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PI3K-mTORC1 Attenuates Stress Response by Inhibiting Cap-independent Hsp70 Translation*[S]

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Jun Sun[‡1], **Crystal S. Conn**[§1], **Yan Han**[‡], **Vincent Yeung**[‡], **and Shu-Bing Qian**[‡§2]

From the [‡]*Division of Nutritional Sciences,* [§]*Graduate Field of Genetics and Development, Cornell University, Ithaca, New York 14853*

Protein synthesis is a key regulated cellular process that links nutrient availability and organismal growth. It has long been known that some cellular proteins continue to be synthesized under conditions where global translation is severely compromised. One prominent example is the selective translation of heat shock proteins (Hsps) under stress conditions. Although the transcriptional regulation of *Hsp* genes has been well established, neither the specific translation-promoting features nor the regulatory mechanism of the translation machinery have been clearly defined. Here we show that the stress-induced preferential translation of *Hsp70* mRNA is negatively regulated by PI3K-mTORC1 signaling. Despite the transcriptional up-regulation, the translation of *Hsp70* mRNA is deficient in cells lacking tuberous sclerosis complex 2. Conversely, Hsp70 synthesis is enhanced under the reduced PI3K-mTORC1 signaling. We found that the 5′ UTR of *Hsp70* mRNA contributes to cap-independent translation without exhibiting typical features of internal ribosome entry site. Our findings imply a plausible mechanism for how persistent PI3K-mTORC1 signaling favors the development of age-related pathologies by attenuating stress resistance.

The eukaryotic translation machinery is a tightly controlled system that regulates protein synthesis based on the availability of growth factors, nutrients, and glucose (1–3). A key pathway that integrates and responds to environmental cues involves the mammalian target of rapamycin (mTOR),[3] a member of the PIKK family of protein kinase conserved from yeast to human (4–6). Recent studies revealed the existence of two mTOR complexes, named mTORC1 and mTORC2, which differ in molecular composition and cellular functions (7–10). Insulin and insulin-like growth factors are major mTORC1 activators that operate through phosphoinositide 3-kinase (PI3K) and the protein kinase AKT (11). Conversely, mTORC1 activity is suppressed by a variety of stress conditions including limited nutrients, hypoxia, and DNA damage (12).

Activation of mTORC1 positively stimulates cap-dependent mRNA translation via its downstream substrates S6Ks and 4E-BPs (7, 8, 10, 13). S6K1 phosphorylation promotes protein synthesis and cell growth presumably by phosphorylating multiple substrates (*e.g.* ribosomal protein S6, translational regulators eIF4B and PDCD4) (1, 14). Phosphorylation of 4E-BP1 results in its dissociation from eIF4E, promoting assembly of the eIF4F complex (15). The recruitment of the eIF4F complex to the mRNA 5′ cap structure is both rate-limiting in translation initiation and is tightly regulated (16). Translation consumes a substantial amount of cellular material and energy. It is thus not surprising that global translation is reduced in response to most, if not all, types of cellular stress (2). However, some cellular proteins continue to be synthesized under conditions where global translation is severely compromised, such as during virus infection, stress, and mitosis (17, 18).

Heat shock proteins (Hsps) are known to protect cells against a wide variety of stresses (19–21). Therefore, the regulation of Hsp production is crucial for cell survival. In mammalian cells, heat shock transcription factor 1 (HSF1) is the major transcription regulator of *Hsp* gene expression (22–24). HSF1 binding to the heat shock elements results in a rapid increase in the rate of transcription (up to ~200-fold) (25). In addition to the up-regulation of *Hsp70* gene transcription, the *Hsp70* mRNA is also robustly translated under stress conditions despite the slowing of global protein synthesis (26, 27). However, neither the specific translation-promoting features of the *Hsp70* mRNA nor the regulatory mechanism of the translation machinery have been clearly defined.

Persistent mTORC1 activation is associated with diverse pathologies such as inflammation, cancer, and diabetes (28). Conversely, inhibition of mTORC1 prolongs lifespan and increases quality of life by reducing the incidence of age-related pathologies (29–32). It has been suggested that the general reduction of protein synthesis lowers the cellular load of erroneously synthesized polypeptides. This situation results in "spare" chaperone function, which may contribute to the observed increase in organism stress resistance and lifespan (33). In contrast, constitutive active mTOR signaling might increase the burden of chaperone molecules by producing more misfolded proteins. Consistent with this notion, a recent study reported that hyperactive mTOR signaling triggered the unfolded protein response in the endoplasmic reticulum (34). However, it is unclear whether unrestrained mTORC1 activation also triggers cytosolic stress response.

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[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: 301 Biotech, Cornell University, Ithaca, NY 14853. Tel.: 607-254-3397; Fax: 607-255-6249; E-mail: sq38@cornell.edu.
[3] The abbreviations used are: mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; S6K1, p70 S6 kinase 1; 4E-BP, eIF-4E binding protein; HSF1, heat shock transcription factor 1; TSC, tuberous sclerosis complex; F-Luc, firefly luciferase; R-Luc, *Renilla* luciferase; IRES, internal ribosome entry site; MEF, mouse embryonic fibroblast.

Here we report our findings that the stress-induced *Hsp70* mRNA translation is deficient in cells with hyperactive mTORC1 activities. Interestingly, although the 5′ UTR of *Hsp70* mRNA contributes to the cap-independent translation, it does not behave as the viral IRES. Our results not only reveal novel aspects of cap-independent translation, but also imply a plausible mechanism about how persistent PI3K-mTORC1 signaling favors the development of age-related pathologies by attenuating stress resistance.

EXPERIMENTAL PROCEDURES

Cells and Reagents—TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs were kindly provided by Dr. David J. Kwiatkowski (Harvard Medical School) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The polio IRES luciferase construct was a generous gift from Peter Bitterman (University of Minnesota). The plasmid expressing Rheb was kindly provided by Dr. Kun-Liang Guan (University of California at San Diego). Rapamycin and LY294002 were purchased from Sigma. Anti-Hsp70 (SPA-810), anti-Hsp90α (SPA-830), anti-Hsp25 (SPA-801), and anti-Hsp40 (SPA-400) antibodies were purchased from Stressgen; antibodies for phosphorylated and total S6K1, 4E-BP1, Raptor, and Rictor from Cell Signaling. siRNA targeting Raptor and Rictor were purchased from Santa Cruz.

Plasmids—The 5′ UTR of mouse *Hsp70* were amplified by RT-PCR using total RNA extracted from TSC2$^{-/-}$ MEFs. The Hsp70 5′ UTR was cloned into HindIII and BamHI sites of pcDNA3.1 (Invitrogen). The firefly luciferase gene was directly removed from pCDNA3-rLuc-polIRES-fLuc into the pcDNA3.1/Hsp70 5′ UTR using BamHI and XbaI restriction sites. For IRES constructs containing the Hsp70 5′ UTR, the polIRES cassette in the bicistronic vector pcDNA3/rLuc-polIRES-fLuc was replaced by the full-length of Hsp70 5′ UTR cloned from TSC2$^{-/-}$ MEFs (231 bp, NM_010479).

