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# RyR1-mediated moderate endoplasmic reticulum stress is required for myogenic differentiation of myoblasts

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**Research Article**

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# Abstract

## Background

Cytosolic $Ca^{2+}$ plays vital roles in myogenesis and muscle development. Key mutations of ryanodine receptor 1 (RyR1), a major $Ca^{2+}$ release channel of endoplasmic reticulum (ER), are main causes of severe congenital myopathies. The role of RyR1 in myogenic differentiation has attracted intense research interest, however, it remains unclear.

## Methods

This study employed RyR1-knockdown myoblasts and CRISPR/Cas9-based RyR1-knockout myoblasts cells to explore the role of RyR1 in myogenic differentiation, myotube formation as well as the potential mechanism of RyR1-related myopathies.

## Results

Cytoplasmic $Ca^{2+}$ concentration was significantly elevated during myogenic differentiation of both primary myogenic cells and myoblasts C2C12 cells, accompanied with a dramatic increase in RyR1 expression and resultant ER stress. Inhibition of RyR1 by siRNA-mediated silence or chemical inhibitor, dantrolene, significantly reduced cytosolic $Ca^{2+}$, alleviated ER stress, and blocked multinucleated myotube formation. Moderate activation of ER stress effectively relieved myogenic differentiation stagnation induced by RyR1 suppression and demonstrated that RyR1 modulates myogenic differentiation via activation of $Ca^{2+}$ -induced ER stress signaling. RyR1 knockout-induced $Ca^{2+}$ leakage led to severe ER stress and excessive unfolded protein response, and drove cell fate from differentiation into apoptosis.

## Conclusions

Therefore, we concluded that dramatic increase in RyR1 expression is required for myogenic differentiation, and RyR1-mediated $Ca^{2+}$ release leading to the activation of ER stress signaling serves a double-edged sword role during myogenic differentiation. This study contributes to a novel understanding of the role of RyR1 in muscle development and related congenital myopathies, and provides a potential target for regulation of muscle regeneration and tissue engineering.

# Background

Ryanodine receptor 1 (RyR1), highly expressed in skeletal muscle, is located on the endoplasmic/sarcoplasmic reticulum (ER/SR) membrane. Intracellular flux of $Ca^{2+}$ mediated by RyR1 is required for skeletal muscle contraction. Various mutations or epigenetic changes in the RyR1 gene have been associated with muscle myopathies mainly including malignant hyperthermia and several congenital myopathies [1–4]. Diagnostic gene-sequencing has been employed to avoid RyR1-related

congenital myopathies. Several disease-modulating therapeutic strategies and salvage therapies have also been developed against RyR1-related myopathies [5–9].

RyR1 attracts intense interest in the field of medicine because it is a $Ca^{2+}$ channel of great clinical significance. The crystal structure of RyR1 has been determined by electron cryomicroscopy to be a 6-transmembrane ion channel with an EF-hand domain for $Ca^{2+}$-mediated allosteric gating and a huge cytoplasmic domain on top of each transmembrane domain [10–12]. $Ca^{2+}$ signaling is important for myogenic gene expression and skeletal muscle differentiation [13]. RyRs-mediated $Ca^{2+}$ release plays a role in the histogenesis of mammalian skeletal muscle, and the block of RyRs selectively inhibits fetal myoblast differentiation [14]. Mutations in RyR1 that results in leaking of the internal $Ca^{2+}$ store can have both physiological and pathological consequences [15].

A naturally occurring single-base mutation of RyR1 closely was associated with malignant hyperthermia of pigs but has been particularly enriched in genetic selections for muscle growth rate and lean body mass [16]. Homozygous RyR1-null mice died after birth and displayed small limbs and abnormal skeletal muscle organization [17, 18]. Presumably, RyR1 is not only associated with myopathies, but is implicated in myogenesis and subsequent muscle development. However, the mechanisms of RyR1 action in myogenesis have not been elucidated.

Alterations in cellular $Ca^{2+}$ dynamics directly trigger ER stress and activate the unfolded protein response (UPR) [19, 20]. ER/SR is a membrane-bound organelle in mammalian cells that is responsible for proper folding, processing, and trafficking of proteins and also plays an important role in cellular $Ca^{2+}$ homeostasis. ER stress and UPR modulation are implicated in various human diseases including sarcopenia [21–23]. In recent decades, roles of ER stress and UPR pathways in skeletal muscle health and disease have received increased research attention [24]. In addition to induction of apoptosis, ER stress positively influences myogenic differentiation and myofiber formation [25, 26]. Therefore, we surmise that ER stress signaling is involved in RyR1-mediated muscle development.

Satellite cells and myoblasts play a pivotal role in the repair and maintenance of skeletal muscle by differentiating into mature myocytes [27]. In this study, we hypothesized that RyR1-mediated $Ca^{2+}$ dynamics delicately balance ER stress-induced apoptosis and myoblast differentiation. To test this theory, we employed RyR1-KO myoblasts established by CRISPR/Cas9 gene-editing and RyR1-weaken cells to explore the role of RyR1 in myogenic differentiation and formation mechanism of RyR1-related myopathies.

# Materials And Methods

### Cell culture and myogenic differentiation

Mouse skeletal myoblast C2C12 cells were purchased from the National Infrastructure of Cell Line Resource in China. Proliferating myoblasts were maintained in DMEM/high glucose medium (Hyclone,

Logan, UT, USA) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) in a humidified $CO_2$ incubator (5% $CO_2$, 37 °C; HF90, Heal Force, Hongkong, China). For myogenic differentiation, myoblasts with 80%~90% confluence were induced by DMEM/high glucose medium supplemented with 2% horse serum (Hyclone, Logan, UT, USA).

Myogenic cells of pigs (n = 3) were isolated using preplate techniques from skeletal muscle according to our previous reports [28]. The cells were cultured in growth medium in a dish coated with collagen I (Sigma-Aldrich) at 37 °C and 5% $CO_2$. The growth medium was composed of DMEM/F12 (Hyclone), 10% FBS (Gibco-BRL, Carlsbad, CA, USA), 2 mM glutamine (Gibco-BRL), and 5 ng/mL bFGF (Peptech, Burlington, MA, USA). As for myogenic induction, cells were cultured for 5 days in DMEM/F12 medium containing 2% horse serum (Hyclone).

### Cellular $Ca^{2+}$ concentration measurement

$Ca^{2+}$ concentration in the cytoplasm or ER was measured using flow cytometry. Cells were collected, washed three times with PBS and HBS respectively, and then incubated in 5μg/mL Fluo-3 acetoxymethyl ester (Cayman, Ann Arbor, MI, USA) or Mag-fluo-AM (GENMED, Shanghai, China) for 30 min at 37 °C in dark. After three washes with PBS supplemented with 1% FBS, cells were resuspended in 200 μL PBS containing 1% FBS. Flow cytometry was carried out immediately using a FACS Calibur Cytometer and Image Cytometry software (BD, Franklin, NJ, USA). Calcium-bound Fluo-3 or Mag-fluo-AM has an emission maximum of 526 nm which was quantified by excitation with a 488-nm laser and signals were collected using a 530/30 nm band-pass filter. Each sample generated 20,000 live gated events. Debris, multicellularity, and dead cells were excluded by forward scatter (FSC) and side scatter. For detecting dynamic change of $Ca^{2+}$ concentration of cells during myogenic differentiation, a blank control combined with a house-keeping control (the proliferative C2C12 cells) was used to correct the deviation caused by the loaded indicator amount and the voltage used in each measurement. Mean fluorescence intensity was determined from the entire cell population and then adjusted by relative cell size calculated according to FSC to represent $Ca^{2+}$ concentration.

### Cell viability and apoptosis assays

Cell vitality was detected using Cell Counting Kit-8 (CA1210, Solarbio, Beijing, China). According to the experimental protocol, cells were cultured in 96-well plates for 24 h, and then CCK-8 reagent was added at 100 uL per well. One hour later, the absorbance of culture medium was analyzed by microplate spectrophotometer. In addition, cell proliferation activity was also measured by Cell-Light™ EdU Apollo®488 Cell Tracking Kit (RIBOBIO, Guangzhou, China). After pre-cultured for 24 h in 96-well plates, cells were cultured continuously for another 2 h in new media supplemented with 50 μmol/L EdU reagent. Then cells were fixed by 4% paraformaldehyde, permeabilized by 0.2% Trutib X-100, and fluorescently-tagged with Hoechst3342 using nucleus staining methods. The newly proliferated cells were visualized by an Apollo reaction system. Cell proliferation rate was analyzed by ImageJ (v1.51h, National Institutes of Health, Bethesda, MD, USA).

Apoptosis was detected using an Annexin V-FITC/PI Apoptosis Detection Kit (Gene Protein Link, Beijing, China) according to the manufacturer's protocol. Briefly, cells were stained with a combination of Annexin V-FITC and propidium iodide in darkness for 15 min at room temperature, and then analyzed by the flow cytometry system.

