

Intertissue Control of the Nucleolus via a Myokine-Dependent Longevity Pathway

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SUMMARY

Recent evidence indicates that skeletal muscle influences systemic aging, but little is known about the signaling pathways and muscle-released cytokines (myokines) responsible for this intertissue communication. Here, we show that muscle-specific overexpression of the transcription factor *Mnt* decreases age-related climbing defects and extends lifespan in *Drosophila*. *Mnt* overexpression in muscle autonomously decreases the expression of nucleolar components and systemically decreases rRNA levels and the size of the nucleolus in adipocytes. This nonautonomous control of the nucleolus, a regulator of ribosome biogenesis and lifespan, relies on *Myoglianin*, a myokine induced by *Mnt* and orthologous to human GDF11 and Myostatin. *Myoglianin* overexpression in muscle extends lifespan and decreases nucleolar size in adipocytes by activating p38 mitogen-activated protein kinase (MAPK), whereas *Myoglianin* RNAi in muscle has converse effects. Altogether, these findings highlight a key role for myokine signaling in the integration of signaling events in muscle and distant tissues during aging.

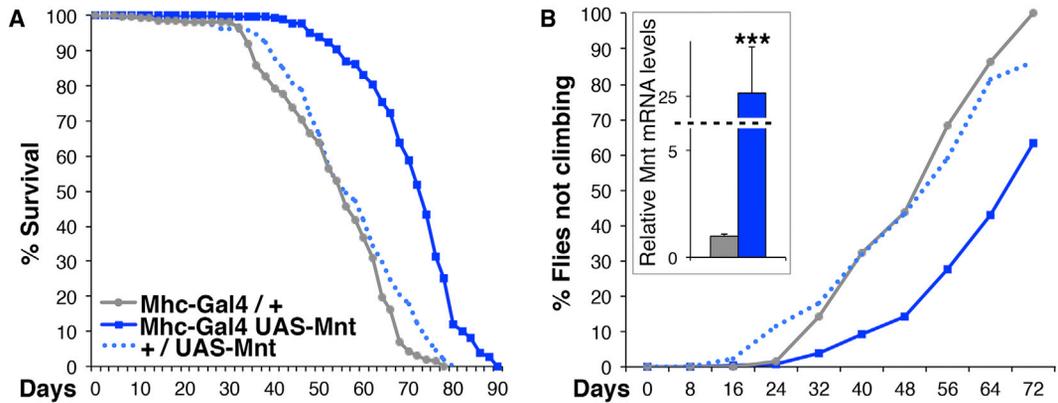
INTRODUCTION

Aging is characterized by changes in multiple organ systems that collectively decrease the ability of the organism to withstand homeostatic perturbations (Kenyon, 2010). Depending on the tissue considered, tissue-specific activation of longevity pathways can result in only local effects (i.e., prevention of tissue aging without systemic effects; Wessells et al., 2004) or also influence aging processes in other tissues via endocrine signals (Durieux et al., 2011; Giannakou et al., 2004; Hwangbo et al., 2004; Karpac et al., 2011; Libina et al., 2003; Rera et al., 2011; Satoh et al., 2013; Taguchi et al., 2007; van Oosten-Hawle et al., 2013; Wang et al., 2005, 2008). This network of tissue interactions has been proposed to coordinate the rate of aging of different tissues within an organism and regulate lifespan

(Panowski and Dillin, 2009; Russell and Kahn, 2007). However, despite its importance, the key tissues and endocrine signals involved are only in part known.

Epidemiological studies in humans indicate that the mortality rate and pathogenesis of many age-related diseases are associated with sarcopenia and the functional status of skeletal muscle (Metter et al., 2002; Ruiz et al., 2008; Swindell et al., 2010). These findings suggest that the muscle is a key tissue with the capacity to influence systemic aging and lifespan (Demontis et al., 2013a, 2013b; Nair, 2005). In line with this hypothesis, several muscle-specific genetic interventions have been recently reported to extend lifespan in mice and in the fruit fly *Drosophila* (Demontis and Perrimon, 2010; Demontis et al., 2013a; Duteil et al., 2010; Gates et al., 2007; Hakimi et al., 2007; Katewa et al., 2012; Owusu-Ansah et al., 2013; Pospisilik et al., 2007). For instance, muscle-specific overexpression of uncoupling protein 1 (*UCP1*) in mice increases the median lifespan and decreases the incidence of several age-related pathologies arising in nonmuscle tissues, such as lymphomas, diabetes, and hypertension (Gates et al., 2007). Moreover, activation of the transcription factor FOXO and its target 4E-BP solely in skeletal muscles preserves muscle function, extends lifespan and prevents the gradual accumulation of polyubiquitin protein aggregates in muscles and other aging tissues via the autonomous and nonautonomous increase in basal autophagy in *Drosophila* (Demontis and Perrimon, 2010; Bai et al., 2013). Although these findings underscore the fundamental role of muscles in regulating systemic aging, the molecular mechanisms involved in this intertissue communication are largely unknown.

Over the past decade, skeletal muscle has been recognized as an endocrine tissue with the capacity to secrete several cytokines and growth factors, known as myokines, which can act on distant tissues such as the adipose tissue, liver, pancreatic β cells, and endothelium (Demontis et al., 2013a; Pedersen and Febbraio, 2012). For example, activity of the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) in muscle protects from obesity and diabetes in mice at least in part via the induction of irisin, a myokine that promotes browning of white adipose tissues (Boström et al., 2012). Whether the emerging role of skeletal muscle in influencing systemic aging and lifespan depends on the action of myokines is largely unknown.

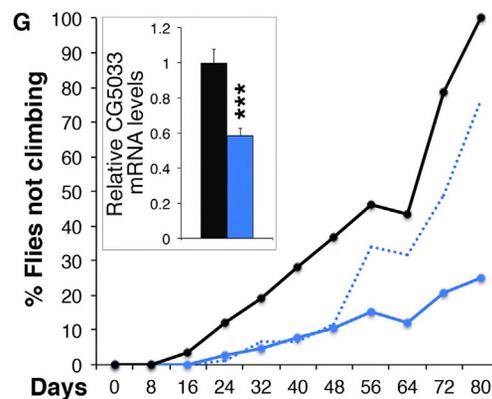
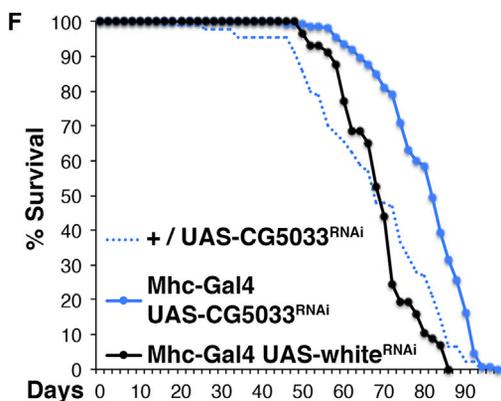
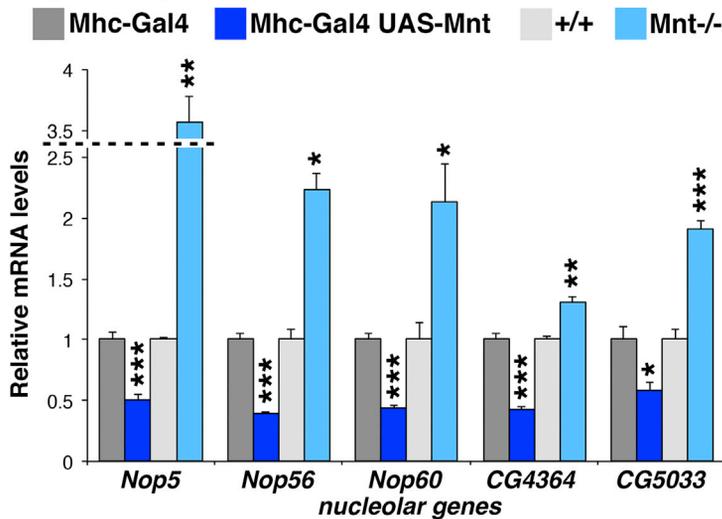


C *Microarray analysis (muscle)*

GO categories with decreased expression in response to Mnt transcriptional activity

GOCCID	P value	Odds Ratio	Exp Count	Count	Size	Term
GO:0005730	1.8522E-05	7.09157562	1.54652595	9	56	nucleolus ←
GO:0000313	0.00014265	5.27509653	1.9883905	9	72	organellar ribosome
GO:0005762	0.00134684	5.59246052	1.24274406	6	45	mitochondrial large ribosomal subunit
GO:0015934	0.00449461	3.35786469	2.6235708	8	95	large ribosomal subunit
GO:0005763	0.0446765	4.12237762	0.80087951	3	29	mitochondrial small ribosomal subunit

D *qRT-PCR (muscle)*



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In this study, we found that overexpression of the transcription factor *Mnt* in skeletal muscle extends lifespan and regulates nucleolar function autonomously in muscle and nonautonomously in adipocytes. In muscle, *Mnt* decreases the expression of nucleolar components and rRNA levels and promotes the expression of *myoglianin*, a secreted protein homologous to human GDF11 and myostatin. In turn, overexpression of *myoglianin* in muscle leads to a systemic decrease in rRNA levels and reduces nucleolar size in adipocytes by activating the stress-sensing kinase p38 mitogen-activated protein kinase (MAPK). Altogether, these findings indicate a key role for myokine signaling in the intertissue regulation of aging.

