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SGCE missense mutations that cause myoclonus-dystonia syndrome impair ε-sarcoglycan trafficking to the plasma membrane: modulation by ubiquitination and torsinA

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Myoclonus-dystonia syndrome (MDS) is a genetically heterogeneous disorder characterized by myoclonic jerks often seen in combination with dystonia and psychiatric co-morbidities and epilepsy. Mutations in the gene encoding ε-sarcoglycan (*SGCE*) have been found in some patients with MDS. *SGCE* is a maternally imprinted gene with the disease being inherited in an autosomal dominant pattern with reduced penetrance upon maternal transmission. In the central nervous system, ε-sarcoglycan is widely expressed in neurons of the cerebral cortex, basal ganglia, hippocampus, cerebellum and the olfactory bulb. ε-Sarcoglycan is located at the plasma membrane in neurons, muscle and transfected cells. To determine the effect of MDS-associated mutations on the function of ε-sarcoglycan we examined the biosynthesis and trafficking of wild-type and mutant proteins in cultured cells. In contrast to the wild-type protein, disease-associated ε-sarcoglycan missense mutations (H36P, H36R and L172R) produce proteins that are undetectable at the cell surface and are retained intracellularly. These mutant proteins become polyubiquitinated and are rapidly degraded by the proteasome. Furthermore, torsinA, that is mutated in *DYT1* dystonia, a rare type of primary dystonia, binds to and promotes the degradation of ε-sarcoglycan mutants when both proteins are co-expressed. These data demonstrate that some MDS-associated mutations in *SGCE* impair trafficking of the mutant protein to the plasma membrane and suggest a role for torsinA and the ubiquitin proteasome system in the recognition and processing of misfolded ε-sarcoglycan.

INTRODUCTION

The sarcoglycans are a family of single pass transmembrane proteins that are part of the dystrophin-associated glycoprotein complex (DGC); a multiprotein complex that links the actin cytoskeleton to the extracellular matrix in cardiac and skeletal muscle (1). The DGC can be separated biochemically into three smaller sub-complexes; the cytoplasmic sub-complex that contains dystrophin, the dystrobrevins and the syntrophins, the dystroglycan sub-complex and the sarcoglycan:sarcospan sub-complex (2). Although the precise function of the sarcoglycan sub-complex is not known, mutations in α-, β-, γ- and δ-sarcoglycan cause different forms of autosomal recessive limb girdle muscular dystrophies (LGMD) demon-

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strating its importance for normal muscle function. Based on studies in heterologous cells, it is apparent that co-synthesis of at least two different sarcoglycans is required for efficient membrane targeting of the sarcoglycan complex (3,4). Moreover, disease-associated sarcoglycan mutations often affect the assembly and trafficking of the entire sarcoglycan subcomplex to the sarcolemma (5). In contrast to the other sarcoglycans (α, β, γ and δ) which are expressed predominantly in striated and smooth muscles, ε-sarcoglycan, a paralogue of α-sarcoglycan, is expressed in a wide range of tissues with the highest levels in heart and lung (6–9). ε-Sarcoglycan is associated with the other sarcoglycans in C2C12 cells (10), vascular smooth muscle (11) and peripheral nerves (12). In the brain, ε-sarcoglycan is found in the midbrain monoaminergic neurons, in cerebellar Purkinje cells and in other brain regions including the hippocampus and cortex (6,8,13). In contrast, very little is known about the core sarcoglycan complex in the brain. Northern and western blot analysis shows that α-, γ- and δ-sarcoglycan and sarcospan only appear to be expressed in cardiac, skeletal and smooth muscles (14). β-Sarcoglycan is expressed weakly in brain, whereas the recently discovered ζ-sarcoglycan appears to be more widely expressed (15,16). From these studies, it is apparent that if a sarcoglycan complex exists in neurons it will differ significantly from the complex found in muscle.

Mutations in the gene encoding ε-sarcoglycan (*SGCE*) cause a form of dystonia called myoclonus-dystonia syndrome (MDS, *DYT11*) (17–19). MDS is a rare dystonia plus syndrome characterized by early-onset myoclonus (shock-like jerks) commonly associated with focal or segmental dystonia and more infrequently, with psychiatric disturbances such as panic attacks and obsessive compulsive disorder (20). Inheritance of MDS is autosomal dominant and typically involves the presence of bilateral myoclonus that affects the arms, neck and trunk (21,22). Although MDS is genetically heterogeneous, mutations in the *SGCE* gene appear to be a significant cause of MDS in some populations. The *SGCE* gene is maternally imprinted (paternally expressed) that explains the reduced penetrance of MDS when the disease is maternally transmitted (17,23). Importantly, the *SGCE* gene is imprinted in both humans and mice such that only the paternal allele is thought to be expressed in the brain and other tissues in the periphery (17,23–25). Mutations associated with other hereditary forms of dystonia have also been found in the genes encoding torsinA (early-onset torsion dystonia, DYT1), GTP cyclohydrolase-1 (GCH1 formerly known as DYT5) and the α3-subunit of the Na^+/K^+-ATPase (ATP1A3) (26).

While a multitude of studies have identified mutations in the *SGCE* gene in patients with MDS (18,19,27–29), the consequences of these mutations on the ε-sarcoglycan protein have not been determined. The majority of *SGCE* mutations reported in MDS families are nonsense mutations that would abolish the synthesis of the full-length protein. Recently, three missense mutations (H60R, H60P and L196R) in the *SGCE* gene have been reported that could provide important insights into the function of ε-sarcoglycan in the central nervous system and the molecular pathology of MDS (18,30). Here, we show that *SGCE* missense mutants reported in patients with MDS produce proteins that are not trafficked to the plasma membrane but are retained intracellularly, ubiquitinated and degraded by the proteasome.

RESULTS

Location of endogenous ε-sarcoglycan

The esg-5009 antibody was used to determine the tissue distribution of ε-sarcoglycan and its regional localization in the brain. On western blots, esg-5009 detects a 50 kDa protein in all tissues tested (Supplementary Material, Fig. S1). The highest levels of ε-sarcoglycan are found in lung, heart and spleen. In agreement with previous studies, ε-sarcoglycan is resolved as a doublet in mouse brain (Supplementary Material, Fig. S1) (6).

To determine the subcellular location of ε-sarcoglycan in neurons and muscle, the esg-5009 antibody was used to stain cultured hippocampal neurons and muscle sections. In hippocampal neurons cultured for 21 days *in vitro* (21 d.i.v.), ε-sarcoglycan is found at the plasma membrane of dendrites and the soma but is also concentrated in discrete cytoplasmic punctae and associated with the Golgi apparatus (Supplementary Material, Fig. S2A). In normal muscle, ε-sarcoglycan is found at the muscle plasma membrane (sarcolemma) and also in blood vessels and peripheral nerves (Supplementary Material, Fig. S2B). In contrast to the other members of the sarcoglycan family, ε-sarcoglycan is retained at the sarcolemma of dystrophin-deficient *mdx* mouse muscle albeit at reduced levels compared with the wild-type C57 control (Supplementary Material, Fig. S2B). These data show that under physiological conditions, ε-sarcoglycan is found at the plasma membrane in neurons and muscle and within intracellular inclusions and the Golgi apparatus of cultured hippocampal neurons.

Missense mutations are associated with intracellular retention of the mutant protein

The majority of *SGCE* mutations that cause MDS are insertions, deletions or nonsense mutations that result in premature termination of the protein. Recently, three missense mutations, H60P, H60R and L196R have been described that should result in the synthesis of full length proteins carrying a single altered amino acid (18,30). H60 is located within the dystroglycan-type cadherin-like (CADG) domain of ε-sarcoglycan (31), while L196 is conserved in the vertebrate orthologues of α- and ε-sarcoglycan. The mouse mutants, H36P/R and L172R used in this study correspond to the human ε-sarcoglycan mutations, H60P/R and L196R, respectively. The three mutations modelled here affect amino acid residues that are conserved between the human and mouse orthologues. Thus, the mouse cDNA is an appropriate source of ε-sarcoglycan for this study. Furthermore, mice with a targeted deletion of *Sgce* exon 4 have myoclonus, motor impairments, hyperactivity, anxiety, depression and some neurochemical abnormalities (25,32). It is noteworthy that the mouse sequence has a shorter predicted signal sequence when compared with the human sequence, but following processing each protein is predicted to start at the same amino acid (VHS/DR). In order to determine the subcellular location

