Cytosolic and mitochondrial tRNA synthetase inhibitors increase lifespan in a GCN4/atf-4-dependent manner

Highlights
- tRNA Synthetase inhibitors significantly extend lifespan
- This lifespan extension is completely dependent on GCN4/atf-4
- Other effects appear separable from lifespan extension
- Lowered overall translation is not sufficient for the observed extension
Cytosolic and mitochondrial tRNA synthetase inhibitors increase lifespan in a GCN4/atf-4-dependent manner

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SUMMARY
Deletion of genes encoding ribosomal proteins extends lifespan in yeast. This increases translation of the functionally conserved transcription factor Gcn4, and lifespan extension in these mutants is GCN4-dependent. Gcn4 is also translationally upregulated by uncharged tRNAs, as are its Caenorhabditis elegans and mammalian functional orthologs. Here, we show that cytosolic tRNA synthetase inhibitors upregulate Gcn4 translation and extend yeast lifespan in a Gcn4-dependent manner. This cytosolic tRNA synthetase inhibitor is also able to extend the lifespan of C. elegans in an atf-4-dependent manner. We show that mitochondrial tRNA synthetase inhibitors greatly extend the lifespan of C. elegans, and this depends on atf-4. This suggests that perturbations of both cytosolic and mitochondrial translation may act in part via the same downstream pathway. These findings establish GCN4 orthologs as conserved longevity factors and, as long-lived mice exhibit elevated ATF4, leave open the possibility that tRNA synthetase inhibitors could also extend lifespan in mammals.

INTRODUCTION
Aging is known to be a modifiable phenotype. Multiple pathways have been identified that are conserved from nonvertebrate models through mice (Blüher et al., 2003; Holzenberger et al., 2003; Kennedy et al., 1995; Kenyon et al., 1993; Klass, 1983; McCormick et al., 2013), and these have now led to drug trials in dogs (Juffer et al., 2017) and humans (Mannick et al., 2014). Furthermore, many model organisms exhibiting increased lifespan also show delayed onset of many phenotypes and diseases of aging (Garigan et al., 2002; Martin-Montalvo et al., 2013; Vora et al., 2013), suggesting that these conserved interventions extend the healthy disease-free period of life, or healthspan, in humans.

In our previously completed genome-wide screen for increased replicative lifespan (RLS) in the budding yeast Saccharomyces cerevisiae, we identified many deletions of ribosomal protein-encoding genes and found that this category of genes is statistically overrepresented among deletions that increase the RLS of yeast (McCormick et al., 2015; Steffen et al., 2008). These ribosomal protein gene deletions lead to increased translation of the nutrient-responsive yeast transcription factor Gcn4, and the increased RLS of these strains is mostly suppressed by the deletion of GCN4 (Steffen et al., 2008, 2012). The Gcn4 pathway is functionally conserved from yeast through humans. In Caenorhabditis elegans, a very successful model organism for the study of aging, the functional ortholog of yeast GCN4 is atf-4, which until recently was referred to as atf-5, and in mammals, it is ATF4.

In addition to being upregulated by the deletion of yeast ribosomal proteins, Gcn4 translation can also be upregulated by the accumulation of uncharged tRNAs (Krupitza and Thireos, 1990; Wek et al., 1989). tRNA Synthetases, which promote the association of an amino acid to the appropriate tRNA, are highly conserved essential proteins, and as such, several species have evolved to form highly specific inhibitors of tRNA synthetase activity (O’Donoghue and Luthey-Schulten, 2003). We reasoned that it might be possible to identify a dose range for these compounds that did not appreciably inhibit growth or fitness by completely blocking translation but still led to an accumulation of uncharged tRNAs. This could...
potentially increase Gcn4 translation and recapitulate the increased lifespan we saw in ribosomal protein-deletion mutants with increased Gcn4. If this were the case, we would expect any increase in lifespan to be GCN4/ATF4 dependent.

Interestingly, work in mice has now shown that multiple types of long-lived mice show increased ATF4 levels (Li and Miller, 2015; Li et al., 2014). Thus, compounds that increased the lifespan in invertebrates by upregulating the Gcn4 pathway might lead to drug treatments that could extend the lifespan in mammals. We identified a biologically derived tRNA synthetase inhibitor, borrelidin, that leads to large increases in Gcn4 translation that are completely dependent on the uncharged tRNA sensor Gcn2. Borrelidin extends yeast RLS specifically at highly Gcn4-inducing doses, and this increase in lifespan depends completely on the presence of GCN4. We then asked whether this phenotype might be conserved and whether uncharged tRNAs generated by specific disruption of mitochondrial translation might trigger this response. Along with borrelidin, we identified another mitochondrial tRNA synthetase inhibitor, mupirocin, that greatly extends the lifespan of C. elegans in a dose-dependent manner. We found that this increased lifespan due to both these inhibitors is completely dependent on the presence of atf-4, the C. elegans functional ortholog of GCN4.

