

Aberrant lysosomal dynamics disrupt myogenesis via mTORC1 signalling in X-linked myotubular myopathy

Kengo Kora¹, Takeshi Yoshida¹, Atsushi Yokoyama¹, Kei Fujiwara^{2,3}, Naoko Yano¹, Taisei Kayaki¹, Satoshi Kajimoto¹, Kinuko Nishikawa¹, Hidetoshi Sakurai², Junko Takita¹

Abstract

X-linked myotubular myopathy is a severe congenital muscle disorder caused by pathogenic variants in the *MTM1* gene, which encodes the phosphoinositide phosphatase myotubularin. Muscle biopsies from patients with X-linked myotubular myopathy exhibit distinctive histopathological features, including small, rounded myofibres with centrally located nuclei, indicating a developmental defect in muscle maturation. While earlier studies have indicated that myotubularin dysfunction causes dysregulation of mechanistic target of rapamycin complex 1 (mTORC1) signalling, the underlying mechanisms and phenotypic impact on human muscle cells remain poorly understood. Currently, there are no approved therapies available for the treatment of this disorder.

In this study, we established an induced pluripotent stem cell-based model of X-linked myotubular myopathy using two pairs of isogenic induced pluripotent stem cells: healthy-control versus *MTM1*-knockout and patient-derived versus gene-corrected induced pluripotent stem cells. Through MyoD-inducible myogenic differentiation, this model successfully recapitulates the key pathological features of X-linked myotubular myopathy, including elevated phosphatidylinositol-3-phosphate levels, hyperactivation of mTORC1 signalling, and increased expression of integrin- β 1 and dynamin 2.

We identified impaired lysosomal dynamics as a novel pathogenic mechanism in X-linked myotubular myopathy. Our induced pluripotent stem cell-derived X-linked myotubular myopathy myotubes exhibited an abnormal redistribution of lysosomes, with peripheral accumulation, leading to abnormally activated mTORC1 signalling. *FYCO1* knockdown, a key regulator of lysosomal trafficking, ameliorated this hyperactivation of mTORC1 signalling. Comprehensive transcriptome analysis revealed distinct gene expression patterns associated with altered mTORC1 signalling and lysosomal localisation in X-linked myotubular myopathy myotubes. Network analysis suggested the central role of the mTORC1 signalling pathway and its connections to disrupted muscle development and differentiation. To investigate the influence of mTORC1 signalling and myotubularin deficiency on myogenic differentiation, we established two mouse myoblast models: one with constitutively activated mTORC1 signalling and another with *Mtm1* knockout. Increased mTORC1 signalling in mouse myoblasts impaired myogenic differentiation, and this impairment was reversed by mTORC1 inhibitor rapamycin. Notably, rapamycin treatment also ameliorated the impaired myogenic differentiation observed in *Mtm1*-knockout mouse myoblasts, supporting the causative role of mTORC1 hyperactivation in X-linked myotubular myopathy pathogenesis.

In conclusion, our findings establish the first human cell model of XLMTM, revealing that myotubularin deficiency leads to impaired lysosomal dynamics, which in turn causes mTORC1 dysregulation, a critical factor in the early stage of myogenic differentiation in X-linked myotubular myopathy. These findings provide new insights into the pathogenesis of X-linked myotubular myopathy and suggest that targeting mTORC1 signalling may be a promising therapeutic strategy for this debilitating disorder.

Author affiliations:

1 Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

2 Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto 606-8507, Japan.

3 Department of Radiation therapy, Osaka Rosai Hospital, Osaka 591-8025, Japan.

Correspondence to: Takeshi Yoshida, MD, PhD

Full address: Department of Pediatrics, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, JAPAN

Japan

E-mail: tayoshi@kuhp.kyoto-u.ac.jp

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Introduction

X-linked myotubular myopathy (XLMTM) is a severe congenital muscle disorder caused by pathogenic variants in the *MTM1* gene, which encodes the protein myotubularin (MTM1). MTM1 is a lipid phosphatase that dephosphorylates phosphatidylinositol-3-phosphate (PI₃P) and phosphatidylinositol-3,5-bisphosphate,¹⁻³ playing crucial roles in a multitude of cellular processes, including endosomal trafficking, autophagy, and membrane remodelling.⁴⁻⁶ XLMTM is characterised by severe muscle weakness and hypotonia, frequently resulting in respiratory failure at or shortly after birth.⁷ Approximately half of the affected individuals do not survive beyond 18 months of age.^{8,9} Muscle biopsies from patients with XLMTM reveal distinctive histopathological features, including small, rounded myofibres with centrally located nuclei and a peripheral halo visible on oxidative staining.^{10,11} These characteristics point to a developmental defect in muscle maturation. Despite the severity of XLMTM, there are currently no approved therapies to treat the disease, highlighting the urgent need for research into potential treatment strategies.

The mechanistic target of rapamycin signalling complex 1 (mTORC1) is a key regulator of cell fate, balancing proliferation and differentiation by integrating environmental signals to regulate protein synthesis, autophagy, and lipid metabolism.^{12,13} Dysregulation of mTORC1 signalling has been linked to various neuromuscular disorders,^{14,15} highlighting its importance in muscle biology. However, the precise influence of this pathway on skeletal muscle differentiation remains unclear. The complex roles of mTORC1 signalling in myogenesis make understanding the pathogenesis of muscle disorders and developing targeted therapies challenging.

Recent studies have indicated a correlation between MTM1 deficiency and dysregulation of the mTORC1 signalling pathway. For instance, *Mtm1* knockout mice exhibit increased mTORC1 activity and impaired autophagy in skeletal muscle.¹⁶⁻¹⁸ However, the precise mechanisms through which alterations in MTM1 lead to muscle dysfunction, particularly through mTORC1 signalling, are incompletely understood. This knowledge gap is largely due to the lack of a human cell model that accurately reflects the disease. While animal models have provided valuable insights,¹⁹⁻²¹ they may not fully capture the complexity of human muscle biology and disease progression.

To address these knowledge gaps, we established a skeletal muscle cell model of XLMTM using induced pluripotent stem cells (iPSCs). This model successfully demonstrated the ability to recapitulate critical disease features, including hyperactivation of mTORC1 signalling. Notably, our model exhibited impaired lysosomal dynamics, which in turn led to mTORC1 activation. Additionally, we demonstrated that aberrant mTORC1 activation is associated with impaired myogenic differentiation, which can be substantially reversed by rapamycin treatment in *Mtm1*-knockout mouse myoblasts. This study reveals a previously unrecognised link between MTM1 deficiency, impaired

lysosomal dynamics, and mTORC1-mediated defects in early myogenic differentiation, providing new insights into XLMTM pathogenesis.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (approval number #R0091) and was conducted according to the guidelines of the Declaration of Helsinki. Informed consent was obtained from the participant or their guardian.

Donor information and iPSC generation

The healthy control iPSC line (iPSC clone ID: CiRA00656) was established from a healthy male. The patient-derived iPSC line (CiRA00496) was established from a 5-year-old boy with severe XLMTM, carrying a p.E48* (NM_000252.3:c.142G>T) nonsense variant in the *MTM1* gene. Both iPSC lines were generated using episomal vectors as described previously.²²

Cell lines and culture

iPSCs were maintained following protocols from previous studies.^{23,24} The cells were cultured in StemFit AK02N medium (Takara). For the first 48 h after seeding, 10 μ M Y-27632 (Nacalai) was added to the culture medium. C2C12 mouse myoblasts were cultured in Dulbeccos' modified Eagle's medium (DMEM; Fujifilm) supplemented with 20% fetal bovine serum (FBS; Sigma) and 0.5% penicillin/streptomycin (Nacalai). For differentiation, cells at 80–90% confluence were switched to DMEM containing 2% horse serum (HS; Sigma).

CRISPR/Cas9-mediated gene editing

MTM1 knockout (KO) and c.142G>T (E48*) variant correction in iPSCs were performed using CRISPR/Cas9 technology. gRNAs were designed as 5' -ATGTTTCTACATTACCGTGC-3' to target exon3 for the *MTM1* knockout and 5' -ATGTTTCTACATTACCGTGC-3' for E48* correction. In both cases, the gRNA sequences were cloned into the pSpCas9(BB)-2A-Puro (PX459) vector (#62988, Addgene), which co-expresses Cas9 and the gRNA. Donor vectors containing a puromycin resistance gene flanked by loxP sites, with either a sequence lacking exon 3 (for KO) or a wild-type sequence (for E48* correction), were used. iPSCs were transfected with the appropriate Cas9-gRNA plasmid and donor vector using NEPA21 (Nepagene) at 125 V and 5 ms. Following puromycin selection and cloning, the integration of only one copy of the puromycin-resistance gene was confirmed by real-time quantitative polymerase chain reaction (PCR). Subsequently, the puromycin resistance genes were removed by transient Cre recombinase expression. In both cases, final verification was performed by PCR, Sanger sequencing, and western blot analysis to confirm the intended genetic modifications and their effects on MTM1 protein expression. *Mtm1* knockout C2C12 cells were also generated using CRISPR/Cas9 technology. Two gRNAs were designed to flank exon 3 and were cloned into a PX459 vector. The sequences were 5' -GGCACAATGATCGGAAATTG-AGG-3' and 5' -TTCTAATCTGGCATTATAGC-AGG-3'. After transfection of the vector by electroporation and cloning, deletion of exon 3 was confirmed by PCR and Sanger sequencing.

Generation of iPSC lines stably expressing Tet-inducible MyoD

iPSC lines stably expressing Tet-inducible MyoD were generated as described previously.²³ Tet-inducible MyoD expressing piggyBac vector without reporter gene (KW879-hMyoD vector) was co-electroporated with the piggyBac transposase vector using NEPA21 at 125 V and 5 ms. After selecting the efficiently differentiated clones from puromycin (Nacalai) -resistant colonies, cells were established

and used for experiments. After establishment of the clones, cells were maintained with supplementation of 0.5 µg/mL puromycin to prevent silencing of Tet-inducible MyoD vector.

MyoD-mediated myogenic differentiation

iPSCs transfected with the KW879-hMyoD vector were differentiated into myotubes using a Tet-inducible MyoD expression as previously described.²³ Briefly, the cells were seeded on Matrigel (BD) coated six-well plates in StemFit + Y-27632 medium at an optical density for each cell line. Following a 24 and 48-h period, the medium was replaced in sequence with Primate ES Cell Medium (PECM; Reprocell) + 10 µM Y-27632 and PECM + 0.3 µg/mL doxycycline (Dox; LKT Labs), respectively. After 2 days, the iPSCs were reseeded onto new Matrigel-coated plates in αMEM (Nacalai) supplemented with 2% HS, 3 µM Y-27632, and 200 mM 2-mercaptoethanol (2-ME; Nacalai). Precisely 2 days after reseeding, the medium was replaced with a 2% HS/αMEM solution containing 200 mM 2-ME, 1.0 µg/mL Dox, 5 µM SB431542 (Wako), and 10 ng/mL IGF-1 (PeproTech). Dox was then removed from the medium, and the cells were maintained in 2% HS/αMEM with 200 µM 2-ME. The medium was refreshed every other day.

PI₃P quantification

PI₃P levels were quantified using a PI₃P mass enzyme-linked immunosorbent assay (ELISA) kit (Echelon Biosciences). To standardise the quantification, cells were replated at a density of 5×10^4 cells/cm² prior to extraction. After the differentiation, lipids were extracted from cells through a series of steps: trichloroacetic acid precipitation, washing with trichloroacetic acid/ethylene diamine triacetic acid solution, neutral lipid removal using a methanol-chloroform mixture, and acidic lipid extraction using a methanol-chloroform-hydrochloric acid solution. The resulting extract underwent phase separation, and the organic phase containing PI₃P was collected and dried. Dried lipids were stored at -30°C until analysis. For the ELISA assay, the lipids were resuspended and analysed according to the manufacturer's instructions. PI₃P concentrations were calculated using the standard curve provided with the kit and normalised to the cell number.

Quantitative analysis of immunofluorescence images

Images were quantified using ImageJ 1.54f (Java 1.8.0_322). The fusion index was calculated as the ratio of nuclei within MyHC-positive myotubes containing at least two nuclei to the total number of nuclei from at least three fields per experiment. Myotube length was measured by tracing the longest axis of myotubes, with at least 40 myotubes analysed from a minimum of three fields per experiment. The measured lengths were then normalised to the average length of myotubes in the control line for each experiment. For protein distribution analysis, the cell peripheral region was defined as a 10-pixel-wide border (approximately 2.84 µm) inward from the cell boundary, with the remaining intracellular area defined as the cytosolic region. The distribution ratio was calculated as the mean intensity ratio (cell peripheral region) / (cytosolic region) with at least 10 cells analysed from a minimum of three fields per experiment. Statistical significance for all quantitative immunofluorescence analyses was determined using Welch's *t*-test, with *p* < 0.05 considered significant.

Lysosomal dynamics assays

Prior to microscopy, iPSCs undergoing differentiation were seeded onto Matrigel-coated µ8-well slides (ibidi). On day 13 of the MyoD-mediated differentiation protocol, cells were stained with Lysotracker Red DND-99 (Thermo Fisher) according to the manufacturer's instructions. The culture medium was then replaced with FluoroBrite DMEM (Thermo Fisher) supplemented with 2% HS. Subsequently, cells were transferred to a Leica SP8 microscope for live cell imaging under controlled conditions at 37°C and 5% CO₂ to ensure cell viability and stability. Images were acquired every 1.3 s at maximum scanning speed for a total of 100–200 images per session. Lysosome detection and tracking were automated using TrackMate,^{25,26} an ImageJ plugin, with the following parameter settings: particle

diameter of 1.0 μm , threshold of 10, and maximum link distance of 4.0 μm . For movement directionality analysis, tracks longer than 10 s were categorised based on the net displacement vector along the longitudinal axis of the myotube as follows: ‘Peripheral’ (net displacement $> 0.5 \mu\text{m}$ away from the nucleus), ‘Nuclear’ ($> 0.5 \mu\text{m}$ towards the nucleus), and ‘Static’ (if neither ‘Peripheral’ nor ‘Nuclear’ criteria were met).

Fluorescence recovery after photobleaching (FRAP) assay was performed during live cell imaging. Ten pre-bleach images were acquired to record the initial intensity at each point of interest. The designated points were subjected to photobleaching using a white laser set to 543 nm at 100% intensity for 500 ms. Post-bleach images were acquired every 1.3 s for 150 s, followed by acquisition every 5.0 s for an additional 500 s. Intensity analysis of sequential images was performed using the Spectral Image Analysis plugin of ImageJ. Intensity normalisation was performed using whole-cell intensity and background in each image with easyFRAP-web.^{27,28} A one-phase exponential association curve-fitting method was applied, and the half-times to reach the maximum recovery level and mobile fractions were calculated.

siRNA transfection

On day 11 of the MyoD-mediated differentiation protocol, cells were transfected with siRNA duplexes using Lipofectamine RNAiMax transfection reagent (Thermo Fisher) following the manufacturer’s instructions. The final concentration of siRNA duplexes was 20 nM. The cells were analysed 24 h after transfection. siRNA targeting *FYCO1* was 5′ -CCAGUGACUGGUACUAUGC-3′ (AM16708, Thermo Fisher). Nontargeting control siRNA (SIC001) was purchased from Sigma-Aldrich.

RNA sequencing and bioinformatics analysis

mRNA samples were extracted from cultured cells using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Sequencing libraries were constructed using the NEBNext Poly(A) mRNA Magnetic Isolation Module, followed by the NEBNext Ultra RNA II Library Prep Kit for Illumina (New England Biolabs). The libraries were sequenced using a DNBSEQ-G400RS (MGI Yech) with a 150-bp paired-end read protocol. Sequence alignment to the human reference genome (GRCh37) and read counting were conducted using the Genomon v2.6.3 pipeline as previously described.²⁹ Low-expressed genes with less than 0.5 counts per million in at least one sample were filtered out. Normalisation of count data and differential gene expression analysis were performed using the variance-stabilising transformation function of the R package DESeq2. Normalised count data were used for clustering analysis and heat map generation using the heatmap.2 function from the gplots R package. Gene ontology (GO) enrichment analysis and network analysis were performed using Metascape³⁰.

RHEB expression study in C2C12 cells

For Ras homolog enriched in brain (RHEB) expression studies, dual-expression vectors were constructed from pcDNA-5/TO vector (V103320, Sigma-Aldrich). These vectors contained either wild-type RHEB or RHEB with the Y35N variant. They were under the control of the CMV promoter, followed by an internal ribosome entry site (IRES) sequence and an enhanced green fluorescent protein (EGFP) reporter gene with a polyadenylation signal (pCMV-RHEB-IRES-EGFP-pA). C2C12 cells were seeded at a density of 2.0×10^4 cells/cm² in 20% FBS/DMEM without antibiotics 24 h before transfection. The plasmids were transfected into the cells using JetOPTIMUS (Polyplus) with a 1:1.5 plasmid-to-reagent ratio, following the manufacturer’s guidelines. The medium was replaced with 20% FBS/DMEM 4 h post-transfection. Precisely 24 h post-transfection, the medium was replaced with a differentiation medium (2% HS/DMEM) and subsequently changed every other day.

