PNAS RESEARCH ARTICLE | CELL BIOLOGY

Exercise suppresses DEAF1 to normalize mTORC1 activity and reverse muscle aging

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Edited by Gerald Shulman, Yale University, New Haven, CT; received April 13, 2025; accepted October 20, 2025

Skeletal muscle is essential for movement, respiration, and metabolism, with mTORC1 acting as a key regulator of protein synthesis and degradation. In aging muscle, mTORC1 becomes overactivated, contributing to sarcopenia, though the mechanisms remain unclear. Here, we identify DEAF1, a FOXO-regulated transcription factor, as a key upstream driver of mTORC1 in aged muscle. Elevated *Deaf1* expression increases *mTOR* transcription, leading to heightened mTORC1 activity, impaired proteostasis, and muscle senescence. Remarkably, exercise suppresses *Deaf1* expression via FOXO activation, restoring mTORC1 balance and alleviating muscle aging. Conversely, FOXO inhibition or *Deaf1* overexpression blocks exercise benefits on muscle health. These findings highlight DEAF1 as a critical link between FOXO and mTORC1 and suggest that targeting the FOXO–DEAF1–mTORC1 axis may offer therapeutic potential to preserve muscle function during aging.

mTORC1 | proteostasis | autophagy | muscle | sarcopenia

Significance

Healthy muscle is essential for movement, metabolism, and overall vitality. With age, the growth regulator mTORC1 becomes chronically overactivated, disrupting protein balance and contributing to muscle loss, but the cause of this remains unclear. We identify the transcription factor DEAF1 as a key link between FOXO signaling and mTORC1 activation in aging muscle. Increased DEAF1 boosts *mTOR* expression, driving protein imbalance and muscle decline. Exercise reverses this process by suppressing DEAF1 through FOXO activation, while blocking FOXO or elevating DEAF1 prevents exercise benefits. These findings reveal how exercise restores muscle health and highlight the FOXO–DEAF1–mTORC1 pathway as a promising target to combat age-related muscle loss.

Sarcopenia, the progressive loss of skeletal muscle mass and function during aging, leads to frailty and reduced mobility in the elderly (1). As skeletal muscle accounts for ~40% of body mass, its function extends beyond movement—it is vital for metabolic regulation and overall protein homeostasis (2, 3). Regular exercise preserves muscle mass and function, making it a key intervention against muscle-wasting conditions such as sarcopenia (4, 5).

The mechanistic target of rapamycin complex 1 (mTORC1) is a central regulator of muscle mass, coordinating essential cellular processes such as protein synthesis, autophagy, and cellular senescence, all of which are critical for muscle growth and maintenance (3). mTORC1 promotes muscle hypertrophy by activating downstream effectors such as S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP), which drive protein synthesis (6–8). mTORC1 also suppresses autophagy, a vital process for the degradation and recycling of damaged cellular components (9–12). While autophagy is crucial for maintaining cellular health (13), excessive autophagy can result in muscle degradation (14). mTORC1 inhibits autophagy by phosphorylating and inactivating ULK1, thereby preventing the initiation of autophagy (14, 15). Besides, mTORC1 also regulates diverse downstream targets to block autophagy (9, 10, 16, 17). Thus, a delicate balance between mTORC1-mediated anabolism and autophagy is essential for maintaining muscle integrity, particularly in aging.

Although mTORC1 activation supports muscle growth, its hyperactivation has been implicated in muscle damage and aging-related dysfunction (18). In *Tsc1* knockout mouse models, excessive mTORC1 activity leads to abnormal mitochondria, oxidative stress, fiber damage, and progressive atrophy (18). Moreover, elevated mTORC1 activity induces cellular senescence, further exacerbating tissue degeneration (19). This paradox highlights the need for tight regulation of mTORC1 signaling to maintain muscle homeostasis.

Although mTORC1 hyperactivation is recognized as a key driver of sarcopenia (3, 18, 20), its upstream mechanisms remain unclear. One potential factor is the increased expression of the IGF-1 receptor observed in aging muscles (21), which may enhance mTORC1 activation. However, sarcopenic patients exhibit reduced circulating levels of IGF-1 (22, 23), suggesting that the upregulation of IGF-1 receptors may act as a compensatory response to declining IGF-1 availability. Notably, AKT phosphorylation remains unchanged in aging muscles (24). These findings suggest that age-related mTORC1 overactivation arises from alternative, IGF-1/AKT-independent mechanisms.