RESULTS

The tRNA synthetase inhibitor borrelidin inhibits yeast growth in a dose-dependent manner

Borrelidin is a biologically synthesized polyketide that inhibits threonyl tRNA synthetase. It is produced by several Streptomyces species and was first isolated from Streptomyces rochei (Berger et al., 1949). We reasoned that at a low-enough dose to prevent lethality, an appropriate tRNA synthetase inhibitor might still lead to accumulation of uncharged tRNAs, thus activating Gcn2, leading to phosphorylation of Sui2 (yeast eIF2α), and thus to increased translation of Gcn4 (Hinnebusch, 2005; Krupitza and Thireos, 1990), as outlined in Figure 1A. In yeast, doubling time, or the mean time for a population to double in number, is a direct and commonly used measure of growth rate. Specifically, during the exponential (“log”) growth phase in yeast, where \( N_0 = N_t \cdot e^{(gR \cdot t)} \) (\( N_0 \), number of yeast at time zero; \( N_t \), number of yeast at time \( t \)), the doubling time \( T_d \) and the growth rate \( g_R \) are related by the formula \( T_d = \ln(2)/g_R \). We first measured the effects of borrelidin on the yeast growth rate, quantified in terms of doubling time, for yeast grown at a wide range of borrelidin concentrations in a standard liquid media (Yeast Extract Peptone +2% glucose at 30°C). The results, shown in Figure 1B, indicate that a borrelidin concentration of 80 μM is able to greatly increase the yeast doubling time without completely stopping growth, while concentrations below 20 μM have no significant effect on the yeast doubling time. This suggests that concentrations above 20 μM should be biologically active for yeast grown in liquid media. Using these data as a starting estimate of the biologically active range of borrelidin concentrations in yeast liquid media, we then measured yeast growth on solid media (YEP agar +2% glucose at 30°C), at a range of borrelidin concentrations, finding that concentrations from 40 to 320 μM were sufficient to greatly inhibit growth, while concentrations below 20 μM did not appreciably slow growth. We repeated these experiments on solid media kept at both 4°C and 30°C for 15 days in order to confirm that the biological activity of borrelidin was not lost under conditions of yeast RLS experiments. Growth on solid media in all cases tested is shown in Figure 1C. The increased amounts of borrelidin needed to produce the same growth inhibition on solid media presumably reflect a less-efficient uptake of the drug by yeast under these conditions. Additionally, our results agree with a large-scale review of drug screening in S. cerevisiae with diverse compounds, in both solid and liquid media, in which the authors noted that solid media-based assays typically require 1–2 orders of magnitude higher drug concentrations than liquid (Smith et al., 2010).

Borrelidin induces Gcn4 translation at concentrations that do not inhibit growth, in a dose-dependent and completely GCN2-dependent manner

Once we had identified biologically active doses of borrelidin in both solid and liquid media, we asked whether these doses could induce translation of Gcn4. Gcn4 is regulated at the level of translation by Gcn2/eIF2α (and thus by uncharged tRNA accumulation), through the activity of upstream open reading frames (uORFs) in the S’ untranslated region of the GCN4 mRNA (Mueller and Hinnebusch, 1986), and this mechanism of translational regulation is conserved through mammals (Vattem and Wek, 2004). We measured Gcn4 translation using a dual-luciferase reporter (Steffen et al., 2008) with the PGK1 (yeast 3-phosphoglycerate kinase) promoter driving Renilla luciferase as an on-plasmid control, as
illustrated in Figure 2A. In liquid media, concentrations of borrelidin from 0.16 to 5 μM showed increasing induction of Gcn4 up to 5-fold over control and then decreased as the concentrations reached those that inhibit yeast growth in liquid media, from 10 to 80 μM, as illustrated in Figure 2B showing fold induction relative to DMSO-only control. In parallel, we measured the effect of borrelidin on gcn2Δ yeast, finding this strain refractory to induction of Gcn4 (Figure 2C), as predicted by the pathway outlined in Figure 1A. This pathway also suggests that increased Gcn4 translation should depend on SUI2 (yeast eIF2α), but this could not be tested directly as SUI2 is an essential gene in yeast (Cigan et al., 1989). We next asked which doses of borrelidin could induce increased Gcn4 translation of yeast grown on solid media, and we found that roughly 8-fold higher doses were required to achieve the same increased translation of Gcn4 relative to liquid media, as observed in the case of growth inhibition (Figure 2D). We determined whether the Gcn4-inducing activity of borrelidin in solid media was decreased after 15 days at 30°C, in order to estimate whether this activity would be likely to be maintained over the course of a yeast RLS experiment. We found that there was no loss in Gcn4 induction by borrelidin in solid media after 2 weeks at 30°C (Figure 2E), again shown as fold induction relative to DMSO-only control. While our dual-luciferase reporter will reflect any changes in both transcription and translation of GCN4, GCN2 is only known to act in the regulation of the translation of GCN4, not transcription. Finally, we measured the overall effects of borrelidin on protein synthesis in both wild-type and gcn4Δ yeast (Figures 2F and 2G). While there is a predictable dose-dependent decrease in protein synthesis in wild-type yeast treated with the tRNA synthetase inhibitor, this effect is not dependent on GCN4. Taken together, these data further suggest that the observed changes in GCN4 are due to changes specifically in translation, which are induced at certain biologically active concentrations of the tRNA synthetase inhibitor, borrelidin via Gcn2.)
Borrelidin increases yeast RLS specifically at the most Gcn4-inducing doses, in a completely GCN4-dependent manner

After identifying borrelidin concentrations that increased Gcn4 translation in yeast grown on solid media and that maintained this activity after 2 weeks at 30°C, we asked whether these concentrations could increase yeast RLS, as was seen previously in ribosomal protein deletion strains that induce Gcn4 translation (Steffen et al., 2008, 2012). As shown in Figure 3A, yeast RLS was increased by borrelidin, at a highly Gcn4-inducing dose as shown in Figure 2D. Perhaps unsurprisingly, very high doses of borrelidin that greatly inhibit yeast growth showed decreased RLS (Figure S1A, related to Figure 3). We also asked whether the increased RLS seen in borrelidin-treated yeast required GCN4, as predicted by the pathway outlined in Figure 1A. Figure 3B shows that in gcn4Δ yeast, borrelidin treatment does not increase yeast RLS at all, and in fact, it slightly shortens it. At very high doses of borrelidin that greatly inhibit yeast growth, RLS is shortened.

