altier et al., 2010). In western blot, WT RFP2-GFP is detectable as a main protein band of about 74 KDa (Figure 31) plus additional weak bands (Lerner M et al., 2007), whereas the dominant negative mutant produces a protein band of about 28 kDa and other weaker bands. The densitometric analysis shown in Figure 31, demonstrates that RFP2 overexpression has no influence in the degradation of V247M, whereas, on the contrary, the inactive Δ131-RFP2 remarkably delays V247M degradation, suggesting the intervention also of this E3 ligase in the disposal of our mutant protein.

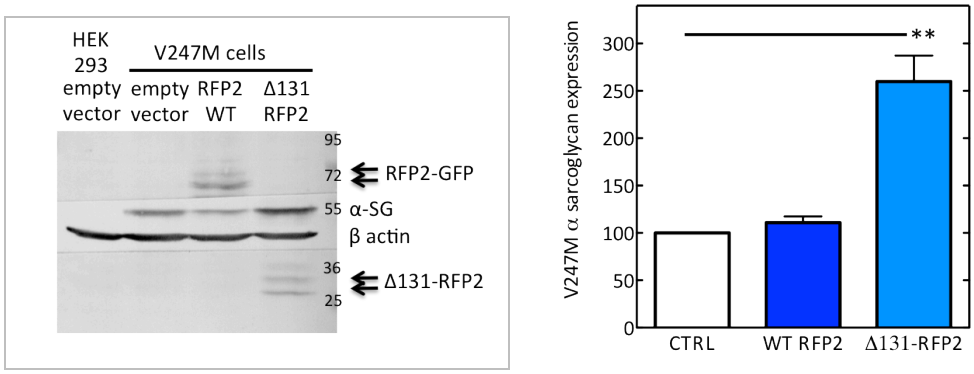


Figure 31. Left: example of western blot of 40 µg of total proteins from HEK-293 or V247M expressing cells transfected with empty vector, WT RFP2-GFP protein, or Δ131-RFP2, the dominant negative mutant, as indicated. Blot was stained with antibodies against RFP2, α-SG and β-actin, used as loading control. Right: densitometric analysis demonstrate that transfection with the dominant negative form of RFP2 substantially delay V247M mutant degradation and results in its accumulation; ** $p<0.01$.

To confirm that the RFP2 E3 ligase is involved in ERAD pathway of V247M mutant protein, IP experiments were performed in the presence of WT RFP2 and after inhibition of proteasome activity by MG132 (Figure 32). Also in this case, two bands, corresponding to the RFP2-GFP protein and the auto-ubiquitinated form, are present in the blot and both co-immunoprecipitated with V247M α-SG.

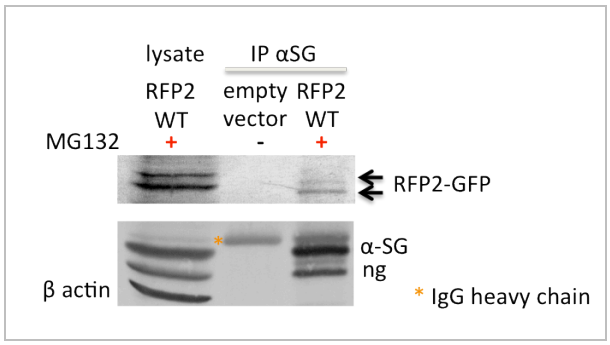


Figure 32. In the first lane 40 µg of proteins lysate from V247M cells transfected with WT RFP2-GFP treated (+) with MG132 before immunoprecipitation. Second and third lanes are samples obtained after IP with α-SG antibody. In the second lane IP sample was from HEK-293 lysate, used as control, in the third lane IP sample was from the same lysate loaded in the first lane. The blot was stained with specific antibodies for RFP2, α-SG and β-actin, used as loading control and to verify the goodness of the IP. The unglycosylated form of V247M α-SG is detectable due to the treatment with 10 µM MG132. The heavy chains of the monoclonal antibody used for the IP were recognized by the murine secondary antibody and are indicated by orange star.

Taking together these data, V247M α-SG might be considered a canonical ERAD-L substrate that needs the HRD1-SEL1L complex but is also supported in its way to disposal by an additional "assistant E3 ligase", RFP2.

To date, the only two substrates reported for RFP2 are unassembled subunits of multimeric complexes (Lerner et al., 2007; Altier et al., 2010). Therefore, RFP2 involvement in the disposal of V247M mutant might be related to this possible peculiar function of the ligase. In fact, α-SG is one of four subunits of the sarcoglycan complex. Further experiments are needed to validate this hypothesis.

Two hands to get out the ER: the AAA-ATPase p97 and Derlin-1

As protein degradation is carried out in the cytosol, misfolded membrane proteins, as α-SG, must be extracted from the ER membrane. Diverse proteins have been proposed to be involved in the retrotranslocation process (including the E3 ligases) but the driving force needed to eradicate most ubiquitinated proteins from the ER seems to be provided by the AAA-ATPase p97 (Ye, 2006; Lilley & Ploegh, 2004). We addressed this topic by using a dominant negative variant (K524A) of p97, a critical substitution in the Walker A domain of p97 that renders non-functional the ATPase domain (Kobayashi et al., 2002; Hanson & Whiteheart, 2005). To verify the involvement of this protein in the retrotranslocation of V247M α-SG, either the wild type p97-GFP or its dominant negative variant were transfected in V247M cells. As demonstrated by western blot and the relative densitometric analysis (Figure 33), the K524A p97 mutant increased V247M α-SG expression delaying its degradation, while overexpression of WT p97 did not modify V247M expression, suggesting that a higher content of p97 does not accelerate substrate degradation.

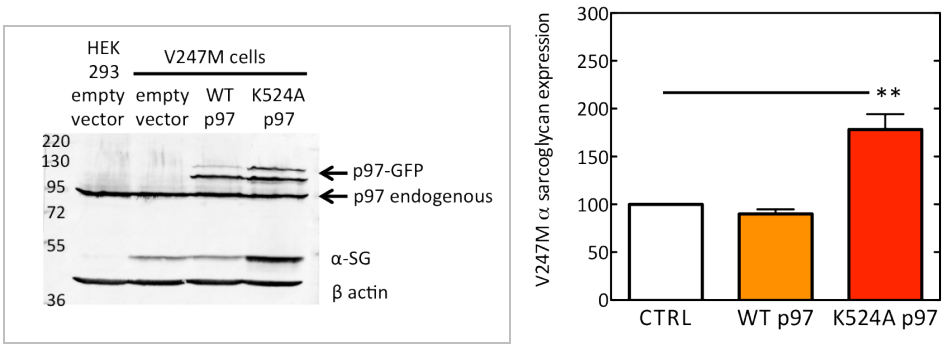


Figure 33. Left: example of western blot of 40 µg of total proteins from HEK-293 cells or V247M cells transfected with the empty vector, WT p97-GFP (WT p97), K524A p97-GFP (K524A p97) the dominant negative form of the AAA-ATPase. Blot was stained with antibodies against p97 α-SG and β-actin antibody. The transfection of WT p97-GFP produces a protein of higher molecular weight compared to the endogenous p97, because of the GFP tag. Above the transfected protein another band is recognized by p97 antibody: this band however is undetectable with GFP antibody (data not shown). Right: densitometric analysis demonstrates that transfection with an inactive form of p97 delayed V247M degradation and results in its accumulation; ** $p<0.01$.