### RNA isolation and qRT-PCR

Cells used for total RNA extraction obtained from 3 separate experiments (different batches of cells and on different days). Total RNA was extracted from cells using HiPure Total RNA Mini Kit (Magen, Beijing, China), and then reverse-transcribed into cDNA using a PrimeScript$^{TM}$ RT reagent Kit with gDNA Eraser (Takara, Osaka, Japan). Synthesized cDNA was used for RT-qPCR analysis by employing a quantitative real-time PCR kit (Takara, Osaka, Japan) with an AJ qTOWER 2.2 Real-Time PCR system (Analytik Jena AG, Jena, Germany) according to standard procedures. All samples were measured in triplicate. The primers used in the experiment were listed in **Additional file 4: Table S1**. For the comparison of RyR1 and RyR3 expression in cells, the amplification efficiency of their primers was used firstly to rectify the qRT-PCR cycle number. GAPDH was used as an internal control. Relative gene expression level was calculated by $2^{-\Delta\Delta Ct}$ method.

### Protein extraction and western blot analysis

The relative abundances of proteins related to ER stress, MAPK signaling pathway, and apoptosis were determined by Western Blot. Cell samples were collected and lysed in RIPA buffer (Huaxingbio, Beijing, China) composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.1% SDS, plus a Halt protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The homogenate was centrifuged at 14,000 × g for 15 min at 4°C and the supernatant was isolated for Western Blot analysis. Protein concentrations were determined using a BCA Protein Assay Kit (Huaxingbio, Beijing, China). Equal amounts of protein (30 μg), together with a pre-stained protein ladder (Thermo Fisher Scientific, Waltham, MA, USA), were electrophoresed on SDS polyacrylamide gel, electro- transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, OH, USA), and blocked for 1 h in 5% non-fat dry milk at room temperature in Tris-Buffered saline and Tween-20 (TBST; 20 mmol/L Tris-Cl, 150 mmol/L NaCl, 0.05 % Tween 20, pH 7.4). Samples were incubated with corresponding primary antibodies overnight at 4°C. After washing with TBST (pH 7.4), membranes were incubated with the secondary antibody (DyLight 800, Goat Anti-Rabbit IgG). Protein bands were detected with the Odyssey Clx kit (LI-COR, Lincoln, NE, USA) and quantified using an Alpha Imager 2200 (Alpha InnoTec, CA, USA). Relative protein expression was calculated by taking GAPDH as an internal standard. Information of antibodies used for Western Blot in this study was listed in **Additional file 5: Table S2**.

### Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA)/PBS for 30 min. After the neutralization of excess formyl group by 2 mg/mL glycine, cells were permeabilized by 0.2% Trutib X-100 in PBS for 10 min. After blocked with 3% BSA/PBS, cells were incubated with primary antibody (anti-RyR1, 1:300, MA3-925,

Thermo Fisher Scientific, Waltham, MA, IL, USA; anti-myosin, 1:300, M4276, Sigma-Aldrich, Louis, MO, USA) overnight and then incubated with Fluorescein-Conjugated secondary antibody (ZF-0311, ZSGB-BIO, Beijing, China) at 1:100. Nuclei were stained with DAPI (Thermo Fisher Scientific, Waltham, MA, USA). Finally, myotubes were visualized by an inverted fluorescence microscope.

### Chemical blockers of $Ca^{2+}$ channels

Two kinds of chemical blocker were used for the experimental treatments. DAN (Stock solution: 200 mM in DMSO, HY-12542A, MedChemExpress, South Brunswick, NJ, USA) was added as a final concentration of 10 μM in culture media. THA (ab120286), purchased from Abcam (Cambridge, UK), was used at a final concentration of 100 nM in culture media. Equal amounts of vehicle (DMSO) were used as the control. During the proliferating period, myoblasts were treated with chemical blocker for 48 h and then collected for further analysis. Upon myogenic induction, myoblasts were treated with chemical blocker for 5 days and then used for mRNA extraction and immunocytochemistry. Each treatment was conducted in three independently repeated experiments.

### Small interfering RNA transfection

RNA interference of RyR1 (mouse, gene ID: 20190) was performed using a 21-base pair small interfering RNA (siRNA) duplex (designed and synthesized by IBSBIO, Shanghai, China). The sense strand nucleotide sequence for RyR1 siRNA was 5’-CCUGCUCUAUGAACUUCUAGC-3’ (sense strand) and 5’-UAGAAGUUCAUAGAGCAGGUU-3’ (anti-sense strand). A scrambled siRNA (siControl, sense strand: 5’-UUCUCCGAACGUGUCACGUTT-3’, anti-sense strand: 5’-ACGUGACACGUUCGGAGAATT-3’) with the same nucleotide composition as RyR1 siRNA but lacks significant sequence homology to the RyR1 was also designed as a negative control. Briefly, myoblasts were plated in a cell culture dish for 24 h, and then transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol with 100 nM siRNA. After transfection for 24 h, myogenic differentiation was induced in cells.

### CRISPR/Cas9 gene-editing

Gene-edited myoblasts with RyR1-knockout were generated via the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated protein 9 (Cas9) system. The plasmid vectors expressing Cas9 protein and guide-RNA (gRNA) were designed and synthesized by Syngentech (Beijing, China). One gRNA among the three gRNA targeting RyR1was selected for using in further study according to their shearing efficiency. The sequences of gRNA-1, gRNA-2, and gRNA-3 are as follows: 5’-GGCGATGATCTCTATTCTTA-3’, 5’-TACAGCCCCTACCCCGGAGG-3’, and 5’-AGCTCAGGCCACCCACCTGA-3’, respectively. The transient transfection of CRISPR-Cas9 vectors was achieved by electroporation using Nucleofector Program B-032 (VCA-1003, Lonza, Basel, Switzerland). Infected myoblasts were selected by incubation with 200 μg/mL hygromycin for 1 week. The stable RyR1-knockout cell line was obtained through single cell clone techniques. Genotype identification and verification of putative off-target sites (**Additional file 6: Table S3**) were conducted via DNA-sequencing technology. The related primers were list in **Additional file 7: Table S4**.

### Statistical analysis

Data were analyzed using t-test procedures of SAS software (Version 9.3, SAS Institute, Cary, NC, USA) and presented as mean ± S.E.M. The criterion for statistical significance was set at $P < 0.05$.

# Results

### Cytoplasmic $Ca^{2+}$ dynamics and expression patterns of $Ca^{2+}$ channels during myogenic differentiation

Upon myogenic induction, myoblasts gradually expressed plenty of myosin from the day 0 to 6 (**Additional file 1: Figure S1A**) and protein expression of RyR1 was significantly increased at day 6 (**Additional file 1: Figure S1B, C**). During the entire period (day 1-5) of myogenic differentiation, cytoplasmic $Ca^{2+}$ concentration (labeled by Fluo-3) of myoblasts was significantly increased (**Additional file 1: Figure S1D, E**).

*Myf5* (myogenic factor 5) and *MyoD1* (myogenic differentiation 1) expression were significantly increased on day 2 relative to day 0, then sharply decreased on day 6 and 8 and even lower than the initial level during myogenic induction (**Figure 1A, B**). *MyoG* (myogenin) expression showed continuous increase and reached a plateau on day 4 (**Figure 1C**).

As for $Ca^{2+}$ transporters, CAV1.1 (also known as CACNA1S, calcium voltage-gated channel subunit alpha1 S), CRACR2B (calcium release activated channel regulator 2B), ITPR1 (inositol 1,4,5-trisphosphate receptor type 1), and ORAI2 (ORAI calcium release-activated calcium modulator 2) showed similar expression pattern with *Myf5* and *MyoD1* (**Figure 1D-G**). The expression patterns of RyR1 and STIM1 (stromal interaction molecule 1) matched MyoG expression pattern well. Notably, RyR1 mRNA expression showed greater 100-fold increase (**Figure 1H, I**), while RyR3 mRNA expression was less than 1/10 of RyR1 in C2C12 cells, and the increase in RyR3 mRNA expression was far below that of RyR1 during myogenic differentiation (**Additional file 2: Figure S2A, B**), therefore, the major role of RyRs involved in myogenesis of C2C12 cells can be attributed to RyR1. Consistently, RyR1 mRNA expression showed almost a 25-fold increase during myogenic differentiation, while the mRNA expression of RyR3 was not changed in myogenic cells derived from pigs (**Additional file 2: Figure S2C-E**). In addition, the mRNA expression of ATP2A2 (ATPase ER/SR $Ca^{2+}$ transporting 2; **Figure 1J**), ATP2B (ATPase plasma membrane $Ca^{2+}$ transporting 1; **Figure 1K**), and CRACR2A (calcium release activated channel regulator 2A; **Figure 1L**) was significantly increased on day 2 and maintained a plateau on day 2-8 during myogenic induction of C2C12 cells.