Figure 2. Borrelidin affects Gcn4 translation
(A) A yeast dual-luciferase reporter plasmid used to measure Gcn4 translation.
(B) Borrelidin increases Gcn4 translation in wild-type yeast in a dose-dependent manner in liquid culture.
(C) Borrelidin causes no increase in Gcn4 translation in gcn2Δ yeast in liquid culture.
(D) Borrelidin increases Gcn4 translation in wild-type yeast grown on solid media, in a dose-dependent manner.
(E) Borrelidin retains Gcn4-inducing activity in solid media after 15 days at 30°C.
(F and G) Borrelidin decreases protein synthesis in a dose-dependent manner independent of GCN4. In B, C, D, and E, values are reported specifically as fold-change relative to DMSO-only control = 1, where error bars represent standard error of the mean ratio, *, p < 0.05; ***, p < 0.001.

Borrelidin increases yeast RLS specifically at the most Gcn4-inducing doses, in a completely GCN4-dependent manner

After identifying borrelidin concentrations that increased Gcn4 translation in yeast grown on solid media and that maintained this activity after 2 weeks at 30°C, we asked whether these concentrations could increase yeast RLS, as was seen previously in ribosomal protein deletion strains that induce Gcn4 translation (Steffen et al., 2008, 2012). As shown in Figure 3A, yeast RLS was increased by borrelidin, at a highly Gcn4-inducing dose as shown in Figure 2D. Perhaps unsurprisingly, very high doses of borrelidin that greatly inhibit yeast growth showed decreased RLS (Figure S1A, related to Figure 3). We also asked whether the increased RLS seen in borrelidin-treated yeast required GCN4, as predicted by the pathway outlined in Figure 1A. Figure 3B shows that in gcn4Δ yeast, borrelidin treatment does not increase yeast RLS at all, and in fact, it slightly shortens it. At very high doses of borrelidin that greatly inhibit yeast growth, RLS is shortened.
in gcn4Δ yeast exactly as it is in wild-type (Figure S1B, related to Figure 3). This suggests that while the extended lifespan at lower doses of borrelidin due to the accumulation of uncharged tRNAs is dependent on GCN4, the shortened lifespan at much higher growth-inhibiting concentrations is a consequence of blocked overall translation and does not act through Gcn4. These data fit a model where tRNA synthetase inhibitor-induced lifespan extension is the result of uncharged tRNAs activating GCN2-eIF2alpha-GCN4 signaling in a GCN4-dependent manner. However, an excessive accumulation of uncharged tRNA can interfere with overall translation in a GCN4-independent manner that does not act through GCN2 or eIF2alpha. Furthermore, these data suggest that any lifespan effects of tRNA synthetase inhibition are likely to be extremely dose dependent. We wanted to determine if this lifespan extension by borrelidin could be genetically modeled. As deletion of THS1 is lethal, we utilized the Tet-Off system that offers a tunable reduction of gene expression by the addition of varying concentrations of doxycycline (Mnaimneh et al., 2004). The yeast Tet-promoters collection offers two different THS1 strains. Both strains have significantly increased lifespan when treated with doxycycline while the control R1158 strain does not (Figure S2, related to Figure 3). While borrelidin is capable of having other biological effects such as roles in the unfolded protein response, synthesis of nitrile, and angiogenesis in mammals (Wakabayashi et al., 1997; Wang et al., 2015), the GCN2 dependence of the Gcn4 upregulation, and GCN4 dependence of the lifespan extension, leads us to favor a model whereby the tRNA synthetase inhibitor activity of borrelidin is key to the increased lifespan we have observed.
Mupirocin delays C. elegans development in a dose-dependent manner that is not dependent on atf-4

Previous work has shown that depletion of C. elegans mitochondrial ribosomal subunits by RNAi knockdown increases lifespan (Hansen et al., 2007; Houtkooper et al., 2013), and very recently, lifespan extension by knockdown of one of these, mrps-5, has been shown to partially depend on atf-4 (Molenaars et al., 2020), providing further evidence of a conserved mechanism. Based on this previous work, we wanted to ask whether the phenotypes we had observed could be recapitulated by the inhibition of mitochondria-specific tRNA synthetases. Mitochondrial translation and mitochondrial tRNA synthetase activity have been recently thoroughly reviewed (D’Souza and Minczuk, 2018), but in brief, the mitochondria is responsible for only translating a small number of mitochondrial proteins including mRNAs encoding proteins required for oxidative phosphorylation. While the mitochondrial genome encodes the mitochondrial tRNAs, the mitochondrial tRNA synthetases responsible for charging tRNAs with their cognate amino acid are coded in the nucleus and transported to the mitochondria. Other proteins, such as initiation factors, required for mitochondrial translation are also encoded in the nucleus and transported to the mitochondria. While mitochondrial and cytosolic tRNA perform the same job in different cellular regions, the mitochondrial amino acid code that the charged tRNA recognizes differs from the universal cytosolic code.