We show that DEAF1 is an upstream activator of mTORC1 that drives age-related muscle atrophy. In *Drosophila*, *Deaf1* overexpression increases protein synthesis, suppresses autophagy, and induces senescence, whereas *Deaf1* knockdown restores autophagy, reduces

Author contributions: S.M.C., K.Y.G., W.X.L., P.D.G.K., and H.-W.T. designed research; S.M.C., K.Y.G., W.X.L., W.J., Q.G., P.D.G.K., S.C.O., K.C., and H.-W.T. performed research; S.M.C., K.Y.G., W.X.L., Q.G., P.D.G.K., N.H., and H.-W.T. analyzed data; and S.M.C., K.Y.G., W.X.L., N.H., and H.-W.T. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2508893122/-/DCSupplemental.

Published November 24, 2025.

translation, and preserves muscle homeostasis. These regulatory roles of DEAF1 on mTORC1 signaling were also observed in C2C12 myotubes, highlighting the evolutionary conservation of DEAF1 function. ChIP-seq analysis revealed that DEAF1 directly binds the *mTOR* promoter, enhancing *mTOR* transcription and mTORC1 activity. DEAF1 is negatively regulated by FOXO, and exercise reduces *Deaf1* via FOXO activation, restoring mTORC1 balance, improving proteostasis, and reversing muscle aging. FOXO inhibition or *Deaf1* overexpression abolishes these exercise benefits. Together, these results establish DEAF1 as a key link between FOXO and mTORC1, revealing a conserved pathway by which exercise rejuvenates aging muscle and suggesting new therapeutic avenues for sarcopenia.

Result

Muscular DEAF1 Regulates Muscle Weakness During Aging. DEAF1, previously identified as a regulator of muscle stem cells (MuSCs) (14), is also expressed in differentiated C2C12 myotubes and mouse skeletal muscles (*SI Appendix*, Fig. S1 *A* and *B*), suggesting DEAF1 may have additional roles in skeletal muscle function. To investigate DEAF1's role in muscle, we manipulated its expression in *Drosophila* muscles using the muscle-specific *Mef2-Gal4* driver. We expressed either *Deaf1* or *Deaf1-RNAi* and assessed muscle function at different ages—week 1 (young), week 3 (middle-aged), and week 6 (old)—by measuring climbing ability, a well-established indicator of muscle weakening and degeneration in *Drosophila* (25) (Fig. 1*A*). Climbing ability declined significantly in week 6 flies, indicating muscle defects associated with aging (Fig. 1*B*). Depletion of *Deaf1* through *Deaf1*-RNAi significantly preserved muscle function and integrity, whereas *Deaf1* overexpression had no effect in weeks 1 to 3 but worsened defects at week 6 (Fig. 1 *B* and *C*). These findings suggest that *Deaf1* plays a role in age-related muscle atrophy and functional decline.

mTORC1 Signaling Functions Downstream of DEAF1 in Muscles. To explore mechanisms of *Deaf1*-induced muscle defects, we performed a small-scale drug screen to identify inhibitors that could mitigate these defects (Fig. 1*D*). Notably, both Rapamycin and Everolimus, mTORC1 inhibitors (3), effectively suppressed *Deaf1*-induced muscle defects and reversed aging-induced muscle weakness (Fig. 1 *D* and *E*), suggesting that mTORC1 may act downstream of DEAF1.