RESULTS

Mnt Activity in Skeletal Muscle Decreases the Expression of Nucleolar Components and Extends Lifespan

Drosophila *Mnt* is a basic helix-loop-helix transcription factor homologous to human MNT/MAD that decreases the growth and proliferation of epithelial cells by directly regulating the expression of several target genes involved in ribosome biogenesis and cellular anabolism (Hurlin and Huang, 2006; Orian et al., 2005). Although *Mnt* null flies are short-lived (Loo et al., 2005), the role of *Mnt* in aging is not understood. In *Drosophila*, *Mnt* protein is detected in the nuclei of several tissues, including skeletal muscle of adult flies (Figure S1). To test whether muscle-restricted overexpression of *Mnt* regulates lifespan, we used the *Mhc-Gal4* driver, which has been previously shown to drive transgene expression specifically in skeletal muscle but not in nonmuscle tissues (Demontis and Perrimon, 2010; Schuster et al., 1996). We crossed the skeletal-muscle-specific *Mhc-Gal4* line with either males carrying a *UAS-Mnt* transgene or isogenic siblings having the same genetic background but no transgene and analyzed the resulting progenies (*Mhc-Gal4/+* and *Mhc-Gal4/UAS-Mnt*). We also analyzed heterozygous *+/UAS-Mnt* flies as additional controls. *Mnt* overexpression

in skeletal muscle extended both the median and maximum lifespan (Figure 1A), and this effect was not associated with changes in muscle mass, body weight, or feeding behavior, as assessed with capillary feeding (CAFE) assays (Figure S2 and data not shown). We next used negative geotaxis assays to test whether *Mnt* regulates age-related muscle functional decay and found that *Mnt* overexpression in muscle lowers the percentage of flies with climbing defects during aging (Figure 1B). Lifespan extension and preservation of climbing ability was also obtained by inducing *Mnt* overexpression with the *dMef2-Gal4* driver under control of the temperature-sensitive *Gal80^{ts}* (Figure S3).

To examine the transcriptional changes induced by *Mnt* in skeletal muscle, we profiled gene expression changes with Affymetrix microarrays. Gene ontology cell-component terms most significantly downregulated by *Mnt* overexpression included ribosomal proteins and components of the nucleolus (Figure 1C), a nuclear compartment necessary for ribosome subunit biogenesis (Dimario, 2004), suggesting that the nucleolus is a key effector of *Mnt* in lifespan regulation. Quantitative RT-PCR (qRT-PCR) confirmed a decrease in the expression of genes encoding nucleolar components (*Nop5*, *Nop56*, *Nop60*, *CG4364*, and *CG5033*) in *Mnt*-overexpressing muscles, whereas muscles from *Mnt^{-/-}* null flies showed an increase in the expression of these genes (Figure 1D). Luciferase assays in *Drosophila* S2R+ cells with reporters based on the promoters of *CG4364* and *CG5033* further confirmed this regulation (Figure 1E).

The nucleolus has been linked with the regulation of replicative senescence in yeast (Guarente, 1997) and is emerging as a key cellular hub for stress sensing in multicellular organisms (Boulon et al., 2010; Mayer et al., 2005; Michel et al., 2011). Interventions that delay cellular aging and extend lifespan, such as rapamycin, AMP-activated protein kinase activity, and decreased target of rapamycin signaling, are known to reduce the size and function of the nucleolus (Grewal et al., 2007; Hoppe et al., 2009; Iadevaia et al., 2012; Zhang et al., 2000). On this basis, we next tested whether the nucleolus is per se a determinant of lifespan acting

Figure 1. *Mnt* Overexpression in Skeletal Muscle Extends Lifespan and Decreases Age-Related Muscle Dysfunction and Nucleolar Protein Gene Expression

(A and B) *Mnt* overexpression in skeletal muscle extended the median and maximum lifespan (A; *Mhc-Gal4/UAS-Mnt*; blue; n = 311; median/maximum lifespan = 72.4/90 days; p < 0.0001) and reduced the percentage of flies with age-related climbing defects (B; p < 0.001; log-rank test) as compared with isogenic control siblings (*Mhc-Gal4/+*; gray; n = 257; median/maximum lifespan = 54.8/78 days) and heterozygous *UAS-transgene-alone* controls (*+/UAS-Mnt*; blue dotted line; n = 236; median/maximum lifespan = 55.7/80 days). Relative *Mnt* mRNA levels are shown in the inset with n = 4 and SEM indicated; ***p < 0.001; unpaired two-tailed Student's t test.

(C) Gene expression profiling of skeletal muscle overexpressing *Mnt* and controls with Affymetrix microarrays. Genes encoding components of the nucleolus and the ribosome are significantly represented among the target genes having decreased expression in response to *Mnt* transcriptional activity (p < 0.05; Exp Count, expected count).

(D) Relative mRNA levels of nucleolar components from skeletal muscles with increased *Mnt* levels, from *Mnt^{-/-}* null flies, and controls. *Mnt* overexpression (dark blue) decreased the mRNA levels of nucleolar components as compared with controls (dark gray). Conversely, *Mnt* loss of function (blue) led to increased expression. SEM is indicated with n = 4; unpaired two-tailed Student's t test.

(E) *Mnt* overexpression (blue) decreased the activity of luciferase reporters based on the promoters of the *CG4364* and *CG5033* genes (which encode nucleolar components) as compared with controls (*YFP* overexpression; gray). SEM is indicated with n = 16.

In (D) and (E), *p < 0.05; **p < 0.01; ***p < 0.001; unpaired two-tailed Student's t test.

(F and G) *CG5033* RNAi in skeletal muscle extended the median lifespan (F; *Mhc-Gal4/UAS-CG5033^{RNAi}*; blue; n = 152; median/maximum lifespan = 81.9/98; p < 0.0001; log-rank test) and reduced the percentage of flies with age-related climbing defects (G; p < 0.01; log-rank test) as compared with a mock RNAi treatment against *white* (*Mhc-Gal4/UAS-white^{RNAi}*; black; n = 57; median/maximum lifespan = 68.6/86) and heterozygous *UAS-transgene-alone* controls (*+/UAS-CG5033^{RNAi}*; blue dotted line; n = 90; median/maximum lifespan = 67.7/94). Relative *CG5033* mRNA levels are shown in the inset with n = 4 and SEM indicated; ***p < 0.001; unpaired two-tailed Student's t test.

See also Figures S1–S3.