Mupirocin is a biologically synthesized inhibitor of isoleucyl tRNA synthetase initially isolated from Pseudomonas fluorescens that shows an 8000-fold greater affinity for prokaryotic Ile-tRNA synthetase over the eukaryotic one (Fuller et al., 1971; Hughes and Mellows, 1980). As a result, the effects in C. elegans should be mediated by inhibition of the C. elegans mitochondrial isoleucyl tRNA synthetase, iars-2, rather than the cytoplasmic isoleucyl tRNA synthetase, iars-1. We first assayed the biological activity of mupirocin on C. elegans by exposing multiple C. elegans eggs in liquid culture to varying concentrations of mupirocin. At all concentrations, eggs developed normally into healthy adults, but they did so at varying rates. Doses below 0.04 mM showed no discernible effect, while doses between 0.1 mM and 5 mM showed increasingly slower development through all larval stages, without any noticeable growth arrest (Figure 4A). Development was measured every 24 h for 10 worms per well per day until adulthood was reached. There was no discernible variation in development within wells. Interestingly, these developmental delays are completely independent of the presence of worm atf-4 (Figure 4B), suggesting that they are a consequence of lowered overall translation during the rapid growth phase of C. elegans development due to tRNA synthetase inhibition and not downstream of the Gcn4/ATF-4/ATF4 pathway signaling. Having confirmed that there was a range of doses that were biologically active on C. elegans in liquid culture, we turned next to solid culture, as this is the media we use in lifespan assays. We found that, again, mupirocin treatment led to delays in development without arrest consistent with liquid developmental delay data, with no discernible delay for doses up to 0.04 mM, and increasingly slowed development for doses from 0.15 to 3.08 mM. This was measured for an average of 34 worms per strain and mupirocin concentration, spread onto four plates, with almost zero variability observed both within and between plates for all worms at a given strain and concentration. Quantification of times to reach adulthood from egg on solid media for wild-type worms at various concentrations of mupirocin is shown in Figure 4C. As with liquid culture, all developmental delays were completely unchanged in worms deleted for atf-4, specifically atf-4(ok576), as shown in Figure 4D.

Representative growth images for wild-type N2 worms on solid media after 72 h at 20°C are shown in Figure 4E, and representative growth images for atf-4(ok576) worms on solid media after 72 h at 20°C are shown in Figure 4F. As mupirocin is thought to act on the mitochondrial Ile-tRNA synthetase, we investigated the effects it has on the development of the already developmentally delayed mitochondrial electron transport chain mutant, isp-1(qm150) (Feng et al., 2001). As seen in Figure 5, related to Figure 4, the developmental delay of mupirocin appears to be additive to the mitochondrial mutant’s developmental delay. At the highest concentrations of mupirocin, not only was this additional developmental delay less consistently dose dependent but many animals failed to develop into adults at all. Taken together, these data show that there is a consistent delay in development in worms treated with tRNA synthetase inhibitors that are independent of atf-4 activity.

Borrelidin and mupirocin greatly extend the lifespan of C. elegans in a dose- and atf-4-dependent manner

Having confirmed that there was a range of doses where mupirocin was biologically active in worms in both liquid and solid media, we then turned to lifespan. All worm lifespans were scored as survival beginning from day 1 of adulthood for that specific cohort of worms, so that any developmental delays did not influence survival times. We observed that mupirocin treatment led to a dramatic increase in mean and
maximum lifespan, with increasing doses of mupirocin leading to increasing lifespan. At no concentrations did we observe any ill effects. This suggests that it is possible that a higher effective dose of tRNA synthetase inhibitor, perhaps through a different route of administration, could possibly increase lifespan even further. Survival curves for worms at increasing concentrations of mupirocin are shown in Figure 5A, and the relationship between dose and mean lifespan is shown in Figure 5B. Significant increases in lifespan in Figure 5A are interpreted relative to the 0-mM control that contains only a DMSO vehicle. This is because DMSO itself (the solvent for mupirocin in these experiments) has previously been shown to more modestly, but significantly, extend lifespan in C. elegans (Frankowski et al., 2013). Survival curves and mean lifespan vs. dose plots also containing the no DMSO and no drug control are shown in Figure S4, related to Figure 5.

Next, we asked whether the increased lifespan seen upon administration of tRNA synthetase inhibitors depended on the presence of the C. elegans functional ortholog of GCN4, atf-4. We tested the effects of mupirocin on lifespan in atf-4(ok576), an atf-4 deletion mutant generated by the C. elegans Deletion Mutant Consortium (C. elegans Deletion Mutant Consortium, 2012). No increase in lifespan was seen at any high concentration of mupirocin in atf-4 deletion mutants, as shown in survival curves in Figure 5C and the dose...
Additionally, we measured the lifespan of the mitochondrial electron transport chain mutant, *isp-1(qm150)*, treated with mupirocin. As seen in **Figure S5A**, related to **Figure 5**, the lifespan extension of *isp-1* mutants is not increased when treated with mupirocin and was lethal at higher concentrations. This is unlike the additive effects seen for developmental delay. We also examined the lifespan effects of mupirocin on a *gcn-2* deletion strain, *gcn-2(ok886)*. As in yeast, this gene functions as
one of the upstream regulators of ATF-4/Gcn4 activity. As seen in Figure S5B, related to Figure 5, at all doses of mupirocin, there is a reduced lifespan extension in gcn-2(ok886) worms compared to wild type, and any extension seen is only significant at the highest concentrations. This slight but significant difference is not seen in atf-4(ok576) worms treated with mupirocin. One potential explanation for this increase is that much of the lifespan extension occurs in a gcn-2-mediated manner, but there may be an additional mechanism involved that acts at higher concentrations in an atf-4-dependent but gcn-2-independent manner. As the C. elegans mitochondrial isoleucyl tRNA synthetase iars-2 is the predicted target of mupirocin, we asked whether RNAi knockdown of iars-2 had any effect on lifespan in wild-type or atf-4(ok576) worms and found that it did not (Figure S6, related to Figure 5). We hypothesize that there is a narrow beneficial range of tRNA synthetase activity reduction, where atf-4-dependent mechanisms are activated yet not offset by deleterious effects of overall lowered translation. It is possible that the reduction of iars-2 activity by RNAi using 1-mM isopropyl β-D-1-thiogalactopyranoside for induction did not induce a level of reduction in this beneficial range. Another possible explanation is that another gene such as iars-1 is able to compensate for the loss of iars-2 activity. In addition to mupirocin, we found that borrelidin is able to significantly extend the lifespan of worms at lower concentrations in a dose-dependent manner (Figure 5E). This lifespan extension is also dependent on atf-4 (Figure 5F). Again, RNAi knockdown of iars-1, the target of borrelidin, is unable to mimic this effect (Figure S6, related to Figure 5). This shows that lifespan extension through drug-induced uncharged cytosolic and mitochondrial tRNA is dependent on atf-4.