To validate these results, we examined the genetic interaction between DEAF1 and mTORC1. *Tsc1* Knockdown, which constitutively activates mTORC1 (9), mimicked *Deaf1*-induced atrophy and exacerbated age-related climbing impairments (Fig. 1 *F* and *G*). However, *Deaf1* depletion did not rescue muscle defects caused by *Tsc1*-RNAi (Fig. 1 *F* and *G*). In contrast, climbing defects and muscle atrophy resulting from *Deaf1* overexpression in aged flies were rescued by co-expression of *Tor*-RNAi (Fig. 1 *F* and *G*). Consistently, knockdown of *Raptor*, a key component of mTORC1, mitigated muscle atrophy induced by either aging or *Deaf1* overexpression (*SI Appendix*, Fig. S1 *C* and *D*). By contrast, depletion of *Rictor*, a core component of mTORC2, had no effect on aging or *Deaf1*-induced muscle atrophy (*SI Appendix*, Fig. S1 *C* and *D*). These findings show DEAF1 promotes age-related muscle weakness via mTORC1.

***Drosophila* DEAF1 Regulates mTORC1 Activity in Muscles.** We examined whether DEAF1 directly modulates mTORC1, which controls protein translation via S6K phosphorylation (3). Immunoblotting revealed increased pS6K levels in aging *Drosophila* muscles, indicating heightened mTORC1 activity during aging (Fig. 2*A*). Overexpression of *Deaf1* further elevated pS6K levels in aged muscles, while *Deaf1* knockdown reduced the age-related increase in pS6K, demonstrating that DEAF1 is critical for mTORC1 hyperactivation in aging muscles (Fig. 2*A*).

Consistent with these findings, immunofluorescence data showed elevated phospho-S6 (pS6) levels in aged muscles. Phospho-S6, which reflects ribosomal protein S6 phosphorylation, serves as a marker for mTORC1-S6K1 activation (18). *Deaf1* knockdown significantly reduced the aging-associated pS6 increase and reversed muscle loss, underscoring its essential role in age-related mTORC1 activation (Fig. 2*B*). Conversely, *Deaf1* overexpression markedly increased pS6 levels in aging muscles, exacerbating muscle fiber loss (Fig. 2*B*). These results demonstrate that DEAF1 positively regulates mTORC1, promoting aging-related muscle deterioration.

***Drosophila* DEAF1-Regulated mTORC1 Controls Muscle Proteostasis and Senescence.** mTORC1 promotes protein translation, inhibits autophagy, and induces senescence, processes linked to aging-related muscle dysfunction (26). To explore whether DEAF1 influences these mTORC1-regulated processes, we assessed its role in protein synthesis, autophagy, proteostasis, and senescence in muscle tissue. Using an ex vivo approach, we labeled newly synthesized peptides in fly muscles with puromycin and quantified protein synthesis levels with an anti-puromycin antibody (Fig. 2*C*). Puromycin incorporation was higher in old muscles compared to young muscles, indicating increased protein translation in aging muscles with elevated mTORC1 activity (Fig. 2*C*). *Deaf1* depletion reduced puromycin signals, while its overexpression enhanced them (Fig. 2*C*). Notably, *Deaf1*-induced puromycin signals were suppressed by Rapamycin treatment (Fig. 2*C*), suggesting that Deaf1 promotes muscle protein synthesis via mTORC1 activation.

Aging muscles also showed reduced autophagy, evidenced by diminished Atg8 puncta even after chloroquine treatment, a lysosomal inhibitor that blocks autophagosome degradation (27) (Fig. 2 *D* and *G* and *SI Appendix*, Fig. S2). *Deaf1* depletion restored autophagy, while its overexpression further inhibited it in old muscles (Fig. 2 *D* and *G* and *SI Appendix*, Fig. S2). Rapamycin reversed this inhibition, suggesting that Deaf1 suppresses autophagic protein degradation through mTORC1 (Fig. 2 *D* and *G* and *SI Appendix*, Fig. S2). Similarly, protein aggregates detected by anti-ubiquitin staining accumulated in aged muscles but were reduced upon *Deaf1* depletion and further increased by its overexpression (Fig. 2 *E* and *G* and *SI Appendix*, Fig. S2). Rapamycin suppressed DEAF1-induced aggregate formation, demonstrating that DEAF1 regulates proteostasis via mTORC1 (Fig. 2 *E* and *G* and *SI Appendix*, Fig. S2).

