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Investigation

An age-downregulated ribosomal RpS28 protein variant regulates the muscle proteome

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Abstract

Recent evidence indicates that the composition of the ribosome is heterogeneous and that multiple types of specialized ribosomes regulate the synthesis of specific protein subsets. In *Drosophila*, we find that expression of the ribosomal RpS28 protein variants *RpS28a* and *RpS28-like* preferentially occurs in the germline, a tissue resistant to aging and that it significantly declines in skeletal muscle during aging. Muscle-specific overexpression of *RpS28a* at levels similar to those seen in the germline decreases early mortality and promotes the synthesis of a subset of proteins with known anti-aging roles, some of which have preferential expression in the germline. These findings indicate a contribution of specialized ribosomal proteins to the regulation of the muscle proteome during aging.

Keywords: aging; ribosome; protein translation; *Drosophila*; germline; skeletal muscle

Introduction

Protein translation is increasingly recognized as an important regulator of aging. Signaling pathways that increase overall protein synthesis promote aging, whereas interventions that reduce protein synthesis extend lifespan and delay multiple aspects of tissue aging across species (Mehta *et al.* 2010).

The ribosome is the central regulator of protein synthesis, and knockdown of core ribosomal proteins delays aging by decreasing overall protein synthesis, which in turn can induce adaptive stress responses and improve proteostasis (Hansen *et al.* 2007; Steffen and Dillin 2016).

Recently, it was found that the core proteins composing the ribosome are heterogeneous and that multiple versions of the ribosome are present within an organism and even within a single cell (Shi *et al.* 2017). Such specialized subpopulations of ribosomes have been proposed to preferentially promote the synthesis of specific proteins (Xue and Barna 2012; Filipovska and Rackham 2013).

An example of specialized mRNA translation became apparent from analysis of *RpL38* mutant mice. Such mice do not display any substantial change in the rate of development or body size, indicating that RPL38 is not required for maintaining overall protein synthesis. However, ribosomes lacking RPL38 are inefficient at translating a subset of *Hox* mRNAs, leading to developmental patterning defects of the skeleton (Kondrashov *et al.* 2011).

Another example of specialized mRNA translation is evident in yeast ribosomes that incorporate RpL7b, RpL12a, RpL22b, and RpL18a ribosomal protein variants but are incapable of translating *ASH1* mRNA. However, ASH1 protein synthesis occurs when paralog ribosomal proteins are incorporated into the ribosomes (Komili *et al.* 2007).

Altogether, evidence that specialized ribosomes can customize protein synthesis is growing, but a comprehensive understanding of this phenomenon is missing (Xue and Barna 2012; Filipovska and Rackham 2013). Moreover, it is unknown whether the abundance of specialized ribosomes changes with aging or whether such ribosomes regulate any aspect of aging.

Materials and methods

qPCR

Total RNA was extracted with the TRIzol reagent (Life Technologies) from at least 30 thoraces from male flies per group, followed by cleanup with the RNeasy kit (QIAGEN) and on-column DNase digestion. Total RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed with the iQ SYBR Green Supermix and a CFX96 Real-Time PCR Detection System (Bio-Rad). *Alpha-Tubulin 84B* was used as a normalization reference. These data were further normalized by the average gene expression of all ribosomal proteins in the same group (*i.e.*, *RpS* or *RpL* genes). Relative quantitation of mRNA levels was determined with the comparative C_T method. The list of ribosomal proteins was obtained from FlyBase (www.flybase.org) and the RPG database (Nakao *et al.* 2004).

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Cloning and fly transgenesis

The coding sequences of *Drosophila RpS28a* and *RpS28-like* were cloned into the pUAST-attB vector with *EcoRI* and *XbaI* restriction enzymes. The resulting vectors were sequenced to ensure the absence of mutations and injected into w^{1118};*attP2;* embryos via phiC31-mediated transgenesis.

Fly stocks

The *O1*, *B3*, *UAS-foxo*, *UAS-Mnt*, and *Mhc-Gal4* fly stocks have been previously described (Demontis and Perrimon 2010; Demontis *et al.* 2014; Parkhitko *et al.* 2016; Hunt *et al.* 2019).

Lifespan analysis

For survival analysis, male flies were collected within 24 h from eclosion and reared at standard density (25 per vial) on cornmeal/soy flour/yeast fly food at 25°C and 60% humidity. Dead flies were counted every 2 days and the food was changed.

