The mTOR Target S6 Kinase Arrests Development in *Caenorhabditis elegans* When the Heat-Shock Transcription Factor Is Impaired

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ABSTRACT The widely conserved heat-shock response, regulated by heat-shock transcription factors, is not only essential for cellular stress resistance and adult longevity, but also for proper development. However, the genetic mechanisms by which heat-shock transcription factors regulate development are not well understood. In *Caenorhabditis elegans*, we conducted an unbiased genetic screen to identify mutations that could ameliorate the developmental-arrest phenotype of a heat-shock factor mutant. Here, we show that loss of the conserved translational activator *rsks-1*/S6 kinase, a downstream effector of mechanistic Target of Rapamycin (mTOR) kinase, can rescue the developmental-arrest phenotype of *hsf-1* partial loss-of-function mutants. Unexpectedly, we show that the rescue is not likely caused by reduced translation, nor by activation of any of a variety of stress-protective genes and pathways. Our findings identify an as-yet unexplained regulatory relationship between the heat-shock transcription factor and the mTOR pathway during *C. elegans* development.

KEYWORDS heat shock factor; development; translation; ribosomal S6 kinase; developmental arrest; *C. elegans*; mTOR; WormBase

THE health and longevity of an organism depends on robust proteostatic machinery to keep proteins functioning properly. One major source of cellular quality control is the heat-shock response. The heat-shock response increases expression of a variety of chaperones in response to many stresses, including heat and heavy metals. These so-called heat-shock proteins are coordinately regulated by one (in yeast and invertebrates) or multiple (in vertebrates) transcription factors called heat-shock factors (HSFs). In addition, heat-shock factors act under normal conditions to promote longevity and developmental growth to adulthood.

In *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila*, a loss of heat-shock factors leads to developmental defects at nonstressful temperatures. In yeast, defects in growth caused by lack of Hsf1 activity can be rescued by

restoring basal expression levels of two heat-shock proteins, Hsp70 and Hsp90, but additional Hsf1 target genes are required for resistance to heat stress (Solís *et al.* 2016). In *Drosophila*, loss of Hsf causes arrest at the first or second larval-instar stage of development, as well as defects in oogenesis (Jedlicka *et al.* 1997). Unlike in yeast, Jedlicka *et al.* found that this essential developmental function in *Drosophila* was not mediated through canonical heat-shock genes. In *C. elegans*, HSF-1 is needed for progression past the L2–L3 larval stage, and it has been shown to regulate a developmental program that is distinct from the heat-shock response by binding to a promoter sequence that is different from the canonical sequence (Li *et al.* 2016).

Heat-shock factors are important but not essential for mouse development. Mouse cells lacking HSF1, or both HSF1 and HSF2, can be cultured and show normal constitutive transcription of heat-shock proteins, although cells without Hsf1 are sensitive to heat stress (McMillan *et al.* 1998; Zhang *et al.* 2002; Solís *et al.* 2016). *In vivo*, *Hsf1* (–/–) mice display additional phenotypes, including growth retardation, female infertility, prenatal lethality (Xiao *et al.* 1999; Christians *et al.* 2000), and neurological defects (Santos and Saraiva 2004; Takaki *et al.* 2006), with the penetrance of prenatal lethality

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partially regulated by the genetic background. *Hsf2*($-/-$) mice also display abnormalities, in both gametogenesis and brain structure (Chang *et al.* 2006).

While these studies establish that a heat stress-independent activity of heat-shock factor is inextricably tied to development, the genetic pathways and mechanisms by which heat-shock factors promote growth and development remain largely unexplored. To address this question in an unbiased way, we conducted a genetic screen in *C. elegans* for suppressor mutations that allow *hsf-1*(*sy441*) mutant animals to grow to adulthood. The *sy441*-mutant HSF-1 protein lacks its transactivation domain, severely blunting its ability to regulate canonical heat-shock response genes (Hajdu-Cronin *et al.* 2004). These animals grow well at lower temperatures (15–20° in our hands), but when grown at temperatures of 25° or higher, they display a developmental phenotype similar to that of *hsf-1* loss-of-function mutants, arresting at the L2–L3 stage (Li *et al.* 2016).

In this study, we show that mutations in multiple complementation groups allow developing *sy441* mutants to reach adulthood. These mutations dramatically postpone, but do not eliminate, growth arrest within the strain's lineage, as the rescued adults produce progeny that fail to progress through development. We show that one of these mutations is a putative null allele of *rsks-1*/S6 kinase, a translational activator regulated by the mechanistic Target of Rapamycin (mTOR). We find that knockdown of various mTOR pathway components can also rescue the phenotype. Unexpectedly, we find no evidence that phenotypic rescue is mediated by inhibition of translation. In addition, rescue does not appear to be due to the activation of other stress responses such as the ER unfolded protein response (ER UPR) or the mitochondrial unfolded protein response (mito-UPR), nor is it due to the activation of various stress resistance pathways known to extend adult life span.

