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Identification and characterization of three novel mutations in the *CASQ1* gene in four patients with tubular aggregate myopathy

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ABSTRACT

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Here we report the identification of three novel missense mutations in the calsequestrin-1 (*CASQ1*) gene in four patients with tubular aggregate myopathy. These CASQ1 mutations affect conserved amino acids in position 44 (p.(Asp44Asn)), 103 (p.(Gly103Asp)) and 385 (p.(Ile385Thr)). Functional studies, based on turbidity and dynamic light scattering measurements at increasing Ca²⁺ concentrations, showed a reduced Ca²⁺-dependent aggregation for the CASQ1 protein containing p.Asp44Asn and p.Gly103Asp mutations and a slight increase in Ca²⁺-dependent aggregation for the p.Ile385Thr. Accordingly, limited trypsin proteolysis assay showed that p.Asp44Asn and p.Gly103Asp were more susceptible to trypsin cleavage in presence of Ca²⁺ in comparison to WT and p.Ile385Thr. Analysis of single muscle fibers of a patient carrying the p.Gly103Asp mutation showed a significant reduction in the response to caffeine stimulation, compared to normal control fibers. Expression of CASQ1 mutations in eukaryotic cells revealed a reduced ability of all these CASQ1 mutants to store Ca²⁺ and a reduced inhibitory effect of p.Ile385Thr and p.Asp44Asn on store operated Ca²⁺ entry. These results widen the spectrum of skeletal muscle diseases associated to *CASQ1* and indicate that these mutations affect properties critical for correct Ca²⁺ handling in skeletal muscle fibers.

Key Words: excitation-contraction coupling; SOCE; tubular aggregate myopathy, calsequestrin; STIM1; ORAI1

INTRODUCTION

Calsequestrin (CASQ) is a protein of 45 kDa localized in the lumen of the sarcoplasmic reticulum (SR) of striated muscles where, thanks to its ability to bind Ca²⁺ with low affinity and high capacity, is responsible for Ca^{2+} storage in relationship with activation of muscle contraction. Two isoforms of CASQ, encoded by two different genes, have been identified: the skeletal isoform CASQ1 (MIM# 114250) expressed in fast skeletal muscle fibers and the cardiac isoform CASQ2 (MIM# 114251) expressed in cardiac muscle and slow skeletal muscle fibers (Schiaffino and Reggiani, 2011). Mutations in the CASO2 gene have been associated to catecholaminergic polymorphic ventricular tachycardia (CPVT) (Lahat et al., 2001). This disorder is usually caused by emotional stress or exercise in young individuals, and is characterized by recurrent syncopal episodes and seizures or sudden death due to the impaired Ca²⁺ handling properties of cardiomyocytes expressing mutated CASQ2 (Faggioni et al., 2012). More recently, we reported the first mutation in CASQ1 in patients with vacuolar aggregate myopathy (MIM# 616231), an autosomal dominant muscle disorder characterized by the presence of large vacuoles containing distinctive inclusions of variable size that result from the aggregation of SR proteins (Tomelleri et al., 2006; Rossi et al., 2014b).

In the SR of skeletal muscle fibers, CASQ1 may exist both as monomers and polymers, where polymerization depends on Ca^{2+} concentrations. Low concentrations of Ca^{2+} induce dimerization by favoring front-to-front interactions between two adjacent monomers. Further increases in Ca^{2+} levels lead to the formation of polymers, mediated by back-to-back interactions, that result in an increased ability to bind Ca^{2+} (Park et al., 2003). CASQ1 participates, together with other proteins as triadin, junctin and the ryanodine receptor Ca^{2+} release channel (RyR1) in the assembly of a large macromolecular machinery dedicated to

 Ca^{2+} release from the SR (Barone et al., 2015). Previous studies have provided evidence that CASQ1 may have an inhibitory effect on the pathways that regulate Ca^{2+} entry (Shin et al., 2003; Zhao et al., 2010). A physical interaction between CASQ1 and the Stromal Interaction Molecule 1 (STIM1), localized on the SR, has been also reported (Wang et al., 2015; Zhang et al., 2016). STIM1, together with the Ca^{2+} release-activated Ca^{2+} modulator 1 (ORAI1) on the plasma membrane, functions to allow the replenishing of depleted intracellular Ca^{2+} stores, a mechanism known as Store-Operated Ca^{2+} Entry (SOCE).

Recently, missense mutations in STIM1 (MIM# 605921) and ORAI1 (MIM# 610277) genes have been identified in patients affected by tubular aggregate myopathy (TAM) (Böhm et al., 2013, Böhm et al., 2017; Endo et al., 2015; Okuma et al., 2016). TAM is a progressive condition characterized by muscle weakness, cramps, myalgia and the presence of tubular aggregates in muscle fibers, that originate from the SR membrane (Engel et al., 1970) and contain numerous SR proteins including, among others, sarco-endoplasmic reticulum Ca²⁺ ATPase (SERCA), triadin and CASQ1 (Salviati et al., 1985; Chevessier et al., 2005; Boncompagni et al., 2011). The processes leading to the formation of these structures are not fully understood. Tubular aggregates formation has been proposed to result from aggregation of SR-derived membranes possibly caused by misfolding and aggregation of SR proteins as a consequence of hereditary or acquired conditions (Kuncl et al., 1989; Chevessier et al., 2005; Agbulut et al., 2000; Schiaffino 2012; Giacomello et al., 2015). Activating mutations in STIMI have been also detected in patients with Stormorken syndrome, who present thrombocytopenia and bleeding diathesis in association with miosis and TAM (Misceo et al., 2014; Nesin et al., 2014). Activating mutations in ORAII have been detected in patients with a Stormorken-like syndrome lacking hematological disorders but presenting with miosis and TAM (Nesin et al., 2014). On the other hand, loss-of-function mutations in ORAII and STIMI have been detected in patients with severe combined immunodeficiency (SCID) 4 This article is protected by copyright. All rights reserved.

syndrome that also present a non-progressive muscle hypotonia (Feske et al., 2006; Maus et al., 2015) indicating that mutations resulting in either decreased or increased Ca^{2+} influx have the potential to affect skeletal muscle tissue (Lacruz and Feske, 2015).

Here we report the first identification of three different mutations in the *CASQ1* gene in patients with TAM. Characterization of these CASQ1 mutations indicated that they have altered Ca^{2+} -dependent polymerization and reduced ability to store Ca^{2+} .

