The asthma-associated ORMDL3 gene product regulates endoplasmic reticulum-mediated calcium signaling and cellular stress

Gerard Cantero-Recasens†, César Fandos†, Fanny Rubio-Moscardo, Miguel A. Valverde and Rubén Vicente‡

Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Edifici PRBB, C/ Dr Aiguader 88, Barcelona 08003, Spain

Received September 9, 2009; Revised and Accepted October 7, 2009

Alterations of protein folding or Ca^{2+} levels within the endoplasmic reticulum (ER) result in the unfolded-protein response (UPR), a process considered as an endogenous inducer of inflammation. Thereby, understanding how genetic factors modify UPR is particularly relevant in chronic inflammatory diseases such as asthma. Here we identified that ORMDL3, the only genetic risk factor recently associated to asthma in a genome wide study, alters ER-mediated Ca^{2+} homeostasis and facilitates the UPR. Heterologous expression of human ER-resident transmembrane ORMDL3 protein increased resting cytosolic Ca^{2+} levels and reduced ER-mediated Ca^{2+} signaling, an effect reverted by co-expression with the sarco-endoplasmic reticulum Ca^{2+} pump (SERCA). Increased ORMDL3 expression also promoted stronger activation of UPR transducing molecules and target genes while siRNA-mediated knock-down of endogenous ORMDL3 potentiated ER Ca^{2+} release and attenuated the UPR. In conclusion, our findings are consistent with a model in which ORMDL3 binds and inhibits SERCA resulting in a reduced ER Ca^{2+} concentration and increased UPR. Thus, we provide a first insight into the molecular mechanism explaining the association of ORMDL3 with proinflammatory diseases.

INTRODUCTION

The ER is essential for the generation of intracellular Ca^{2+} signals, functioning as a regulated Ca^{2+} store (1,2). There are Ca^{2+} release channels (e.g. inositol trisphosphate receptor and ryanodine receptor) that control the exit of Ca^{2+} from the ER into the cytoplasm and pumps [sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA)] that return Ca^{2+} to the ER. In this sense, the activity of SERCA determines the rate of removal of cytosolic Ca^{2+}, shaping the Ca^{2+} signal and helping to maintain low cytosolic Ca^{2+} levels. SERCA activity also influences Ca^{2+}-dependent cellular responses by determining the ER [Ca^{2+}] that is available for release in response to the next stimulus (2). Consistent with the key role of SERCA in Ca^{2+} homeostasis, several pathological conditions presenting ER Ca^{2+} dysregulation are associated with SERCA dysfunction (3), including asthma (4) and Alzheimer disease (5,6), although in the latter case ER Ca^{2+} dysregulation has been also attributed to a Ca^{2+} leak via presenilin (7) or via the ryanodine receptor (8).

The assembly and folding of numerous proteins also occurs at the ER, a process that requires appropriate ER Ca^{2+} levels (9–11). Alterations of protein folding or Ca^{2+} levels within the ER result in the unfolded-protein response (UPR) (12–16). The UPR involves different signaling pathways that sense conditions of ER stress and trigger the subsequent cellular homeostatic response (17). Although the UPR has been characterized in different cell models, it is particularly relevant in cells from the immune system where it is related to lymphocyte development (18) as well as the generation and function of antibody-secreting plasma cells (19). The UPR can also trigger the activation of NF-κB and JNK (20–22), key molecules in the onset of inflammation. Together, the UPR has been considered a condition closely related to the inflammatory...
response of specialized cells (17), either through the differentiation of inflammatory cells or the production of molecules involved in the onset of inflammation (23).