To demonstrate that p97 physically interacts with V247M mutant in the retrotranslocation process, IP experiments were carried out. In the same experiments, the involvement of Derlin-1, known to associates with p97 (Lilley & Ploegh, 2005), was also evaluated. Derlin-1 is proposed to facilitate the exposure of ERAD substrates (Greenblatt et al., 2001) because, as mentioned before, it has a rhomboid architecture that compresses and deforms the lipid bilayer to help the transfer of membrane proteins during retrotranslocation. To initiate dislocation, Derlin-1 recognizes misfolded but not ubiquitinated substrates (Sun et al., 2006). Derlin-1 is in fact an integral ER membrane protein, while p97, a cytosolic protein, can associate with polyubiquitinated species only.

Hence, V247M cells were transfected either with empty vector, wild type p97-GFP, or K524A p97-GFP; untransfected HEK-293 cells were used as control. 48 hours after transfection cells were lysed and proteins subjected to IP with the anti-α-SG antibody. To favour IP, cells transfected with empty vector and with WT p97 were treated with the proteasome inhibitor MG132 to increase the amount of available V247M. As shown in Figure 34, the expression of endogenous p97 and of β-actin was not affected by the diverse transfection experiments (left panel). Transfected p97-GFP appears as a double band of about 130 KDa. Expression of the upper p97-GFP band is variable, being reduced when samples are treated with MG132 and elevated when the mutant p97 is expressed (compare Figure 33 with Figure 34). The reason for the doublet is still under investigation.

Figure 34, right panel, shows that V247M α-SG mutant co-immunoprecipitated with transfected WT p97 and K524A p97, as well as endogenous Derlin-1. Interestingly, V247M α-SG interacts with Derlin-1 even in cells in which the sole endogenous p97 is present (cells tranfected with the empty vector, 2nd lane of the right panel). Unfortunately Derlin-1 co-migrates with the IgG light chains however, the intensity of the band of lanes 2, 3 and 4 is more intense compared to the control sample, indicating that Derlin-1 is effectively co-immunoprecipitated by the α-SG antibody.

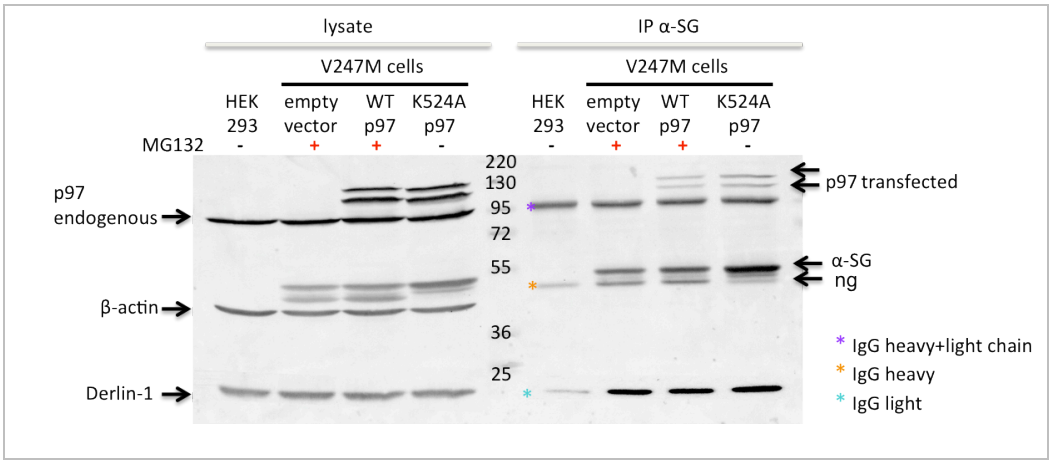


Figure 34. Western blots of 40 µg proteins lysates before immunoprecipitation with the α-SG monoclonal antibody (left panel). In the middle, numbers indicate the molecular weights of pre-stained markers. On the left western blot of proteins immunoprecipitated by the α-SG antibody. V247M cells were transfected either with empty vector, WT p97 or the K524A p97 inactive form and treated where indicated (+) with the proteasome inhibitor MG132 (10 µM). Blots were probed with antibodies specific for p97, Derlin-1, α-SG and β-actin, used as a loading control. ng, indicates the non-glycosylated α-SG that becomes evident when proteasome activity is inhibited. Colored asterisks indicated the immunoglobulins (heavy and light chains) from the monoclonal antibody used for the IP assay that were recognized by the murine secondary antibody.

The immunoprecipitation experiments demonstrate that p97 and Derlin-1 cooperate in the extraction from the ER membrane of V247M α-SG, suggesting that p97 and Derlin-1 form a complex that deforming the bilayer (Derlin-1) and, utilizing ATP as driving force (p97), extract this type I membrane protein from ER.

To confirm the role of p97 and Derlin-1 in the ERAD pathway of V247M α-SG, selected RNAi experiments were also performed. Unfortunately, the shRNAs used in the experiments were ineffective in knocking down p97 and Derlin-1 protein expression. As expected, these shRNAs were also ineffective in the mutant rescue (Figure 35 and Figure 36). Additional experiments with different target sequences for shRNA will be thus necessary.

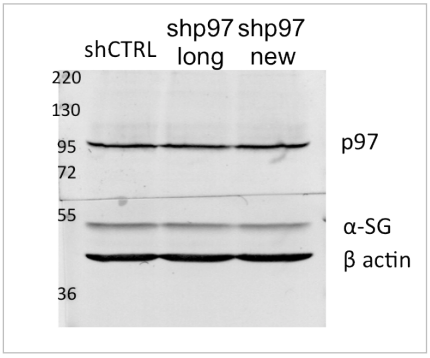


Figure 35. Example of western blot of 40 µg of total proteins from V247M cells transfected with the indicated shRNAs. shp97 long and shp97 new indicate two different shRNAs utilized to target p97. On the left are indicated the molecular weights of pre-stained markers. Blot was stained with specific antibodies that recognize endogenous p97, α-SG and β-actin, used as loading control. It is evident the ineffectiveness of p97 silencing with both constructs. The expression of V247M mutant was unchanged, as expected.

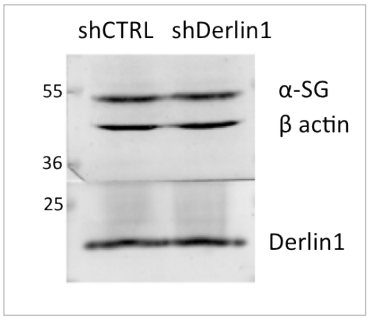


Figure 36. Example of western blot of 40 µg of total proteins from V247M cells transfected with the indicated shRNAs. In first lane proteins from cells transfected with shRNA CTRL, while in the second lane those from cells transfected with shRNA against Derlin-1. On the left are indicated the molecular weights of pre-stained markers. The blot was stained with specific antibodies that recognize Derlin-1, α-SG and β-actin, used as loading control. As shown, the silencing was ineffective and thus the expression of V247M was unchanged.

V247M's nursing chaperones

To guarantee an efficient retrotranslocation, misfolded proteins do not have to aggregate inside the ER. ER-QC chaperones and lectins are competent for both the recognition and protection from aggregation of ERAD substrates. To determine which chaperones are involved into this process, pilote RNAi experiments were carried out on candidate ER proteins. Figure 37 shows that down regulation of GRP94, a chaperone known to deliver misfolded proteins to OS9 and then to the HRD1-SEL1L complex (Christianson et al., 2008), reduces the degradation of V247M α-SG.

I tested the role of BiP, known to prevent aggregation of ERAD substrates and to promote their folding (Hegde et al., 2006), particullary of non-glycosilated misfolded proteins (Hosokawa et al., 2008). BiP acts also on glycosylated misfolded protein in a complex with EDEM and ERdj5 (Hoseki et al., 2010). However, down regulation of BiP was without effects on V247M disposal. Further more efficient RNAi experiments are needed to ascertain or not the involvement of BiP. The down regulation of OS9, a lectin that together with XTP3B escort ERAD substrates to E3 complexes, probably did not influence the level of V247M mutant.