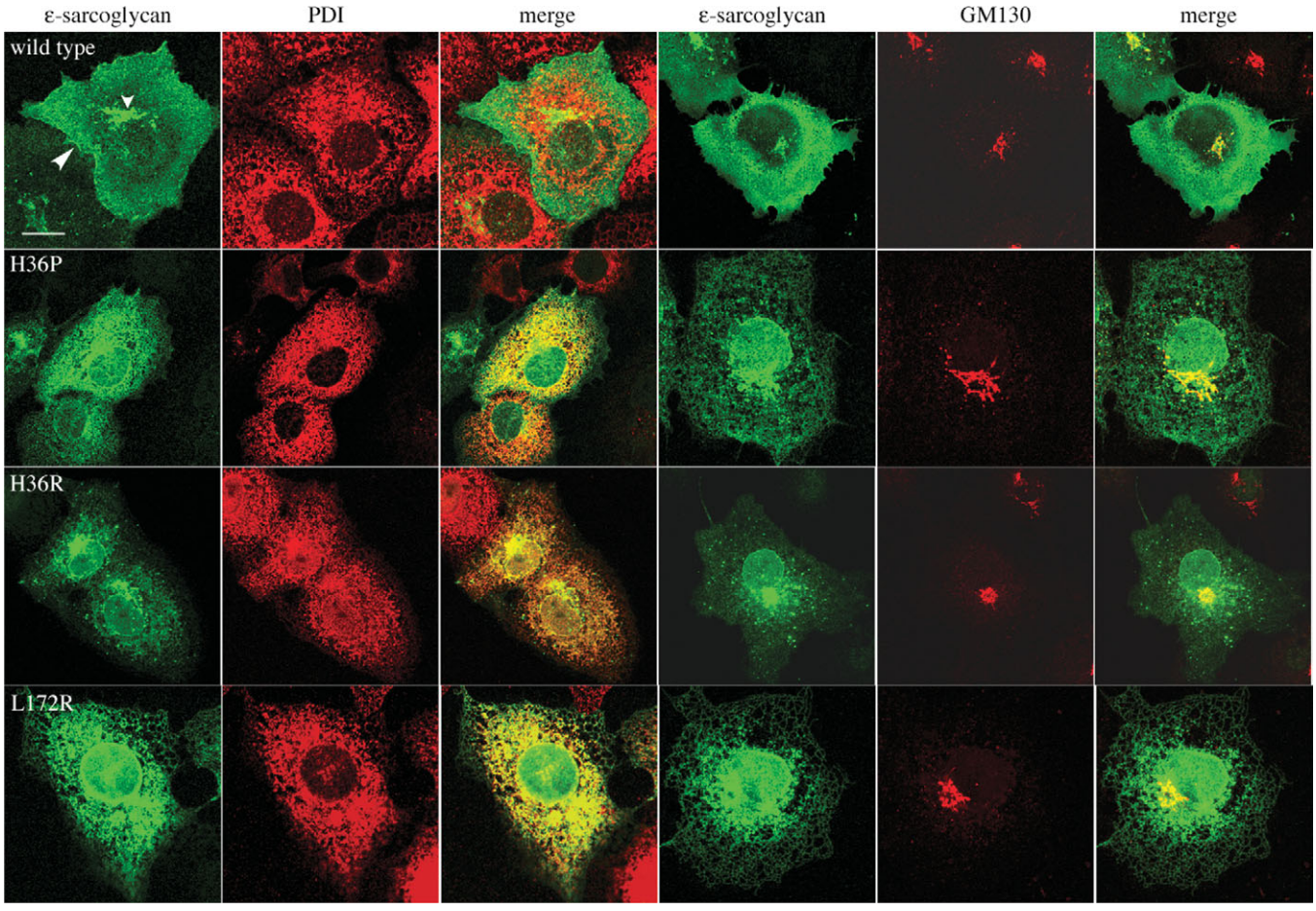

Figure 1. Intracellular retention of ε-sarcoglycan mutants in COS-7 cells. Cells expressing wild-type ε-sarcoglycan or ε-SG-H36P, ε-SG-H36R and ε-SG-L172R were counterstained with anti-GM130 (Golgi apparatus) or anti-PDI (ER) antibodies. Wild-type ε-sarcoglycan is targeted to the plasma membrane (large arrowhead) but is also found in intracellular compartments including the Golgi apparatus (small arrowhead). The MDS-associated mutants are found ostensibly in intracellular compartments, notably the ER, where they co-localize in part with PDI. The H36R mutant is also found in the Golgi apparatus and as small punctae within the ER. For each mutant there is no evidence of plasma membrane labelling. Scale bar, 20 μm.

of wild-type ε-sarcoglycan and the three mutants, constructs encoding each protein were transfected into COS-7 cells and stained with the esg-5009 antibody. As shown in Figure 1, wild-type ε-sarcoglycan localizes to the plasma membrane and some intracellular structures including the Golgi-apparatus (co-localizing with the Golgi-matrix protein, GM130) and post-Golgi vesicles. This distribution clearly differs from the endoplasmic reticulum (ER) labelled with an anti-protein disulphide isomerase (PDI) antibody. In contrast, each of the MDS-associated ε-sarcoglycan mutants were mislocalized and retained intracellularly in the ER (Fig. 1). The ER localization of H36P/R and L172R was confirmed by co-localization with PDI. The H36R mutant was also found in punctae within the ER that could correspond to the formation of small aggregates (Fig. 1). Both untagged and enhanced yellow fluorescent protein (EYFP)-tagged constructs gave the same distribution pattern in transfected cells.

While many studies have been conducted on the function of torsinA in heterologous cells (33,34), it is important to determine whether the MDS-associated mutants were mislocalized in cell types with neuronal phenotypes. EYFP-tagged constructs were transfected into cortical neurons (Fig. 2) and SH-SY5Y neuroblastoma cells (Fig. 3). Each of these cell types have been used to study the function and immunolocalization of torsinA (35,36). In cultured cortical neurons, wild-type ε-sarcoglycan is found throughout the dendrites and soma where it partially co-localizes with GM130 (Fig. 2). Discrete punctae, reminiscent of transport vesicles, are visible within the diffuse staining in both dendrites and soma. These punctae do not co-localize with GM130 or PDI. In contrast, the mutants H36P and H36R are diffusely distributed within the soma and proximal dendrites of cortical neurons co-localizing with PDI (Fig. 2). There is no evidence that either mutant forms punctae in distal parts of the cell. L172R-YFP forms discrete punctae in the soma and proximal dendrites of cortical neurons (Fig. 2). These punctae co-localize precisely with PDI in cells expressing the L172R mutant. L172R-YFP does not co-localize with GM130. Thus, it appears that in neurons, L172R can form ER-associated aggregates. Each MDS-associated ε-sarcoglycan mutant is therefore mislocalized in cortical neurons. Finally, we examined the distribution of ε-sarcoglycan and the MDS-associated

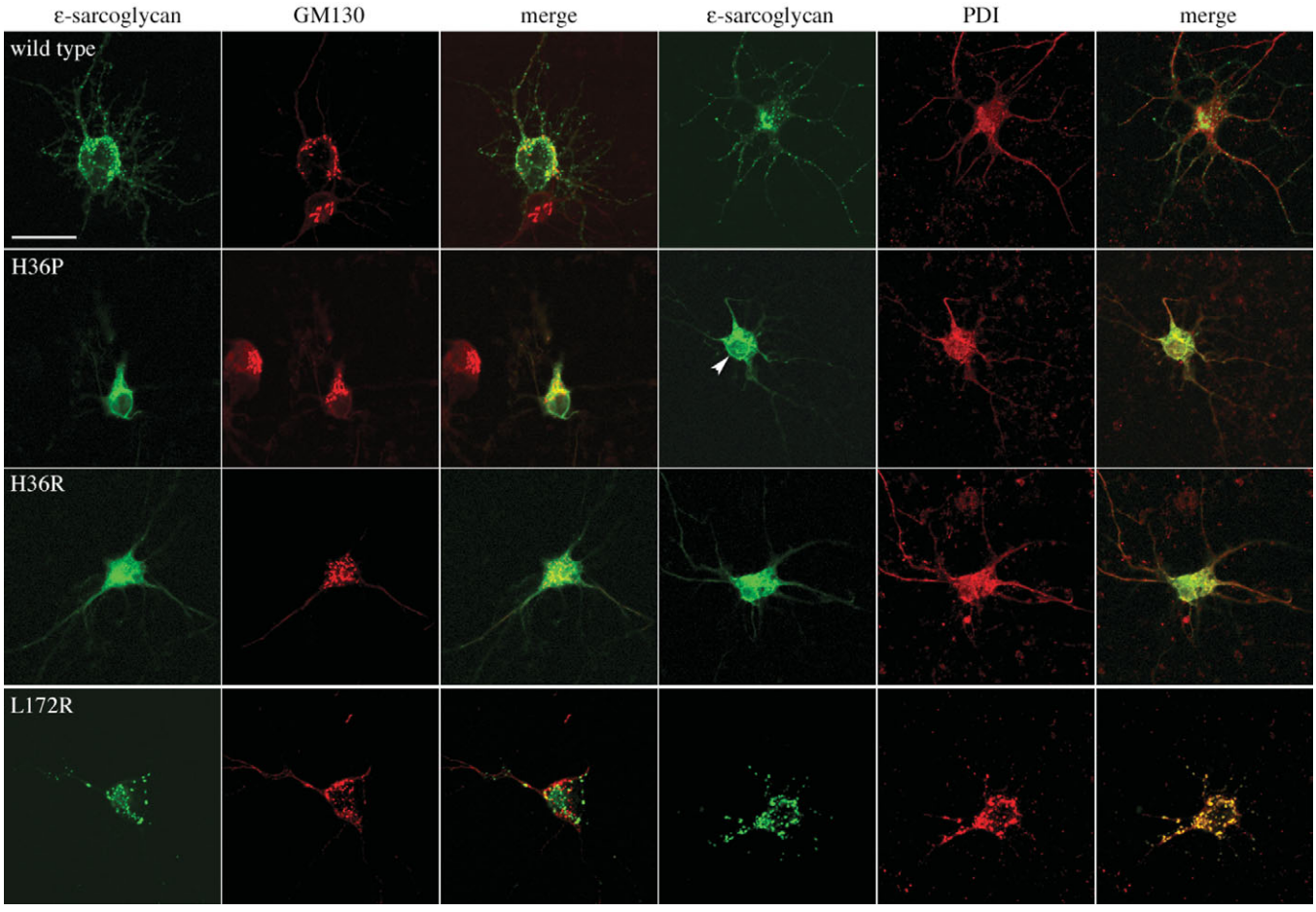

Figure 2. Mislocalization of YFP-tagged ε-sarcoglycan mutants in cortical neurons. Cells expressing EYFP-tagged wild-type ε-sarcoglycan or ε-SG-H36P, ε-SG-H36R and ε-SG-L172R were counterstained with anti-GM130 or anti-PDI antibodies. YFP-tagged, wild-type ε-sarcoglycan is found in the soma and dendrites of neurons and co-localizes in part with GM130. PDI was distributed diffusely in the soma and dendrites of neurons. Among the diffuse labelling, prominent punctae are visible both within the soma and in most dendrites. These punctae did not co-localize with PDI. In contrast to the wild-type protein, ε-SG-H36R and ε-SG-H36P were localized diffusely in the soma and dendrites of neurons. These mutants co-localized with PDI but failed to co-localize with the GM130. The L172R mutant formed discreet punctae within the neuronal soma and proximal dendrite. These punctae did not co-localize with GM130 but did co-localize perfectly with PDI that appeared to be redistributed into punctae within the neuronal soma and proximal dendrite. The nuclear membrane (arrowhead) is visible in cells expressing ER-retained mutants. Scale bar, 20 μm.

mutants in SH-SY5Y cells (Fig. 3). The wild-type protein is detected at the plasma membrane of neuroblastoma cells but is also found in the Golgi apparatus, co-localizing with GM130 and within vesicular structures (Fig. 3). Each of the MDS-associated mutants is mislocalized in SH-SY5Y cells and are found within the ER partially co-localizing with PDI. The H36R mutant can form perinuclear punctae reminiscent of ER-associated aggregation (Fig. 3). Thus, each MDS-associated ε-sarcoglycan mutant is mislocalized in SH-SY5Y cells.