We have found that mupirocin is unable to increase lifespan in yeast in glucose- or glycerol-containing media (Figure S7, related to Figure 5). We propose that this could be due to the mitochondrial-specific nature of the drug. As yeast do not require the use of their mitochondria for fermentation, it is possible that a drug that primarily effects a mitochondrial tRNA synthetase might be less likely to lead to a phenotype in yeast.

**Mupirocin acts primarily during adulthood on a worm’s lifespan**

As mupirocin has the most significant lifespan extension, we determined the life stages during which mupirocin predominantly exerts its lifespan-extending effects in worms. This was in part due to the effects of mupirocin on developmental timing although these were clearly shown to be completely independent of atf-4. While the lifespan shown in Figures 5A and 5C depicts treated worms from hatching until death, we next compared treatments at different times. One condition, adult-only, involved eggs hatched and grown to adulthood on solid media with no drug and then transferred at adulthood to plates containing varying concentrations of mupirocin. The other, development-only, involved eggs grown on varying concentrations of mupirocin from hatching to adulthood and then transferred to plates containing no drug from then until death. The survival curves for the adult-only mupirocin treatment are shown in Figure 6A, and those for development-only mupirocin in Figure 6B. As before, lifespan differences were compared to the 0-mM DMSO-only control, but complete data with no drug and no DMSO control can also be found in Figure S8, related to Figure 6. These results suggest that the lifespan-extending effects of mupirocin treatment are largely separable from the developmental delays, as adult-only treatment extends lifespan less than whole-life treatment, and development-only treatment extends lifespan still less than adult-only treatment. The fact that adult-only treatment can clearly still extend lifespan again supports a model in which the developmental delay effect of mupirocin is separable from its effect on lifespan. At the same time, we propose that the decreasing effect on lifespan seen by whole-life treatment > adult-only treatment > development-only treatment can be explained by the relative times that worms are exposed to mupirocin in these three treatments: longest for whole-life, then for adult-only, and shortest for development-only.

**DISCUSSION**

Aging is a common underlying risk factor for many of the most significant causes of mortality and morbidity in most countries, such as Alzheimer’s disease, cancer, and heart disease. By studying pathways that can delay aging, we may be able to eventually delay the onset of these and other diseases and perhaps uncover the underlying causes of aging itself. Of the pathways now known to greatly affect aging, several, such as TOR signaling (Jia et al., 2004; Kaaberlein et al., 2005; Kapahi et al., 2004; Powers et al., 2006), insulin/IGF-1 signaling (Blüher et al., 2003; Clancy et al., 2001; Holzenberger et al., 2003; Kenyon et al., 1993), and chemo-sensation of food (Alcedo and Kenyon, 2004; Libert et al., 2007) involve nutrient sensing. Gcn4 is a nutrient-sensing protein that responds in part to nitrogen starvation, which can be upregulated by accumulation of uncharged tRNAs (Krupitza and Thireos, 1990; Wek et al., 1989) or by the deletion of cytosolic ribosomal proteins in yeast (Steffen et al., 2008, 2012). Mutations in genes encoding proteins in the cytosolic ribosome
have also been shown to extend lifespan in *C. elegans* (Curran and Ruvkun, 2007; Hansen et al., 2007; Reis-Rodrigues et al., 2012). The data presented here suggest a model (Figure 7) in which accumulation of uncharged tRNAs, whether due to inhibition of mitochondria-specific tRNA synthetases or cytosolic ribosome tRNA synthetases, can lead to activation of the Gcn4/ATF-4/ATF4 nutrient-sensing pathway, and from there to increased lifespan in multiple organisms. Another group has recently shown that inhibition of the integrated stress response extends lifespan in *C. elegans* (Derisbourg et al., 2021); however, we have clearly shown that the lifespan extension caused by tRNA synthetase inhibitors is dependent on the activity of this very pathway. While these compounds can clearly have an effect on other proteins, we have shown a clear Gcn4 dependence on lifespan extension. It is likely that the inhibition of tRNA synthetases will affect the translation of other proteins, and a search for epistatic modifiers could identify other translational changes that are important to the observed increased lifespan.