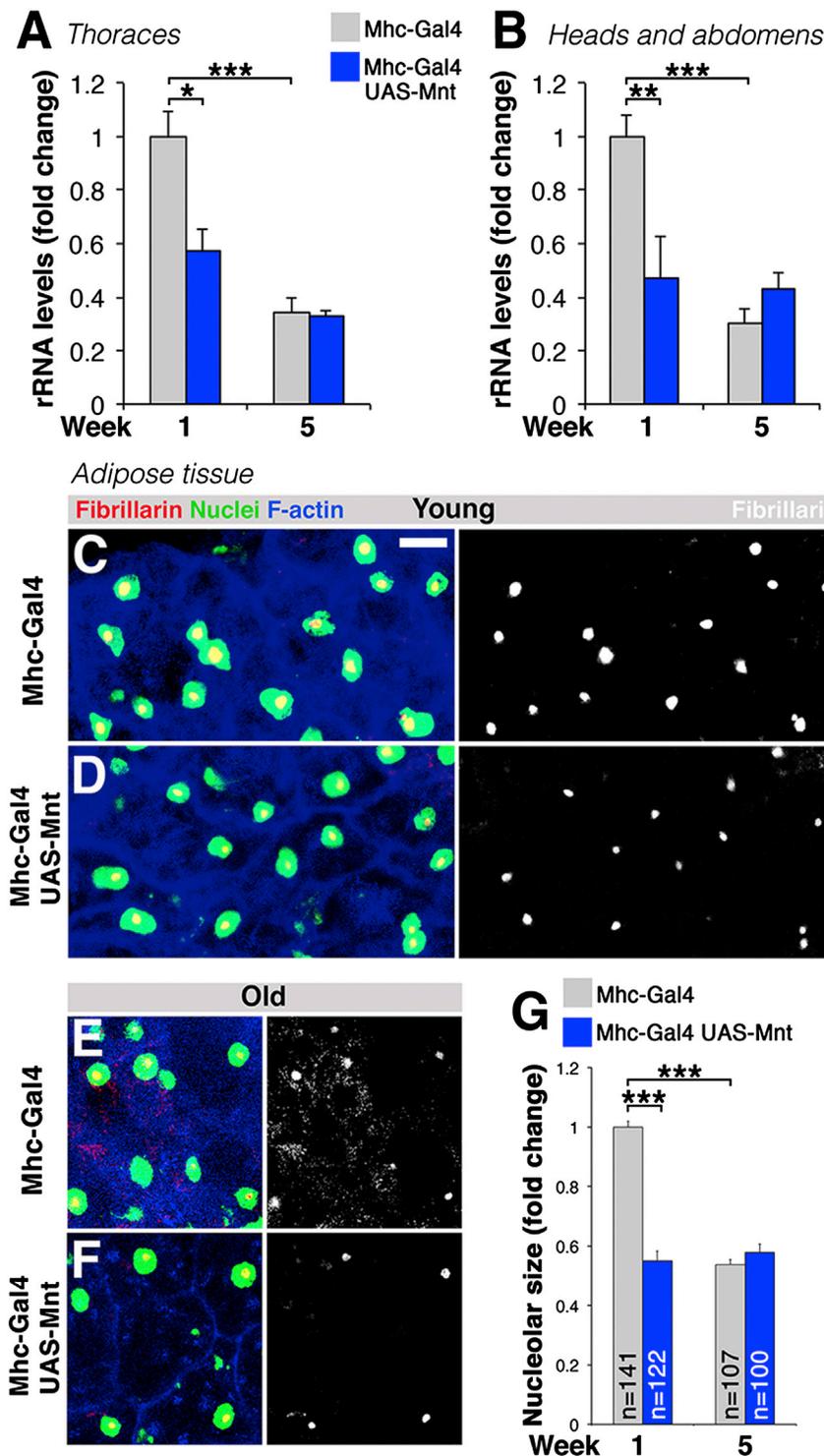


Figure 2. *Mnt* Overexpression in Muscle Systemically Regulates rRNA Levels and Nonautonomously Reduces Nucleolar Size in Adipocytes

(A and B) qRT-PCR analysis of rRNA levels in fly thoraces (which consist mostly of skeletal muscle) and heads and abdomens (which consist mostly of nonmuscle tissues) from flies overexpressing *Mnt* specifically in muscle with the *Mhc-Gal4* driver. rRNA levels decline during aging in thoraces, heads, and abdomens. Flies overexpressing *Mnt* in skeletal muscle (*Mhc-Gal4/UAS-Mnt*) display low levels of rRNA already in young age in both thoraces (A) and heads and abdomens (B) in comparison with control flies (*Mhc-Gal4/+*). SEM and n are indicated with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; two-way ANOVA followed by Tukey's post test.

(C–F) As compared with controls (C and E), overexpression of *Mnt* in skeletal muscle decreases the nucleolar size of adipocytes already in young age (D), anticipating the decline typically observed in old age (E and F). The nucleolar marker fibrillarins (red) is shown together with DAPI staining for nuclei (green) and F-actin (phalloidin; blue). Scale bar is 10 μm .

(G) Nucleolar size quantification is shown with SEM and n indicated; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; two-way ANOVA followed by Tukey's post test.

See also Figure S4.

median lifespan as compared to *white* RNAi and upstream activating sequence (UAS)-RNAi alone controls (Figures 1F and 1G; Figure S3), suggesting that a partial reduction in nucleolar function can delay aging.

***Mnt* Activity in Skeletal Muscle Nonautonomously Decreases Nucleolar Size in Adipocytes and rRNA levels**

Considering that *Mnt* decreases the expression of several nucleolar components (Figure 1), we next tested whether *Mnt* also reduces nucleolar function. By measuring ribosomal 28S rRNA levels, which are indicative of the functional status of the nucleolus, we found that *Mnt* overexpression in muscle resulted in lower 28S rRNA levels when compared to controls (Figure 2A). We also found that rRNA levels decrease during

aging, indicating that *Mnt* prematurely induces this decline (Figure 2A). Aging is characterized by the occurrence of degenerative changes as well as by compensatory stress responses that counteract aging itself (Haigis and Yankner, 2010). Considering that RNAi interventions mimicking decreased nucleolar function

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extend the median lifespan (Figure S3), a reduction in rRNA levels is most likely part of a protective stress response activated during aging and induced by Mnt.

While *Mnt* overexpression in muscle led to lower rRNA levels in thoraces, which consist mostly of muscle (Figure 2A), it also induced a similar response in heads and abdomens, which are enriched in nonmuscle tissues (Figure 2B). The observation that Mnt activity solely in muscle regulates rRNA levels systemically (Figure 2B) suggests that Mnt contributes to the intertissue communication that has been proposed to be an important determinant of aging and lifespan in multiple species.

On this basis, we next asked whether the Mnt-induced systemic regulation of rRNA levels also entails cellular changes in distant tissues. Specifically, we tested whether muscle-restricted overexpression of *Mnt* regulates nucleolar size in the adipose tissue, a key target tissue for metabolic crosstalk with muscle in mammals and *Drosophila* (Demontis et al., 2013a). The size of the nucleolus is representative of its functional status and can be monitored with antibodies against the nucleolar component fibrillarin/Nop1p (Boulon et al., 2010; Dimario, 2004). Consistent with a nonautonomous role for Mnt in regulating the nucleolus, *Mnt* overexpression in skeletal muscle led to smaller nucleoli in adipocytes of young flies (Figures 2D and 2G) as compared with controls (Figure 2C).

By comparing adipocytes from young and old control flies, we also found that a decrease in nucleolar size is observed during aging (Figures 2C, 2E, and 2G), and that Mnt prematurely induces this response (Figures 2C–2G). Conversely, adipocytes from *Mnt*^{-/-} null flies, which are short-lived (Loo et al., 2005), had larger nucleoli than controls throughout aging (Figure S4).

Altogether, these findings indicate that Mnt activity in skeletal muscle nonautonomously regulates nucleolar size in adipocytes and that the nucleolus is dynamically regulated during aging.

Transgenic RNAi Screening Identifies Myoglianin as a Myokine Regulating Lifespan and Induced by Mnt

The observation that Mnt activity in muscle nonautonomously regulates the nucleolus in adipocytes suggests a possible role of factors released by muscles and induced by Mnt. Several cytokines and growth factors, known as myokines, are known to be secreted by skeletal muscle and transduce signals from muscle to distant tissues in humans (Demontis et al., 2013a; Pedersen and Febbraio, 2012). To identify myokines regulating lifespan in *Drosophila*, we used the skeletal muscle-specific *Mhc-Gal4* driver to perform a transgenic RNAi screen with a set of *UAS-RNAi* transgenes encoding known secreted proteins (Figure 3A). No developmental or growth defects were observed under any of the RNAi treatments, consistent with the previous finding that the *Mhc-Gal4* line used drives only minimal transgene expression during skeletal muscle development (Demontis and Perrimon, 2010). To identify lifespan regulators, the survival of cohorts of male flies with a given RNAi treatment in muscle was monitored during aging and compared to a control syngenic RNAi treatment against *white* (Figure 3A). RNAi knockdowns that decreased the median lifespan included 2 independent RNAi