Recently, the ORMDL3 gene, coding for the ER-resident transmembrane protein ORMDL3 (24), has been associated to asthma (25–29), a chronic inflammatory disease of the airways (30,31). ORMDL3 shows a wide distribution in different tissues, being particularly high its expression in cells participating in the inflammatory response (24,25). A common C/T polymorphism (rs7216389) controlling ORMDL3 expression was associated to asthma (25), but neither the function of ORMDL3 or the underlying mechanism for its association with asthma is known at present. Therefore, considering that: (i) there is a close relationship between UPR and inflammation; (ii) different transmembrane proteins of the ER participate in the sensing and initiation of the UPR; (iii) perturbation of ER calcium stores induces the UPR and (iv) diminished SERCA expression contributes to airway remodeling (4); we examined whether ORMDL3 may modulate ER-mediated Ca\(^{2+}\) signals and/or participate in the UPR, thereby providing a plausible pathophysiological mechanism explaining its association to asthma.

**RESULTS AND DISCUSSION**

**Expression and topology of ORMDL3**

Computer-based protein sequence analysis of the ER-resident human ORMDL3 protein originally predicted four membrane-spanning domains with different hydrophobicity scores (24). To obtain more detailed topological information, we used a fluorescence protease protection assay (32). Three versions of ORMDL3 tagged with fluorescence proteins (FP) were generated: at the N-terminus; C-terminus and amino acid 79 of the protein (Fig. 1A). HEK293 cells were transfected with different combinations of ORMDL3-CFP or ORMDL3-YFP, ER targeting sequence of calreticulin fused to Discosoma sp. red fluorescent protein, STIM1-YFP and TRPV4-CFP were treated with 20 \(\mu\)M digitonin. Images were taken before (B) and after (C) application of trypsin (2 min). The FP color combination was selected to code cytosolic fluorescent markers in blue and intrareticular fluorescent markers in yellow for transmembrane proteins and red for globular proteins. (D) Schematic structure cartoon based on predictions obtained using TMHMM-2 (www.cbs.dtu.dk/services/TMHMM-2.0), PSIPRED V2.3 (bioinf.cs.ucl.ac.uk/psipred) and the topology experiments shown in a-c. Scale bars 10 \(\mu\)m.

Figure 1. Localization and topology of ORMDL3. (A) Schematic cartoon of YFP-tagged ORMDL3 constructs and cellular localization of different molecules with fluorescent protein (FP) tags. HEK293 cells co-expressing different combinations of ORMDL3-CFP or ORMDL3-YFP, ER targeting sequence of calreticulin fused to Discosoma sp. red fluorescent protein, STIM1-YFP and TRPV4-CFP were treated with 20 \(\mu\)M digitonin. Images were taken before (B) and after (C) application of trypsin (2 min). The FP color combination was selected to code cytosolic fluorescent markers in blue and intrareticular fluorescent markers in yellow for transmembrane proteins and red for globular proteins. (D) Schematic structure cartoon based on predictions obtained using TMHMM-2 (www.cbs.dtu.dk/services/TMHMM-2.0), PSIPRED V2.3 (bioinf.cs.ucl.ac.uk/psipred) and the topology experiments shown in a-c. Scale bars 10 \(\mu\)m.
tagged at amino acid 79, suggesting that the central region of ORMDL3 faces the ER lumen. Based on these results and the fact that only two domains, of around 20 amino acids each (Supplementary Material, Fig. S1), show considerable hydrophobicity scores (>80% probability), we proposed a topology model for ORMDL3 consisting of two transmembrane domains, with N- and C-terminus facing the cytoplasm and a large loop within the ER lumen (Fig. 1D).

Impact of ORMDL3 on cellular Ca$^{2+}$ homeostasis

The impact of heterologous expression of ORMDL3 on ER-mediated intracellular Ca$^{2+}$ signals was evaluated in HEK293 cells. Cytosolic Ca$^{2+}$ signals (using fura-2 calcium sensitive dye) were recorded from GFP- and ORMDL3-transfected cells in response to carbachol, a muscarinic agonist that triggers the release of Ca$^{2+}$ from inositol trisphosphate (IP$_3$)-sensitive