To rule out any variation from cytoplasmic background effects, all crosses were set up with female virgins from the w^{1118};*Mhc-Gal4*. To avoid any contribution of genetic background mutations to the observed lifespan phenotypes, *UAS-RpS28a* and *UAS-RpS28-like* transgenes were injected into a w^{1118} background, and each line was maintained as a mixed population of isogenic siblings carrying either a *UAS-* or no transgene (distinguished by eye color: *white+* and *white−*, respectively). Male siblings carrying either a *UAS-* or no transgene and having the same genetic background were then crossed to homozygous w^{1118};*Mhc-Gal4* (*rosy+ white−*) females and the resulting male progenies were sorted (based on eye color) into isogenic transgene-expressing and transgene-nonexpressing cohorts before lifespan analysis. *UAS-*transgene expression obtained with Mhc-Gal4 was confirmed by qRT-PCR from fly thoraces, which consist mostly of skeletal muscle.

Proteome profiling by TMT-LC/LC-MS/MS

The analysis was performed essentially following our previously optimized method (Bai *et al.* 2017). *Drosophila* thoraces, consisting mostly of skeletal muscle, were lysed by a denaturing 8 M urea-based buffer. Specifically, ∼20 mg of tissue was lysed with ∼400 µl of the lysis buffer in a NextAdvance bullet blender at 4°C, with addition of ∼200 µl of glass beads. The lysate was further centrifuged to remove any remaining cuticle fragments. Protein concentration was determined in the resulting supernatant by a previously described short gel-based staining method (Xu *et al.* 2009). Each sample was digested and desalted, followed by TMT labeling. The labeled samples were equally mixed and fractionated by basic pH reverse-phase liquid chromatography (LC). The LC fractions were collected and further analyzed by acidic pH reverse-phase LC-MS/MS. During ion fragmentation, the TMT regents were cleaved to produce reporter ions for quantification. The collected raw MS data were searched against a database to identify peptides using a hybrid search engine (Wang *et al.* 2014b). While the peptides were identified by MS/MS, the quantification was achieved by the fragmented reporter ions in the same MS/MS scans. The peptide quantification data were then corrected for mixing errors and summarized to derive protein quantification results. Statistical analysis (ANOVA) was performed to determine cutoff for altered proteins (Niu *et al.* 2017).

Computational analyses of mRNA features

A table linking transcript IDs to gene IDs for *Drosophila* genes was obtained from ftp://ftp.flybase.net/releases/current/precomputed_files/genes/fbgn_fbtr_fbpp_fb_2016_04.tsv.gz. Gene sequences were downloaded from the UCSC Genome Browser table browser (Karolchik *et al.* 2004) after selecting the ensGene table from the Ensembl Genes track. 5′ UTR, CDS and 3′ UTR sequences were separately downloaded. The genes were also split into lists according to their increased translation in response to RpS28a. The 5′ UTR, CDS, and 3′ UTR sequences for the genes in each of these lists were analyzed for GC content and length. The results were loaded into R software (v3.2.2) and plotted in boxplots using ggplot. Analysis for motif enrichment was performed with DREME, Discriminative Regular Expression Motif Elicitation (http://meme-suite.org/tools/dreme).

Statistical analyses

Statistical analysis was performed with Excel and the GraphPad Prism software. Unpaired two-tailed Student's *t*-test was used to compare the means of two independent groups with each other.

OASIS (https://sbi.postech.ac.kr/oasis/surv/) and the Fisher's Exact Test were used for the statistical analysis of lifespan data.

Data availability

The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, Supplemental figures, and Supplementary tables. The mass-spectrometry data are provided in Supplementary Table S1. Other primary data are provided in Supplementary Table S2. Supplementary files are available at figshare: https://doi.org/10.25387/g3.14515938.

Results

Expression of RpS28a and RpS28-like declines during skeletal muscle aging

Ribosomes have long been proposed to undergo senescence (Miquel and Johnson 1979) but it is currently unknown whether their composition changes with aging, as observed in other developmental and disease contexts (Filipovska and Rackham 2013).

To determine whether the expression of ribosomal proteins changes with aging in *Drosophila*, we profiled the expression of components of the 60S large and 40S small ribosomal subunits by qPCR in skeletal muscle, a major tissue for lifespan determination (Demontis and Perrimon 2010; Demontis *et al.* 2013; Hunt *et al.* 2021; Rai *et al.* 2021). The expression of most genes encoding ribosomal proteins remained relatively constant in both young and old flies but the mRNA levels of *RpL10Aa*, *RpS28a*, and *RpS28-like* significantly declined with aging (Figure 1A and Supplementary Figure S1).