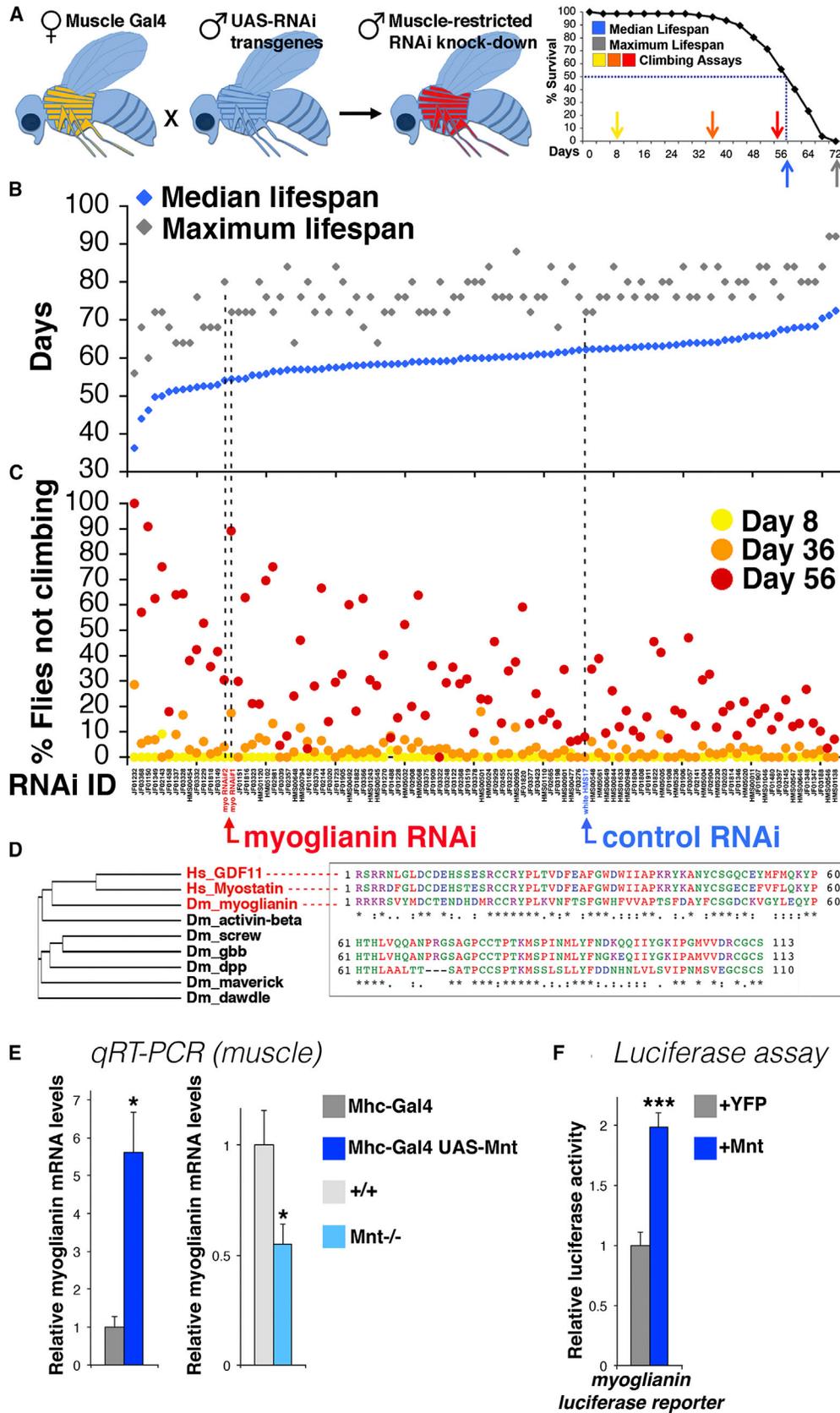
lines against *myoglianin* (Figures 3B and S5), which also increased the percentage of flies with climbing defects in old age as compared to controls (Figures 3C and S5). Myoglianin is a secreted transforming growth factor β (TGF- β) ligand expressed primarily in glial cells and skeletal muscle in *Drosophila* (Awasaki et al., 2011; Lo and Frasch, 1999; Serpe and O'Connor, 2006) and orthologous to human Myostatin/Growth Differentiation Factor-8 (GDF8) and the related factor GDF11 (Figure 3D). In mammals, Myostatin inhibits muscle growth and regeneration (Lee, 2004; McPherron et al., 1997; Sartori et al., 2013), but neither Myostatin nor GDF11 has been implicated in lifespan determination.

Given the high degree of sequence conservation of this myokine across species and its role in lifespan determination in *Drosophila* (Figure 3), we next tested whether Myoglianin is a myokine regulated by Mnt. As indicated by qRT-PCR, *myoglianin* expression increased in skeletal muscle overexpressing *Mnt*, whereas muscle from *Mnt*^{-/-} null flies had lower *myoglianin* mRNA levels (Figure 3E). To further confirm the role of Mnt in regulating *myoglianin* expression, we generated luciferase transcriptional reporters based on the *myoglianin* gene promoter. In agreement with *in vivo* findings (Figure 3E), *Mnt* overexpression in *Drosophila* S2R+ cells resulted in higher *myoglianin*-luciferase reporter activity than *yellow fluorescent protein* (YFP) overexpression (Figure 3F). Taken together, these results indicate that the *Drosophila* Myostatin/GDF11 ortholog Myoglianin is a myokine that is induced by Mnt.

Mnt/Myoglianin Signaling Regulates Lifespan

We next asked whether *myoglianin* overexpression regulates lifespan. Similar to *Mnt* (Figure 1), *myoglianin* overexpression in muscle extended both the median and maximum lifespan (Figure 4A) and resulted in a lower percentage of flies with climbing defects during aging (Figure 4B) as compared with isogenic controls. Lifespan extension and preservation of climbing ability was also obtained by overexpressing *myoglianin* in adulthood with *dMef2-Gal4* under the temporal control of *Gal80^{ts}* (Figure S3). Opposite to *myoglianin* overexpression, RNAi against *myoglianin* shortened the median lifespan (Figure 4C) and resulted in a higher percentage of age-related climbing defects (Figure 4D) as compared to RNAi against *white*. Notably, heterozygous *UAS-transgene* alone controls did not cause changes in lifespan and climbing without the *Mhc-Gal4* driver (Figures 4A–4D). In addition, no significant changes in muscle mass, body weight, and feeding behavior were observed upon *myoglianin* overexpression and RNAi with the *Mhc-Gal4* driver (Figure S2 and data not shown).

Considering that Mnt regulates *myoglianin* levels (Figure 3) and that both Mnt and Myoglianin extend lifespan (Figures 1 and 4), we next asked whether Myoglianin contributes to the lifespan extension that follows Mnt activation in muscles. To this end, a recombined line carrying the *Mhc-Gal4* and *UAS-Mnt* transgenes was crossed with RNAi lines targeting either *myoglianin* or *white* (control). A decrease in *myoglianin* mRNA levels reduced the lifespan extension and preservation of muscle function associated with *Mnt* overexpression (Figures 4E, 4F, and S6), indicating that Myoglianin contributes in part to the longevity effects of Mnt.



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Muscle-Derived Myoglianin Regulates rRNA Levels Organism-wide

In mammals, both Myostatin and GDF11 are detected in the circulation (Souza et al., 2008; Zimmers et al., 2002), from where they have been proposed to act on nonmuscle cells (McPherron, 2010). Considering that Myoglianin acts downstream of Mnt in regulating lifespan (Figures 3 and 4), we asked whether muscle-derived Myoglianin regulates rRNA levels in muscle and non-muscle tissues as observed following *Mnt* overexpression in muscle. In agreement with this scenario, rRNA levels decreased in young age in thoraces (Figure 5A) and heads and abdomens (Figure 5B) in response to *myoglianin* overexpression in muscle, anticipating the age-related decline in rRNA levels observed in control flies (Figures 5A and 5B).

We next asked whether *myoglianin* knockdown in muscle also elicits systemic effects. Opposite to its overexpression, *myoglianin* RNAi in muscle led to higher rRNA levels in old age in both thoraces and heads and abdomens, partially preventing the decline in rRNA levels observed during aging (Figures 5C and 5D). Taken together, these findings indicate that Myoglianin regulates rRNA levels in the whole organism during aging.

Muscle-Derived Myoglianin Regulates Nucleolar Size in Adipocytes via p38 MAPK

Given the role of Myoglianin in regulating rRNA levels, we next tested whether muscle-derived Myoglianin regulates nucleolar size in adipocytes. To this purpose, *myoglianin* was overexpressed specifically in skeletal muscle with the *Mhc-Gal4* line. As compared with controls (Figures 6A, 6B, and 6I), *myoglianin* overexpression in muscle led to smaller nucleoli in adipocytes (Figures 6C and 6D). Conversely, RNAi-mediated knockdown of *myoglianin* specifically in muscle led to increased nucleolar size in adipocytes throughout aging (Figures 6G and 6H) as compared to RNAi against *white* (Figures 6E, 6F, and 6J). Altogether, these findings indicate that Myoglianin is a myokine that regulates nucleolar size in adipocytes during aging.