Figure 2. Decreased ER Ca$^{2+}$ signal in cells overexpressing ORMDL3. (A) Changes in cytosolic [Ca$^{2+}$]$_{cyt}$ (fura-2 ratio) evoked by 5 μM carbachol in Ca$^{2+}$-containing solutions. (B) ORM DL3 overexpression slows the clearance of cytosolic Ca$^{2+}$ following carbachol stimulation in Ca$^{2+}$-free bath solutions. Traces show mean ratio normalized to peak Ca$^{2+}$ signals in GFP and ORM DL3 transfected HEK293 cells (n > 50 for each condition). Time constants for Ca$^{2+}$ clearance are 1.47 min and 3.7 min for GFP- and ORM DL3-transfected cells, respectively. (C and D) Changes in [Ca$^{2+}$]$_{ER}$ (mag fura-2 ratio) in GFP- (C) and ORM DL3-transfected cells (D) exposed to 5 μM IP$_3$. Traces show normalized mean ratio Ca$^{2+}$ signals in GFP and ORM DL3-transfected HEK293 cells. Time constants for Ca$^{2+}$ reuptake are 15 ± 2 min for GFP- (n = 16) and 26 ± 5 min (n = 18) for ORM DL3-transfected cells. *P < 0.05 (versus GFP transfected cells), Student’s t-test.
ER (Fig. 2A). The Ca\textsuperscript{2+} signal generated, which mainly reflects the amount of Ca\textsuperscript{2+} stored in the ER, was quantified by calculating the mean area under the curve (inset). ORMDL3 overexpression also slowed the decay rate of cytosolic Ca\textsuperscript{2+} clearance following carbachol stimulation (Fig. 2B), indicative of decreased SERCA activity (5,35–37), as this Ca\textsuperscript{2+} pump is in charge of replenishing the stores with Ca\textsuperscript{2+} pumped from the cytosol into the ER (38). This observation was further confirmed by direct measurements of intra-ER Ca\textsuperscript{2+} levels using a low-affinity Ca\textsuperscript{2+}-sensitive dye (mag fura-2) trapped within the ER in cells permeabilized with digitonin (39). Higher resting mag fura-2 ratios were observed in GFP- (Fig. 2C) than ORMDL3-transfected HEK293 cells (Fig. 2D). Mean basal ratios are shown in Fig. 2E. When permeabilized cells were stimulated with 5 μM IP\textsubscript{3}, the ratio decreased rapidly in both GFP- and ORM DL3-transfected cells. Upon IP\textsubscript{3} removal, ratio increased, indicating reuptake of Ca\textsuperscript{2+} into the IP\textsubscript{3}-sensitive store (Fig. 2C and D), but with a slower kinetics in ORM DL3-cells. Exponential fitting of Ca\textsuperscript{2+} reuptake gave mean time constants of 15 ± 2 min (n = 16) and 26 ± 5 min (n = 18) for GFP- and ORM DL3-expressing cells (P < 0.05), respectively (Fig. 2F). Addition of the SERCA inhibitor thapsigargin (TG) (3,38) prevented Ca\textsuperscript{2+} reuptake into the intracellular organelle following a second IP\textsubscript{3} challenge (Fig. 2C and D). This approach using mag fura-2 that only reports on Ca\textsuperscript{2+} signal within the ER, minimized the effect of Ca\textsuperscript{2+} extrusion through the plasma membrane on calcium clearance that may be present in the experiment shown in Fig. 2B. In other words, while in Figure 2B, time constant of Ca\textsuperscript{2+} clearance from the cytosol may be influenced by both Ca\textsuperscript{2+} reuptake into the ER and Ca\textsuperscript{2+} extrusion through the plasma membrane, data shown in Figure 2F only refers to the time constants of Ca\textsuperscript{2+} reuptake into the ER.