As shown in **Figure 2A, B**, cytoplasmic $Ca^{2+}$ concentration was significantly decreased by treatment with dantrolene (DAN), an inhibitor of RyR1, which blocks the release of $Ca^{2+}$ from SR/ER [29]. RyR1 knockdown by siRNA significantly decreased cytoplasmic $Ca^{2+}$ concentration of C2C12 cells (**Figure 2C, D**). The protein expression of RyR1 was effectively suppressed by siRNA interference (**Figure 2E, F**). Functional constraints of RyR1 realized by either DAN or siRyR1 dramatically inhibited the formation of

multinucleated myotubes of myoblasts (**Figure 2G, H**). Upon myogenic induction, DAN effectively blocked *MyoD1* expression on day 2 and both *MyoG* and *Mymk* (myomaker, myoblast fusion factor) expression on day 4 (**Figure 2I**). At the day 4 during myogenic induction, siRyR1-knockdown significantly reduced RyR1, *MyoG*, and *Mymk* expression without effects on *Myf5* and *MyoD1* expression (**Figure 2J**).

**RyR1-induced ER stress is indispensable for myogenic differentiation**

During myogenic differentiation of C2C12 myoblasts, the mRNA expression of protein disulfide isomerases including ERP44 (ER protein 44), PDIA3 (protein disulfide isomerase associated 3), and PDIA4 (protein disulfide isomerase associated 4) which are present in ER, was significantly increased relative to those before differentiation (**Figure 3A**). Endoplasmic membrane proteins ATF6 (activating transcription factor 6) and EIF2AK3 (eukaryotic translation initiation factor 2 alpha kinase 3), considered as typical ER stress markers, also showed significantly higher mRNA expression (**Figure 3B**). Furthermore, the mRNA expression of apoptosis- and heat stress- related proteins including BAX (BCL2-associated X protein), caspase-12, DDIT3 (DNA-damage inducible transcript 3), HSP90B1 (heat shock protein 90, beta, member 1), and HSPA5 (heat shock protein 5) was also significantly stimulated during myogenic differentiation (**Figure 3C**). Similarly, significantly increased mRNA expression of protein disulfide isomerases, ERP44 and PDIA3, were also observed in primary myogenic cells derived from skeletal muscle of pigs during myogenic differentiation relative to those before differentiation (**Figure 3D**). Simultaneously, mRNA expression of ER stress markers, ATF6 and EIF2AK3, increased significantly (**Figure 3E**). The mRNA expression of apoptosis- and heat stress- related proteins including caspase-9, DDIT3, and HSPA5 was also significantly stimulated (**Figure 3F**). The phosphorylation of IRE1α and PERK was significantly reduced by DAN treatment compared with the control, while EIF2α was not altered (**Figure 3G, H**). For MAPK signaling (**Figure 3I, J**), phosphorylation level of Erk1/2 (extracellular regulated protein kinases) was significantly increased by DAN treatment, while JNK (stress-activated protein kinase/Jun-amino-terminal kinase) was decreased.

THA treatment significantly increased cytoplasmic $Ca^{2+}$ concentration (**Figure 4A, B**), but did not influence the mRNA expressions of myogenic-specific genes including *Myf5*, *MyoD1*, *MyoG* and *Mymk* (**Figure 4C**). RyR1-knockdown realized by siRNA interference was independent on THA treatment (**Figure 4D, E**). As shown in **Figure 3F, G**, on day 4 during myogenic induction, RyR1-silencing significantly decreased phosphorylation of IRE1α, PERK, and EIF2α. Meanwhile, THA did not influence IRE1α and EIF2α phosphorylation, but tended to increase PERK phosphorylation in myoblasts. Obviously, THA recovered the decreased phosphorylation level of IRE1α, PERK, and EIF2α caused by siRyR1-knockdown.

On day 4 during myogenic induction, siRyR1-knockdown significantly increased *Myf5* and *MyoD1* mRNA expression but decreased *MyoG* and *Mymk* expression. Alterations in *Myf5*, *MyoD1*, *MyoG* and *Mymk* mRNA expressions caused by siRyR1-knockdown were effectively eliminated by THA treatment. In addition, mRNA expression of *Myf5*, *MyoD1*, *MyoG* and *Mymk* was not influenced by TAH in myoblasts with absence of siRyR1-knockdown (**Figure 5A**). Accordingly, THA treatment did not affect myogenic

differentiation of myoblasts with absence of siRyR1-knockdown. In particular, myotube formation was significantly inhibited by siRyR1-knockdown, which was effectively recovered by THA (**Figure 5B, C**).

### Effects of RyR1-knockout on cell proliferation and differentiation

As shown in **Additional file 3: Figure S3**, the frame-shift mutation of RyR1 was realized by the CRISPR/Cas-9 gene editing system targeting Exon 18 of RyR1 via gRNA-3, which showed the highest shearing efficiency among three designed gRNA. Homozygote and heterozygote of RyR1-knockout cells, named as $RyR1^{-/-}$ and $RyR1^{+/-}$, were obtained by monoclonal cultivation and identified via gene sequencing on the target site and putative off-target sites of gRNA-3. Relative to the wild type cells (WT), $RyR1^{-/-}$ showed higher $Ca^{2+}$ concentration in cytoplasm, but lower $Ca^{2+}$ level in the ER (**Figure 6A-C**). The mRNA expression of ATP2A2, ATP2B, CRACR2B, and ORAI1 was significantly increased, while CAV1.1 and ORAI2 expression were decreased in $RyR1^{-/-}$ or $RyR1^{+/-}$ relative to WT (**Figure 6D**).

Cell proliferation viability (the proportion of $EdU^{+}$ cells) of $RyR1^{-/-}$ or $RyR1^{+/-}$ significantly declined relative to WT (**Figure 6E, F**), which was also demonstrated by the CCK-8 test (**Figure 6G**). As shown in **Figure 6H**, the mRNA expression of *Myf5*, *MyoD1*, and *MyoG* was significantly increased in $RyR1^{-/-}$ or $RyR1^{+/-}$ relative to WT. RyR1-knockout significantly reduced phosphorylation of Erk1/2 and JNK, as well as the total protein of JNK (**Figure 6I, J**).

### Apoptosis triggered by RyR1-knockout

On day 2 during myogenic induction, apoptosis instead of myotube formation was accelerated in both $RyR1^{-/-}$ and $RyR1^{+/-}$. The apoptosis rate of $RyR1^{-/-}$ and $RyR1^{+/-}$ was significantly increased relative to WT (**Figure 7A, B**). The protein level of cyclin D1 and caspase-3 was significantly lower in $RyR1^{-/-}$ or $RyR1^{+/-}$, but the expression of cleaved caspase-3 was significantly increased as compared with WT (**Figure 7C, D**).

ER stress level in $RyR1^{-/-}$ and $RyR1^{+/-}$ was evaluated by Western Blot (**Figure 7E**). Both phosphorylated and total protein expression of IRE1α and PERK were dramatically elevated by RyR1-knockout, while phosphorylation of EIF2α was significantly decreased (**Figure 7F**). As to several apoptosis-related proteins, the protein abundance of CHOP (also known as DDIT3), caspase-9, and caspase-12 in $RyR1^{-/-}$ or $RyR1^{+/-}$ was significantly increased relative to WT, while the expression of ERP44 and HSPA5 was not influenced (**Figure 7G, H**).

## Discussion

Skeletal muscle mass is maintained by myogenic differentiation of myogenic progenitors [30], in which cytosolic $Ca^{2+}$ regulation plays a vital role [28]. Myotube formation requires net $Ca^{2+}$ influx into myoblasts [31-33]. RyR1, serving as a major $Ca^{2+}$ release channel of ER, has attracted the most intense research interest among intracellular $Ca^{2+}$ channels. However, the role of RyR1 in myogenic differentiation remains unclear.

RyR1 protein expresses during differentiation in C2C12 cells which provides an appropriate model for investigations of RyR1 function during myogenic differentiation [34]. In the current study, we observed that cytoplasmic $Ca^{2+}$ concentration of C2C12 myoblasts was significantly elevated during myogenic differentiation. K Qiu, D Xu, L Wang, X Zhang, N Jiao, L Gong, et al. [28] also observed increased $Ca^{2+}$ concentration in primary myogenic cells of pigs. Cellular $Ca^{2+}$ is tightly regulated by channels and transports [35]. Relative to the extracellular matrix and ER, cytoplasmic $Ca^{2+}$ concentration is maintained at very low levels (10-100 nM) under resting conditions. $Ca^{2+}$ is released from the ER, the main storage site of intracellular $Ca^{2+}$, through the transmembrane channels RyR1 and ITPR1 [36, 37]. Cytoplasmic $Ca^{2+}$ influx from extracellular matrix occurs through plasma membrane channels, CAV1, CAV2, and CAV3 [38]. To maintain the resting state, excessive amounts of cytoplasmic $Ca^{2+}$ re-accumulates in the ER by SR/ER $Ca^{2+}$-ATPase (SERCA, also called ATP2A) [39] or is expulsed in the external milieu by plasma membrane $Ca^{2+}$-ATPase (PMCA, also called ATP2B) [40, 41]. Furthermore, store-operated calcium entry (SOCE), mediated by STIM (ER $Ca^{2+}$ sensors), activates CRAC and ORAI channels located at plasma membrane to maintain cellular $Ca^{2+}$ homeostasis [42, 43].