Finally, senescence-associated β-galactosidase (SPiDER-β-gal) activity, a marker of senescence, was elevated in old muscles (Fig. 2 *F* and *G*). *Deaf1* depletion abolished this signal, whereas its overexpression intensified it (Fig. 2 *F* and *G*). Rapamycin treatment significantly reduced *Deaf1*-induced senescence, further linking DEAF1 to mTORC1-mediated aging phenotypes (Fig. 2 *F* and *G*). Together, these results demonstrate that DEAF1 drives age-related changes in protein synthesis, autophagy, proteostasis, and senescence via mTORC1.

Mammalian DEAF1 Modulates mTORC1 Activity in Myotubes. Given its evolutionary conservation (15), we tested whether DEAF1 regulates mTORC1 in mammalian muscles. In C2C12 myotubes, *Deaf1* overexpression increased p-S6 signaling, which was attenuated by Rapamycin treatment (Fig. 3 *A* and *B*). Conversely, knockdown of *Deaf1* reduced p-S6 signaling, an effect that was reversed by the concurrent knockdown of *Tsc2* (Fig. 3 *A* and *B*), confirming that mammalian DEAF1 activates mTORC1 in myotubes.

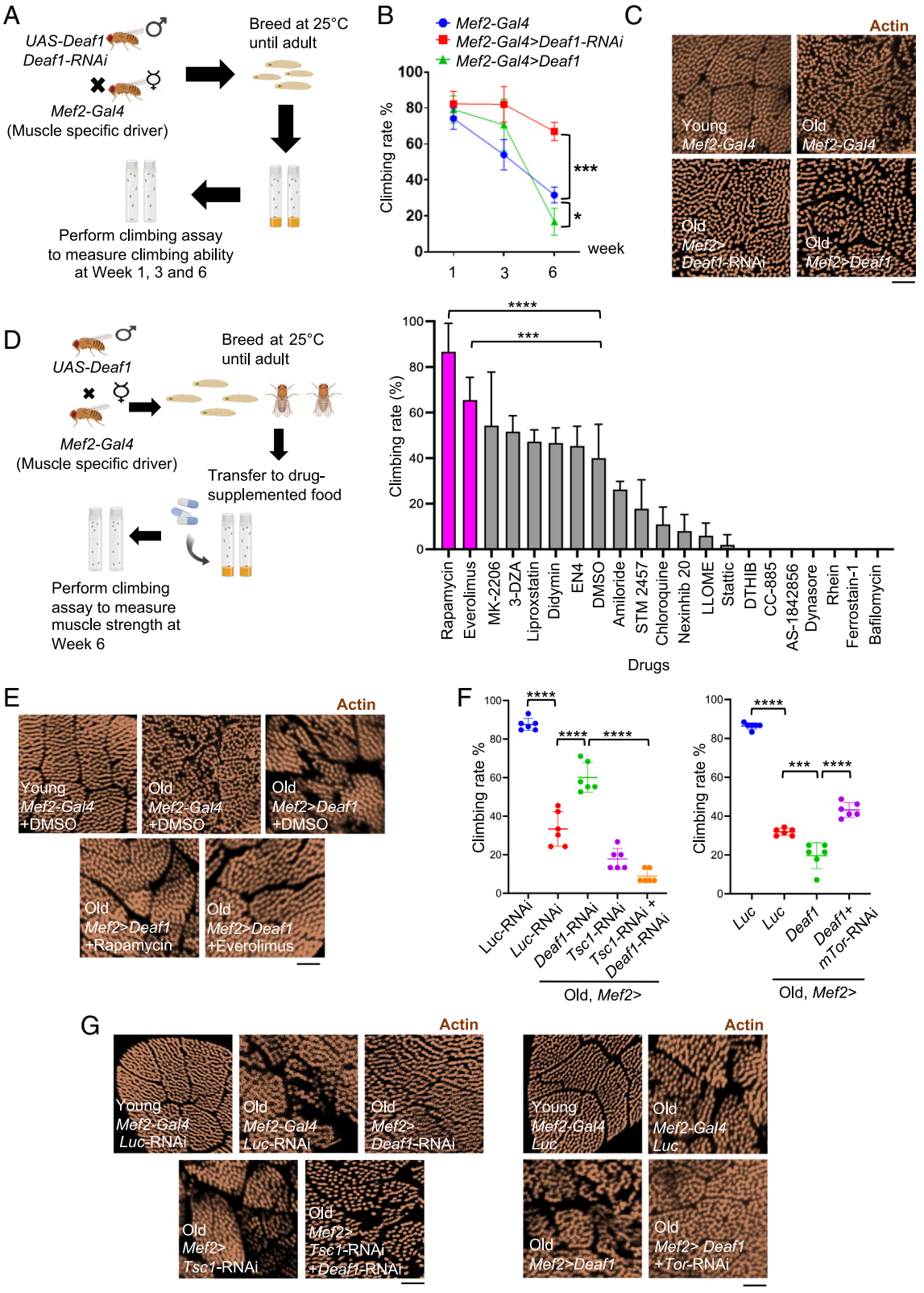