The effect of other stimuli and knockdown of endogenous ORM DL3 with siRNA was also tested. ORM DL3 overexpression decreased Ca\textsuperscript{2+} response to the SERCA inhibitor cyclopiazonic acid (CPA, 10 μM), which promotes passive release of Ca\textsuperscript{2+} from ER (3,38), whereas ORM DL3 siRNA potentiated the response to CPA (Fig. 3B), recorded in the absence of extracellular Ca\textsuperscript{2+} to avoid possible contribution of Ca\textsuperscript{2+} influx across the plasma membrane. Mean curve areas obtained following CPA treatment in cells transfected with GFP, control siRNA, ORM DL3 and ORM DL3 siRNA are shown in Figure 3C. The fact that similar effect was observed in ORM DL3-transfected cells exposed to an IP\textsubscript{3}-generating stimulus and the SERCA inhibitor CPA, suggests that the effect shown in Figure 2A is mainly produced by inhibition of Ca\textsuperscript{2+} reuptake into the ER, rather than inhibition of the IP\textsubscript{3} receptor. Although, at present, we cannot unequivocally discard the latter possibility.

Note the inverse correlation between ORM DL3 levels and CPA-induced, ER-mediated Ca\textsuperscript{2+} response: the highest Ca\textsuperscript{2+} signal corresponded with lowest ORM DL3 levels. On the other hand, a positive correlation was detected between basal cytosolic Ca\textsuperscript{2+} concentration and ORM DL3 levels (Fig. 3D). Similar results were obtained with ionomycin, an ionophore that releases Ca\textsuperscript{2+} from most intracellular stores in the absence of external Ca\textsuperscript{2+} (Fig. 3E). Overexpression of ORM DL3 was always associated with a significant reduction in the ER Ca\textsuperscript{2+} response while knocking down ORM DL3 increased such response.

**ORM DL3 interacts with and modulates SERCA activity**

ORM DL3-GFP co-localized with endogenous SERCA2b in HEK293 cells (Fig. 4A) and SERCA2b (either native or overexpressed) co-immunoprecipitated ORM DL3-GFP in HEK293 cells (Fig. 4B). Expression of the proteins of interest was probed by western blotting in the same cell lysates (input) used for immunoprecipitation (Supplementary Material, Fig. S2). These results suggested that the modulatory effect of ORM DL3 upon SERCA may involve a direct association. As with other SERCA-interacting proteins that regulate pump activity (40–42), co-immunoprecipitation of ORM DL3 and SERCA2b was stronger at 5 mM Ca\textsuperscript{2+}.

Next, we attempted reverting ORM DL3-induced phenotype by co-expressing SERCA and ORM DL3. Cells overexpressing ORM DL3 and stimulated with 1 μM TG—that also promotes passive release of Ca\textsuperscript{2+} from ER—showed reduced Ca\textsuperscript{2+} release from the ER, a phenotype that was reverted by co-expressing SERCA2b (Fig. 5A and B). Basal cytosolic Ca\textsuperscript{2+} was also returned to control conditions in cells co-expressing SERCA2b and ORM DL3 (61 ± 1 μM, n = 80; P = 0.1 versus GFP transfected cells).

Our results showed: lower ER Ca\textsuperscript{2+} levels and release from the ER; slower Ca\textsuperscript{2+} reuptake into the ER and cytosolic Ca\textsuperscript{2+} clearance; and higher basal cytosolic Ca\textsuperscript{2+} concentrations. Besides, co-localization and co-immunoprecipitation of both ORM DL3 and SERCA suggest a physical interaction between both proteins. Together, these results obtained from ORM DL3 overexpressing cells are consistent with the inhibition of the SERCA pump that contributes to maintain low cytosolic Ca\textsuperscript{2+} concentrations by pumping Ca\textsuperscript{2+} from the cytosol into the ER (3,38). Although at present we cannot fully discard that ORM DL3 may also present certain channel activity, as reported for other modulators of SERCA (7).

In our attempt to identify ORM DL3 functional motifs, we deleted the last nine amino acids of ORM DL3 that contain a failure to avoid possible contribution of Ca\textsuperscript{2+} influx across the plasma membrane.