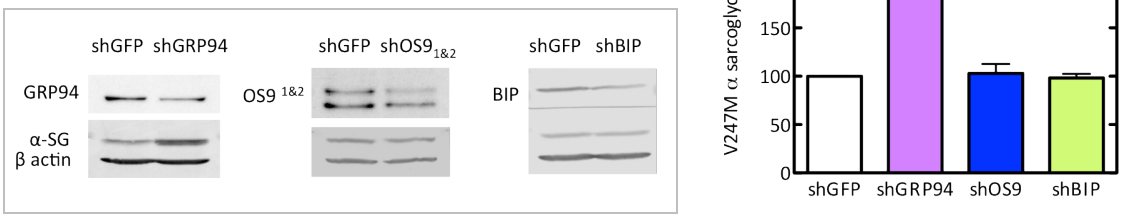


Figure 37. Left: example of western blot of 40 µg of total proteins from V247M cells transfected with the indicated shRNAs. In the first lane transfection was always performed with shRNA CTRL, while the second lane is with shRNA against the indicated ER-QC component (first blot GRP94, second blot OS9, third blot BIP). Blots were probed with antibody specific for GRP94, OS9, BiP, α-SG and β-actin, used as loading control. The bar chart on the left shows the densitometric analysis of western blots. A significative rescue in V247M expression is evident only after GRP94 down regulation; ** $p<0.01$.

OS9 and XTP3-B are known to be interchangeable or even dispensable in the disposal of some ERAD-L substrates (Bernasconi et al., 2010). Moreover it has been proposed that

ERAD substrates utilizing the HRD1 pathway do not share a strict dependence on subnetwork components (Christianson et al., 2011). It is therefore possible that, like for other reported ERAD substrates, the lectin involved in α-SG mutant binding could be XTP3-B or a still unidentified one(s). Additional experiments with more efficient molecular tools are therefore needed to dissect the chaperones and lectins involved in the ERAD pathway of V247M mutant.

Concluding remarks

In summary, these results demonstrate that the V247M α-SG misfolded mutant is recognized by the ER-QC (GRP94 is probably involved) and delivered to degradation through an ERAD-L pathway leaded by the HRD1-SEL1L complex. The UBC6e E2 ubiquitin conjugating enzyme is also a critical component of the complex. Importantly, an additional E3 ubiquitin ligase, RFP2 cooperates in the disposal of V247M α-SG. Dissecting the molecular interaction between these two E3 ligases will represent a stimulating challenge for the future. Retrotranslocation of V24M α-SG needs the fundamental action of the AAA-ATPase p97 and Derlin-1.

Investigating the ubiquitination sites of V247M mutant, we discovered that none of the five lysine residues are sites of ubiquitylation, stimulating the quest to uncover α-SG's cryptic ubiquitination residue(s).

Altogether, these data depicted for the first time the ERAD pathway of a naturally occouring mutant of the type I membrane protein, α-sarcoglycan, and permit to identify numerous potential drug targets for the recovery of such mutant.

SUPPLEMENTARY RESULTS

The fate of a calcium pump: the R164H SERCA1a mutant

Clinical symptoms, genetic and biochemical data indicated that Chianina cattle pseudomyotonia (PMT) is the counterpart of human Brody's disease and the cow represents an ideal non-conventional animal model to investigate the pathogenesis of this rare human disease (Drögemüller et al., 2008).

The goal of this side project was to understand if the loss of SERCA1a protein in PMT is due to degradation by the ubiquitin-proteasome system and if it is the lack of the protein the reason for the disease. The working hypothesis is that the missense mutation causing the disease does not alter the pumping activity of SERCA1a. Consequently, the project was aimed to evaluate wheteher, by preventing mutant degradation, it is possible to rescue both expression and localization of the calcium pump and restore the missing function. To this end, the cDNA of SERCA1b (the neonatal isoform available in the lab) was modified in SERCA1a (adult isoform) and inserted in a pcDNA3 vector (see materials and methods). The R164H mutation was then engineered by site directed mutagenesis.

In the experiments, transfections and treatments were carried out in HEK-293 cells; compared to usual protocols, the amount of DNA transfected was reduced to avoid the formation of high molecular weight aggregates that a massive overexpression could produce. For the same reason, cells were lysed 24 hours after transfection, an adequate time to get a good protein expression.

As shown in Figure 38, the antibody specific for SERCA1 recognizes a protein band of about 110 kDa, present only in cells transfected with wild type and mutated SERCA 1a. Consistent with the reduced level observed in muscles of cows affected by PMT (Drögemüller et al., 2008), expression of R164H missense mutant protein in HEK-293 cells is about one third that of WT protein.

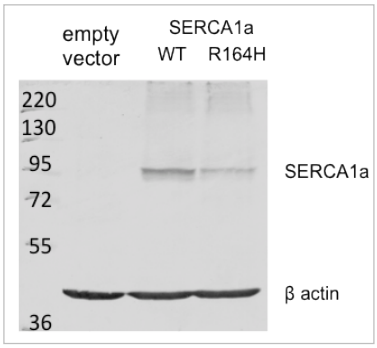


Figure 38. Western blot analysis of proteins from cells transfected either with empty vector, WT SERCA1a or R164H SERCA1a. The blot was probed with antibodies specific for SERCA1 and β-actin, used as loading control. On the left, the molecular weights (kDa) of pre-stained protein markers.

To verify whether the reduced R164H expression is due to degradation by the ubiquitin-proteasome system, the experiment was repeated and, eight hours before cell lysis, the proteasome inhibitor MG132 was added. Figure 39 shows that in the presence of the proteasome inhibitor MG132 there is a significative increase of R164H SERCA1a mutant, at levels comparable to that of WT protein. These data point to a clear involvement of ERAD and the ubiquitin-proteasome system, in the pathogenesis of PMT.

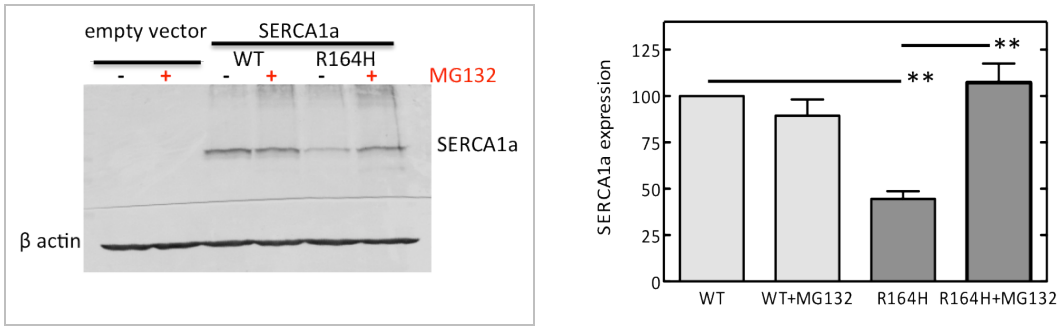


Figure 39. Western blot (left) and densitometric (right) analysis of proteins from cells transfected with empty vector (lanes 1 and 2), WT SERCA1a (lanes 3 and 4) or R164H SERCA1a (lanes 5 and 6), with (+) or without (-) MG132 (10 μM) treatment, to assess the possible role of proteasome in the degradation of R164H mutant. β-actin staining was used as loading control. Right: densitometric and statistical analysis. The expression of WT SERCA1 was not affected by the treatment with MG132, whereas the expression of R164H was significantly increased after proteasomal inhibition; ** $p<0.01$.