Expression of RpS28a and RpS28-like is enriched in the germline *vs* somatic tissues and increases in response to anti-aging interventions

Previous studies have suggested that ribosome heterogeneity may arise from changes in the expression of ribosomal protein variants in different cell types and developmental stages (Xue and Barna 2012; Filipovska and Rackham 2013). Therefore, we interrogated the ModEncode gene expression dataset to define whether age-regulated ribosomal proteins also have a distinct pattern of tissue expression in *Drosophila*. Interestingly, whereas most ribosomal proteins were highly expressed in all tissues, the ribosomal protein variants *RpS28a* and *RpS28-like* were preferentially expressed in the germline (testis) and had low expression in somatic tissues (Figure 1A). Importantly, RpS28a and RpS28-like have high sequence similarity with the more highly expressed

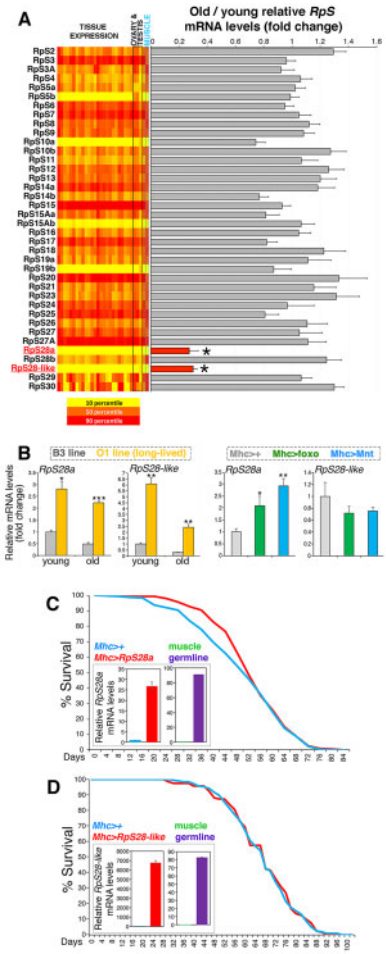


Figure 1 Role of germline- and age-regulated ribosomal RpS28 proteins in longevity. (A) Age-associated changes in ribosomal *RpS* gene expression. Old versus young fold change in *RpS* gene expression reveals a significant decline in *RpS28a* and *RpS28-like* gene expression ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$). ModEncode tissue expression data are reported for a panel of *Drosophila* tissues and developmental stages (see legend of Supplementary Figure S1 for complete listing), including the expression in the germline (ovary and testis; black box), and RNAseq data for skeletal muscle (blue box). Low (yellow) to high (red) expression is indicated. (B) *RpS28a* and *RpS28-like* ribosomal genes are more highly expressed at both young and old age (1 and 6 weeks) in the long-lived O1 *Drosophila* strain (yellow), obtained from multiple rounds of selection for postponed senescence from the B3 strain (grey; $n = 4$, with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). *RpS28a* expression also increases in response to the activity of the anti-aging FoxO and Mnt transcription factors ($n = 4$, with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$), whereas *RpS28-like* levels are not modulated. (C) RpS28a extends lifespan. *RpS28a* overexpression in skeletal muscle with the UAS/Gal4 system and the muscle-specific *Mhc-Gal4* driver results in muscle levels of *RpS28a* closer to those seen in the germline ($n = 4$, $^{***}P < 0.001$) and in increased survival in the first half of the lifespan, compared with isogenic controls (*P*-value at 25% <0.0001, Fisher's Exact test; RpS28a^{OE}: *Mhc>RpS28a*, $n = 492$; isogenic control: *Mhc>+*, $n = 465$). Reduced early mortality upon *RpS28a* overexpression was also seen in another independent trial (Supplementary Figure S3). (D) *RpS28-like* overexpression at levels similar to those in the germline ($n = 4$, $^{***}P < 0.001$) does not regulate lifespan, as compared with isogenic controls (control: *Mhc>+*, $n = 147$; RpS28-like^{OE}: *Mhc>RpS28-like*, $n = 75$).

RpS28b protein (Supplementary Figure S2), suggesting that these alternative RpS28 variants may substitute for RpS28b in ribosome assembly. Altogether, these findings suggest that subpopulations of ribosomes comprising RpS28a and/or RpS28-like are relatively more abundant in the germline than in somatic tissues and that their expression declines during aging in muscle.