Transfections—Plasmid and siRNA transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Immunoblotting—Cells were lysed on ice in TBS buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA) containing protease inhibitor mixture tablet (Roche Applied Science) and 1% Triton X-100. After incubating on ice for 30 min, the supernatants were heated for 5 min in SDS-PAGE sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol). Proteins were resolved on SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were blocked for 1 h in TBS containing 5% blotting milk, followed by incubation with primary antibodies. After incubation with horseradish peroxidase-coupled secondary antibodies, immunoblots were developed using enhanced chemiluminescence (Amersham Biosciences).

Ribosome Profiling—Sucrose solutions were prepared in polysome gradient buffer (10 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 100 μg/ml of cycloheximide, 5 mM DTT, 20 units/ml of SUPERase_In). Sucrose density gradients (15–45% w/v) were prepared in SW41 ultracentrifuge tubes (Fisher) using a BioComp Gradient Master (BioComp Instruments) according to the manufacturer's instructions. Cells were lysed in ice-cold polysome lysis buffer (10 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 100 μg/ml of cycloheximide, 5 mM DTT, 20 units/ml of SUPERase_In, 2% Triton), about 650 μl of supernatant was loaded onto gradients, followed by centrifugation for 100 min at 38,000 × *g* at 4 °C in an SW41 rotor. Gradients were fractionated at 0.375 ml/min using a fractionation system (Isco), which continually monitored OD_{254} values. Fractions corresponding to 60-s intervals were collected.

RT-PCR and qPCR—Total RNA was extracted from whole cell lysates or fraction samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using Superscript III kit (Invitrogen). PCR was performed under the following conditions: 30 s, 94 °C; 30 s, 55 °C; 30 s, 72 °C, 22 cycles. The primer pair for the *Hsp70* gene was 5′-GCAAGGCCAACAAGATCAC-CAT-3′ and 5′-GGCGCTCTTCATGTTGAAGGC-3′. The primer pair for *β-actin* gene was 5′-TTGCTGACAGGATG-CAGAAG-3′ and 5′-ACTCCTGCTTGCTGATCCACAT-3′. For real-time PCR analysis, a SYBR Green PCR kit (Invitrogen) was used on a LightCycler 480 II Q-PCR machine (Roche Applied Science). Raw data were analyzed using the LightCycler 480 Software (version 1.5.0, Roche Applied Science).

mRNA In Vitro Transcription—mRNAs with normal m7G cap or analog ApppG were synthesized using the mMessage mMachine T7 Ultra kit (Ambion), followed by purification using the MEGAclear kit (Ambion), according to the manufacturer's instructions.

Luciferase Reporter Assay—For the non-real time luciferase assay, transfected MEFs were lysed and luciferase activity was measured using a luciferase reporter assay system (Promega) on a Synergy™ HT Multi-detection Microplate Reader (BioTek Instruments). For real time luciferase assay, cells were plated on 35-mm dishes and transfected with plasmid or mRNA containing the luciferase gene. Immediately after transfection, luciferase substrate D-luciferin (1 mM) was added into the culture medium. Luciferase activity was recorded at 37 °C with 5% CO_2 using Kronos Dio Luminometer (Atto).

Cell Viability Assays—TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs were grown to 90% confluence, followed by incubation at 45 °C for various times. Cells were then returned to 37 °C for a 20-h recovery. The cells were then counted via trypan blue staining. For the rescue experiment, MEFs were infected with recombinant adenoviruses expressing Hsp70, Hsp90, or GFP control using 20 multiplicity of infection 24 h after infection, cells were heat shocked and viability was measured via cell counting.

RESULTS

TSC2 Null Cells Are Defective in Heat Shock-induced Hsp70 Expression—TSC2 serves as a GAP for the small GTPase Rheb, which activates mTORC1 (35). Cells lacking a functional TSC-Rheb-GAP exhibit constitutive activation of mTORC1 signaling, which is not increased further by insulin. To test whether TSC deficiency activates cytosolic stress response, we used a luciferase reporter to evaluate the transcriptional activity of HSF1 in TSC2$^{-/-}$ MEFs after heat

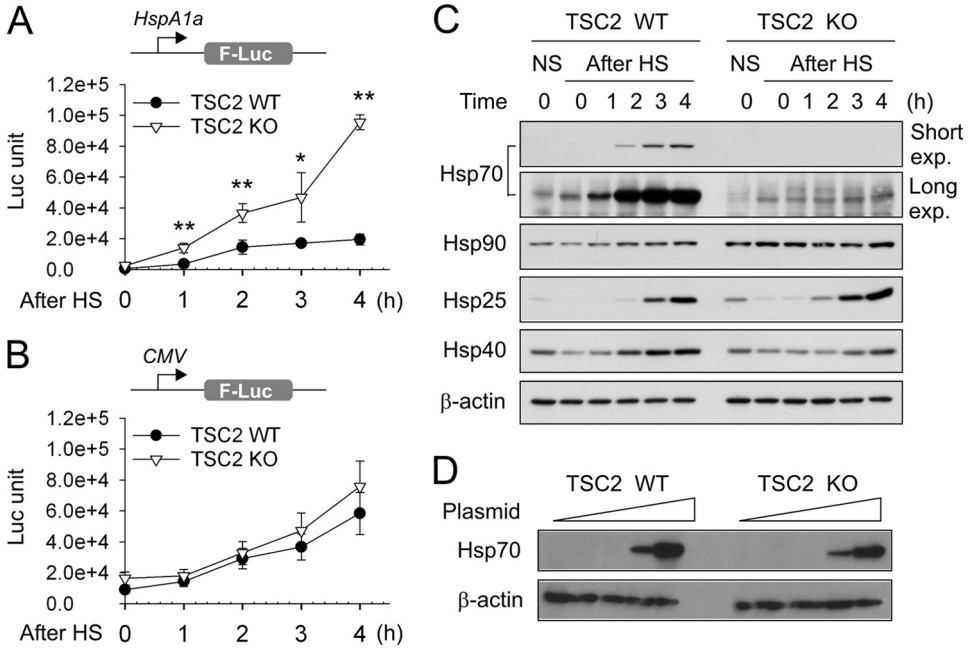

FIGURE 1. **TSC2 null cells are defective in heat shock-induced Hsp70 expression.** *A*, TSC2 wild type (*WT*) and knock-out (*KO*) cells were heat shocked at 42 °C for 1 h and recovered at 37 °C for the times as indicated. HSF1 activity was measured by using a F-Luc reporter driven by the *HspA1a* promoter. The experiments were repeated 5 times. *Error bar*, S.E. *, $p < 0.01$; **, $p < 0.001$ (Student's *t* test, two tails). *B*, general protein synthesis in cells as *A* was determined by using a control F-Luc reporter driven by the CMV promoter. The experiments were repeated 5 times. *C*, molecular chaperone levels in cells as *A* were determined by immunoblotting analysis using the antibodies as indicated. *D*, TSC2 WT and TSC2 KO cells were transfected with plasmids encoding Hsp70 with different doses (0, 0.1, 0.5, and 2.5 μg) in a 6-well plate. 24 h after transfection, whole cell lysates were immunoblotted with antibodies as indicated.

shock (36). After a 1-h incubation at 42 °C, TSC2$^{-/-}$ MEFs exhibited significantly higher HSF1 activity than did TSC2$^{+/+}$ MEFs (Fig. 1*A*). This was not due to the general increase of luciferase protein synthesis, because the control plasmid (CMV-Luc) showed no significant increase in luciferase expression in TSC2$^{-/-}$ MEFs (Fig. 1*B*).