ε-Sarcoglycan mutants are degraded by the proteasome

Proteins in the secretory pathway are scrutinized by an elaborate quality control system in the ER such that misfolded proteins are retrotranslocated from the ER and degraded in the cytoplasm by the proteasome. The intracellular retention of the H36R/P and L172R mutants suggested that the proteins were possibly misfolded and therefore could be authentic substrates for the proteasome. ε-Sarcoglycan is a type 1 transmembrane protein whose N-terminus has a single conserved consensus site, NIT (amino acids 176–178), for N-linked glycosylation. Thus the presence of N-linked glycans on ε-sarcoglycan and the mutants would indicate that each protein had entered the ER lumen. Accordingly, both wild-type ε-sarcoglycan and the MDS-associated mutants H36P/R and L172R are glycosylated in HEK 293T cells (Fig. 4A). Each protein is sensitive to the glycosidases Endo H and PNGase F, indicating that ε-sarcoglycan and the MD-associated mutants possess high mannose structures (Fig. 4A). Furthermore, this pattern of glycosidase sensitivity is also found in ε-sarcoglycan purified from mouse brain indicating that HEK 293T cells process and traffic ε-sarcoglycan in a similar manner to brain (Fig. 4B). Treatment with each glycosidase reduces the apparent relative molecular mass of ε-sarcoglycan by ∼2 kDa. As described previously for β-dystroglycan, a reduction of 2 kDa corresponds to the occupancy of a high mannose glycan at a single sequon (37). These

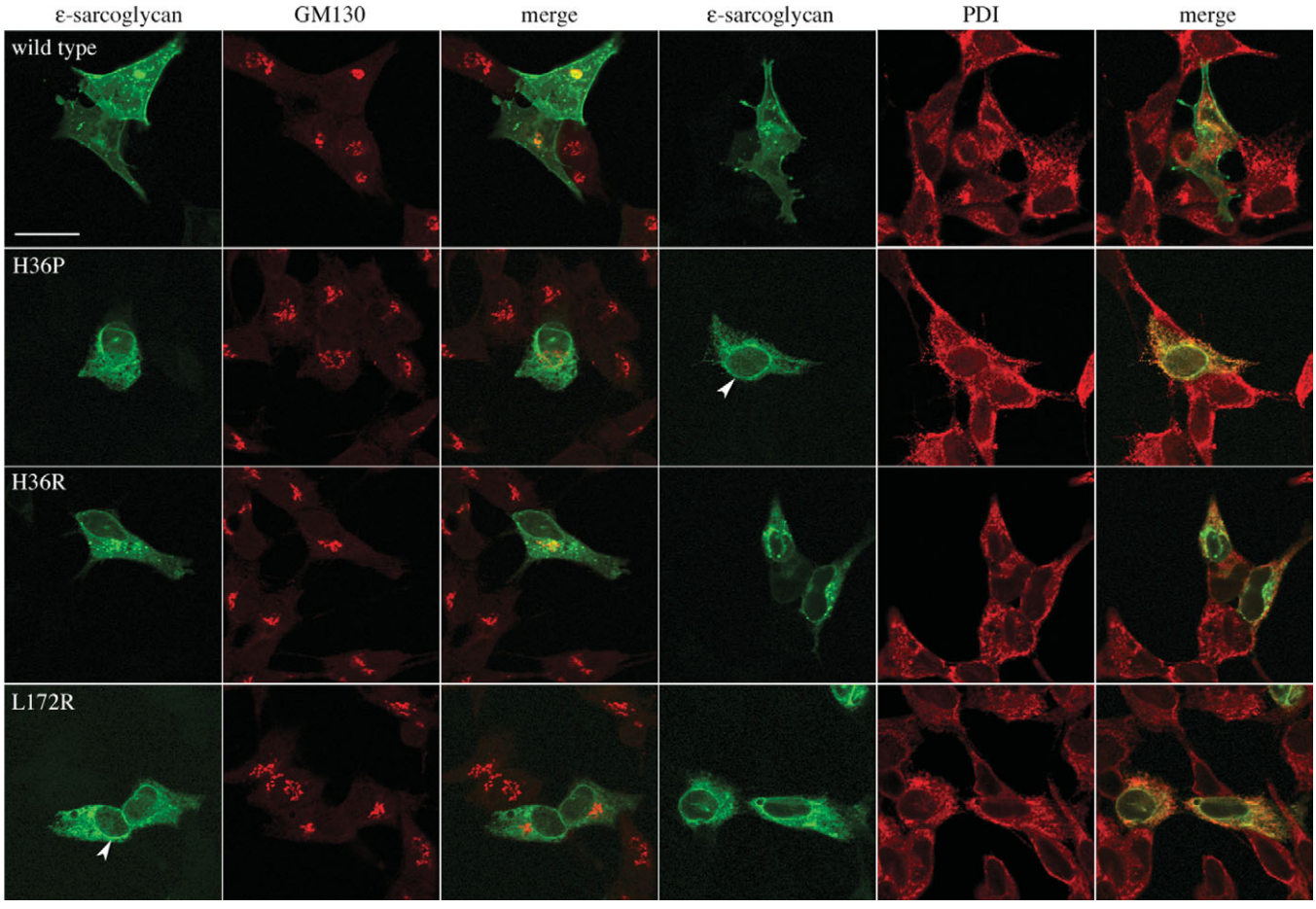

Figure 3. Mislocalization of YFP-tagged ε-sarcoglycan mutants in SH-SY5Y neuroblastoma cells. Cells expressing EYFP-tagged wild-type ε-sarcoglycan or ε-SG-H36P, ε-SG-H36R and ε-SG-L172R were counterstained with anti-GM130 or anti-PDI antibodies. Wild-type ε-sarcoglycan is targeted to the plasma membrane but is also found in intracellular compartments including the Golgi apparatus and small vesicular structures. The tagged MDS-associated mutants are not targeted to the plasma membrane but are retained in the ER where they co-localize in part with PDI. In SH-SY5Y cells, the H36R mutant is also found within small perinuclear vesicular structures. The nuclear membrane (arrowhead) is prominent in cells expressing ER-retained mutants. Scale bar, 20 μm.

data demonstrate that ε-sarcoglycan and the three missense mutants can enter the ER.

Quantitative western blot analysis of the MDS-associated mutants showed that the steady-state expression levels of the mutants H36P, H36R and L172 were consistently lower than the wild-type protein. If an arbitrary level of 100 ± 4.0% (SEM) is assigned to wild-type ε-sarcoglycan, the relative levels of each MDS-associated mutant are as follows; H36P, 25.4 ± 6.9%, H36R, 26.1 ± 0.6% and L172R, 85 ± 3.0%. To determine whether the observed reduction in the levels of the ε-sarcoglycan mutants was due to proteasome-mediated degradation, HEK 293T cells were transfected with either wild-type ε-sarcoglycan or the MDS-associated mutants and HA-tagged ubiquitin (a kind gift from Prof. Dirk Bohmann) in the presence and absence of the proteasome inhibitor lactacystin (Fig. 4C). Ubiquitination was assessed by immunoprecipitation of ε-sarcoglycan and the mutants from transfected cells followed by western blotting of the immunoprecipitates with the anti-HA and esg-5009 antibodies. The highest levels of polyubiquitinated ε-sarcoglycan were detected in immunoprecipitates from cells expressing the H36P/R mutants and to a lesser extent the L172R mutant when incubated with lactacystin (Fig. 4C, upper panel). Polyubiquitinated H36P-ε-sarcoglycan could also be detected in the absence of lactacystin (Fig. 4C, upper panel). Polyubiquitinated wild-type ε-sarcoglycan can also be detected with the proteasome inhibitor suggesting that a proportion of this protein may be destroyed by the proteasome. The presence of polyubiquitinated ε-sarcoglycan was also detected with the esg-5009 antibody (Fig. 4C, middle panel).

To determine the effect of MDS-associated mutations on the stability of ε-sarcoglycan, quantitative western blots were used to determine the half-lives of each protein in the presence and absence of the proteasome inhibitor MG132 (Fig. 5). In these experiments, protein synthesis was inhibited with cycloheximide and ε-sarcoglycan levels were normalized to endogenous α-tubulin on the same blot using two-colour imaging. MG132 was used in preference to lactacystin because we found that lactacystin combined with cycloheximide was toxic to transfected HEK 293T cells. Under these conditions, wild-type ε-sarcoglycan had a half-life of 5.0 h that increases to 7.8 h with the addition of MG132. Similarly,