Previous studies have shown that the germline is a tissue resistant to aging at least in part due to its increased capacity to maintain proteostasis and mount stress responses (Smelick and Ahmed 2005; Curran *et al.* 2009). For example, reduction in insulin-like signaling causes activation of the transcription factor FOXO and the somatic misexpression of the germline genes *pie-1* and *pgl* in the *C. elegans* intestine. In turn, somatic cells with acquired germline characteristics are protected from genotoxic stress (Curran *et al.* 2009).

Because the *Drosophila* germline had higher *RpS28a* and *RpS28-like* expression than did somatic tissues (Figure 1A), we asked whether these ribosomal proteins, which are also age-downregulated, are differentially expressed in fly strains with distinct lifespans. To this purpose, we analyzed the mRNA levels of *RpS28a* and *RpS28-like* in muscle tissue from the B3 and O1 *Drosophila* lines. The O1 line was derived from the B3 line via multiple rounds of selection for negligible senescence (Rose 1984; Wilson *et al.* 2006) and displays a ~75% increase in the median lifespan (Hunt *et al.* 2019). Interestingly, O1 flies express higher levels of *RpS28a* and *RpS28-like* than do B3 flies at both young and old age (Figure 1B), suggesting that these alternative ribosomal components may contribute to the extreme longevity of the O1 strain. Moreover, overexpression of the transcription factors *foxo* and *Mnt* in muscle, which increases lifespan in *Drosophila* (Demontis and Perrimon 2010; Demontis *et al.* 2014), significantly promoted the expression of *RpS28a*, whereas *RpS28-like* mRNA levels were unaffected (Figure 2B). Altogether, these findings suggest that anti-aging pathways may extend lifespan by altering the expression of ribosomal protein variants.

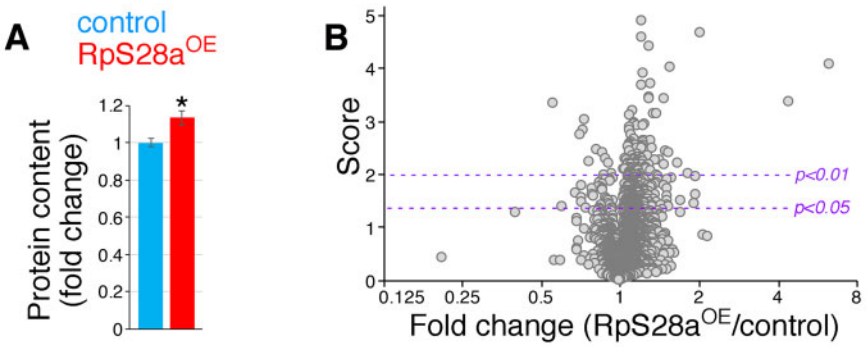
Muscle-specific RpS28a overexpression improves early survival during aging

To test the hypothesis that RpS28a and RpS28-like ribosomal proteins delay aging, we employed the UAS/Gal4 system and the muscle-specific *Mhc-Gal4* driver to increase *RpS28a* expression in skeletal muscle to levels similar to those seen in the germline (Figure 1C). Compared with isogenic siblings with no transgene expression, flies with muscle-specific *RpS28a* overexpression exhibited increased survival in the first half of their lifespan (Figure 1C and Supplementary Figure S3). In contrast, *RpS28-like* overexpression did not extend lifespan (Figure 1D). These findings suggest that RpS28a-containing ribosomes may have unique properties that delay early mortality during aging.

Previous studies have shown that an overall reduction in protein synthesis delays aging across species (Mehta *et al.* 2010; Demontis *et al.* 2014). Therefore, *RpS28a* may delay aging by contributing to the assembly of specialized ribosomes with reduced capacity for overall protein synthesis. To test this hypothesis, we analyzed the total protein content of muscle with *RpS28a* overexpression and found it to be significantly higher than in controls (Figure 2A). This suggests that *RpS28a* overexpression promotes protein synthesis rather than decreasing it, which we also observed with *RpS28-like* overexpression (Supplementary Figure S4). This finding is in line with the observation that RpS28 is indeed necessary for rRNA processing and ribosome assembly (Robledo *et al.* 2008; Gripp *et al.* 2014; Kim *et al.* 2017). Therefore, a mechanism other than reduction in protein synthesis must be responsible for the anti-aging effects of RpS28a.