The recovery of R164H SERCA1a mutant after MG132 treatment can be also visualized in the immunofluorescence analysis shown in Figure 40. The comparison of confocal immunofluorescence signals in cells transfected with WT (panel a and b) and R164H SERCA1a (panel c and d) clearly evidences a reduced fluorescence intensity in cells expressing the mutant. After proteasomal inhibition, the mean fluorescence intensity of cells expressing R164H SERCA1a (d) increased, reaching an intensity comparable to that of WT (a and b).

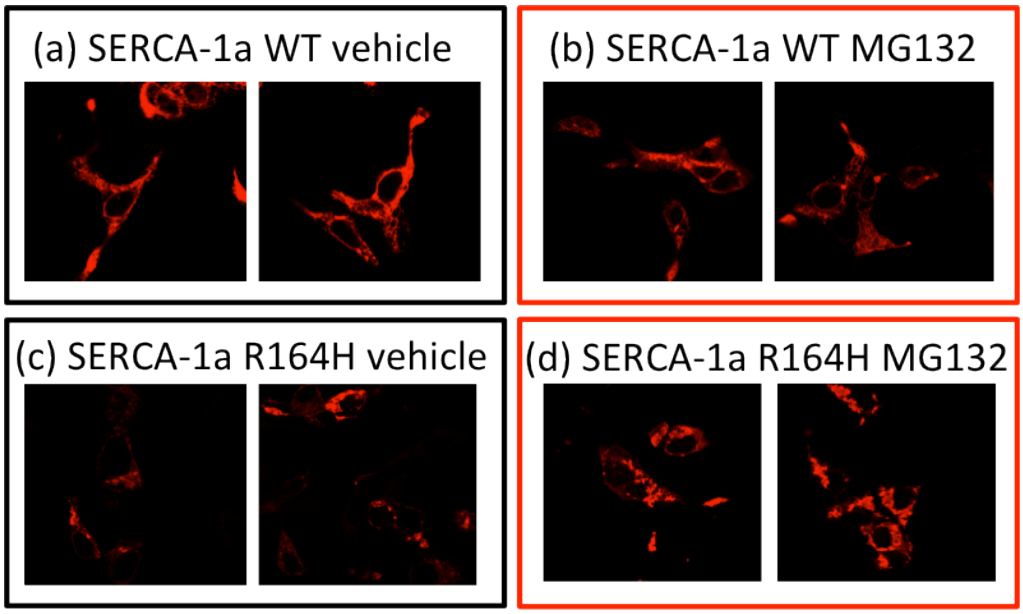


Figure 40. Immunofluorescence performed on permeabilized cells transfected with WT SERCA1a (a and b) or R164H SERCA1a (c and d), cells were treated with MG132 (b and d) or vehicle (a and c). Cells were fixed and stained with SERCA1 specific antibody. The secondary antibody was TRITC-conjugated.

To confirm the ER localization of SERCA1a, as suggested by the distribution of the immunofluorescence signal in Figure 40, cells transfected with SERCA1a were immunodecorated with specific antibodies to SERCA1 and calreticulin (CRT), a luminal ER

marker. Figure 41 shows that SERCA1a protein (red fluorescence), expressed by the two cells, nicely co-localized with calreticulin (green fluorescence), as demonstrated by the overlapping yellow signal.

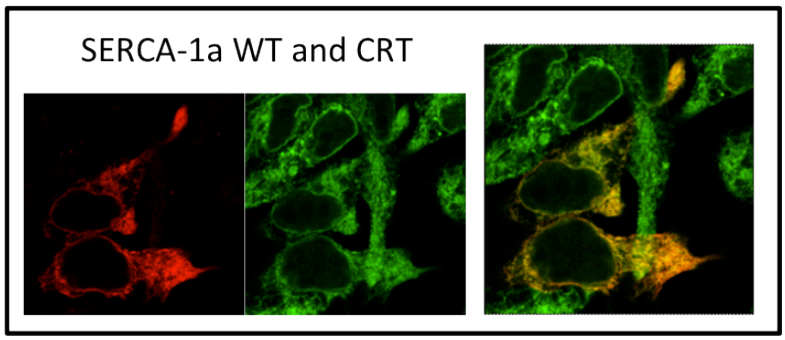


Figure 41. Immunofluorescence staining performed on permeabilized cells transfected with WT SERCA1a. Cells were fixed and then stained with the specific antibody against SERCA1 and calreticulin (CRT), a secondary antibody TRITC-conjugated was used to localize SERCA1a while a FITC-conjugated antibody was used to localize CRT. A yellow signal, derived by the overlapping of green (FITC) and red (TRITC) signals, denotes proteins co-localization.

Cell fractionation experiments on transfected HEK-293 cells, conclusively confirmed the correct localization of both WT and R164H SERCA proteins in the ER fraction (data not shown, experiments carried on by R. Sacchetto).

To verify whether the rescue of R164H mutant also permits the recovery of its function, a number of experiments were performed to evaluate the calcium pumping activity of SERCA1a mutant. To this end, cells transfected with WT and R164H SERCA proteins were co-transfected with the cytosolic Ca^{2+}-sensitive photoprotein Aequorin (AEQcyt). Figure 42 reports the peaks of ER calcium release, upon 500 nM carbachol stimulation, from cells transfected with the different constructs and treated or not with MG132. The ER calcium release from cells transfected with WT SERCA1 (with or without MG132) was significatly lower compared to that of cells transfected with the empty vector. This is probably the consequence of the faster re-entry of calcium into ER because of the additional pumping activity provided by the transfected SERCA1a. On the contrary, the peak of ER calcium release from R164H mutant transfected cells, did not significantly differ from that observed in control cells. This result might be explained by either an impaired calcium pump activity of the SERCA mutant or loss of activity due to the reduced amount of the mutant (Figure 38). However, interestingly, proteasomal inhibition determined a strong reduction of cytosolic calcium in carbachol-stimulated cells expressing R164H SERCA1, at levels comparable to those of cells expressing WT SERCA. Experiments and analyses were performed by M. Brini's group.

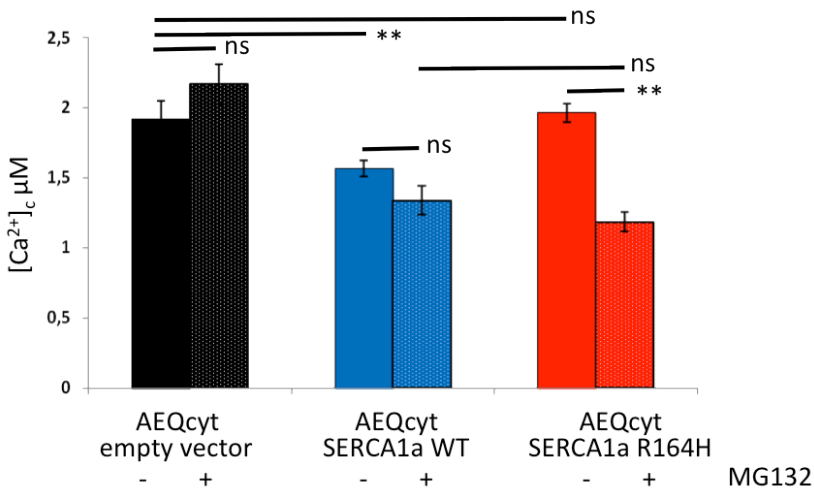


Figure 42. Cytosolic calcium measurements performed on cells transfected with cytosolic aequorin (AEQcyt) and either with empty vector, WT SERCA1a, or R164H SERCA1a. Cells were treated with vehicle (-) or MG132 (+). Release of calcium was stimulated by challenging cells with 500 nm carbachol. ns, not significant; ** $p<0.01$.