We next examined protein levels of molecular chaperones in both MEFs after heat shock using immunoblotting. As expected, TSC2$^{+/+}$ MEFs exhibited a robust induction of Hsp70 and Hsp25 after heat shock (Fig. 1*C*). To our surprise, there was no Hsp70 induction in cells lacking the *Tsc2* gene. A closer look at the overexposed immunoblotting revealed that Hsp70 was only detectable shortly after heat shock with little accumulation in TSC2$^{-/-}$ MEFs. This deficiency was not due to the lack of *Hsp70* transcription, because the *Hsp70* mRNA levels were comparable in both MEFs as measured by real time PCR (supplemental Fig. S1). Adding proteasome inhibitor MG132 did not rescue the Hsp70 expression in TSC2$^{-/-}$ MEFs (supplemental Fig. S2), excluding the possibility that there is an accelerated Hsp70 degradation in these cells. Further supporting this notion, ectopic expression of *Hsp70* by plasmid showed no difference in both MEFs (Fig. 1*D*). Notably, the *Hsp70* gene was directly cloned from TSC2$^{-/-}$ MEF cells, excluding the possibility that there are mutations in the endogenous *Hsp70* gene. To further substantiate the role of TSC2 in stress-induced Hsp70 expression, we performed siRNA-mediated TSC2 knockdown in HeLa cells. Despite the high basal levels of Hsp70 in HeLa cells, TSC2 knockdown largely blunted the heat shock-induced Hsp70 expression (supplemental Fig. S3). Therefore, the lack of heat shock-induced Hsp70 expression in TSC2$^{-/-}$ MEFs is likely due to the deficiency of translational regulation.

Deficient Hsp70 mRNA Translation in TSC2 Null Cells after Heat Shock—To examine the translational status of *Hsp70* mRNA before and after heat shock in both MEFs, we performed ribosome sedimentation analysis. Actively translated mRNAs are distributed to polysomes, whereas inactive mRNAs are associated with monosomes (37). Under normal growth conditions, TSC2$^{-/-}$ MEFs exhibited much more polysome formation than the wild type, with a corresponding decrease of monosome peak (Fig. 2, *A* and *B*). This feature is consistent with the increased cap-dependent mRNA translation in cells lacking TSC2. To analyze the polysome localization of specific mRNAs, we performed qPCR for every ribosome fraction. As expected, *β-actin* mRNA was mainly localized in the heavier polysome fractions of both MEFs (Fig. 2, *A* and *B*, *grey bar*). *Hsp70* mRNA was barely detectable in ribosome fractions because the basal levels were low in both cells under normal conditions (Fig. 2, *A* and *B*, *black bar*).

To investigate the translational status of stress-induced *Hsp70* mRNA, we applied heat shock to both TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs. As expected, the polysome formation was largely suppressed in both cell types with higher sensitivity in TSC2$^{-/-}$ MEFs. In line with the efficient *Hsp70* mRNA translation after heat shock (26, 38), there was an enrichment of *Hsp70* message in polysome fractions of wild type MEFs. However, the ribosome fractions from TSC2$^{-/-}$ MEFs showed only basal levels of *Hsp70* mRNA, despite the total amount of the message was comparable in both MEFs (Fig. 2,

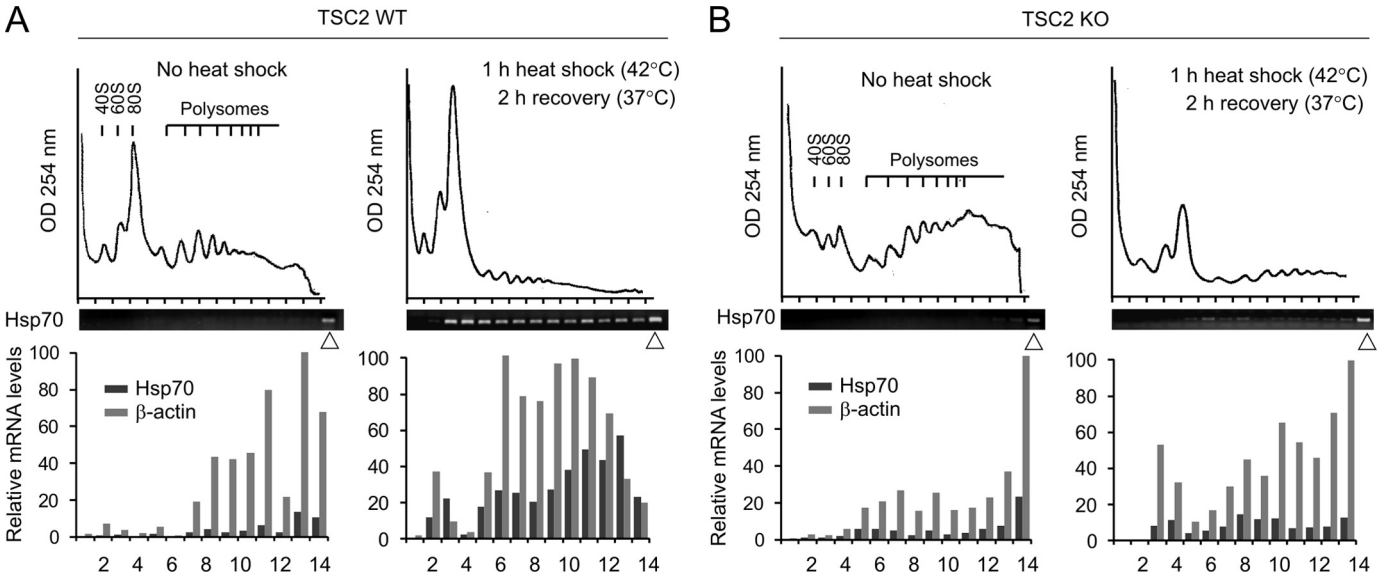

FIGURE 2. **Deficient *Hsp70* mRNA translation in TSC2 null cells after heat shock.** *A*, ribosome profiling of TSC2 WT cells before and after heat shock. Cell lysates were sedimented on a 15–45% sucrose gradient followed by fractionation. The positions of the 40 S, 60 S, 80 S, and polysomal peaks were indicated. Total RNA was extracted from each fraction and subject to RT-PCR and qPCR analysis. *Hsp70* RT-PCR results were shown in the middle without concentration normalization. The *Hsp70* mRNA levels in whole cell lysates before sucrose gradient are indicated by the *triangle*. qPCR results of *Hsp70* (*black bar*) and *β-actin* (*grey bar*) were normalized based on RNA concentration of each fraction. The highest level was arbitrarily set as 100 and the relative mRNA levels were presented in all polysome fractions. *B*, ribosome profiling of TSC2 KO cells before and after heat shock. RT-PCR and qPCR were performed as described under *A*.

