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A non-translational role of threonyl-tRNA synthetase in regulating JNK signaling during myogenic differentiation

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Abstract

Aminoacyl-tRNA synthetases (aaRSs) are house-keeping enzymes that are essential for protein synthesis. However, it has become increasingly evident that some aaRSs also have non-translational functions. Here we report the identification of a non-translational function of threonyl-tRNA synthetase (ThrRS) in myogenic differentiation. We find that ThrRS negatively regulates myoblast differentiation in vitro and injury-induced skeletal muscle regeneration in vivo. This function is independent of amino acid binding or aminoacylation activity of ThrRS, and knockdown of ThrRS leads to enhanced differentiation without affecting the global protein synthesis rate. Furthermore, we show that the non-catalytic new domains (UNE-T and TGS) of ThrRS are both necessary and sufficient for the myogenic function. In searching for a molecular mechanism of this new function, we find the kinase JNK to be a downstream target of ThrRS. Our data further reveal MEKK4 and MKK4 as upstream regulators of JNK in myogenesis and the MEKK4-MKK4-JNK pathway to be a mediator of the myogenic function of ThrRS. Finally, we show that ThrRS physically interacts with Axin1, disrupts Axin1-MEKK4 interaction and consequently inhibits JNK signaling. In conclusion, we uncover a non-translational function for ThrRS in the maintenance of homeostasis of skeletal myogenesis and identify the Axin1-MEKK4-MKK4-JNK signaling axis to be an immediate target of ThrRS action.

Keywords

JNK signaling; myoblast; myogenesis; skeletal muscle regeneration; threonyl-tRNA synthetase

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AUTHOR CONTRIBUTIONS

C.D. and J.C. designed the study. C.D. and A.R.-O. performed the experiments. C.D., J.-S.Y., and J.C. analyzed the data. C.D. and J.C. wrote the manuscript. A.R.-O. and J.-S.Y. edited the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of the article at the publisher's website.

DISCLOSURES

The authors declare that there is no disclosures.

1 | INTRODUCTION

Skeletal myogenesis is a fundamental process in both embryonic muscle development and adult muscle regeneration. During myogenesis, muscle progenitor cells or satellite cells become proliferating myoblasts, which then exit the cell cycle, differentiate into mononucleated myocytes, and fuse to form multinucleated myotubes/myofibers.[1,2] This well-coordinated process is regulated by a transcriptional network controlled by a plethora of extracellular and intracellular signals.[3,4] Failure of proper myogenic regulation can exacerbate pathological conditions, such as muscular dystrophy, cachexia, and sarcopenia.[5,6]

Regulation of myogenic differentiation entails numerous signaling pathways, such as mTOR,[7] PI3K/Akt,[8] Jak/STAT,[9] and p38,[10] to name a few. As a versatile kinase, JNK can promote myogenic differentiation through the downstream AP-1 complex that contains Fra-2 and c-Jun or JunD.[11] JNK also promotes myogenesis by phosphorylating SMAD2 at the linker region, which prevents SMAD2 from entering the nucleus and concomitantly decreases the activity of myostatin, a negative myogenic regulator.[12]

Aminoacyl-tRNA synthetases (aaRSs) are essential for life due to their fundamental role in protein synthesis. With hydrolysis of ATP, aaRSs charge tRNAs with cognate amino acids. Interestingly, aaRS functions are not limited to processing genetic information. Decades ago lysyl-tRNA synthetase was found to synthesize 5′,5′-diadenosine tetraphosphate in *Escherichia coli*.[13] In recent years noncanonical functions of aaRSs have been increasingly recognized to play diverse roles in cellular regulation.[14,15] For instance, glycyl-tRNA synthetase (GlyRS, or GARS1) can be secreted by immune cells to suppress cancer cell pro-liferation.[16] Leucyl-tRNA synthetase (LeuRS, or LARS1) senses intracellular leucine levels and induces mTORC1 activation.[17] During evolution aaRSs are expanded by the addition of new domains in higher eukaryotes, and it is believed that those domains may be responsible for non-translational functions of aaRSs.[18,19] Importantly, aaRSs have also been found to be directly involved in human diseases. For instance, in a subtype of the Charcot-Marie-Tooth diseases mutations in GlyRS are found to confer a gain of function to bind the neuropilin-1 receptor and sub-sequently antagonize motor neuron survival.[20]

Threonyl-tRNA synthetase (ThrRS or TARS1) contains a unique UNE-T domain at the N-terminus, followed by an-other new domain, TGS (ThrRS, GTPase, and SpoT), which structurally resembles ubiquitin.[21] The UNE-T domain has recently been reported to interact with eIF4E homologous protein and mediate ThrRS regulation of VEGF translation.[22] ThrRS is also found to be secreted by human vascular endothelial cells upon TNF-α or VEGF stimulation, and exogenous ThrRS can stimulate endothelial cell migration and angiogenesis although a molecular mechanism is not known.[23] Here, we report a new non-translational role of ThrRS in myogenic differentiation, which is dependent on its new domains. We have also uncovered a direct mechanism of ThrRS action through an Axin-MEKK4-MKK4-JNK signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Antibodies and other reagents

Antibodies for MHC (clone MF20) and myogenin (clone F5D) were obtained from the Developmental Studies Hybridoma bank (developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa, Department of Biological Sciences). Anti-Flag-BioM2 (F9291), anti-MEKK4 (M7194), and gelatin were purchased from Sigma. Anti-tubulin (ab11304) and anti-ThrRS (ab236903) were from Abcam. Antibodies for the following proteins were obtained from Cell Signaling Technology: JNK (9252), phospho-JNK (Thr183/Tyr185; 9255), MKK4 (9152), phospho-MKK4 (Ser257/Thr261; 9156), MKK7 (4172), phospho-MKK7 (Ser271/Thr275; 4171), HA tag (3724), and Axin1 (2087). Peroxidase-conjugated anti-rabbit (115-036-003) and anti-mouse (111-036-003) IgG and FITC-conjugated anti-mouse IgG (115-096-003) were from Jackson ImmunoResearch Laboratories. EdU (5-Ethynyl-2-deoxyuridine) was from Carbosynth Limited, and 5-FAM-azide was from Lumiprobe.

2.2 | Plasmids

ThrRS cDNA (Cat #: MG51548-G) was purchased from Sinobiological and then subcloned into pcDNA3 containing a Flag tag (pcDNA8). pcDNA8-Flag-ThrRS was then used to generate all point mutants of ThrRS using Q5® Site-Directed Mutagenesis Kit (New England Biolabs) and truncation mutants using PCR. The following plasmids were obtained from Addgene: pCMV5-MEKK4alpha (#12187) and M50 Super 8 × TOPFlash (#12456). pRL-SV40 (Cat #: E2231) was from Promega.

2.3 | Cell culture

C2C12 myoblasts were a gift from S. Kaufman at the University of Illinois and originally obtained from ATCC. Cells were maintained in DMEM containing 4.5 g/L glucose, 10% fetal bovine serum, and 1% penicillin–streptomycin, at 37°C in a humidified atmosphere of 92.5% air and 7.5% CO_2. To induce differentiation, cells were plated on tissue culture plates coated with 0.2% gelatin, grown to 100% confluence, and cultured in a differentiation medium (DMEM containing 2% horse serum) thereafter. The cells were replenished with fresh differentiation medium daily for 3 days. Myoblasts were transfected by using TransIT-LT1 (Mirus) or Lipofectamine 3000 according to the manufacturers' recommendations. Cells were selected in 3 μg/ml puromycin or 1.0 mg/ml G418 as needed.

