Fyn Activation of mTORC1 Stimulates the IRE1α-JNK Pathway, Leading to Cell Death*

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Background: Skeletal muscle-specific Fyn transgenic mice show severe muscle wasting phenotype concomitant with increased mTORC1 activity.

Results: Overexpression of Fyn stimulates mTORC1 and IRE1α phosphorylation, and rapamycin treatment represses Fyn- and ER stress-induced cell death.

Conclusion: Fyn plays a role in ER stress-induced cell death at least partially through regulation of mTORC1.

Significance: Our findings indicate that Fyn drives pro-apoptotic signaling by activating the unfolded protein response.

We previously reported that the skeletal muscle-specific overexpression of Fyn in mice resulted in a severe muscle wasting phenotype despite the activation of mTORC1 signaling. To investigate the bases for the loss of muscle fiber mass, we examined the relationship between Fyn activation of mTORC1, JNK, and endoplasmic reticulum stress. Overexpression of Fyn in skeletal muscle in vivo and in HEK293T cells in culture resulted in the activation of IRE1α and JNK, leading to increased cell death. Fyn synergized with the general endoplasmic reticulum stress inducer thapsigargin, resulting in the activation of IRE1α and further accelerated cell death. Moreover, inhibition of mTORC1 with rapamycin suppressed IRE1α activation and JNK phosphorylation, resulting in protecting cells against Fyn- and thapsigargin-induced cell death. Moreover, rapamycin treatment in vivo reduced the skeletal muscle IRE1α activation in the Fyn-overexpressing transgenic mice. Together, these data demonstrate the presence of a Fyn-induced endoplasmic reticulum stress that occurred, at least in part, through the activation of mTORC1, as well as subsequent activation of the IRE1α-JNK pathway driving cell death.

The endoplasmic reticulum (ER) is responsible for the folding, maturation, and trafficking of most secretory and membrane proteins, as well as for autophagosome biogenesis, glycosylation, and lipid synthesis (1). ER stress happens when the nascent protein loading exceeds the ER folding capacity under circumstances such as virus infection, gene mutation, calcium flux perturbation, or glucose deprivation, and it has been linked with diseases such as cancer, neurodegeneration, inflammation, metabolism, aging, and muscle dysfunction (2, 3). ER stress activates the unfolded protein response (UPR) to suppress general protein synthesis and increase the ER folding capacity and misfolded protein degradation, thereby restoring the cell back to homeostasis. However, if the stress response is prolonged or beyond the adaptive range, UPR may also lead to cell death (1).

The UPR is mediated by three pathways, RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (4, 5). PERK is an ER-resident type I transmembrane protein kinase. PERK phosphorylation at Ser-51 of eukaryotic translation initiation factor 2α (eIF2α) in general inhibits cap-dependent protein translation with the notable exception of activating transcription factor 4 (ATF4). As a transcription factor, ATF4 expression is activated by phosphorylation of eIF2α, which in turn promotes the transcription of CCAAT/enhancer-binding protein homologous protein (CHOP), which is important in ER stress-induced apoptosis by regulating calcium signaling and cytochrome c release from mitochondria (6). Initially located at the ER as a type II transmembrane protein, ATF6 is transferred to the Golgi, where it goes through cleavage by site-1 protease and site-2 protease. The cleaved N-terminal ATF6 fragment enters the nucleus and increases the transcription of adaptive chaperons, such as Bip. IRE1 is a type I transmembrane protein with both endoribonuclease and kinase activity. IRE1α is ubiquitously expressed, whereas the other isoform, IRE1β, is found only in the epithelial cells of gastrointestinal track (7) and airway (8). The endoribonuclease activity of IRE1α cleaves a 26-nucleotide intron from X-box-binding protein 1 (XBPI) to generate spliced XBPI (XBPIs), a potent transcription factor that participates in pro-survival response but that declines during prolonged progression of ER stress (9). IRE1α also forms a complex with the adaptor protein TNFR-associated factor 2 (TRAF2) to activate apoptosis signal-regulating kinase 1.