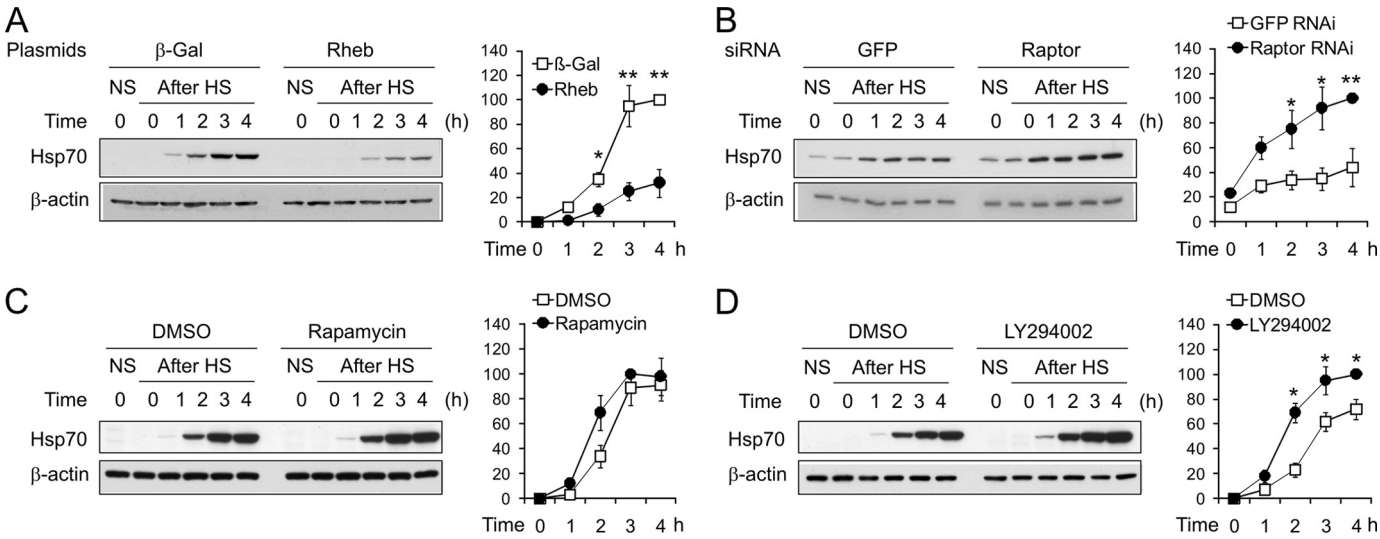

FIGURE 3. **PI3K-mTORC1 negatively regulates Hsp70 mRNA translation.** *A*, TSC2 WT cells were transfected with plasmids encoding β-Gal or Rheb. 48 h after transfection, cells were incubated at 42 °C for the times as indicated. Whole cell lysates were immunoblotted using Hsp70 and β-actin antibodies. Relative Hsp70 levels were quantitated by densitometry. $n = 3$, *error bar*, S.E.; **, $p < 0.01$; *, $p < 0.05$. *B*, TSC2 WT cells were transfected with siRNA targeting Raptor or GFP as control. 48 h after transfection, cells were incubated at 42 °C for the times as indicated. Whole cell lysates were immunoblotted using Hsp70 and β-actin antibodies. Relative Hsp70 levels were quantitated by densitometry. $n = 3$, *error bar*, S.E.; **, $p < 0.01$; *, $p < 0.05$. *C*, TSC2 WT cells were incubated at 42 °C for the times as indicated in the presence of 20 nM rapamycin or DMSO as control. Whole cell lysates were immunoblotted using Hsp70 and β-actin antibodies. Relative Hsp70 levels were quantitated by densitometry. $n = 3$, *error bar*, S.E. *D*, TSC2 WT cells were incubated at 42 °C for the times as indicated in the presence of 50 μM LY294002 or DMSO as control. Whole cell lysates were immunoblotted using Hsp70 and β-actin antibodies. Relative Hsp70 levels were quantitated by densitometry. $n = 3$, *error bar*, S.E.; *, $p < 0.05$.

triangle). Thus, *Hsp70* mRNA translation was largely deficient in TSC2 null cells, despite up-regulation of HSF1 transcriptional activity after heat shock.

PI3K-mTORC1 Negatively Regulates Hsp70 mRNA Translation—Having found the unexpected deficiency of *Hsp70* mRNA translation in cells lacking TSC2, we were interested in assessing whether altered mTORC1 signaling in general affects the translation of *Hsp70* mRNA. We first transfected cells with plasmids encoding Rheb, a direct activator of mTORC1 (39, 40). Indeed, Rheb overexpression enhanced mTORC1 signaling as evidenced by increased phosphorylation of S6K1 at Thr^{389} (supplemental Fig. S4*A*). Consistent with TSC2 null MEFs, cells overexpressing Rheb showed a significant reduction in Hsp70 expression after heat shock as compared with cells overexpressing β-Gal (Fig. 3*A*). The *Hsp70* transcript levels were indistinguishable in these