2.4 | Lentivirus-delivered RNA interference

shRNAs in the pLKO.1-puro vector were purchased from Sigma-Aldrich (MISSION TRC). Clone IDs are: shThrRS#1, TRCN0000075969; shThrRS#2, TRCN0000075972; shMKK4#1, TRCN0000025266; shMKK4#2, TRCN0000025268; shMKK7#1, TRCN0000012608; shMKK7#2, TRCN0000012609; shMEKK4#1, TRCN0000000849; shMEKK4#2, TRCN0000000850. A hairpin of scrambled sequence (shScramble) used for a negative control and lentivirus packaging plasmids were described previously.[24] C2C12 cells were transduced with lentiviruses in a growth medium containing

8 μg/ml Polybrene and selected in 3 μg/ml puromycin for 2 days, followed by plating into 12-well plates for differentiation.

2.5 | Cell lysis, western blotting, and immunoprecipitation

For western blotting, cells were rinsed with PBS and lysed in SDS sample buffer containing 5% β-mercaptoethanol or in MIPT buffer as described previously.[25] Proteins were resolved on SDS-PAGE, transferred onto PVDF membrane (EMD Millipore), and incubated with various antibodies according to the manufacturers' recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and visualized using an iBright CL1000 Imaging System. Quantification of western results was performed by densitometry in ImageJ (NIH). For immunoprecipitation, transiently transfected 293T cells in 6-cm dishes were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1% (v/v) NP-40, and 1 mM protease inhibitor cocktail. The cell lysates were cleared with a microcentrifuge at 16 249 *g* for 10 min at 4°C. Flag-tagged and HA-tagged proteins were immunoprecipitated from the lysates with ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich; A2220) and anti-HA-Agarose (Millipore Sigma; A2095), respectively. Immunoprecipitates were analyzed by western blotting as described above.

2.6 | Immunofluorescence microscopy and quantitative analysis of myocytes

C2C12 cells differentiated in 12-well plates were fixed and stained for MHC with DAPI as previously described.[25] The stained cells were observed with a Leica DMI 4000B fluorescence microscope (Leica), and the fluorescence images were captured using a RETIGA EXi camera (QImaging) equipped with Image Pro Express (Media Cybernetics). Images were analyzed by using ImageJ (NIH). The fusion index was calculated as the ratio of the number of nuclei in multinucleated cells to the total number of nuclei on a given plate of cells. Each data point was generated by quantifying all cells in five randomly chosen microscopic fields.

2.7 | EdU labeling

C2C12 myoblasts or differentiating myocytes were incubated with 5-ethynyl-2′-deoxyuridine (EdU, final concentration 1 μM) for 2 h and then fixed with 3.7% formaldehyde. Cells were treated with 10 μM 5-FAM azide, 1 mM $CuSO_4$, and 100 mM ascorbic acid in PBS for 30 min. After the treatment, nuclei were stained by DAPI for 10 min. Fluorescence microscopy and quantification were performed as described above.

2.8 | Determination of protein synthesis rate by metabolic labeling

C2C12 myoblasts (3.5×10^5 cells) were pre-incubated in methionine/cysteine-free DMEM medium with 10% FBS at 37°C for 30 min. ^{35}S-methionine/cysteine (Perkin-Elmer) was added into the medium at a final concentration of 200 μCi/ml and incubated at 37°C for 30 min. Labelled cells were collected in PBS and lysed, and cellular proteins were precipitated with trichloroacetic acid and subjected to scintillation counting to determine ^{35}S incorporation.

2.9 | Injury-induced muscle regeneration and intramuscular gene knockdown

Male and female C57BL/6 wild-type mice (Envigo) aged 10–12 weeks were randomly allocated to the different experimental groups. Muscle injury was induced by injecting 20 μl of 2% (w/v) $BaCl_2$ dissolved in saline into mouse hindlimb TA muscles as previously described.[26] To knock down ThrRS, 20 μl lentivirus was concentrated to 1×10^7–1×10^8 IU/ml via ultracentrifugation and co-injected with $BaCl_2$ into the TA muscles. Every mouse was injected with shScramble in one leg and shThrRS in the other leg. For JNK inhibitor experiments, 30 mg SP600125 per kg body weight was delivered by intraperitoneal injection daily. The injured muscles were collected at 7 or 14 days after injury.

2.10 | Muscle tissue cryosection and analysis

Isolated TA muscles were frozen in liquid nitrogen-cooled 2-methylbutane and embedded in a TBS tissue freezing medium (Thermo Fisher Scientific). Sections of 10 μm thickness were made with a Microm HM550 (Thermo Fisher Scientific) at −20°C, placed on uncoated slides, and stained with hematoxylin and eosin (H&E). Five to ten images of the injured areas were randomly captured from the stained sections with a 20× dry objective (Fluotar, numerical aperture 0.4; Leica) on a Leica DMI 4000B microscope. The images were then analyzed for cross-sectional area (CSA) of centrally nucleated regenerating myofibers. A total area of ~750 000 μm^2 from the regenerating regions of each TA muscle was subjected to the measurement using ImageJ (NIH). Because injection was always performed with shScramble in one leg and shThrRS in the other leg, the data were paired, with shScramble as reference.

2.11 | Luciferase assay

Cells grown in 12-well plates were transfected with the M50 Super 8 × TOPFlash and control pRL-SV40 plasmids for 24 h and then lysed by incubating with 1 × passive lysis buffer (Cat #: E1910, Promega) and gently shaking at room temperature for 15 min. The lysate was cleared in a tabletop centrifuge (17 000*g*; 30 s), and the supernatant was subjected to assay using Dual-Luciferase Reporter Assay System (Cat #: E1910, Promega) on a SPECTRA max GEMINI XPS plate reader.

2.12 | Statistical analysis

All quantitative values were expressed as mean ± SEM. The exact sample size for each experiment is described in figure legends. Whenever necessary, the statistical significance of the data comparison was analyzed by performing two-tailed paired, unpaired or one-sample *t* tests, or two-way ANOVA followed by Student–Newman–Keuls post hoc test for multiple comparison. The exact methods of analysis are described in figure legends. Differences between groups were considered significant when $p < .05$. Statistical analyses were performed using Excel and SigmaPlot 14.0.

3 | RESULTS

3.1 | ThrRS plays a negative role in myogenic differentiation

We investigated a potential role of ThrRS in myogenic differentiation of the mouse C2C12 myoblasts. Myoblast differentiation was induced by growth factor deprivation and measured by the degree of fusion to form myosin heavy chain (MHC)-positive myotubes. We found that knockdown of ThrRS by lentivirus-delivered shRNA resulted in enhanced myoblast differentiation. As shown in Figure 1A, myotube formation quantified by fusion index was increased upon treatment by two independent shRNAs compared to the scramble shRNA control. Conversely, transient expression of a Flag-tagged recombinant ThrRS led to a 20% increase in the total ThrRS protein and decreased myoblast differentiation (Figure 1B). These observations suggest that ThrRS may play a negative role in myogenic differentiation.