**ORM DL3 facilitates UPR**

A decrease in ER Ca\textsuperscript{2+} (using SERCA inhibitors) triggers the UPR, characterized by activation of signaling molecules and, ultimately, increased transcriptional activation of immediate-early genes and others directly related with the onset of inflammation (17). UPR signaling pathways at the ER usually follow the activation of one or more of the known protein sensors: pancreatic endoplasmic reticulum kinase (PERK), inositol-requiring 1 α (IRE1α) or activating transcription factor 6 (ATF6). The levels of ER stress were evaluated by analyzing the phosphorylation of eukaryotic initiation factor 2 α (eIF2α), an early marker of UPR downstream of PERK activation (15,17). ORM DL3 overexpression induced significantly higher basal eIF2α
phosphorylation, without changing total eIF2α, whereas ORM DL3 knock-down with siRNA significantly reduced the phosphorylated eIF2α levels (Fig. 5E and F). Overexpression of ORM DL3-Δ145–153 did not significantly affect eIF2α phosphorylation (Fig. 4E and F). Therefore, expression of functional ORM DL3 correlated with the level of phosphorylated eIF2α.

We also evaluated the influence of ORM DL3 on two UPR target genes downstream of eIF2α, BIP and EGR-1. Similar to what we observed with eIF2α, overexpression of ORM DL3 augmented BIP while overexpression of ORM DL3-Δ145–153 did not significantly affect BIP expression (Fig. 5G). Loss of ORM DL3—with siRNA—also attenuated BIP expression (Supplementary Material, Fig. S4A). Similar results were obtained when evaluated EGR-1 transcription (Supplementary Material, Fig. S4B). Typically, ER stress activates different UPR signaling pathways (22), although preferences may exist.
cells, ORMDL3 overexpression in Jurkat cells decreased the Ca\(^{2+}\) response to the SERCA inhibitor CPA (30 \(\mu\)M) (Fig. 6A). Phosphorylation of eIF2\(\alpha\) was also evaluated in Jurkat cells transfected with CFP or ORMDL3-CFP. Due to the low transfection rate of Jurkat cells, eIF2\(\alpha\)-P was analyzed on individual cells by immunofluorescence confocal microscopy (Fig. 6B). Mean normalized levels of eIF2\(\alpha\)-P and percentage of responding Jurkat cells are clearly increased in ORMDL3-transfected cells, compared with CFP transfected Jurkat cells (Fig. 6C and D).

**Conclusions**

Our data reports on the involvement of ORMDL3 on ER-mediated Ca\(^{2+}\) signaling and facilitation of ER-mediated inflammatory responses. Besides, our study increases the understanding of the cellular and molecular mechanisms underlying the reported association of ORMDL3 with inflammatory diseases such as asthma (25) and Crohn’s disease (44). Interestingly, the increased risk of asthma conferred by ORMDL3 variants has been recently associated to tobacco smoke (29), being this environmental disease modifier an inducer of UPR (45). In conclusion, our observation offers a novel target for the study of ER Ca\(^{2+}\) signaling and its impact on disease pathophysiology.

**Materials and Methods**

**Plasmids and cell transfection**

Human ORMDL3 and pig SERCA2b expression vectors were a kind gift from Drs R. Gonzalez-Duarte (University of Barcelona) and M. Brini (University of Padova), respectively; pDsRed-ER was obtained from CLONTECH. ORMDL3 tagged with CFP at the N- and C-terminus was generated by subcloning ORMDL3 cDNA into pCDA3-CFP and pEFP-C1 vectors, respectively. ORMDL3 tagged with YFP at amino acid position 79 and ORMDL3-\(\alpha\) were analyzed by western blots with anti-GFP (bottom) to detect ORMDL3. Mean immunoprecipitated signals are shown for each lane (n = 3).