Lineage commitment and differentiation of myoblasts are governed by the programmed expression and functional activation of myogenic regulatory transcription factors (*MRFs*) [44, 45]. In this study, we clearly demonstrated the expression pattern of $Ca^{2+}$ transporters as well as *MRFs* during myogenic differentiation and especially discovered RyR1 expression was increased more than 100-fold in C2C12 cells and almost 25-fold in myogenic cells of pigs during myogenic differentiation. CAV1.1 is a physiological activator of RyR1 in the excitation–contraction coupling of skeletal muscle [46]. In this study, the expression of CAV1.1 was not significantly increased as RyR1 during myogenic differentiation, which indicated that RyR1 expression in myogenic cells is not activated by the expression of CAV1.1 but probably induced by the surroundings, such as myogenic medium and cellular density. Notably, RyR1 restriction via either DAN treatment or siRNA interference significantly decreased cytoplasmic $Ca^{2+}$ concentration, and then blocked formation of multi-nuclei myotubes and expression of MRFs. As a major $Ca^{2+}$ release channel of ER, the dramatic increase of RyR1 expression should be responsible for the significant elevation of cytosolic $Ca^{2+}$ concentration. Therefore, we deduced that dramatic increase of RyR1 expression is required for myogenic differentiation.

Alterations in $Ca^{2+}$ dynamics can trigger ER stress and activation of the UPR [19, 20]. In the current study, expressions of protein disulfide isomerases in the ER including ERP44, PDIA3, and PDIA4, directly reflecting the oxidation state of ER [47], and were significantly increased during myogenic differentiation. Moreover, we observed that ER transmembrane proteins including protein kinase RNA-like ER kinase (PERK/EIF2AK3) and activating transcription factor 6 (ATF6), involved in the UPR process as sensors [48], were elevated upon myogenic induction. Therefore, ER stress signaling was activated by the dramatic increase of RyR1 expression in myoblasts during myotube formation.

IRE1α, PERK and ATF6 function as UPR stress sensors. UPR transmits information about the protein-folding status in the ER lumen to the nucleus and cytosol, and buffers fluctuations in unfolded protein load via UPR stress sensors. Upon activation of ER stress, PERK phosphorylates initiation factor eukaryotic translation initiator factor 2α (eIF2α) and attenuates general protein synthesis [48]. ATF6-transmitted ER stress signaling results in apoptosis during muscle development [26]. ER stress is always accompanied by activation of a series of stress-induced protein phosphorylation involved in the MAPK signaling pathways. For example, JNK protein kinases can be activated by the coupling of ER stress and transmembrane protein kinase IRE1 [49]. In addition, the activation of the Erk1/2 pathway is important for cells to avoid apoptosis caused by physical stress [50]. In our study, inhibition of RyR1 function by DAN treatment significantly reduced phosphorylation levels of IRE1α, PERK, and JNK proteins, but obviously increased Erk1/2 phosphorylation, demonstrating that DAN-induced RyR1 function restriction effectively and specifically alleviated ER stress.

Thapsigargin (THA), a well-characterized ER stress-inducing agent, was used to trigger ER stress through inhibiting microsomal $Ca^{2+}$-ATPase, disrupting cellular $Ca^{2+}$ homeostasis, and accumulating unfolded proteins in the ER lumen [51]. In the current study, the dose of THA was far below that used in previous studies [52, 53] to gain a moderate ER stress status, and guaranteed that THA did not influence the efficiency of siRyR1-knockdown. As a result, THA treatment did not trigger severe ER stress compared with the control. Furthermore, THA treatment significantly increased cytoplasmic $Ca^{2+}$ concentration but did not affect expression of myogenic specific genes. We observed that ER stress alleviated by RyR1 suppression was dramatically intensified by THA addition. At the same time, myotube formation inhibited by siRyR1-knockdown was also recovered by THA treatment, accompanied by the expression of *MRFs*. Therefore, RyR1-mediated ER stress was indispensable for myogenic differentiation.

C/EBP homologous protein (CHOP) encoded by DDIT3 gene is a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factor, whose expression is stimulated during ER stress [54]. Caspase-12 is specifically activated during ER stress-mediated apoptosis especially caused by the disruption of ER $Ca^{2+}$ dynamic [55]. Caspase-9 activates apoptotic signals when mitochondria are damaged [56]. The localization of BAX from cytoplasm to mitochondria is a necessary process of apoptosis [57]. The HSPA5 gene encodes the binding immunoglobulin protein (BiP), a member of the heat shock protein 70 (HSP70) family localized in the ER lumen. Once ER stress overwhelms, BiP can initiate the UPR and decrease unfolded/misfolded protein load of the ER to assist cell survival by avoiding the activation of apoptosis machinery [58, 59]. HSP90B1 (also called GRP94 or gp96), located in the ER, plays important roles in stabilizing and folding other proteins [60]. ERP44, a ER folding assistant of the thioredoxin family, is induced during ER stress for protein quality control [61]. In our study, myogenic induction not only increased expression of proteins related to ER stress-induced apoptosis, including DDIT3, caspase-12, and BAX, but also enhanced expression of proteins that mitigate ER stress, such as ERP44, HSP90B1, and HSPA5. Under the pressure of ER stress-induced apoptosis, cells express high levels of heat stress proteins (HSPA12A, HSP90B1, HSPA4, HSPA5 and HSPA6) to increase their adaptability [62]. Therefore, we deduced that a certain degree of ER stress-induced apoptosis was

triggered during myogenic differentiation but balanced by the enhanced protection mechanism against ER stress per se.

To further explore the roles of RyR1 in myogenic differentiation and muscle development, a model of RyR1-KO myoblasts established by CRISPR/Cas9 gene-editing was employed in this study. RyR1 $Ca^{2+}$ release channels are composed of macromolecular complexes consisting of a homotetramer of 560-kDa RyR1 subunits that form scaffolds for proteins that regulate channel function including protein kinase A (PKA) and the phosphodiesterase PDE4D3 (both of which are targeted to the channel via the anchoring protein mAKAP), PP1 (targeted via spinophilin), and calstabin1 (FKBP12) [63, 64]. The deletion of RyR1 probably resulted in the dysfunction of the protein complex, losing the control of $Ca^{2+}$ flowing out of the ER and accelerated $Ca^{2+}$ flooding into the cytoplasm from the ER, which also distorted the expression patterns of other $Ca^{2+}$ transporters. This observation is consistent with previous studies that demonstrated both over-activation and mutations of RyR1 resulted in leakage of $Ca^{2+}$ from the SR [9, 65]. Cytoplasmic $Ca^{2+}$ elevation directly activated ER stress [19, 20], and has a strong influence on differentiation through oxidative signaling and G0/G1 cell cycle arrest [66]. In the current study, expression of Cyclin D1 was abolished and viability of RyR1-KO cells was decreased, demonstrating that cell cycle and metabolism was suppressed by the enhanced ER stress caused by RyR1-KO mediated cytoplasmic $Ca^{2+}$ elevation.

ER stress signaling can give rise to myogenic differentiation and apoptosis [25]. In the present study, differentiation potential of RyR1-KO myoblasts reflected by *MRFs* expression was dramatically enhanced. However, the differentiation process of RyR1-KO myoblasts was interrupted by programmed cell death through apoptosis during myogenic induction, which indicated that RyR1 knockout reduced the capacity of cells to adapt to the surroundings, such as the differentiation medium containing 2% horse serum. UPR was a protective response for cells under stress, while excessive or prolonged UPR can cause apoptosis [67]. Caspase-3, belonging to a highly conserved family of cysteinyl aspartate-specific proteases, is an essential regulator of apoptosis. In the current study, cleaved activation of caspase-3 in RyR1-KO cells was significantly stimulated, which is supported by a previous study of RyRs-mediated ER stress [68]. The total and phosphorylated protein expressions of IRE1α and PERK were sharply increased in RyR1-KO cells, indicating that RyR1 deletion activated serious ER stress through ER $Ca^{2+}$ leakage. Furthermore, aggravated ER stress significantly increased expression of CHOP, caspase-9, and caspase-12. Nonetheless, the positive effects of ERP44 and HSPA5 against ER stress were not enhanced in RyR1-KO myoblasts. In addition, phosphorylation levels of JNK and Erk1/2, whose activations are beneficial for the resistance to ER stress-induced apoptosis [49, 50], were also significantly reduced in RyR1-KO cells. Therefore, we deduced that RyR1 deletion led to more serious ER stress and excessive UPR than the tolerance thresholds of cells, and accounted for the apoptosis of RyR1-KO myoblasts upon myogenic induction.