To assess whether ThrRS also played a myogenic role in vivo, we made use of a well-established mouse model of injury-induced muscle regeneration. Injury of the tibialis anterior (TA) muscle was induced by injection of barium chloride ($BaCl_2$), followed by de novo formation of new myofibers marked by centrally located nuclei (Figure 1C). When lentivirus expressing shThrRS was co-injected with $BaCl_2$, the average size of regenerating myofibers as measured by the cross-sectional area (CSA) increased by ~18% compared to the control (a scrambled sequence in the hairpin; shScramble) on day 7 after injury (AI); the difference was no longer significant by day 14 AI (Figure 1C). Knockdown of ThrRS at the protein level in the injected muscle on day 7 AI was evident as shown by western blotting but not on day 14 AI (Figure 1C), which could explain a lack of phenotype at the later stage of regeneration. This data suggests that knockdown of ThrRS enhances muscle regeneration, consistent with the enhanced differentiation in vitro.

Next, we asked at which stage of myogenic differentiation ThrRS may function. ThrRS knockdown enhanced the expression of both the early myogenic marker myogenin and the late marker MHC as assessed by western blotting (Figure 2A) and quantification of the results (Figure 2B), implying that ThrRS may regulate an early event of myogenesis. It was noted that the protein level of ThrRS did not change throughout the course of differentiation in the shScramble-treated cells (Figure 2C). To examine one of the earliest events of myogenesis, cell cycle withdrawal, we performed EdU incorporation assay and found no effect from ThrRS knockdown (Figure 2D).

3.2 | Myogenic function of ThrRS is independent of protein synthesis and dependent on the new domains

A cytoplasmic threonyl-tRNA synthetase-like protein, ThrRS-L (or TARSL2), is highly homologous to ThrRS and shown to have tRNA aminoacylation and editing activities.[27,28] Mutation of either of two conserved residues in the active site based on the *E. coli* threonyl-tRNA synthetase structure[29] is found to drastically reduce the aminoacylation activity of ThrRS-L.[28] We mutated those two residues in ThrRS, C412 and R441 (Figure S1A and Figure 3A), in order to assess whether the canonical activity of ThrRS is involved in its myogenic function. Overexpression of either of the single point mutants (C412A and R441A) dampened differentiation to the same degree as that of WT ThrRS (Figure 3B),

suggesting that the aminoacylation activity of ThrRS is dispensable for its function in myogenesis.

Next, we asked which domain(s) of ThrRS may be responsible for its myogenic function by examining several truncation mutants of ThrRS (Figure 3A). As shown in Figure 3C, expression of a truncated ThrRS containing only UNE-T and TGS domains conferred a differentiation phenotype similar to that of WT, whereas ThrRS devoid of either UNE-T or TGS (or both) no longer had an effect on differentiation. These data suggest that the new domains are both necessary and sufficient for ThrRS myogenic function.

Our observations thus far strongly imply that ThrRS regulation of myogenesis is independent of its translational function. Since the process of differentiation requires protein synthesis, the enhanced differentiation upon ThrRS knockdown led us to suspect that the global protein synthesis rate may not have been affected by the partial depletion of ThrRS in the knockdown cells. To directly test this possibility, we performed metabolic labeling assays to examine the global protein synthesis rate. Indeed, we found protein synthesis to be unaffected by ThrRS knockdown under our experimental conditions (Figure 3D). Taken together, the collective evidence indicates that ThrRS exerts a non-translational function in the regulation of myogenic differentiation.

3.3 | ThrRS regulation of myogenesis is mediated by JNK signaling

In search of a mechanism of the novel function of ThrRS, we examined several known myogenic signaling pathways (Figure S2 and Figure 4A) and found that ThrRS knockdown led to a consistent increase of JNK phosphorylation levels throughout myoblast differentiation (Figure 4A). Previous studies suggested that loss of ThrRS might lead to activation of the unfolded protein response (UPR) pathway.[30] However, ThrRS knockdown in C2C12 cell did not affect phosphorylation of eIF2 (Figure S2), a marker of UPR activity.[31] To assess whether JNK may mediate ThrRS regulation of myogenesis, we treated C2C12 cells with the JNK inhibitor SP600125 during the first 24 h of differentiation. As shown in Figure 4B, ThrRS knockdown promoted differentiation in the presence and absence of SP600125. However, two-way ANOVA analysis revealed a significant interaction between the effects of SP600125 treatment and ThrRS knockdown ($p < .05$). Indeed, SP600125 significantly reduced the effect of ThrRS knockdown on differentiation (Figure 4B, right graph). Another JNK inhibitor, JNK-IN-8, had a similar effect (Figure S3). These results suggest that JNK signaling is an important mediator of ThrRS function even if it may not be the sole mediator.

To validate the role of JNK in ThrRS regulation of myogenesis in vivo, we again took advantage of the injury-induced muscle regeneration model. As shown in Figure 4C, administration of SP600125 completely eliminated the effect of shThrRS on day 7 AI. Collectively, our data strongly suggest that JNK is an important downstream mediator of the myogenic function of ThrRS.

3.4 | A MEKK4-MKK4-JNK pathway is downstream of ThrRS in myogenic regulation

As a MAP kinase (MAPK), JNK can be activated by multiple upstream MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs). Despite the established role of JNK

in myogenic regulation, it is not known which MAPKK or MAPKKK is the relevant activator. MKK4 and MKK7 are two MAPKKs that can directly activate JNK.[32] We asked whether ThrRS might impact the activity of MKK4 or MKK7 in muscle cells. Indeed, we observed that the phosphorylation (and thus activity) of both MKK4 and MKK7 increased upon ThrRS knockdown throughout differentiation (Figure 5A). To test whether MKK4 or MKK7 may be involved in ThrRS regulation of JNK, we first asked if either kinase regulates myogenic differentiation, which had not been reported before. Interestingly, knockdown of MKK4 decreased differentiation, whereas knockdown of MKK7 enhanced differentiation (Figure 5B). We also found that depletion of MKK7 was accompanied by up-regulation of endogenous MKK4 expression (but not vice versa) (Figure 5C), suggesting a compensatory regulation between MKK4 and MKK7. A simple model consistent with all these observations is that MKK4 has a positive role in myogenic differentiation, making it a strong candidate for mediating ThrRS function upstream of JNK. Indeed, when MKK4 was knocked down, the effects of ThrRS knockdown on differentiation (Figure 5D) and JNK activity (Figure 5E and Figure S4) were eliminated. These results suggest that MKK4 is necessary for ThrRS regulation of JNK activity and myogenic differentiation, and that MKK7 may not play any significant role in the myogenic function of ThrRS.

Next, we asked what may be upstream of MKK4 in the ThrRS pathway. Both MEKK1 and MEKK4 are known MAPKKKs upstream of JNK. MEKK1 was reported to suppress myogenic differentiation,[33] which would not be consistent with the positive role of JNK signaling in myogenesis, whereas a function of MEKK4 in muscle cells was not yet known. Therefore, we set out to examine the potential involvement of MEKK4. We found that MEKK4 knockdown in C2C12 cells decreased JNK phosphorylation (Figure 6A) and reduced fusion index (Figure 6B), indicating a positive role of MEKK4 in myogenic differentiation. Moreover, even though ThrRS knockdown still promoted differentiation in the presence of MEKK4 knockdown, two-way ANOVA analysis revealed a significant interaction between the effects of ThrRS knockdown and MEKK4 knockdown ($p < .05$) (Figure 6C, left graph). Indeed, MEKK4 knockdown significantly reduced the effect of ThrRS knockdown on differentiation (Figure 6C, right graph). Taken together, our observations suggest that ThrRS negatively regulates myogenic differentiation at least in part by inhibiting the MEKK4-MKK4-JNK pathway.