Figure 4. ORMDL3 interacts with SERCA. (A) Colocalization of ORMDL3 and SERCA2b in HEK293 cells transfected with ORMDL3-GFP. ORMDL3-GFP signal (green) and SERCA2b (1:250) immunostaining in permeabilized cells (red). Merge images appear yellow in colocalization areas. Scale bar 20 \(\mu\)m. (B) Co-immunoprecipitation of SERCA2b and ORMDL3 in HEK293 cells transfected with ORMDL3-GFP (lane 2) or ORMDL3-GFP + SERCA2b (lanes 1 and 3 to 5) was analyzed under different conditions: control nominal free calcium (lane 3), 5 \(\mu\)m Ca\(^{2+}\) (lane 4) or 5 \(\mu\)m EGTA (lanes 5). Immunoprecipitation lanes in the absence (lane 1) or presence of \(\alpha\)-SERCA2b polyclonal antibody (lanes 2 to 5). Immunocomplexes were analyzed by western blots with anti-GFP (bottom) to detect ORMDL3. Mean immunoprecipitated signals are shown for each lane (n = 3).

Figure 5. ORMDL3 modulates SERCA activity and UPR. (A) ER Ca\(^{2+}\) release evoked by 1 \(\mu\)M TG in Ca\(^{2+}\)-free solutions in HEK293 cells transfected with GFP (filled circle), ORMDL3 (open circle) or ORMDL3+SERCA (filled square). (B) Average ER Ca\(^{2+}\) release (area under the curve) obtained by integrating Ca\(^{2+}\) signals of individual cells. (C) Schematic structure of ORMDL3-\(\Delta\)145–153 construct and ER Ca\(^{2+}\) release evoked by 5 \(\mu\)m carbobal in Ca\(^{2+}\)-free solutions in HEK293 cells transfected with GFP (filled circle) or ORMDL3 (open circle) or ORMDL3-\(\Delta\)145–153 (filled circle). (D) Average ER Ca\(^{2+}\) release (area under the curve) obtained by integrating Ca\(^{2+}\) signals of individual cells. Data are expressed as the mean \(\pm\) SEM (N values shown for each bar). (E) Total and phosphorylated eIF2\(\alpha\) were detected by western blot analysis of whole extracts from EGFP, ORMDL3, ORMDL3 siRNA and ORMDL3-\(\Delta\)145–153 transfected cells. (F) Mean relative levels of phosphorylated eIF2\(\alpha\)/total eIF2\(\alpha\) obtained from four independent experiments. (G) Messenger RNA levels of BIP (relative to Rlp19) were analyzed by quantitative RT–PCR in cells transfected with GFP and ORMDL3 and ORMDL3-\(\Delta\)145–153. Data are expressed as the mean \(\pm\) SEM (n values shown for each bar). *P < 0.05 (versus GFP transfected cells), one way ANOVA and Tukey post hoc for comparison of multiple conditions.
Measurement of intracellular $[\text{Ca}^{2+}]$

Cytosolic $\text{Ca}^{2+}$ signal was determined at RT in cells loaded with 4.5 $\mu$M fura-2-AM (20 min) as previously described (46). Cytosolic $[\text{Ca}^{2+}]$ increases are presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm, relative to the ratio measured prior to cell stimulation.

**Figure 6.** Effect of ORMDL3 on T cell ER $\text{Ca}^{2+}$ signaling and stress. (A) Changes in cytosolic $\text{Ca}^{2+}$ evoked by 30 $\mu$M CPA in Jurkat cells overexpressing CFP (filled circle) or human ORMDL3-CFP (open circle). Insets show average $[\text{Ca}^{2+}]$ increases (Area) obtained by integrating the $\text{Ca}^{2+}$ signals of individual cells. (B) Confocal CFP (left) and phosphorylated elf2$\alpha$ (right) immunofluorescence images of Jurkat cells transfected with CFP or human ORMDL3-CFP. Arrows mark CFP and elf2$\alpha$-P signals in the same cell. (C) elf2$\alpha$-P fluorescence signal normalized by area in non-transfected cells (NT), CFP and ORMDL3-CFP transfected cells. Data are expressed as the mean ± SEM (N values shown for each bar). *$P < 0.05$ (versus non-transfected cells), one way ANOVA and Tukey post hoc. CFP versus ORMDL3 $P < 0.05$. (D) Percentage of Jurkat cells showing increased elf2$\alpha$-P signal (>30% of the signal detected in NT cells). Number of positive cells over total shown for CFP and ORMDL3-CFP transfected cells. Scale bars 20 $\mu$m.
(fura-2 ratio 340/380). Absolute basal Ca^{2+} concentration was obtained from the fluorescence ratios using an on-cell calibration protocol (47). All experiments were carried out at room temperature and cells were bathed in an isotonic solution containing (in mM): 140 NaCl, 5 KCl, 1.2 CaCl2, 0.5 MgCl2, 5 glucose, 10 HEPES (300 mosmol/l, pH 7.4 with Tris). Ca^{2+}-free solutions were obtained by replacing CaCl2 with equal amount of MgCl2 plus 0.5 mM EGTA.