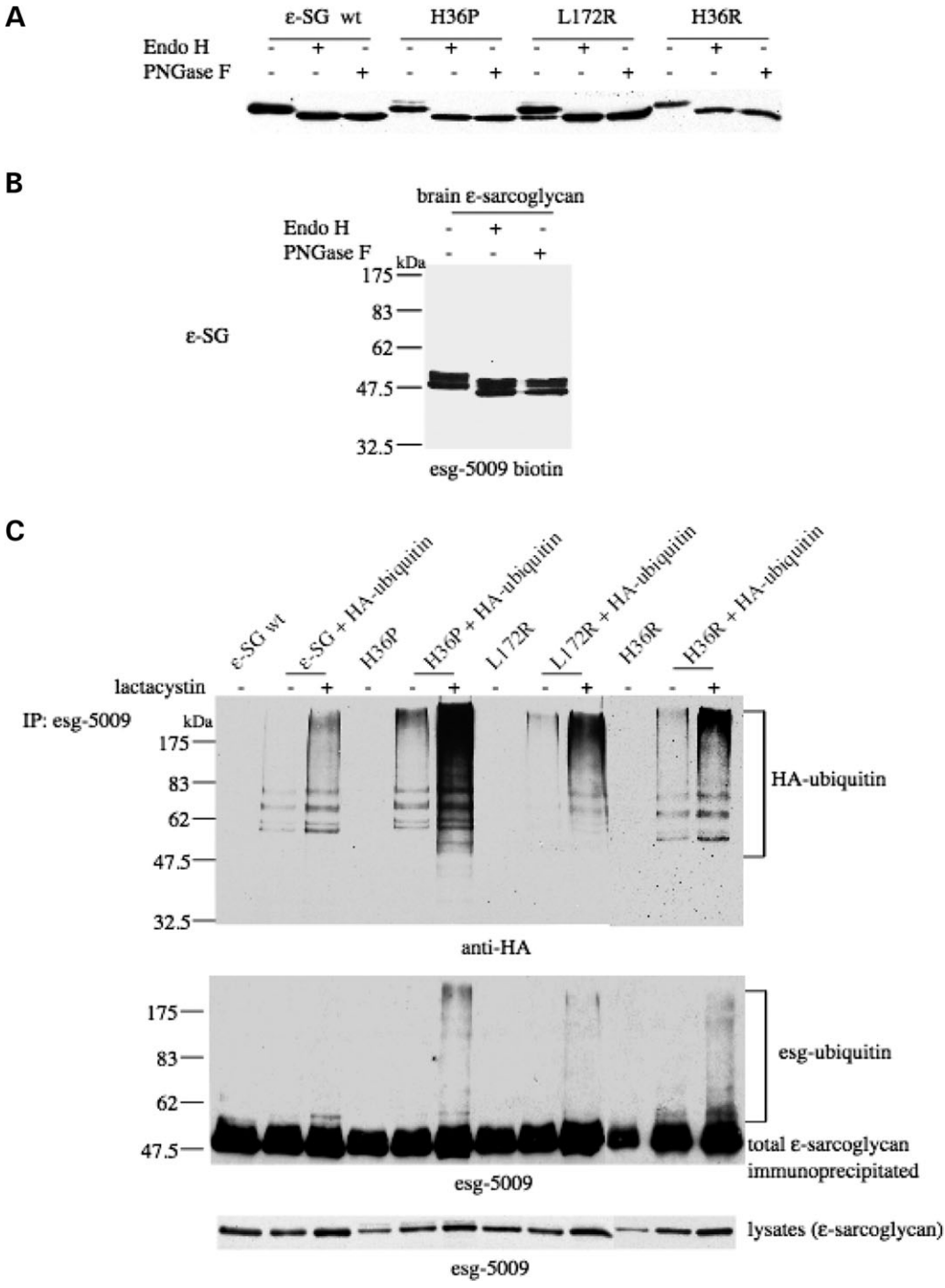

Figure 4. Polyubiquitination of MDS-associated ε-sarcoglycan mutants. (**A**) Western blot using anti-esg 5009 antibody showing that ε-sarcoglycan, H36P/R and L172R are glycoproteins and have therefore entered the ER. Proteins prepared from transfected HEK 293T cells expressing the indicated constructs were digested with Endo H and PNGase F. In each case glycosidase treatment reduced the apparent relative molecular mass of each protein by ∼2 kDa. (**B**) Glycosidase sensitivity of brain-derived ε-sarcoglycan. ε-Sarcoglycan was immunoprecipitated from mouse brain and treated as indicated. Endo H and PNGase F reduced the apparent relative molecular mass of each protein by ∼2 kDa. Brain-derived ε-sarcoglycan exists as a dimer due to alternative splicing of the C-terminus [see Supplementary Material, Fig. S1 and (6)]. (**C**) Ubiquitination of ε-sarcoglycan. ε-Sarcoglycan was immunoprecipitated from HEK 293T cells expressing the constructs as indicated. The upper panel shows that the anti-HA antibody detects polyubiquitinated ε-sarcoglycan in the presence of lactacystin (+) and even when the drug was omitted (−) in the case of the H36P/R mutants. The presence of ε-sarcoglycan in each immunoprecipitation (middle panel) and the cell lysates (lower panel) are shown for comparison. Note that polyubiquitinated ε-sarcoglycan is also detected with the esg-5009 antibody (middle panel).

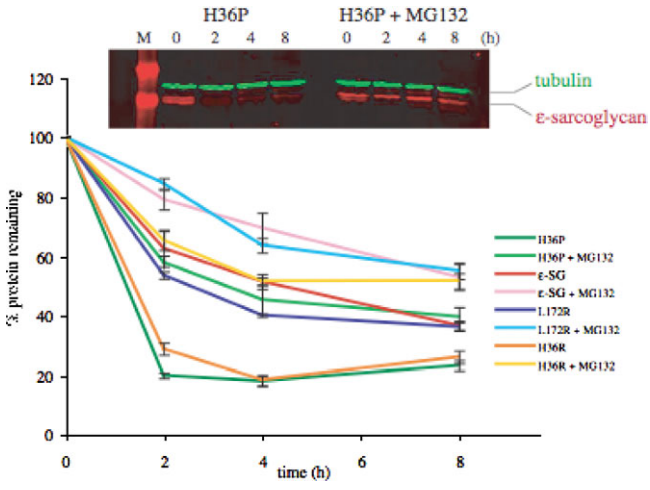

Figure 5. MDS-associated ε-sarcoglycan mutants have shorter half-lives. Graphical representation of the protein levels of wild-type ε-sarcoglycan, H36P/R and L172R over time. The protein levels of wild-type ε-sarcoglycan, H36P/R and L172R were normalized to α-tubulin following the addition of cycloheximide (0 h) in the presence or absence of the proteasome inhibitor, MG132. The inset shows a representative, quantitative immunoblot of H36P (red) and α-tubulin (green) with and without MG132. The error bars show the SEM from four to six independent experiments.

the half-life of the L172R mutant is 4.2 h in untreated cells but increases to 7.6 h after proteasome inhibition. The H36P and H36R mutants that are expressed at lower levels and heavily polyubiquitinated relative to the wild-type protein and the L172R mutant (Fig. 5) had half-lives <2.0 h. Incubation of H36P and H36R with MG132 increased the half-lives of the protein to 4.6 h and 4.8 h, respectively.

Impaired plasma membrane targeting of mutant ε-sarcoglycan

To demonstrate that intracellular retention of the mutated proteins and preferential degradation by the proteasome affected plasma membrane delivery, cell surface biotinylation was used to establish whether each protein was trafficked to the surface of transfected HEK 293T cells. These experiments were performed in the presence and absence of the proteasome inhibitor lactacystin. Wild-type ε-sarcoglycan is targeted to the plasma membrane in the absence of lactacystin, while incubation with lactacystin increased the levels of the protein at the cell surface (Fig. 6A). In contrast, no surface-expressed protein could be detected with any of the MDS-associated mutants that were expressed under the same conditions and even in the presence of lactacystin (Fig. 6A). Control experiments showed that ε-sarcoglycan was efficiently immunoprecipitated in each case and that incubation with lactacystin dramatically increased the levels of the H36P mutant (and to a lesser extent wild-type ε-sarcoglycan and the L172R and H36R mutants) in both the immunoprecipitates and cell lysates (Fig. 6A). Immunoblotting with an anti-α-tubulin antibody showed that each transfection contained similar levels of a protein that was unaltered by incubation with lactacystin (Fig. 6A). These data support our hypothesis that some ε-sarcoglycan mutations produce proteins that have impaired intracellular transport and cannot reach the plasma membrane. Thus, ε-sarcoglycan can be efficiently trafficked to the cell surface without co-synthesis of the other sarcoglycans (4). While the esg-5009 detects human ε-sarcoglycan in brain extracts (Supplementary Material, Fig. S3A), endogenous ε-sarcoglycan was not detected in COS-7 or HEK 293T cells (Supplementary Material, Fig. S3A and Fig. 3B). This is in agreement with previous studies that failed to detect endogenous sarcoglycans in heterologous cell lines including HEK 293 and COS-1 (38,39). Furthermore, we were unable to detect α- and β-sarcoglycan in COS-7 cells, suggesting that the plasma membrane trafficking of ε-sarcoglycan in this cell type is not dependent upon the presence of the other sarcoglycans (Supplementary Material, Fig. S3B). To determine whether other components of the DGC were in a complex with ε-sarcoglycan, we immunoprecipitated ε-sarcoglycan from mouse brain extracts prepared in RIPA buffer (Supplementary Material, Fig. S4). While ε-sarcoglycan was highly enriched in these preparations, components of the core DGC such as dystrophin, Dp71, the dystrobrevins and β-dystroglycan were not co-purified. These data suggest that in the brain, ε-sarcoglycan may not be a component of the DGC-like complexes found in neurons (40).

It has previously been shown that mutations in one member of the sarcoglycan sub-complex can affect the trafficking of the other components to the plasma membrane (3–5). Since *SGCE* is a maternally imprinted (paternally expressed) gene, the expression of ε-sarcoglycan will be monoallelic if the locus is faithfully imprinted. The vast majority of MDS patients only express a single mutant allele suggesting that loss of function may be the underlying cause of the disease. However, there are cases in the literature where imprinting has been lost and the individuals with MDS are thought to express both a untagged and mutant *SGCE* allele (23). Given that mutations in the gene encoding ε-sarcoglycan cause MDS that is inherited in an autosomal dominant manner we sought to determine whether the ε-sarcoglycan mutants could behave in a dominant-negative manner by impairing trafficking of the wild-type protein to the cell plasma membrane. To investigate a dominant-negative effect on ε-sarcoglycan trafficking to the cell surface, wild-type ε-sarcoglycan and the mutants H36P and L172R were co-expressed with ε-sarcoglycan:EYFP that, like the wild-type protein, is normally trafficked efficiently to the cell surface. The ε-sarcoglycan:EYFP chimera has a higher relative molecular mass when compared with the untagged protein that can be easily resolved by SDS–PAGE (Fig. 6B). The same experimental paradigm was applied as described above except that the wild-type and mutant ε-sarcoglycans were co-transfected with ε-sarcoglycan:EYFP. Surface biotinylation of proteins expressed in cells co-transfected as shown in Figure 6B showed that comparable levels of ε-sarcoglycan:EYFP are present at the plasma membrane in each transfection. In cells expressing untagged ε-sarcoglycan and ε-sarcoglycan:EYFP, both proteins can be readily detected at the surface by biotinylation (Fig. 6B). When ε-sarcoglycan:EYFP is co-expressed with the untagged mutants, low levels of the mutant become biotinylated indicating that, under these conditions, they can be trafficked to the plasma membrane. Thus the wild-type protein may promote the trafficking of the mutant protein to the membrane as part of a homotypic protein complex. These data show that MDS-associated