# Conclusions

In summary, dramatically increased RyR1 expression which activated ER stress signaling through increased cytoplasmic $Ca^{2+}$ was absolutely indispensable for myogenic differentiation. We discovered a novel role of RyR1 acting as a double-edged sword in myogenic differentiation by swaying the fate of myoblasts between differentiation and apoptosis. Our study contributes to the knowledge of the role of RyR1 in myogenic differentiation and related congenital myopathies, and provides a potential target to regulate muscle regeneration and related tissue engineering.

# Abbreviations

| | |
|---|---|
| ATF6 | Activating transcription factor 6 |
| ATP2A2 | ATPase ER/SR $Ca^{2+}$ transporting 2 |
| ATP2B | ATPase plasma membrane $Ca^{2+}$ transporting 1 |
| BAX | BCL2-associated X protein |
| BiP | Binding immunoglobulin protein |
| C/EBP | CCAAT/enhancer-binding protein |
| Cas9 | CRISPR associated protein 9 |
| CASP3 | Caspase-3 |
| CAV1.1 | Calcium voltage-gated channel subunit alpha1 S |
| CHOP | C/EBP homologous protein |
| CRACR2A | Calcium release activated channel regulator 2A |
| CRACR2B | Calcium release activated channel regulator 2B |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| DAN | Dantrolene |
| DDIT3 | DNA-damage inducible transcript 3 |
| EIF2AK3 | Eukaryotic translation initiation factor 2 alpha kinase 3 |
| eIF2α | Eukaryotic translation initiation factor 2α |
| ER | Endoplasmic reticulum |
| Erk1/2 | Extracellular regulated protein kinases |
| ERP44 | ER protein 44 |
| FKBP12 | Calstabin1 |
| FSC | Forward scatter |
| gRNA | Guide-RNA |
| HSP70 | Heat shock protein 70 |
| HSP90B1 | Heat shock protein 90, beta, member 1 |
| HSPA5 | Heat shock protein 5 |
| ITPR1 | Inositol 1,4,5-trisphosphate receptor type 1 |
| JNK | Stress-activated protein kinase/Jun-amino-terminal kinase |
| MRFs | Myogenic regulatory transcription factors |
| Myf5 | Myogenic factor 5 |
| Mymk | Myomaker |
| MyoD1 | Myogenic differentiation 1 |
| MyoG | Myogenin |

| | |
|---|---|
| ORAI2 | ORAI calcium release-activated calcium modulator 2 |
| OTS | Off-target sites |
| PDIA3 | Protein disulfide isomerase associated 3 |
| PDIA4 | Protein disulfide isomerase associated 4 |
| PERK/EIF2AK3 | Protein kinase RNA-like ER kinase |
| PKA | Protein kinase A |
| RyR1 | Ryanodine receptor 1 |
| SOCE | Store-operated calcium entry |
| SR | Sarcoplasmic reticulum |
| STIM1 | Stromal interaction molecule 1 |
| TBST | Tris-Buffered saline and Tween-20 |
| THA | Thapsigargin |
| UPR | Unfolded protein response |
| WT | Wild type |

# Declarations


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Not applicable.


### Authors' contributions

JY obtained financial support and oversaw the study; JY and KQ conceptualized this study, analyzed and interpreted the data, and drafted the manuscript; KQ, YW, DX, LH, XZ, and EY performed the experiments. All authors contributed, commented, and approved the final content of the manuscript.


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### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

### Declarations

Ethics approval and consent to participate

All studies using pigs were conducted in accordance with the guidelines of, and approved by, the Institutional Animal Care and Use Committee of China Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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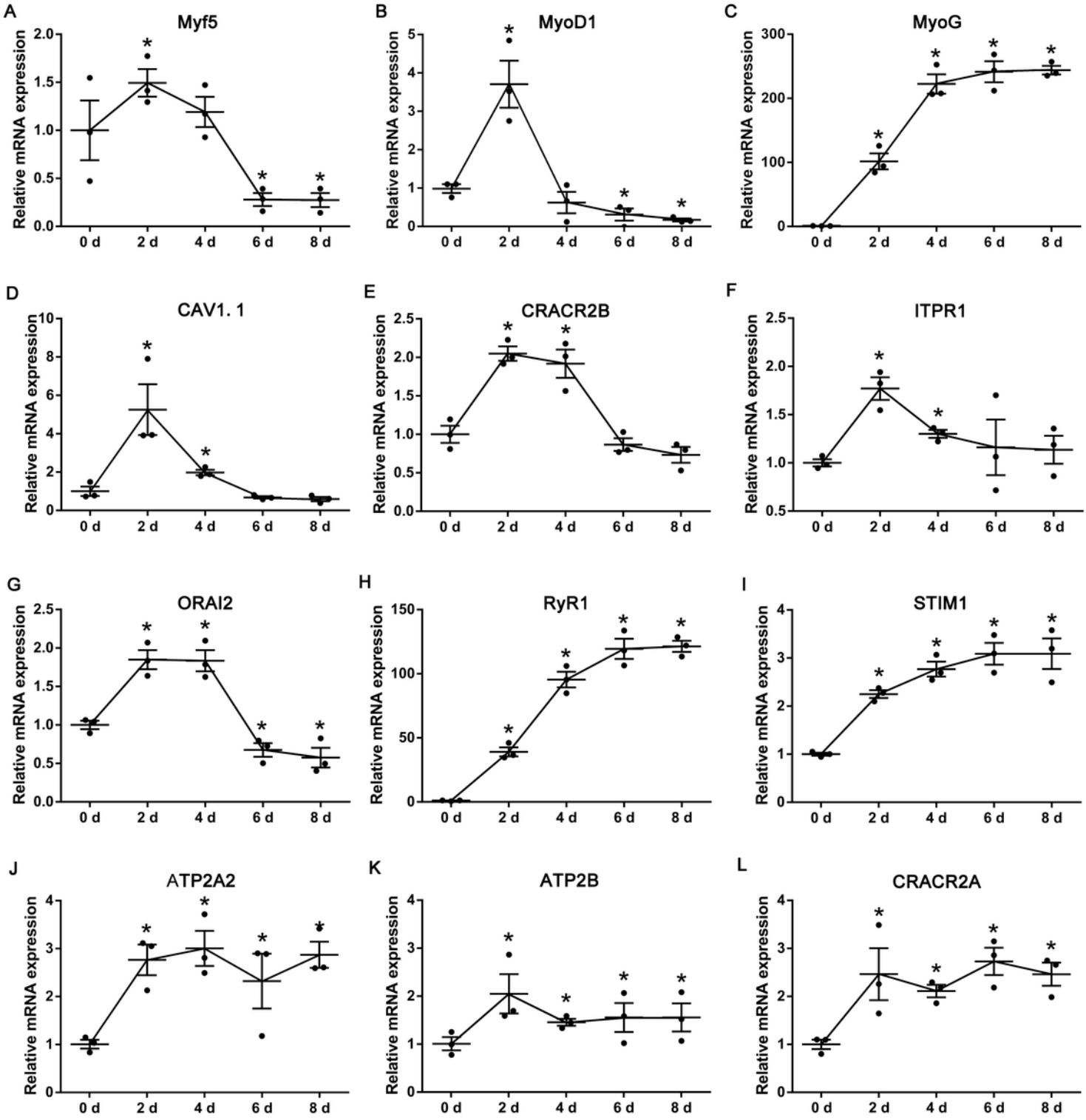
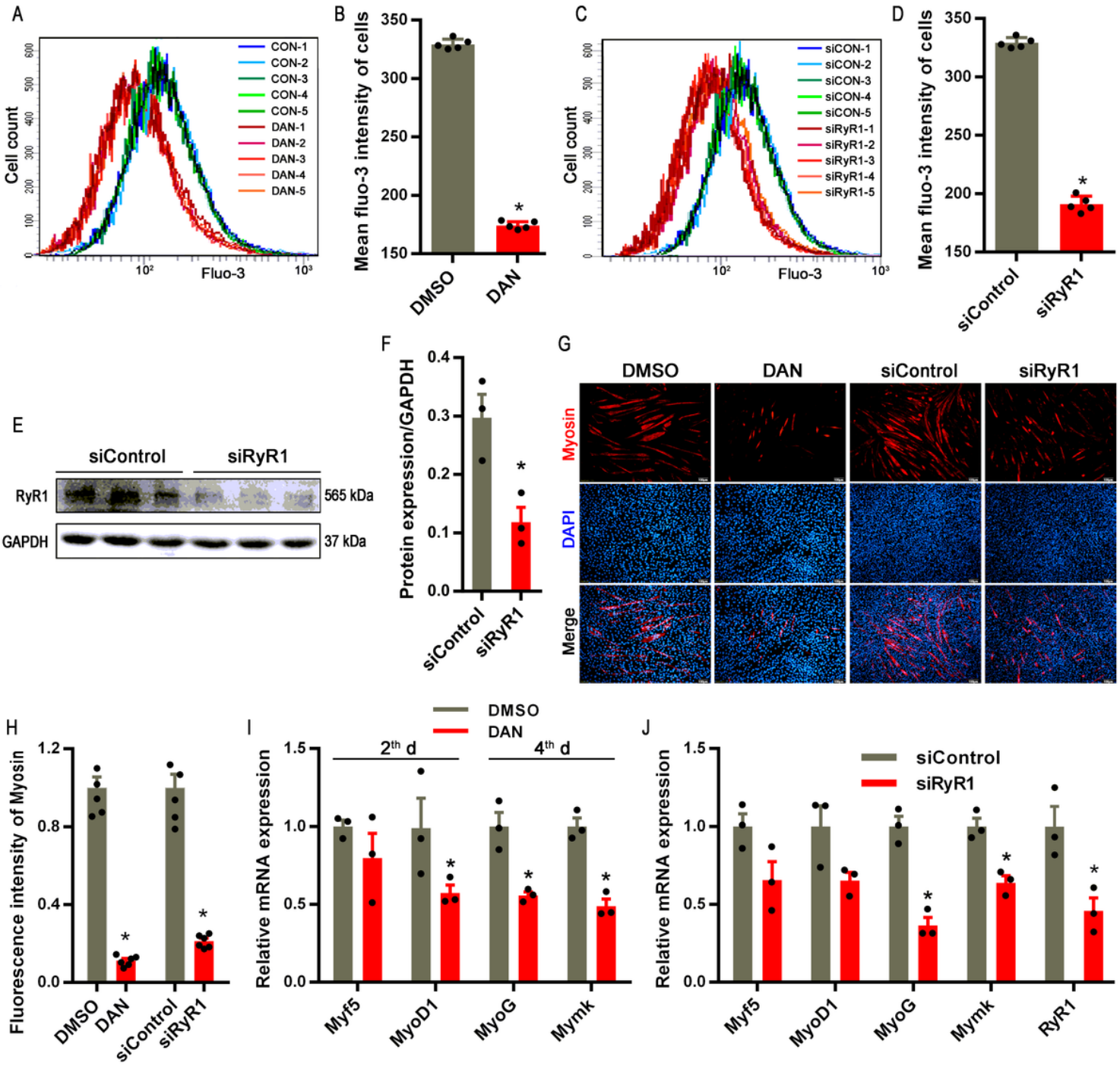
# Figures