MATERIALS AND METHODS

Ethics statement

Ethics committee approval and written informed consent was obtained for all patients. This study complies with the ethical standards laid down in the 1964 Declaration of Helsinki.

Morphological analysis of patients' muscle biopsies

All patients underwent an open biopsy of vastus lateralis muscle. Serial 10 µm-thick transversal or longitudinal sections were stained with standard histological and histochemical methods including hematoxylin and eosin (H&E), reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH), succinate dehydrogenase (SDH), cytochrome-c oxidase (COX). For immunofluorescensce experiments, 8 µm-thick sections were incubated with the following primary antibodies: anti-CASQ1 mouse monoclonal antibody (clone MA3-913; Thermo Scientific); anti-CASQ1 rabbit polyclonal antibody (Thermo Scientific); anti-STIM1 mouse monoclonal antibody (clone 44/GOK; BD Biosciences), anti-ORAI1 mouse monoclonal antibody (clone 6D11A11; Thermo Scientific), anti-SERCA mouse monoclonal antibody (clone IIH11; Thermo Scientific) and anti-RyR1 rabbit polyclonal antibody (Rossi et al., 2014a). Cy2- or Cy3-conjugated antimouse or antirabbit secondary antibodies (Jackson ImmunoResearch Laboratories) were used for immunofluorescence detection. All procedures for histochemical and electron microscope analysis have been previously described (Rossi et al., 2014b; Tomelleri et al., 2006).

Genetic analysis

Genomic DNA was extracted from peripheral blood leucocytes by standard procedures. All CASQ1 exons amplified were using specific primers designed with Primer3 software (http://bioinfo.ut.ee/primer3). Amplified DNA fragments were directly sequenced using an ABI Prism 310 apparatus (Applied Biosystem) as previously described (Rossi et al., 2014b). DNA mutation numbering was based on cDNA reference sequence (NM_001231.4), taking nucleotide +1 as the A of the ATG translation initiation codon. The mutation nomenclature used follows that described at http://www.hgvs.org./mutnomen/. The CASQ1 mutations have been submitted to the locus-specific database (www.lovd.nl/CASQ1).

Whole-exome sequencing (WES) was performed at CRIBI Biotechnology Center (Padova, Italy). Briefly, DNA libraries were constructed following the standard protocol of the Ion AmpliSeq[™] Exome RDY Kit (ThermoFisher Scientific). Starting from 100ng of gDNA, a multiplex-PCR with 12 primer pools was perfomed, allowing the amplification of 24,000 amplicons per pool, covering the exonic regions of the genomes (about 58 Mb). Further, 9 µl of 100 pM barcoded libraries were amplified by emulsion PCR in the OneTouch2 instrument.

Finally, the barcoded samples were loaded into Ion Proton P1 v3 chip and sequenced on the Ion Proton instrument using the Ion Proton HiQ Sequencing kit. The bioinformatic analysis was performed on the Ion Torrent Server and the Torrent SuiteTM (<u>https://ts-pgm.epigenetic.ru/ion-docs/Torrent-Variant-Caller-Plugin.html</u>) for variant calling and to align the reads to the human genome reference 19 (hg19, http://grch37.ensembl.org/). Data were then analyzed by QueryOR web platform (http://queryor.cribi.unipd.it/cgi-bin/queryor/mainpage.pl) (Bertoldi et al., 2017) and genetic variants (missense, nonsense, indels) filtered for a global minor allele frequency ≤0.001,

shared by the affected individuals but not present in five healthy controls. Multiple protein alignment was performed using the ClustalW software (<u>http://www.ebi.ac.uk/Tools/msa/clustalo/</u>). Structural analysis of CASQ1 mutations was performed using the " PyMOL molecular visualization system" (http://pymol.org) on the crystal structure of hCASQ1 (PDB code: 3UOM).

Generation and purification of recombinant CASQ1

The human CASQ1 cDNA (NM_001231.4) was amplified by Polymerase Chain reaction (PCR) using specific primers with BamHI and SalI restriction sites (Forward: 5' cgg gat cca tgg ggc cca gag ctg tgc cg; Reverse: 5' gac gtc gac cta gtc atc atc atc gtc). The cDNA was cloned in frame with GST in the plasmid vector pGEX-4T-1 (GE Healthcare) digested with the same restriction enzymes. Site-directed mutagenesis of double-stranded cDNA was performed using the Q5 Site-Directed Mutagenesis Kit (NEB Inc.). All the constructs were sequenced using an ABI Prism 310 apparatus (Applied Biosystems). Recombinant plasmids were transformed by electroporation in the BL21 E. Coli strain. Expression and purification of recombinant proteins have been previously described (Rossi et al., 2014a). Thrombin cleavage was performed to separate GST from the protein of interest using the Thrombin Cleavage Capture Kit (Millipore) according to manufacturer's instructions.

Limited trypsin proteolysis assay

Wild type (WT) and mutant CASQ1 proteins were incubated in 20 mM Tris-HCl, pH 7.4 at 25°C for 20 minutes in a reaction volume of 100 μ l in presence or absence of 0-1-3 mM Ca²⁺. 5 μ g of protein were taken before trypsin (Sigma-Aldrich) was added at a protease:CASQ1 ratio of 1:500 (w/w). Samples (5 μ g) were taken at specific time points of 5, 30 and 120 minutes, mixed with sample buffer 4X to stop trypsin proteolysis and heated at 95°C for 3 minutes before the analysis on 15% SDS-PAGE. The gels were stained with Blue-Coomassie. Experiments were repeated at least 3 times.

Turbidity assay, Dynamic Light Scattering and structural analysis

Turbidity assay was performed to study CASQ1 aggregation as a function of Ca²⁺ concentration (Scherer et al., 2012). Turbidity was detected using a spectrophotometer and measuring the absorbance at 350 nm. In particular, 2.5 μ M of protein was incubated in 100 μ l of a solution containing 20 mM Tris-HCl and 150 mM KCl, pH 7.4 in a quartz micro cuvette. Increasing Ca²⁺ concentrations from 0 to 10 mM were added in the sample and mixed by pipetting. Absorbance measurements were recorded at intervals of 10 minutes at 25°C. Experiments were repeated at least 3 times. The Ca²⁺-dependent aggregation of the proteins was analyzed also by dynamic light scattering (DLS), using a Zetasizer NanoTM (Malvern Instruments) and a low volume quartz cuvette. Samples were centrifuged at 12000 rpm for 10 minutes and filtered using 0.45 μ m filters prior to data collections. Measurements were performed at 25 °C with 3 mg/ml of protein buffered in 20 mM Tris-HCl, 300 mM KCl, pH 7.4, with the following Ca²⁺ concentrations: 0, 3, 5 and 10 mM. To evaluate the reversibility of the aggregation process, 10 mM EGTA was added at the sample with 10 mM Ca²⁺.