3.5 | ThrRS regulates MEKK4 by interacting with Axin1

We were interested in a more detailed mechanism by which ThrRS regulates the JNK pathway. We tested whether ThrRS physically interacted with any component or regulator of this pathway by performing co-immunoprecipitation (co-IP) in HEK293T cells, but did not find ThrRS to interact with JNK or MKK4. Axin1 was reported to bind and activate MEKK4,[34] and we wondered whether ThrRS might interact with Axin1. Indeed, we observed that a recombinant ThrRS interacted with the endogenous Axin1 in co-IP, and a recombinant Axin1 also interacted with endogenous ThrRS (Figure 7A). This interaction was observed in C2C12 cells as well (Figure S5). Furthermore, we found that the new domains of ThrRS are necessary and sufficient for the interaction with Axin1 (Figure 7B), in full agreement with the myogenic role of the new domains (see Figure 3C). As shown in Figure 7C, we also confirmed the reported interaction between Axin1 and

MEKK4. Therefore, we hypothesized that ThrRS binding to Axin1 might inhibit MEKK4 by disrupting the interaction between Axin1 and MEKK4. To test this hypothesis, we overexpressed ThrRS and found a reduced amount of Axin1 associated with MEKK4 (Figure 7D). Conversely, ThrRS knockdown enhanced MEKK4-Axin1 interaction (Figure 7E). Furthermore, overexpression of Axin1 not only enhanced JNK phosphorylation but also rendered it insensitive to the inhibitory effect of recombinant ThrRS expression (Figure 7F). Taken together, these observations are consistent with a model where ThrRS directly inhibits Axin1 activation of the MEKK4-MKK4-JNK pathway (Figure 7G).

4 | DISCUSSION

In this study, we have uncovered a non-translational function of ThrRS in myogenic regulation. Two domains newly added to ThrRS in higher eukaryotes during evolution are necessary and sufficient for this non-canonical function. We also find that ThrRS regulation of myogenic differentiation is mediated by the kinase JNK. Furthermore, we have revealed the positive roles of MKK4 and MEKK4 in myogenic regulation and in mediating signal transduction from ThrRS to JNK. Finally, we show that ThrRS binds to Axin1 and disrupts Axin1 interaction with and activation of MEKK4, which in turn inhibits JNK signaling. In conclusion, ThrRS negatively regulates myogenesis via an Axin1-MEKK4-MKK4-JNK pathway (Figure 7G).

Myogenesis is a process that requires a considerable level of protein synthesis. Knockdown of ThrRS in our experiments did not impair global protein synthesis rate, suggesting that a fraction of cellular ThrRS is sufficient to support its canonical function. This is consistent with previously reported observations that protein synthesis remained intact upon knockdown of other aaRSs in various cell types.[35,36] An attractive idea is that the cellular abundance of many of these aaRSs may reflect their involvement in functions unrelated to protein synthesis. The aaRSs may utilize their canonical catalytic domains or the non-catalytic new domains to perform non-canonical functions. In the current study, we provide a striking example of an aaRS using exclusively its new domains (UNE-T and TGS) to carry out a non-translational function. Those two domains are highly conserved between the mouse and human proteins (Figure S1B), suggesting that our observations made in the mouse system can potentially be translated to humans. A recent report uncovered a non-canonical function of ThrRS as a scaffold to help assemble the translation initiation complex and facilitate translation of selected mRNAs.[22] Both the new domains and the catalytic domains of ThrRS are involved in the scaffolding, unlike the myogenic function of ThrRS which requires only its new domains. However, the shared use of the new domains could potentially connect the two mechanisms. Interestingly, a naturally occurring ThrRS splice variant encodes an N-terminal fragment of the protein that encompasses most of the new domains.[37] This fragment, if expressed in muscle cells, could compete with full-length ThrRS to disrupt the translation of selective mRNAs, and potentially influence the synthesis of myogenic proteins. It will be interesting to probe this possibility in future studies.

Axin1 is a scaffold protein in the canonical Wnt signaling pathway, critical for β-catenin down-regulation in the absence of Wnt ligands by assembling adenomatous polyposis coli, Gsk-3β, casein kinase, and β-catenin.[38] Canonical Wnt activation of the Frizzled receptors,

leading to the stabilization and nuclear translocation of β-catenin, regulates satellite cell differentiation during adult muscle regeneration.[39] Axin1 together with Axin2 has been reported to maintain satellite cell proliferation and prevent precocious differentiation.[40] Independent of its role in Wnt signaling, Axin1 activates JNK signaling by interacting with MEKK1 and MEKK4 via distinct domains.[34,41] We did not detect any change in the activity of a β-catenin reporter upon ThrRS knockdown in either C2C12 or HEK293T cells (Figure S6), suggesting that the ThrRS-Axin1 interaction may regulate only JNK signaling and not canonical Wnt signaling. One potential possibility is that ThrRS may specifically interact with the MEKK4 binding site on Axin1.

We find ThrRS knockdown to result in an increase of regenerating TA myofiber CSA by ~18% in an injury-induced mouse model. This degree of enhancement is remarkable. As a reference, the knockout of myostatin, a well-known endogenous inhibitor of muscle growth, led to a 14% increase of TA muscle CSA.[42] The effect of ThrRS knockdown on regeneration is significant on day 7 but not on day 14 after injury, possibly because the knockdown was not persistent. This is similar to the regeneration phenotype of LeuRS knockdown.[36] Alternatively, ThrRS knockdown may accelerate the regeneration process without affecting the ultimate regenerative capacity. Whether or how ThrRS may be regulated during myogenesis is not known. The protein levels of ThrRS remain unchanged during myoblast differentiation. Nevertheless, it may be informative to examine ThrRS expression levels in muscular diseases in future studies. Elevated ThrRS expression has been found to correlate with angiogenic markers in ovarian cancer and late stage of the disease,[30] although a link between ThrRS and any pathological mechanism has not been reported. ThrRS is also one of the eight autoantigens in the antisynthetase syndrome, characterized by multiple organ involvement and often associated with inflammatory myopathies.[43] Although the role of ThrRS or its autoantibody in the pathology is yet to be established, the myogenic function of ThrRS discovered in our study could have a potential connection to those diseases, as increased autoantigen expression in regenerating muscles has been well documented (e.g., Ref. [44]).