Fig. 1. *Drosophila* DEAF1 regulates aging-related muscle weakness via mTORC1. (*A*–*C*). *Deaf1* knockdown alleviates, while overexpression worsens, aging-induced muscle wasting. Experimental design using *Mef2-Gal4*, *Mef2-Gal4>UAS-Deaf1*, and *Mef2-Gal4>Deaf1-RNAi* (A). Muscle function was assessed by climbing assay at 1, 3, and 6 wk (*B*). Muscle cross-sections were stained with phalloidin to visualize actin organization and assess muscle morphology. [Scale bar, 20 μm (*C*)]. (*D*–*E*) mTORC1 inhibition mitigates *Deaf1*-induced muscle atrophy in aged flies. Experimental design testing drug effects on climbing ability (*D*). Phalloidin-stained muscle sections showing morphology. [Scale bar, 20 μm (*E*).] (*F*–*G*) Genetic interaction between *Deaf1* and *Tor* revealed by climbing performance and muscle morphology. (Scale bar, 20 μm.)

In addition, *Deaf1* overexpression significantly enhanced protein translation efficiency, as assessed by a puromycin incorporation assay (Fig. 3*C*), inhibited LC3 puncta formation (Fig. 3*E* and *SI Appendix*, Fig. S3), induced protein aggregate accumulation (assessed using ProteoStat dye) (Fig. 3 *F* and *G*), and promoted cellular senescence, as indicated by elevated *p16* and *p21* levels