Patient's single fiber Ca²⁺ release and force measurement

Small fragments of muscle biopsies were immersed in glycerol-containing skinning solution (Salviati and Volpe, 1988). Single fibers were manually dissected and segments of approximately 1-mm in length were cut and mounted with small aluminum clips (T clips) at both ends. The experimental setup has been previously described (Doria et al., 2011). The ability of the SR to store and release Ca²⁺ was tested with a protocol of cyclic SR loading in the presence of ATP at low pCa²⁺ and releasing Ca²⁺ with caffeine in low Mg²⁺ and low EGTA (Rossi et al 2001, Rossi et al 2014b). Caffeine concentrations tested were in the range 0.5 mM to 20 mM, the latter being sufficient to induce a maximal release, virtually emptying SR (Fryer and Stephenson, 1996; Lamb et al., 2001; Lamboley et al., 2015). Therefore, maximal response to caffeine provided an approximation of the total Ca²⁺ content of the SR, while the curve linking the amount of Ca²⁺ release to the caffeine concentration was taken as an indication of the ryanodine receptor channel properties. Tension time area,

normalized to the amplitude of the contractile response elicited by a direct maximal activation with pCa 4.3 was adopted as an indication of the amount of Ca²⁺ released (Endo and Iino, 1988; Makabe et al 1996, Fryer and Stephenson 1996, Lamb et al., 2001; Lamboley et al., 2015).

Cell culture, DNA transfection and Ca²⁺ measurement

HeLa cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum as previously described (Giurisato et al., 2014). Cells were plated on 4-wells glass chambers slides (Sarstedt, Nümbrecht, Germany) at the density of 4x10⁴ cells/chamber. After 24 hours, cells were cotransfected with pcDNA3.1 vectors expressing CASQ1 and GFP using Lipofectamine-Plus reagent following the manufacturer's instructions. Changes in cytosolic Ca²⁺ concentration were measured with Fura-2/AM (Life Technologies). Transfected HeLa cells were loaded with 3 µM Fura-2 as described (Giurisato et al., 2014). Transfected cells were identified by detection of GFP fluorescence and only cells expressing comparable levels of GFP fluorescence were selected. For Ca²⁺ measurements Fura-2 fluorescence was analyzed with a MetaFluor imaging system; experiments were carried out at room temperature in Ca²⁺-free solution containing 0.5 mM EGTA. Ca²⁺ release from the endoplasmic reticulum (ER) was induced by 1µM Thapsigargin (Tg, Sigma-Aldrich). Ca²⁺ influx was evaluated following the addition of 1.5 mM CaCl₂. The ratio (R) between fluorescence signals 340/380 nm was used to calculate cytosolic Ca^{2+} concentration according to the following formula: $[Ca^{2+}]_i = Kd * [(R-Rmin)/(Rmax-R)] * Sf/Sb; Rmax was obtained by treating the cells with 5 <math>\mu$ M ionomycin and 10 mM Ca^{2+} and Rmin by adding 5 μ M ionomycin and 20 mM EGTA in Ca^{2+} -free Hepes buffer. Sf and Sb are the emission intensities at 380 nm for Ca²⁺ free and Ca²⁺ bound Fura-2, respectively. Kd for Fura-2 at 25°C was assumed 135 nM. In order to quantify the Ca²⁺ released from the ER, the Area Under the Curve (AUC), which represents the Ca²⁺ released in 5 min after the addition of Tg was measured. An indication of Ca²⁺ influx was calculated measuring the difference between $[Ca^{2+}]_i$ peak after Ca^{2+} re-addition and basal $[Ca^{2+}]_i$. Statistical significance was estimated by

the two-tailed unpaired Student's test with systematic Welch correction for variance with GraphPad Prism 6.0.

Time-lapse live imaging and image analysis

GFP-fusion proteins were obtained by cloning WT and mutant CASQ1 cDNAs in the pEGFP expression vector (Clontech). HeLa cells were co-transfected with vectors expressing WT or mutant GFP-CASQ1 and mRFP-STIM1 (Giurisato et al., 2014). Cells were plated on 4-chamber glass bottom dishes (In vitro Scientific, Mountain View, CA, USA) and treated with 1 μ M Tg to induce SOCE in Hepes buffer medium. Time-lapse live imaging was performed with a confocal laser scan microscopy (Zeiss LSM 510 Meta). Single cells were selected and top to bottom focal planes were acquired at a time interval of three minutes, at a 1024 x 1024 resolution and with a maximum voxel size of 0.11 x 0.11 x 1 μ m (X, Y and Z dimensions). The distribution of the CASQ1 GFP fluorescence signal was evaluated by counting the number of cells showing linear structures, round structures and a homogeneous distribution in the ER at t = 0 and t = 12 minutes after Tg addition. The relative percentage of each class of distribution was calculated and expressed as average value from at least four independent experiments. Statistical analysis was performed with GraphPad Prism 6.0 software and the Holm-Sidak's multiple comparisons test. Image analysis was performed with ImageJ software v.10.2 (https://imagej.nih.gov/ij/). For evaluation of the average size of mRFP-STIM1 clusters, selected images were corrected for background fluorescence. The ImageJ "Auto Threshold" function (version 1.14) of the GDSC plugin was set on corrected images and average size of clusters was calculated using the ImageJ "Analyze Particles" plugin. Statistical analysis was performed with GraphPad Prism 6.0 software and the Holm-Sidak's multiple comparisons test.