Taken together, these results strongly suggest the involvement of the ubiquitin-proteasome system in the disposal of SERCA1a mutants and provide new information on the pathogenetic mechanism of Pseudomyotonia, thus including the disease in the growing list of the UPDs. More interestingly, data demonstrate that proteasomal inhibition allows the rescue of the mutant protein to normal values and, more importantly, the protein mutant maintains its calcium pump activity. Even though obtained in a cellular model, these data are very promising for the future development of therapeutic approaches to treat Pseudomyotonia and Brody's disease.

MATERIALS AND METHODS

All reagents, if not differently specified, were purchased from Sigma Aldrich.

Cells culture

HEK293 were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen), and then maintained in a humidified 5% CO_2 atmosphere at 37°C. Every 2 days, when at 80% confluence, cells were diluted. To do this, the culture medium was removed, cells washed once with PBS 1X (140 mM NaCl, 6.4 mM Na_2HPO_4, 2.7 mM KCl, 1.76 mM K_2HPO_4, pH 7.4) and then detached by trypsin solution (trypsin 0.05% in PBS) addition. Trypsinization occurs in a couple of minutes after which culture medium was added to stop the enzymatic activity and cells were centrifuged at 800 rpm for 5 minutes. Cell pellet was subsequently resuspended in an appropriate volume of medium and then seeded at the required dilution. To determine cell concentration a Burker counting chamber was used.

When needed, cells can be frozen. After trypsinization and centrifugation, cell pellet was resuspended in cold Freezing Medium (10% DMSO for cell culture, 90% heat inactivated FBS), placed in freezing vials, stored at -80°C ON (overnight) and then long-term stored in liquid nitrogen.

Transfections

For transfection experiments, were seeded in plastic Petri dish at 50.000 cells/cm^2, and transiently transfected the day after with Transit293 (MirusBio) following manufacturer's instruction. Briefly, for 6 well plate, 4 µl of Transit were added to 200 µl of DMEM mixed and incubated at room temperature for 5 minutes. 2 µg of plasmid DNA were added, vortex again and incubated 30 minutes. The mixture was then added drop wise to the cell culture.

Cells were subjected to immunoblotting or immunofluorescence staining at least 48 hours after transfection.

A cell population stable expressing V247M α-SG was selected by supplementing culture medium with 2.5 mg/L of G418 (Calbiochem) in PBS 1X for 3 weeks. The selected cells have been used in chosen experiments.

Cells' treatments

Cells were treated, when indicated, 40 or 44 hours after transfection depending on the length of treatment and then lysed at 48 hours. Compounds at the working dilution are: 10 µM MG132 in DMSO for 8 hours to block the proteasome, 40 µg/ml cycloheximmide in DMSO for 4 hours to inhibit protein neosynthesis, 100 nM Bafilomycin A in DMSO for 8 hours to prevent the maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes, 20 µg/ml leupeptin in ddH_2O for 8 hours to inhibit lysosomal proteases and accumulate autophagosomes.

Plasmids

V247M α-SG plasmid construct was generated as previously described (Gastaldello S. et. al., AJP 2008 and Gastaldello S's PhD thesis).

V247M α-SG K>R mutants were generated by site-directed mutagenesis as described in the next paragraph.

Murine p97-GFP and K524A p97-GFP mutant in pCMX-AFAP vector was provided by R. Piccirillo; human HRD1 and HRD1-C1A mutant in pcDNA3.1 vector were from E. Wiertz; murine gp78 and C337S-C374S gp78 (RFM) mutant in pcDNA3.1 vector from K. Nagata; human UBC6e and C91S UBC6e in pcDNA3.1 vector from H.L. Ploegh; human Δ131-RFP2 mutant in pcDNA3.1 vector from G.W. Zamponi. Human RFP2 was provided in pUC57 vector, the cDNA of the protein cut with EcoRI and BamHI and then sub-cloned in pEGFPN1 vector, in frame with GFP.

shRNA plasmids were constructed, as described below, in pSUPER vector. P. Ballar provided shRNA against human gp78 and human gp78-HA protein used to confirm the effectiveness of silencing.

Rabbit SERCA1b (neonatal) in pMT2 vector, gift of D.H. MacLennan, was sub-cloned in pcDNA3 and manipulated by mutagenesis PCR to obtain SERCA1a (adult) and by site directed mutagenesis to obtain the mutant form containing the R164H amino acid substitution. cytAEQ-HA was a gift of P. Pizzo.

All these plasmid constructs were confirmed by sequencing (BMR genomics).

DNA preparation and manipulation

To check the goodness and correctness but also to remove or insert a fragment in a plasmid, restriction reactions were performed. All used enzymes were purchased from Promega and reactions occurred in the equipped buffer diluted at 1X using 1 U of each enzyme for 1 µg of plasmid DNA. When more then one enzyme was used, the compatibility of the reaction buffer and temperature was checked, if they were not compatible, a two-step reaction was performed. After carrying out the first digestion, the reaction products should be purified (Qiaquick PCR purification kit) following manufacturer's instruction, and then the second digestion was performed. The reaction was stopped adding 1X final concentration of loading buffer (1 mM TRIS, 10 mM EDTA, 5% glycerol w/v, 0.2% xylene cianol and bromophenol blue). To separate by size the digested DNA a 1% agarose gel electrophoresis was performed and 100 bp and/or 1Kb DNA marker (Fermentas) used to compare sample molecular weights. Agarose was dissolved, jellified and then the electrophoresis performed in TAE 1X buffer (2.93 mM TRIS, 2.3 mM sodium acetate, 2.31 mM EDTA disodium salt, 1.12 ml glacial acetic acid, pH 8) applying a current of 150 mA for 1 hour. To visualize DNA with the ultraviolet light, 0.5 µg/ml of ethidium bromide was added into the agarose solution before jellification as a fluorescent dye of DNA. After run, if a fragment of the digested DNA had to be subsequently cloned in another vector, the cut DNA was extracted from agarose exciding the selected agarose band with a blade an hence purified with QIAquick gel extraction kit (QIAGEN) following manufacturer's instruction.

To construct shRNA plasmids the two complementary oligonucleotides were synthetized (MWG), resuspended in TE buffer (10 mM TRIS, 1 mM EDTA pH 7.5), denatured by heating in water at 95°C for 3 min and then annealed by slowly cooled water to 25°C. Phosphorylation of 100 ng of the annealed oligos was done using 10 U of T4 PNK (Promega) in 1X buffer supplemented with 10 mM MgATP for 1 hour at 37°C. The annealed and phosphorylated oligos (dsDNA fragment) were diluted and ligated into the pSUPER vector previously linearized with BglII and HindIII and dephosphorylated.
Dephosphorylation of DNA vector was carried out using 5 µg of plasmid DNA and 20 U of Alkaline Phosphatase (Roche) in 1X buffer in 100 µl final volume. Ligation reaction was done with a molar ratio of 1 : 2 of dephosphorylated vector to DNA fragment at 16°C ON.
To create shRNA the sense and antisense sequences (underlined in table 3) of each target were linked by a standard TTCAAGAGA loop structure for mammalian cells.

TARGET	Sequence and its complementary, that will form the hairpin, are underlined
HRD1 (A)	GATCCCCGGAGACTGCCACTACAGTTGTTTCAAGAGAACAACTGTAGTGGCAGTCTCCTTTTTA
HRD1 (C)	GATCCCCGAGACAGTTTCAGATGATTTTCAAGAGAAATCATCTGAAACTGTCTCTTTTTA
SEL1L	GATCCCCGGCTATACTGTGGCTAGAATTCAAGAGATTCTAGCCACAGTATAGCCTTTTTA
$OS9_{1\&2}$	GATCCCCCATCATCCAGGAGACAGAGTTCAAGAGACTCTGTCTCCTGGATGATGTTTTTA
GRP94	GATCCCCGGCTCAAGGACAGATGATGTTCAAGAGACATCATCTGTCCTTGAGCCTTTTTA
Bip	GATCCCCCCTTCGATGTGTCTCTTCTTTCAAGAGAAGAAGAGACACATCGAAGGTTTTTA
p97 (long)	GATCCCCGGGCACATGTGATTGTTATTTCAAGAGAATAACAATCACATGTGCCCTTTTTA
p97 (new)	GATCCCCAGGGCACATGTGATTGTTATGTTCAAGAGACATAACAATCACATGTGCCCTTTTTTA
GFP (ctrl)	GATCCCCGAAGTCGTGCTGCTTCATGTTCAAGAGACATGAAGCAGCACGACTTCTTTTTA

Table 3. The oligonucleotides used to create shRNA.