We find that the rescue mediated by loss of S6 kinase is dependent upon residual activity of the mutant HSF-1(*sy441*) protein, and that transgenically increased expression of the *hsf-1*(*sy441*) allele is sufficient on its own to rescue development. However, we see no evidence that the S6 kinase mutation increases expression of *hsf-1* nor any of a variety of target genes that we examined. We conclude that loss of S6 kinase could potentially elevate other targets of *hsf-1* or, alternatively, provide other factors that allow low levels of HSF-1 activity to sustain growth to adulthood.

Materials and Methods

C. elegans strains

Wild-type (N2).

VB654: *rsks-1*(*sv31*); *svEx136*[*unc-36*(+) *rsks-1*(+) *sur-5::gfp*].

AGD794: *hsf-1*(*sy441*); *uthIs225*[*sur-5p::hsf-1*(*sy441*); *myo2p::tdTomato*].

CF3951: *hsf-1*(*sy441*).

CF4522: *hsf-1*(*sy441*) with an unidentified suppressor mutation.

CF4523: *hsf-1*(*sy441*) with an unidentified suppressor mutation.

CF4524: *hsf-1*(*sy441*) with an unidentified suppressor mutation.

CF4525: *hsf-1*(*sy441*); *rsks-1*(*mu482*).

CF4526: *hsf-1*(*sy441*) with an unidentified suppressor mutation.

CF4540: *hsf-1*(*sy441*); *rsks-1*(*sv31*).

CF4542: *hsf-1*(*sy441*); *rsks-1*(*mu482*); *svEx136*[*unc-36*(+) *rsks-1*(+) *sur-5::gfp*].

CF4543: *hsf-1*(*sy441*); *T24F1.4*(*tm5213*).

Development assay

Arrested L1 larvae were spotted onto plates preheated to 25.8°. After spotting, plates were placed back at 25.8° in an open Tupperware container for 1 hr to reequilibrate temperature. After 1 hr, the container was closed (except for one corner) to prevent drying for 4 days. Each condition utilized four plates containing roughly 25 animals each. After 4 days, conditions were blinded and scored on a qualitative five-point scale for developmental stage. Animals with a score of three had passed the point of vulval eversion (transition to adulthood) but had not yet produced any eggs. Therefore, animals with a score of three or higher were classified as adults in the figures and tables shown here.

EMS mutagenesis and screening

Mutant *hsf-1*(*sy441*) worms grown at 20° on OP50 were bleach-prepped, and the eggs were incubated overnight in M9. The next day, a total of 10,000 L1 larvae were divided among four 10 cm plates containing OP50 (~2500/plate) and incubated at 20°. Once this P₀ population reached early L4, the worms were collected from the plates, washed three times, and resuspended in 15 ml of M9 buffer. In a separate 50-ml conical tube, 100 μ l of EMS (#M-0880; Sigma [Sigma Chemical], St. Louis, MO) was mixed into 5 ml of M9. The worm suspension was then added to this new conical tube containing EMS, the top was parafilm, and the tube was placed in a rotator at 20° for 4 hr. The final concentration of EMS under these conditions was ~50 mM, for an expected mutation rate of 5×10^{-4} mutations/gene/gamete (Brenner 1974). Next, the worms were pelleted, the supernatant was removed, and the worms were washed thrice with 15 ml of M9. Lastly, the worm pellet was split equally among four 10-cm plates containing OP50 and recovered at 20° for 24 hr.

At the end of this recovery period, the P₀ animals were collected from the plates and bleached using a standard protocol. Assuming five viable eggs were obtained per P₀, this yielded a total of ~50,000 F₁ animals, representing 50 $\times$ genomic coverage. These eggs were plated evenly onto 20 separate 10-cm plates containing OP50 (~2500/plate) and grown to adulthood at 20°. The gravid populations on each of these 20 plates were then collected individually and bleached, and each resulting batch of F₂ eggs was plated onto

its own 15-cm plate containing OP50 (25,000/plate). The F₂ worms were then grown at 25°. On day 5, the plates were screened for any worms that had developed beyond the L3 arrest phase, and these candidates were collected onto individual 3-cm OP50 plates and recovered at 20°.

Validation and phenotypic analysis of screen hits

All candidates recovered from the screen were validated by determining whether the suppression-of-*hsf-1*-arrest phenotype bred true to the next generation. Those that failed this test were discarded. The validated lines were then genotyped for reversal of the *hsf-1*(*sy441*) point mutation, with intent to discard true genetic revertants.

To narrow down the 17 remaining mutant lines, we further characterized them for penetrance and expressivity. First, we measured the percentage of suppression of developmental arrest that each line displayed. This was done by picking 50 eggs onto a 3-cm OP50 plate, incubating for 4 days at 25°, and then counting the number of worms that developed past L3 arrest. Investigation was continued only for lines with 50% or higher population rescue. Next, we determined the furthest developmental stage each line could achieve at 25°, using the same experimental set-up just described, and only pursued those that could produce gravid adults.

Genetic characterization of screen hits

We characterized the allelic nature of the nine remaining lines. Males were generated for each, as well as for *hsf-1*(*sy441*) single mutants, and reciprocal crosses were carried out to identify any dominant or sex-specific mutations. Given that all suppressors were found to be recessive, the males generated above were then used to perform complementation assays. To ensure independence, all lines isolated from the same plate (of the original 20) were interrogated with reciprocal crosses, looking for heterozygous progeny that maintained the suppression-of-*hsf-1*-arrest phenotype. Second, reciprocal complementation crosses were carried out between all remaining lines (across the original 20 populations), with the intent of again keeping only one from each complementation group. This yielded a set of five lines with unique, recessive, and penetrant mutations that allowed *hsf-1*(*sy441*) worms to reach gravid adulthood at 25°. Four of these five strains exhibited extended life span at 20°, but this phenotype was lost upon backcrossing.