It makes sense that cells might interpret uncharged tRNAs as a sign of amino acid starvation and translational stress, whether these arise from problems with cytosolic translation or with mitochondrial translation. Indeed, given the mechanism of uncharged tRNA sensing by Gcn2 and its orthologs (Krupitza and Thireos, 1990; Wek et al., 1989), it is hard to imagine how this sensor protein can distinguish tRNAs from these two different origins. This suggests more broadly that uncharged tRNAs may signal mitochondrial translation defects and that some types of translation stress can lead to the same transcriptional and cellular responses, whether they are cytosolic or mitochondrial in origin. Other work has also indicated methods

![Figure 6. Mupirocin primarily acts during adulthood to extend lifespan](image)

(A) Lifespans of worms treated with mupirocin during adulthood only.
(B) Lifespans of worms treated with mupirocin during development only. Legends indicate (mean lifespan, number of worms scored). *, p < 0.01; **, p < 0.001. See also Figure S8.
of tRNA transport including export from the mitochondria to the cytosol where Gcn2 and its orthologs may be able to encounter uncharged mitochondrial tRNA (Maniataki and Mourelatos, 2005). These transport mechanisms may be responsible for the activation of Gcn2 and upregulation of Gcn4 from uncharged mitochondrial tRNA, but this will need further investigation. While our current model is that mupirocin only acts as an inhibitor of mitochondrial Ile-tRNA synthetase as it shows a strong preference for prokaryotic tRNA synthetase, we have not ruled out the possibility that mupirocin could also act on the cytosolic Ile-tRNA synthetase, or even on other previously undiscovered targets. Previous work supports our model as mupirocin-resistant parasites developed mutations in their organelle-encoded tRNA synthetases rather than their cytosolic forms (Istvan et al., 2011). Further studies will be needed to discover any additional activities of this drug.

We have shown by our use of pharmacological agents that there is a fine-tuned concentration in order to achieve the optimal effect and observed changes in lifespan without halting translation altogether or to a deleterious extent. Deletion of many tRNA synthetases in yeast and worms is lethal, and we have found that the use of the Tet-off system in yeast or RNAi in worms is not capable of eliciting the same effect in our hands and is perhaps sensitive enough to mimic the precise effect. Other work has also shown that tRNA synthetase RNAi knockdown is able to extend lifespan in C. elegans, particularly under starvation conditions (Webster et al., 2017), or in the case of mitochondrial tRNA synthetase RNAi, it is able to impair mitochondrial function and extend lifespan (Lee et al., 2003). Others have shown RNAi knockdown of methionyl-tRNA synthetase can increase lifespan in Drosophila (Suh et al., 2020), further suggesting a conserved mechanism of lifespan extension.

While inhibition of both cytosolic and mitochondrial tRNA synthetases using mupirocin and borrelidin is able to significantly extend lifespan in C. elegans, only borrelidin has been able to increase yeast-RLS under
conditions we have tested. We show that both compounds extend lifespan through Gcn4/ATF-4, but the
differences in drug activity will need to be further investigated. The differences between single and multi-
cellular organisms are an obvious potential cause of these observed reactions. Yeast and worms also differ
in their need for mitochondrial respiratory function. Yeast is capable of surviving without the respiratory
function of their mitochondria, and this biological quirk could also be responsible for the lack of mupirocin
effects seen in yeast.

These findings have broader impacts, as several longevity-inducing interventions in mammals are associ-
ated with increased levels of the mammalian Gcn4/ATF-4 ortholog ATF4 (Li and Miller, 2015; Li et al., 2014).
Given that many antibiotics target mitochondrial translation and are well-tolerated, these findings suggest
new strategies for interventions to impact lifespan and healthspan. Moreover, they call for a more complete
understanding of the role of ATF4 in mammalian aging.

Limitations of the study
Our interpretation of the results reported here makes the assumption that for both borrelidin and mupir-
ocin, the phenotypes we have observed are primarily due to their activities as inhibitors of tRNA synthetase
activity. While some of the genetic experiments, such as dependence specifically on the uncharged tRNA-
sensing kinase GCN2/gcn-2, are supportive of this interpretation, we have not directly demonstrated any
change in levels of charged and uncharged tRNA upon drug treatment. This leaves open the possibility that
rather than tRNA synthetase inhibition, other previously reported targets or activities of these two drugs,
which we have drawn attention in the main text, or even other previously unknown targets or activities could
be responsible for the phenotypes we have observed.

STAR+METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105410.