Rapamycin treatment

Rapamycin (Adipogen) was dissolved in dimethylsulfoxide to prepare a 10 mM stock solution. For experiments, rapamycin was diluted in culture medium to final concentrations ranging from 0.1 to 10

nM. Control cells were treated with an equivalent volume of dimethylsulfoxide. Cells were treated with rapamycin throughout the differentiation period, and the medium was changed every 2 days.

Additional experiments

Detailed protocols for western blotting, immunofluorescence analyses, and mitochondrial imaging are provided in the Supplementary Materials.

Statistical analysis

All experiments were performed at least three times independently. Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software. Comparisons between the two groups were made using Welch's *t*-test. A *p*-value < 0.05 was considered statistically significant.

Results

Generation of isogenic XLMTM and control iPSC lines

Two pairs of isogenic iPSC lines were established (Fig. 1A). The first pair comprised a healthy control iPSC line (referred to as "HC") and an *MTM1*-knockout iPSC line ("KO") generated from HC. The second pair consisted of an iPSC line derived from a patient with XLMTM carrying a p.E48* nonsense variant in the *MTM1* gene ("E48*"), and a corresponding gene-corrected line ("corr-E48*"). Sanger sequencing confirmed the successful repair of the nonsense variant in the *MTM1* gene in the corr-E48* line (Supplementary Fig. 1A). We also verified the absence of MTM1 protein in both the KO and E48* lines and its restoration in the corr-E48* line (Fig. 1B). For our study, we used HC and corr-E48* as the isogenic control group ("control group"), and KO and E48* as the XLMTM disease group ("XLMTM group"). Immunofluorescence analysis confirmed the expression of undifferentiated stem cell markers, including Oct 3/4 and TRA1-81, in all iPSC lines (Supplementary Fig. 1B).

MyoD-mediated myogenic differentiation of iPSC lines

To enhance myogenic differentiation, a previously established system of tetracycline-inducible expression of MyoD, a master regulator of myogenesis, was employed.^{23,31,32} Clones with high myogenic differentiation potential were selected from each iPSC line. The iPSC lines were differentiated into myotubes using Dox-inducible MyoD expression (Fig. 1C). After 14 days of differentiation, myotubes from all four iPSC lines exhibited a spindle-shaped morphology and were predominantly positive for myosin heavy chain (MyHC), a marker of mature myocytes (Fig. 1D and E). The differentiation efficiency, defined as the percentage of MyHC-positive cells relative to total cells, ranged from 85% to 95%. Furthermore, the fusion index, a measure of differentiation maturity, varied between 18% and 25%. No significant differences in these myogenic differentiation indices were observed between the control and XLMTM groups (Fig. 1F and G). These results indicate that our myogenic differentiation strategy allows for a comparative investigation between the two groups without confounding influences related to differences in differentiation efficiency and maturity.

Recapitulation of XLMTM pathological features in iPSC-derived myotubes

To validate our iPSC-derived skeletal muscle model, we examined key pathological features of XLMTM, focusing on MTM1 substrate PI₃P levels,^{19,33} mTORC1 signalling,¹⁶ and other features reported in the muscles of affected patients, such as those related to integrin signalling,^{34,35} dynamin 2 (DNM2) expression,^{36–38} desmin expression,^{39,40} and mitochondrial morphology and distribution.^{10,39}

iPSC-derived myotubes in the XLMTM group exhibited significantly elevated PI₃P levels (Fig. 2A). Additionally, western blot analysis of the mTORC1 signalling pathway revealed increased phosphorylation of p70 S6K (Thr389), 4E-BP1 (Thr37/46), and AKT (Ser473) in the XLMTM group (Fig. 2B and C). These changes in phosphorylation status indicate hyperactivation of the mTORC1 signalling in the XLMTM iPSC-derived myotubes.

Further, total integrin- β 1 protein levels were significantly elevated in the XLMTM group (Fig. 2D and E), along with an increased number of intracellular integrin- β 1-positive vesicles (Supplementary Fig. 2A and B). However, the activation status of integrin signalling and one of its downstream key effectors, ERK signalling, assessed using FAK (Tyr576/577) and ERK1/2 (Thr202/Tyr204) phosphorylation ratios, did not exhibit significant alterations (Fig. 2D and F).

DNM2, which plays a crucial role in membrane trafficking and T-tubule formation by interacting with MTM1 and amphiphysin-2 (BIN1) in skeletal muscle, shows abnormal accumulation and distribution in muscle biopsies of patients with XLMTM.^{36–38} In our iPSC-derived myotubes, elevated levels of *DNM2* mRNA expression were observed based on RNA-seq data (Fig. 2G), and increased DNM2 protein expression was confirmed by western blotting (Fig. 2H and I). In contrast, the mRNA expression of *BIN1*, which collaborates with MTM1 and DNM2 in membrane trafficking, was not markedly altered in the XLMTM group (Supplementary Fig. 2C). Desmin, a muscle-specific intermediate filament, is known to misfold and aggregate in XLMTM muscle.^{39,40} Our iPSC-derived myotubes showed no obvious changes in desmin mRNA expression, protein levels, or localisation (Supplementary Fig. 2D–F). Similarly, the morphology and subcellular distribution of the mitochondria did not exhibit significant changes between the two groups, as confirmed using live cell imaging of the iPSC-derived myotubes stained with MitoTracker (Supplementary Fig. 2G).

In summary, our iPSC-derived myotubes effectively recapitulated several hallmark pathological characteristics of XLMTM, including elevated PI₃P levels, hyperactivation of mTORC1 signalling, and overexpression of integrin- β 1 and DNM2. However, some features, such as integrin signalling dysregulation, desmin accumulation and mislocalisation, and mitochondrial morphological changes, were not observed.

Impaired lysosomal positioning and mobility in XLMTM iPSC-derived myotubes

The MTM1 substrate PI₃P plays a critical role in regulating the trafficking of organelles, including lysosomes.^{41,42} As lysosomal localisation is a key regulator of mTORC1 signalling,^{43,44} there may be a link between elevated PI₃P levels and mTORC1 hyperactivation through altered lysosomal dynamics in the context of XLMTM pathology. This hypothesis was supported by the observation that the inhibition of PI₃P synthesis suppressed hyperactivated mTORC1 signalling in our iPSC-derived XLMTM myotubes (Supplementary Fig. 3A). Based on these findings, we proceeded to investigate the lysosomal dynamics in our model.

Immunostaining of iPSC-derived myotubes with lysosomal-associated membrane protein 2 (LAMP2), a lysosomal marker, revealed distinct patterns of lysosomal distribution between the groups. While lysosomes predominantly localised around the nucleus in the control group, the XLMTM group exhibited a marked abundance of lysosomes in close proximity to the plasma membrane (Fig. 3A and B). The peripherally distributed lysosomes appeared to outline the cell shape. This observation is consistent with previous reports indicating that elevated PI₃P levels activate motor proteins, leading to lysosomal translocation to the cell periphery.^{41,42} The dynamic behaviour of lysosomes was examined using live cell imaging. The automated tracking of lysosomes revealed a significant decrease in their movement velocities in the XLMTM group compared to the control group (Fig. 3C and D). Further directional analysis indicated that this phenomenon was associated with a higher proportion of immobile ('Static') lysosomes and a lower proportion of lysosomes moving towards the nucleus ('Nuclear') in the XLMTM group (Fig. 3E and F).

Additionally, we conducted FRAP analysis, a technique used to measure the mobility of fluorescent molecules within living cells, to assess lysosomal diffusion. The rate of fluorescence recovery was notably slower in the XLMTM group, requiring more time to reach half of the relative fluorescence intensity compared to the control group (Fig. 3G–I). In contrast, the mobile fraction, which represents the proportion of lysosomes capable of movement and engagement in the recovery process, did not differ significantly between the XLMTM and control groups (Fig. 3J). These results suggest that while the velocity of lysosomal movement was reduced in the XLMTM group, the total pool of mobile lysosomes remained unchanged.

FYCO1-mediated lysosomal positioning and mTORC1 signalling

To further elucidate the mechanism by which altered lysosomal distribution leads to increased mTORC1 signalling in XLMTM, we examined the localisation and function of FYVE and coiled-coil domain containing 1 (FYCO1), a PI₃P-dependent adaptor protein that links lysosomes to kinesin-1. FYCO1 facilitates lysosomal trafficking along microtubules toward the plus-end, which typically corresponds to the cell periphery.^{41,45}

Immunostaining of iPSC-derived myotubes with FYCO1 and LAMP2 revealed that FYCO1 was enriched at the cell periphery, similar to the distribution of LAMP2, with a close localisation with LAMP2 in the KO (Fig. 4A and B). A quantitative co-localisation analysis revealed a higher Manders' coefficient of LAMP2 to FYCO1, indicating that more lysosomes were linked to FYCO1 in the KO compared to the HC (Fig. 4C). Furthermore, siRNA-mediated knockdown of *FYCO1* in the KO significantly reduced its protein levels (Fig. 4D and E) and ameliorated the peripheral lysosomal accumulation (Fig. 4F and G). This effect was correlated with residual intracellular FYCO1 levels (Fig. 4H). Moreover, *FYCO1* knockdown alleviated the increased mTORC1 signalling in the KO myotubes (Fig. 4D and E). These results suggest FYCO1 as a driver of both lysosomal mislocalisation and mTORC1 dysregulation in our XLMTM model. However, this FYCO1-mediated lysosomal repositioning did not affect the expression or phosphorylation status of key focal adhesion molecules such as integrin- β 1 or FAK (Supplementary Fig. 3B and C).

Collectively, these results suggest that the peripheral distribution of lysosomes contributes to increased mTORC1 signalling through FYCO1-mediated recruitment of lysosomes to the cell periphery in our XLMTM model.

Transcriptional signatures of dysregulated mTORC1 signalling and lysosomal localisation

To gain insight into the observed mTORC1 signalling and lysosomal abnormalities from a transcriptional perspective, we conducted an RNA sequencing (RNA-seq) analysis on iPSC-derived myotubes. The results of the hierarchical clustering analysis and principal component analysis of the RNA-seq data demonstrated that the samples exhibited distinct clustering patterns based on their isogenic backgrounds rather than whether the samples were control or XLMTM (Supplementary Fig. 4A and B).

Subsequently, we focused on specific gene sets related to mTORC1 signalling and lysosome localisation, selected based on GO annotations. In contrast to the overall pattern of gene expression, the hierarchical clustering of these gene sets revealed distinct patterns between XLMTM and control samples (Fig. 5A). Notably, XLMTM samples exhibited elevated expression levels of these genes and clustered separately from control samples. These findings indicate activated mTORC1 signalling and impaired lysosomal localisation in our iPSC-derived myotubes.

GO enrichment and gene network analysis in XLMTM

A differential gene expression analysis identified 602 upregulated and 841 downregulated differentially expressed genes (DEGs) in the KO compared to the HC pair, and 217 upregulated and 166

downregulated DEGs in the E48* compared to the corr-E48* pair (Fig. 4B, Supplementary Tables 3 and 4). While most DEGs were unique to each isogenic pair, 18 genes were commonly upregulated, and 20 genes were commonly downregulated in both disease lines. Based on these results, we proceeded to analyse the DEGs within each isogenic pair to identify XLMTM-specific gene expression changes. GO enrichment analysis using Metascape³⁰ revealed a number of commonly altered biological processes between the two XLMTM iPSC-derived myotubes (KO and E48*) compared to their respective controls (Fig. 5C, D, Supplementary Fig. 5 and 6).

GO enrichment analysis of the upregulated DEGs in both XLMTM lines revealed significant enrichment of terms related to focal adhesion-phosphatidylinositol 3-kinase-AKT-mechanistic target of rapamycin (PI3K-Akt-mTOR) signalling pathway, growth regulation, skeletal system development, tube morphogenesis, and tissue morphogenesis (Fig. 5C). The upregulation of these pathways likely reflects the effects of activated PI3K-AKT-mTOR signalling on cell growth and morphogenesis or compensatory responses to impaired myogenic differentiation. Additionally, terms associated with extracellular matrix (ECM)-related processes were identified, which aligns with previous findings that MTM1 deficiency results in integrin- β 1 mislocalisation and vulnerability of cell-matrix interactions.^{34,46} The diverse developmental terms identified in our analysis point to broader alterations in cell fate determination and differentiation processes.

GO enrichment analysis of the downregulated DEGs in both XLMTM lines revealed significant suppression of muscle differentiation-related processes, including muscle cell differentiation, tissue development, and structure development (Fig. 5D). Additionally, the expressions of key fundamental processes that are essential for mature muscle function, including calcium signalling, ion transport, and growth factor responses decreased. These findings suggest that MTM1 deficiency impacts both myogenic differentiation and the establishment of fundamental muscle cell functions, indicating a broad defect in muscle maturation.

Network analysis of DEGs between the XLMTM and control groups revealed multiple interconnected gene clusters, with the PI3K-AKT-mTOR signalling pathway showing close associations with diverse cellular processes (Fig. 5E and Supplementary Fig. 7). Genes involved in the focal adhesion-PI3K-Akt-mTOR signalling pathway were connected to those involved in muscle cell differentiation, skeletal system development, and integrin- β 1 cell surface interactions (PID_INTEGRIN1_PATHWAY), which were further linked to muscle structure development, muscle system processes, and monoatomic ion transport regulation. This interconnectedness suggests that mTORC1 signalling, a downstream component of the PI3K-AKT-mTOR signalling pathway, influences multiple aspects of muscle biology in the context of XLMTM.

Impact of mTORC1 signalling levels on myogenic differentiation

Although our iPSC-derived myocyte model has provided valuable insights, it has inherent limitations in assessing the impact of mTORC1 signalling on early myogenesis. This is because forced MyoD induction may bypass some of the abnormalities in the early stages of myogenesis affected by MTM1 deficiency. To overcome this challenge and evaluate the effects of mTORC1 signalling throughout myogenic differentiation, the C2C12 mouse myoblast cell line was used in the following experiments.

A RHEB-based approach was employed. RHEB, a key upstream activator of mTORC1, was transiently expressed in the form of either wild-type RHEB (RHEB^{WT}) or constitutively active RHEB (RHEB^{Y35N}), along with an EGFP reporter (Fig. 6A). The RHEB Y35N mutant constitutively activates mTORC1 regardless of its nucleotide-binding state,^{47,48} thus enabling the modelling of mTORC1 hyperactivation.^{49,50} Flow cytometric analysis of EGFP expression confirmed comparable transfection efficiencies of approximately 35% for both constructs (Supplementary Fig. 8A and B). Additionally, cells expressing RHEB^{Y35N} showed increased phosphorylation of p70 S6K (Thr389), confirming mTORC1 hyperactivation (Fig. 6B and C).

During the differentiation process, compared to RHEB^{WT} controls, RHEB^{Y35N}-expressing myotubes exhibited a significant reduction in length and a diminished MyHC-positive area relative to the EGFP-positive cell area (Fig. 6D–F). To ascertain whether the abnormal differentiation was directly

caused by mTORC1 hyperactivation, the cells were treated with rapamycin, an mTORC1 inhibitor, at varying concentrations (0.1 to 10 nM). In RHEB^{Y35N}-expressing cells, treatment with 1.0 nM rapamycin resulted in a significant improvement in myotube length (Fig. 6G and H) and a restoration of p70 S6K phosphorylation to levels comparable to those observed in untreated RHEB^{WT} controls (Fig. 6B and C). In contrast, in RHEB^{WT}-expressing cells, rapamycin treatment impaired differentiation in a dose-dependent manner, indicating that insufficient mTORC1 activity is deleterious to myogenic differentiation.

These results demonstrate that optimal mTORC1 activity is crucial for normal myogenic differentiation, as both excessive activation and inhibition disrupt this process.

Therapeutic potential of mTORC1 inhibition in XLMTM model cells

To validate the findings that the hyperactivation of mTORC1 signalling impairs myogenic differentiation in the context of XLMTM, *Mtm1*-knockout C2C12 cells were generated (*Mtm1*-KO).

The hyperactivation of the mTORC1 signalling pathway in *Mtm1*-KO was confirmed by the increased phosphorylation ratio of p70 S6K (Thr389) and AKT (Ser473). Phosphorylation of 4E-BP1 (Thr37/46) also exhibited an increasing trend, although it did not reach statistical significance in our experiments (Fig. 7A and B). Immunostaining for LAMP2 demonstrated an abundance of lysosomes in close proximity to the plasma membrane in *Mtm1*-KO (Fig. 7C and D). These findings were consistent with our iPSC model findings. Subsequently, the myogenic differentiation of *Mtm1*-KO cells was markedly diminished compared to the wild-type, as evidenced by myotube length and reduced fusion index (Fig. 7E–G). These observations align with previous reports indicating that *Mtm1* knockout impairs the myogenic differentiation of C2C12 cells.^{40,51}

Finally, we examined the effect of rapamycin on the impaired myogenic differentiation in *Mtm1*-KO. Significantly, rapamycin treatment ameliorated the defects of myogenesis in *Mtm1*-KO, including increased myotube length and a higher fusion index. In contrast, myogenic differentiation of wild-type was inhibited in a rapamycin concentration-dependent manner (Fig. 7E–G), consistent with the results of the RHEB expression experiments described above. These findings indicate that mTORC1 activation in XLMTM adversely affects myogenic differentiation.