Previously, we reported that LeuRS plays a negative role in myoblast differentiation and muscle regeneration.[36] Despite the similar phenotypes and both proteins being in the family of aaRS, the noncanonical functions of ThrRS and LeuRS in myogenesis are unrelated, mediated by two independent signaling pathways—JNK and mTORC1, respectively. It is fascinating that nature has made use of these house-keeping proteins in diverse ways. Whether other aaRSs may also be involved in myogenic regulation and whether there is an evolutionary advantage for the utilization of aaRSs as myogenic regulators are intriguing questions to be explored in the future. Regardless, our findings to date reveal potential opportunities of targeting non-canonical functions of the house-keeping aaRSs for therapeutic development against muscular diseases involving regenerative defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

aaRS	aminoacyl-tRNA synthetase
GlyRS	glycyl-tRNA synthetase
IP	immunoprecipitation
LeuRS	leucyl-tRNA synthetase
TA	tibialis anterior
TGS	ThrRS, GTPase, and SpoT
ThrRS	threonyl-tRNA synthetase

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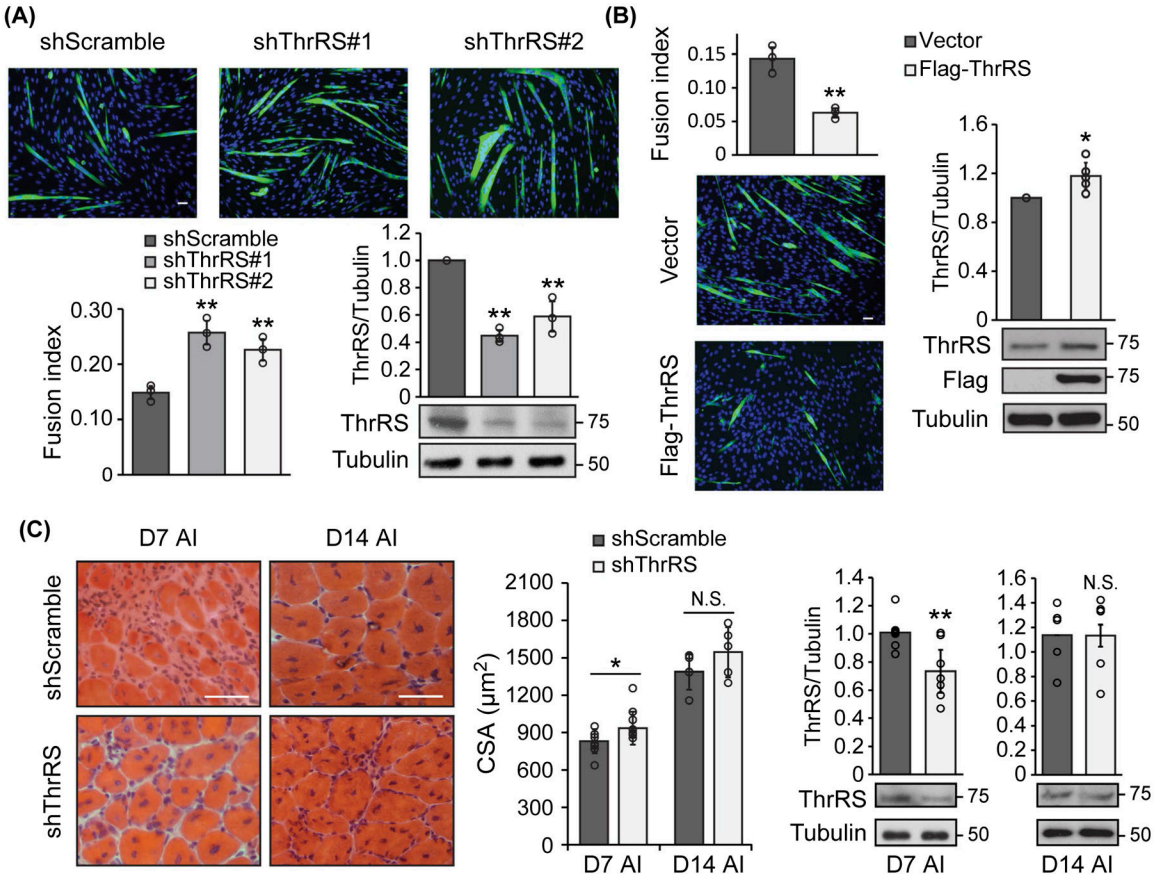

FIGURE 1.

ThrRS negatively regulates myogenesis. (A) C2C12 myoblasts were transduced with lentiviruses expressing shThrRS or shScramble as negative control and selected by puromycin, followed by differentiation for 3 days. Representative images of myotubes (green: MHC, blue: DAPI) and quantification of fusion index are shown, along with ThrRS knockdown levels quantified from western blotting ($n = 3$). (B) C2C12 myoblasts were transfected with Flag-ThrRS or empty vector control, followed by 3-day differentiation. Data are presented as in A ($n = 3$). (C) Mouse TA muscles were co-injected with $BaCl_2$ and lentiviruses expressing shThrRS or shScramble. Injected muscles were isolated on day 7 or day 14 AI and subjected to cryosection and preparation of homogenates. Upon H&E staining, regenerating myofiber CSA was quantified ($n = 8$ for D7AI, $n = 5$ for D14AI). Muscle homogenates were subjected to western blotting and quantification for relative levels of ThrRS protein. Molecular weight markers in kDa are indicated on all western blots. All quantitative data are presented as mean ± SEM. Two-tailed paired *t* test was performed to compare shThrRS to shScramble. Scale bars: 50 μm. $*p < .05$, $**p < .01$. NS: not significant

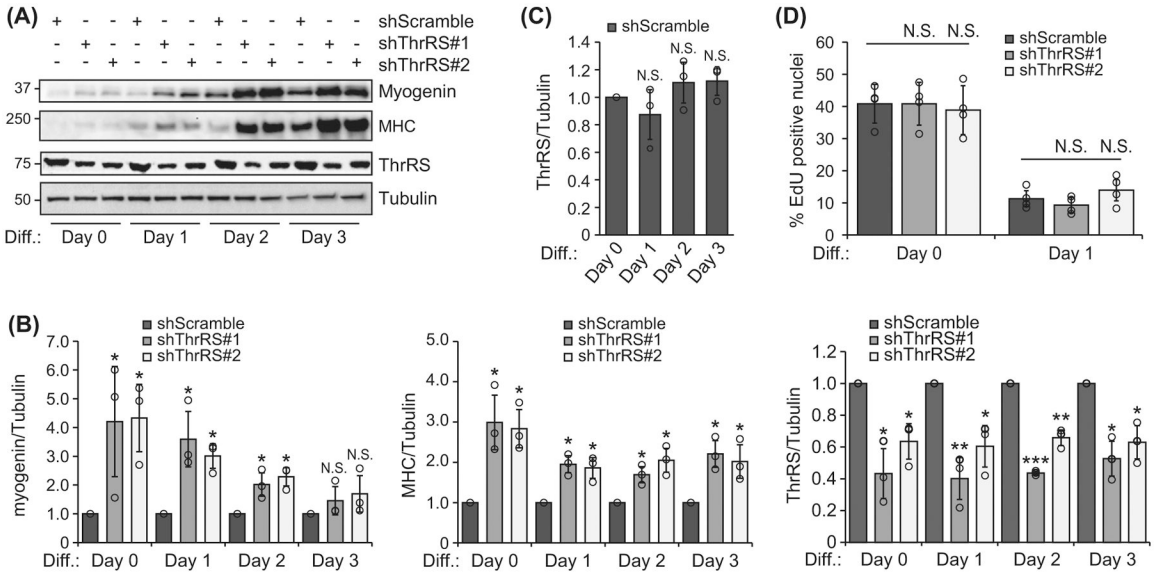

FIGURE 2.