## Figure 1

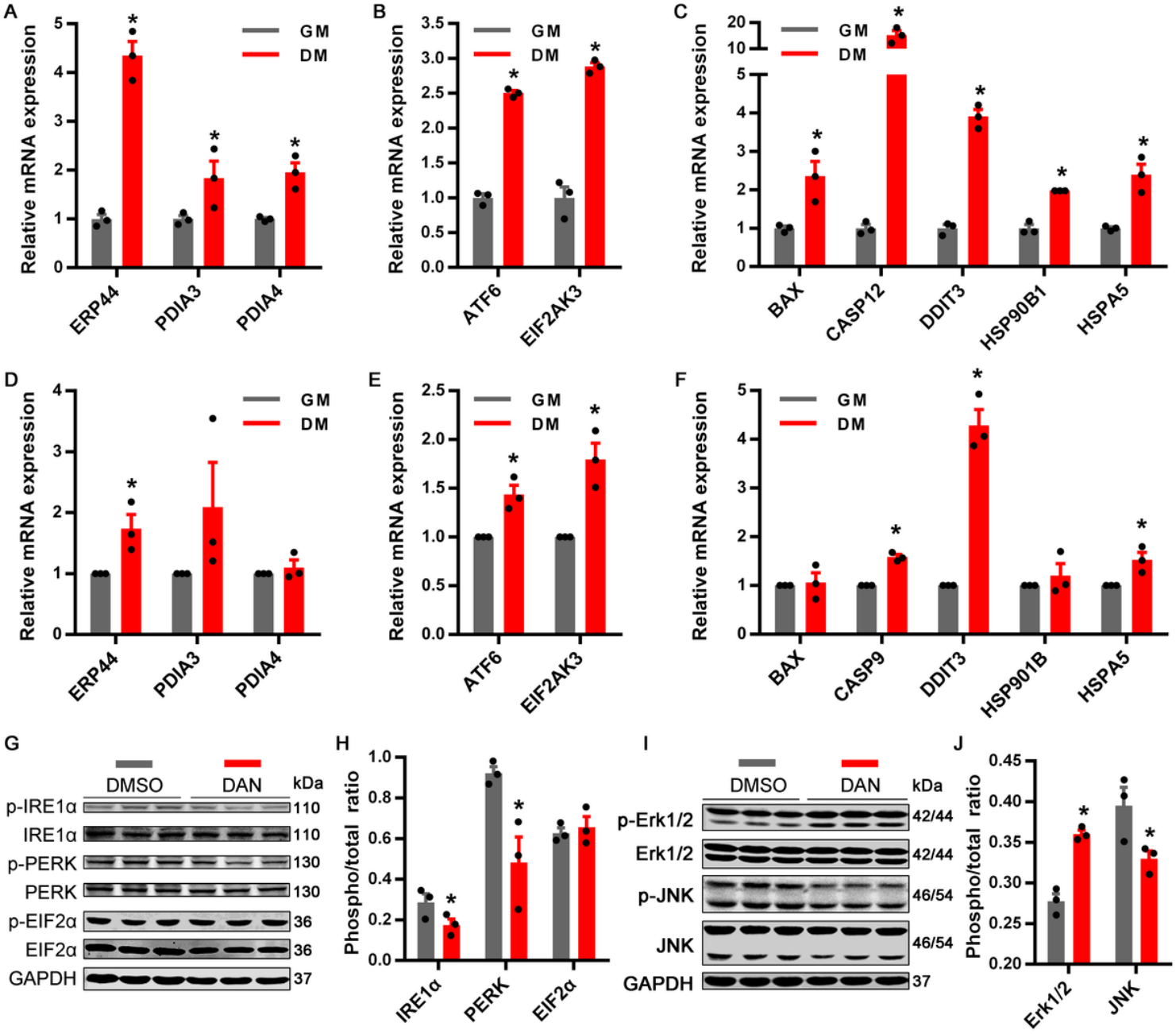
Relative mRNA expression of Myf5 (A), MyoD1 (B), MyoG (C), CAV1.1 (D), CRACR2B (E), ITPR1 (F), ORAI2 (G), RyR1 (H), STIM1 (I) ATP2A2 (J), ATP2B (K), and CRACR2A (L), on day 0, 2, 4, 6, and 8 during myogenic differentiation of C2C12 cells (n = 3). The data are presented as the mean ± SEM. *Represents significant difference with the value on day 0 ($P < 0.05$).




## Figure 2

The effects of RyR1 restriction on myogenic differentiation of C2C12 myoblasts. (A-B) The concentration of cytoplasmic Ca2+ labeled by Fluo-3 treated with Dantrolene (DAN, 10 μM, RyR1 inhibitor) for 48 h (n = 5). (C-D) The concentration of cytoplasmic Ca2+ labeled by Fluo-3 in C2C12 cells transfected with siRyR1 for 72 h (n = 5). (E-F) The proteins expression of RyR1 in C2C12 cells after myogenic induction for 5 days with siRyR1 transfection (n = 3). (G) Immunostaining with myosin antibody of C2C12 cells after myogenic induction for 5 days with DAN treatment or siRyR1 transfection. (H) Fluorescence intensity of myosin in G (n = 6). (I) Relative mRNA expression of related genes in C2C12 cells treated with DAN on day