In conclusion, the amelioration of the impaired myogenic differentiation in *Mtm1*-KO following rapamycin treatment strongly supports the role of mTORC1 hyperactivation in XLMTM pathogenesis.

Discussion

This study presents the first human cell model of XLMTM using iPSCs, identifying a previously unrecognised link between MTM1 deficiency, impaired lysosomal dynamics, and dysregulated mTORC1 signalling. Furthermore, we demonstrated that the impaired myogenic differentiation in both constitutively mTORC1-activated mouse myoblasts and *Mtm1*-knockout mouse myoblasts was ameliorated by rapamycin, an mTORC1 inhibitor.

The abnormal lysosomal positioning and dynamics observed in our XLMTM models offer a causal link between MTM1 dysfunction and mTORC1 signalling dysregulation. Lysosomes are not only recognised as terminal degradative organelles but also as cellular centres for regulating signalling such as metabolic signalling, cell adhesion, and cell growth and differentiation through dynamic changes in their positioning.^{44,45} In our iPSC model, we observed elevated PI₃P levels, consistent with observations in patients with XLMTM³³ and various animal models.^{16,19,21,52} PI₃P on the lysosomal membrane plays a critical role in controlling lysosomal dynamics by recruiting FYCO1,⁴¹ thereby influencing mTORC1 signalling. Our findings demonstrate that FYCO1 drives peripheral accumulation of lysosomes, with an increase in static lysosomes and a decrease in lysosomes moving towards the nucleus, suggesting that lysosomes become trapped in the cell periphery. This peripheral retention likely contributes to mTORC1 hyperactivation, which aligns with the current understanding that peripheral

lysosomes activate mTORC1 owing to their proximity to phospho-active AKT, a facilitator of growth factor signalling.^{53,54} The suppression of mTORC1 hyperactivation following *FYCO1* knockdown supports the evidence that FYCO1-mediated abnormal lysosomal behaviour is a key factor in the dysregulation of mTORC1 signalling in XLMTM.

Our transcriptome analysis of XLMTM iPSC-derived myotubes further corroborates these findings and provides additional insights into the molecular mechanisms underlying XLMTM pathogenesis. The upregulation of genes associated with the focal adhesion-PI3K-Akt-mTOR signalling pathway aligns with our observations of mTORC1 hyperactivation. Conversely, the downregulation of genes involved in muscle cell differentiation and function provides a molecular basis for the impaired myogenic differentiation and muscle weakness characteristic of XLMTM. The pivotal role of PI3K/AKT/mTOR signalling in XLMTM pathogenesis is emphasised by its central position in the network analysis, with links to various muscle-related, ECM, and integrin signalling pathways. Given the recognised interrelationship between integrin signalling and mTORC1 signalling,^{55–57} our findings, specifically the increased integrin- β 1 protein levels and the network connections linking the PI3K-Akt-mTORC1 axis to integrin-related pathways, suggest a potentially complex interplay between these pathways in XLMTM pathology. These findings not only support our experimental observations but also highlight potential therapeutic targets, suggesting that modulation of mTORC1 signalling may simultaneously address multiple aspects of XLMTM pathology.

Our study demonstrated that dysregulation of mTORC1 signalling is a key feature of XLMTM pathology, observed in both iPSC-derived and C2C12 cell models. The impact of mTORC1 signalling on muscle differentiation appears to be dependent on the differentiation stage.⁵⁸ While its activation is essential for myotube maturation,^{59,60} excessive activation during early myogenesis is detrimental, as evidenced by our RHEB-expressing experiments and previous studies.^{61,62} Given that satellite cells in XLMTM mouse models display impaired functions,⁶³ including increased cell death and limited engraftment, it is reasonable to posit that mTORC1 signalling, which regulates cell survival and differentiation, may be involved in the early stages of muscle cell pathogenesis in XLMTM. Of note, the amelioration of the defects in myogenic differentiation in *Mtm1*-KO cells through rapamycin treatment provides direct evidence for the causative role of mTORC1 hyperactivation in XLMTM pathogenesis. This dysregulation of mTORC1 signalling might contribute to the characteristic maturation defects observed in muscle pathology from patients with XLMTM, such as the presence of small, rounded myofibres with centrally located nuclei, by interfering with the normal progression of myogenic differentiation and muscle fibre maturation.

Our research has significant clinical implications. The iPSC-derived human cell model that we developed offers a valuable platform for screening new drugs and developing targeted therapies, which could potentially accelerate the translation of these findings into clinical practice. Moreover, the rapamycin-induced alleviation of the myogenic defects in our *Mtm1*-KO C2C12 cells suggests mTORC1 inhibitors as potential therapeutics. However, translating these observations to the complex *in vivo* context of XLMTM requires careful consideration, given the limited efficacy of mTORC1 inhibition in XLMTM mouse models,¹⁶ where mTORC1 dysregulation was characterised at later disease stages.⁶⁴ This discrepancy may reflect complex factors, including stage-dependent rapamycin sensitivity, or the multifaceted roles of mTORC1 signalling in muscle differentiation, maturation, and maintenance. Given this complexity, it would be necessary to refine the potential therapeutic strategies targeting the mTORC1 pathway. Further investigation into the molecular consequences of mTORC1 hyperactivation may enhance our understanding of the pathogenesis of XLMTM and facilitate the discovery of new therapeutic targets.

It is important to acknowledge the limitations of our study. Although our iPSC-derived model replicates several aspects of XLMTM pathology, it may not fully capture the complexity of the disease *in vivo*. This discrepancy may be attributed to the relative immaturity of iPSC-derived muscle cells and the differences between the *in vitro* environment and living tissue. Our model has inherent limitations in assessing the pathological mechanisms occurring during late-stage muscle development and the maintenance of mature muscle fibres. The absence of mechanical forces, innervation, and tissue-

specific environmental factors in our *in vitro* system may affect the complete manifestation of the disease phenotype. Furthermore, while our findings demonstrate the impaired lysosomal dynamics associated with mTORC1 hyperactivation in patient iPSC-derived myotubes, whether similar lysosomal mislocalisation occurs in XLMTM mouse models or in patient muscle tissue remains to be determined. These limitations highlight the need for complementary studies using animal models and primary human tissue to fully understand the disease mechanisms throughout different stages of muscle development and maintenance.

Despite these limitations, our study represents a significant advancement and provides a foundation for future investigations in XLMTM research. Building upon our findings, future directions include validating the observed lysosomal alterations *in vivo* and establishing their precise temporal relationship with other pathological hallmarks. Confirming these aspects is important to understand their role in the pathogenesis of XLMTM. Moreover, further research should focus on further elucidating the molecular mechanisms linking MTM1 dysfunction, PI₃P accumulation, and altered lysosomal dynamics to mTORC1 dysregulation and the resulting myogenic defects. Additionally, exploring alternative therapeutic strategies that provide similar benefits to mTORC1 inhibition without potential adverse effects would be valuable. Long-term studies in animal models and, eventually, clinical trials will be necessary to fully assess the safety and efficacy of mTORC1-targeted therapies for XLMTM.

In conclusion, our study provides new insights into the pathogenesis of XLMTM, emphasising the potential involvement of abnormal lysosomal dynamics and mTORC1 dysregulation in impaired muscle differentiation and maturation. These findings not only enhance our understanding of this rare neuromuscular disorder but also suggest potential avenues for therapeutic intervention. This work advances us towards developing effective treatments for patients with XLMTM.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Data availability

The computational tools and code utilized in this study are based on previously reported methods, as detailed in the Materials and methods section. The minimum dataset necessary to independently verify the research results reported in this manuscript is available as Supplementary Materials. The raw data generated during this study are available from the corresponding author upon reasonable request.

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Figure 1

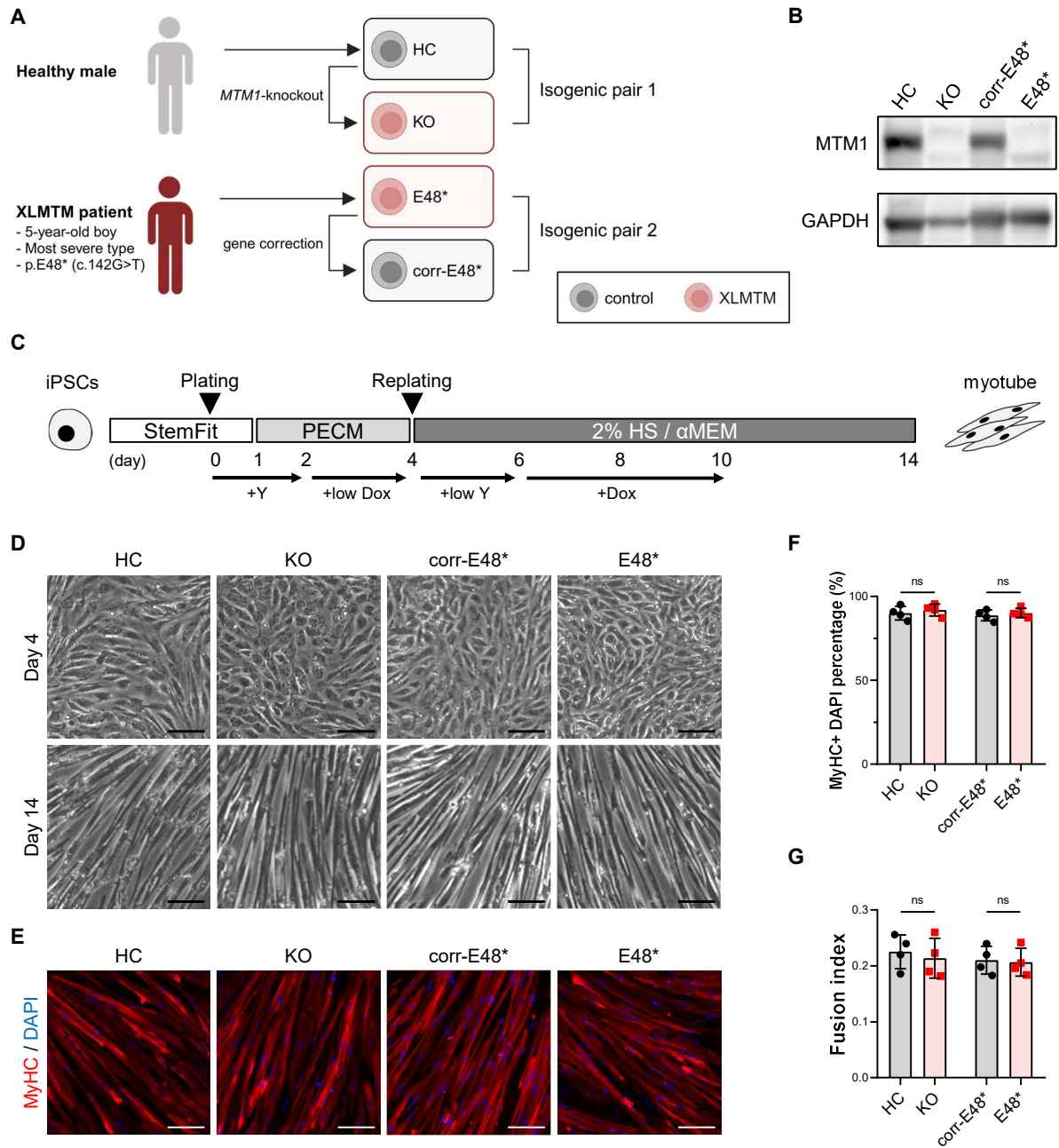


Figure 1. Generation and characterisation of XLMTM and isogenic control iPSCs and their differentiation into myotubes. (A) Schematic representation of the iPSC lines used in this study. (B) Western blot analysis of MTM1 protein expression in the iPSC lines. GAPDH serves as a loading control. (C) Schematic of the myogenic differentiation protocol. (D) Phase-contrast microscopy images of iPSC-derived myotubes at days 4 and 14 of differentiation. Scale bar: 100 μ m. (E) Immunofluorescence images of iPSC-derived myotubes at day 14 stained for the mature muscle marker MyHC (red). Nuclei are stained with DAPI (blue). Scale bar: 100 μ m. (F) Quantification of the MyHC-positive DAPI in iPSC-derived myotubes. (G) Quantification of the fusion index in iPSC-derived myotubes. (F and G) Data are presented as mean $\pm$ standard deviation ($n = 4$ independent experiments). Statistical difference was evaluated using Welch's t -test. Abbreviations: HC, healthy control; KO, knock out; PECM, Primate ES Cell Medium; HS, horse serum; α MED, alpha Modified Eagle Minimum Essential Medium; Y, Y-27632; Dox, doxycycline; ns, not significant; iPSCs, induced pluripotent stem cells; MyHC, myosin heavy chain; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Figure 2

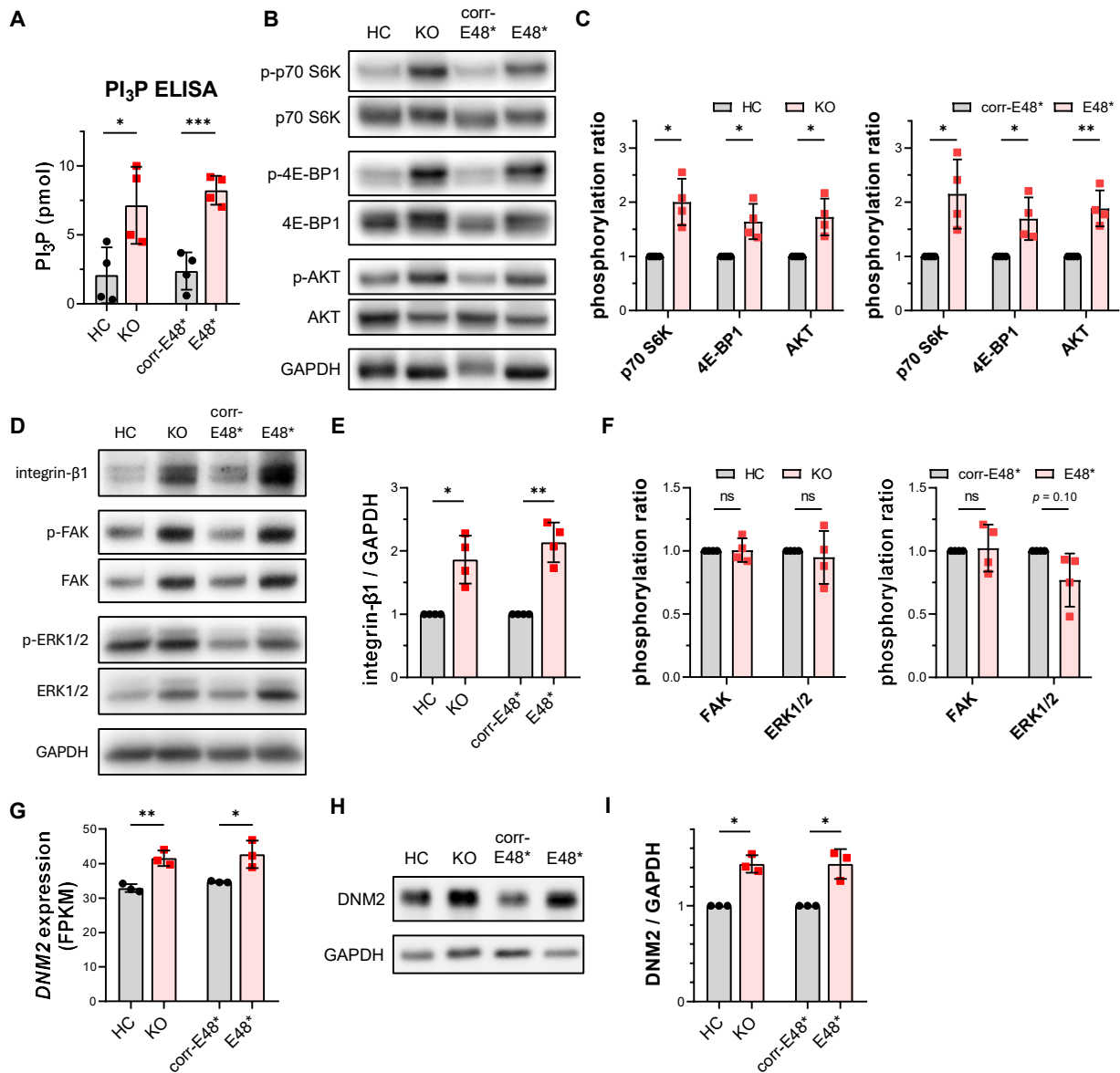


Figure 2. Characterisation of XLMTM pathological phenotypes in iPSC-derived myotubes. (A) Quantification of PI₃P levels by ELISA in iPSC-derived myotubes. (n = 4 independent experiments). (B) Representative Western blot analysis of mTORC1 signalling pathway components, including phosphorylated and total levels of p70 S6K (Thr389), 4E-BP1 (Thr37/46), and AKT (Ser473) in iPSC-derived myotubes. GAPDH serves as a loading control. (C) Quantification of the ratios of phosphorylated to total protein levels for p70 S6K, 4E-BP1, and AKT in iPSC-derived myotubes (n = 4 independent experiments). (D) Representative western blots of integrin and ERK signalling components, including integrin-β1, p-FAK (Tyr576/577), total FAK, p-ERK1/2 (Thr202/Tyr204), and total ERK1/2 in iPSC-derived myotubes. GAPDH served as a loading control. (E) Relative expression of integrin-β1 normalised to GAPDH in iPSC-derived myotubes (n = 4 independent experiments). (F) Quantification of the ratios of phosphorylated to total protein levels for FAK and ERK1/2 in iPSC-derived myotubes (n = 4 independent experiments). (G) Quantification of *DNM2* RNA expression by RNA-sequencing in iPSC-derived myotubes. Raw read count data are normalised to FPKM (n = 3 independent experiments). (H) Western blot analysis of DNM2 and GAPDH protein expression in iPSC-derived myotubes. (I) Quantification of DNM2/GAPDH ratio in iPSC-derived myotubes (n = 3 independent experiments). (C, E, F, and I) The ratios were normalised to the corresponding control lines (HC for KO, and corr-E48* for E48*) within each experiment. (A, C, E, F, G, and I) Data are presented as mean ± standard deviation. Statistical difference was evaluated using Welch's *t*-test **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Abbreviations: PI₃P, phosphatidylinositol-3-phosphate; ELISA, enzyme-linked immunosorbent assay; HC, healthy control; KO, knock out; p-p70 S6K, phosphorylated p70 S6K; p-4E-BP1, phosphorylated 4E-BP1; p-AKT, phosphorylated AKT; p-FAK, phosphorylated FAK; p-ERK, phosphorylated ERK; FPKM, fragments per kilobase of exon per million mapped reads; iPSCs, induced pluripotent stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Figure 3