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Accepted Article

Missense variants in the *CASQ1* gene are present in four patients affected by tubular aggregate myopathy

Sequence analysis of the CASQ1 gene (NC 000001.11) in a group of twenty-one patients with a clinical history of molecularly undiagnosed myopathy resulted in the identification of a NM 001231.4:c.130G>A variant, predicting an amino acid change from aspartic acid to asparagine (p.(Asp44Asn), RNA not analyzed), in a 32-year-old male (patient 1), who complained of fatigue and diffuse exercise-induced myalgia. Electromyography (EMG) showed a myopathic pattern in the muscles examined (bilateral anterior tibial, rectus femoris, deltoid and biceps brachii, and right vastus median). Repetitive stimulation excluded neuromuscular junction disorders. Echocardiography and electrocardiogram were normal. At the histological level patient 1 presented features of tubular aggregates compatible with a diagnosis of TAM (Fig. 1A). Based on this evidence, we extended the analysis to seven unrelated patients with a diagnosis of TAM. This analysis resulted in the identification of two additional CASQ1 heterozygous missense variants in three patients (Fig. 1B). The NM 001231.4:c.308G>A variant, predicting an amino acid change from glycine to aspartic acid (p.(Gly103Asp), RNA not analyzed), was found in two different probands, a 67-year-old male (patient 2) who complained of fatigue, and a 52-year-old woman (patient 3) with a tenyear history of exercise-induced muscle pain, stiffness and early fatigue. The NM 001231.4:c.1154T>C variant, predicting an amino acid change from isoleucine to threonine (p.(Ile385Thr) RNA not analyzed), was detected in an 82-year-old woman (patient 4) suffering of myalgia and proximal muscle weakness (Fig. 1B). The serum CK was normal in all patients except for patient 2, who exhibited occasional elevation of CK serum (752 UI/l). No family history has been documented for these patients.

Light microscopy analysis of muscle biopsies of all probands revealed, as a distinguishing feature, the presence of tubular aggregates mainly in type II fibers that stained strongly with NADH, whereas they were negative to SDH or COX staining, indicating a reticular rather than a mitochondrial origin of the aggregrates (Fig. 1A). Biopsies of patients 2, 3 and 4 were also stained with H&E, which stains tubular aggregrates in pink. Electron microscopy analysis was performed on specimens from patients 1, 2 and 3 and showed the typical presence of cytoplasmic aggregrates of swollen membranous tubules. Immunostaing of cross sections from all patients' biopsies with antibody against SERCA confirmed the SR origin of these aggregrates (Fig. 1A). Additional analysis performed on sections from patients 3 and 4 biopsies, carrying the p.(Gly103Asp) and the p.(Ile385Thr) mutations, respectively, revealed that additional SR proteins such as CASQ1, RYR1 and STIM1 were present in the aggregrates (Fig. 2). ORAl1 was usually not detectable in CASQ1-positive tubular aggregrates or only at much lower levels (Fig. 2).

All the variants identified affect amino acid residues conserved across species (Fig. 1C). Two of the variants identified in TAM patients are reported in the ExAC exome database (exac.broadinstitute.org) as rs140253806 (c.130G>A) and rs371278891 (c.1154T>C) with a minor allele frequency established of 0,002 and 0,00011, respectively. It is worth noting that the frequency of these variants, being higher than that reported for TAM cases, may reflect an underdiagnosis of TAM in the general population and/or that some individuals might be asymptomatic carrier, thus making it difficult to determine the real frequency of this disorder. The other variant, c.308G>A, has not been described, although two distinct rare substitutions affecting the same codon (rs567336006) have been reported. Damage prediction on protein function was assessed using established online tools (SIFT [http://sift.bii.a-star.edu.sg/index.html] and Polyphen-2 [http://genetics.bwh.harvard.edu/pph2/index.shtml]). This analysis indicated the p.(Asp44Asn) substitution as deleterious (SIFT: 0.0; PolyPhen2: 1.0). SIFT and PolyPhen2 analysis indicated the p.(Gly103Asp) and p.(IIe385Thr) as tolerated (SIFT: 0.553; PolyPhen2: 0.007 and SIFT:

0.117; PolyPhen2: 0.593, respectively). These predictions are based exclusively on the CASQ1 sequence and on the degree of amino acid residues conservation in closely related sequence alignments. The effects of these mutations on the tertiary structure as well as possible consequences on polymeric forms of the protein are not considered. To exclude mutations in other genes, whole exome sequencing was performed on the four patients. No mutations were detected in *STIM1* and *ORAI1* genes. Moreover, variants were filtered for a frequency ≤ 0.001, shared among the affected individuals and not present in five healthy controls. This analysis revealed no other potentially pathological variants in the four probands. The position of these three TAM mutations is reported in Fig. 1D, along with that of the p(Asp244Gly) mutation identified in patients with vacuolar aggregate myopathy, using a model of polymerization based on the crystal structures of WT and p.Asp244Gly CASQ1 (Wang et al., 1998; Lewis et al., 2015). Interestingly, these mutations are located at sites of back-to-back (p.Asp244Gly and p.Ile385Thr) or front-to-front interactions (p.Asp44Asn and p.Gly103Asp).

p.Asp44Asn and p.Gly103Asp proteins are more susceptible to proteolysis

Proteases can be used at low concentrations to distinguish folded from unfolded proteins, based on the observation that unfolded proteins are cut more rapidly than structured proteins due to the inability of proteases access buried sites. То investigate CASQ1 to proteins conformation/aggregation at increasing Ca²⁺ concentrations, recombinant CASQ1 proteins containing the three different TAM mutations were purified from bacteria together with the WT and the p.Asp244Gly previously reported in vacuolar aggregate myopathy (Rossi et al., 2014b; Di Blasi et al., 2015). CASQ1 aggregation propensity was analyzed by limited trypsin proteolysis in absence or presence of increasing Ca²⁺ concentrations. In the absence of Ca²⁺ all proteins were highly sensitive to trypsin digestion. The addition of Ca²⁺ reduced the proteolysis of WT and mutated CASQ1 proteins demonstrating appropriate changes in the aggregation state of these proteins. Densitometric analysis of the intact protein at 0 minutes (input) compared to the different trypsin incubation times

revealed, at 1 and 3 mM Ca²⁺, that p.Asp44Asn and p.Gly103Asp remained more susceptible to trypsin proteolysis, although this tendency decreased for p.Gly103Asp. No differences were detected for the p.Ile385Thr and p.Asp244Gly mutations. In particular, in presence of 1 mM Ca²⁺, the amount of residual p.Asp44Asn was 46% and 32% and the amount of residual p.Gly103Asp was 56% and 37%, whereas WT, displayed a maximum value of residual protein of 67% and 52% at 30 and 120 minutes of incubation, respectively (Fig. 3).