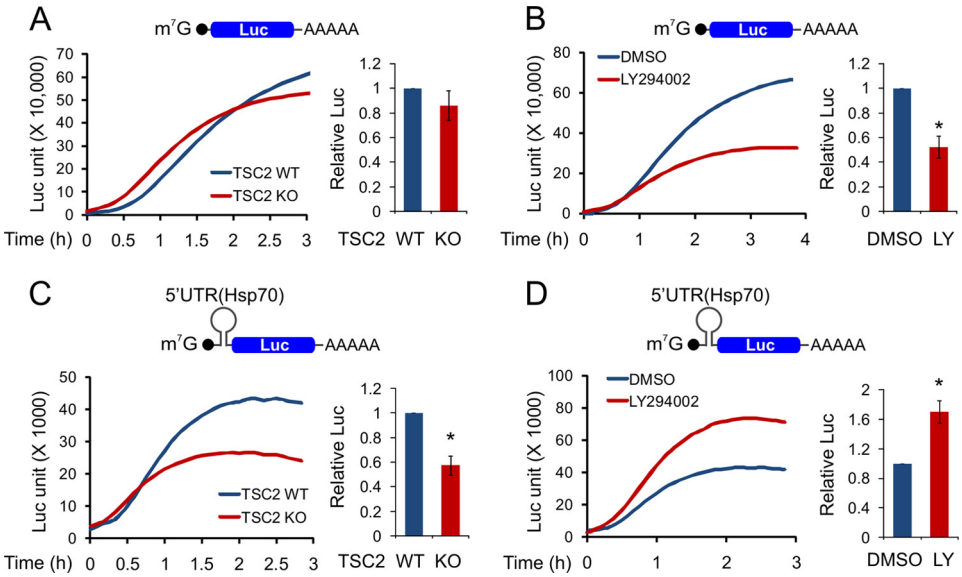

FIGURE 4. **Hsp70 5′ UTR responds to the PI3K-mTORC1 signaling.** *A*, luciferase mRNA (*Luc*) was synthesized using *in vitro* transcription followed by 5′ end capping and 3′ end polyadenylation. mRNA transfection was performed on TSC2 WT and TSC2 KO cells. Real time luciferase activity was recorded immediately after mRNA transfection (*left panel*). Relative Luc expression (3 h) in TSC2 KO cells was normalized against the wild type (*right panel*). $n = 5$, *error bar*, S.E. *B*, *Luc* mRNA transfection was performed on TSC2 WT cells treated with 50 μM LY294002 or DMSO as control. Real time luciferase activity was recorded immediately after mRNA transfection (*left panel*). Relative Luc expression (3 h) after LY294002 treatment was normalized against the DMSO control (*right panel*). $n = 5$, *error bar*, S.E. *, $p < 0.01$. *C*, *Luc* mRNA bearing the *Hsp70* 5′ UTR was synthesized using *in vitro* transcription followed by 5′ end capping and 3′ end polyadenylation. mRNA transfection was performed in cells as in *A*. $n = 5$, *error bar*, S.E. *, $p < 0.01$. *D*, *Hsp70* 5′ UTR *Luc* mRNA transfection was performed on TSC2 WT cells treated with 50 μM LY294002 (*LY*) or DMSO as control. $n = 5$, *error bar*, S.E. *, $p < 0.01$.

cells, suggesting that hyperactive mTORC1 signaling inhibits stress-induced *Hsp70* mRNA translation.

We next tested whether decreasing mTORC1 signaling would augment the heat shock-induced *Hsp70* mRNA translation. mTORC1 activity was reduced in cells via siRNA-mediated knockdown of Raptor, a defining component of mTORC1 (8). Cells with Raptor knockdown exhibited 90% reduction of Raptor levels and consequently lower levels of S6K1 phosphorylation as compared with cells transfected with control siRNA (supplemental Fig. S4*B*). As shown in Fig. 3*B*, cells with Raptor knockdown demonstrated a much higher Hsp70 induction after heat shock. In contrast to Raptor knockdown, we observed no difference in heat shock-induced Hsp70 expression in cells with Rictor knockdown that specifically reduces the mTORC2 signaling (supplemental Fig. S4*C*).

Rapamycin is a potent mTORC1 inhibitor and can achieve complete attenuation of S6K1 phosphorylation within 5 min of rapamycin treatment (41). We expected that the presence of rapamycin should increase the heat shock-induced Hsp70 expression by suppressing mTORC1 signaling. However, the presence of rapamycin only caused a marginal increase of Hsp70 expression with a slightly faster induction (Fig. 3*C*). Prolonged rapamycin treatment had little effects in promoting Hsp70 induction (supplemental Fig. S5). In contrast to rapamycin, inhibition of PI3K by LY294002 was able to augment Hsp70 expression after heat shock (Fig. 3*D*). The discrepancy between rapamycin and LY294002 suggests the existence of rapamycin-resistant mTORC1 function (42, 43). Taken together, PI3K-mTORC1 signaling plays a negative role in the regulation of *Hsp70* mRNA translation.

Hsp70 5′ UTR Responds to the PI3K-mTORC1 Signaling—In many cases, features in the 5′ UTR of mRNAs are important for translational control (44). To test the role of 5′ UTR of *Hsp70* mRNA in responding to PI3K-mTORC1 signaling, we used a real time luciferase reporter assay. In contrast to the conventional end point assays, the real time luciferase assay permits continuous measurement of luciferase activity at multiple time points for the same cells. Thus, it allows us to precisely monitor the translational status of the reporter mRNA (*F-Luc*) in live cells under different PI3K-mTORC1 signaling. In addition, mRNA transfection was selected over plasmid expression as it eliminates any transcriptional variances. To mimic the natural mRNAs in cells, *in vitro* transcribed *F-Luc* mRNA was capped with m7GpppG at the 5′ end followed by polyadenylation at the 3′ end. In the absence of 5′ UTR, TSC2$^{-/-}$ MEFs showed little increase of luciferase translation as compared with the wild type cells (Fig. 4*A*, *left panel*). Remarkably, inclusion of the 5′ UTR of *Hsp70* mRNA resulted in ~50% reduction of *F-Luc* translation in cells lacking TSC2 (Fig. 4*C*, *left panel*). This reduction was not due to increased mRNA turnover in TSC2$^{-/-}$ MEFs, as qPCR analysis showed the similar turnover of transcripts within 3 h of transfection (supplemental Fig. S6). Further supporting the negative role of mTORC1 signaling in the translation of mRNAs bearing the *Hsp70* 5′ UTR, cells overexpressing Rheb exhibited a similar pattern of *F-Luc* mRNA translation as TSC2$^{-/-}$ MEFs (supplemental Fig. S7).

We next examined the effects of reduced PI3K-mTORC1 signaling in the translation of *F-Luc* mRNA. Although the PI3K inhibitor LY294002 significantly inhibited the translation of F-Luc in the absence of the Hsp70 5′ UTR (Fig. 4*B*), the same treatment caused a 35% increase of the translation of *F-Luc* bearing the *Hsp70* 5′ UTR (Fig. 4*D*). In agreement with the concept that PI3K acts upstream of TSC, LY294002 treatment had limited effects on mTORC1 signaling in cells