Identification of candidate genes

These five suppressor strains were backcrossed to the control *hsf-1*(*sy441*) strain a total of six times before genomic sequencing. The suppressor strains were then backcrossed a total of nine times before use in all other experiments.

Genomic DNA from each mutant strain was isolated from roughly 300 µl of pelleted worms from a mixed population. DNA was sheared using a Covaris M220 sonicator to an average length of 350 bp. After shearing, DNA was quantified using the Qubit dsDNA high-sensitivity assay kit. DNA libraries were prepared from genomic DNA utilizing the Bio

Scientific NEXTflex Rapid DNA-Seq Library Prep Kit. Library quality was assessed utilizing the Agilent high-sensitivity DNA analysis kit. Libraries were sequenced by the University of California, San Francisco Institute for Human Genetics Core Facility, according to the manufacturer's protocol, using an Illumina HiSeq 2500.

Genomic information from the Human Genetics Core Facility was analyzed using the Galaxy platform through Amazon Web Services. Genes with high-quality protein-coding point mutations were then examined for effects on the development-arrest phenotype.

Life span analysis

Life span analysis was performed at 20° as described previously (Apfeld and Kenyon 1998). Animals were grown on OP50 *Escherichia coli* and transferred to fresh plates on the first day of adulthood ("day 1") and every 2 days thereafter. Every 2 days, starting on day 1, animals were scored. Animals that moved were scored as alive; animals that did not move, even after being prodded with a platinum wire, were scored as dead; and animals that could not be found, or displayed phenotypes such as rupturing or bagging, were censored.

Translation inhibiting drugs

Salubrinal (SML0951) and homoharringtonine (SML1091) were purchased from Sigma. Cycloheximide (#94271) was purchased from VWR Life Sciences. Drugs were diluted and spotted on plates with bacteria to a final DMSO concentration of 0.19% (unless otherwise noted), and allowed to diffuse into the agar for 1 day before animals were plated for development experiments.

Real-time qPCR

RNA extraction, cDNA preparation, and real-time quantitative (RT-q) PCR were performed as described previously (Van Gilst *et al.* 2005) from three biological replicates of each condition, with ≥ 500 animals included in each condition. For larval experiments, animals were grown from arrested L1 larvae at 25.8° until > 50% of the population had grown to the L1 lethargus stage between the L1 and L2 larval stages (~13 hr for the wild-type background and ~16 hr for the *hsf-1*(*sy441*) background), before being washed and flash-frozen for RNA extraction. For heat-shock experiments, animals were grown from arrested L1 larvae at 20° until > 50% of the population had reached young adulthood, after which the animals were separated into heat-shock conditions, which were placed at 30°, and nonheat-shock conditions, which were placed at 20°, for 1 hr before being washed and flash-frozen for RNA extraction. Data were standardized to three control primers: *tba-1*, *cdc-42*, and *pmp-3*. For *rps-6* dilution experiments, data were standardized to *tba-1* and *cdc-42* only. Primer sequences are listed in Supplemental Material, Table S3.

RNA interference

HT-115 RNA interference (RNAi) bacteria were obtained from existing Ahringer and Vidal libraries. The control for

all RNAi experiments was L4440 vector. For each experiment, single colonies were grown overnight at 37° in 3 ml LB with 100 µg/ml carbenicillin and 10 µg/ml tetracycline. The next day, the resulting stationary-phase cultures were diluted 10-fold with LB containing 100 µg/ml carbenicillin and grown for 2 hr at 37°. Next, 1 M IPTG was added to the culture to reach a final concentration of 2 mM, after which 100 µl of the resulting culture were spotted onto nematode growth media plates. The bacteria were grown on these plates overnight at 30°. Animals were placed on plates the following day in all experiments except those involving a drug condition, in which case drugs were added and left to diffuse into the plate for an additional day before the addition of experimental and control animals.

Size measurement

Arrested L1 larvae were spotted onto plates preheated to 20° (for young-adult experiments) or 25.8° (for larval experiments). For young-adult experiments, animals were grown for 3 days at 20°, and for larval experiments, animals were grown until L1–L2 lethargus at 25.8°. Animals were then washed off plates, immobilized in 10 mM levamisole, and imaged at ×80 magnification. Animal size was then quantified in ImageJ by measuring the area inside a polygon drawn around each animal.

Statistical analyses

On all dot plots, bars represent the mean and error bars represent the SEM. For developmental assays of multiple genes in parallel, significance was measured across three independent experiments using Cochran–Mantel–Haenszel tests with Bonferroni corrections unless otherwise noted. For life span assays, log-rank tests with Bonferroni corrections were used. For RT-qPCR experiments and animal size experiments, one-way ANOVAs with Tukey post-tests were used on three biological replicates.

Data availability

Strains are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Supplemental material available at Figshare: <https://doi.org/10.25386/genetics.7094729>.