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‡ The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; PERK, RNA-dependent protein kinase-like ER kinase; ATF, activating transcription factor; CHOP, CCAAT/enhancer-binding protein homologous protein; IRE1, inositol-requiring enzyme 1; XBPI, X-box binding protein 1; mTOR, mechanistic target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; S6K, S6 ribosomal protein kinase; AMPK, AMP-activated protein kinase; TH, thapsigargin; TNFR, TNF receptor; KD, kinase-defective; SH, Src homology; PI, propidium iodide.
Experimental Procedures

Antibodies and Reagents—The phospho-IRE1α antibody was purchased from Novus Biologicals (Littleton, CO). Antibodies for detecting GAPDH and V5 were purchased from Marine Biological Laboratory (Woods Hole, MA), and all the other antibodies were purchased from Cell Signaling (Boston, MA). Hoechst was purchased from Life Technologies. Propidium iodide, thapsigargin, and tunicamycin were obtained from Sigma-Aldrich. Rapamycin for in vitro experiments was purchased from Sigma-Aldrich, and rapamycin for in vivo injection was purchased from LC Laboratories (Woburn, MA). JNK inhibitor was purchased from Calbiochem.

Fyn Transgenic Mice—The skeletal muscle-specific FynB transgenic and littermate control mice were generated as described previously and maintained on a C57BL6/129svj background (26). The mice were fed ad libitum with chow diet containing 20% protein and 9% fat (PicoLab Rodent Diet 20, catalog number 5058). All animal studies were approved by and performed following the guidelines from the Institutional Animal Care and Use Committee (IACUC) of Albert Einstein College of Medicine.

Cell Culture—HEK293T cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Life Technologies). Cells were incubated at 37 °C in a moisturized incubator containing 5% CO₂. Cell transfection was performed using the FuGENE HD transfection reagent according to the manufacturer’s instructions (Promega, Fitchburg, WI). Plasmids of pcDNA, pcDNA-Fyn-WT-V5, and pcDNA-Fyn-KD-V5 were constructed before (26).

Hoechst, Propidium Iodide, and TUNEL Analysis—Hoechst reagent and propidium iodide at 4 and 1 μg/ml were applied to the cells grown in 6-well plates followed by incubation at 37 °C for 5–10 min. The incorporation of label was determined by fluorescent light microscopy. TUNEL assay was applied to the cells grown on cover slips after 4% paraformaldehyde fixation, followed by using the ApopTag® fluorescein in situ apoptosis detection kit as described by the manufacturer’s instructions (Millipore, Billerica, MA).

Microscopic Analysis—The live cells were directly observed in 6-well plates under transmitted light microscopy using a Zeiss Axiovert 40c microscope with a 10 × Aplan 0.25 objective, equipped with a Canon Powershot A640 digital camera. Fluorescent images for Hoechst and propidium iodide and corresponding translight images were obtained from live cells using an Olympus 1X71 inverted microscope with a LUCPlanFLN 10 × 0.30 or 20 × 0.45 objective and exported by Olympus DP2-BSW application software. Fluorescent images of TUNEL staining were obtained using a Leica SP5 AOB5 confocal microscope with a 63 × 1.4 oil objective and exported by the Leica LAS-AF software. All images were captured at room temperature.

Immunoblotting—Cell and tissue extracts were prepared in lysis buffer as described by Cao et al. (27) containing protease inhibitor cocktail set V (Calbiochem) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). Protein concentration was mea-
sured using the BCA protein assay kit (Thermo Scientific). 

total lysates were separated in 8 or 15% SDS-PAGE overnight or 

semidry transferred to PVDF membranes at 4 °C and soaked in 

blocking buffer (GenDEPOT, Barker, TX) for 2 h at room 

temperature. The incubation of primary antibodies proceeded 

at 4 °C overnight, and the incubation of secondary antibodies 

(Thermo Scientific and LI-COR Biosciences, Lincoln, NE) 

proceeded at room temperature for 30 min before development 

using ECL or the Odyssey infrared imaging system (9140-01 

Odyssey® CLx infrared imaging system, LI-COR Biosciences). 