To obtain α-SG V247M K>R mutants site directed mutagenesis was performed using QuikChange II site directed mutagenesis kit (Stratagene) following manufacturer's instruction. Primers between 16 and 24 bases in length were designed with the mutation in the middle with a T_m greater than or equal to 78°C. The formula used for estimating the T_m that should be grater than or equal to 78°C was 81.5 + 0.4 (n°GC) − 675/N, in which N is the primer length in bases and does not include the bases which are being changed.
Reaction mixture used was composed by 2 ng of supercoiled DNA, 5 µl buffer 10X, 0.2 mM of provided dNTPs, 150 ng of each primer resuspended in TE buffer, 5 U of PfuTurbo® DNA polymerase and ddH_2O to reach 50 µl. The reaction cycles performed were: 95°C for 30'' and then the repetition for 12 times of the scheme 95°C for 30'', 55°C for 1', 68°C 1 minute/Kb of plasmid length. At the end of the amplification the reaction was kept on ice and then 10 U of DpnI enzyme added to digest supercoiled metilated DNA. Bacteria were then transformed as described in the next paragraph to obtain mutated DNA.
Oligonucleotides and its complementary sequence (mutated bases introduced are underlined) to perform mutagenesis are in table 4.

TARGET	Sequence
K193R	GGGCCGAAGGGAAGGG
K199R	GTATACATTAGGGTGGGTT
K211R	CTTGCCTGAGGATGGTG
K252R	GGTGGATAGGTCAGTGC
K321R	AGGGAAGGCTGAGGAGAGACCTGG
C313A	CTATGTCATGGCCTGCCGGCGG
C326A	GACCTGGCTGCCTCCGAC
S327A	CTGGCTACCGCCGACATCC
T326A with S327A	GACCTGGCTGCCGCCGACATCC
T367A	GCTCACCTGCGTGCACATTG

Table 4. The oligonucleotides used to perform mutagenesis of V247M α-SG.

Each mutation was carried out in the smaller pBSK$^+$ vector and after sequencing the mutated fragment was cut and re-inserted into well-known pcDNA3 mammalian expression vector.

To produce single point mutants of K193R, K199R, K211R, K252R, pcDNA3 vector containing V247M α-SG was cut with BamHI: obtained BamHI fragment was sub-cloned in pBSK$^+$ vector. To obtain K321R, C313A, T326A, S327A, T326A-S327A and T367A mutated protein, mutagenesis was performed on EcoRI-XbaI fragment of pcDNA3 α-SG V247M inserted into pBSK$^+$.

To generate K199R-K211R mutant, the K211R mutagenesis was performed on pBSK$^+$ containing K199R mutated BamHI fragment. To construct K199R-K211R-K252R mutant, mutagenesis was performed on pBSK$^+$ containing K199R-K211R mutated fragment with K252R oligos. To construct 4K mutant K193R oligos were used on K199R-K211R-K252R mutated cDNA. To obtain the 5K mutant an EcoRI-BamHI restriction was performed on pBSK$^+$ BamHI fragment containing K193R-K199R-K211R-K252R mutations. This fragment was ligated into pBSK$^+$ containing the EcoRI-XbaI fragment with K321R point mutation after the digestion with EcoRI-BamHI. All the fragments containing one to five mutations were re-ligated in V247M pcDNA3.

To obtain SERCA1a cDNA, from the gifted vector containing SERCA1b, the EcoRI restriction fragment was inserted in the well-known pcDNA3 vector previously cut with EcoRI and dephosphorylated. To determine the orientation of the single cut EcoRI fragment cloned in pcDNA3, a digestion with KpnI was performed. The correctly orientated cDNA was then sequenced. Unrelated sequences at both the 5' and the 3' ends were present, containing a long stretch of poly G at 5' end and of poly T at 3' end. To eliminate these unwanted residues and change SERCA1b in 1a, two Polymerase Chain Reactions (PCR) were performed: one on the first 1800 bp of the cDNA and the other on the last 1200 bp. Used primers for the first PCR were HEADfwd_CAAGAAGCTTGCGCA*ATG*GAG and HEADrev_AGCTGGATGGAGCCCATG. The HEADfwd primer was designed to insert a HindIII restriction site (underlined) just before the *ATG* site, to facilitate the re-cloning and HEADrev primer to anneal several

nucleotides downstream the SphI restriction site. Used primers for the PCR performed on the last 1200 bp were TAILfwd: ATCCTCTCCGTGATCAAGGAG and TAILrev: CTTTCGAATTCTTAACCTTCCAGGTAGTTCC. The TAILfwd primer annealed upstream the SphI restriction site while the TAILrev primer has been designed to anneal from the triplet 997. This primer changed E994G and transformed triplet 995 in a stop codon, eliminating the last seven amino acids present in SERCA1b but in the SERCA1a form.
To reconstruct the SERCA1a cDNA both PCR fragments cut respectively with HindIII/SphI and SphI/EcoRI, were cloned by two steps in pBS^+ vector. After complete sequencing the SERCA1a cDNA was then extracted and ligated in HindIII/EcoRI pcDNA3. To generate R164H single point mutant, HindIII/Kpn fragment was removed from pBS^+ vector containing the entire SERCA cDNA and inserted in $pBSK^+$ were the mutagenesis, with CTGCAGACATCCACATCCTGTCTATC and its complementary sequence, as previously described, was performed.

Bacteria

Bacteria were the host in which the DNA plasmid, used for the transfection of human cells, were replicated and also conserved. Bacteria can acquire the competence to take up extracellular DNA from the environment. *E. Coli* XL1-blue competent for electroporation and calcium chloride competent cells were prepared from previously acquired cells stored at -80°C (XL1-blue super competent cells, Stratagene). Electrocompetent cells were used after ligation because of their higher capability to uptake DNA, while for routinely procedures calcium chloride competent cells were chosen.

For both preparation cells were initially plated on a LB agar plate (8 gr Tripton, 5 gr Yeast extract, 5 gr NaCl, 15 gr Agar (from Sacco), and ddH_2O to reach a final volume of 1 L) and incubated at 37°C overnight. The next day, a colony was picked up and suspended in 5 ml of LB (same recipe of the solid medium, without Agar) and the tube placed at 37°C under 220 rpm rotation in the GallenKamp ON. Day after, 3 ml of this mixture was added to 300 ml of LB and then incubated at 37°C under rotation to reach an OD (optical density) of 0.5 at 550 nm. Bacteria were then incubated on ice for 15 minutes and pelleted at 3500 rpm for 15 minutes at 4°C.

For electrocompetent cells the supernatant was discarded and the bacteria resuspended in the same volume of cold sterile ddH_2O. The pellet obtained by the next centrifugation at 3500 rpm, 4°C for 15 minutes was resuspended in 150 ml of cold sterile ddH_2O. The bacteria were centrifuged again at 3500 rpm 4°C for 15 minutes and the pellet resuspended in 6 ml of cold sterile 10% glycerol. The next centrifugation was done at 4000 rpm 4°C for 15 minutes and the pellet resuspended in 0.6 ml of cold sterile 10% glycerol. Finally 40 µl of the obtained suspension was aliquotated into sterile tubes, frozen in liquid nitrogen and stored at -80°C until use.