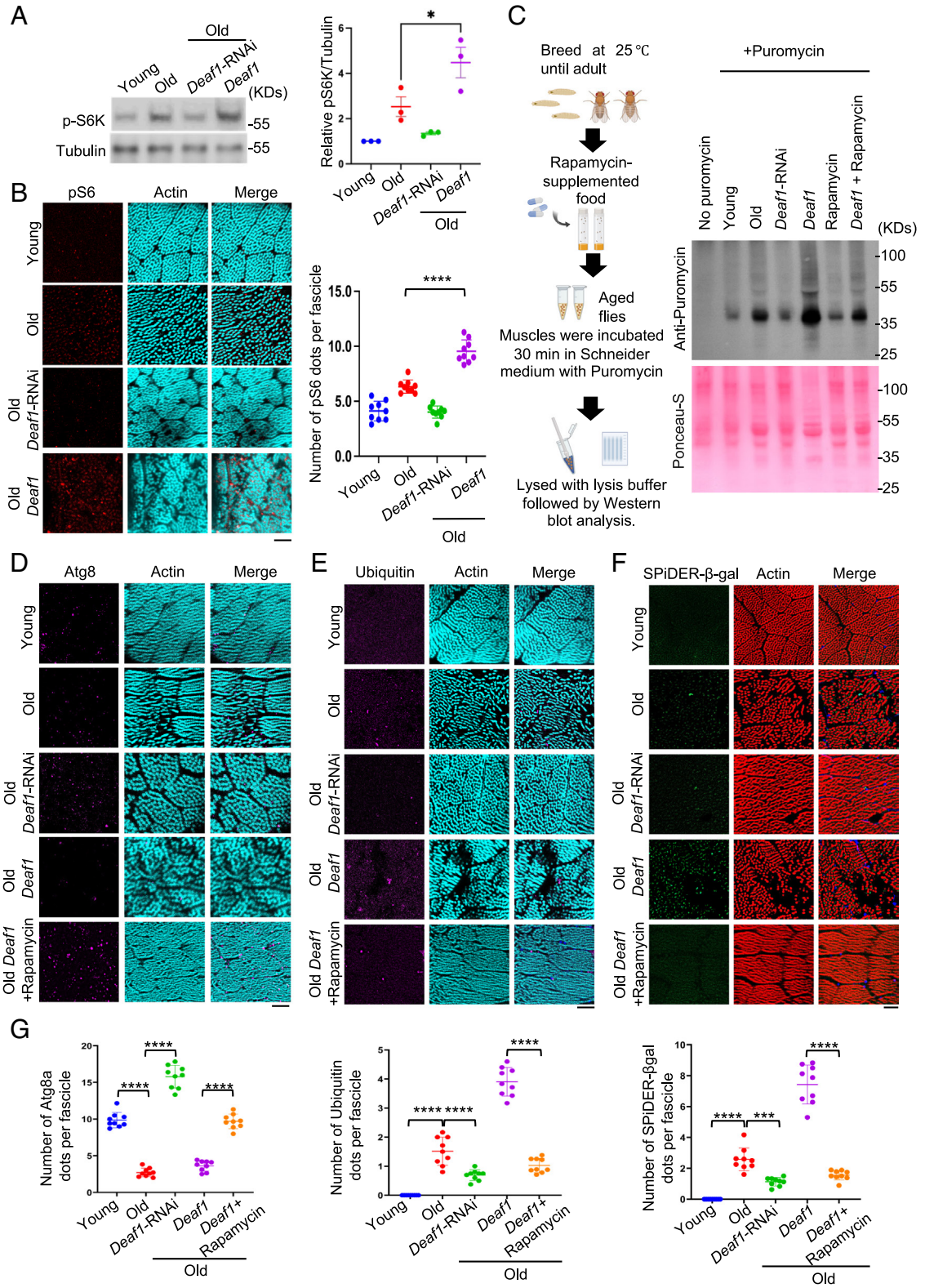


Fig. 2. DEAF1 regulates mTORC1 and mTORC1-mediated proteostasis and senescence in *Drosophila* muscles. (*A* and *B*) DEAF1 is required for age-dependent mTORC1 activation, shown by phospho-S6K immunoblotting (*A*) and phospho-S6 immunofluorescence (*B*) in 1- and 6-wk-old muscles expressing *Deaf1* or *Deaf1*-RNAi. (*C*) Protein synthesis in adult muscles was assessed via puromycin incorporation ± Rapamycin. (*D*–*G*) DEAF1 suppresses autophagy, increases protein aggregates, and accelerates senescence. Muscle sections were stained for Atg8 (*D*), ubiquitin (*E*), SPiDER-βGal (*F*), and phalloidin; quantifications are shown in (*G*). (Scale bar, 20 μm.)

(Fig. 3 *H* and *I*). These *Deaf1*-induced effects were reversed by Rapamycin treatment (Fig. 3 *A*–*I* and *SI Appendix*, Fig. S3).

Conversely, *Deaf1* depletion reduced translation, enhanced autophagy, decreased aggregates, and suppressed senescence, effects rescued by *Tsc2* knockdown (Fig. 3 *A*–*I* and *SI Appendix*, Fig. S3). Together, these findings show that DEAF1's regulation of mTORC1 and associated proteostasis and senescence is conserved from *Drosophila* to mammals.