Results

A genetic screen identifies mutations that rescue the *hsf-1(sy441)* developmental arrest

To investigate how *hsf-1* regulates development, we conducted an unbiased forward mutagenesis screen in *C. elegans* to rescue the growth-arrest phenotype displayed by *hsf-1(sy441)* mutants grown at 25°. Following EMS mutagenesis (Brenner 1974), we recovered 17 independent mutants that, when shifted from 20 to 25.8° at the mid-L4 (final) larval stage, produced progeny that grew to adulthood. We picked

Figure 1 Mutants discovered in a mutagenesis screen for rescue of the developmental arrest of *hsf-1(sy441)* mutants. (A) Appearance of wild-type, *hsf-1(sy441)*, and multiple suppressor strains after 4 days at 25.8° from arrested L1 larvae cultured at 20°, imaged at ×100 magnification. Strains CF4522, CF4523, CF4524, and CF4526 contain additional suppressors from the screen, but were not analyzed further in this study. (B) Appearance of wild-type, *hsf-1(sy441)*, and CF4525 suppressor strain grown at 25.8° to various time points from arrested L1 larvae cultured at 20°, imaged at ×600 magnification.

five strains (see methods) to backcross and sequence to discover candidate genes. *hsf-1(sy441)* mutants carrying the suppressors reached adulthood exhibiting apparently normal anatomy, but they displayed a reduced body size and could not be cultivated past a single generation at 25.8° (Figure 1, A and B). The progeny of some strains grown at 25.8° produced eggs that did not hatch, whereas others produced progeny that arrested at L1. If adult animals were shifted back to 20°, they produced offspring that developed normally.

When parents were shifted to 25.8° at the L4 stage, 100% of *hsf-1(sy441)* progeny without suppressor mutations arrested at the L1–L3 stage, whereas 100% of progeny homozygous for the five suppressor mutations reached adulthood. If, instead, eggs were bleach-extracted from the gonads of the parental generation at 20°, allowed to hatch into minimal medium (causing L1 developmental arrest), and then placed on 25.8° plates, a small percentage of *hsf-1(sy441)* single-mutant animals could reach early adulthood but rarely produced eggs, while 100% of progeny homozygous for the suppressor mutations reached adulthood. To detect subtle shifts in the percentage of animals that reached adulthood, all of the assays described here utilized arrested L1 larvae hatched at 20° and then shifted to the higher temperature.

Figure 2 Loss of *rsk-1*/S6 kinase or reduction of mTOR function was sufficient to rescue the developmental arrest of *hsf-1*(*sy441*). (A) Loss of *rsk-1* rescued the developmental arrest of *hsf-1*(*sy441*), *** $P < 0.001$ compared to *hsf-1*(*sy441*) using the Cochran–Mantel–Haenszel (CMH) test with three replicates. (B and C) Transgenic overexpression of *rsk-1::GFP* in *hsf-1*(*sy441*); *rsk-1*(*mu482*) double mutants suppressed the rescue. All animals are *hsf-1*(*sy441*); *rsk-1*(*mu482*) double mutants with the stochastically inherited transgene *svEx136[unc[36(+)] rsk-1(+)] sur-5::gfp*. Strain identity was blinded and development was scored, and then groups were categorized based on whether GFP was visible, *** $P < 0.001$ compared to animals without GFP using the CMH test with three experiments; animals were imaged at $\times 100$ magnification. (D) RNA interference (RNAi) knockdown of genes in the mTOR pathway also rescued the developmental arrest of *hsf-1*(*sy441*). * $P < 0.05$ and *** $P < 0.001$ compared to *hsf-1*(*sy441*) using the CMH test with three replicates (four for *daf-15*).

Because our suppressor mutations rescued the developmental growth-arrest phenotype of *hsf-1*(*sy441*) animals, we tested whether they could also rescue another well-documented *hsf-1*(*sy441*) phenotype, shortened adult life span (Garigan *et al.* 2002; Hajdu-Cronin *et al.* 2004). None of the mutants extended the shortened life span that *hsf-1*(*sy441*) mutant animals exhibit at the temperatures permissive for growth (Figure S1).

Loss of the gene *rsk-1* rescues the *hsf-1*(*sy441*) developmental arrest

Genomic sequencing revealed that one of the suppressor strains (CF4525) contained a premature stop codon in the gene *rsk-1* at the 295th base pair, *rsk-1*(*mu482*). The *rsk-1* gene encodes the *C. elegans* ribosomal S6 kinase ortholog, which promotes normal levels of translation. We determined that this mutation was the causative factor for the developmental rescue in two ways. First, we confirmed the developmental rescue with a previously isolated null allele, *rsk-1*(*sv31*), and with RNAi-mediated knockdown of *rsk-1* (Figure 2A). Second, we showed that transgenic reexpression of *rsk-1* in the suppressor strain blocked the rescue phenotype (Figure 2, B and C). While there was variation in the baseline percentage of *hsf-1*(*sy441*) animals that reached adulthood (Tables S1 and S2), this variation did not obfuscate

the rescue phenotype of *rsk-1* RNAi. In 44 out of 46 trials, we saw a significant difference in the percentage of *hsf-1*(*sy441*) animals that developed to adulthood between animals grown on vector-only control RNAi bacteria and those grown on *rsk-1* RNAi bacteria (Figure S2).