TAM-associated CASQ1 mutations show altered Ca²⁺-induced aggregation properties

To study the aggregation properties of the CASQ1 mutants, turbidity measurements were performed exposing purified CASQ1 proteins to increasing Ca^{2+} concentrations and measuring the absorbance at 350 nm. Increasing absorbance was detected with WT CASQ1 across the tested Ca^{2+} concentrations, as expected. The p.Asp244Gly mutation aggregated more than WT, displaying an increase in absorbance that started between 1 and 2 mM Ca^{2+} and that remained higher until 10 mM Ca^{2+} , in agreement with reported data (Lewis et al., 2015). TAM-associated CASQ1 mutations have a different behavior compared to WT and p.Asp244Gly. The p.Ile385Thr mutant showed an increase in turbidity consistently higher, although not significantly different from WT. On the contrary, turbidity values observed between 1 and 10 mM Ca^{2+} with both p.Gly103Asp and p.Asp44Asn were constantly lower than WT (Fig. 4A). All aggregations were fully reversed by the addition of EGTA (not shown).

The Ca^{2+} -dependent aggregation tendency of the proteins was also evaluated by DLS, a technique highly sensitive to the presence of aggregates as the signal depends on the hydrodynamic properties of the scattering object in solution and hence on its dimensions (both size and shape). As shown in Fig. 4B, WT protein did not appreciably change its aggregation state between 0 and 3 mM Ca^{2+} . At 5 mM Ca^{2+} , the signal revealed a more

aggregated sample and at 10 mM Ca^{2+} the sample appeared even more aggregated. The p.Asp244Gly mutation had the highest tendency to aggregate, showing aggregation already at 3 mM Ca^{2+} , with the correlogram shifted to higher correlation times. The mutation p.Ile385Thr showed a similar behavior, starting aggregating from 3 mM Ca^{2+} on. In contrast, mutations p.Gly103Asp and p.Asp44Asn did not change their aggregation state up to 5 mM Ca^{2+} and were found aggregated only at 10 mM Ca^{2+} . With all proteins, aggregation was fully reversed by the addition of EGTA.

Single muscle fibers from patient with the p.Gly103Asp mutation show altered response to caffeine stimulation

To correlate SR alterations to the myopathic phenotype, the tension elicited by stimulation with increasing concentrations of caffeine was studied in single muscle fibers isolated from a biopsy of patient 3 (p.Gly103Asp) and compared with fibers dissected from 4 healthy subjects of comparable age, as described in Materials and Methods. Examples of response to 2 mM and 20 mM caffeine are given in Fig. 5A: the response induced by 2 mM caffeine (Fig. 5A1 and 5A3) and by 20 mM caffeine (Fig. 5A2 and 5A4) were much lower in a fiber of the patient 3 (Fig. 5A3 and 5A4) compared to a fiber of a control subject (Fig. 5A1 and 5A2). As can be seen in Fig. 5B, average dose-response curves show that the maximal response was significantly reduced in a balanced pool of fast and slow fibers of the patient 3 in comparison to healthy control fibers of comparable age, although the responsiveness to caffeine measured as log EC50% was not reduced.

Altered Ca²⁺ handling in HeLa cells expressing CASQ1 mutations

To directly analyze the ability of CASQ1 mutations to increase the ER Ca^{2+} storage capability, the WT and CASQ1 mutations were expressed in HeLa cells and intracellular Ca^{2+} levels were measured following Fura-2 loading. Transfected cells were treated with Tg to measure the total content of Ca^{2+} in the ER. Expression of WT CASQ1 increased the

intracellular Ca²⁺ content, as expected (Fig. 5C-D). Transfection of the three novel TAMassociated mutations and of the p.Asp244Gly, responsible for vacuolar aggregate myopathy, resulted in a significant lower increase in the amount of Tg-releasable Ca²⁺ revealing that all CASQ1 mutants analyzed have a reduced ability in increasing the intracellular Ca²⁺ store content compared to WT CASQ1. Earlier work has shown that CASQ expression has an inhibitory effect on the pathways that regulate SOCE, the mechanism needed to refill intracellular Ca²⁺ store in eukaryotic cells (Shin et al., 2003; Zhao et al., 2010; Wang et al., 2015). Therefore, we measured the peak phase following the addition of extracellular Ca^{2+} to measure the amount of Ca^{2+} entering cells after emptying stores with Tg (Fig. 5E-F). In agreement with reported data, following addition of 1.5 mM extracellular Ca²⁺, expression of WT CASQ1 inhibited SOCE. However, under these conditions, the expression of the TAMassociated p.Asp44Asn and p.Ile385Thr mutations did not result in a significant reduction of Ca²⁺ influx, suggesting a reduced ability to inhibit SOCE. On the contrary, expression of the p.Glv103Asp mutation found in TAM patients and of the p.Asp244Gly mutation, found in patients with vacuolar aggregate myopathy, resulted in an inhibition of Ca²⁺ influx comparable to that obtained with WT CASQ1.

Expression of WT and mutant CASQ1 proteins does not affect the subcellular localization of STIM1 in transfected cells

CASQ1 was reported to inhibit SOCE by preventing STIM1 aggregation (Zhang et al., 2016). We thus investigated the effects of CASQ1 mutants on STIM1 clusters assembly. HeLa cells were co-transfected with expression plasmids encoding mRFP-STIM1 and GFP fusion proteins of WT or mutant CASQ1. Cells were treated with 1 μ M Tg to induce activation of SOCE. STIM1 aggregation and CASQ1 depolymerization were analyzed by live imaging confocal microscopy on four to five focal planes to image the cortical ER close to the plasma membrane and the regular ER positioned in the interior of the cell (Supp. Fig. S2). Under resting conditions, WT CASQ1-GFP formed linear and round 16 This article is protected by copyright. All rights reserved.

aggregates in the ER, which reflect the assembly of large CASQ1 polymers at physiological Ca²⁺ concentration, while in a minority of cells CASQ1 proteins were more homogeneously diffused in the ER lumen (Supp. Table S1). Similarly, transfection of the p.Asp44Asn-GFP, p.Gly103Asp-GFP, p.Ile385Thr-GFP mutants resulted in the assembly of linear aggregates and clusters, with a remaining fraction of cells showing a CASQ1-GFP signal diffused in the ER. In contrast, expression of the p.Asp244Gly-GFP protein resulted in assembly of round aggregates in the majority of the cells, with only a minor fraction of cells showing linear polymers (Supp. Table S1). Imaging of the mRFP-STIM1 in these cells resulted in fluorescence signal compatible with ER localization, both in cells expressing wild type and mutant CASQ1, thus showing no co-localization with the linear and round aggregates of CASQ1 (Supp. Fig. S2).