Semi-quantitative and Real-time Quantitative PCR—

The RNeasy mini kit (Qiagen, Valencia, CA) was used to isolate total 

RNA from cells or tissues. cDNA was generated by reverse tran-

scription using a SuperScript VILO cDNA synthesis kit (Invit-

rogen) and served as a template for semi-quantitative and quan-

titative PCR. Semi-quantitative PCR for detecting XBP1 

splicing was performed by using the GoTaq Green master mix 

(Promega) on a T1000™ thermal cycler (Bio-Rad). Primer 

sequences for XBP1 and PCR condition were described previ-

ously (9), and GAPDH was amplified as internal control 

together with the following primers: forward, 5’-ACC ACA 

GTC CAT GCC ATC AC-3’, and reverse, 5’-TCC ACC ACC CTG TTG CTG TA-3’. PCR products were resolved by 

electrophoresis on a 2.5% agarose (Bio-Rad)/Tris-acetate-EDTA 

gel. Real-time quantitative PCR reactions were run on a 

7900HT Fast Real-Time PCR system with a 384-well block 

module (Life Technologies). Gene expression of Bip, Chop, 

and Fyn was amplified by using TaqMan gene expres-

sion assays with the ΔΔCt method for quantification (Applied 

Biosystems, Branchburg, NJ). Spliced XBP1 was amplified by 

the Integrated DNA Technologies PrimeTime quantitative 

PCR assay (Integrated DNA Technologies, Coralville, IA) as 

described previously (28, 29). GAPDH was amplified in each 

experiment and served as the endogenous internal control.

Results

Fyn Overexpression in Skeletal Muscle Increases ER Stress—

Previously, we observed that transgenic mice overexpressing 

Fyn in skeletal muscle resulted in a marked increase of 

mTORC1 activation (26), which is important in regulation of 

ER stress-induced cell death (23, 24). To investigate the corre-

lation between Fyn- and ER stress-induced cell death, in which 

the latter could be responsible for the muscle wasting pheno-

type of SKM-Fyn mice, we examined the effect of Fyn on the 

indicators of three UPR pathways. Western blot analyses of 

SKM-Fyn mice demonstrated a marked increase in IRE1α-

phosphorylation (Fig. 1A). The increased IRE1α phosphor-

ylation was associated with the increase in XBP1 splicing and 

JNK phosphorylation (Fig. 1, A and C). In parallel, the downstream targets of ATF6 and PERK stimulation 

(Bip and Chop mRNA, respectively) were also elevated, indi-

cating that all three arms of the UPR were activated (Fig. 1C).

Generation of Stable Knockdown Cell Lines—The stable cell 

line with endogenous IRE1α knockdown was achieved by len-

tivirus infection using a pLKO.1 vector containing IRE1α-tar-

geting shRNA (TRCN0000000530). Control cells were infected 

with lentivirus containing the empty vector (pLKO.1). Virus 
generation and cell infection were performed following the proto-

col from Addgene. Puromycin (2.5 μg/ml) was applied after 

24 h of infection for cell selection of positive infection.

Quantification and Statistical Analysis—The numbers of the 

fluorescent puncta from microscopy analysis were counted by 

using the ImageJ software (National Institutes of Health, 

Bethesda, MD). All the results present were representatives 

from at least three repeats, and quantified data were present as 

mean ± S.E. Significant data were defined as p < 0.05 using 

analysis of variance followed by the Tukey’s multiple compari-

son or Student’s t test.
Thr-389 is the mTORC1-specific site of S6K phosphorylation, which is the major event promoting S6K activation (30). Consistent with our previous results, Fyn overexpression also led to the mTORC1 activation (26), shown by the increased phosphorylation of S6K at Thr-389 and its downstream S6 phosphorylation (Fig. 1A). 4EBP1 is another direct mTORC1 substrate that is phosphorylated on multiple sites, represented by three bands (H9251, H9252, H9253) in SDS-polyacrylamide gels (31, 32). In accordance with increased S6K phosphorylation, SKM-Fyn transgenic mice also demonstrated increased H9252 and H9253 bands with decreased H9251 band indicative of mTORC1 activation (Fig. 1B).