ThrRS may regulate an early stage of myogenic differentiation but not cell cycle withdrawal. (A) C2C12 myoblasts were transduced with lentiviruses expressing shThrRS or shScramble as negative control and selected by puromycin, followed by differentiation for 3 days. Cell lysates were made on various days of differentiation followed by western blotting, and representative data from three independent experiments are shown. (B) Western blots in A were quantified for levels of myogenin, MHC, and ThrRS relative to shScramble at each time point ($n = 3$). Molecular weight markers in kDa are indicated on western blots. (C) Protein levels of ThrRS in shScramble cells throughout the course of differentiation were compared. (D) C2C12 myoblasts were transduced with lentiviruses and selected with puromycin, followed by differentiation. At day 0 or day 1 of differentiation the cells were subjected to EdU labeling. Percentage of cells with EdU incorporation was calculated ($n = 3$). All quantitative data are presented as mean ± SEM. Two-tailed paired *t* test was performed to compare shThrRS to shScramble at each time point. *$p < .05$, **$p < .01$, ***$p < .001$. N.S: not significant

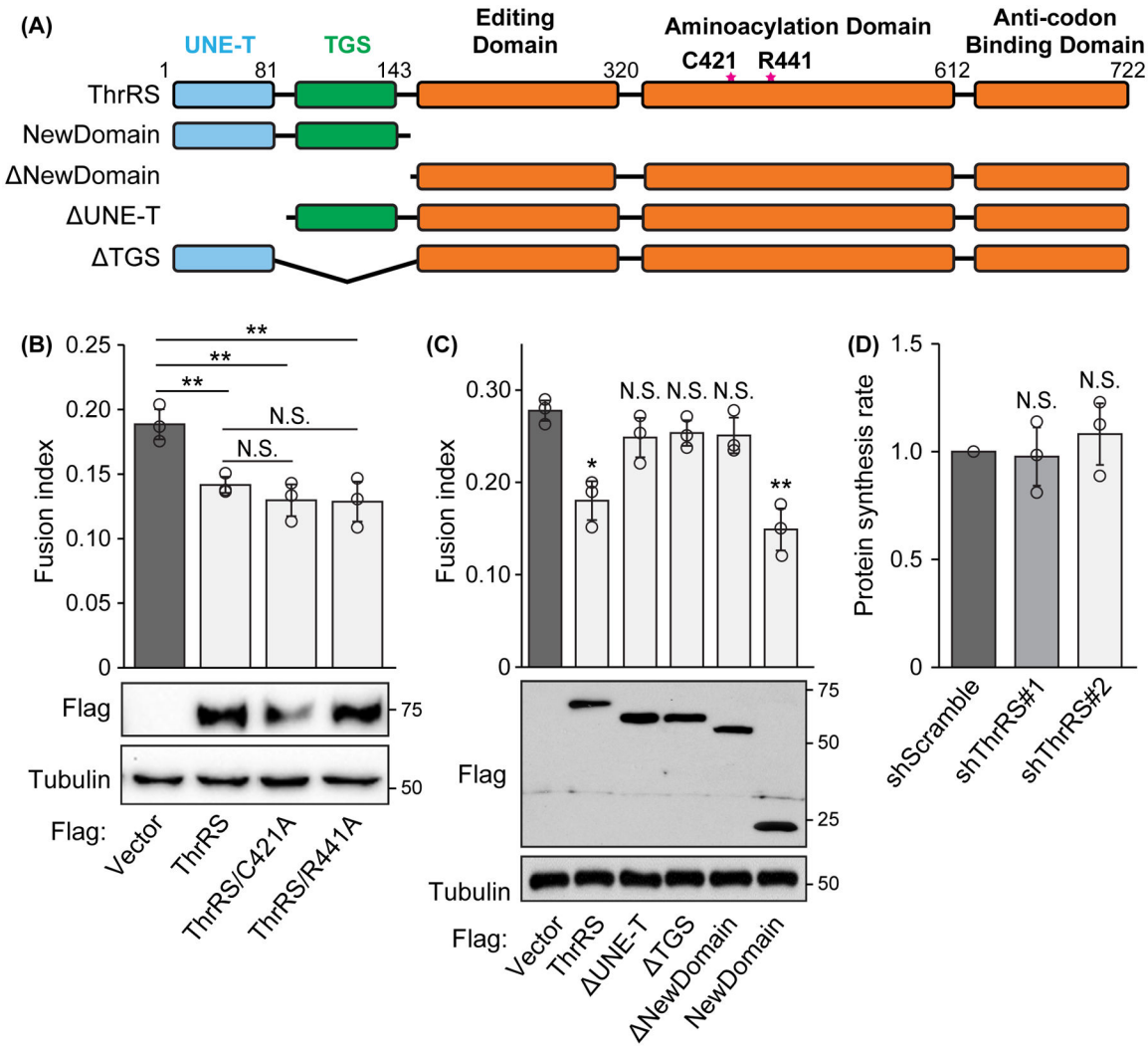

FIGURE 3.

Myogenic function of ThrRS is non-translational. (A) Schematic diagram of ThrRS showing its domains and two point-mutations that lead to loss of aminoacylation activity. (B,C) C2C12 myoblasts were transfected with WT or mutant ThrRS (B) or various fragments of ThrRS (C), followed by G418-selection for 2 days and then differentiation for 3 days and measurement of fusion index ($n = 3$). Protein expression is shown by representative western blots. Molecular weight markers in kDa are indicated on western blots. (D) C2C12 cells were transduced with lentiviruses expressing shThrRS or shScramble, followed by puromycin-selection for 2 days. Confluent cells were subjected to measurement of protein synthesis rate by ^{35}S-Met/Cys metabolic labeling ($n = 3$). All quantitative data are presented as mean ± SEM. For B, one-way ANOVA analysis was performed, followed by Student–Newman–Keuls post hoc test for multiple comparisons. For C and D, two-tailed paired *t* test was performed to compare data to the first data point in each graph. $*p < .05$, $**p < .01$. N.S: not significant

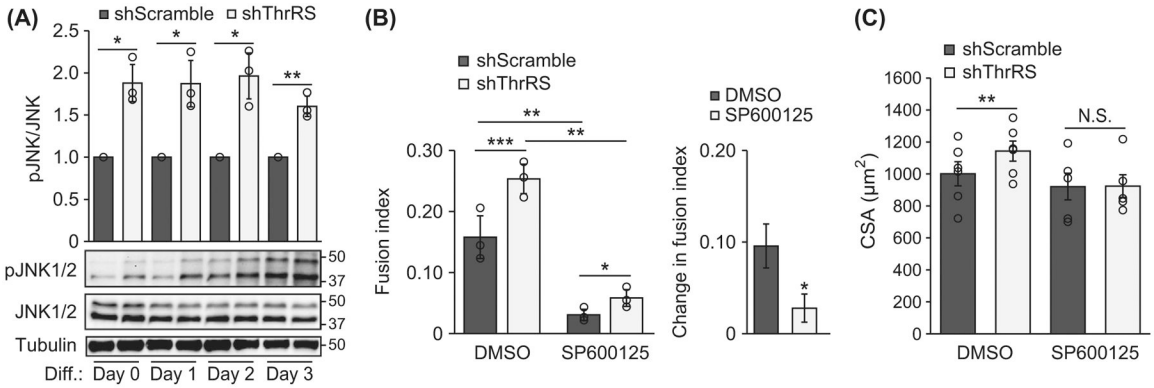

FIGURE 4.