Muscle-specific RpS28a overexpression increases the protein levels of a subset of proteins, some of which are germline-enriched and/or have anti-aging functions

Previous studies in yeast have shown that mutations in RpS28 influence translation accuracy (Anthony and Liebman 1995)



C

% increase (RpS28^{OE}/control)		enriched in the germline	Human homolog
RpS28a	+526%		RPS28
CG3397	+340%		SRM spermidine synthase
CG10553	+102%		N/A
CG12975	+95%		TRMT112 tRNA methyltransferase 11-2 homolog
CG5145	+94%		N/A
CecA1	+93%		N/A
Ics	+83%		N/A
CG5776	+70%		SPATA5 spermatogenesis associated 5
CG30110	+66%		N/A
CG7601	+58%		DHRS7B dehydrogenase/reductase (SDR family) member 7B
CG7628	+55%		SLC20A1 solute carrier family 20 (phosphate transporter)
CG18508	+54%		C18orf32
CG4580	+54%		ECE2 endothelin converting enzyme 2
CG15715	+52%		ZNF706 zinc finger protein 706
N	+51%		Notch
CG9733	+51%		N/A
CG13611	+51%		MAOA monoamine oxidase A
CG8097	+50%		DALRD3 DALR anticodon binding domain containing 3

D

% increase (RpS28^{OE}/control)		Protein name	Role in aging
CG3397	+340%	CG3397 related to spermidine synthase	synthesis of anti-aging metabolites
CG5776	+70%	SPATA5 spermatogenesis associated 5	mitochondrial function and integrity
CG5144	+36%	creatine kinase	ATP energy homeostasis
Uch-L3	+35%	26S proteasome regulatory complex subunit p37A	protein degradation
GstD10	+30%	Glutathione S transferase D10	oxidative stress resistance
CG14757	+25%	succinate dehydrogenase assembly factor 2-A	mitochondrial function
GstD1	+24%	Glutathione S-transferase 1-1	oxidative stress resistance
MRG15	+24%	NuA4 complex subunit EAF3 homolog	DNA repair
boca	+22%	LDLR chaperone	chaperone
CG8539	+22%	carboxypeptidase A2	protein degradation
CoRest	+22%	REST corepressor	stress resistance
Nep2	+22%	Neprilysin 2	protein degradation
tefu	+22%	Serine/threonine-protein kinase ATM	DNA repair
Ndc1	+21%	Nucleoporin Ndc1	nuclear integrity
Atg8a	+20%	Autophagy-specific gene 8a	protein degradation

Figure 2 Specialized regulation of protein synthesis by RpS28a. (A) *RpS28a* overexpression (OE) increases overall protein content in skeletal muscle when compared with isogenic controls ($P < 0.05$; control: *Mhc*>+, $n = 4$; RpS28a^{OE}: *Mhc*>*RpS28a*, $n = 3$). (B) *RpS28a* overexpression induces proteomic changes limited to a subset of proteins. A significance score, defined as –log10(*P*-value) is indicated. (C) *RpS28a* overexpression induces an increase in the synthesis of several proteins of which half have preferential or enriched expression in the germline (testis and/or ovary; purple). As expected, *RpS28a* overexpression increased RpS28a protein levels. Percent increase in protein levels is indicated ($P < 0.05$; control: *Mhc*>+, $n = 4$; RpS28a^{OE}: *Mhc*>*RpS28a*, $n = 3$). (D) *RpS28a* overexpression increases the synthesis of several proteins which have been implicated in anti-aging responses ($P < 0.05$). The full mass-spectrometry data are provided in Supplementary Table S1.

suggesting that, despite their high sequence homology (Supplementary Figure S2), RpS28 variants may confer distinct accuracy to protein synthesis and consequently regulate lifespan. However, additional anti-aging effects of RpS28a-containing ribosomes are also possible, including the preferential synthesis of specific protein subsets. To test this hypothesis, TMT mass-spectrometry was utilized to profile the proteomic changes induced in skeletal muscle by *RpS28a* overexpression. Overall, 5951 proteins were detected, of which 494 were significantly regulated ($P < 0.05$; Figure 2B and Supplementary Table S1). Analysis of a more stringent set of 128 regulated proteins ($P < 0.05$ and >20% change) indicated that RpS28a promotes the synthesis of a subset of 114 proteins whereas only 14 proteins had lower levels in response to *RpS28a* overexpression (Figure 2B and Supplementary Table S1). Therefore, a primary effect of RpS28a is to promote the synthesis of a subset of proteins. Consultation of the ModEncode tissue expression dataset revealed that 9 out of the 18 most upregulated proteins ($P < 0.05$ and >50% change) had preferential or enriched

expression in the *Drosophila* germline (testis and/or ovary; Figure 2C), suggesting that RpS28a reinforces the synthesis of a subset of germline proteins (Figure 2C).