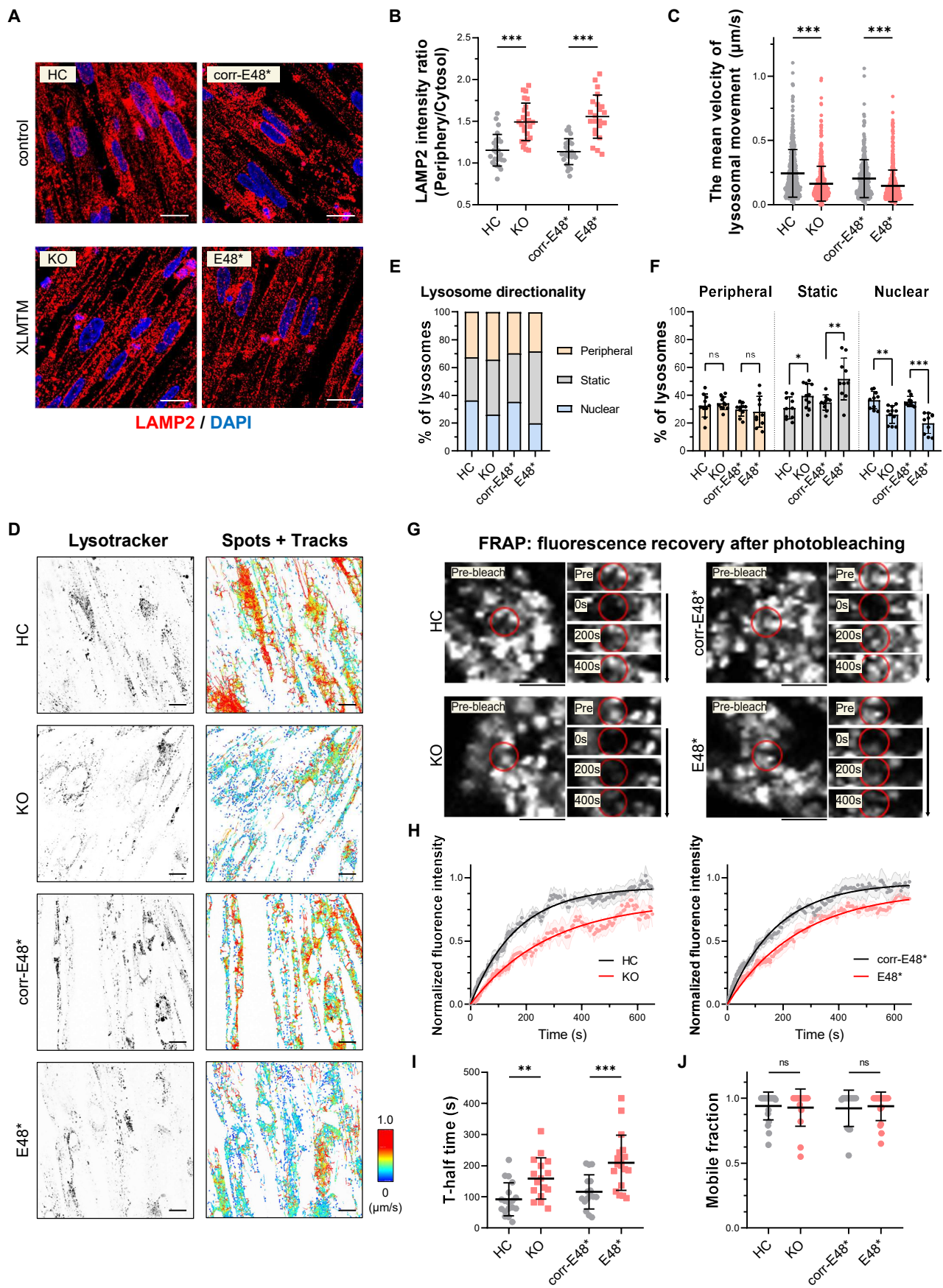


Figure 3. Abnormal lysosomal localisation and dynamics in XLMTM iPSC-derived myotubes. (A) Immunofluorescence images of iPSC-derived myotubes stained for LAMP2 (red). Nuclei are stained with DAPI (blue). Scale bar: 20 μ m. (B) Quantification of LAMP2 fluorescence mean intensity ratio (cell peripheral region / cytosolic region) in iPSC-derived myotubes ($n > 20$ myotubes from three independent experiments). (C) Quantification of lysosomal moving velocity by analysing time-lapse images and automated tracking ($n > 500$ lysosomes from three independent experiments). (D) Representative images of iPSC-derived myotubes stained with LysoTracker (left panels). Time-lapse analysis of lysosomal movement. (right panels). Individual lysosomes are marked by colour-coded dots with their trajectories, where colours represent average movement speeds. Scale bar: 20 μ m. (E and F) Directional analysis of lysosomal movement in iPSC-derived myotubes, classifying lysosomes as ‘Peripheral’ (outward), ‘Static’ (immobile), or ‘Nuclear’ (inward) ($n \geq 10$ myotubes analysed per group from three independent experiments). (E) Stacked bar graph showing the average proportion (%) in each category. (F) Percentage of lysosomes per category, with individual data points representing each measurement from the analysed region. (G) Representative time-lapse images of FRAP in iPSC-derived myotubes. Red circles indicate photobleached areas. Numbers at the left upper position in each panel represent the duration after photobleaching. (H) FRAP curves of LysoTracker-stained lysosomes in iPSC-derived myotubes. Points represent mean fluorescence intensity normalised to both pre-bleach (set as 1.0) and post-bleach (set as 0) intensities at each time point ($n > 15$ photobleached regions from three independent experiments). Lines represent one-phase exponential association curve-fitting. The shaded area indicates the mean $\pm$ standard error of the mean. Quantification of the half-time of fluorescence recovery (I) and mobile fraction (J) in iPSC-derived myotubes ($n > 15$ regions per group). (B, C, F, I, and J) Data are presented as mean $\pm$ standard deviation. Statistical difference was evaluated using Welch’s *t*-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: HC, healthy control; KO, knock out; LAMP2, lysosomal-associated membrane protein 2; DAPI, 4',6-diamidino-2-phenylindole; ns, not significant; FRAP, fluorescence recovery after photobleaching

Figure 4

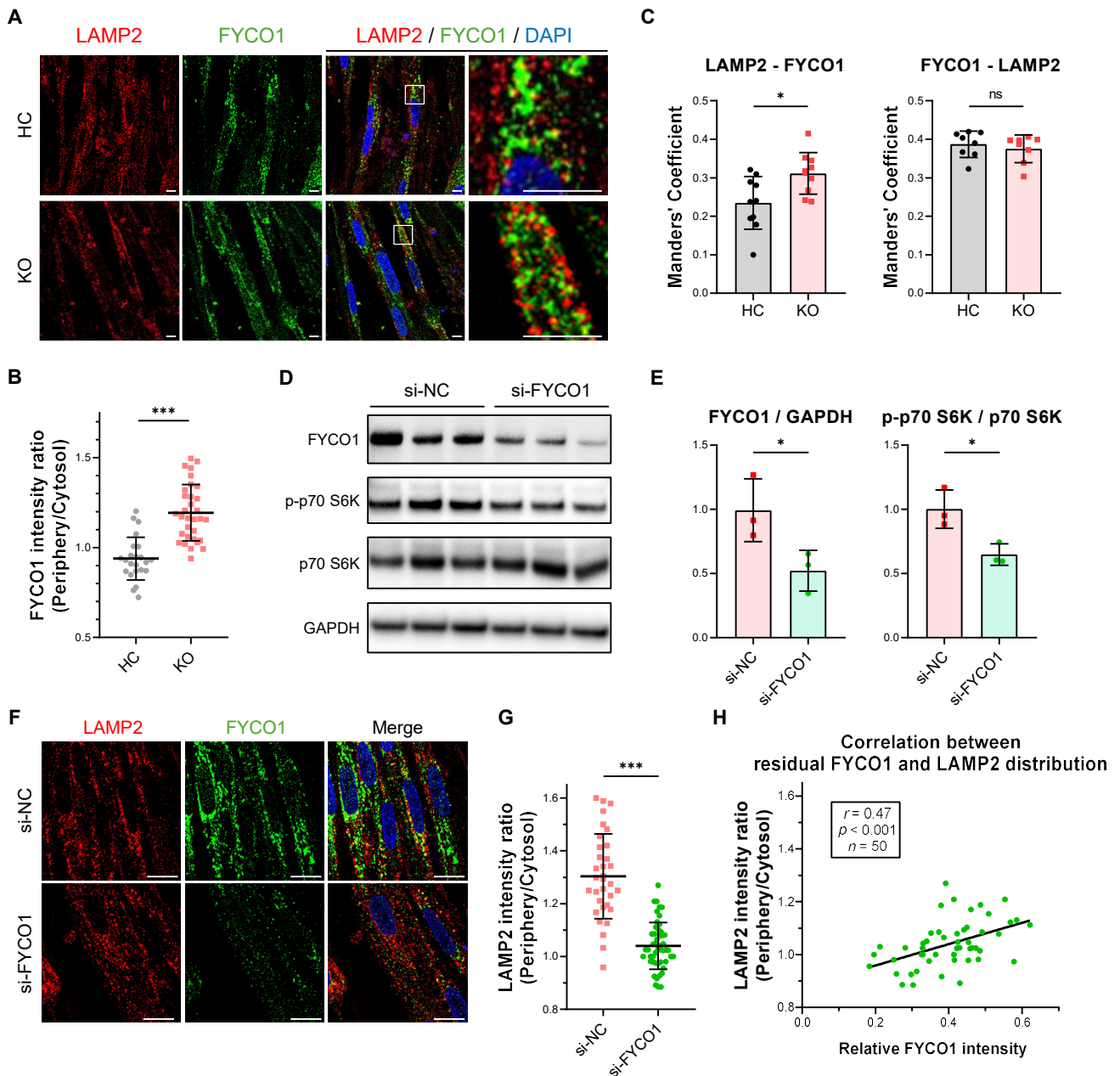


Figure 4. FYCO1-mediated lysosomal positioning contributes to mTORC1 hyperactivation in XLMTM. (A) Immunofluorescence images of iPSC-derived myotubes stained for LAMP2 (red) and FYCO1 (green). Nuclei are stained with DAPI (blue). The right panels show higher magnification images of the boxed regions. Scale bar: 10 μ m. (B) Quantification of FYCO1 fluorescence mean intensity ratio (cell peripheral region / cytosolic region) in iPSC-derived myotubes ($n > 20$ myotubes from three independent experiments). (C) Quantification of co-localisation between LAMP2 and FYCO1 using Manders' coefficients. Left: proportion of LAMP2 signal co-localised with FYCO1. Right: proportion of FYCO1 signal co-localised with LAMP2 ($n > 10$ fields from three independent experiments). (D) Western blot analysis of FYCO1, p-p70 S6K (Thr389), total p70 S6K, and GAPDH (loading control) expression in the KO myotubes treated with si-NC or si-FYCO1 from three independent experiments. (E) Quantification of FYCO1 protein levels normalised to GAPDH (left) and phosphorylation levels of p70 S6K (Thr389) normalised to total p70 S6K (right) in the KO myotubes treated with si-NC or si-FYCO1 ($n = 3$ independent experiments). (F) Immunofluorescence images of the KO myotubes treated with si-NC or si-FYCO1, stained for LAMP2 (red) and FYCO1 (green). Nuclei are stained with DAPI (blue). Scale bar: 20 μ m. (G) Quantification of LAMP2 fluorescence mean intensity ratio (cell peripheral region / cytosolic region) in the KO myotubes treated with si-NC or si-FYCO1 ($n > 30$ myotubes from three independent experiments). (H) Correlation between residual intracellular FYCO1 intensity and the LAMP2 peripheral/cytosol ratio for each myotube quantified in Fig. 4G. Each point represents an individual myotube ($n = 50$ from three independent experiments). The x-axis presented the relative FYCO1 fluorescence intensity normalised to si-NC-treated cells for each experiment. Pearson correlation coefficient (r) and p-values are indicated. (B, C, E, and G) Data are presented as mean $\pm$ standard deviation. Statistical difference was evaluated using Welch's t -test * $p < 0.05$, *** $p < 0.001$. Abbreviations: LAMP2, lysosomal-associated membrane protein 2; FYCO1, FYVE and coiled-coil domain containing 1; DAPI, 4',6-diamidino-2-phenylindole; ns, not significant; NC, negative control; p-p70 S6K, phosphorylated p70 S6K; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 5

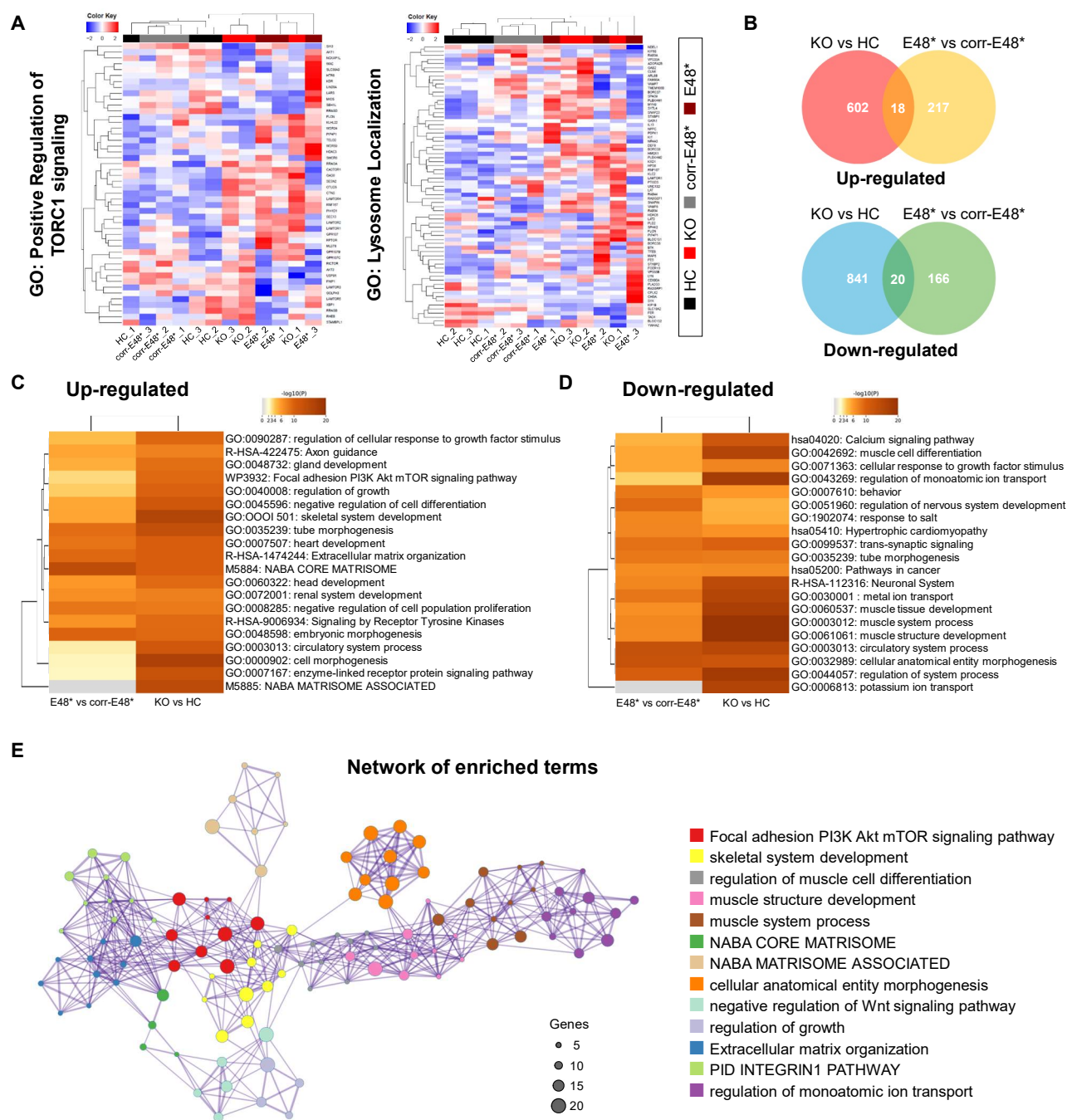


Figure 5. RNA sequencing analysis reveals altered gene expression profiles in XLMTM iPSC-derived myotubes. (A) Hierarchical clustering of specific biological processes based on RNA-sequencing data ($n = 3$ independent experiments). The processes include positive regulation of mTORC1 signalling (left) and lysosomal localisation (right). Red indicates high expression levels, while blue indicates low expression levels. (B) Venn diagram showing the overlap between two isogenic pairs for upregulated DEGs and for down-regulated DEGs in iPSC-derived myotubes. The numbers within the diagram indicate the number of DEGs in each region. (C and D) Heatmap of enriched GO terms of upregulated DEGs and down-regulated DEGs in XLMTM iPSC-derived myotubes. The bar plot displays the $-\log_{10}(p\text{-value})$ for each enriched GO term, with the colour scale representing the level of significance. (E) Network analysis of DEGs in XLMTM iPSC-derived myotubes. The network nodes represent enriched GO terms, and the edges represent the overlap between the gene sets associated with each term. The colour of the nodes indicates the category of the GO term, while the size of the nodes reflects the number of genes associated with each term. The network analysis reveals interconnected clusters of genes related to various cellular processes. Abbreviations: DEG, differentially expressed gene; GO, gene ontology; PID, Pathway Interaction Database