Following treatment with Tg, the linear structures formed by WT CASQ1 as well as p.Asp44Asn and p.Ile385thr mutants disappeared and the GFP fluorescence signal redistributed to the ER and, to a lesser extent, in round aggregates. At variance, linear aggregates formed by the p.Gly103Asp mutant disappeared and the signal was mainly found in round clusters and to a lesser extent diffused in the ER. Finally, the distribution of p.Asp244Gly did not significantly change following Tg treatment, suggesting a reduced ability of this protein to depolymerize (Supp. Fig. S2). In parallel, the formation of STIM1 clusters at the plasma membrane was analyzed in cells co-expressing WT and mutant CASQ1. Measurement of the average size of STIM1 clusters formed following Tg treatment showed that clusters assembled in cells expressing WT or p.Asp244Gly were apparently larger compared to those measured in cells expressing mutants p.Asp44Asn, p.Gly103Asp and p.Ile385Thr, although statistical analysis did not reveal significant differences (Supp. Table S2). Analysis of top to bottom focal planes acquired in resting conditions and following Tg treatment showed that the mRFP-STIM1 fluorescence signal progressively increased from median to top focal planes resembling the assembly of cortical ER structures induced by Ca²⁺ store depletion and SOCE activation. On the contrary, the GFP-CASQ1 fluorescence signal did not apparently follow the assembly of cortical ER

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structures and remained mainly in median planes even at the end of Tg treatment (Suppl. Fig. S2). No significant co-localization between STIM1 and WT and CASQ1 mutants before and after Tg treatment was detected by Pearson coefficient analysis.

DISCUSSION

Here we report the first identification and characterization of three missense mutations in the *CASQ1* gene in four unrelated patients with TAM with diffuse myalgia, post-exercise fatigue and typical cytoplasmic tubular aggregates. Until now, only mutations in *STIM1* and *ORA11* have been identified in patients with TAM. The TAM patients with CASQ1 mutations have a milder phenotype compared to TAM patients with mutations in STIM1 and ORA11 and to patients with vacuolar aggregate myopathy, a related disorder that is caused by a different mutation in CASQ1. No one of the TAM patients carrying mutations in CASQ1 presented additional signs related to Stormorken syndrome. Functional characterization of these CASQ1 mutations revealed altered polymerization tendency and a common reduced ability to increase intracellular Ca²⁺ stores. However, the ability of CASQ1 mutations to inhibit Ca²⁺ entry pathways was maintained in only one of the three mutations, suggesting a further potential level of complexity in the pathomechanisms of TAM due to mutations in *CASQ1*.

CASQ1 mutations are localized at sites of interactions between CASQ1 subunits.

The extraordinary ability of CASQ1 to bind Ca^{2+} with high-capacity appears to depend on its propensity to form large polymers in a highly regulated, Ca^{2+} -dependent process. Indeed, the current model for CASQ1 polymerization indicates that low concentrations of Ca^{2+} induce formation of dimers by favoring front-to-front interactions between monomers, while increasing Ca^{2+} concentrations result in the assembly of tetramers, through back-to-back interactions, which will then result in large, complex CASQ1 polymers that contain 18 This article is protected by copyright. All rights reserved. additional Ca²⁺ binding sites at the interfase between CASQ1 dimers and tetramers (Park et al., 2003, Kumar et al., 2013). Initial studies on the p.Asp244Gly revealed an extremely rapid aggregation and a significant reduction of the Ca^{2+} binding properties of this protein (Lewis et al., 2015). We observed altered aggregation ability in all three CASQ1 mutations identified in patients with TAM. Aggregation of the p.Ile385Thr mutation was more sensitive to Ca²⁺ than WT CASQ1, even if much less than the p.Asp244Gly mutation. In contrast, both p.Asp44Asn and p.Gly103Asp mutations were less susceptible to aggregate at rising Ca²⁺ concentrations than WT CASQ1, similarly to what reported for the p.Met87Thr mutation (Lewis et al., 2015). Using the available crystal structures of WT and p.Asp244Gly CASQ1 (Wang et al., 1998; Lewis et al., 2015), we noticed the three CASQ1 mutations identified in TAM patients are located at sites that can affect the interactions between CASQ1 subunits. Initial studies with the p.Asp244Gly mutation (Lewis et al., 2015) indicated that it may decrease the association of dimers through linear back-to-back interactions. As shown in Fig. 1D. the p.Ile385Thr mutation is located at the interface of "lateral" back-to-back contacts that are responsible for the formation of the branched polymers formed by WT CASQ1 (Perni et al., 2013). Moreover, the negatively charged C-terminal tail, immediately downstream of Ile385, has been shown to be important to hamper the formation of not-specific polymerization products (Park et al., 2004; Beard and Dulhunty, 2015). Hence, it can be hypothesized that the p.Ile385Thr mutation favors the assembly of atypical, not-fully functional, polymeric forms. On the other hand, residues 44 and 103 are positioned at the interface responsible for the formation of CASQ1 dimers (Fig. 1D, Supp. Fig. S1). Accordingly, the p.Asp44Asn and p.Gly103Asp mutations, can affect the interactions that strengthen the front-to-front dimerization of the protein, resulting in dimers less efficient in supporting the formation of back-to-back tetramers, similarly to what proposed for the p.Met87Thr mutation (Lewis et al., 2015, Supp. Fig. S1). It can be noted that the CASQ1

mutations that have increased polymerization (p.Asp244Gly and p.Ile385Thr) are located at sites of back-to-back interactions, while CASQ1 mutations that have a reduced polymerization (p.Asp44Asn and p.Gly103Asp) are positioned at sites of front-to-front interactions.

CASQ1 mutations impair intracellular Ca²⁺ homeostasis

Analysis of single muscle fibers isolated from patient 3, carrying the p.Gly103Asp mutation, revealed a significant reduction in tension developed following maximal stimulation with caffeine, a result that suggests a possible reduced ability to store Ca^{2+} in these fibers. Indeed, all three TAM CASQ1 mutations, when transfected in HeLa cells, showed a reduced ability to increase the intracellular Ca^{2+} store content compared to WT CASQ1. Given that either increased or decreased polymerization tendency may reduce the ability of CASQ1 to bind Ca^{2+} , we hypothesize that the altered polymerization properties of the TAM CASQ1 mutations may explain their reduced capacity to enhance intracellular Ca^{2+} stores in muscle fibers and in transfected cells.