To better understand whether RpS28a-mediated translational regulation is responsible for the effect of RpS28a on lifespan determination, we next examined whether proteins upregulated by RpS28a have been implicated in aging. Interestingly, some Rps28a-upregulated proteins are known to contribute to several protective responses (Figure 2D) that delay aging and extend lifespan, including preservation of mitochondrial function and integrity (CG5776/SPATA5; CG14757/succinate dehydrogenase assembly factor), stress resistance (CoRest/REST corepressor; GstD1 and GstD10/glutathione S-transferase D1 and D10), DNA repair (tefu/ATM kinase; MRG15/NuA4 complex subunit), protein degradation (Atg8a/autophagy-specific gene 8a; CG8539/carboxypeptidase A2; Uch-L3/26S proteasome regulatory complex subunit p37A), and energy homeostasis (CG5144/creatine kinase). These findings indicate that RpS28a may delay aging by promoting the synthesis of a subset of proteins with anti-aging functions (Figure 2D).

RpS28 is part of the translation initiation complex of eukaryotic ribosomes and is located at the ribosome's exit site, in which it interacts with the 5′ UTR of the mRNA initiating translation (Pisarev *et al.* 2008; Anger *et al.* 2013). Specialized ribosomes with different RpS28 protein variants may therefore have a propensity to translate mRNAs with specific features, as observed for other specialized ribosomes (Xue and Barna 2012; Filipovska and Rackham 2013).

To investigate the basis of preferential protein translation following *RpS28a* overexpression, the GC content and length of the 5′ UTR, coding sequence, and 3′ UTR of the mRNAs corresponding to the upregulated proteins were compared with those of all detected proteins. However, the GC content and length were not significant different (Supplementary Figure S5). Further analyses identified two motifs that were significantly enriched in the 3′ UTR of the mRNAs corresponding to RpS28a-upregulated proteins (Supplementary Figure S5). The 3′ UTR is known to regulate translational efficiency via multiple mechanisms, including mRNA circularization and interaction between the 3′ UTR and 5′ UTR (Mazumder *et al.* 2003), suggesting that RpS28a-containing ribosomes may more efficiently translate mRNAs with certain 3′ UTR features.

Discussion

In this study, we have found that the expression of some ribosomal proteins varies during skeletal muscle aging in *Drosophila*. RpS28a and RpS28-like are the only components of the small ribosomal subunit that decline with aging, suggesting that these RpS28 ribosomal protein variants may contribute to aging. Whereas RpS28-like overexpression does not impact lifespan, we find that RpS28a overexpression in skeletal muscle reduces mortality in the first half of the lifespan. Interestingly, other genes have been previously found to promote survival in the first part of the lifespan without impacting later mortality in *C. elegans* (Wang *et al.* 2014a), suggesting that RpS28a activity in muscle induces a similar response.

Although many mechanisms may explain the impact of RpS28a on aging, we have found that RpS28a overexpression changes the levels of a subset of proteins (494), some of which have known anti-aging roles. Apart from RpS28a, no other ribosomal proteins were upregulated in muscle with RpS28a overexpression (Supplementary Table S1) but most RpS28a-induced changes consisted of upregulated proteins (450), which may contribute to explain the increase in protein levels seen in response to RpS28a overexpression, in addition to the capacity of RpS28 to promote rRNA processing and ribosome assembly (Robledo *et al.* 2008; Gripp *et al.* 2014; Kim *et al.* 2017).

Because RpS28a is a ribosomal protein, it is possible that proteomic changes observed upon its overexpression stem from its direct role in promoting the preferential translation of certain mRNAs. Alternatively, they may derive from indirect effects of Rps28a overexpression on other cellular processes that regulate protein levels, such as protein degradation. Moreover, although we have found preferential enrichment of certain motifs in the mRNAs that encode for RpS28a-upregulated proteins, their significance remains undetermined and experimental testing of such mRNA motifs will be needed to assess whether they are necessary for RpS28a-mediated modulation of mRNA translation.

Several RpS28a-upregulated proteins and RpS28a itself are preferentially expressed in the germline compared to somatic tissues. On this basis, it is possible that expression of RpS28a in the germline may help increase the levels of proteins that define this tissue and its capacity to resist aging.

In summary, this study identifies a previously unanticipated role for age-downregulated Rps28a in regulating the levels of a subset of proteins with known anti-aging roles in skeletal muscle.

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Conflicts of interest

None declared.

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