Figure 6

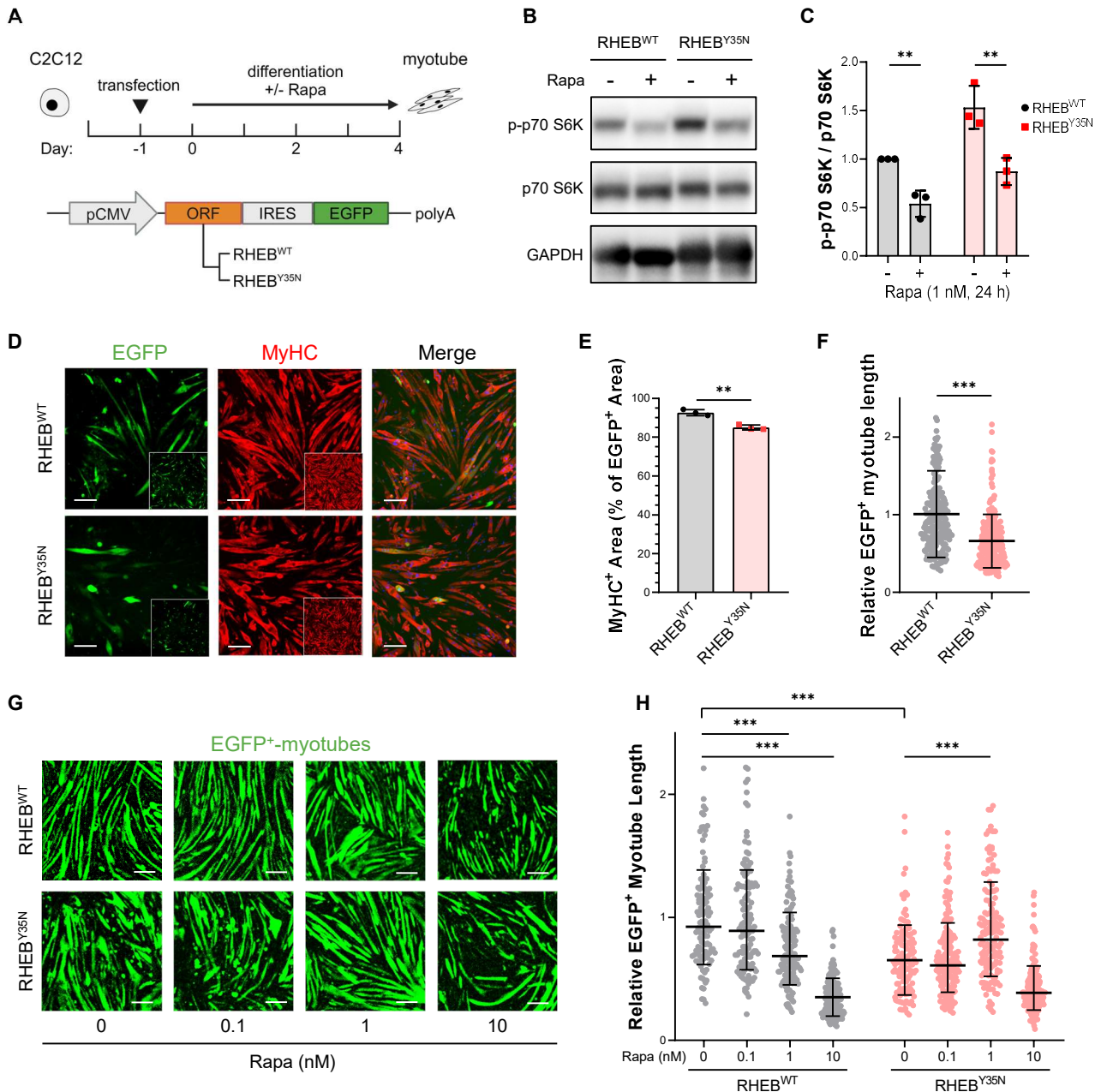
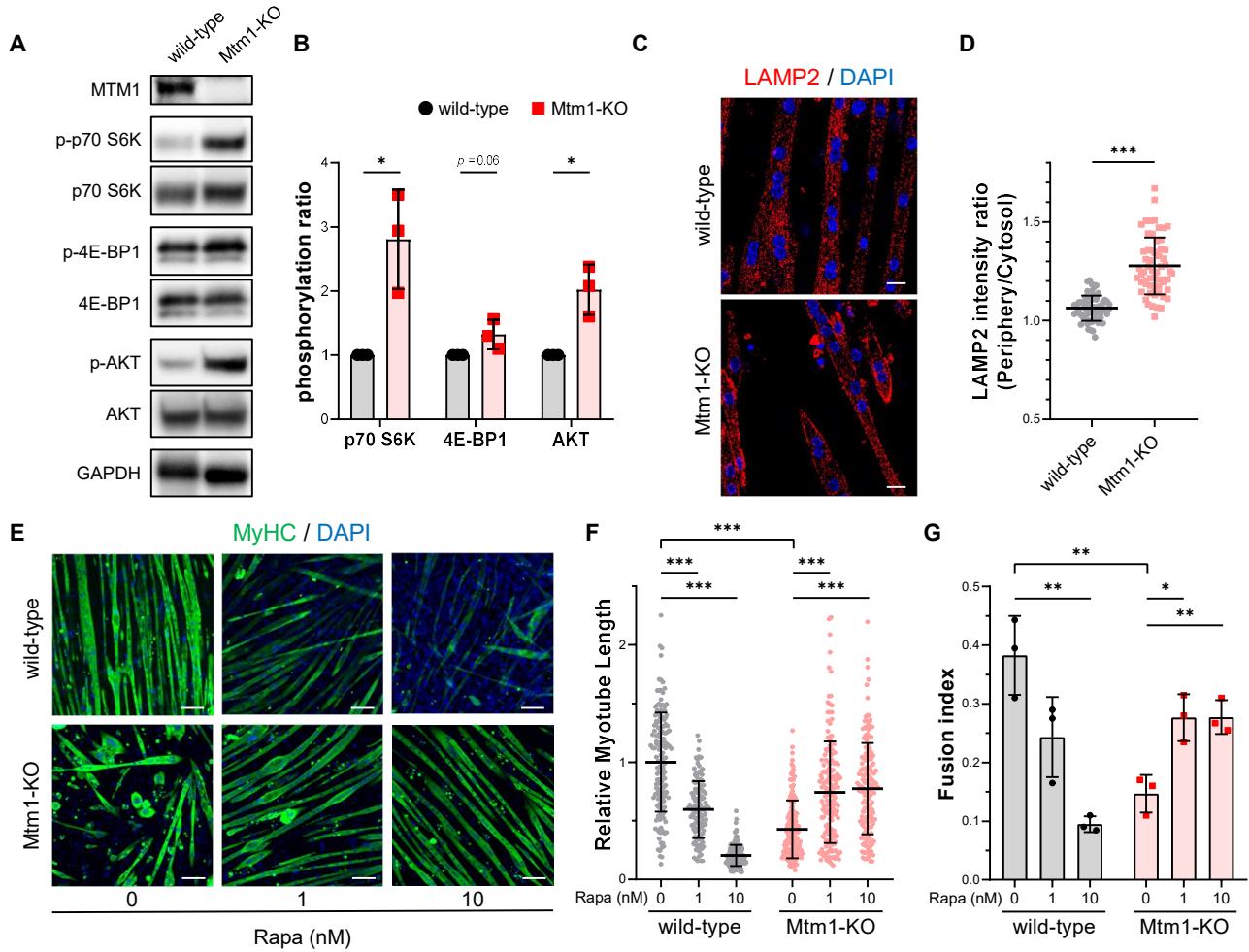


Figure 6. mTORC1 hyperactivation impairs myogenic differentiation in mouse myoblasts. (A) Schematic representation of the experimental design (top) and the construct of a RHEB transfection vector (bottom). Cells were transfected 1 day before the initiation of differentiation. Differentiation was initiated on day 0 by changing to the differentiation medium. (B) Western blot analysis of phosphorylated and total levels of p70 S6K (Thr389). GAPDH serves as a loading control. (C) Quantification of the ratio of phosphorylated to total protein levels for p70 S6K from Western blot analysis. Ratios are normalised to the corresponding values in myotubes expressing RHEB^{WT} for each independent experiment ($n = 3$ independent experiments). (D) Representative fluorescence images in C2C12-derived myotubes. EGFP is shown in green, MyHC is shown in red, and nuclei are stained with DAPI (blue). Scale bar: 100 μ m. (E) Quantification of the percentage of MyHC-positive area within the EGFP-positive area ($n = 3$ independent experiments, ≥ 3 fields per experiment). (F) Quantification of EGFP-positive myotube length. All values are normalised to the corresponding mean length in myotubes expressing RHEB^{WT} for each experiment ($n > 200$ from three independent experiments). (G) Fluorescence images of EGFP-positive myotubes treated with different concentrations of rapamycin. EGFP is shown in green. Scale bar: 100 μ m. (H) Quantification of relative myotube length. All values are normalised to the corresponding mean length in RHEB^{WT} without rapamycin for each independent experiment ($n > 100$ from three independent experiments). (C, E, F, and H) Data are presented as mean $\pm$ standard deviation. Statistical difference was evaluated using Welch's t -test $**p < 0.01$, $***p < 0.001$. Abbreviations: pCMV, CMV promoter; ORF, open reading frame; IRES, internal ribosome entry site; Rapa, rapamycin; p-p70 S6K, phosphorylated p70 S6K; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MyHC, myosin heavy chain; DAPI, 4',6-diamidino-2-phenylindole

Figure 7



Supplementary materials and methods

Western blot analysis

Cultured cells were lysed using M-PER™ Mammalian Protein Extraction Reagent (Thermo Fischer) containing a Protease Inhibitor Cocktail (Nacalai) and PhosSTOP (Roche). Cell pellets were sonicated three times on ice, and protein concentrations were determined using the Bradford method with Bio-Rad Protein Assay Kit (Bio-Rad). Samples were separated on Bullet PAGE One Precast Gels (Nacalai) and transferred onto polyvinylidene fluoride membranes (Millipore). Membranes were blocked for 1 h in 3% bovine serum albumin in tris-buffered saline with 0.1% Tween® 20 detergent (TBS-T) and incubated overnight at 4°C with primary antibodies (Supplementary Table 1). After washing with TBS-T, membranes were incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using Clarity Western ECL Substrate (Bio-Rad), and bands were digitally detected and quantified using ChemiDoc XRS+ and Image Lab software (Bio-Rad).

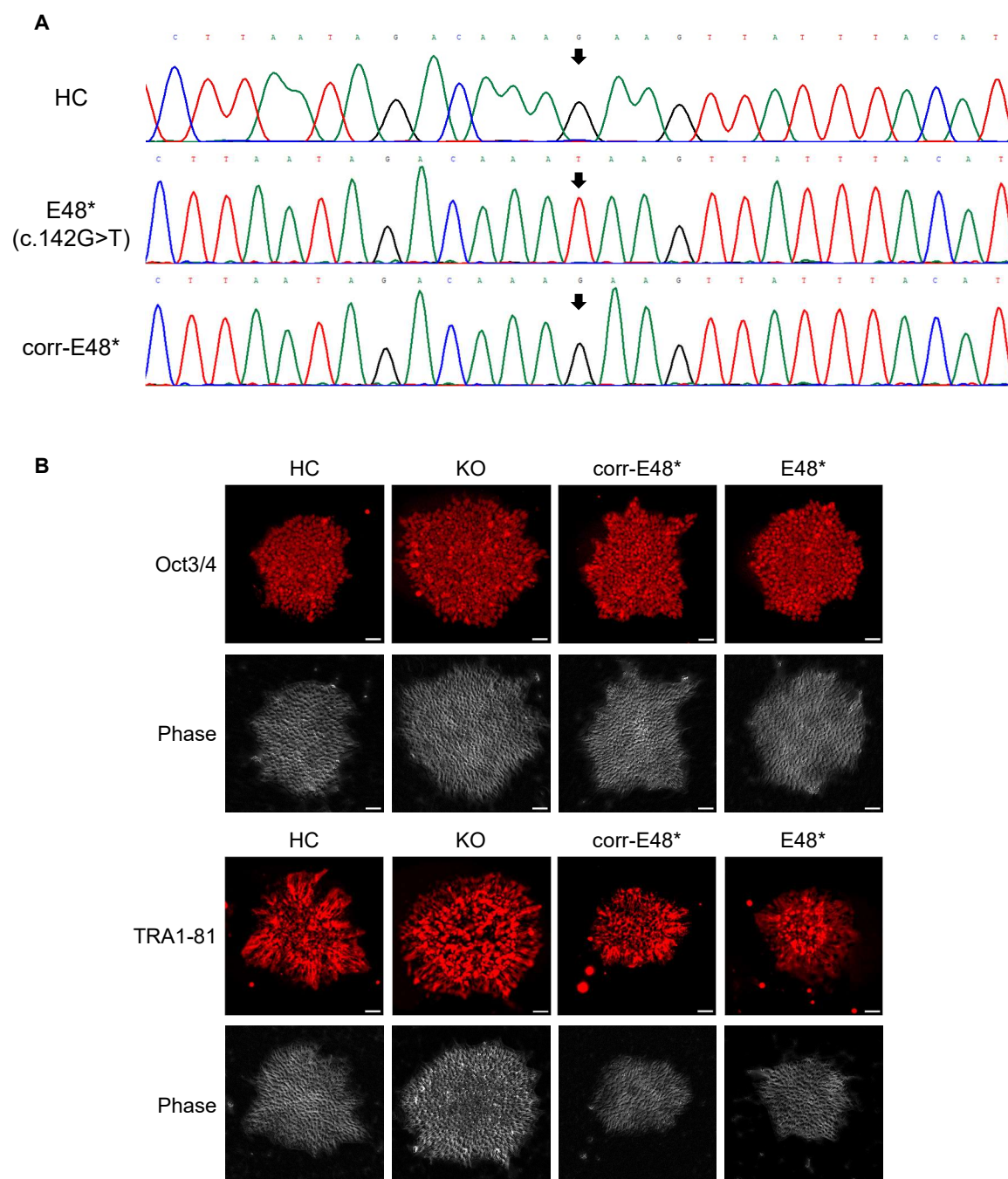
Immunofluorescence and microscopy

Cultured cells were fixed with 2% paraformaldehyde (Wako) for 10 min at room temperature (RT). After washing three times with phosphate-buffered saline (PBS), permeabilisation was performed with 0.1% Triton X-100 in PBS for 15 min, or 0.0025% digitonin (Nacalai) in PBS for 5 min (only for integrin-β1 staining) at RT. The cells were again washed three times with PBS. Blocking was conducted using Blocking One solution (Nacalai) for 40 min at RT. Primary antibodies (Supplementary Table 1) diluted in 20% Blocking One in PBS were incubated with the cells for 1 h at RT. After washing three times, the cells were incubated with secondary antibodies (Supplementary Table 2) diluted in 20% Blocking One in PBS for 40 min at RT. After washing three times, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Nacalai) for 15 min at RT. Images were acquired using a BZ-X microscope (Keyence) or an SP8 confocal microscope (Leica).