To prepare calcium chloride competent cells after the incubation on ice and first centrifugation the supernatant was discarded and the bacteria resuspended in 150 ml of sterile and cold 0.1 M $CaCl_2$ and incubated 30 minutes on ice. The pellet obtained by the next centrifugation at 3500 rpm, 4°C for 15 minutes was resuspended in 6 ml of sterile

and cold 0.1 M $CaCl_2$. The bacteria were then incubated on ice ON. The day after 1.2 ml of cold glycerol was added and gently stirred: 200 µl of the mixture was finally aliquotated into sterile tubes, frozen in liquid nitrogen and stored at -80°C until use.

To transform electrocompetent bacteria, the electroporation was performed with 100 to 500 ng of dialyzed DNA (0.025 µm membrane, Millipore). The electric shock used was 1500 V, 50 mA, 50 W, 50 mF and 150 mΩ. After that, bacteria were quickly suspended in 800 µl of SOC non-selective medium (200 µl 20% glucose, 100 µl 1 M $MgCl_2$, 1 M $MgSO_4$ in LB medium to a final volume of 10 ml) and recovered at 37°C under rotation at 220 rpm for 1 hour.

To transform calcium chloride competent cells, bacteria should be incubated on ice with 100 to 500 ng of DNA for 40 minutes and mildly stirred every 10 minutes. The bacteria suspension was then subjected to 42°C heat for 90 seconds: after this procedure 800 µl of SOC was quickly added and they were recovered at 37°C under rotation at 220 rpm for 1 hour.

For both transformations, after recover, bacteria were plated on pre-warmed LB-agar plates containing selective antibiotic (Ampicillin 100 µg/ml or Kanamycin 30 µg/ml, both in ddH_2O, depending on the resistance present in the plasmid) and let them grow ON. The day after only bacteria in which the transformation occurred would grown on plate and so single colonies can be picked up and incubated ON in LB with selective antibiotic at 37°C under 220 rpm. The following day plasmid DNA can be extracted from bacteria using QIAprep Miniprep Kit (QIAGEN) and analyzed. After verifying, with restriction and sequence analysis, that the plasmid contained in the bacteria holds the proper DNA, bacteria can be grown in a large vessel to obtain ~~a~~ huge large amount of DNA by using QIAprep Midi or Maxiprep Kit. At the end of the preparation, DNA was quantified using a spectrophotometer (Eppendorf, BioPhotometer Plus) and its purity evaluated: the ratio 260/280 should be between 1.8-2 and the ratio 260/230 should be between 2 and 2.2.

Proteins' extraction and quantification

At least 48 hours after transfection, cells were washed twice with PBS 1X and then lysed with an appropriate amount of lysis buffer: about 200 µl was used for each well in a 6 well plate, this volume was scaled up proportionally when needed.

For total protein lysate, cells were lysed with DOC 5% w/v (Calbiochem) in ddH_2O supplemented with Complete Protease Inhibitor (Roche). Cells were scraped and the lysate recovered in a tube. Deoxycholic acid is a strong detergent, capable to destroy all cells' membranes, including the nuclear one. Free DNA in the sample, that renders solution too viscous to be quantified and to worked with, can be mechanically fragmented by passing the solution through a 0.45 µM needle.

To perform immunoprecipitation experiments it's fundamental to preserve the protein-protein interaction. To this aim cells were lysed with 50 mM TRIS pH 7.4, 150 mM NaCl, 5 mM EDTA pH 7.4, 2 mM NEM in EtOH and either triton X100 1% or NP40 1% with DOC 0.5%, supplemented with Complete Protease Inhibitor. Subsequent analyses were

performed using the ON soluble fraction after centrifugation at 14000 rcf, 4°C, 20 minutes.

In order to quantify protein lysate, the BCA protein assay kit (Thermo Scientific) was used according the manufacturer instructions. The BCA protein assay is a colorimetric, fast and sensitive microplate test in which it is possible to compare samples concentration to increasing amount of Bovine Serum Albumine (BSA) in a standard curve.

Electrophoresis and western blotting

To assess protein expression and modulation a SDS polyacrylamide gel electrophoresis (PAGE) was performed. With this technique it is possible to separate proteins by size: samples were supplemented 1X with Laemmli loading solution (5X solution: 0.3 M TRIS pH 6.8, 25% β-mercaptoethanol v/v (PLUSone), 11.5% SDS w/v (Biochemical), 50% glycerol w/v, 0.02 % bromophenol blue) in order to achieve denaturation and S-S reduction, then loaded into a gel and the electrophoresis was hence performed. For this purpose a 4% "stacking" gel in which the comb was inserted was poured on the top of a 10% "running" gel. Recipe used to prepare two stacking gels was: 0.5 ml of a 40% solution of acrylamide:bisacrylamide 29:1 (BDH), 1.25 ml of solution M (1.5 mM TRIS pH 6.8, 0.4% SDS w/v), 0.92 ml of 50% glycerol w/v, 2.35 ml of ddH_2O, 33.5 µl of 10% APS w/v, 8.5 µl of TEMED (Jannsen). To make two running gels the mixture was composed by: 2.5 ml of a 40% solution of acrylamide:bisacrylamide 29:1, 2.5 ml of solution L (1.5 mM TRIS pH 8.8, 0.4% SDS w/v), 3.75 ml of 50% glycerol w/v, 1.25 ml of ddH_2O, 25 µl of 10% APS w/v, 10 µl of TEMED. 40 µg of total lysate of each sample was loaded in the wells produced by the comb and gels were run in a Running Buffer composed by 25 mM TRIS, 192 mM glycine for electrophoresis, 0.1% SDS w/v. A pre-stained protein marker (Protein Sharpmass V prestained 11-250 kDa, Euroclone) was always loaded to monitor the migration process and allow, by comparison, an approximate molecular weight estimation of the separated proteins.

Electrophoresis was carried out at constant voltage of 60 V during the focusing, and then the voltage was doubled during the running phase. After run, samples were blotted on a 0.45 µm nitrocellulose filter to perform further analysis. For this purpose, gel should be unassembled and soaked for a couple of minutes in cold Transfer Buffer (25 mM TRIS, 192 mM glycine, 10% methanol v/v purchased from Sigma). In the meantime the transfer "sandwich" was prepared assembling the transfer cassette, a 3MM Whatman paper wetted with Transfer buffer, the nitrocellulose, the gel and again the wetted paper: the transfer cassette can thus be closed. Bubbles between the membrane and the gel should be carefully removed, in order to obtain perfect proteins transfer.

The transfer was achieved inserting the transfer cassette with the membrane facing the positive pole in the Transfers cell (BIO-RAD) in Transfer Buffer and applying 500 mA constant current for 2 hours at 4°C. After transfer, the nitrocellulose filter was stained with Ponceau Red solution (0.2% Ponceau S w/v, 3% TCA w/v). This is a rapid and reversible stain used to detect the effectiveness of protein transfer: after a rinse with ddH_2O protein bonds would appear red on a white background. The stain could be

removed with water and the membrane used for subsequent analysis through immunodetection.

Proteins immunoprecipitation

To isolate α-SG and proteins that are able to bind and interact with protein of interest an immunoprecipitation assay was performed. To this aim, 300 µg of the soluble fraction lysate were incubated ON while tumbling at 4°C with the specific antibody. The following day 35 µl of Protein G-magnetic beads were added and the mixture incubated 1 hour in the same condition. Beads were spun and extensively washed with the same lysis buffer, finally aspirated to dryness. Laemmli buffer was added 1X directly on the beads: samples were then ready to be loaded on the gel, and a standard western blotting was performed.