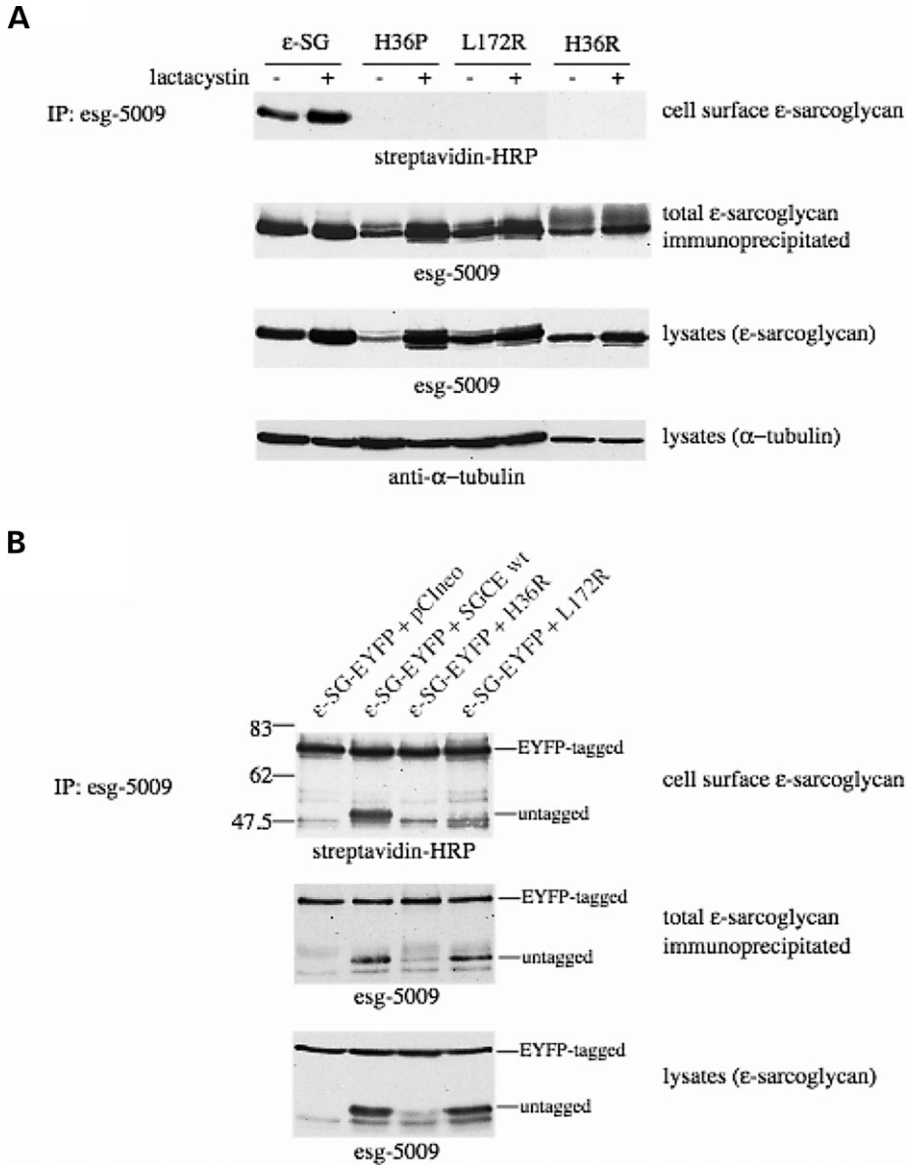

Figure 6. ε-Sarcoglycan mutants are poorly targeted to the plasma membrane. (**A**) Twenty-four hours after transfection, HEK 293T cells expressing the designated constructs were treated with lactacystin (+) or left untreated (−) and incubated for a further 18 h. Following cell surface biotinylation, ε-sarcoglycan proteins were immunoprecipitated with the esg-5009 antibody cross-linked to protein G-Sepharose beads. The streptavidin–horseradish peroxidase (HRP) blot detects biotinylated protein and shows that ε-sarcoglycan is found at the cell surface. Furthermore, the levels of ε-sarcoglycan at the cell surface are increased by proteasome inhibition. No cell surface labelling of the MDS-associated mutants could be detected even when the cells were pre-treated with lactacystin. The lysates were blotted with the anti-α-tubulin antibody to show equal amounts of protein. (**B**) MDS-associated mutants do not have a dominant-negative effect on the trafficking of the wild-type protein. Cell surface biotinylation of HEK 293T cells expressing ε-sarcoglycan:EYFP with wild-type and MDS-associated ε-sarcoglycan mutants as indicated. Tagged (EYFP) and untagged ε-sarcoglycan was immunoprecipitated using the esg-5009 antibody. The upper panel shows that the levels of ε-sarcoglycan:EYFP at the plasma membrane remain similar even in the presence of the MDS-associated mutants. The middle panel shows the levels of ε-sarcoglycan in the immunoprecipitates, while the lower panel shows the presence of each of the proteins in the lysates. As described previously, the H36R mutant is rapidly degraded by the cell and therefore present at reduced levels when compared with the other proteins.

mutants do not behave in a dominant-negative manner to impair plasma membrane delivery of the wild-type protein (Fig. 6B).

Intracellular mobility of ε-sarcoglycan and mutants

Fluorescence recovery after photobleaching (FRAP) was used to determine the mobility of the wild-type and mutant ε-sarcoglycans in the ER (Fig. 7A). It has been demonstrated that some misfolded membrane proteins have altered rates of diffusional mobility associated with the formation of mixed disulphide bonds with ER-resident chaperones when ATP is depleted (41). EYFP-tagged constructs were transfected into COS-7 cells and used to measure FRAP rates in cells incubated with brefeldin A to block protein transport in the secretory pathway. In cells expressing the wild-type protein, no significant difference in FRAP rates was found when

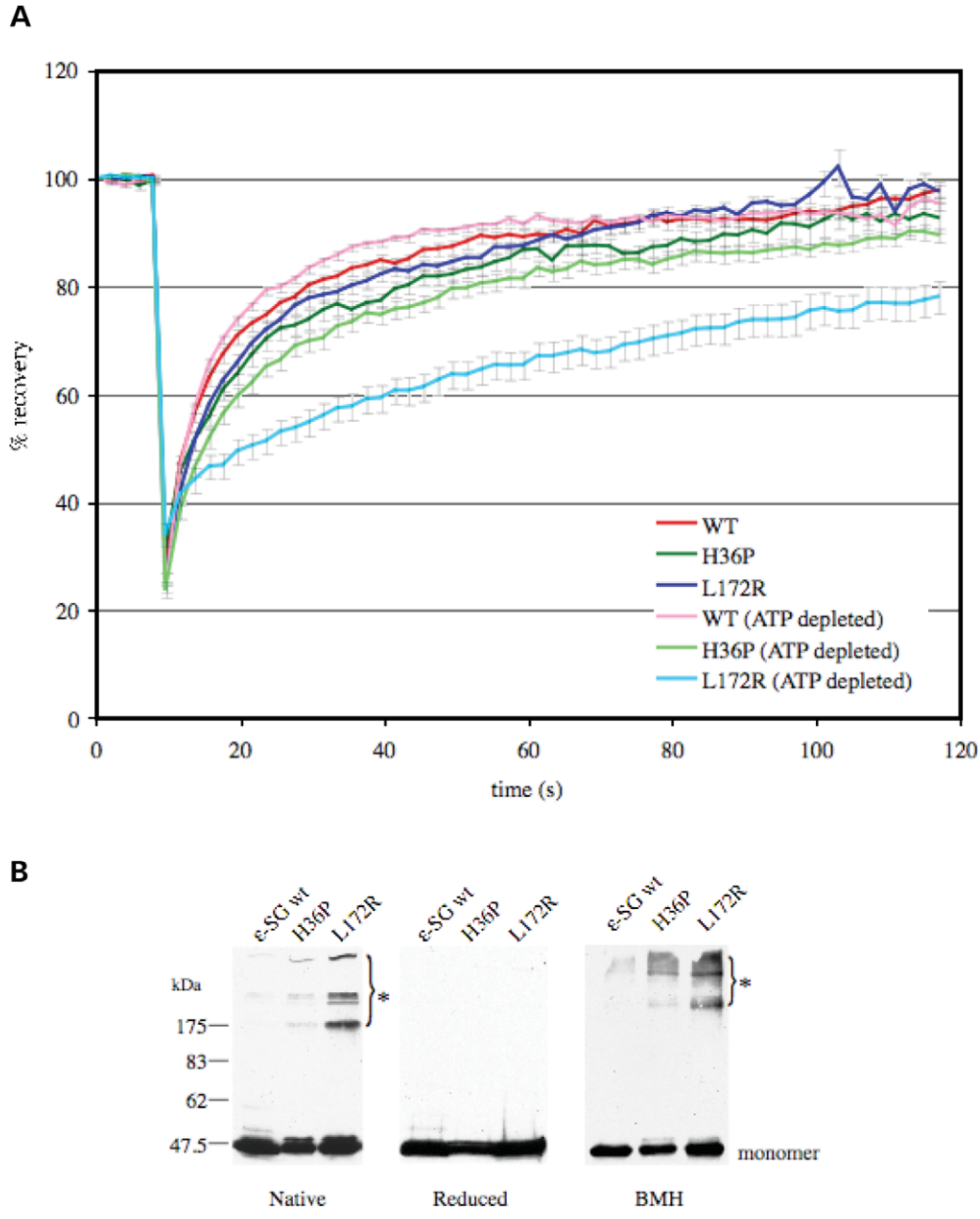

Figure 7. MDS-associated mutations affect protein dynamics. (**A**) FRAP analysis of ε-sarcoglycan and MDS-associated mutants. Living COS-7 cells expressing EYFP-tagged constructs were photobleached with high intensity laser light. The recovery of the bleached area was monitored for several minutes to examine the rate of fluorescence recovery. The FRAP recovery curves for wild-type ε-sarcoglycan, H36P and L172R are shown as indicated. The experiments were performed under two conditions: (i) in normal HBSS containing 1 mM glucose or (ii) in HBSS without glucose and containing 2-deoxyglucose and sodium azide to deplete ATP (ATP depleted). The error bars show the SEM on data obtained from at least 10 independent cells. (**B**) Biochemical detection of high molecular weight ε-sarcoglycan aggregates. Proteins were extracted from cells expressing the indicated constructs and separated under native and reduced PAGE conditions. In addition to the prominent 50 kDa ε-sarcoglycan monomer, the mutants H36P and L172R show high molecular weight adducts that are removed by pre-incubation with 5% 2-mercaptoethanol. The thiol-selective cross-linker BMH is also able to cross-link the MDS-associated mutants into non-reducible high molecular weight aggregates similar to those detected by native PAGE.