Live cell imaging of mitochondria

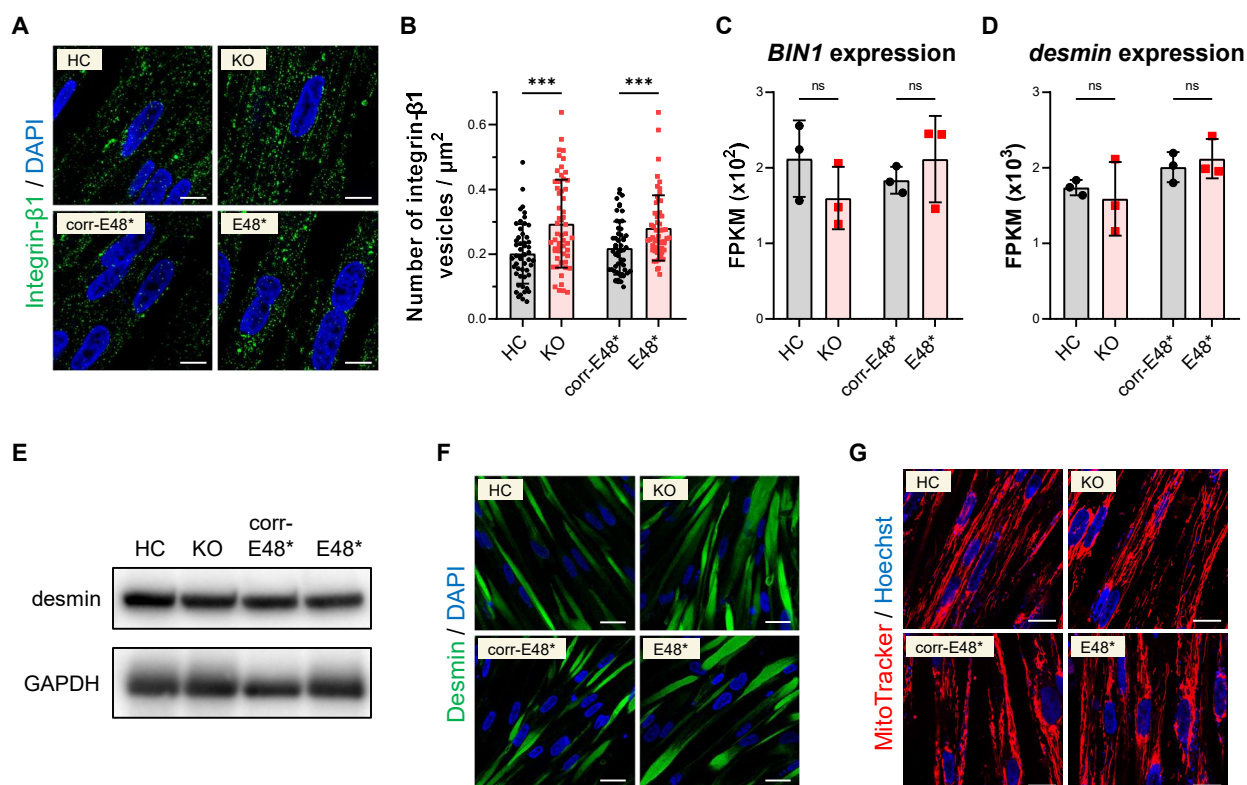
Prior to microscopy, iPSCs undergoing differentiation were seeded onto Matrigel-coated μ8-well slides (ibidi). On day 13 of the MyoD-mediated differentiation protocol, cells were stained with MitoTracker Red CMXRos (Thermo Fisher) and 5 μg/ml Hoechst 33342 (Thermo Fisher) according to the manufacturer's instructions. The culture medium was then replaced with FluoroBrite DMEM (Thermo Fisher) supplemented with 2% HS. Subsequently, cells were transferred to a Leica SP8 microscope for live cell imaging, and images were acquired under controlled conditions at 37°C and 5% CO₂ to ensure cell viability and stability.

Supplementary Figure 1



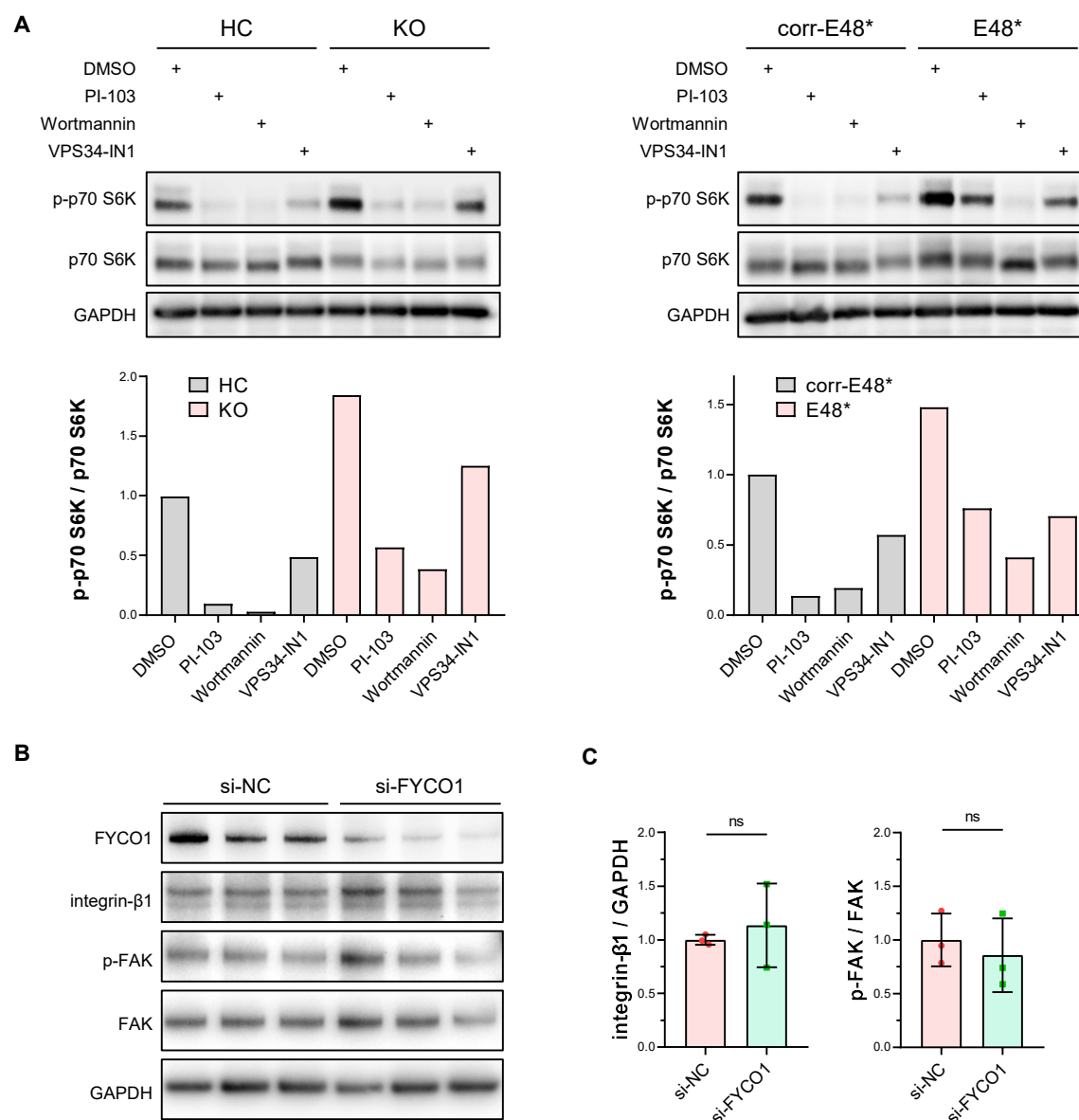
Supplementary Figure 1. Validation of gene correction and pluripotency in XLMTM iPSC lines. (A) Sanger sequencing chromatograms showing the *MTML* c.142G>T (p.E48*) variant in E48* and the successful gene correction of the variant in corr-E48*. The HC sequence is shown for comparison. Black arrows indicate the position of the variant. (B) Representative immunofluorescence images of pluripotency markers Oct3/4 and TRA1-81 (red) in HC, KO, corr-E48*, and E48* iPSC lines. Phase contrast images show colony morphology with defined borders. All lines maintain expression of pluripotency markers and exhibit characteristic iPSC morphology. Scale bar: 200 μ m.

Supplementary Figure 2



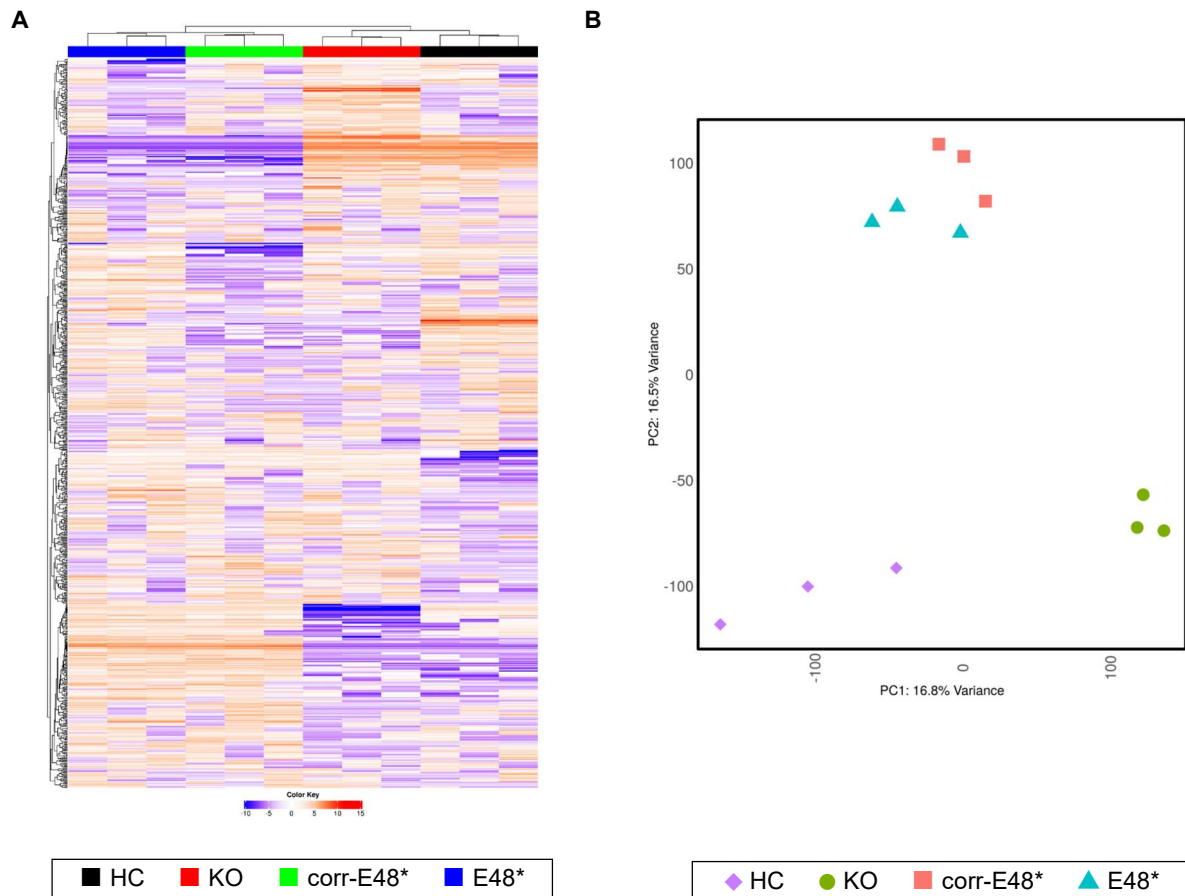
Supplementary Figure 2. Further characterization of XLMTM iPSC-derived myotubes. (A) Immunofluorescence images of iPSC-derived myotubes stained for integrin- β 1 (green) and nuclei with DAPI (blue). Scale bars: 10 μ m. (B) The number of intracellular integrin- β 1-positive vesicles per μ m². ($n > 30$ myotubes from three independent experiments). (C and D) RNA-seq analysis showing expression levels of *BIN1* (C) and *desmin* (D) in iPSC-derived myotubes. Data are normalised to FPKM ($n = 3$ independent experiments). (E) Representative Western blot showing desmin protein expression, with GAPDH used as a loading control. (F) Immunofluorescence images showing desmin localization (green) and nuclear staining (DAPI, blue) in iPSC-derived myotubes. Scale bar: 50 μ m. (G) Representative live cell images showing mitochondrial localisation and morphology in iPSC-derived myotubes. Cells were stained with MitoTracker Red CMXRos (red) and Hoechst 33342 (blue). Images were acquired using live cell confocal microscopy. Scale bar: 20 μ m. (B, C, and D) Data are presented as mean $\pm$ standard deviation. Statistical significance was evaluated using Welch's t -test. *** $p < 0.001$ (B); ns, not significant (C and D).

Supplementary Figure 3



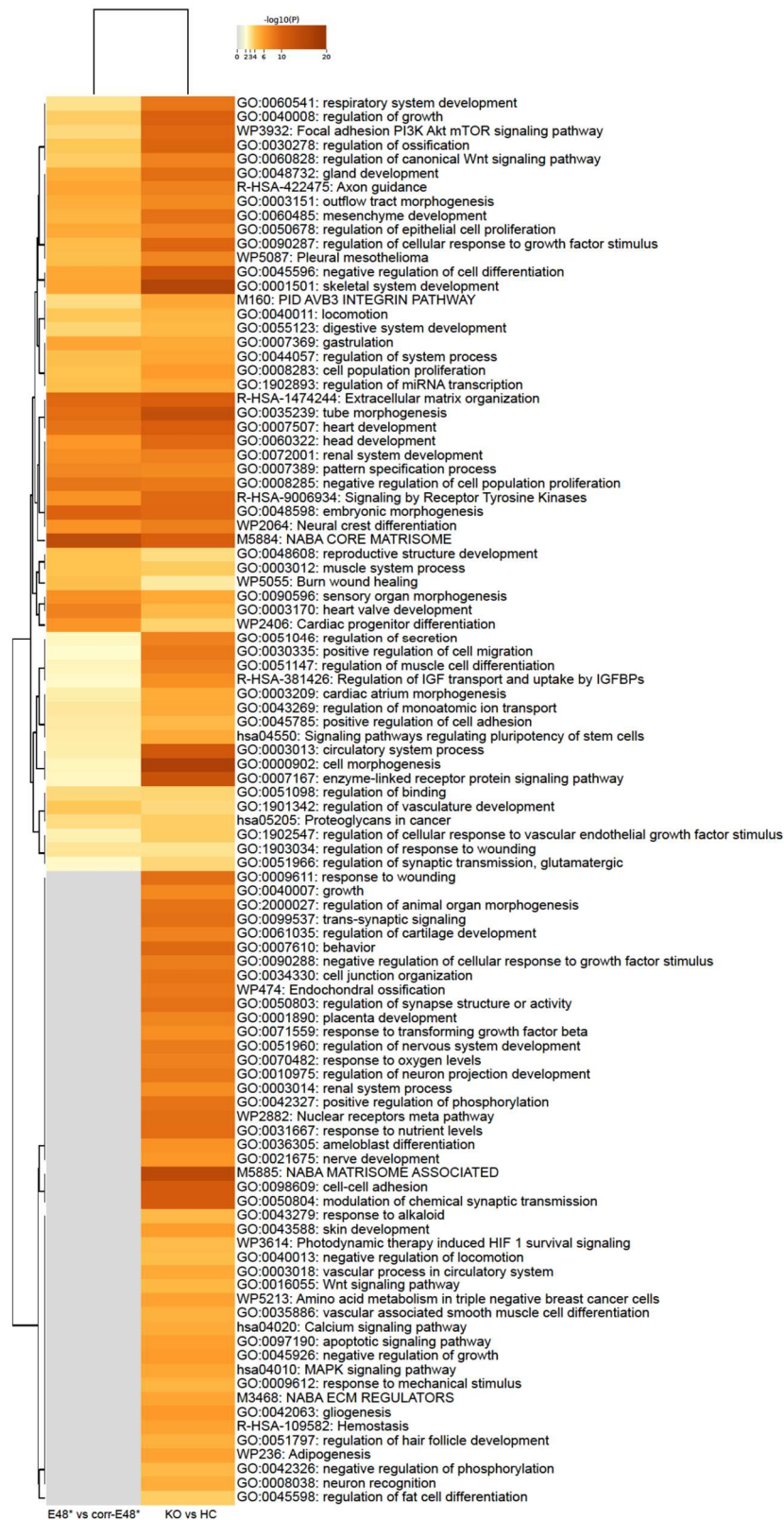
Supplementary Figure 3. Effects of PI₃P pathway inhibition on mTORC1 signalling and *FYCO1* knockdown on focal adhesion molecules. (A) Western blot analysis of the phosphorylation of p70 S6K in iPSC-derived myotubes treated with PI₃P synthesis inhibitors (500 nM PI-103, 500 nM Wortmannin) or VPS34 inhibitor (1 μM VPS34-IN1) for 2 h. Blots (top) and quantification (bottom) are shown for the HC and KO pair (left) and the corr-E48* and E48* pair (right). GAPDH serves as a loading control. (B) Western blot analysis of FYCO1, integrin-β1, p-FAK (Tyr576/577), total FAK, and GAPDH (loading control) expression in the KO myotubes treated with si-NC or si-FYCO1 from three independent experiments. (C) Quantification of relative protein expression levels of integrin-β1/GAPDH and p-FAK(Tyr576/577)/FAK ratio normalised to si-NC-treated cells. Data are presented as mean ± SD from three independent experiments. ns; not significant by Welch's *t*-test. Abbreviations: DMSO, dimethyl sulfoxide; p-p70 S6K, phosphorylated p70 S6K; NC, negative control; p-FAK, phosphorylated FAK.