Myostatin and GDF11 can signal via the activation of several intracellular effectors in mammals (Lee, 2004). We therefore sought to determine the Myoglianin signal transducers that regulate nucleolar function in this context. In response to Mnt/Myoglianin signaling, we detected substantial changes in the phosphorylation and hence activity of the stress-sensing kinase p38 MAPK (Figure 7A), a known transducer of Myostatin and TGF- β signaling in mammals (Li et al., 2008; Moustakas and Heldin, 2005; Philip et al., 2005). Interestingly, p38 MAPK protects from age-dependent motor deficits and extends lifespan in multiple species (Vrailas-Mortimer et al., 2011; Youngman et al., 2011), suggesting that it may play an important role downstream of Mnt/Myoglianin signaling. Increased phospho-p38 MAPK levels were detected in both thoracic fractions (consisting of transgene expressing muscles) and head and abdominal fractions (enriched in nonmuscle tissues with no transgene expression) from young and old flies with either *Mnt* or *myoglianin* overexpression in muscles in comparison to controls (Figure 7A). Conversely, *myoglianin* knockdown in muscles led to a decline in phospho-p38 MAPK levels in both muscle and nonmuscle tissues compared with mock RNAi treatments against *white* (Figure 7A). Similar changes in phospho-p38 MAPK levels were observed in muscle overexpressing *Mnt* and having *myoglianin* knockdown (Figure S7). There were no changes in phospho-p38 MAPK in UAS-alone controls in the absence of Gal4-driven transgene expression (Figure S7). Moreover, total p38 levels did not change in response to *Mnt* and *myoglianin* overexpression and RNAi, indicating that Mnt and Myoglianin regulate p38 activity rather than total p38 protein levels (Figure S7). In addition, no changes in phospho-p70 S6K levels were detected (Figure S7). Taken together, these findings suggest that Mnt activity in muscles and Myoglianin levels are sensed systemically via p38 MAPK phosphorylation and activation in target tissues.

To investigate whether p38 MAPK regulates nucleolar size in adipocytes, we overexpressed wild-type (WT) or dominant-negative (kinase-dead [KD]) p38 MAPK in adipocytes with *ppl-Gal4*. Increased levels of p38 WT led to smaller nucleoli

Figure 3. Muscle-Restricted RNAi Screening Identifies Myoglianin, a Myokine Regulating Lifespan and Induced by Mnt

(A) Scheme of the RNAi screen for myokines regulating lifespan and age-related muscle dysfunction in the fruit fly *Drosophila melanogaster*. Females carrying the *Mhc-Gal4* driver (specific for skeletal muscles) were crossed with males from a collection of Gal4-responsive UAS-RNAi transgenes, generated in a similar genetic background, to knock down a given cytokine or growth factor specifically in the skeletal muscle of the progeny. Climbing ability (indicative of neuromuscular function) was measured in young (day 8, yellow arrow) and older (day 36, orange arrow; and day 56, red arrow) ages, together with the median lifespan (the day at which 50% of flies are alive; blue arrow) and the maximum lifespan (the day at which the last fly in a cohort is alive; gray arrow).

(B) RNAi screen results ordered by ascending median lifespan. Each blue dot represents the median lifespan (indicated in days on the y axis) of a cohort of typically 60 flies with a muscle-specific RNAi intervention (TRiP RNAi ID is shown on the x axis of the graph in C, see also Figure S5). Corresponding gray dots represent the maximum lifespan of the same cohort. Two different RNAi treatments in skeletal muscles against the TGF- β ligand *myoglianin* (arrow and name in red) shortened the median lifespan as compared with a control RNAi against *white* (arrow and name in blue). RNAi in muscle for several Insulin-like peptides extended lifespan (Figure S5).

(C) The percentage of flies with climbing defects at day 8 (yellow), 36 (orange), and 56 (red) for the cohorts of flies with lifespan reported in (B). *Myoglianin* RNAi increased the percentage of flies with climbing defects in old age as compared with a control RNAi against *white*. Note that none of the RNAi treatments resulted in climbing defects in young age (day 8), indicating that the differences observed in older age are not due to developmental defects. See Supplemental Experimental Procedures and Figure S5 for statistical analysis of the screen.

(D) Phylogenetic analysis of *Drosophila* BMP and TGF- β ligands shows that Myoglianin is the sole *Drosophila* homolog of the highly related human TGF- β ligands Myostatin (GDF8) and GDF11. Alignment of the mature *Drosophila* Myoglianin and human GDF11 and Myostatin peptides shows extensive sequence identities (47%; *) and similarities (69%; ;).

(E) *Mnt* overexpression in skeletal muscles significantly increased *myoglianin* expression, whereas muscles from *Mnt*^{-/-} null flies had lower *myoglianin* mRNA levels. SEM is indicated with n = 4; *p < 0.05; unpaired two-tailed Student's t test.

(F) Overexpression of *Mnt* (blue) in *Drosophila* S2R+ cells resulted in higher *myoglianin*-luciferase reporter activity than controls (*YFP* overexpression; gray). SEM is indicated with n = 16; ***p < 0.001; unpaired two-tailed Student's t test.

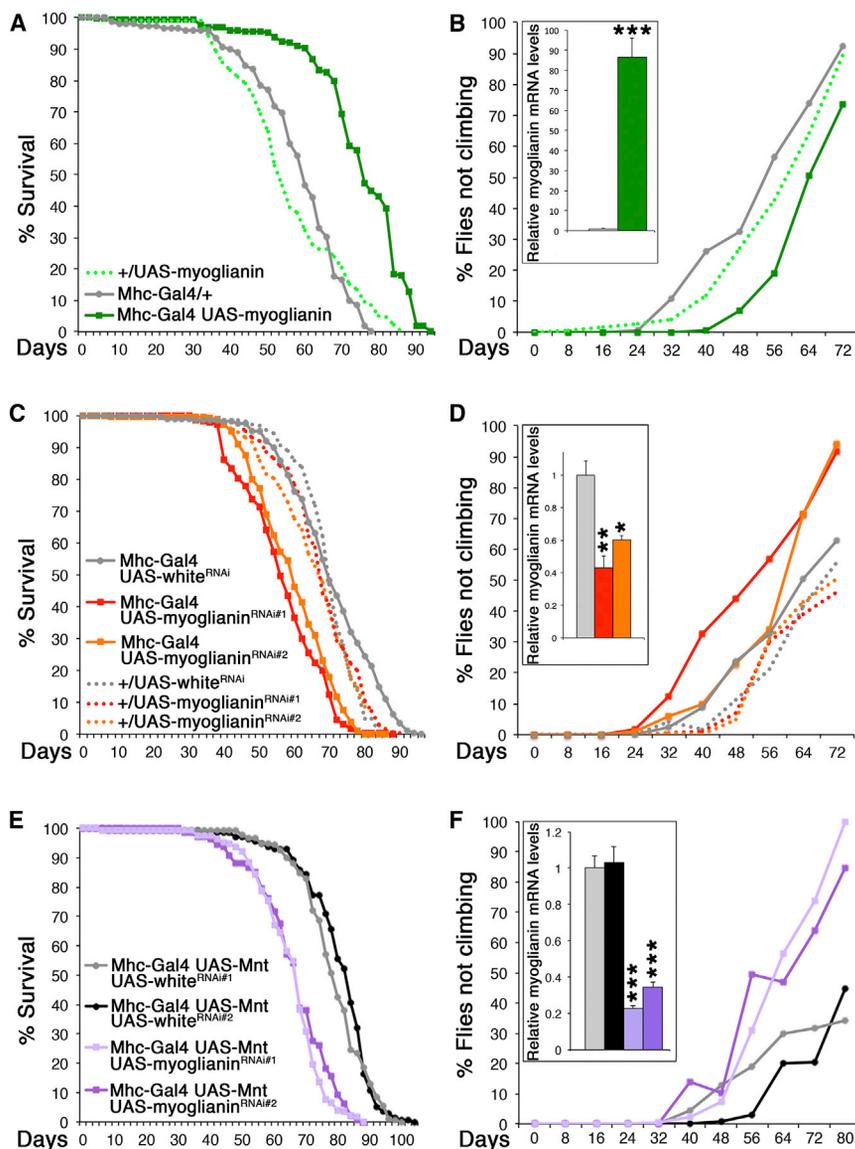


Figure 4. Mnt/Myoglianin Signaling Regulates Lifespan and Muscle Function during Aging

(A and B) *Myoglianin* overexpression (*Mhc-Gal4/UAS-myoglianin*; green; $n = 458$; median/maximum lifespan = 75.5/94 days) in skeletal muscles extended the median and maximum lifespan (A; $p < 0.0001$; log-rank test) and reduced the percentage of flies with age-related climbing defects (B; $p < 0.01$; log-rank test) as compared with isogenic control siblings (*MhcGal4/+*; gray; $n = 152$; median/maximum lifespan = 59.3/78 days) and heterozygous UAS-transgene-alone controls (*UAS-myoglianin/+*; green dotted line; $n = 370$; median/maximum lifespan = 53/86 days).