ATP was depleted with 2-deoxyglucose (Fig. 7A). Cells expressing L172R showed a significant reduction in FRAP times upon ATP-depletion when compared with the wild-type control (Fig. 7A). Interestingly, the H36P mutant behaved similarly to the wild-type protein when ATP was depleted suggesting that ER-retention of this protein was not dependent on disulphide bond formation (Fig. 7A).

Biochemical techniques were used to confirm the findings of the FRAP studies as follows. The N-terminus of ε-sarcoglycan has two pairs of cysteine residues that could

form disulphide bonds in the native protein. An additional cysteine close to the N-terminus of ε-sarcoglycan is located within the signal peptide and is therefore unlikely to be present in the mature protein. The formation of mixed disulphide bonds that retain proteins in the ER was demonstrated using native polyacrylamide gel electrophoresis (PAGE) and cross-linkers. Figure 7B shows that a proportion of the L172R, and to a lesser extent H36P/R forms high molecular weight aggregates on native PAGE that are absent when the protein is reduced with 2-mercaptoethanol. In contrast, wild-type ε-sarcoglycan is essentially resolved as a single correctly folded protein on both native and reducing polyacrylamide gels. The susceptibility of the protein to the thiol-reactive cross-linker bismaleimidohexane (BMH) was also used to show the presence of free thiols in the mutants. BMH is able to oxidatively cross-link free thiol groups to form a stable thioether linkage. When the cell lysates are treated with BMH, the mutants L172R and H36P are associated with high molecular weight, cross-linked adducts while the wild-type protein is unmodified (Fig. 7B). Importantly, the BMH cross-linked proteins are resistant to chemical reduction and can be separated by reduced PAGE (Fig. 7B). The diffuse pattern of high molecular weight aggregates is consistent with our hypothesis that the MDS-associated ε-sarcoglycan mutants co-associate with intracellular chaperones rather than forming homotypical aggregates.

Interaction with torsinA and effect on ε-sarcoglycan trafficking and steady-state levels

Although each of the ε-sarcoglycan mutants are retained intracellularly and degraded by the proteasome, there are several important differences between the behaviour of the L172R mutant compared with the H36P/R mutants. These differences in behaviour prompted us to look for a potential interaction between ε-sarcoglycan and torsinA because the L172R mutation was found in association with a torsinA mutation (42). To determine whether ε-sarcoglycan and torsinA interact, we co-expressed the wild-type protein and H36P, H36R and L172R mutants with wild-type and mutant torsinA. The mouse torsinA mutant, torsinAΔE304, used in this study corresponds to the common mutation ΔE302/303(Δgag) described in DYT1 patients (43). TorsinA and torsinAΔE304 co-immunoprecipitated with the ε-sarcoglycan mutants when co-expressed in HEK 293T cells (Fig. 8A). Very little wild-type ε-sarcoglycan is found in the torsinA immunoprecipitates, however, this does not negate the possibility that the wild-type proteins bind transiently in the ER, a common finding with chaperone–substrate interactions. Furthermore, ε-sarcoglycan and torsinA could not be immunoprecipitated from mouse brain extracts. This is not surprising because we have shown that torsinA preferentially binds misfolded conformations of ε-sarcoglycan (Fig. 8A). In control experiments we examined the potential association between torsinA, α-sarcoglycan and the ER-retained mutant, H77C-α-sarcoglycan (44) (Supplementary Material, Fig. S5). TorsinA could not be co-immunoprecipitated with either EYFP-tagged α-sarcoglycan construct and had no apparent affect upon the steady-state levels of each protein when co-expressed in heterologous cells (Supplementary Material, Fig. S5).

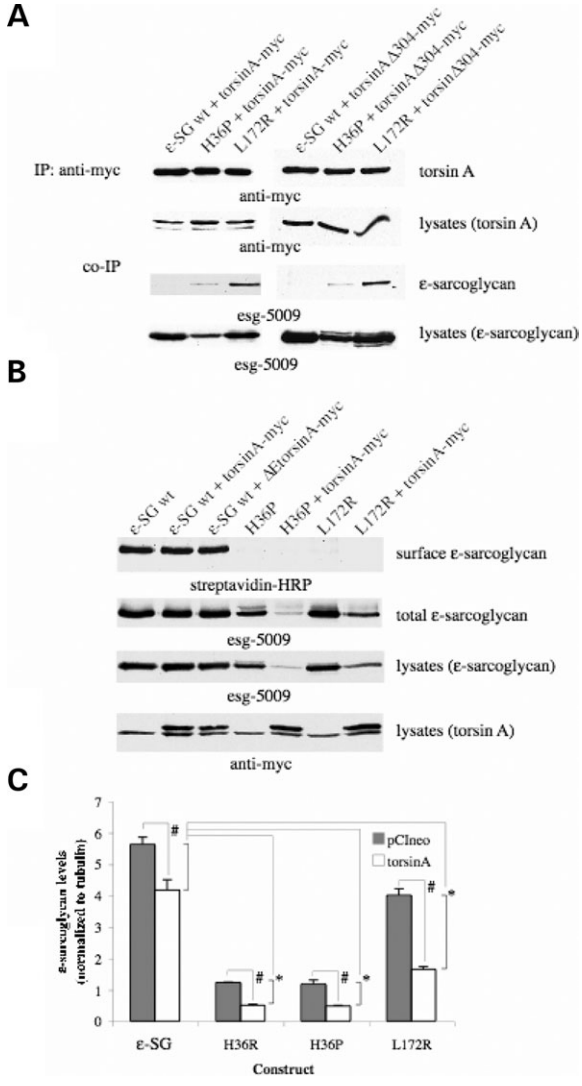

Figure 8. Interaction of ε-sarcoglycan mutants with torsinA. (**A**) HEK 293T cells were transfected as indicated. Western blot analysis of co-immunoprecipitated proteins reveals that the ε-sarcoglycan mutants, H36P and L172R are associated with torsinA and torsinAΔE304 (middle panel). TorsinA is expressed uniformly and immunoprecitated in each transfection (upper panel) however, as described previously, the H36P mutant is poorly expressed (lower panel). (**B**) Effect of torsinA on the cell surface targeting of ε-sarcoglycan and MDS-associated mutants. The presence of ε-sarcoglycan at the plasma membrane of HEK 293T cells co-expressing the indicated constructs was determined by cell surface biotinylation on non-permeabilized cells. TorsinA and torsinAΔE304 have no apparent effect on the surface expression of ε-sarcoglycan. Furthermore, torsinA was unable to restore surface expression of H36P and L172R. TorsinA co-expression dramatically reduces the steady-state levels of the MDS-associated ε-sarcoglycan mutants (middle panels). Quantitation of ε-sarcoglycan levels in cells co-expressing torsinA. (**C**) The steady-state levels of ε-sarcoglycan and α-tubulin were determined in COS-7 cells expressing the indicated constructs (wild-type ε-sarcoglycan and mutants co-transfected with pCIneo or torsinA-myc). The levels of ε-sarcoglycan were normalized to α-tubulin and are expressed in arbitrary units. Co-expression of torsinA and ε-sarcoglycan results in a highly significant ($^{\#}P < 0.005$) reduction in the levels of ε-sarcoglycan and the MDS-associated mutants, H36R/P and L172R. Pairwise comparison of the observed reduction in the levels of ε-sarcoglycan and mutants when co-expressed with torsinA shows a statistically significant ($^{*}P < 0.05$) difference on the steady-state levels of ε-sarcoglycan when compared with the mutants. The error bars show the SEM from six independent experiments.

We also examined the effect of torsinA on the trafficking of ε-sarcoglycan to the cell surface. Using conventional chemiluminescence detection of proteins on western blots, co-expression of wild-type torsinA and torsinAΔE304 with ε-sarcoglycan had no apparent effect upon ε-sarcoglycan trafficking to the cell surface or the levels of the protein (Fig. 8B). Furthermore, co-expression of torsinA and the mutants H36P and L172R did not enhance trafficking of mutant ε-sarcoglycan to the plasma membrane. However, co-expression of torsinA and the ε-sarcoglycan mutants H36P and L172R caused a dramatic reduction in the steady-state levels of the mutant proteins (Fig. 8B). To quantify the differential effect of torsinA co-expression on the steady-state levels of mutant relative to the wild-type protein, quantitative two-colour immunoblotting was used to determine protein levels relative to α-tubulin in transfected HEK 293T cells. Co-expression of torsinA with ε-sarcoglycan and the MDS-associated mutants caused a statistically significant ($P < 0.005$) reduction in the steady-state levels of each protein when compared with control groups where ε-sarcoglycan and the mutants were co-transfected with pCIneo (Fig. 8C). However, torsinA expression caused a much greater reduction in the levels of the three mutants compared with the wild-type that was statistically significant ($P < 0.05$). While the levels of the wild-type protein were reduced by 27% when torsinA is co-expressed, the levels of H36P, H36R and L172R were reduced by 58.3, 58.9 and 58.9%, respectively, when co-expressed with torsinA (Fig. 8C). This effect is independent of the total protein synthesized because the percentage change is expressed relative to the levels of each protein in a pair-wise comparison (e.g. wild-type ε-sarcoglycan compared with wild-type ε-sarcoglycan and torsinA).

These data suggest that torsinA may augment the clearance of ε-sarcoglycan from the cell and could indicate a role for torsinA in the quality control of protein folding in the ER.

DISCUSSION

It is now well established that mutations in the gene encoding the plasma membrane protein, ε-sarcoglycan cause MDS. While genetics has been invaluable in determining the aetiology of MDS, little is known about the function of ε-sarcoglycan in the central nervous system and its role in the molecular pathology of MDS. In this study, we show that three different single amino acid changes that cause MDS produce proteins that are not targeted to the plasma membrane and are degraded by the ubiquitin proteasome system. We also show that torsinA can interact with the MDS-associated ε-sarcoglycan mutants to augment their removal from the cell possibly via the proteasome supporting the suggestion made by others that torsinA, in common with other members of the AAA+ protein family, may have intrinsic chaperone activity (45–47).