**Fyn Overexpression Activates mTORC1 and IRE1α in HEK293T Cells in Culture**—To examine the role of Fyn in mediating ER stress in a more experimentally tractable system, we transfected wild type Fyn (Fyn WT) and a kinase-defective Fyn mutant (Fyn KD) in HEK293T cells. Expression of Fyn WT increased IRE1α and JNK phosphorylation as well as that of the mTORC1 substrate S6K (Fig. 2A). In contrast, expression of Fyn KD had no significant effect (Fig. 2A). Neither expression of Fyn WT nor expression of Fyn KD had any statistically significant effect on Bip or Chop mRNA levels (Fig. 2, B and C). These data indicate that the effect of Fyn overexpression on IRE1α phosphorylation in mice is recapitulated in acute Fyn-transfected HEK293T cells. However, the lack of effect on Bip or Chop probably reflects the additional presence of ER stress inducers in vivo that are not present in tissue culture. To address this possibility, HEK293T cells were transfected with and without the Fyn and subsequently treated with TG, a well established ER stress inducer (33). As expected, TG induced both Bip and Chop mRNA that were not further increased by the expression of Fyn WT or Fyn KD (Fig. 3, A and B). Although TG treatment also increased XBP1 splicing, this response was further enhanced by the expression of Fyn WT (Fig. 3, C and D). These data indicate that Fyn regulates the IRE1α signaling pathway of UPR.

**Fyn Overexpression Induces Cell Death in HEK293T Cells in Culture through IRE1α-JNK Pathway**—As visually apparent, acute expression of Fyn resulted in significant morphological changes that include cell shrinkage, increased rounding, and reduced adherence to the substratum (Fig. 4A). In contrast, expression of Fyn KD had no significant morphological features when compared with empty vector control-transfected cells. To assess whether the Fyn WT-induced morphology was associated with cell death, the cells were treated with Hoechst to visualize condensed nuclei and propidium iodide (PI) to assess cell permeability. Hoechst staining demonstrated a large increase in condensed nuclei cells, and PI demonstrated a large increase in permeable cells following acute Fyn WT transfection. Quantification of the PI
staining is shown in Fig. 4. The Fyn WT- but not Fyn KD-induced cell death was further confirmed by TUNEL staining (Fig. 5, A and B).

To assess the IRE1α dependence of Fyn-induced cell death, we generated a stable cell line with IRE1α deficiency by infection with a lentivirus shRNA and a control cell line infected...
with the empty lentiviral vector (pLKO.1). PI staining demonstrated a lower degree of Fyn-induced cell death in the IRE1α knockdown cells (Fig. 6, A and B).

JNK phosphorylation is a downstream consequence of IRE1α activation leading to cell death through phosphorylation of Bcl-2 family members (6). Consistent with the IRE1α dependence of JNK phosphorylation, knockdown of IREα1 reduced the extent of JNK phosphorylation that occurs in Fyn-overexpressing cells (Fig. 6C). Treatment of Fyn-transfected cells with a JNK inhibitor resulted in fewer condensed nuclei and PI-positive stained cells (Fig. 7, A and B). Together these data indicate that the Fyn-induced cell death occurs at least partially through activation of the IRE1α-JNK pathway.

Thapsigargin Potentiates Cell Death Induced by Fyn Overexpression—To examine the effects of general ER stress with Fyn, we next examined cell death by TG in cells overexpressing Fyn. As observed previously, following transfection with Fyn WT but not Fyn KD, there were obvious visual morphological changes indicative of cell death initiation (Fig. 8). Treatment of control cells with TG also induced some morphological changes that were not as visually apparent when compared with that observed in the Fyn WT-transfected cells. In any case, the combination of TG treatment in cells overexpressing Fyn WT displayed a large increase in cell detachment with the remaining cells highly rounded, indicative of cell death.