(C) Muscle-specific knockdown of *myoglianin* (RNAi#1: red; $n = 275$; median/maximum lifespan = 55.9/88 days; RNAi#2: orange; $n = 731$; median/maximum lifespan = 59.1/86 days) shortened the median lifespan ($p < 0.0001$; log-rank test) as compared with *white* knockdown (gray, control; $n = 290$; median/maximum lifespan = 69.5/96 days) and heterozygous flies carrying UAS-RNAi transgenes alone (*UAS-myoglianin^{RNAi#1}/+*; red dotted line, $n = 367$; median/maximum lifespan = 67.2/92 days; *UAS-myoglianin^{RNAi#2}/+*; orange dotted line, $n = 323$; median/maximum lifespan = 67.1/92; and *UAS-white^{RNAi}/+*; gray dotted line, $n = 307$; median/maximum lifespan = 69.6/88).

(D) The percentage of flies with climbing defects progressively increased during aging in controls but to a greater extent in flies with *myoglianin* knockdown in muscles ($p < 0.01$; log-rank test).

(E) *Myoglianin* knockdown (RNAi#1: pink, $n = 142$; median/maximum lifespan = 66.5/88 days; and RNAi#2: violet, $n = 192$; median/maximum lifespan = 66.6/88 days) reduced the lifespan extension resulting from *Mnt* overexpression in muscles ($p < 0.001$; log-rank test) as compared with control *white* knockdowns (RNAi#1: gray, $n = 140$; median/maximum lifespan = 78.2/100 days; and RNAi#2, black, $n = 179$; median/maximum lifespan = 82.4/104).

(F) A higher percentage of flies with climbing defects was observed during aging when *myoglianin* levels were reduced in skeletal muscles overexpressing *Mnt* ($p < 0.01$; Log-rank test).

In all panels, relative *myoglianin* mRNA levels are shown in insets with $n = 4$ and SEM indicated; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; unpaired two-tailed Student's *t* test.

See also Figures S3 and S6.

(Figures 7B, 7C, and 7H) than those in controls (Figures 7D, 7E, and 7H). Conversely, *p38 KD* overexpression led to increased nucleolar size throughout aging (Figures 7F–7H), even when *myoglianin* was concomitantly overexpressed (Figure 7I), indicating that p38 MAPK activity is a key regulator of nucleolar size in adipocytes downstream of Myoglianin.

DISCUSSION

In multicellular organisms, crosstalk between tissues and organs is responsible for the homeostatic regulation of many physiological functions. Recent evidence suggests that intertis-

sue signaling is also an important determinant of systemic aging and lifespan (Panowski and Dillin, 2009; Russell and Kahn, 2007). However, the key tissues, signaling pathways, and endocrine factors involved in this long-distance communication are only in part known. In this study, we have identified an intertissue myokine-based longevity pathway that mediates the crosstalk between skeletal muscle and adipose tissue during aging via the transcription factor Mnt and the myokine Myoglianin (Figure 7J). The activity of the transcription factor Mnt in skeletal muscles leads to both local and systemic responses that delay whole-organism aging. Cell autonomously, Mnt reduces the function of the nucleolus by decreasing the

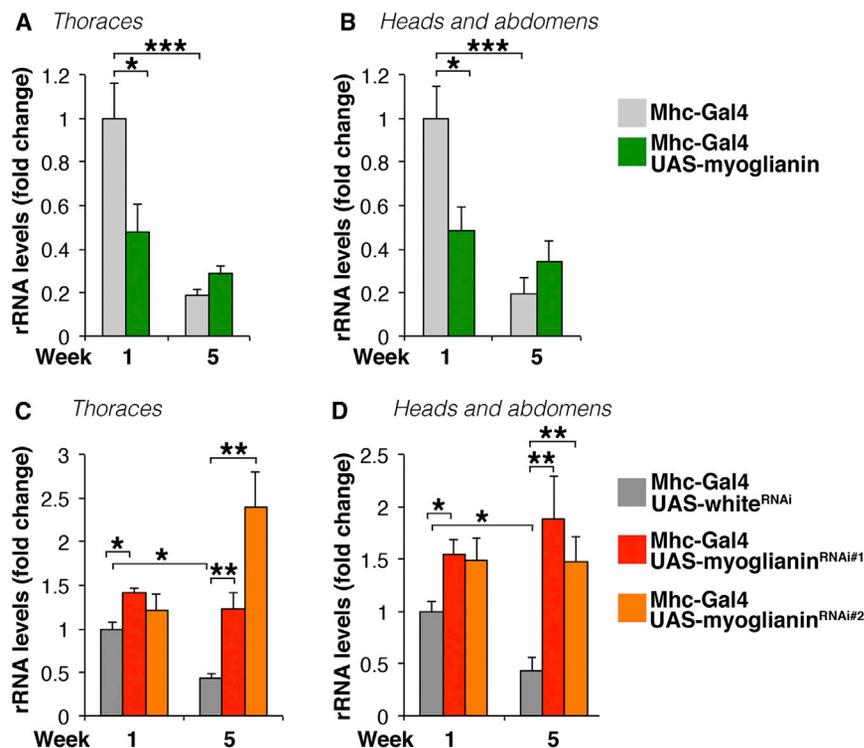


Figure 5. Muscle-Derived Myoglianin Regulates Nucleolar Function during Aging

qRT-PCR analysis of rRNA levels in fly thoraces (which consist mostly of skeletal muscle) and heads and abdomens (which consist mostly of nonmuscle tissues) from flies expressing different levels of *myoglianin* in skeletal muscle.

(A and B) rRNA levels decline during aging in thoraces, heads, and abdomens. Flies overexpressing *myoglianin* in skeletal muscle (*Mhc-Gal4/UAS-myoglianin*) display low levels of rRNA already in young age in both thoraces and abdomens in comparison with control flies (*Mhc-Gal4/+*).

(C and D) rRNA levels do not decline during aging in thoraces, heads, and abdomens from flies with *myoglianin* RNAi in skeletal muscle (*Mhc-Gal4/UAS-myoglianinRNAi*) in comparison with control flies (*Mhc-Gal4/UAS-whiteRNAi*).

In all panels, n = 4 and SEM is indicated; *p < 0.05, **p < 0.01, and ***p < 0.001; two-way ANOVA followed by Tukey's post test.

expression of nucleolar genes and rRNA levels in muscle. In addition, Mnt increases the expression of *myoglianin* in muscle and this myokine has both local and systemic effects. In muscle, Myoglianin reduces rRNA levels and age-related muscle dysfunction. Systemically, muscle-restricted *myoglianin* overexpression leads to lower rRNA levels and reduces nucleolar size in adipocytes by modulating the activity of the stress-sensing kinase p38 MAPK. Direct binding of Myoglianin to its cellular receptor and activation of TAK1 (TGF- β -activated kinase 1) may be responsible for the regulation of p38 MAPK activity in muscle and the adipose tissue, as previously observed in mammals (Li et al., 2008; Moustakas and Heldin, 2005; Philip et al., 2005).

We found that a prominent cellular response induced by Mnt/Myoglianin/p38 MAPK signaling is the regulation of nucleolar function. The nucleolus is a key promoter of cellular anabolism (Dimario, 2004), and its activity is reduced in response to several genetic and pharmacological interventions that are known to extend lifespan (Grewal et al., 2007; Hoppe et al., 2009; Iadevaia et al., 2012; Zhang et al., 2000). In line with the notion that a moderate reduction in cellular anabolism and protein translation delays aging (Hansen et al., 2007; Kenyon, 2010; Pan et al., 2007; Steffen et al., 2008; Syntichaki et al., 2007), we have found that decreasing nucleolar function is a protective response induced to counteract aging and sufficient to extend lifespan.