The correct folding of proteins in the secretory pathway is essential for physiological delivery to the plasma membrane or secretion (48). Human genetic diseases that affect proteins that utilize the secretory pathway are frequently associated with the intracellular retention and degradation of the mutant protein. These diseases are diverse and include cystic fibrosis (49), retinochisis (50), Robinow syndrome (51) and hereditary emphysema (52). Herein, we show that missense mutations in the *SGCE* gene produce proteins that are retained intracellularly and degraded by the proteasome. Using FRAP and PAGE, we provide complementary evidence that the L172R mutant is probably misfolded. It has been shown that misfolded proteins can be locked into immobilized complexes in the ER when cells are depleted of ATP (41). ATP is required for the hydrolysis of disulphide bonds formed between chaperones such as the PDIs and misfolded proteins (53,54). Furthermore, misfolded proteins have a propensity to form aggregates that can be resolved when separated by native PAGE and can be cross-linked with different cross-linkers including the thiol-selective homobifunctional reagent, BMH (54). While L172R has the hallmarks of a typical misfolded protein, the mutant H36P (and H36R) does not appear to be processed by the cell in the same manner. The H36P mutant was indistinguishable from the wild-type protein by FRAP and was not immobilized by ATP depletion (Fig. 7A). High molecular weight adducts were obtained by native PAGE and BMH cross-linking (Fig. 7B) suggesting that a proportion of H36P is bound to chaperones. However, H36P was more rapidly degraded by the cell when compared with the L172R mutant and had the highest level of polyubiquitination (Figs 4C and 5). These data could suggest that the cell processes the two mutants differently and that the H36P mutant may be too labile to form stable complexes with chaperones.

Many of the disease-associated mutations in the *SGCE* gene are nonsense mutations that cause the premature termination of the protein. The most common MDS-associated nonsense mutation in the *SGCE* gene is R102X that has been described in at least nine different individuals (see http://www.dmd.nl/sgce_seqvar.html (55). This mutation truncates ε-sarcoglycan before the transmembrane domain and produces low levels of protein possibly through nonsense-mediated decay of the mutant transcript (Waite and Blake, unpublished data). The overwhelming majority of MDS-associated mutations are found in the extracellular part of the molecule. The extracellular region of ε-sarcoglycan contains an N-linked glycosylation site and several conserved cysteine residues that are likely to form intramolecular disulphide bonds. Glycosylation and disulphide bond formation are initiated in the lumen of the ER and represent major quality control checkpoints for proteins in the secretory pathway. We have found that co-expression of the MDS-associated mutants with wild-type ε-sarcoglycan does not prevent trafficking of the wild-type protein to the cell surface and therefore precludes a dominant gain-of-function role for the mutant in plasma membrane delivery. From the data presented in this article, we conclude that MDS is probably caused by the loss of ε-sarcoglycan function at the plasma membrane. Importantly, ε-sarcoglycan (and torsinA) is expressed in a wide range of tissues, yet the primary defect in the dystonias appears to reside within neurons of the basal ganglia and/or cerebellum (43). Furthermore, MDS patients with *SGCE* mutations do not show any signs of muscle disease. It is therefore conceivable that, in this context, ε-sarcoglycan may co-operate with other membrane proteins to modulate the activity of neurons in the basal ganglia and cerebellum.

A mutation in the δ-sarcoglycan gene, *S151A*, has been reported to cause autosomal dominant cardiomyopathy (56).

Furthermore, a second mutation, N211Y, found in a family with mild proximal myopathy also appears to be dominantly inherited (57). Each of these amino acids is located in the extracellular domain of δ-sarcoglycan. It is possible that these diseases are dominantly inherited because the wild-type protein could traffic the altered protein to the membrane (Fig. 6B). This could disrupt the assembly and function of the muscle-expressed sarcoglycan complex leading to a disease state. It is also noteworthy that an extracellular deletion of γ-sarcoglycan is trafficked to sarcolemma but disrupts the function of the sarcoglycan complex in a family with LGMD and cardiomyopathy (58). It is therefore possible that mutations in individual sarcoglycans that can by-pass the normal quality control pathway mechanism in the secretory pathway could cause disease through a dominant negative mechanism.

Intracellular trafficking has been postulated to play an important role in the molecular pathology of dystonia. The AAA(+) protein torsinA is found in the lumen of the ER and can regulate the plasma membrane delivery of some polytopic membrane proteins including the dopamine transporter (34,59). Co-expression of torsinA and a number of polytopic proteins caused the intracellular retention of the polytopic protein and reduced targeting to the plasma membrane (34). Torres *et al*. (34) also showed that co-expression of torsinAΔE302/303 and the wild-type protein caused the sequestration of the wild-type protein into oligomeric complexes that formed aggregates. Co-expression of wild-type and mutant torsinA reversed the effect on polytopic membrane protein trafficking presumably through a dominant-negative effect (34). In this study, we have found that torsinA reduces the levels of both wild-type and mutant sarcoglycan when co-expressed *in vitro*. The extent of this effect is dependent upon protein conformation, such that torsinA expression reduces steady-state levels of the ε-sarcoglycan mutants by ∼60% compared with the wild-type control that is only reduced by 30%. Thus, torsinA could co-operate with the proteasome to augment the clearance of misfolded proteins from the cell. This hypothesis is supported by a study in *Caenorhabditis elegans* which showed that torsinA suppresses polyglutamine-induced protein aggregation (46). Caldwell *et al*. found that the *C. elegans* orthologue of torsinA and ubiquitin were targeted to the sites of protein aggregation (46). Finally, many members of the AAA(+) protein family have been shown to have chaperone functions related to their ATP-dependent ability to unfold proteins during degradation [see (60) for recent review]. It should be noted that torsinA has also been shown to play an important role in maintaining the integrity of the nuclear envelope (61–63). Furthermore, protein aggregates produced by transfection of torsinA mutants may be dependent upon expression levels because cells expressing low levels of mutant torsinA show a distinct perinuclear (nuclear envelope) staining pattern, whereas aggregates predominate in cells expressing high levels of the mutant (62,63).

In conclusion, we have shown that MDS-associated missense mutations in the extracellular region of ε-sarcoglycan impair trafficking to the plasma membrane. The mutant proteins are retained intracellularly, show evidence of misfolding and are degraded by the ubiquitin proteasome system. Co-expression of torsinA appears to reduce the steady-state levels of mutant ε-sarcoglycan but has little effect on the wild-type protein.

MATERIALS AND METHODS

Antibodies

The anti-ε-sarcoglycan antibody, esg-5009, was raised in rabbits immunized with a thioredoxin fusion protein containing the last 98 amino acids of mouse ε-sarcoglycan. The antisera was purified by affinity chromatography using the antigen coupled to Sulfolink (Pierce, Rockford, IL, USA) as described previously (64). Biotinylated esg-5009 was prepared by reacting 1 mg of affinity-purified antibody with sulfo-NHS-LC-biotin following the manufacturer's instructions (Pierce). Commercial antibodies were purchased from BD Bioscience (GM130), Stressgen, San Diego, CA, USA (PDI), Covance Research Products, Berkley, CA, USA (HA.11 anti-HA and B34, anti-GFP), Novocastra Laboratories Ltd, Newcastle-Upon-Tyne, UK (α-sarcoglycan, NCL-a-SARC; β-sarcoglycan, NCL-b-SARC and β-dystroglycan, 43DAG 8D5 monoclonal antibodies), Rockland Immunochemicals, PA, USA (IRDye™ 800 anti-mouse IgG), Invitrogen (Alexa Fluor 488, 568 and 680) and Sigma (α-tubulin). The anti c-myc monoclonal antibody 9E10, was produced in-house from a hybridoma and was purified and concentrated by protein G-sepharose chromatography (GE Healthcare). The anti-dystrophin 2166 and anti-dystrobrevin βCT-FP were produced in-house and are described elsewhere (40,65).

Expression constructs and site-directed mutagenesis

Mouse ε-sarcoglycan was amplified from a brain cDNA library by PCR using the primers esg-*Sal*I (5′-CGCG TCGACCTTGGACGGGAAAGGGTCG) and esg-*Not*I (5′-CGCGCGGCCGCTCAGGGATACCATTTACCTGTAG). The PCR product was digested and cloned into the *Sal*I/*Not*I sites of pCIneo (Promega). EYFP-tagged ε-sarcoglycan and mutants thereof were produced by PCR with the primers, 5′-CGGAAGCTTCTCAAGATGAGCCCCGCG and 5′-CCGGGATCCCGGGGATACCATTTACCTGTAGT, digested with *Hind*III and *BamH*I and subcloned into the corresponding sites in pEYFP-N1 (Clontech). The QuickChange site-directed mutagenesis kit (Stratagene) was used to introduce point mutations in the *SGCE* cDNA. The mutagenic primers are shown below with mutated codons italicized. L172Rf, 5′-CAGCCAGAGCGC*CGC*AACGCCATAAACAT; L172Rr, 5′-ATGTTTATGGCGTT*GCG*GCGCTCTGGCTG; H36Pf, 5′-CAGGTGTCCTCTTTGTT*CCG*GTGTTGGAGA GAGAGTA; H36Pr, 5′-TACTCTCTCTCCAACAC*CGG*AA CAAAGAGGACACCTG; H36Rf, 5′-AGGTGTCCTCTTT GTT*AGA*GTGTTGGAGAGAGAG; H36Rr, 5′-CTCTCTCT CCAACAC*TCT*AACAAAGAGGACACCT. A torsinA construct with a C-terminal myc-tag (torsinA-myc) was generated by PCR using the primers, 5′-CGGGAATTCGGGTCCGGTT ATGAAGCT and 5′-CGCGTCGACTCACAAGTCCTCTT CAGAAATGAGCTTTTGCTCGTCATCCAGGTAGTAGT CCA. The PCR product was digested with *Eco*RI and *Sal*I and cloned into the corresponding sites in pCIneo (Promega). TorsinAΔE304 was made by site-directed mutagenesis using the primers, DYT1ΔE304-F, 5′-CAGCAAGGTAGCGGAAA TGACGTTCTTCCCC and DYTΔE304-R, 5′-GGGGAA GAACGTCATTTCCGCTACCTTGCTG. The mouse α-sarcoglycan (αSG-EYFP) construct with a C- terminal EYFP

fusion was generated using the primers, 5′-TATGAA TTCTGATGGCAGCAGCAGTAACTTGG and 5′-ACCGT CGACTGGTGCTGGTCCAGGATAAGAG. The PCR product was digested with *Eco*RI and *Sal*I and cloned into the corresponding sites in pEYFP-N1. The mutant H77C-EYFP was generated by site-directed mutagenesis using the primers H77Cf 5′-GCCCAGGTGGCTGTGCTACACACAGCGCAG and H77Cr 5′-CTGCGCTGTGTGTAGCACAGCCACCTGGGC. All constructs were verified by sequencing. Human α-, β- and ε-sarcoglycan cDNA clones were purchased from OriGene Technologies Inc. (Rockville, MD, USA). All constructs were verified by sequencing. The HA-ubiquitin plasmid pMT123 was kindly provided by Professor Dirk Bohmann, University of Rochester Medical Center (66).