CASQ1 has been shown to inhibit SOCE by interacting with STIM1 and by reducing STIM1 aggregation (Wang et al., 2015; Zhang et al., 2016). Surprisingly, we observed different effects among the TAM CASQ1 mutations on the mechanisms of Ca^{2+} entry. Indeed, p.Asp44Asn and p.Ile385Thr showed loss of the ability to inhibit Ca^{2+} influx, while p.Gly103Asp presented an inhibitory effect similar to that of WT and of p.Asp244Gly. The observed effects on Ca^{2+} influx, did not correlate with Ca^{2+} -induced polymerization ability, or trypsin resistance, or ability to increase intracellular Ca^{2+} stores of the CASQ1 mutations (Supp. Table S3). It is worth noting that the observed increase in total Ca^{2+} content observed in transfected cells expressing WT or CASQ1 mutants is due to Ca^{2+} bound to CASQ1 in the SR, thus it should not affect intracellular levels of free Ca^{2+} or influence SOCE (Rios, 2010).

In spite of the contrasting effects on SOCE observed with WT and mutant CASQ1 proteins, we did not notice significant differences between expression of WT and mutant CASQ1 proteins on STIM1 localization and aggregation before or after Tg treatment. Altogether, these data suggest that changes in the ability to inhibit SOCE might not be an obligatory event in the pathogenic mechanism of TAM-associated CASQ1 mutations, at variance with the mechanisms of disease for TAM- associated mutations in *STIM1* and *ORAI1*. It is possible that identification of more subtle changes in the properties of CASQ1 mutants may help to understand the mechanisms whereby CASQ1 inhibits SOCE. Additional studies are needed to further address this point.

CASQ1 mutations in tubular aggregate myopathy and vacuolar aggregate myopathy.

Although the exact mechanisms responsible for inducing tubular aggregates formations are not fully understood, it is currently accepted that these structures may result from proliferation of the SR membrane triggered by conditions of ER stress due, among other causes, to misfolding of SR proteins resulting in the formation of protein aggregates and/or by altered Ca^{2+} homeostasis (Chevessier et al., 2005; Schiaffino 2012). The results reported here indicate that, similarly to mutations in *STIM1* and *ORAI1*, TAM-associated CASQ1 mutations can also affect intracellular Ca^{2+} homeostasis. It is therefore possible that the SR proteins aggregates observed in the biopsies of TAM patients may result as a consequence of impaired Ca^{2+} homeostasis or of the modified polymerization properties of CASQ1 mutations, or of a combined effect of the two.

TAM patients with CASQ1 mutations refer symptoms like diffuse myalgia, muscle weakness, post-exercise fatigue, and, at the histological level, present typical aggregates of membrane tubules in type II fibers. Patients with vacuolar aggregate myopathy, who all carry the p.Asp244Gly *CASQ1* mutation, in addition to the symptoms described above for TAM

patients with CASQ1 mutations, report also cramps and, more frequently, present elevated plasma creatine kinase levels. At the histological level, the distinctive hallmark of vacuolar aggregate myopathy are large vacuoles containing compact electron-dense inclusions of variable size and shape, that do not stain with several routine histochemical techniques, like the NADH reaction, which in contrast stains tubular aggregates.

All three CASQ1 mutations identified in TAM patients have either a mild increase in Ca²⁺dependent polymerization ability (p.Ile385Thr) or are less able to polymerize than WT CASQ1 (p.Asp44Asn and p.Gly103Asp). This differs from the property of the p.Asp244Gly mutation that shows a strong increase in Ca^{2+} -dependent polymerization (Lewis et al., 2015). The p.Asp244Gly mutation also displays a reduced ability to increase intracellular Ca²⁺ store content, a property common to all CASQ1 TAM mutations analyzed, and an inhibitory effect on SOCE similar to that of WT and p.Gly103Asp mutation. Interestingly, all TAM CASQ1 mutations can form elongated polymers, similar to those formed by WT CASQ1, that differ in their structure and dynamics properties from the aggregated polymers formed by the p.Asp244Gly mutation (Rossi et al., 2014b and Supp. Fig. S1). Thus, the major difference between p.Asp244Gly and the TAM CASQ1 mutations is represented by its exaggerated tendency to aggregate in irregular CASQ1 polymers (Rossi et al., 2014b; Lewis et al., 2015). Accordingly, we hypothesize that the large vacuoles of several µm in diameter that represent the hallmark of vacuolar aggregate myopathy (Tomelleri et al., 2006) develop as a result of the large dimensions of the p.Asp244Gly aggregates that, given their size, cannot be accommodated within the lumen of tubular aggregates, which usually have a diameter of 60-100 nm, and only more rarely of 200-400 nm.

Conclusions

We report here the discovery of three novel missense mutations in the *CASQ1* gene in four unrelated patients with tubular aggregate myopathy. These findings identify *CASQ1* as a new gene responsible for inducing TAM, in addition to *STIM1* and *ORA11*. Characterization of these mutations indicated that they all modify the polymerization properties of CASQ1 and have a reduced ability to increase intracellular Ca²⁺ stores, compared to WT CASQ1. A reduction in caffeine response, which also points to reduced intracellular Ca²⁺ stores content, was also observed in fibers form a patient carrying the p.(Gly103Asp) mutation. We propose that altered polymerization ability of the CASQ1 mutations diminish their capacity to attain maximal Ca²⁺ binding and this might explain the decreased Ca²⁺ content of intracellular stores. Less clear remains, at the moment, the relationship between the above reported properties and the various effects of CASQ1 mutation on Ca²⁺ entry. More work will help to better understand the multiple mechanisms operated by CASQ1 in maintaining physiological skeletal muscle Ca²⁺ homeostasis and function, and how mutations in *CASQ1* modify these mechanisms in tubular aggregate myopathy and vacuolar aggregate myopathy.

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Author Contribution

VB, VDR, AG, VP, EC, LG, SS, EP, DR, NV, GB performed experiments. EC and RB performed DLS experiments and protein modelling. LT and CR performed patient's single fiber force measurements. AM, GR, GS, GV and GT performed clinical evaluation of patients. VB and VS conceived and designed the study and supervised experiments. VB and VS wrote the manuscript. RF, CR, RB and VS critically revised the manuscript.