Supplementary Figure 4



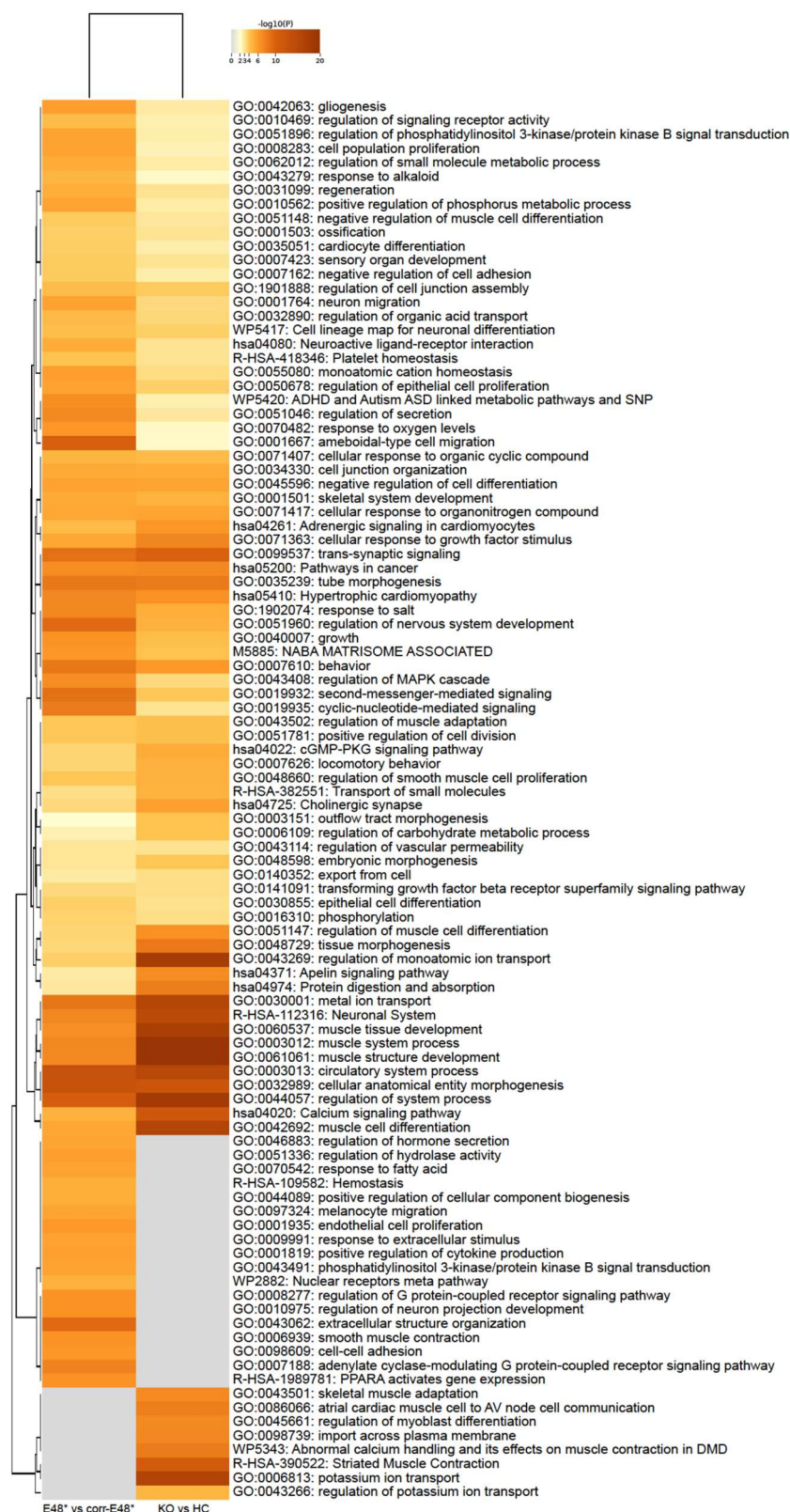
Supplementary Figure 4. RNA-seq analysis shows isogenic background-dependent clustering. (A) Hierarchical clustering analysis of RNA-seq data from iPSC-derived myotubes. The color scale represents the normalized expression values, with red indicating high expression and blue indicating low expression. Each sample is clustered based on their isogenic backgrounds. (B) PCA analysis demonstrates that the samples can be separated based on their isogenic backgrounds, rather than based on whether the samples are control or XLMTM. The first two principal components (PC1 and PC2) account for 16.8% and 16.5% of the total variance, respectively.

Supplementary Figure 5



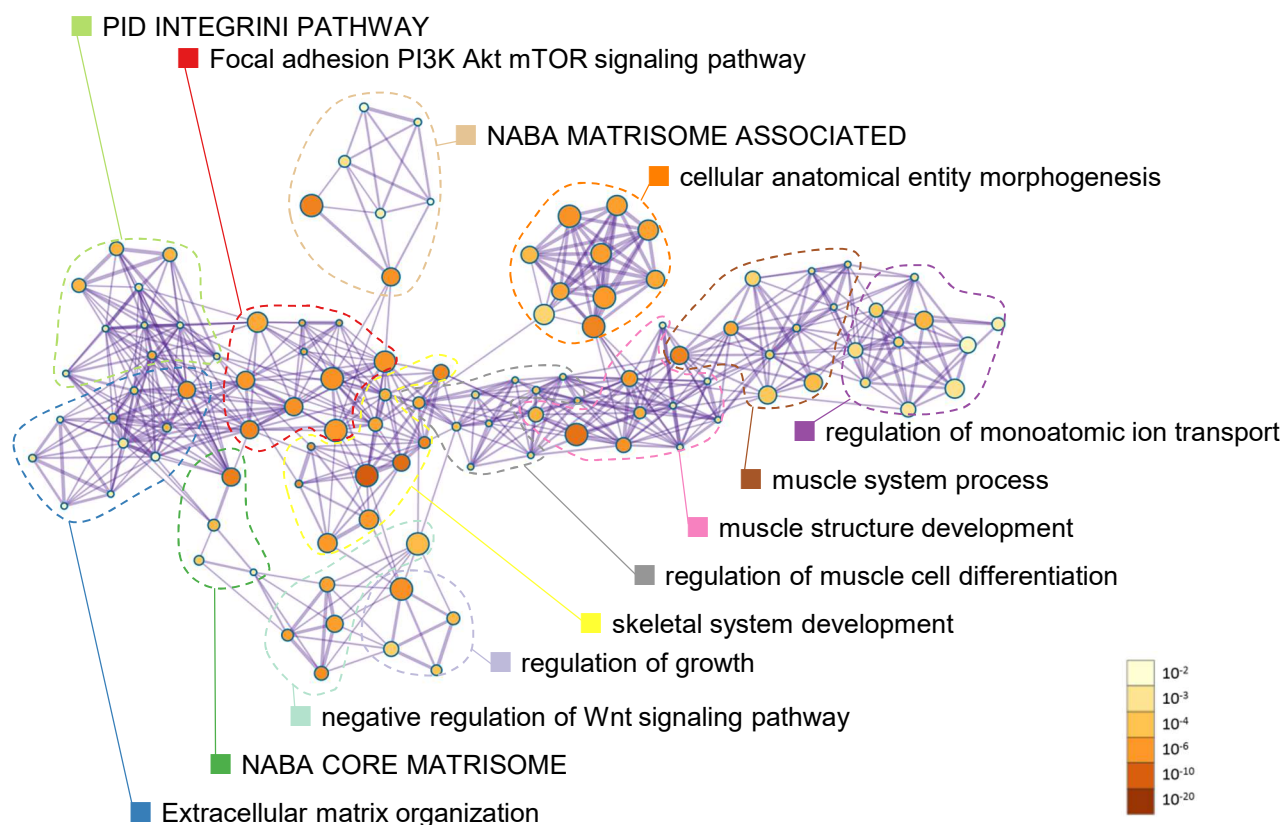
Supplementary Figure 5. Heatmap of enriched GO terms of up-regulated DEGs in XLMTM. Many biological processes including Focal adhesion-PI3K-Akt-mTOR signaling pathway and various developmental processes are shared between both XLMTM models (KO vs HC and E48* vs corr-E48*). The color intensity reflects statistical significance ($-\log_{10}$ P-value), with darker orange representing higher significance. Abbreviations: GO, gene ontology; DEG, differentially expressed gene.

Supplementary Figure 6



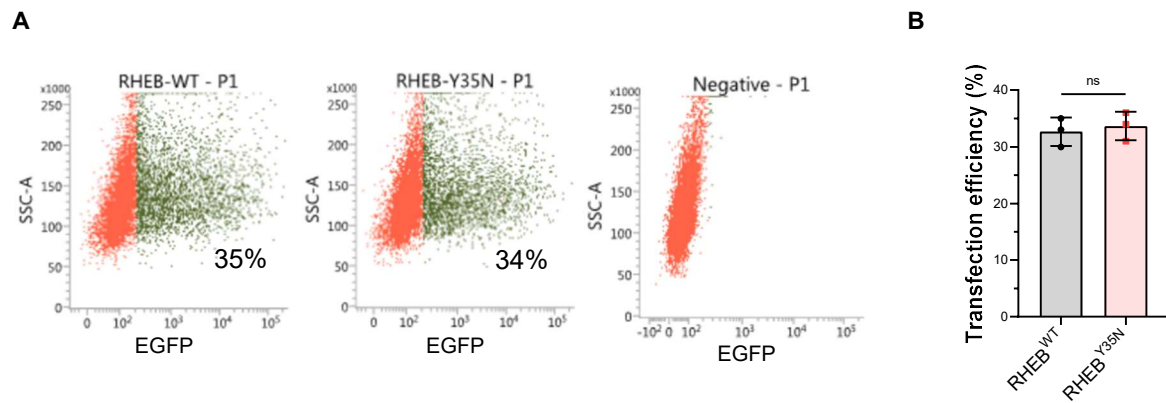
Supplementary Figure 6. Heatmap of enriched GO terms of down-regulated DEGs in XLMTM. Many pathways and processes essential for mature muscle function, including muscle cell differentiation, calcium signaling are shared between both XLMTM models (KO vs HC and E48* vs corr-E48*). The color intensity reflects statistical significance ($-\log_{10}$ P-value), with darker orange representing higher significance. Abbreviations: GO, gene ontology; DEG, differentially expressed gene.

Supplementary Figure 7



Supplementary Figure 7. Network analysis reveals interconnected gene clusters associated with mTORC1 signaling in XLMTM. Network visualization of enriched Gene Ontology (GO) terms and their relationships in XLMTM iPSC-derived myotubes. Nodes represent GO terms with colors indicating different functional categories. Node size reflects the number of associated genes, and edge connections indicate shared genes between GO terms. The color intensity of nodes represents statistical significance, ranging from light yellow ($p = 10^{-2}$) to dark brown ($p = 10^{-20}$). The network demonstrates interconnected clusters centered around Focal adhesion-PI3K-Akt-mTOR signaling pathway, linking to muscle development, differentiation, and ion transport processes.

Supplementary Figure 8



Supplementary Figure 8. Analysis of transfection efficiency in RHEB-expressing cells. (A) Representative flow cytometry plots showing EGFP expression in C2C12 cells transfected with RHEB^{WT}-IRES-EGFP, RHEB^{Y35N}-IRES-EGFP, or empty vector (Negative). SSC-A versus EGFP fluorescence is shown, with percentages indicating EGFP-positive cells. (B) Quantification of transfection efficiency based on EGFP-positive cells. Data are presented as mean $\pm$ SD ($n = 3$ independent experiments). Abbreviations: SSC-A, side scatter area; ns, not significant by Welch's *t*-test.

Supplementary Tables

Antibody	Source	Catalog No.	Host	WB	IF
4E-BP1	Cell Signaling	9452S	Rabbit	1:1000	-
AKT	Cell Signaling	4691S	Rabbit	1:1000	-
Desmin	Cell Signaling	#5332	Rabbit	1:1000	1:100
Dynamin2	Abcam	ab3457	Rabbit	1:1000	-
ERK1/2	Cell Signaling	#9102	Rabbit	1:1000	-
FAK	Cell Signaling	#13009	Rabbit	1:1000	-
FYCO1	Proteintech	25730-1-AP	Rabbit	1:1000	1:200
GAPDH (peroxidase conjugated)	FUJIFILM Wako	015-25473	Mouse	1:10000	-
Integrin- β 1	Sigma-Aldrich	MAB1997	Rat	1:1000	1:150
LAMP2	Proteintech	66301-1-Ig	Mouse	-	1:200
MTM1	Invitrogen	PA5-17972	Goat	1:500	-
Myosin Heavy Chain (MYH3)	Invitrogen	14-6503-82	Mouse	-	1:200
Oct-3/4	Santa Cruz	sc-5279	Mouse	-	1:150
phospho-ERK1/2 (Thr202/Tyr204)	Cell Signaling	9101S	Rabbit	1:1000	-
phospho-FAK (Tyr576/577)	Cell Signaling	3281p	Rabbit	1:500	-
phospho-4EBP1 (Thr37/46)	Cell Signaling	9459S	Rabbit	1:1000	-
phospho-AKT (Ser473)	Cell Signaling	4060S	Rabbit	1:1000	-
phospho-S6K (Thr389)	Cell Signaling	9234S	Rabbit	1:1000	-
S6K	Cell Signaling	2708S	Rabbit	1:1000	-
TRA-1-81	Sigma-Aldrich	MAB4381	Mouse	-	1:150

Supplementary Table 1. List of primary antibodies.

Antibody	Source	Catalog No.	Conjugate	IF
Goat anti-Mouse IgG (H+L)	Invitrogen	A-11001	Alexa Fluor 488	1:200
Goat anti-Mouse IgG (H+L)	Invitrogen	A-11004	Alexa Fluor 568	1:200
Goat anti-Rabbit IgG (H+L)	Invitrogen	A-11008	Alexa Fluor 488	1:200
Goat anti-Rabbit IgG (H+L)	Invitrogen	A-11011	Alexa Fluor 568	1:200
Goat anti-Rat IgG (H+L)	Invitrogen	A-11006	Alexa Fluor 488	1:200

Supplementary Table 2. List of secondary antibodies.

Abbreviations: WB, Western blot; IF, immunofluorescence; -, not applicable.