ThrRS regulation of myogenesis is mediated by JNK. (A) C2C12 myoblasts were transduced with lentiviruses expressing shThrRS or shScramble and selected by puromycin, followed by differentiation for 3 days. Cell lysates were made on various days of differentiation followed by western blotting and quantification of relative pJNK levels (n = 3). Molecular weight markers in kDa are indicated on western blots. (B) C2C12 myoblasts were treated as in A, with 5 μM SP600125 or DMSO as vehicle control present during the first 24 h of differentiation. Differentiation was quantified by fusion index (n = 3). The change in fusion index upon ThrRS knockdown was compared between DMSO and SP600125 treatment (right graph). (C) Mouse TA muscles were co-injected with $BaCl_2$ and lentiviruses expressing shThrRS or shScramble, followed by daily intraperitoneal injection of SP600125 or DMSO. Injected muscles were isolated on day 7 AI and subjected to cryosection. Upon H&E staining, regenerating myofiber CSA was quantified (n = 6 for DMSO, n = 5 for SP600125). All quantitative data are presented as mean ± SEM. Two-tailed paired t test was performed to compare data in A and the right graph in B. For data in B (left graph) and C, two-way ANOVA analysis was performed, followed by Student–Newman–Keuls post hoc test for multiple comparisons. *p < .05, **p < .01, ***p < .001. N.S: not significant

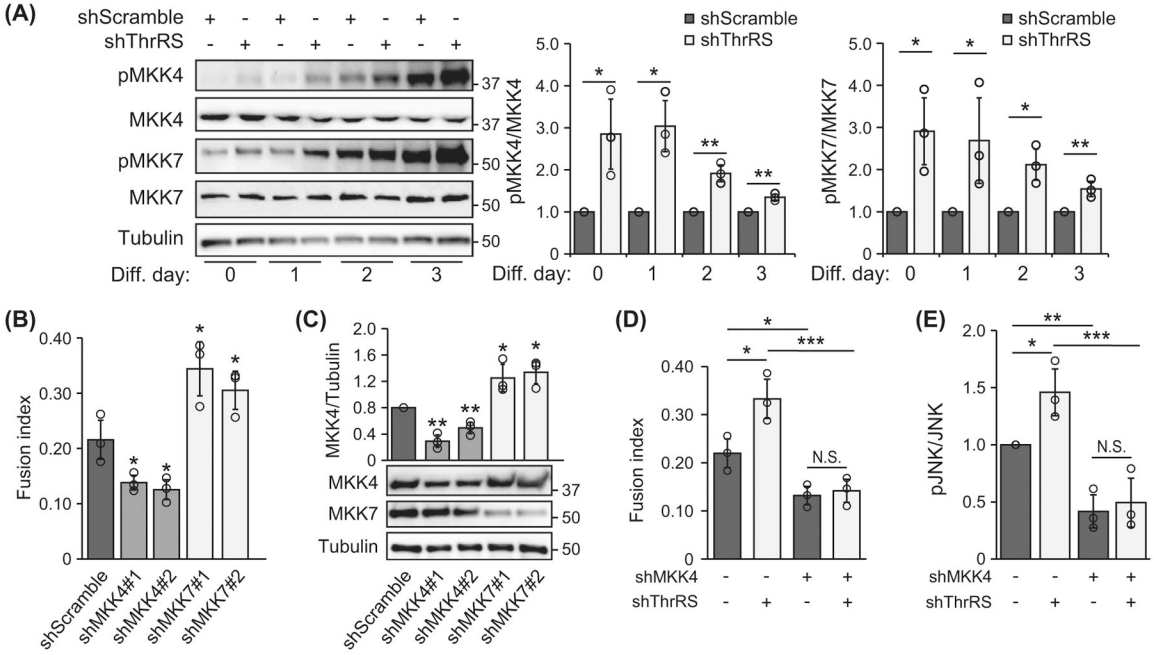

FIGURE 5.

MKK4 mediates ThrRS regulation of JNK and myogenesis. (A) C2C12 myoblasts were transduced with lentiviruses expressing shThrRS or shScramble and selected by puromycin, followed by differentiation for 3 days. Cell lysates were made on various days of differentiation followed by western blotting and quantified. Levels of protein phosphorylation are shown, relative to shScramble at each time point ($n = 3$). (B-C) C2C12 myoblasts were transduced with lentiviruses expressing shMKK4, shMKK7, or shScramble and selected by puromycin, followed by differentiation for 3 days. Differentiation was quantified by fusion index (B), and protein levels were assessed by western blotting (C) ($n = 3$). (D-E) C2C12 myoblasts were transduced with lentiviruses expressing shThrRS with or without shMKK4 and selected by puromycin, followed by differentiation for 3 days. Differentiation was quantified by fusion index (D), and pJNK level was assessed by western blotting (E) ($n = 3$). Molecular weight markers in kDa are indicated on all western blots. All quantitative data are presented as mean ± SEM. Two-tailed paired *t* test was performed to compare data to control (shScramble) in A, B, and C. For data in D and E, two-way ANOVA analysis was performed, followed by Student–Newman–Keuls post hoc test for multiple comparisons. $*p < .05$, $**p < .01$, $***p < .001$. N.S: not significant