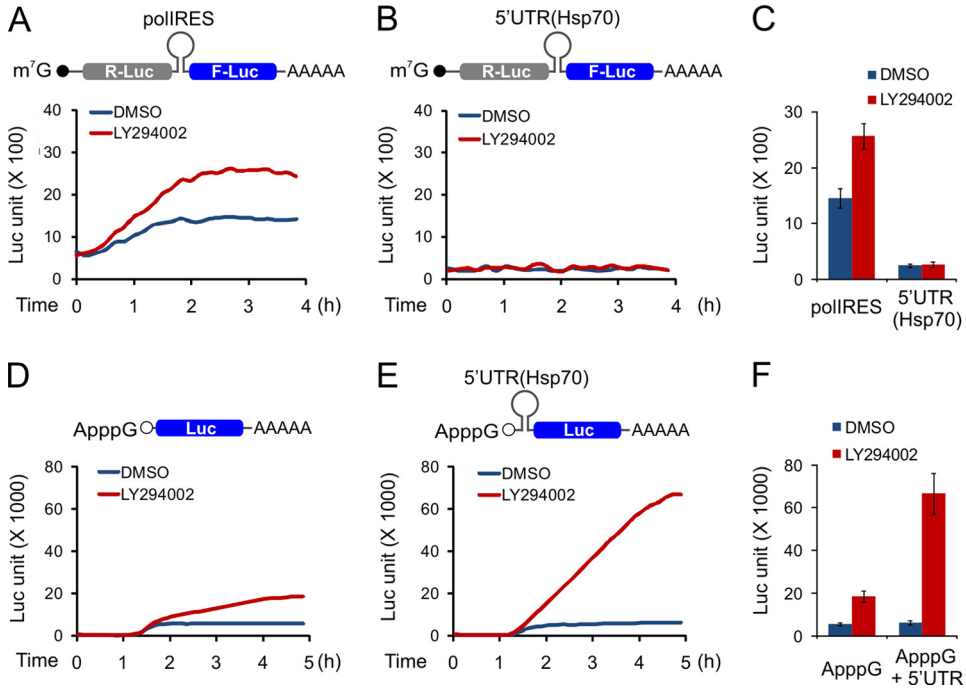

FIGURE 5. ***Hsp70* 5′-UTR differs from IRES in mediating cap-independent translation.** *A*, bicistronic *Luc* mRNA driven by polIRES was synthesized using *in vitro* transcription followed by 5′ end capping and 3′ end polyadenylation. mRNA transfection was performed on TSC2 WT cells treated with 50 μM LY294002 or DMSO. Real time luciferase activity was recorded immediately after mRNA transfection. *B*, bicistronic *Luc* mRNA driven by *Hsp70* 5′ UTR was synthesized using *in vitro* transcription followed by 5′ end capping and 3′ end polyadenylation. mRNA transfection and real time luciferase measurements were the same as *A*. *C*, Luc expression after a 3-h transfection of mRNAs containing polIRES or *Hsp70* 5′ UTR in the presence or absence of LY294002. *Error bar*, S.E. *D*, *in vitro* synthesized *Luc* mRNA was capped at the 5′ end with a non-functional analog (ApppG) followed by 3′ end polyadenylation. mRNA transfection and real time luciferase measurements were the same as *A*. *E*, *in vitro* synthesized *Luc* mRNA bearing *Hsp70* 5′ UTR was capped at the 5′ end with a non-functional analog ApppG followed by 3′ end polyadenylation. mRNA transfection and real time luciferase measurements were the same as *A*. *F*, Luc expression after a 3-h transfection of ApppG-capped mRNAs in the presence or absence of LY294002. *Error bar*, S.E.

lacking TSC2. For example, control mRNAs without *Hsp70* 5′ UTR showed little response to LY294002 treatment in TSC2 KO cells (supplemental Fig. S8). These results indicate that the 5′ UTR of *Hsp70* mRNA is responsible for the mTORC1-mediated translational regulation.

Hsp70 5′ UTR Differs from IRES in Mediating Cap-independent Translation—mTORC1 acts a "master regulator" of the cap-dependent translation in cells (1). A prevalent hypothesis posits that *Hsp70* mRNA is translated by a cap-independent mechanism (45). The cap-independent translation is thought to be mediated by an RNA structure named IRES, which recruits the ribosome independent of both the cap and the entire eIF4F complex (46). The bicistronic test has been employed as the "gold standard" to demonstrate the presence of an IRES feature for a 5′ UTR. In the bicistronic assay, the expression construct is engineered to contain two cistrons with the putative IRES element inserted between them. The first cistron is translated by the cap-dependent scanning mechanism, whereas translation of the second cistron does not happen unless internal initiation at the IRES element occurs. We used a well characterized polio virus IRES (polIRES) as a positive control, in which the IRES element was inserted between *Renilla* luciferase (*R-Luc*) and firefly luciferase (*F-Luc*) (Fig. 5*A*). Consistent with the notion that the IRES-mediated cap-independent translation will be selectively up-regulated when the cap-dependent translation is inhibited (46, 47), we observed a significant increase in F-Luc mRNA translation when PI3K signaling was inhibited by LY294002 (Fig. 5*A*).

We next replaced the polIRES with the whole 5′ UTR of the *Hsp70* mRNA (Fig. 5*B*). In contrast to the polIRES element, Hsp70 5′ UTR was unable to drive F-Luc expression by either plasmid or mRNA transfection. Furthermore, the presence of LY294002 showed little effect on translation of the *F-Luc* mRNA (Fig. 5*B*). This result suggests that the 5′ UTR of *Hsp70* mRNA does not act as the classic IRES element by internally recruiting ribosome machinery. This finding leaves open the question whether translation of the *Hsp70* mRNA is cap-dependent or cap-independent. To address this question, we synthesized *F-Luc* mRNA capped with the non-functional analog ApppG. In contrast to normal capped mRNA (Fig. 4), translation efficiency of ApppG *F-Luc* mRNA, with or without *Hsp70* 5′ UTR, was extremely low (Fig. 5, *D* and *E*), suggesting a strong cap dependence in translation of *F-Luc* mRNA under normal growth conditions. Remarkably, inhibiting PI3K signaling by adding LY294002 significantly increased the translation of ApppG *F-Luc* mRNA bearing the *Hsp70* 5′ UTR, but not in the absence of the 5′ UTR (Fig. 5*E*). We conclude that the *Hsp70* 5′ UTR differs from IRES in mediating cap-independent mRNA translation.

Hsp70 5′ UTR-mediated Cap-independent Translation Is Sensitive to 4E-BP1—To elucidate how PI3K-mTOR signaling controls the balance between cap-dependent and -independent translational mechanisms, we investigated the two well established mTORC1 downstream targets S6Ks and 4E-BPs. We first examined the translation of *Hsp70* mRNA in S6K1/2 double knock-out MEFs (S6K DKO) (48), in which general