To measure free [Ca^{2+}]_{iER} inside the ER cells were incubated 45 min at 37°C with the low-affinity Ca^{2+} dye mag fura-2-AM (5 μM) in isotonic medium containing 0.02% pluronic F-127 (39). To wash out the cytosolic dye, plasma membrane was permeabilized in ATP-free intracellular-like media (in mM: 120 KCl, 25 NaCl, 0.1 MgCl2, 0.75 CaCl2, 0.5 EGTA (~100 mM [Ca^{2+}]) and 10 HEPES; pH 7.2 with KOH, 300 mOsm) containing 8 μM digitonin. Following permeabilization, digitonin was removed and 1 mM ATP added to the intracellular solution for at least 15 min, until stable mag fura-2 340/380 ratio levels were obtained, which are proportional to [Ca^{2+}]_{iER}.

**Fluorescence protease protection assay**

Twenty-four hours after transfection, live cells were permeabilized with 20 μM digitonin at RT for 3 min. After permeabilization, we incubated the cells with 2 mM trypsin for 2 min. The subcellular localization of tagged proteins was analyzed before and after trypsin treatment under a 40 × 1.32 Oil Ph3 CS objective, LCS Leica Confocal software and Argon (488 nm, JDS Uniphase Corporation) and HeNe (555 and 633 nm, JDS Uniphase Corporation and LASOS Lasertecnik GmbH, respectively) lasers using an inverted Leica SP2 Confocal Microscope, as previously described (32). Images were taken at room temperature and were not further processed except to adjust brightness, contrast and color balance.

**Expression knock-down and quantitative RT–PCR analysis**

Cells were seeded in 6-well plates at 90% confluence and exposed to 100 pmol of ORMDL3 siRNA (5'T-TAAGTAGCCACCGATCCATT-3') (Qiagen) or control siRNA (5'T-AATTCCTGGACCGTACGT-3') (Qiagen) diluted into 100 μl serum-free medium. Cells were transfected by a Lipofectamine 2000 (Invitrogen) procedure following the manufacturer’s instructions, as described previously (48). RNA extraction (Nucleospin RNA II kit, Macherey-Nagel) was carried out 48 h after transfection and RT–PCR was performed as described previously (48) using SuperScript-RT system (Invitrogen) and aliquots of 1 μg cDNA were used as template for quantitative PCR. Quantitative RT–PCR was performed on an ABI Prism 7900HT (Applied Biosystems) with SYBR-Green (SYBR-Green Power PCR Master Mix, Applied Biosystems) and ORMDL3 QuantiTect Primer Assay (Qiagen). Other primers used included: BIP 5'-CCGG CAAAGATGTGTCAGGAAA-3' and 5'-TTCGGAACCGGTCTTATGAGAC-3'; EGR-1 5'-CAGACCCCTCCACCCTCAG-3' and 5'-AGCGGGCAGTATAGGCTAG-3'. Beta-actin or ribosomal protein Rpl19 served as an internal normalization standard (20). PCR conditions for ORMDL3 and EGR-1 were: 95°C for 5 min; 95°C for 30 s; 60°C for 30 s, 72°C for 30 s; 72°C for 5 min; with 40 cycles of amplification. PCR conditions for BIP were: 95°C for 5 min; 95°C for 30 s; 60°C for 30 s, 72°C for 1 min; 78°C for 10 s; 72°C for 5 min, with 40 cycles of amplification.