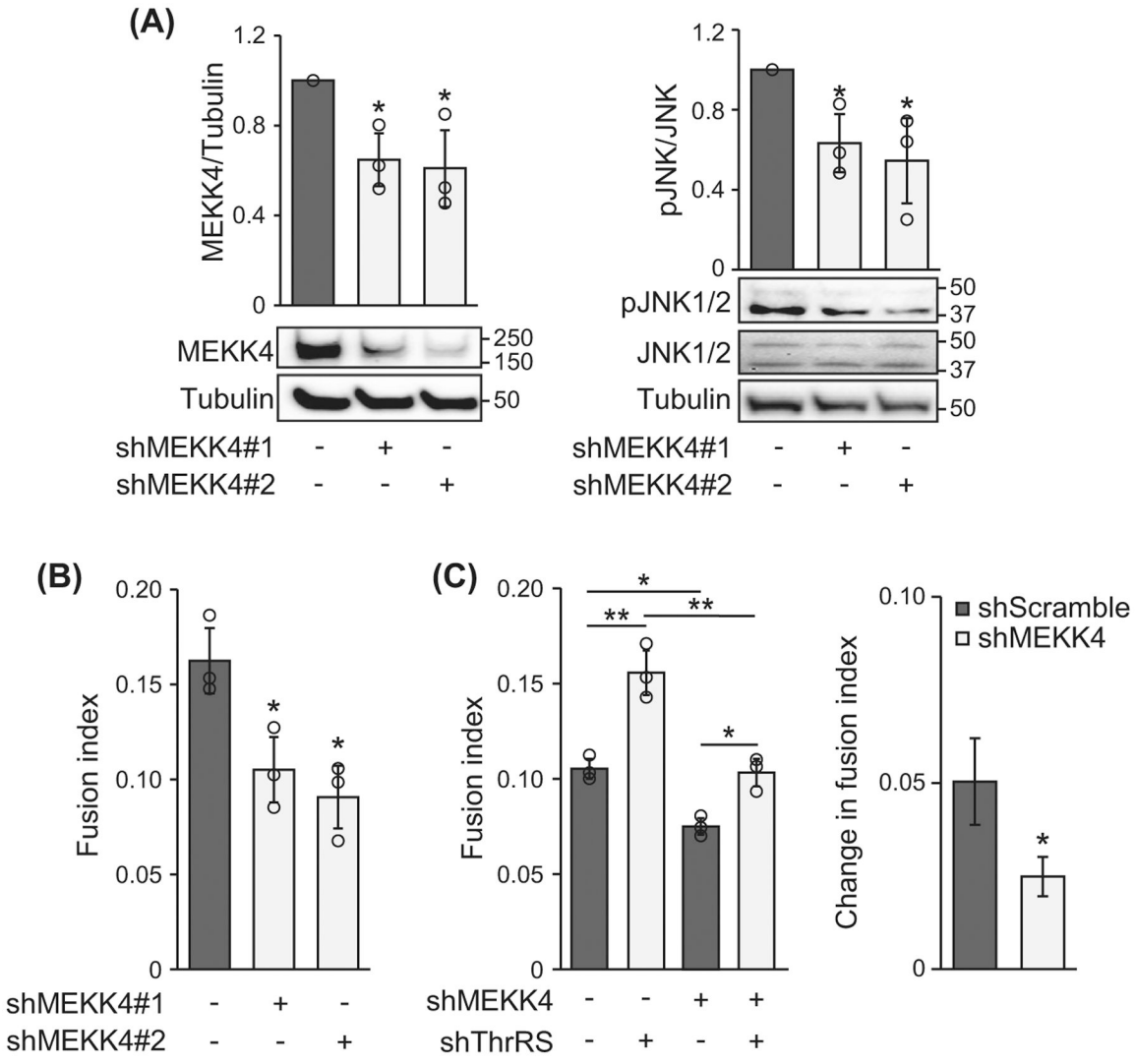

FIGURE 6.

MEKK4 mediates ThrRS regulation of JNK and myogenesis. (A-B) C2C12 myoblasts were transduced with lentiviruses expressing shMEKK4 or shScramble and selected by puromycin, followed by differentiation for 3 days. Protein expression and phosphorylation levels were assessed by western blotting (A) and differentiation was quantified by fusion index (B) ($n = 3$). Molecular weight markers in kDa are indicated on western blots. (C) C2C12 myoblasts were transduced with lentiviruses expressing shThrRS, with or without shMEKK4 and selected by puromycin, followed by differentiation for 3 days. Differentiation was quantified by fusion index. The change in fusion index upon ThrRS knockdown was compared between with and without shMEKK4 (right graph) ($n = 3$). All quantitative data are presented as mean ± SEM. Two-tailed paired *t* test was performed to compare data to control (shScramble) in A, B, and right graph of C. For data in C (left graph), two-way ANOVA analysis was performed, followed by Student–Newman–Keuls post hoc test for multiple comparisons. *$p < .05$, **$p < .01$. N.S: not significant

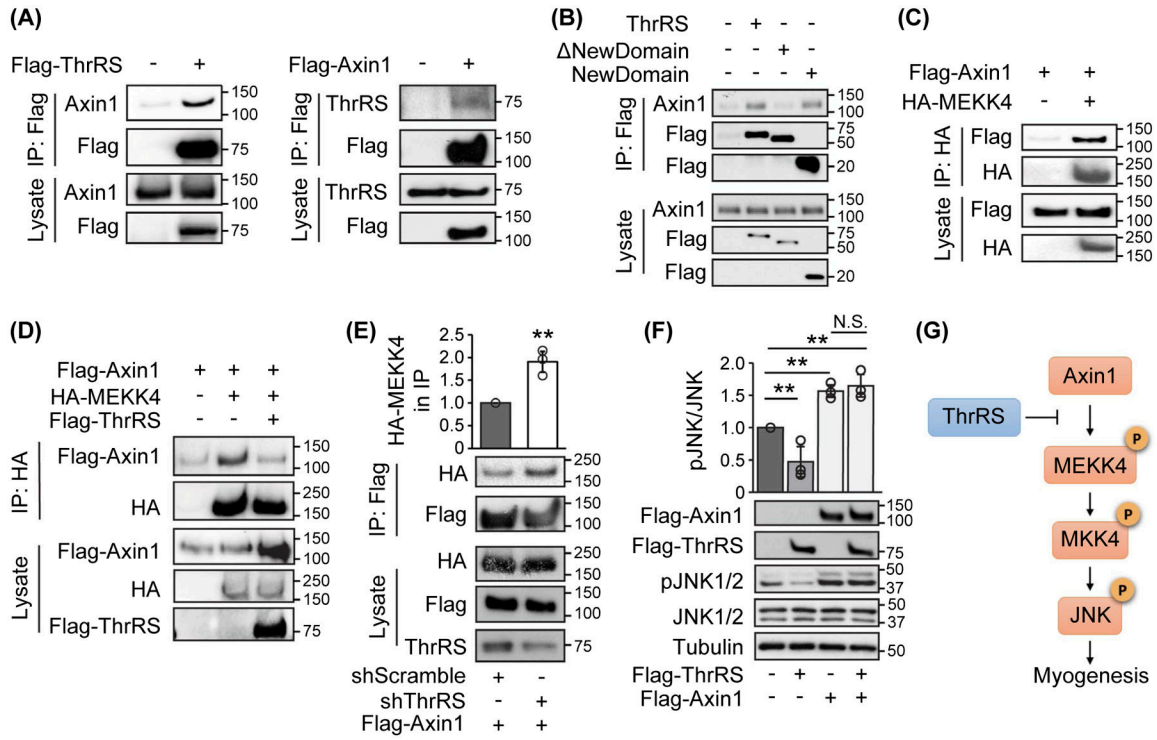

FIGURE 7.

ThrRS regulates MEKK4 by interacting with Axin1. (A) HEK293T cells were transfected to express Flag-ThrRS or Flag-Axin1with empty vector as control. Flag-ThrRS or Flag-Axin1 was pulled down by anti-Flag M2 affinity gel followed by western blotting of endogenous Axin1 or ThrRS, respectively ($n = 3$). (B) Cells were transfected with Flag-tagged WT or fragments of ThrRS with empty vector as control. IP was performed with anti-Flag M2 affinity gel, followed by western blotting of endogenous Axin1 ($n = 3$). (C) Cells were transfected to express Flag-Axin1 with or without HA-MEKK4. IP was performed with anti-HA-agarose followed by western blotting ($n = 3$). (D) Cells were transfected to express Flag-Axin1 with or without HA-MEKK4 or Flag-ThrRS. IP was performed with anti-HA-agarose followed by western blotting ($n = 3$). (E) Cells were transduced with lentiviruses expressing shThrRS or shScramble and then transfected to express Flag-Axin1 and HA-MEKK4. IP was performed with anti-Flag M2 affinity gel followed by western blotting ($n = 3$) (F) Cells were transfected to express Flag-ThrRS with or without Flag-Axin1 for 24 h, followed by cell lysis and western blot analysis of pJNK ($n = 3$). (G) A proposed model of ThrRS regulation of myogenesis. Molecular weight markers in kDa are indicated on all western blots. In E and F, quantitative data are presented as mean ± SEM. Two-tailed paired *t* test was performed to compare the data as indicated. $^{**}p < .01$. N.S: not significant