Antibodies and immunodetection

Nitrocellulose membrane was saturated for 1 hour RT (room temperature) in TBST (20 mM TRIS, 150 mM NaCl, pH 8) supplemented with 0.1% Tween 20 v/v and 10% milk (Weight Watchers) to prevent nonspecific binding of the antibody. All the primary antibodies were diluted at their own working concentration in TBST with 5% milk. The incubation with primary antibody was always done at 4°C, ON, under gently shaking. A number of primary antibodies were used, alone or together, to reveal specific proteins.

α-SG antibody was purchased from DBA and diluted 1:400; OS9, Derlin-1, p97, BIP, GFP, UBC6e and gp78 were obtained from Abcam and diluted respectively to 1:1000, 1:1000, 1:2000, 1:400, 1:10000, 1:500; 1:500; β-actin, HRD1, SEL1L from Sigma and used at 1:30000, 1:2500, 1:2500 dilution; anti RFP2 were acquired from Santa Cruz Biotechnology and used both at 1:200; anti mono and polyubiquitinylated conjugates clone FK2 was from Enzo Life Science and its working dilution was 1:1000; GRP94 was a generous gift from L. Gorza, used at 1:3000. SERCA1a was purchased from Biomol and used 1:5000.

After ON incubation with the primary antibody the blot should be washed 5 times at RT for 5 minutes with TBST with 5% milk and then the secondary antibody can be added and incubated. Secondary antibodies were anti mouse and rabbit labeled with Alkaline Phosphatase (AP) or Horse Radish Peroxidase (HRP), all used at a working dilution of 1:10000 in TBST 5% milk and incubated at RT for 1 hour. At the end of the incubation time, the membrane should be washed 5 times at RT for 5 minutes with TBST with 5% milk and then the signal can be develop. To detect the signal of the two different conjugated secondary antibodies, different procedures were done depending on the antibody used. To develop AP signals an additional rinse with AP Buffer (0.1 M TRIS, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) was done to allow the alkalization of the membrane and therefore permit the activity of the enzyme. After AP buffer removal, blots were incubated with the staining solution (5 mg BCIP dissolved in 100 µl of DMF and 1 mg NBT in 30 ml of AP Buffer) for several minutes. To stop the reaction washing with ddH_2O was done.

To develop HRP signals an additional rinse with TBST was necessary to remove excess milk. The membrane was then incubated with a ratio of 1:1 of the two reagents of the LiteAblot Plus kit (Euroclone). Its principle relies on the enzymatic conversion of a luminol-like molecule by horseradish peroxidase (HRP) that generates light in the presence of hydrogen peroxide. This method was used when higher sensitivity was required.

The Re-Blot Plus Western Blot Mild Antibody Stripping Solution (Millipore) was used to remove antibodies from membrane that have been developed with chemiluminescence to re-incubate the membrane with other antibodies. Provided solution is 10X concentrated and should be diluted at 1X with ddH_2O and then used to submerge blot. After 15 minutes incubation with gentle mixing, stripping solution was removed and the membrane washed 5 times for 5 minutes each with the saturating solution and finally probed with next antibody.

Immunofluorescence and confocal microscopy

To perform immunofluorescence (IF) 50000 cells were seeded on 13 mm diameter cover glass that was previously threated for 30 minutes with Poly-L-Lys 20 µg/ml in ddH_2O in a 24 well plate. Poly-L-Lys treatment was performed to coat plates to improve cells attachment. After transfection, the immunofluorescence staining can be performed. Cells were washed twice with PBS 1X and then fixed with 4% paraformaldehyde (PFA, purchased from BDH) in PBS 1X for 15 minutes at RT. PFA fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid lysine. This procedure was carried out to preserve the sample as close as to living tissue as possible preventing autolysis, and also to attach permanently cells on the glass on which the IF was carried out. To block the reaction that, if protracted, could prevent antibody binding chemically masking the recognition site, cells were washed twice with PBS 1X and incubated 15 minutes at RT with 50 mM NH_4Cl. To ensure free access of the antibody to its antigen, cells were permeabilized with 0.5% Triton X-100 v/v in PBS 1X. The primary antibody staining was performed by dilution in 1% BSA in PBS 1X and incubating for 2 hours at RT. The excess of primary antibody was removed through 3 washes with PBS 1X and then the secondary antibody incubation was performed. A Tetramethyl Rhodamine Iso-Thiocyanate (TRITC) conjugated secondary antibody was used, diluted 1:200 in 1% BSA in PBS 1X, and incubated 1 hour at RT. Also Fluorescein Iso-Thiocyanate (FITC) conjugated secondary antibody was used, diluted 1:200 in 1% BSA in PBS 1X, and incubated 1 hour at RT. The two antibodies were used together in selected experiments. After incubation the excess of secondary antibody was removed through 3 washes with PBS 1X and cover glasses can finally mounted on a microscope slide taking advantage of a Mounting Medium (Dako) that reduce fading of immunofluorescence during microscopy. TRITC dye, labeled to secondary antibody, can be excited at 546 nm and the confocal microscope Leica SP3 can therefore detect its emissions with a peak around 576 nm while FITC dye labeled to secondary antibody, can be excited at 494 nm and its emissions peak around 518 nm.

Cytosolic calcium measurements

Aequorin is a Ca^{2+}-sensitive photoprotein derived from a marine organism, the jellyfish Aequorea victoria (Shimomura et al., 1962). Aequorin is composed of an apoprotein that includes three high- affinity Ca^{2+}-binding sites and a hydrophobic prosthetic group, coelenterazine. Ca^{2+} binding causes the rupture of the covalent link between the apoprotein and the prosthetic group, a reaction associated with the emission of one photon. The rate of this reaction depends on the Ca^{2+} concentration to which the photoprotein is exposed. Thus, the Ca^{2+} concentration of a sample can be calculated from luminescence data (Chiesa et al., 2001). The reconstitution in the active form was made adding the prosthetic group coelenterazine just before measurements to permit the reaction as in Figure 43.

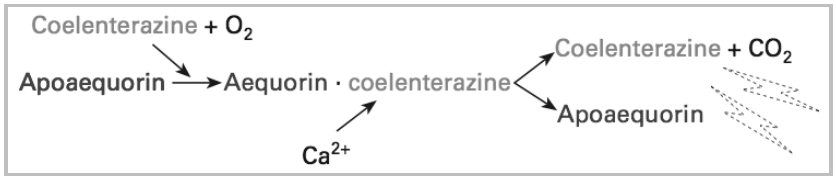


Figure 43. The aequorin reaction and reconstitution (Chiesa A et al., 2001).

During the experiments the cells were stimulated with an agonist, carbachol, to allow the mobilization and release of calcium from the intracellular stores. Application of stimuli induces a Ca^{2+} transient, which peaks and then gradually declines thanks to the action of the Ca^{2+} pumps and the cytosolic buffering proteins. At the end of each experiment, cells were lysed and calcium signals from cells transfected with empty vector, WT SERCA1a or R164H SERCA1a mutant, were compared.

To perform calcium measurement 50000 cells were seeded on 13 mm diameter cover glass that was previously threated for 30 minutes with Poly-L-Lys 20 µg/ml in a 24 well plate. The transfection with the aequorin targeted to the cytosol (cytAEQ) was performed with empty control vector pcDNA3, with WT SERCA1a or R164H SERCA1a in a 1:1 ratio. 6 hours before calcium measurements, cells were treated with proteasome inhibitor MG132, then calcium measurements were performed by M. Brini's group as reported in Brini, 2008.

Statistical analysis

Data from at least three independent experiments are expressed as ± SEM. Statistical differences were determined by unpaired 2-tailed Student's t-test or ANOVA (using GraphPad Prism 5), * for $P<0.05$, ** for $P<0.01$.

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