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C.E.R., B.W., B.K.K., and M.A.M. conceived the experiments and wrote the manuscript. C.E.R., B.P., D.L.S., and M.A.M. generated the yeast strains and contributed to execution and data analysis of all experiments. B.K.K. and M.A.M. provided reagents and support for the studies. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                      | SOURCE                                           | IDENTIFIER |
|------------------------------------------|--------------------------------------------------|------------|
| **Bacterial and virus strains**          |                                                  |            |
| *E. coli* OP-50                          | Caenorhabditis genetic center                    | OP50       |
|                                          | https://cgc.umn.edu/                             |            |
| iars-2 RNAi *E. coli*                    | Ahringer RNAi library, Horizon                   | iars-2 RNAi|
| tars-1 RNAi *E. coli*                    | Ahringer RNAi library, Horizon                   | tars-1 RNAi|
|                                          |                                                  |            |
| **Chemicals, peptides, and recombinant proteins** |                                                  |            |
| Borrelidin CAS# 7184-60-3                 | BioViotica                                       | Cat#BVT-0098|
| Mupirocin CAS# 12650-69-0                | BOC Sciences                                     | Cat#80084-056590|
| **Critical commercial assays**           |                                                  |            |
| Promega Dual Luciferase Reporter Assay System | Promega                                          | Cat#E1980  |
| **Experimental models: Organisms/strains** |                                                  |            |
| WT MATa *S. cerevisiae*                  | Yeast deletion collection, Horizon               | BY4741     |
|                                          | Discovery, Boulder, CO, USA                      |            |
| WT MATa *S. cerevisiae*                  | Yeast deletion collection, Horizon               | BY4742     |
|                                          | Discovery, Boulder, CO, USA                      |            |
| gcn4J MATa *S. cerevisiae*               | Yeast deletion collection, Horizon               | gcn4J MATa|
|                                          | Discovery, Boulder, CO, USA                      |            |
| gcn4J MATa *S. cerevisiae*               | Yeast deletion collection, Horizon               | gcn4J MATa|
|                                          | Discovery, Boulder, CO, USA                      |            |
| gcn2J MATa *S. cerevisiae*               | Yeast deletion collection, Horizon               | gcn2J MATa|
|                                          | Discovery, Boulder, CO, USA                      |            |
| gcn2J MATa *S. cerevisiae*               | Yeast deletion collection, Horizon               | gcn2J MATa|
|                                          | Discovery, Boulder, CO, USA                      |            |
| gcn2J MATa *S. cerevisiae*               | Yeast deletion collection, Horizon               | gcn2J MATa|
|                                          | Discovery, Boulder, CO, USA                      |            |
| N2 *C. elegans*                          | Caenorhabditis genetics center                   | N2         |
|                                          | https://cgc.umn.edu/                             |            |
| atrf-4(ok576) *C. elegans*               | Caenorhabditis genetics center                   | RB790      |
|                                          | https://cgc.umn.edu/                             |            |
| gcn-2(ok886) *C. elegans*                | Caenorhabditis genetics center                   | RB980      |
|                                          | https://cgc.umn.edu/                             |            |
| isp-1(qm150) *C. elegans*                | Caenorhabditis genetics center                   | MQ887      |
|                                          | https://cgc.umn.edu/                             |            |
| THS1 tet-off *S. cerevisiae*             | Yeast tet-off collection, Horizon                | TH3726     |
|                                          | Discovery, Boulder, CO, USA                      |            |
| THS1 tet-off *S. cerevisiae*             | Yeast tet-off collection, Horizon                | TH3727     |
|                                          | Discovery, Boulder, CO, USA                      |            |
| R1158 tet-off control *S. cerevisiae*    | Yeast tet-off collection, Horizon                | R1158      |
|                                          | Discovery, Boulder, CO, USA                      |            |
| **Recombinant DNA**                      |                                                  |            |
| pVW31 dual-luciferase reporter plasmid    | Steffen et al., (2008)                           | pVW31      |

(Continued on next page)
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mark McCormick (mmccormick@salud.unm.edu).

Materials availability
No new reagents were generated in this study. All worm strains used are available from the Caenorhabditis Genetics Center https://cgc.umn.edu/. E. coli OP-50 is available from the Caenorhabditis genetics center https://cgc.umn.edu/. HT115, iars-2 RNAi, and tars-1 RNAi E. coli are available in the Ahringer RNAi library from Horizon Discovery, Boulder, CO, USA. All yeast strains are included in either the yeast deletion collection available from Horizon Discovery, Boulder, CO, USA, or the yeast tet-off promoter collection available from Horizon Discovery, Boulder, CO, USA.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All yeast strains were derived from the parent strains of the haploid yeast ORF deletion collections BY4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), with further deletions (e.g. gcn4Δ or gcn2Δ) marked with the KanMX cassette (Winzeler et al., 1999). The entire collection, as well as individual wild type, gcn4Δ, and gcn2Δ strains in both mating types, are available directly from Horizon Discovery, Boulder, CO, USA. Cells were grown on standard YPD containing 1% yeast extract, 2% peptone and 2% glucose, at 30°C, unless otherwise stated.

All worm strains were maintained at 20°C on NGM plates containing E. coli strain OP-50 as a food source (Brenner, 1974). Wild-type N2 worms, atf-4 deletion worms RB790 atf-4(ok576), isp-1 mutant worms MQ887 isp-1(qm150), and gcn-2 deletion worms RB980 gcn-2(ok886) as well as E. coli strain OP-50 are all available directly from the Caenorhabditis Genetics Center https://cgc.umn.edu/.

METHOD DETAILS

Yeast replicative lifespan
Yeast RLS assays were performed as described in (Kaeberlein et al., 2004; Steffen et al., 2009). Specifically, virgin daughter cells were isolated from each strain and then allowed to grow into mother cells on YPD (yeast extract peptone dextrose) or other media as specified. These cells were micromanipulated into each day in round intervals corresponding to the cell division time at that stage of the RLS (yeast divide more slowly as they age), while grown at 30°C in a Thermo/Thelco 6DG laboratory incubator. These yeast were transferred to 4°C overnight each day. This was done 7 days a week, continuously for the entire RLS, using a 40–160X Trinocular Infinity-corrected Microscope with LED Koehler Illumination (T620A, AmScope, Irvine, CA) and an attached micromanipulation apparatus (Tetrad Manipulator AxioScope A1, w/Stage, Carl

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Software and algorithms | R | https://cran.r-project.org/ |
| Other | Bioscreen C Growth Curve Analyzer | Growth Curves USA, Piscataway, NJ, USA |
| Victor NIVO Multimode Microplate Reader | PerkinElmer | https://www.perkinelmer.com/product/victor-nivo-advanced-f-abs-filter-1u-hh35000500 |

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Zeiss Microscopy, Thornwood, NY), with attached 50 micron optical fiber dissection needles (1050, Styles Lab Supplies, Talent, OR). For each mother cell the corresponding daughters were microdissected and counted, until the mother cell could no longer divide.