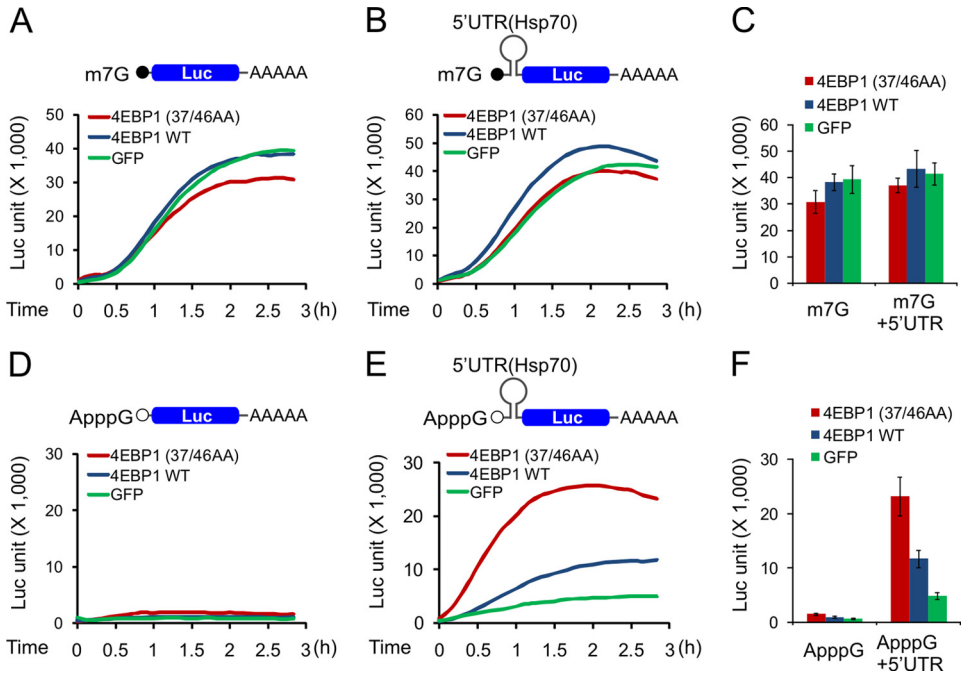

FIGURE 6. ***Hsp70* 5′ UTR-mediated cap-independent translation is sensitive to 4E-BP1.** *A, in vitro* synthesized Luc mRNA was capped at the 5′ end with a non-functional analog ApppG followed by 3′ end polyadenylation. mRNA transfection was performed on TSC2 WT cells pre-transfected with plasmids encoding 4E-BP1 (S37A/S46A), 4E-BP1, or GFP. Real time luciferase activity was recorded immediately after mRNA transfection. *B*, *in vitro* synthesized *Luc* mRNA bearing the *Hsp70* 5′ UTR was capped at the 5′ end with non-functional analog ApppG followed by 3′ end polyadenylation. mRNA transfection and real time luciferase measurements were the same as *A*. *C*, Luc expression after a 3-h transfection of m7G-capped mRNAs in cells transfected with plasmids encoding 4E-BP1 (S37A/S46A), 4E-BP1, or GFP. *Error bar*, S.E. *D*, *in vitro* synthesized *Luc* mRNA was capped at the 5′ end with non-functional analog ApppG followed by 3′ end polyadenylation. mRNA transfection and real time luciferase measurements were the same as *A*. *E*, *in vitro* synthesized *Luc* mRNA bearing the *Hsp70* 5′ UTR was capped at the 5′ end with non-functional analog ApppG followed by 3′ end polyadenylation. mRNA transfection and real time luciferase measurements were the same as *A*. *F*, Luc expression after a 3-h transfection of ApppG-capped mRNAs in cells transfected with plasmids encoding 4E-BP1 (S37A/S46A) (*37/46AA*), 4E-BP1, or GFP. *Error bar*, S.E.

protein synthesis is reduced. However, there was little difference in heat shock-induced Hsp70 expression in these cells (supplemental Fig. S9).

4E-BP1 effectively inhibits cap-dependent translation by binding eIF-4E and inhibiting the formation of eIF-4F. Consequently it frees up the protein synthesis machinery for the selective translation of IRES-containing transcripts (47). We used a dominant-negative 4E-BP1 with alanine mutations at Thr^{37}/Thr^{46}, which is more potent in inhibiting cap-dependent translation (49). In cells overexpressing 4E-BP1 (S37A/S46A), we observed a slight decrease of cap-dependent translation of *F-Luc* mRNA (Fig. 6*A*). However, translation of *F-Luc* mRNA containing the *Hsp70* 5′ UTR was similar in these cells as compared with the ones expressing the GFP control (Fig. 6*B*). It is likely that the increased cap-independent translation was masked by the decreased cap-dependent translation when the 5′ cap is intact. We then tested the 4E-BP1 responsiveness when the normal m7G cap is replaced with the non-functional cap analog ApppG. As expected, no translation occurred in the absence of normal cap for *Luc*-mRNA (Fig. 6*D*). However, the presence of the *Hsp70* 5′ UTR was able to drive an efficient translation of F-Luc mRNA in cells expressing the dominant-negative 4E-BP1 (S37A/S46A) (Fig. 6*E*). Notably, overexpressing wild type 4E-BP1 was also able to boost the translation of F-Luc mRNA driven by the *Hsp70* 5′ UTR. Similar results were also observed in cells lacking TSC2 (supplemental Fig. S10). Thus, the 5′ UTR of *Hsp70* mRNA can efficiently initiate a cap-independent translation mechanism in response to the reduced cap-dependent translation by dominant-negative 4E-BP1.

Deficient Hsp70 Translation Contributes to the Attenuation of Stress Resistance in TSC2 Null Cells—It is well established that Hsp70 molecules protect cells against a wide variety of stresses including heat shock (50, 51). TSC mutant cells are also defective in coping with various stresses. We reasoned that the deficient Hsp70 translation might contribute to the attenuation of stress resistance in TSC2 null cells. To test this possibility, we examined the vulnerability of $TSC2^{-/-}$ MEFs to heat shock. After incubation at 45 °C for 1 h, more than 50% of $TSC2^{-/-}$ MEFs were dead as measured by trypan blue staining (Fig. 7*A*). By contrast, wild type MEFs only showed about 40% cell death ($p = 0.018$). Remarkably, adding back Hsp70, but not Hsp90, using recombinant adenovirus largely rescued the viability of $TSC2^{-/-}$ MEFs after heat shock stress. As Hsp70 protects cells from apoptosis during stress (52), we analyzed the molecular indicators of apoptosis of these cells. In $TSC2^{-/-}$ MEFs, exposure to heat shock resulted in a marked increase in caspase-3 cleavage compared with wild type (Fig. 7*B*). Once again, adding back Hsp70 largely suppressed the caspase-3 cleavage. These results demonstrated that the deficient Hsp70 translation is responsible for the hypersensitivity of $TSC2^{-/-}$ MEFs to heat shock-induced cell death.