As observed previously, expression of Fyn WT increased IRE1α and JNK phosphorylation along with increased phosphorylation of the mTORC1 downstream targets, S6K and S6 (Fig. 9A). Although TG treatment also activated these pathways, it was not as strong as Fyn WT and was further potentiated in the cells overexpressing Fyn WT treated with TG. Similarly, TG induced XBP1 splicing that was enhanced in cells overexpressing Fyn WT (Fig. 9B). Previous studies have reported that inhibition of mTORC1 with rapamycin can suppress the ER stress response (23, 24). Consistent with these studies, we also observed that rapamycin suppressed the phosphorylation of IRE1α and JNK as well as the expected mTORC1 target substrates (Fig. 9A). The inhibition of IRE1α activation was also observed by the inhibition of XBP1 splicing (Fig. 9B). However, rapamycin did not significantly inhibit the induction of Bip and Chop mRNA, further supporting a specific effect of Fyn on the IRE1α pathway through the activation of mTORC1 (Fig. 9C).

As shown in Fig. 8, thapsigargin treatment of Fyn WT-transfected cells resulted in a substantial detachment of cells characteristic of cell death. However, rapamycin treatment completely protected against the Fyn WT-induced loss of cells (Fig. 10). Moreover, rapamycin treatment of the SKM-Fyn transgenic mice in vivo not only inhibited the Fyn-induced activation of mTORC1 (S6 phosphorylation) but also suppressed IRE1α and JNK phosphorylation (Fig. 11A) and resulted in a concomitant decrease of XBP1s, Bip, and Chop mRNA levels (Fig. 11, B–D). These data are consistent with a Fyn-induced activation of mTORC1 that drives ER stress-induced cell death, at least partially through IRE1α.
Discussion

Skeletal muscle is the largest organ of mammals. It undertakes indispensable functions in locomotion, posture, breathing, and whole body metabolism (34). Calcium signaling profoundly participates in skeletal muscle physiology of contraction and pathology of muscle dystrophy (35, 36), which puts ER, the reservoir of calcium, in a more vital position in skeletal muscle than in other tissues. For example, loss of skeletal muscle mass with decreased strength is a major contributor to frailty that occurs during aging (37), which is correlated with increased ER stress (38). In addition, other muscle wasting pathologies also occur during states of denervation and immobilization (disuse-related atrophy) and in states of cancer cachexia, sepsis, and diabetes (nutrient-related atrophy). Several pathways have been identified as stimulating skeletal muscle wasting in different pathophysiologic conditions including activation of the atrogin E3 ligase responsible for proteasome-mediated degradation, STAT3 activation during cancer cachexia, and inhibition of macroautophagy (26, 39–41). Previously, we observed that skeletal muscle-specific overexpression of Fyn also resulted in severe muscle wasting associated with mTORC1 activation and inhibition of AMPK activation (26). Although Fyn expression also suppressed macroautophagy, the causality relationship between mTORC1 activation and skeletal muscle wasting was not determined.