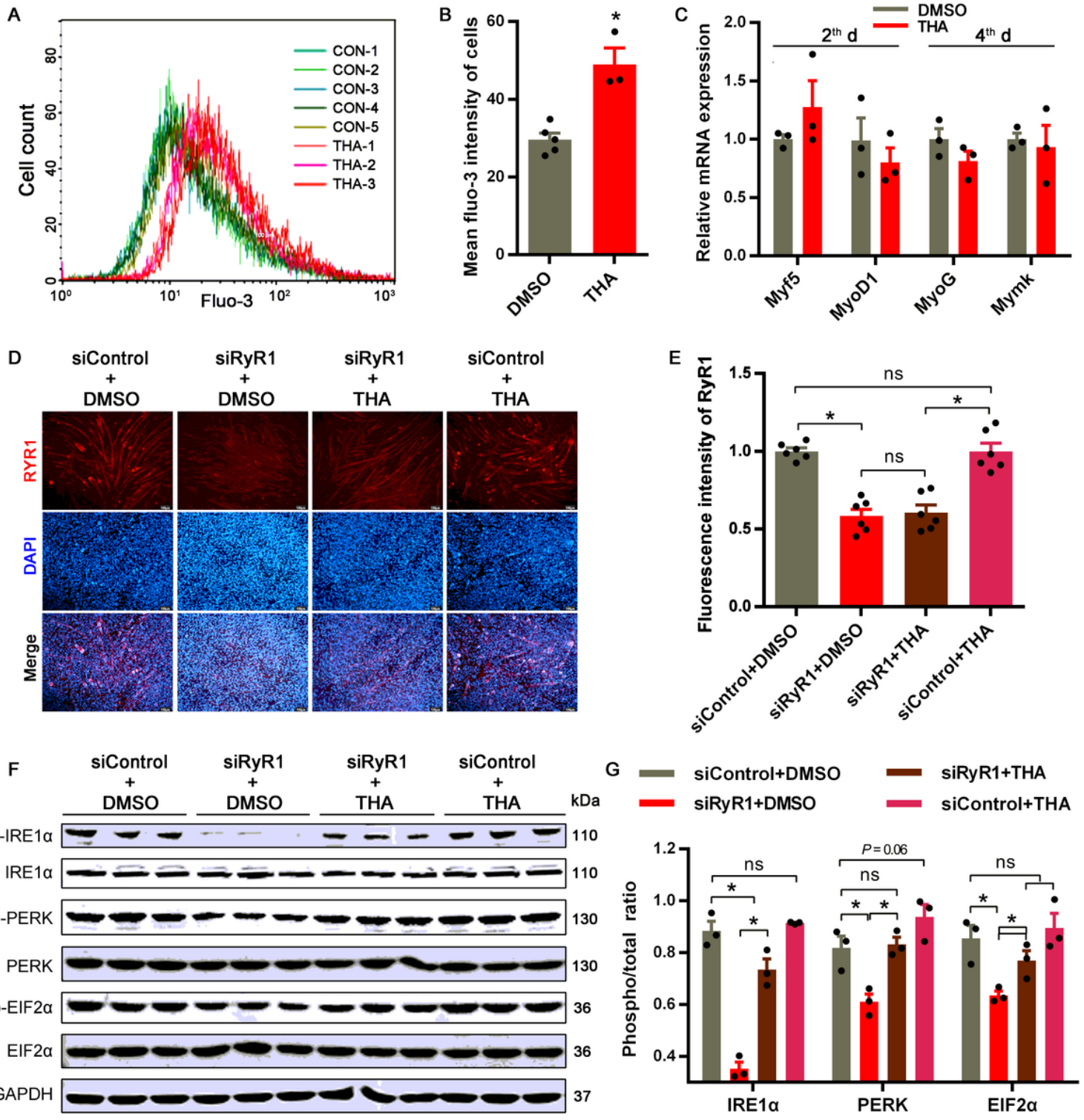
2 (Myf5 and MyoD1) and 4 (MyoG and Mymk) of myogenic differentiation (n = 3). (J) Relative mRNA expression of RyR1, Myf5, MyoD1, MyoG, and Mymk in C2C12 cells transfected with siRyR1 for 72 h (n = 3). *Represents significant difference between the two groups (P < 0.05).




## Figure 3

Endoplasmic reticulum (ER) stress of myoblasts during proliferation and myogenic differentiation (n = 3). (A-C) Relative mRNA expression of genes related ER stress, unfolded protein response, and apoptosis respectively in C2C12 cells. (D-F) Relative mRNA expression of genes related ER stress, unfolded protein response, and apoptosis respectively in skeletal muscle derived myoblasts of pigs. (G-J) The expression of proteins related to ER stress and MAPK signaling in C2C12 cells during proliferation treated with Dantrolene (DAN, 10 μM, RyR1 inhibitor) for 48 h (n = 5). GM, growth medium, representing cells cultured in growth medium before myogenic induction; DM, differentiation medium, representing cells on day 4

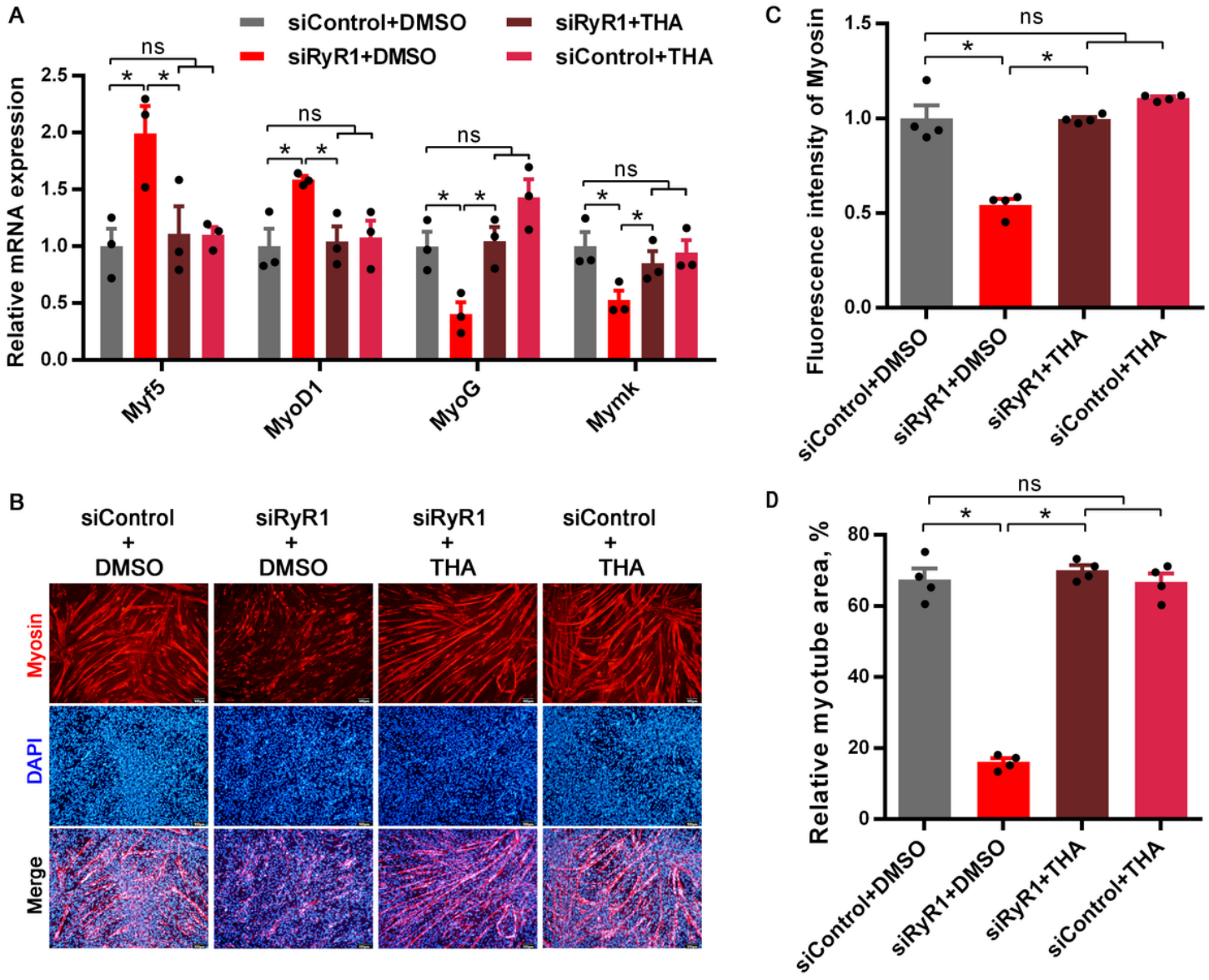
during myogenic differentiation cultured in differentiation medium. The data are presented as the mean ± SEM. *Represents significant difference between the two groups (P < 0.05).