Cell culture, transfection and drug treatment

COS-7 and HEK 293T were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with Glutamax, 10% fetal calf serum (FCS) and penicillin/streptomycin. Cells grown in six-well plates with and without coverslips were transfected with 1 μg of each expression construct using Fugene 6 reagent (Roche) according to the manufacturer's instructions. SH-SY5Y human neuroblastoma cells were cultured in F-12 (Ham)/DMEM (1:1) supplemented with Glutamax, 15% FCS and penicillin/streptomycin. Neuroblastomas were grown in six-well plates with coverslips and transfected with 4 μg of each expression construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The proteasome inhibitors, lactacystin (10μM, Sigma) and MG132 (50μM, Sigma) were applied to the cells 24 h after transfection as described previously (37). Cells were incubated with the drugs for 16–24 h and were then processed as described below. Hippocampal and cortical neuronal cultures were prepared from 18-day-old embryonic rats and grown on coverslips in 24-well-plates or in 6-well-plates as described previously (67). Briefly, pregnant rats were decapitated, the embryos removed and the cortices or hippocampi dissected followed by trypsinization and trituration to dissociate the cells. Neurons were then plated on poly-D-lysine coated coverslips at a density of 15 000 cells/cm^2 for immunocytochemistry. Neurons were initially maintained in DMEM containing 10% FCS, 1× antibiotic/antimycotic (Life Technologies) and 2 mM glutamine. From the second day, hippocampal neurons were maintained in neurobasal medium containing B12 supplement, 1× antibiotic/antimycotic and 0.5 mM glutamine. Cortical neurons were grown in neurobasal media containing 2% B27, 0.5 mM L-glutamine, 25 μM glutamate, 0.05% gentamicin. To restrict the proliferation of non-neuronal cells, after 4 days the media were changed to neurobasal media supplemented with 2% B27, 0.5 mM L-glutamine, 3 μM cytosine β-D-arabinofuranoside and 0.05% gentamicin. Cortical neurons were transfected at 7 d.i.v with 4 μg of each expression construct using Lipofectamine 2000 (Invitrogen).

Immunocytochemistry and confocal microscopy

For immunocytochemistry, neurons were rinsed in phosphate-buffered saline (PBS) after removal of cell culture media and directly post-fixed in 4% (w/v) paraformaldehyde for 15–20 min at room temperature. The fixed cells were washed twice with 25 mM glycine in PBS and then blocked with 10% (v/v) horse serum, 3% (w/v) bovine serum albumin (BSA) and 0.2% (v/v) Triton X-100 in PBS (blocking solution) for 1 h. For immunostaining, cells were incubated overnight at 4°C with primary antibody diluted in blocking solution. After careful rinsing in PBS, cultures were incubated with the secondary antibody (Alexa Fluor 488 or 568 conjugates) diluted in blocking solution without Triton X-100 for 1 h at room temperature. Cells were washed with PBS and mounted in Vectashield (Vector Laboratories). The stained cells were visualized using a Nikon Eclipse TE300 inverted microscope equipped with a Hamamatsu digital camera and images visualized using Simple PCI (Digital Pixel) software. A similar protocol was used for immunostaining of COS-7 and SH-SY5Y cells, however, fixation was performed in methanol (−20°C) to preserve the structure of the ER when the anti-PDI antibody was used.

Fluorescent recovery after photobleaching

COS-7 cells were grown on cover slips and transfected with either EYFP-tagged ε-sarcoglycan, H36P-EYFP or L172R-EYFP. Twenty-four hours after transfection, cells were washed with HEPES buffered Hank's buffered salt solution (HBSS) containing 0.33 mM D-glucose. Wild-type ε-sarcoglycan was treated with 1 μM brefeldin-A for 1 h before use, to ensure that the majority of protein was retained in the ER, and brefeldin-A was added to the HBSS for the duration of the FRAP experiments. ATP depletion of the cells was carried out using 2-deoxyglucose and 0.02% sodium azide treatment of the cells for 30 min, as described by Nehls *et al*. (41) in glucose-free HBSS and the FRAP experiments were performed at 30°C. Preliminary experiments showed that the effect of 2-deoxyglucose treatment of the cells was dose-dependent and the lowest effective concentration was found to be 0.5 mM, and this was used throughout the experiments. ATP depleted cells were not harmed by the treatment, as they showed full FRAP recovery if replaced into tissue culture medium for 1 h after the experiments were completed. Photo bleaching was carried out using the 514 nm argon laser line of an LSM 510 Zeiss confocal microscope at 75% of full power equivalent to 17 mW, recovery was monitored at 1% of this laser output. The bleached regions of interest were generally 50 pixels in diameter and seven iterations were performed to bleach the region. Unbleached regions of the same cell were used to control for photobleaching during the recovery period, though this was generally minimal. For statistical analysis, the recovery curve for each cell was fitted to the equation for diffusion rates and mobile fractions as described in (68) using Graphpad Prism 4 software. The differences in the calculated diffusion rates and fractional recoveries were analyzed using the same software and the Mann–Whitney *t*-test.

Western blotting

Western blots were performed as described previously (64). Samples were prepared for native SDS–PAGE in native

PAGE buffer (1×, 67 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 10 mM iodoacetamide). For quantitative, two-colour western blot analysis, protein extracts were separated by SDS–PAGE and electroblotted onto nitrocellulose membranes before incubation with the esg-5009 polyclonal antibody and a mouse monoclonal anti-α-tubulin antibody. After washing, the membranes were incubated with IR fluorophore-conjugated secondary antibodies, IRDye™ 800 anti-mouse IgG and Alexa Fluor 680 anti-rabbit IgG. Membranes were then washed with Tris-buffered saline (TBS) containing 0.1% (v/v) Tween20 and rinsed in TBS. Simultaneous two-colour detection was performed using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Quantification of ε-sarcoglycan was performed using the Odyssey® imager application v1.2, normalized to α-tubulin fluorescence as an internal reference. Two-tailed Student's *t*-tests were used for the statistical analysis of protein levels.

Immunoprecipitation

Immunoprecipitations from heterologous cells were performed using either anti-c-myc 9E10 monoclonal antibody conjugated to protein G-sepharose beads or esg-5009 conjugated protein G-sepharose beads. The antibodies were covalently cross-linked to protein G-sepharose using dimethyl pimelimidate as described previously (69). Transfected HEK 293T cells were washed once with PBS and lysed and solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 1 mM EGTA) at 4°C for 30 min. Cell debris was removed by centrifugation. Lysates were precleared with unconjugated protein G-sepharose at 4°C for 1 h. For immunoprecipitation, the lysates were mixed with the antibody-protein G-sepharose beads for 3 h at 4°C, after which the beads were washed three times with RIPA buffer. The immunoprecipitated proteins were eluted from the beads by boiling in SDS–PAGE sample buffer for 5 min. ε-Sarcoglycan was immunoprecipitated from whole mouse brain RIPA extracts (1 g in 20 ml) using 4 μg of esg-5009 as described previously (40).

Glycosidase treatment

For glycosidase treatment of endogenous ε-sarcoglycan, mouse brains from eight-week old male CD1 mice were homogenized in RIPA buffer containing 0.1% SDS (w/v) and incubated at 4°C for 30 min. Lysates were clarified by centrifugation at 100 000*g* for 30 min. The brain lysate was precleared with protein G-sepharose at 4°C for 3 h followed by incubation with esg-5009 (10 μg) for 5 h at 4°C. After overnight incubation at 4°C, immune complexes were captured with goat anti-rabbit IgG-Magnabind beads (Pierce) and washed four times with RIPA buffer. Brain-derived ε-sarcoglycan was resuspended directly in the appropriate glycosidase buffer. Endoglycosidase (Endo) H and peptide N-glycosidase (PNGase) F were purchased from New England Biolabs and were used as described previously (37). Glycosidase treatment on proteins produced in transfected cells was performed as described previously (37).

Ubiquitination assays

HEK 293T cells were transfected singly with wild-type ε-sarcoglycan and the MDS mutants, and in combination with HA-ubiquitin. The proteasome inhibitors lactacystin (10 μM) or MG132 (50 μM) were applied to cells 24 h after transfection and incubated for a further 16 h. Transfected cells were washed once with PBS and solubilized in lysis buffer (100 mM Tris pH 8.0, 0.5% (w/v) SDS, 5 mM DTT). Lysates were briefly sonicated, boiled for 5 min and cooled on ice. A sample of each lysate was retained before diluting the sample 1:10 with RIPA buffer. ε-Sarcoglycan was immunoprecipitated with 4 μg of esg-5009 for 5 h at 4°C. Immune complexes were captured with goat anti-rabbit IgG Magnabind beads (Pierce) at 4°C overnight. Immunoprecipitated proteins were washed three times with RIPA buffer and eluted by boiling for 5 min in SDS–PAGE sample buffer.

BMH cross-linking

Transfected COS-7 cells were lysed in PBS containing 1% (v/v) Triton X-100 in the presence of the cross-linker BMH (Pierce) at a final concentration of 1 mM. The reaction was incubated for 30 min at room temperature before quenching by the addition of an equal volume of 2× SDS–PAGE sample buffer containing DTT to a final concentration of 20 mM.

Cell surface biotinylation

Transfected COS-7 and HEK 293T cells were washed three times with ice-cold PBS and incubated for 30 min at 4°C with gentle agitation in the presence of the membrane impermeable sulfo-NHS-biotin reagent (Pierce) prepared in PBS (1 mg/ml); 1 ml of reagent was used per well on a 6-well plate. The cells were then washed for 10 min at room temperature with 100 mM glycine prepared in PBS to quench the cross-linker and remove excess biotin. Cells were rinsed in PBS and lysed in RIPA buffer. ε-Sarcoglycan was immunoprecipitated from the biotin-labelled cells using esg-5009 conjugated protein G-sepharose beads. Samples were separated by SDS–PAGE and western blotted with the esg-5009 or horseradish peroxidase–streptavidin (GE Healthcare).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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