**Immunoprecipitation assay and immunodetection**

Co-immunoprecipitation experiments were run as previously described (46). HEK293 cells were transiently transfected with human ORMDL3-GFP (or ORMDL3:ORMDL3-GFP ratio 1:1) and pig SERCA2b plasmids were lysated with immunoprecipitation buffer (0.5% Triton plus protease inhibitor cocktail in nominal free Ca^{2+} HBS or HBS containing 1 mM TG, 5 mM Ca^{2+} or 5 mM EGTA) and centrifuged at 10000 g to collect total protein in supernatant. Then 1000 μg total protein were incubated at 4°C overnight with anti-SERCA2b antibody (Abcam). After 2 h incubation with 30 μl of G protein (Amersham) at RT, immunocomplexes were washed with immunoprecipitation buffer five times and boiled for 5 min with loading buffer. Co-immunoprecipitation of ORMDL3 was detected with anti-GFP antibody (1:1000, mouse monoclonal antibody, Clontech Laboratories, Inc.). For western blotting of eIF2α, 50 μg of total protein obtained from HEK293 cells 48 h after transfection with GFP, ORMDL3, ORMDL3-A145–153 or ORMDL3 siRNA were separated on 4–10% gradient polyacrylamide gel and transferred to PVDF membranes. Primary antibodies used were mouse anti-eIF2α (1:500) and rabbit anti-phosphoS51-eIF2α (1:500) from Abcam. Secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:3000, GE Healthcare). Immunoreactive signal was detected with SuperSignal West Pico Chemiluminiscent substrate (Pierce) and visualized by Molecular Imager Chemidoc XRS system (Biorad).

For immunodetection of phosphorylated eIF2α, 24 h after transfection of Jurkat cells with ORMDL3-CFP or pCFP vectors, cells were attached for 30 min to poly-l-lysine coated coverslips, fixed with 4% PFA, permeabilized with 0.1% Triton x-100 in PBS and blocked with 1% BSA, 2% FBS, 0.05% Triton in PBS. Samples were stained for phospho-eIF2α using anti-phosphoS51-eIF2α antibody (1:50 in blocking solution). Secondary antibody was a goat anti-rabbit Alexa Fluor555 (Molecular Probes). Images were acquired using an inverted Leica SP2 Confocal Microscope with a 40 × 1.32 Oil Ph3 CS objective and analyzed using ImageJ software. Intensity/area ratio of every transfected cell was normalized to the intensity/area mean of the surrounding non transfected cells in the same image. Endogenous SERCA immunodetection was carried out following the same procedure using SERCA 2b antibody (1:250, Genetex).

**Statistics**

All data were expressed as means ± SEM of N (number of cells analyzed) or n (number of experiments carried out). Statistical analysis was performed with Student’s unpaired tests, or one-way analysis of variance (ANOVA) using Sigma-Plot or OriginPro software. Bonferroni’s or Tukey’s tests were
used for post hoc comparison of means. The criterion for a significant difference was a final value of $P < 0.05$.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**ACKNOWLEDGEMENTS**

We thank C. Plata for technical support, R. Gonzalez-Duarte (University of Barcelona) for ORMDL3 plasmid, M. Brini (University of Padova) for SERCA2b plasmid and S. Muallen (University of Texas) for STIM1 plasmid.

Conflict of Interest statement. None declared.

**FUNDING**

This work was supported by Spanish Ministry of Science and Innovation (SAF2006-04973, SAF2009-09848); Fondo de Investigación Sanitaria (Red HERACLES RD06/0009); Generalitat de Catalunya (SGR05-266); and Fundació la Marató de TV3 (061331 and 080430). M.A.V. is the recipient of an ICREA Academia Award.

**REFERENCES**