## Figure 4

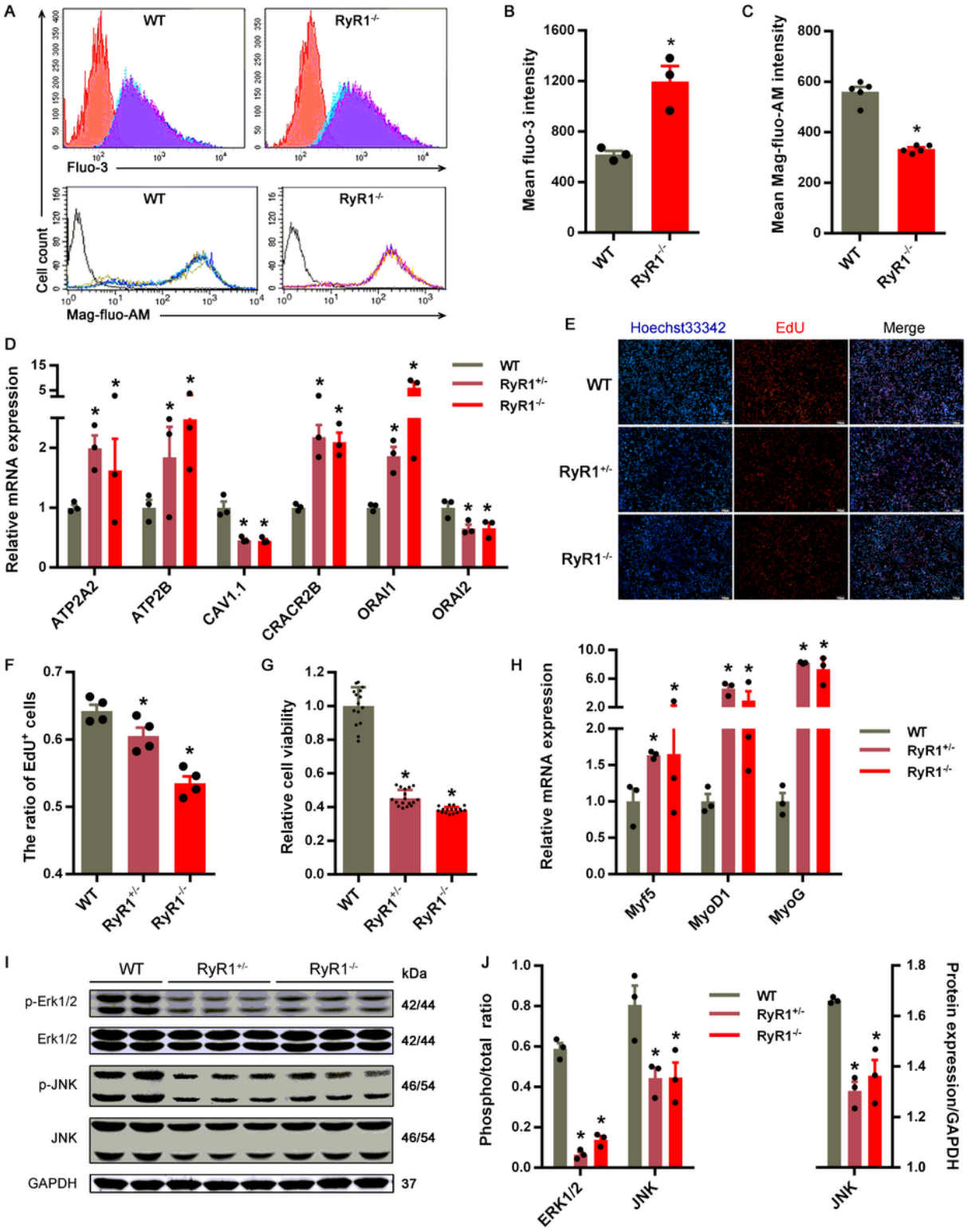
Controlling of RyR1-mediated endoplasmic reticulum stress. (A-B) The concentration of cytoplasmic Ca2+ labeled by Fluo-3 in C2C12 cells treated with Thapsigargin (THA) for 48 h (n = 3). (C) Relative mRNA expression of related genes in C2C12 cells treated with THA on day 2 (Myf5 and MyoD1) and 4

(MyoG and Mymk) during myogenic differentiation (n = 3). (D) Immunostaining with RyR1 antibody of C2C12 cells after myogenic induction for 5 days treated with siRyR1 or THA. (E) Fluorescence intensity of RyR1 (n = 5). (F-G) The expression of proteins related to ER stress in C2C12 cells after myogenic induction for 5 days treated with siRyR1 or THA (n = 3). The data are presented as the mean ± SEM. *Represents significant difference between the two groups (P < 0.05).




## Figure 5

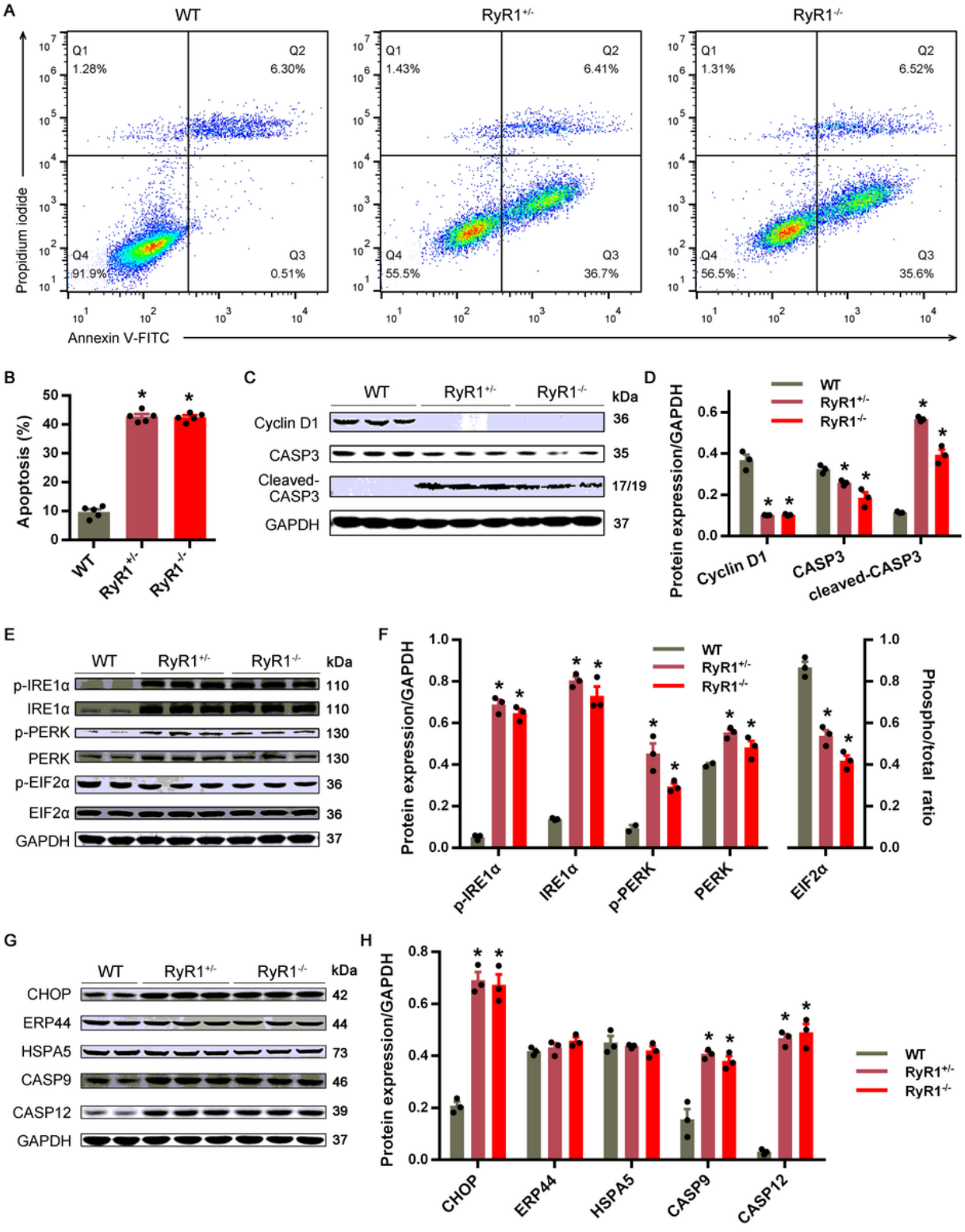
Activation of endoplasmic reticulum stress signaling was indispensable in RyR1-mediated myogenic differentiation of C2C12 myoblasts. (A) Relative mRNA expression of Myf5, MyoD1, MyoG, and Mymk after the treatments with siRyR1 or Thapsigargin (THA) after myogenic induction for 4 days (n = 3). (B) Immunostaining with myosin antibody after myogenic induction for 5 days treated with siRyR1 or THA. (C) Fluorescence intensity of myosin (n = 4). (D) The relative myotube area. The data are presented as the mean ± SEM. *Represents significant difference between the two groups (P < 0.05).




## Figure 6

The effects of RyR1-knockout on cellular Ca2+ dynamics and differentiation of C2C12 myoblasts. (A-C) The concentration of Ca2+ in cytoplasm (labeled by Fluo-3, n = 3) and endoplasmic reticulum (labeled by Fluo-4, n = 5) of RyR1-KO cells. (D) The effect of RyR1-KO on the mRNA expression of Ca2+ channels (n = 3). (E-G) Proliferation and viability of RyR1-KO cells measured by EdU staining or CCK-8 test (n = 10). (H) Relative mRNA expression of Myf5, MyoD1, and MyoG in RyR1-KO cells (n = 3). (I-J) The expression of

proteins related to MAPK signaling in RyR1-KO cells (n = 3). RyR1-/- and RyR1+/- represent homozygote and heterozygote of RyR1-knockout respectively. The data are presented as the mean ± SEM. *Represents significant difference between the two groups (P < 0.05).




## Figure 7

Endoplasmic reticulum stress-associated apoptosis in C2C12 cells was induced by RyR1-knockout (n = 3). (A-B) Apoptosis detection of RyR1-null cells through flow cytometry. (C-D) The protein expression of

Cyclin D1, caspase-3 (CASP3), and cleaved-CASP3 in RyR1-KO cells. (E-H) The expression of proteins related to ER stress or ER stress-induced apoptosis in RyR1-KO cells. RyR1-/- and RyR1+/- represent homozygote and heterozygote of RyR1-knockout, respectively. The data are presented as the mean ± SEM. *Represents significant difference between the two groups ($P < 0.05$).

## Supplementary Files

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