The coordinated regulation of the nucleolus across aging tissues via Myoglianin may represent an assurance mechanism to delay tissue aging systemically and to robustly extend lifespan in response to localized activity of the transcription factor Mnt in muscle. Considering that skeletal muscle constitutes the

major reservoir of proteins in the organism (Wolfe, 2006) and that it has high levels of ribosome biogenesis (Drummond et al., 2009), which occurs in the nucleolus, sensing and regulating the

functional status of the nucleolus in this tissue may be of particular relevance for coordinating the aging of different tissues and determining lifespan. The Myoglianin orthologs Myostatin and GDF11 may regulate lifespan and have similar endocrine roles in humans since they are circulating factors detected in the bloodstream (Souza et al., 2008; Zimmers et al., 2002) and may act on several target tissues, including adipocytes (Feldman et al., 2006; McPherron, 2010). In agreement with this scenario, GDF11 has been recently shown to act systemically to delay heart aging in mice (Loffredo et al., 2013). Future studies in mice shall test whether GDF11 exerts this function via the modulation of p38 MAPK and the nucleolus.

In addition to systemic effects, we found that Mnt and Myoglianin have cell-autonomous/autocrine effects in preserving muscle function during aging. Specifically, overexpression of *Mnt* and *myoglianin* in muscle preserves the flies' climbing ability during aging, whereas *myoglianin* RNAi has converse effects. Considering that there are no changes in muscle mass in the adult *Drosophila* (Piccirillo et al., 2014) and in response to Mnt/Myoglianin signaling in adult flies (Figure S2), the prevention of age-related motor dysfunction by Mnt/Myoglianin signaling in this context does not entail changes in muscle mass but rather derives at least in part from the activation in preexisting myofibers of p38 MAPK, which is known to prevent age-related muscle functional decline in *Drosophila* (Vrailas-Mortimer et al., 2011). In line with these findings, Myostatin knockout mice have decreased force generation when normalized to the cross-sectional area of the muscle (specific force) and may thus experience proportionally higher myofiber functional decay during aging than control mice, although they

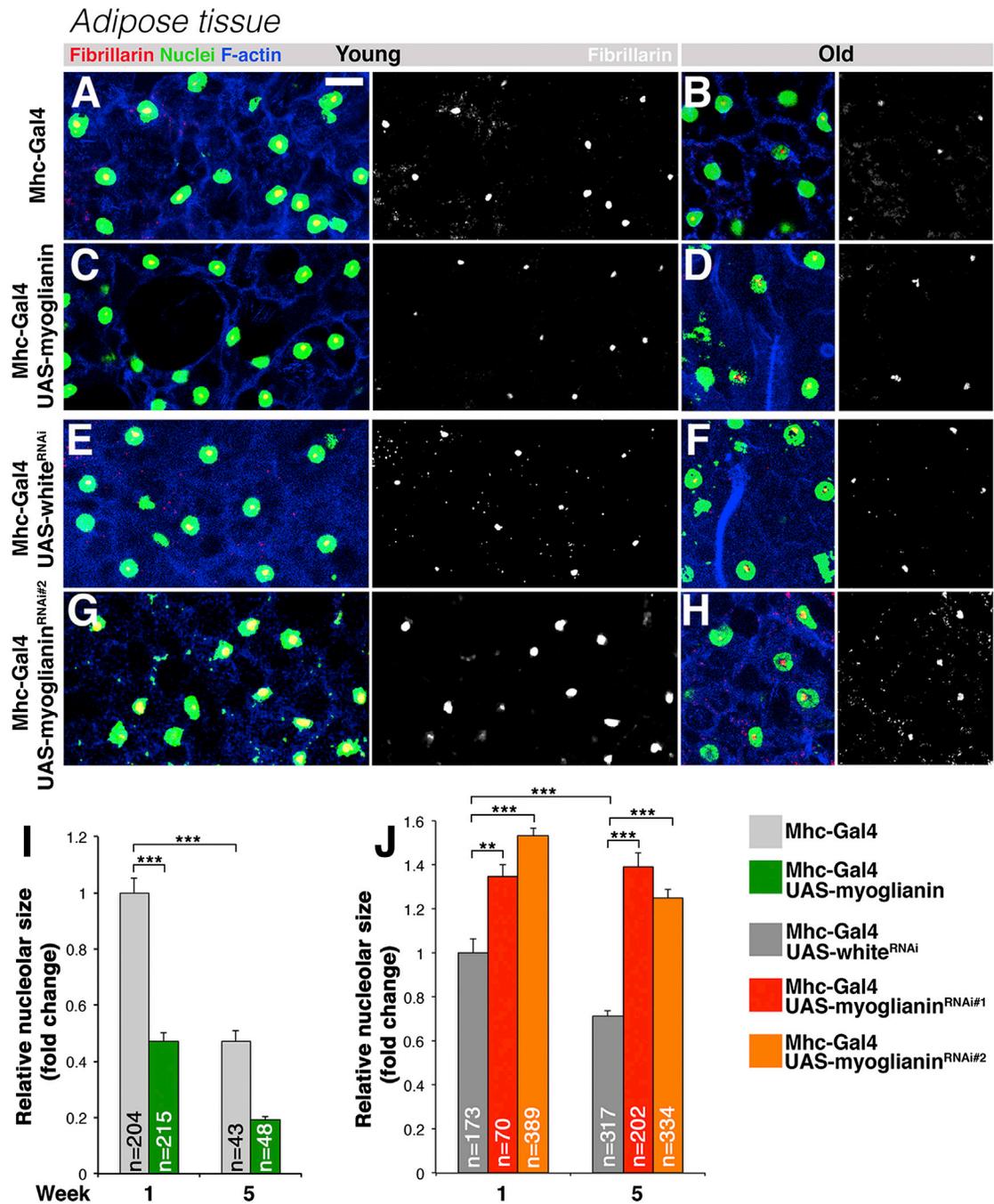


Figure 6. Muscle-Derived Myoglianin Regulates Nucleolar Size in Adipocytes

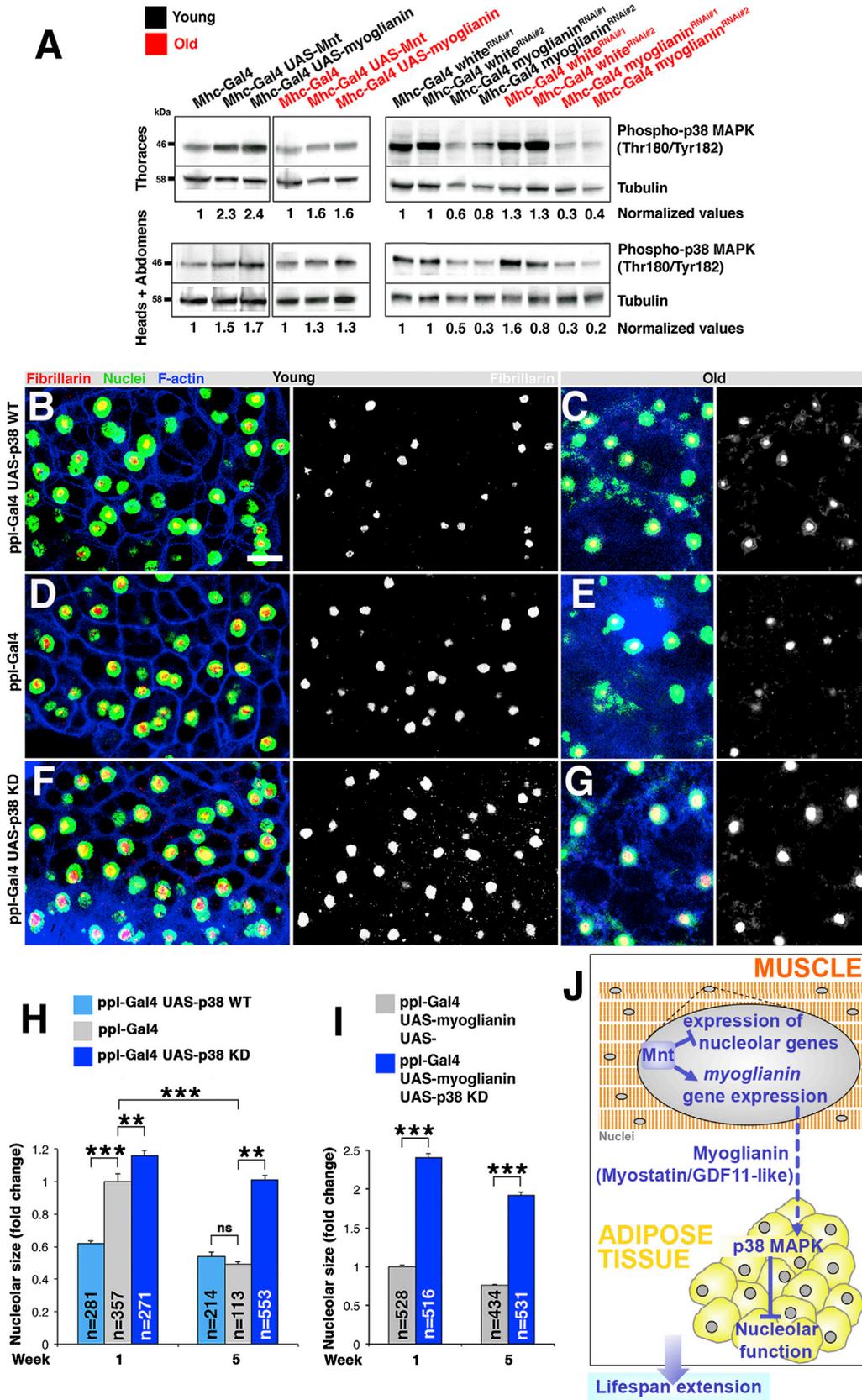
(A–H) Immunostaining of adipose tissue for the nucleolus (fibrillarlin; red), F-actin (phalloidin; blue), and nuclei (DAPI; green). (C and D) Overexpression of *myoglianin* in skeletal muscles decreased the nucleolar size of adipocytes already in young age as compared with controls (A), anticipating the decline in nucleolar size typically observed in old age (B). (G and H) Muscle-restricted *myoglianin* RNAi increased nucleolar size in adipocytes in young and old age, in comparison with a control RNAi against *white* (E and F). Scale bar is 10 μ m.