Reduction in mTOR-pathway function also rescues the *hsf-1*(*sy441*) developmental arrest

What is the mechanism by which *rsk-1* loss influences *hsf-1* mutants? S6 kinase is a key target of mTOR kinase. To determine if the developmental rescue phenotype could be produced by a reduction in mTOR activity more broadly, we used RNAi to knock down *daf-15*/RAPTOR, a component of the mTORC1 complex, as well as *ragc-1*/RAG GTPase, a positive regulator of mTORC1 (Jia *et al.* 2004; Fukuyama *et al.* 2012). Reduced expression of both of these genes significantly rescued the *hsf-1*(*sy441*) developmental arrest (Figure 2D), albeit to a lower degree than did RNAi knockdown of *rsk-1*.

rsk-1(–)-mediated *hsf-1*(*sy441*) rescue was not mimicked by a reduction in translation

Loss of *rsk-1* causes a reduction in translation (Hansen *et al.* 2007). One possible explanation for the mechanism of developmental rescue conferred by *rsk-1* mutation is that

Figure 3 The ability of *rsk-1*(–) to rescue the development of *hsf-1(sy441)* mutants is not explained by a reduction in translation. (A) *rps-6* RNA interference (RNAi) reduces the size of *hsf-1(sy441)* young-adult animals grown at 20°. Serial twofold dilution of *rps-6* RNAi produced incrementally smaller effects (50% denotes 1:1 *rps-6*:vector control RNAi bacteria). (B) Dilutions of *rps-6* RNAi bacteria with vector control RNAi bacteria that show size reductions similar to *rsk-1(mu482)* mutants significantly reduce the percent of *hsf-1(sy441)* animals that develop to adulthood at 25.8° (50% denotes 1:1 *rps-6*:vector control RNAi), *** $P < 0.001$ compared to control *hsf-1(sy441)* by Cochran-Mantel-Haenszel (CMH) test with three replicates. (C) None of multiple dilutions of *rps-6* RNAi bacteria with vector control RNAi bacteria rescued developmental arrest at 25.8° (50% denotes 1:1 *rps-6*:vector control RNAi), *** $P < 0.001$ compared to control *hsf-1(sy441)* by CMH test with three replicates. (D) Concentrations of *rps-6* RNAi bacteria that reduce baseline development to adulthood of *hsf-1(sy441)* animals do not activate stress-response ER unfolded protein response (UPR), mitochondrial UPR, or heat-shock response pathways in animals grown at 25.8° and harvested at the L1–L2 lethargus stage, by one-way ANOVA with Tukey post-test on three biological replicates.

control *hsf-1(sy441)* by CMH test with three replicates. (D) Concentrations of *rps-6* RNAi bacteria that reduce baseline development to adulthood of *hsf-1(sy441)* animals do not activate stress-response ER unfolded protein response (UPR), mitochondrial UPR, or heat-shock response pathways in animals grown at 25.8° and harvested at the L1–L2 lethargus stage, by one-way ANOVA with Tukey post-test on three biological replicates.

hsf-1(sy441) animals, predicted to have reduced proteostatic capability, are overburdened by the amount of protein being translated during development at 25°, and the reduced rate of translation incurred by mutating *rsk-1* reduces that burden to a manageable level. To investigate this hypothesis, we reduced translation in other ways: by treatment with several chemical inhibitors of translation and via various RNAi treatments. If our hypothesis were correct, one would expect the following: at high concentrations these treatments would be lethal and at low concentrations they would have no effect, but at some range of intermediate concentrations they would lower the translation rate to a level permissible for developmental rescue.

We first used RNAi to knockdown *rps-6*, encoding a small ribosomal protein that is phosphorylated by S6 kinase. Because a reduction in translation is known to result in reduced body size (Pan *et al.* 2007), we assessed the efficacy of translational knockdown indirectly by measuring the size of the young-adult animals grown at 20° on *rps-6* RNAi diluted with vector control to achieve multiple levels of knockdown (Figure 3A). At concentrations that significantly reduced animal size close to that of *rsk-1(mu482)* mutants, we found that *rps-6* RNAi reduced the number of *hsf-1(sy441)* animals that reached adulthood (Figure 3, B and C), showing that a reduction in translation may actually be harmful to *hsf-1(sy441)* development. To see whether *rps-6* RNAi treatment on its own was perhaps stressful or toxic to the animals, we

measured expression of levels of canonical genes from the ER UPR, mito-UPR, or heat-shock response pathways using RT-qPCR, and found that none were significantly altered (Figure 3D and Figure S3A). This was despite seeing a significant, if smaller, effect of *rps-6* inhibition on animal size at this time point (Figure S3B).