DISCUSSION

Until now it was unclear how PI3K-mTOR signaling regulated the intracellular stress response. A recent study reported

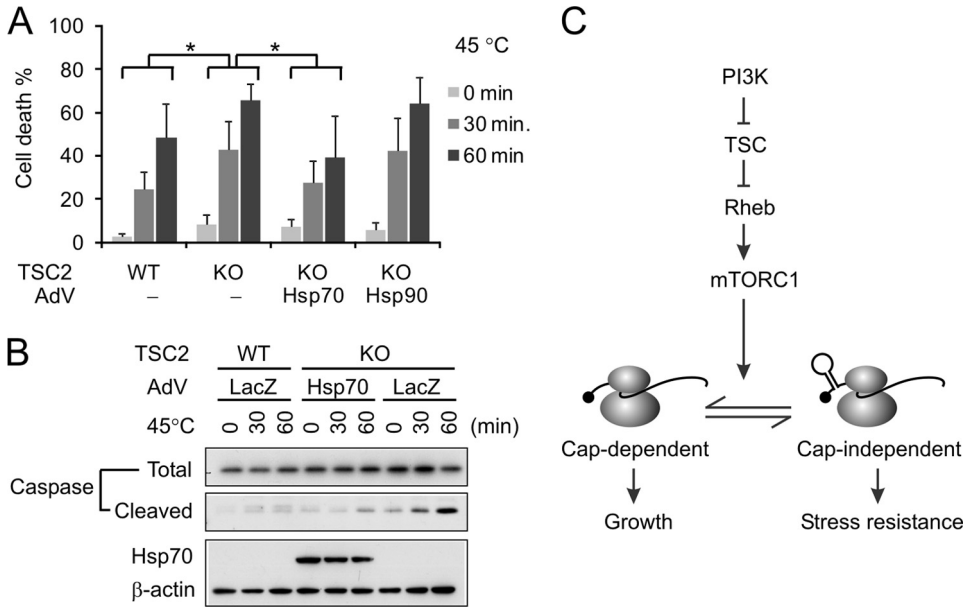

FIGURE 7. **Deficient Hsp70 translation contributes to the attenuation of stress resistance in TSC2 null cells.** *A*, TSC2 WT, TSC2 KO, and adenovirus (AdV)-infected TSC2 KO cells were incubated at 45 °C for various times as indicated. Cell viability was measured by trypan blue counting. $n = 4$, *error bar*, S.E. *, $p < 0.05$ (Student's *t* test, two tails). *B*, AdV-infected TSC2 WT and TSC2 KO cells were incubated at 45 °C for various times followed by immunoblotting using antibodies as indicated. *C*, a schematic model for PI3K-mTORC1-controlled translational balance between cap-dependent and -independent mechanisms.

that a hyperactive unfolded protein response occurred in the ER of MEFs lacking TSC (34). It has been suggested that hyperactive mTOR activity triggers the stress response because higher levels of protein synthesis increased the cellular load of erroneously synthesized polypeptides. To our surprise, we observed a defective cytosolic stress response in these cells. Despite the up-regulated HSF1 transcriptional activity, there is a clear deficiency in heat shock-induced Hsp70 expression in MEFs lacking TSC2. In addition, Hsp70 expression is also significantly reduced in cells overexpressing Rheb. Importantly, decreasing mTORC1 signaling by raptor knockdown or PI3K inhibition augments the heat shock-induced Hsp70 expression. Therefore our results demonstrate a critical role for PI3K-mTOR signaling in controlling the synthesis of one of the most prominent stress-inducible chaperones in cells.

Although much is known about chaperone gene transcription in response to heat stress, relatively little is known about post-transcriptional events. The transcriptional regulation of Hsp70 gene expression has been well established as a prototype of the evolutionary conserved stress response mechanism (53, 54). However, many recent studies using comparative genomic and proteomic profiling of cells have documented a lack of correlation between the mRNA and protein levels of numerous genes (55). This indicates that post-transcriptional control is more important in the regulation of gene expression than is often assumed. Here we report a clear discrepancy between *Hsp70* transcription and translation in cells with hyperactive mTORC1 signaling. Our results, for the first time, uncovered an intimate connection between nutrient signaling and the stress response.

The untranslated regions of *Hsp70* mRNA have been reported to contain elements important to the post-transcriptional regulation of this key component of the stress response. For instance, the 3′ UTR of both the *Drosophila* and the human *Hsp70* mRNA have been shown to control mRNA stability during heat shock as well as during recovery (56, 57). The 5′ UTR of the *Drosophila Hsp70* mRNA allows efficient translation at high temperature when other non-heat shock mRNAs are poorly translated (38, 44). Interestingly, the 5′ UTR of the *Drosophila Hsp70* mRNA is strikingly enriched in adenylic residues (>50%), which suggests a relative absence of secondary structure in this 5′ UTR that is imperative for efficient translation. By contrast, the 5′ UTR of mammalian *Hsp70* mRNA is generally GC rich (~70%), suggesting a relatively high degree of secondary structure. A relaxed cap dependence of translation of this mRNA strongly suggests a translational feature of IRES (45). However, no IRES activity has so far been validated in the *Hsp70* mRNA 5′ UTR (58). Here we show that the 5′ UTR of mouse *Hsp70* mRNA has little effects in driving translation when placed in a bicistronic expression construct. Notably, all eukaryotic mRNAs are both monocistronic and capped with m7GpppN. Therefore, the bicistronic assay cannot faithfully mimic physiological situations of cap-independent translation.

Cap-independent translation was first established for picornavirus viral mRNAs, which do not possess a cap (59). Examples of cap-independent translation have also been documented for some capped cellular mRNAs (37). Accumulating evidences indicate that a down-regulation of cap-dependent translation is associated with up-regulation of cellular IRES-dependent mRNA translation *in vivo* (47). How does the 5′ UTR of Hsp70 mRNA drive the cap-independent translation without acting as an IRES? It is possible that the presence of a 5′ proximal mRNA structure (such as in the artificial bicistronic constructs) prevents the direct recruitment of the ribosome by *Hsp70* 5′ UTR. Another interesting question is how *Hsp70* mRNA adopts the cap-independent translation when

all the eukaryotic mRNAs are synthesized in a capped form. Most recently, it has been reported that the expression of several decapping enzymes was enhanced during heat stress (60). This phenomenon could lead to the selective translation of *Hsp70* mRNA due to unique features of the *Hsp70* 5′ UTR in mediating cap-independent translation.

Our findings may have critical implications for the pathologies associated with PI3K-mTORC1 dysregulation. The stress-induced switch between cap-dependent and -independent translation of Hsp70 represents an important cellular adaptation, which is largely disrupted when mTORC1 signaling is dysregulated (Fig. 7*C*). Significantly, the deficiency of Hsp70 translation in cells with hyperactive mTOR signaling contributes to their stress vulnerability. Unrestrained mTORC1 activity in mammals is associated with the occurrence of disease states including inflammation, cancer, and diabetes (28). By contrast, decreased mTOR signaling by a genetic approach has been shown to extend the lifespan in a variety of organisms (29–32). Interestingly, a robust stress response is required for lifespan extension in these organisms (61–63). We demonstrate that reducing PI3K-mTOR signaling increases stress resistance by promoting cap-independent Hsp70 translation, thereby increasing the availability of proteolytic and chaperone functions that may contribute to the observed increase in organism stress resistance and lifespan. With the demonstration of the mechanistic connection between nutrient signaling and stress resistance, our findings will shed light on therapeutic interventions of aging and age-associated pathologies.

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