(I and J) Nucleolar size quantification is shown with SEM and n indicated; **p < 0.01; ***p < 0.001; two-way ANOVA followed by Tukey's post test.

have a higher muscle mass than controls (Amthor et al., 2007; Gentry et al., 2011).

Altogether, this study highlights a key role for myokine signaling in lifespan regulation and in the integration of signaling

events in different tissues during aging. Therapeutic interventions aimed at modulating myokine levels and their systemic signaling may extend healthy lifespan by simultaneously delaying age-related changes in muscle and other aging tissues.



(legend on next page)

EXPERIMENTAL PROCEDURES

Lifespan Analysis

For survival analysis, male flies were collected within 24 hr from eclosion and reared at standard density (20 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. The median lifespan was calculated by interpolation and corresponds to the day at which 50% of the flies in a cohort are alive. The maximum lifespan is the day at which the last fly in a cohort dies. To rule out any variation due to cytoplasmic background effects, all crosses were set up with female virgins from the *w¹¹¹⁸;Mhc-Gal4*. To avoid any contribution of genetic background mutations to the observed lifespan phenotypes, *UAS-Mnt* and *UAS-myoglianin* transgenes were backcrossed through more than six generations against *y¹w¹¹¹⁸* to obtain isogenic male 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 *w¹¹¹⁸;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. Heterozygous *UAS-* transgene-alone controls were also analyzed to exclude that the observed phenotypes are due to differences in the genetic background. These flies were obtained by crossing *UAS-* males with female *w¹¹¹⁸* virgins isogenic to the *w¹¹¹⁸;Mhc-Gal4* stock. All the progenies analyzed consisted of males. Transgenic RNAi lines used for the screen and for lifespan studies were generated in a *y¹v¹* background (Ni et al., 2011) and were crossed with female virgins from the *w¹¹¹⁸* and the *w¹¹¹⁸;Mhc-Gal4* isogenic stocks. RNAi against *white* was used as control to account for any aspecific effects deriving from the engagement of the RNAi machinery. RNAi-mediated knockdown in muscle was confirmed by qRT-PCR from fly thoraces. Heterozygous *UAS-RNAi* transgene-alone controls were also analyzed and obtained as described above. Note that the RNAi lines are on a genetic background that is different from the *y¹w¹¹¹⁸* (*yellow white*) genetic background of *UAS-Mnt* and *UAS-myoglianin* lines. All the experiments were done with males.

Climbing Assays

Negative geotaxis assays were performed as previously described (Rhodenz et al., 2008). In brief, flies were gently tapped to the bottom of a plastic vial, and the number of flies that could climb to the top of the vial after 20 s was scored.

Whole-Mount Immunostaining

Whole-mount immunostaining of *Drosophila* adipose tissues (peripheral fat body of the abdomen) was done as described previously (Demontis and Perrimon, 2010; Hunt and Demontis, 2013).

qRT-PCR and Microarray Gene Expression Profiling

Total RNA was extracted with the TRIzol reagent (Life Technologies) from at least 15 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. A primer pair for the *28S rRNA* was used to measure rRNA levels. Relative quantitation of mRNA levels was determined with the comparative C_T method. For microarray analysis, RNA integrity was assessed with an Agilent 2100 Bioanalyzer before cRNA synthesis and hybridization to Affymetrix *Drosophila* Genome 2.0 Arrays. Samples from three independent biological replicates per genotype were collected and analyzed.

Luciferase Assay

Luciferase assays were done as before (Demontis and Perrimon, 2009, 2010). See Supplemental Experimental Procedures for detailed information and a list of DNA plasmids used.

ACCESSION NUMBERS

Gene expression profiling data have been deposited in the NCBI Gene Expression Omnibus and are available under accession number GSE39877.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.05.001>.

AUTHOR CONTRIBUTIONS

F.D. conceived the project, designed and did most of the experiments, and wrote the manuscript with input from N.P. V.K.P. did the transgenic RNAi screen. W.R.S. analyzed microarray and RNAi screen data.

Figure 7. Mnt Activity in Muscle Remotely Activates p38 MAPK via Myoglianin to Regulate Nucleolar Size in Adipocytes

(A) Western blot analysis of fractions from either thoraces (consisting mainly of muscles; upper panels) or heads and abdomens (consisting mainly of nonmuscle tissues; lower panels) from controls and flies overexpressing *Mnt* and *myoglianin* (left panels) or with RNAi-mediated knockdown of *myoglianin* or *white* (right) in skeletal muscles from young (1 week) and old (8 weeks) flies. *Mnt* and *Myoglianin* increase the levels of active phospho-p38 MAPK in both muscle and nonmuscle tissues as compared with controls (left), whereas *myoglianin* knockdown had converse effects (right). Relative quantification of phospho-p38 MAPK levels normalized to α -tubulin is indicated. See also Figure S7.

(B–H) Adipocytes labeled with the nucleolar marker fibrillar (red), phalloidin (F-actin; blue), and DAPI (nuclei; green). (B) In young age (1 week), wild-type (WT) *p38 MAPK* overexpression in adipocytes with the *ppl-Gal4* driver led to significantly smaller nucleoli than in controls (D and H), leading to a premature decline in nucleolar size which is otherwise normally observed in old age (C, E, and H). Conversely, bigger nucleoli were observed in young (F) and old age (G and H) after overexpression of dominant-negative, kinase-dead (KD) *p38 MAPK*.

(I) Similar results were obtained also when *myoglianin* was concomitantly overexpressed with *p38 KD* (*ppl-Gal4/UAS-p38KD;UAS-myoglianin/+*), in comparison with overexpression of a *UAS-myoglianin* transgene together with an empty *UAS-* that drives no transgene expression and which is used to control for Gal4 titration effects in the different genotypes analyzed (*ppl-Gal4/UAS-*; *UAS-myoglianin/+*). Scale bar is 10 μ m.

Statistical analysis was done with two-way ANOVA followed by Tukey's post test in (H) and (I). In (H) and (I), n is indicated with SEM. **p < 0.01; ***p < 0.001; ns, not significant.

(J) Summary scheme. Muscle-specific overexpression of *Mnt* extends lifespan and prevents age-related muscle functional decay. In the nuclei (gray) of myofibers (orange), the transcription factor *Mnt* reduces rRNA levels and the expression of components of the nucleolus, a regulator of aging. In addition, *Mnt* increases the expression of the TGF- β ligand *myoglianin*, the *Drosophila* ortholog of human Myostatin and GDF11. In turn, muscle-specific overexpression of *myoglianin* extends lifespan and reduces rRNA levels in the whole organism and nucleolar size in adipocytes (yellow) by activating p38 MAPK, a known transducer of TGF- β signaling, while *myoglianin* RNAi in muscle has converse effects. In addition to inducing systemic effects, *Myoglianin* acts in an autocrine manner to reduce rRNA levels in muscle and preserves muscle function during aging. These findings highlight that signaling events in skeletal muscle can influence systemic aging and lifespan via muscle-derived secreted proteins, i.e., myokines. Myokine-based coordination of nucleolar function across aging tissues may represent an assurance mechanism for robust lifespan determination in aging organisms.

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