We also tested the effects of other translation-inhibiting conditions, including RNAi of *ifg-1*, a gene that promotes translation additively with S6 kinase (Pan *et al.* 2007), salubrinal, a chemical inhibitor of translation initiator, and homoharringtonine, a chemical inhibitor of elongation (Figure S3, C–F and Table S1). Similar to knockdown of *rps-6* treatment with homoharringtonine caused a reduction in the percent of animals that developed to adulthood at concentrations that also significantly reduced animal size. Animals treated with higher concentrations of salubrinal and *ifg-1* RNAi showed a similar reduction. Only *hsf-1(sy441)* animals grown on 45 nM salubrinal showed a marginally significant increase in development to adulthood. Thus, while multiple distinct methods for reducing translation could cause reductions in animal size, no translation-inhibiting conditions mimicked the rescuing effect of *rsk-1* inactivation in terms of either penetrance or expressivity on the development of *hsf-1(sy441)* animals. Together, these data suggest that reduced translation alone cannot explain the *rsk-1(mu482)* mutation's rescue of *hsf-1(sy441)* mutants' development.

Figure 4 Stress responses in *hsf-1(sy441)* animals carrying the *rsk-1(mu482)* suppressor. (A) *hsf-1(sy441); rsk-1(mu482)* double mutants are more sensitive to RNA interference (RNAi) of *xbp-1*, an activator of the ER unfolded protein response (UPR), than are wild-type animals, *** $P < 0.001$ using the Cochran–Mantel–Haenszel (CMH) test with three replicates. (B) Canonical ER UPR and mitochondrial UPR chaperones were not upregulated in *hsf-1(sy441); rsk-1(mu482)* double mutants, using one-way ANOVA with Tukey post-test on three biological replicates. (C) Inhibition of the *daf-2* insulin/IGF-1 receptor or the mitochondrial electron transport chain, which extend the life span of wild-type animals, failed to rescue the developmental arrest of *hsf-1(sy441)* mutants, *** $P < 0.001$ using CMH test with three replicates. (D) Transcription factors required for mTOR reduction-of-function to extend life span are not required for *rsk-1* mutation to rescue *hsf-1(sy441)* developmental arrest, using CMH test with three replicates.

rsk-1(-) mutants do not activate canonical unfolded-protein response pathways

Because we could not attribute the rescuing effects of *rsk-1* mutation to a simple lowering of the translational burden, we hypothesized that it might be increasing the animals' capabilities of managing proteotoxic stress through non-heat-shock response pathways such as the mitochondrial or ER UPR pathways. Like the heat-shock response, these pathways upregulate the expression of protein chaperones such as BIP/HSP-70, which facilitate protein folding (Shen *et al.* 2001).

The development of *hsf-1(sy441); rsk-1(mu482)* double mutants was partially inhibited by knockdown of the ER UPR pathway gene *xbp-1* (Figure 4A), an intervention that has no effect on wild-type worms, showing that the animals are sensitive to disruption of additional proteostatic systems beyond the heat-shock response. This suggests the possibility that *rsk-1* loss upregulates *xbp-1* transcriptional target genes, such as *hsp-4* (the *C. elegans* BIP ortholog). However, when assayed using RT-qPCR, *hsp-4* did not appear to be upregulated by loss of *rsk-1* in *hsf-1(sy441)* mutants (Figure 4B). Likewise, RT-qPCR of the mito-UPR target gene *hsp-6* revealed that the mito-UPR was not upregulated. These findings suggest that an increase in UPR function is not the mechanism by which *rsk-1* loss rescues development.

The developmental rescue of *hsf-1(sy441)* mutants is mTOR-specific but does not act through canonical life span pathways that interact with mTOR

mTOR and heat-shock factor are intimately tied to life span regulation and stress resistance. We wondered whether activating other pathways known to increase life span and proteostasis could also rescue the development of *hsf-1(sy441)* animals. Both *daf-2* (insulin/IGF-1 receptor) and *cco-1/cox-5B* (mitochondrial electron transport gene) RNAi extend life span (Kenyon *et al.* 1993; Dillin *et al.* 2002), but neither rescued the block in *hsf-1(sy441)* development (Figure 4C). In fact, *daf-2* RNAi seemed to have a negative effect on development. To further investigate, we used RNAi to inhibit the transcription factors *daf-16*, *pha-4*, and *skn-1*. Each of these genes promotes *C. elegans* stress resistance and life span extension in mTOR reduction-of-function conditions and/or other long-lived mutants (Sheaffer *et al.* 2008; Robida-Stubbs *et al.* 2012; Seo *et al.* 2013), but none blocked the ability of *rsk-1* loss to rescue the *hsf-1(sy441)* developmental arrest (Figure 4D). We then tested a subset of genes in the published literature that have been shown to be a part of the genetic networks associated with *rsk-1* or *hsf-1*, including those from microarray data sets (Magnuson *et al.* 2012; Chen *et al.* 2013; Baird *et al.* 2014). Of the 65 additional genes tested, we saw no evidence of RNAi knockdown either rescuing the development of *hsf-1(sy441)* mutants or having

Figure 5 *hsf-1(sy441)* activity is necessary and can be sufficient to rescue developmental arrest, but *rsk-1(mu482)* does not appear to affect expression of *hsf-1* or its canonical targets. (A) *hsf-1* RNA interference (RNAi) treatment prevented two *rsk-1/S6* kinase null mutations from rescuing the developmental arrest of *hsf-1(sy441)* mutants, *** $P < 0.001$ using the Cochran–Mantel–Haenszel (CMH) test with three replicates. (B) Overexpression of the *hsf-1(sy441)* allele rescued the developmental arrest of *hsf-1(sy441)* mutants, *** $P < 0.001$ using the CMH test with four replicates. (C) Expression levels of canonical heat-shock genes and developmentally regulated *hsf-1*-response genes are unaffected by the *rsk-1(mu482)* mutation, as measured by real-time quantitative PCR; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by one-way ANOVA with Tukey multiple comparison post-test measured for each gene independently with three biological replicates.

a more severe reduction-of-development effect on *hsf-1(sy441); rsk-1(mu482)* double mutants vs. wild-type (Table S2).