Uncropped versions of immunoblots No.1

Figure 1B

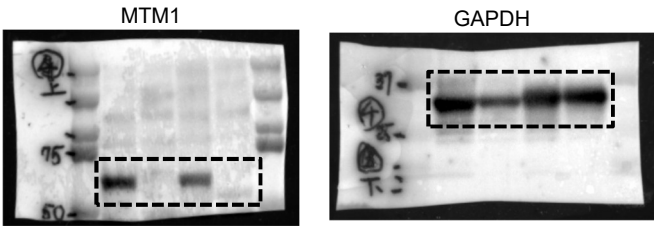


Figure 2B

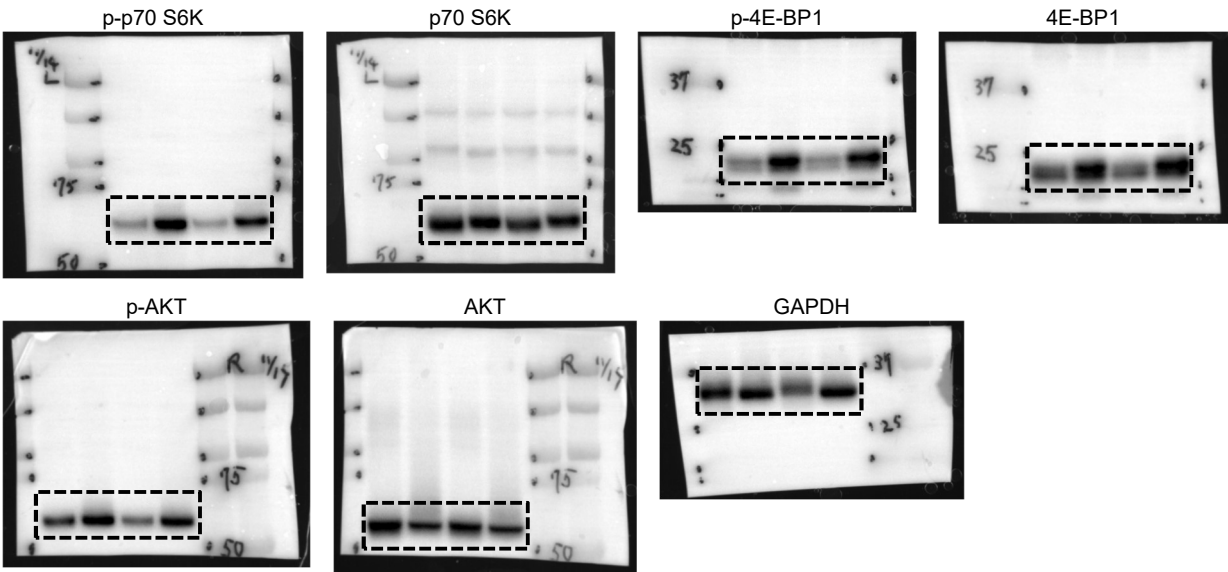
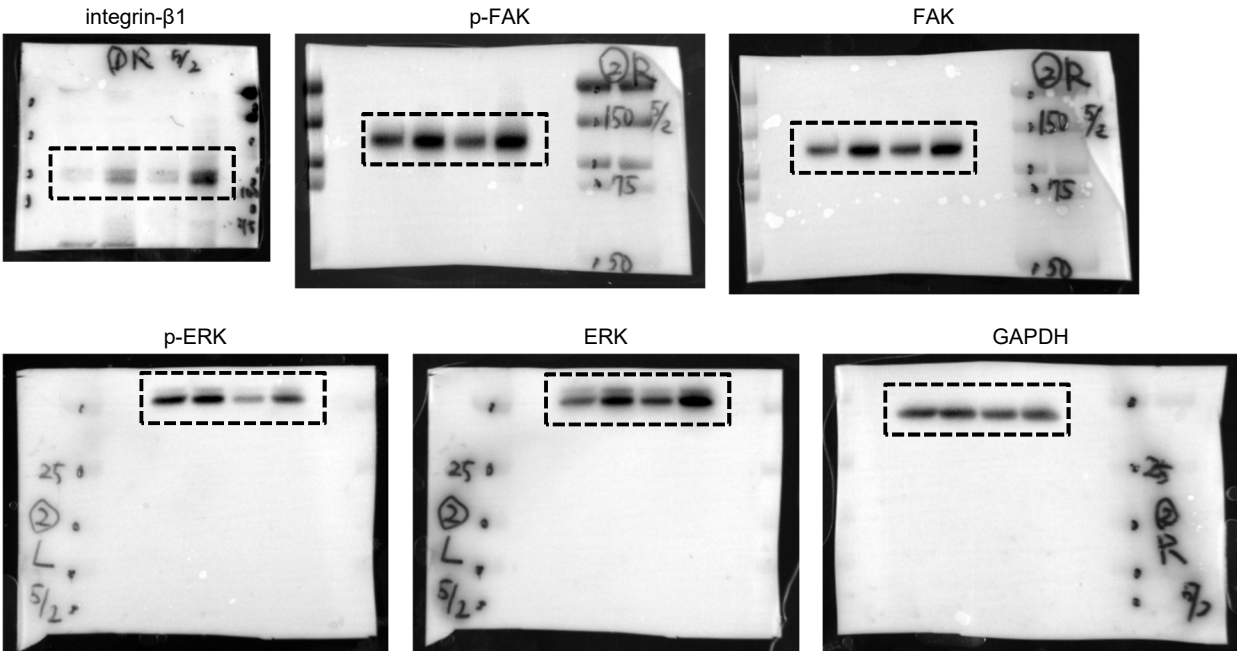


Figure 2D



Uncropped versions of immunoblots No.2

Figure 2H

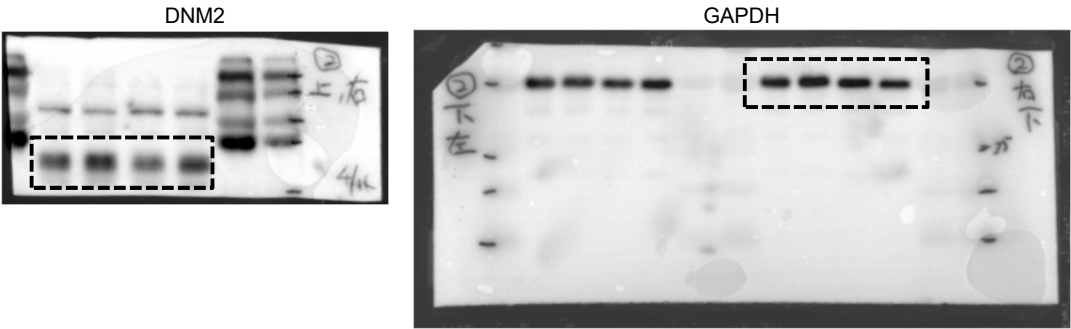


Figure 4D

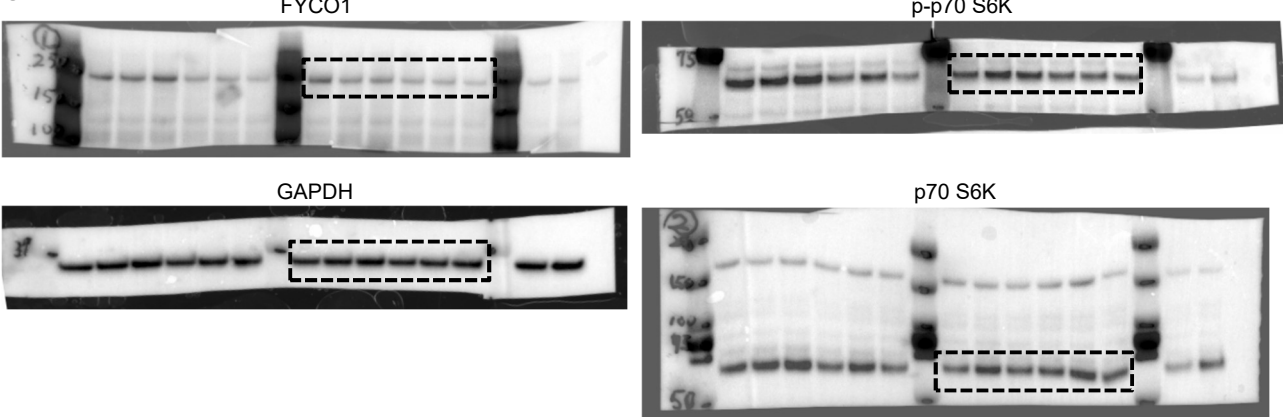
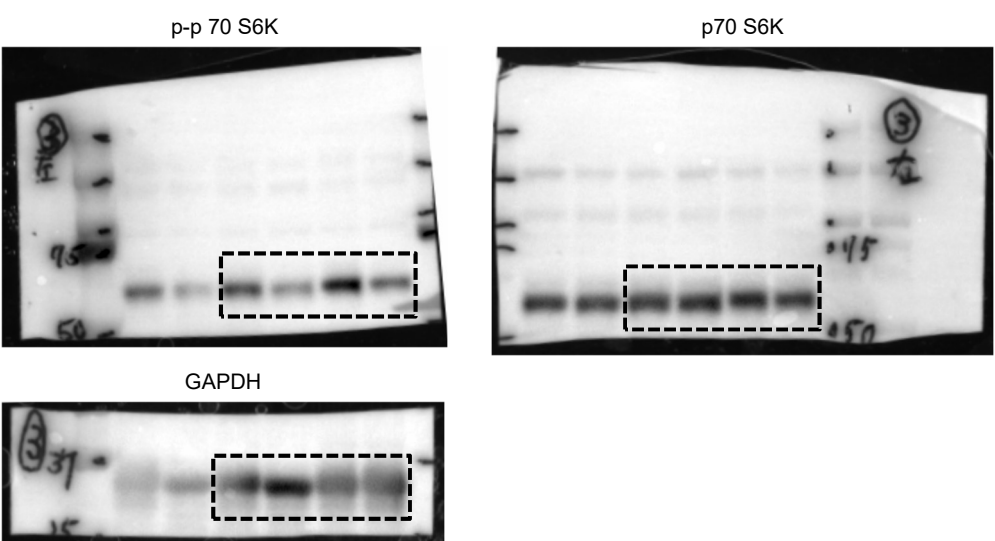
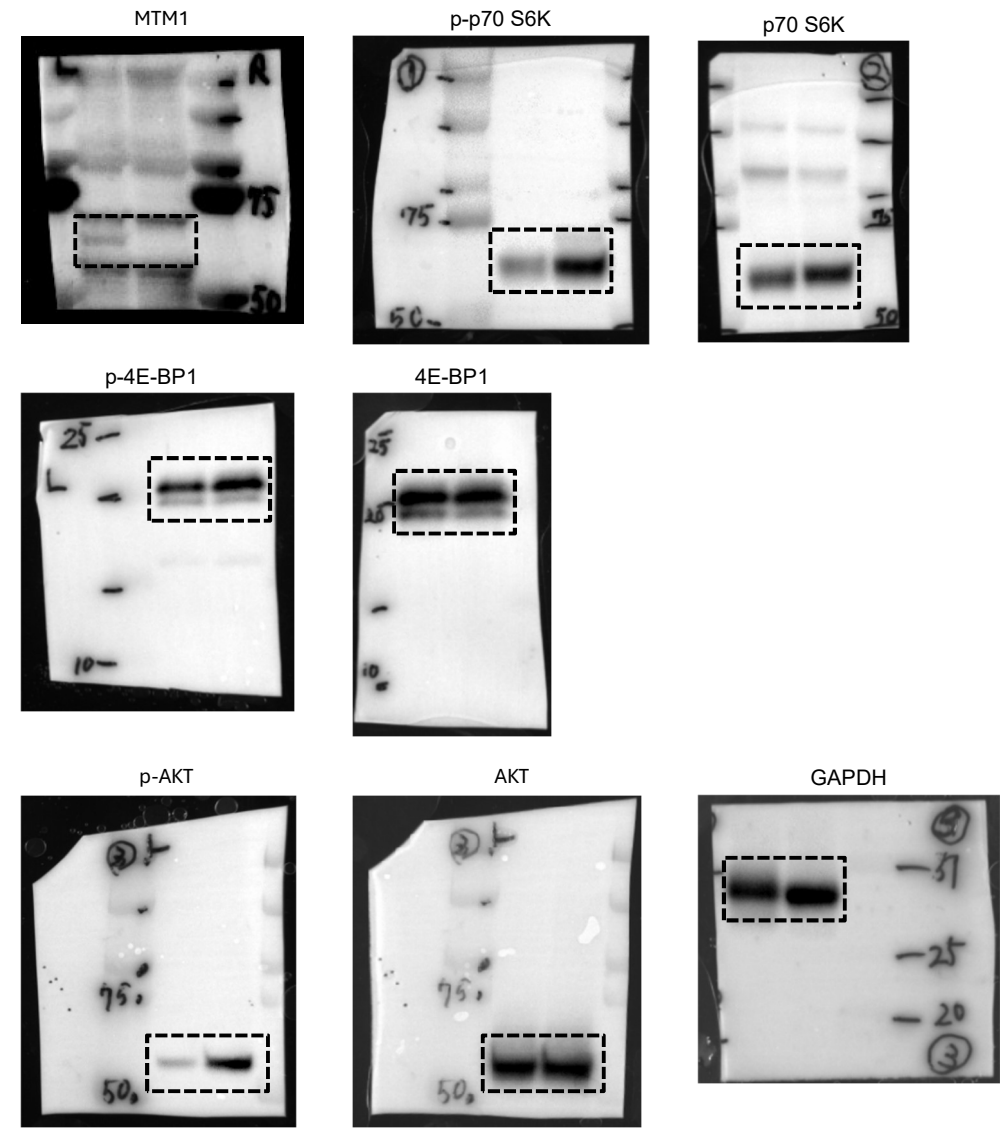


Figure 6B

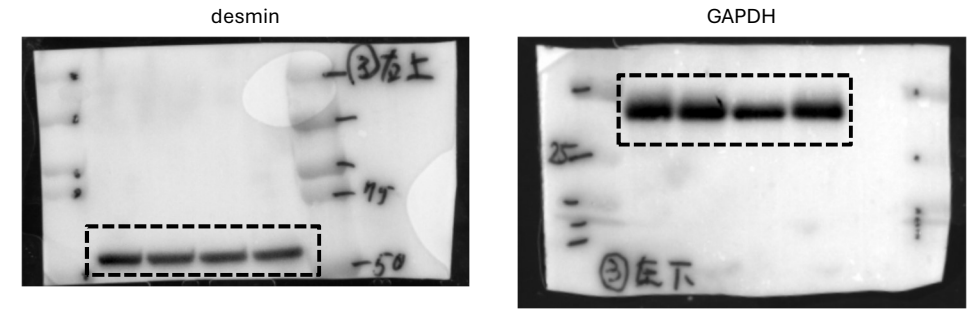


Uncropped versions of immunoblots No.3

Figure 7A

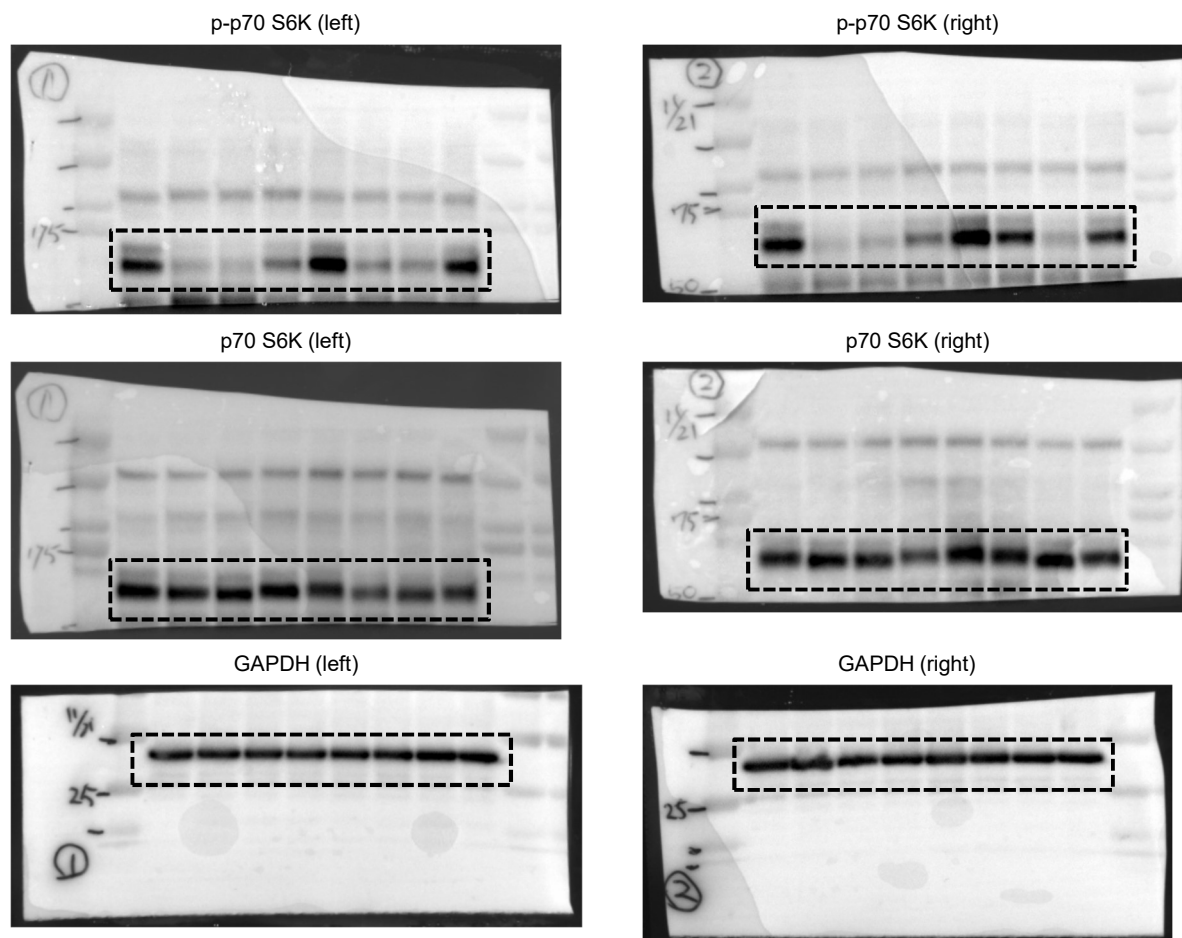


Supplementary Figure 2C



Uncropped versions of immunoblots No.4

Supplementary Figure 3A



Supplementary Figure 3B