**Yeast growth assays**

All yeast growth rates in liquid media were analyzed in the OD$_{420-580}$ range in the Bioscreen C automated microbiology growth curve analysis system (Growth Curves USA, Piscataway, NJ, USA) using the Yeast Outgrowth Data Analyzer (YODA) software (Olsen et al., 2010). For growth on solid media, 50µL of yeast at an OD$_{600}$ of 1 were plated onto 1 mL of YEP agar +2% glucose per well in 24-well plates (CLS3738, Corning, Tewksbury, MA, USA) and growth was photographed every 24h for 120h. Images were quantified using FIJI/ImageJ (Schindelin et al., 2012).

**Dual luciferase assay**

Gcn4 translation was measured using a dual-luciferase reporter plasmid pVW31 (Steffen et al., 2008). Strains were transformed with pVW31, then grown in synthetic glucose minimal medium lacking uracil and containing required amino acids as well as isoleucine and valine (Min D+) (Lucchini et al., 1984). Promega Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA) was used with a Victor X3 or Victor NIVO Multimode microplate reader (PerkinElmer, Waltham, MA, USA).

**Protein synthesis assay**

Total protein synthesis was measured in wild type and gcn4D yeast using a non-radioactive metabolic labeling assay, Click-iT HPG Alexa Fluor 488 Protein Synthesis Assay Kit (Thermo Fisher Scientific) (Mittal et al., 2017). Specifically, this assay utilizes a methionine analog, L-HPG, containing an Alexa Fluor 488 azide. Protein synthesis is determined based on the amount of HPG-Alexa Fluor 488 incorporated and the mean fluorescent intensity was measured by flow cytometry using a BD AccuriTM C6 Flow Cytometer (BD Biosciences) with the support of the UNM Flow Cytometry Shared Resource Facility. Samples were prepared by growing yeast overnight in YPD (yeast extract peptone dextrose) media until mid-log phase at 30°C in a New Brunswick model G25-KC Refrigerated/Illuminated Incubator Shaker at 30°C and 300RPM. Yeast were then washed and re-suspended in synthetic media containing 2% glucose and lacking methionine, and were treated with the indicated concentration of borrelidin for 4h shaking in a New Brunswick model G25-KC Refrigerated/Illuminated Incubator Shaker at 30°C and 300RPM. After 4h, samples were treated and processed exactly according to the protocol provided by the Click-iT HPG Alexa Fluor 488 Protein Synthesis Assay Kit (Thermo Fisher Scientific) (Mittal et al., 2017). For this assay, only BY4742 MATa background yeast were used as the corresponding BY4741 MATa strains are auxotrophic for methionine (met15) and are unable to grow in the required methionine-free media. Assays were conducted in triplicate with each sample reflecting the mean fluorescent intensity of 100,000 events.

**Worm developmental timing**

*C. elegans* eggs were added to standard S-Complete buffer with 6 mg/mL of UV-killed *E. coli* strain OP-50 (available from the Caenorhabditis Genetics Center https://cgc.umn.edu/), and grown at 20°C, with observations taken every 24h. For solid media experiments, *C. elegans* eggs were added to standard *C. elegans* NRM media (Brenner, 1974) in 6cm plates (T3306, Tritech Research, Los Angeles, CA, USA) with UV-killed *E. coli* OP-50, and staged every 24h until adulthood.

**Worm lifespans**

Lifespan analysis was conducted at 20°C unless otherwise stated (Hsin and Kenyon, 1999). Specifically, all lifespans were done on 6cm plates (T3306, Tritech Research, Los Angeles, CA, USA) containing standard NRM media (Brenner, 1974) using on-plate UV-killed bacterial food (*E. coli* strain OP-50 unless otherwise specified) that was first plated at 50µL per lifespan plate in the center of each plate, allowed to grow for 72 h at 25°C, and then treated to 3 x 9999µm Joules x 100 using a UV Stratalinker 2400 (Stratagene, San Diego, CA) on uncovered plates. In all lifespans 0.1mM FUDR (5-fluorodeoxyuridine), (518265, Bio-World, Dublin, Ohio) was added to plates at Day 1 of adulthood in order to suppress development of progeny. All drug treatments were added to plates after UV treatment and before addition of eggs. Each day, all worms were scored for movement using a 6.7X-45X Trinocular Zoom Stereo Microscope with attached incandescent reflected transillumination base (ZM-2T-EB, AmScope, Irvine, CA), and any dead worms removed using a pick made from a 5.75 inch plain soda-lime glass pasteur pipette handle (Thermo-Fisher) and a platinum wire tip.
(Tritech Research, Los Angeles, CA, USA). Bagged, missing (crawled off), or exploded worms were noted and censored daily.

QUANTIFICATION AND STATISTICAL ANALYSIS

All survival curves were plotted using Kaplan-Meier survival curves (Kaplan and Meier, 1958). Statistical significance was determined by calculating p-values using the Wilcoxon Rank-Sum test (Wilcoxon, 1946). Comparisons for developmental timing used Student’s t-test (Student, 1908). Comparisons for yeast doubling time in liquid media and growth on solid media are all versus DMSO only control using Dunnett’s test (Dunnett, 1955). All fold-induction of Gcn4 relative to control is displayed as fold-change with standard error of mean ratio (Sul Lee and Forthofer, 2005). Any noted statistical significance of differences is reported in the corresponding Figure legend. All analysis was performed using R (R Core Team, 2018).