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Figure 1. Histological, immunohistochemical and ultrastructural characterization of patients' muscle biopsies. A: Tubular aggregates were basophilic following hematoxylin and eosin (H&E). Tubular aggregates stained in dark blue by nicotinamide adenine dinucleotide-tetrazolium reductase reaction (NADH), whereas they were not detected by either succinate dehydrogenase (SDH) and cytochrome-c oxidase (COX) staining. Arrows point to tubular aggregates. Asterisks denote the same fiber in serial biopsy sections. All patients' biopsies displayed tubular aggregates that were stained by anti-SERCA1 antibody. Electron microscopy (EM) analysis confirmed the presence of tubular aggregates. EM micrographs were not available for patient 4. Bars: 50µm in H&E, NADH, COX and SERCA1 pictures; 1 μ m in EM micrographs. B: Electropherogram of the CASQ1 gene sequence of patients heterozygous for the c.130G>A (patient 1), c.308G>A (patients 2 and 3) and c.1154T>C (patient 4). C: protein sequence alignment of CASQ1 regions of different species: the aspartic acid (D) in position 44, the glycine (G) in position 103 and the isoleucine (I) in position 385 are highlighted in yellow and are conserved among species ranging from snakes to humans. D: Proposed model of polymerization based on the crystal structure of CASQ1 in presence of high Ca²⁺ (PDB-ID 3UOM). Dimers of CASQ1 held together by front-to-front interactions (yellow and magenta monomers) can interact with other dimers both via "linear" back-to-back contacts (dimers green/cyan and red/grey) and via "branching" back-to-back contacts (dimer blue/olive green), originating a branched polymer. Mutation in position 244 (blue spheres for Asp244) interferes with linear back-to-back contacts while in position 385 interferes (red spheres for Ile385) with branching back-to-back contacts. Residues Asp44 and Gly103 are indicated by light blue and green spheres, respectively and are both localized at the front-to-front interaction surface where they can interfere with dimer formation.

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Figure 2. Immunofluorescence experiments on cross sections of biopsies from patients 3 and 4 revealed that nearly all tubular aggregates contained CASQ1. The vast majority of CASQ1 positive tubular aggregates were also positive for STIM1 and RYR1, whereas ORAI1 was rarely detected and at much lower extent in CASQ1 positive aggregates. Bars: 20 μm.



Figure 3. Limited trypsin proteolysis assay. WT CASQ1 and mutants were subjected to proteolysis in the absence of $Ca^{2+}(A)$, in the presence of 1 mM $Ca^{2+}(B)$ or 3 mM $Ca^{2+}(B)$. D: densitometric analysis of the intact protein (arrows) at 30 and 120 minutes of trypsin incubation, in presence of 1 mM Ca^{2+} . P values ≤ 0.05 and 0.005 are indicated by * and ** respectively. Experiments were repeated 3 times.



Figure 4. Ca^{2+} -dependent aggregation of purified CASQ1 proteins. **A:** CASQ1 turbidity is measured as absorbance at 350 nm (blue circle WT, orange diamond p.Asp44Asn, green triangle p.Gly103Asp, red square p.Asp244Gly, violet inverted triangle p.Ile385Thr). Experiments were carried out in a buffer containing 20 mM Tris-HCl, 150 mM KCl (pH 7.4) and 0 to 10 mM Ca²⁺. Data are presented as mean ± SEM and statistical analysis versus WT was performed by two-tailed unpaired Student's test. P values ≤ 0.005 and 0.0005 are indicated by ** and ***, respectively. **B** DLS measured correlation curves at increasing Ca²⁺ concentrations (red 0, green 3, blue 5 and black 10 mM Ca²⁺) for WT and CASQ1mutants. The right-shifting of the profiles to higher correlation times is correlated to the increase in the mean size of the samples, i.e. to the formation of aggregates. In absence of Ca²⁺ the correlograms are very similar for the five samples, indicating a similar initial state. In all cases the aggregation process induced by Ca²⁺ is reversible as indicated by the effect of the addition of 10 mM EGTA.





Figure 5. Response to caffeine of muscle fibers of patient 3 and analysis of intracellular Ca²⁺ stores and Ca²⁺ entry in HeLa cells expressing WT and mutant CASQ1. A: Response to caffeine of muscle fibers from patient 3 and from healthy controls. Examples of fibers response to the administration of 2 mM caffeine (panels 1 and 3: control and patient 3, respectively) and 20 mM caffeine (2 and 4: control and patient 3, respectively). Each release induced a wave of contraction, whose amplitude was expressed as tension-time area and was normalized to the amplitude of the response induced by maximal direct activation with pCa 4.3. The greater amplitude of the response induced by a given concentration of caffeine in control (panels 1 and 2) compared to patient 3 (panels 3 and 4) is clearly detectable. B: Caffeine dose-response curves of permeabilized muscle fibers from control healthy subjects (black dashed line, open circles) and patient 3 (continuous black line, filled circles). Contraction, analyzed as an indirect measure of Ca²⁺ release, was induced by concentrations of caffeine in the range from 0.1 to 20 mM (expressed in logarithmic scale). Curves were interpolated by a sigmoidal Hill equation: $Y = B + {(Amax - B)/(1+10^{([log EC50-X]*n)})}$. C: Representative traces of Ca²⁺ release from intracellular stores of HeLa cells untransfected (CTR) or transfected with WT or different CASQ1 mutations and loaded with Fura-2 following treatment with Thapsigargin (Tg) in the absence of extracellular Ca²⁺. **D**: Quantification of Ca²⁺ release. Mean values ± SEM of Area Under the Curve (AUC) measurements were calculated from traces of all experiments. E: Representative traces of Ca^{2+} influx from single cells evoked by the re-admission of 1.5 mM Ca^{2+} in HeLa cells pre-treated with Tg. **F**: Quantification of Ca²⁺ influx. Bars indicate Δ [Ca²⁺]₁ (nM) ± SEM. Data presented in panels D and F are from 172, 166, 99, 260, 130 and 141 cells for CTR, WT, and p.Asp44Asn, p.Gly103Asp, p.lle385Thr and p.Asp244Gly mutants, respectively. Measurements were obtained from at least 16 independent experiments. Data are mean ± SEM and statistical evaluation of the data in D and F was performed by the two-tailed unpaired Student's test with systematic Welch correction for variance. Statistically significant differences versus WT are indicated by * (p-values ≤ 0.05), **(p-values ≤ 0.005) and *** (p-values≤0.0005).