Developmental rescue requires residual *hsf-1* function but does not act through canonical heat-shock-regulated genes

Despite the lack of its transactivation domain, the fact that the *hsf-1(sy441)* allele does not cause arrest at 20°, as the null mutation does (Li *et al.* 2016), shows that the HSF-1(*sy441*) mutant protein retains some functionality. To test whether endogenous levels of the mutant protein are required for *rsk-1* null mutations to rescue development at 25.8°, we knocked down *hsf-1* using RNAi in the *hsf-1(sy441); rsk-1(mu482)* double mutant. RNAi knockdown of residual HSF-1(*sy441*) function blocked the *rsk-1* rescue phenotype (Figure 5A). In addition, we found that transgenically increasing the *hsf-1(sy441)* gene dosage was sufficient to rescue the developmental arrest of *hsf-1(sy441)* mutants (Figure 5B). However, despite the fact that residual levels of HSF-1(*sy441*) are necessary for developmental rescue and overexpression of HSF-1(*sy441*) can be sufficient for developmental rescue, we saw no evidence through RT-qPCR that *rsk-1* mutation elevated the expression levels of the *hsf-1* gene nor genes known to be regulated by HSF-1 in heat shock or developmental contexts (Figure 5C) (Li *et al.* 2016). Thus, the hypothesis that, directly or indirectly, loss of S6 kinase promotes growth to adulthood by enhancing the

effectiveness of HSF-1(*sy441*) remains an attractive, though unproven, model.

Three findings from our RT-qPCR experiments were surprising. First, we found that *hsf-1(sy441)* animals exhibited a higher level of *hsf-1* expression compared to wild-type. Second, we found that *rsk-1* mutation did not increase expression of *hsp-16.2* or the *hsp-70* gene F44E5.5, in contrast to a previous report (Seo *et al.* 2013). Third, one might suspect *hsp-16.2* and *hsp-70* (F44E5.5) expression levels to be higher in wild-type than in *hsf-1(sy441)* mutants because 25.8° is higher than the ambient temperature for *C. elegans*, but this was not the case. To more closely replicate previously published experiments, we conducted new RT-qPCR experiments on young-adult animals that were grown at 20° and then heat shocked at 30°, or left at ambient temperature for 1 hr. Under these conditions, as shown previously, the increase in *hsp-16.2* expression upon heat shock was severely blunted in the *hsf-1(sy441)* background (Figure S4A). Curiously, *hsp-70* (F44E5.4) expression was unaffected (Figure S4B), suggesting that the *sy441* allele affects only a subset of heat-shock target genes. Contrary to the findings of Seo *et al.*, we did not observe an increase in the expression of *hsp-16.2* or *hsp-70* (F44E5.4) in *rsk-1* mutant animals compared to wild-type (Figure S4C), but we did see a possible increase in *hsp-70* (F44E5.4) expression after heat shock in *rsk-1* mutant animals. This suggests to us that loss of *rsk-1* can affect the regulation of heat-shock genes, but the mechanism

may be complicated by factors such as genetic background or differences between *rsks-1* mutant alleles. Finally, we found that the increase in *hsf-1* expression seen in *hsf-1(sy441)* mutant larvae was no longer significant in adulthood (Figure S4D).

Discussion

hsf-1 and its orthologs have been studied extensively in the contexts of stress, aging, and human pathologies such as cancer and neurodegenerative diseases (Li *et al.* 2017). Despite characterization of the requirement for a functioning heat-shock factor during development, the specific molecular roles it plays in development remain largely unknown. In this paper, we show that the mTOR pathway, and more specifically the downstream mTOR target ribosomal S6 kinase, acts to truncate development in *hsf-1(sy441)* reduction-of-function mutants.

Interestingly, life span extension caused by inhibiting S6 kinase or mTOR activity is known to be blocked by the *hsf-1(sy441)* mutation (Seo *et al.* 2013). Consistent with this finding, our S6 kinase suppressor mutation failed to extend the life span of *hsf-1(sy441)* mutants. While this finding shows that HSF-1 is required for *rsks-1* loss to extend life span, here, we find another reciprocal relationship, namely, that *rsks-1* loss can rescue the developmental arrest caused by reduction of HSF-1 function. In one case, low levels of S6 kinase cause endogenous levels of HSF-1 to extend life span, whereas in the other case, low levels of HSF-1 cause endogenous levels of S6 kinase to arrest development. The details of these reciprocal interactions remain elusive; nevertheless, our findings reveal a recurring, but potentially complex, relationship between these two important regulators of development, stress resistance, and life span.