In this regard, it was shown that the loss of the tuberous sclerosis complex, an upstream inhibitor of mTORC1, induced the ER unfolded response to activate apoptosis (24). As mTORC1 integrates macroautophagy (42) and ER stress signals, we speculated that Fyn activation of mTORC1 would contribute to ER stress induction mediating UPR and skeletal muscle wasting. To test this hypothesis, we examined the ER stress responses in skeletal muscle overexpressing Fyn and observed activation of mTORC1 along with all three branches of the UPR. Moreover, treatment of the SKM-Fyn mice with the specific mTORC1 inhibitor, rapamycin, suppressed UPR. Although Fyn expression in HEK293T cells also induced UPR, in this case, the IRE1\(\alpha\)-JNK branch was the only one activated. Several groups have observed a role for mTORC1 in activating UPR, and whether this occurs for all three branches or selectively for the IRE1\(\alpha\)-JNK pathway is still under debate (23, 43). Neverthe-
less, mTORC1 activation of UPR has been shown to induce cellular apoptosis (23). In this regard, we also observed a Fyn-dependent induction of cellular apoptosis that was suppressed by rapamycin treatment. Moreover, TG treatment increased UPR and potentiated the Fyn induction of apoptosis that was prevented by rapamycin.
It is well accepted that the IRE1α plays an important role in cell fate determination under ER stress, and IRE1α phosphorylation is required for its activity (44), including XBP1 splicing and JNK phosphorylation (23, 45). Currently, we do not know the basis for the differential effect of Fyn expression on the three UPR branches in skeletal muscle versus cultured HEK293T cells. One difference between these two systems is the timeframe of Fyn expression. In vivo, Fyn was expressed at the beginning of skeletogenesis and remained expressed during embryonic development and into adulthood. In contrast, the HEK293T cells were transiently transfected with Fyn that was expressed for only a few days. Thus, compensational crosstalk between UPR branches may be activated only under chronic or prolonged stress conditions. It is also possible that the effect of Fyn activation of mTORC1, as well as the subsequent UPR activation, occurs in a cell context-dependent manner.

In addition, rapamycin as an effective inhibitor of mTORC1 was only partially effective in inhibiting JNK activation in HEK293T cells. This suggests the presence of an mTORC1-independent pathway responsible for JNK activation. In this regard, the small GTP-binding protein RhoA has also been reported to promote apoptosis via JNK signaling (46).

In any case, JNK is a stress-activated kinase and a downstream target of mTORC1 activation that is associated with insulin resistance (47) and aging (34). JNK activation is also a potent activator of cellular apoptosis (48), and our data demonstrate that Fyn stimulates JNK activation site phosphorylation in vitro and in vivo. Moreover, treatment with a JNK inhibitor partially repressed Fyn-induced cell death in vitro. These findings provide strong evidence that Fyn activates the IRE1α-JNK signaling pathway and that it is the JNK activation that is the proximal event responsible for inducing cell death. Additionally, we have also observed that during aging, the skeletal muscle levels of Fyn protein are increased with increased mTORC1 activation,3 consistent with disrupted ER homeostasis in aged animals (38, 49), suggesting the involvement of a Fyn-mTORC1-IRE1α pathway-induced cell death in sarcopenia (age-related muscle atrophy).

3 Y. Wang, E. Yamada, H. Zong, and J. E. Pessin, unpublished results.
Taken together, these data indicate a novel role for Fyn as an activator of ER stress mediated through IRE1α and JNK activation. As unresolved ER stress and JNK activation are established activators of cellular apoptosis, this can account for the skeletal muscle wasting induced by Fyn expression. Future studies are now needed to molecularly determine the basis for the loss of muscle fiber protein.

FIGURE 10. Rapamycin protects against Fyn and thapsigargin-induced cell death in HEK293T cells. HEK293T cells were transfected with either the empty vector (pcDNA) or Fyn wild type (Fyn). Forty-eight hours later, the cells were treated with 1 μM TG with or without 100 nm rapamycin for 32 h. Representative transmitted light microscopy images are presented from three independent experiments.

FIGURE 11. Rapamycin suppresses mTORC1, IRE1α, and JNK activation in SKM-Fyn mice. Three-week-old wild type (WT) and SKM-Fyn transgenic (Tg) mice were intraperitoneally injected with vehicle or rapamycin (2 mg/kg) once a day for 4 days. The mice were then fasted overnight, and gastrocnemius muscles were isolated. A, extracts were prepared and subjected to Western blotting against the proteins indicated. These are representative immunoblots independently performed four times. p indicates phosphorylated form. B–D, extracts were prepared, and the levels of spliced XBP1 (XBP1s), Bip, and Chop mRNA were determined by quantitative RT-PCR. The data are presented as mean ± S.E. from four independent experiments. Non-identical letters (a and b) indicate results that are statistically different from each other at p < 0.05.
Fyn Activation of the mTORC1-IRE1α-JNK Pathway

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