It is interesting to note that when *hsf-1(sy441)* homozygotes are shifted to 25° at the L4 larval stage, they lay eggs that hatch but fail to reach adulthood. However, when L1 larvae are starvation arrested at 20°, and then fed and shifted to 25.8°, some of those larvae reach adulthood. In other words, the general “arrest point” in the life cycle is different under these two conditions. This finding indicates that *hsf-1* loss does not cause a growth blockade at one specific point in the life cycle. Instead, this finding suggests the model that loss of HSF-1 function impacts a time-dependent accumulation of proteostatic damage rather than a specific developmental requirement. The opposite situation occurs in *Drosophila*, where HSF-1 activity is dispensable specifically after passing the first two larval stages (Jedlicka *et al.* 1997). Because no *hsf-1(sy441); rsks-1(mu482)* double-mutant animals arrest in the first generation but do produce progeny that then arrest, we hypothesize that loss of *rsks-1* does not bypass HSF-1 function, but rather delays or reduces the damage caused by the reduction of HSF-1 function.

It is surprising that we were unable to phenocopy the effects of *rsks-1* mutation through any mechanism other than mTOR knockdown, a condition predicted to reduce *rsks-1* activity. While we cannot know for certain whether a

very specific amount of translation inhibition could rescue the developmental arrest, we have established that multiple methods of reducing translation at multiple concentrations are insufficient to rescue development, and indeed at levels of translation inhibition that significantly reduce animal size, we tended to find a harmful effect on development. If reduced translation were the mechanism of growth arrest suppression, then the situation we found in this study would contrast dramatically with the effects that a similar scan of translation-RNAi knockdowns produced on another phenotype, life span extension. In that case, a wide variety of RNAi knockdowns scored positively, even without careful dose-response analysis (Hansen *et al.* 2007).

Another potential way that translation could be involved in this phenotype is if inhibiting S6 kinase fails to inhibit, or even promotes, the translation of a specific subset of genes. Many stress responses, such as the heat-shock response and integrated stress response, activate a subset of genes while inhibiting translation generally. If S6 kinase inhibition causes a similar effect, then chemical or RNAi-mediated inhibition of translation alone, without activation of specific targets, would not be enough to rescue development.

Other than the small inhibition of rescue caused by *xbp-1* knockdown, and the rescue caused by knockdown of *daf-15* and *ragc-1*, we did not identify other genes known to interact with *rsks-1* that are related to this phenotype. The finding that *daf-2* and *cco-1* knockdown do not rescue development makes it unlikely that the rescue is mediated by some general increase in stress resistance or life span-increasing pathways. Even so, it was surprising to us that knockdown of the transcription factors *daf-16*, *pha-4*, and *skn-1* failed to prevent the rescue phenotype. These transcription factors are essential for stress resistance and life span extension produced by inhibiting components of the mTOR pathway. In addition, we saw no evidence across 65 genes, either predicted to be targets of S6 kinase or proteins known to interact functionally with S6 kinase, of RNAi inhibition rescuing development of *hsf-1(sy441)* mutants or having a more severe effect on the development of *hsf-1(sy441); rsks-1* double mutants vs. wild-type. While we cannot be sure from these experiments of the efficacy of each individual RNAi clone, in each experiment positive controls were present, and in some cases a visible phenotype (other than rescue) was produced by the RNAi clone, such as loss of eggs in animals with inhibited *pha-4*.

Despite the *rsks-1* mutants requiring some level of *hsf-1* function to rescue the *hsf-1(sy441)* arrest phenotype, we saw no evidence of an activation of known *hsf-1*-activated genes or other stress-response pathways. In yeast, only two heat-shock proteins are required to rescue the loss of *hsf-1* for development (Solís *et al.* 2016); therefore, it is possible that one or more unmeasured or noncanonical heat-shock proteins are independently regulated by *hsf-1* and *rsks-1*, and it is through this unknown gene(s) that development is being rescued. For instance, Baird *et al.* (2014) previously showed that overexpression of the *sy441* allele improves cytoskeletal integrity through upregulation of *pat-10*. We did not test the

effects on *rsks-1* mutation on cytoskeletal integrity, so it remains a possible explanation for the rescue phenotype caused by *rsks-1* mutation.

Most of the experiments in this study involved either modulating the expression levels of genes with RNAi or measuring expression levels through RT-qPCR. As a kinase, it is possible that protein S6 kinase modulates the activity of proteins directly without affecting expression levels. If such an activity increase activated the heat-shock response, the ER UPR, or the mito-UPR broadly, we would expect to measure it through an increased expression of canonical target genes, but it is possible that S6 kinase phosphorylates specific proteins in these pathways to mediate developmental arrest. This would be interesting to investigate in the future.

In conclusion, a genetic screen for suppressors of the developmental growth arrest of *hsf-1(sy441)* partial loss-of-function mutants revealed a previously unknown relationship between heat-shock factor and mTOR/S6 kinase activity. Reducing S6 kinase activity allows animals with insufficient HSF-1 activity to progress much further through the life cycle than would otherwise be possible. Because heat-shock factor is known to enhance proteostasis under conditions of heat stress, we propose that loss of S6 kinase postpones arrest, either by reducing the levels of damaged macromolecules produced in the cell, or by increasing the cell's ability to remove or repair them.

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