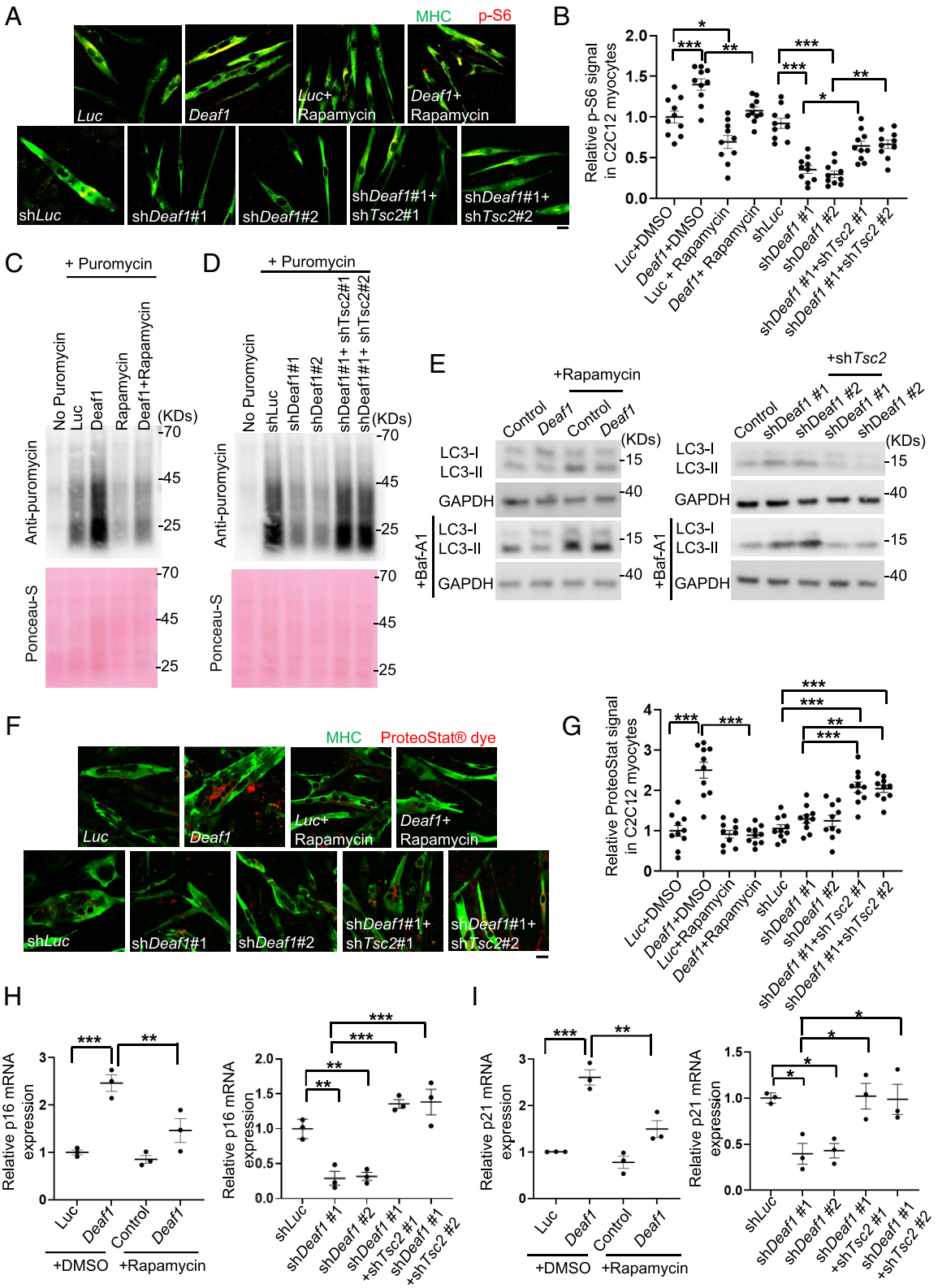
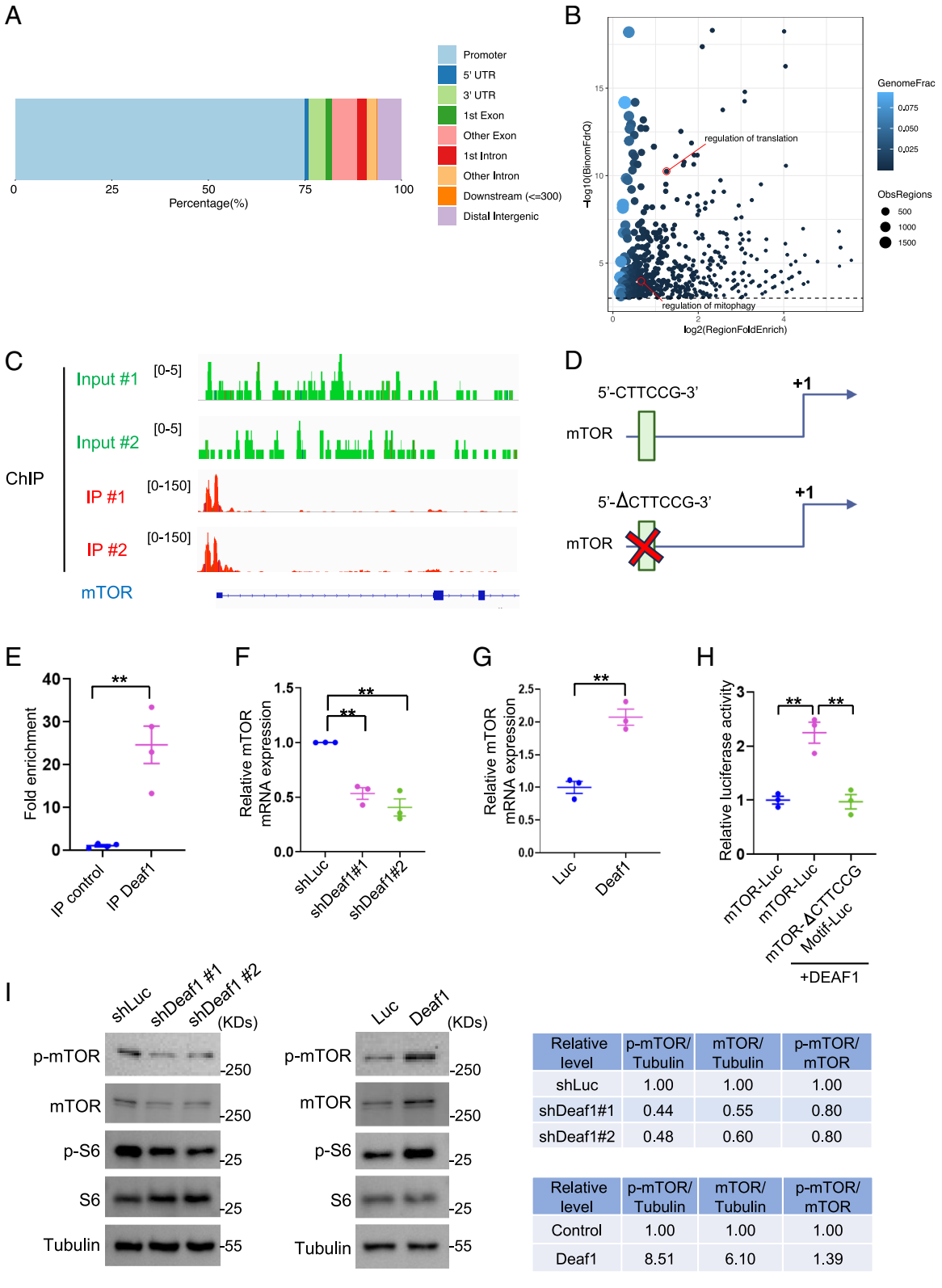


Fig. 3. Mammalian DEAF1 regulates mTORC1 activity in C2C12 myotubes. (*A* and *B*) DEAF1 acts upstream of mTORC1. pS6 immunostaining of myocytes expressing *Deaf1* or sh*Deaf1* ± sh*Tsc2* ± Rapamycin (*A*). Quantification in (*B*). (Scale bar, 20 μm.) (*C*–*G*) DEAF1 regulates protein homeostasis in an mTORC1-dependent manner. Puromycin incorporation (*C* and *D*), LC3 flux ± Baf-A1 (*E*), and ProteoStat staining (*F* and *G*) were analyzed. (Scale bar, 20 μm.) (*H* and *I*) DEAF1-mTORC1 modulates senescence markers p16 and p21 by qPCR.

Mammalian DEAF1 Binds the *mTOR* Promoter and Enhances Its Transcription. To elucidate how DEAF1 regulates mTORC1 signaling, we performed chromatin immunoprecipitation sequencing (ChIP-seq) in C2C12 myotubes. This identified 16,611 DEAF1 binding sites (IDR < 5%), with 75% located at gene promoters (Fig. 4*A* and Dataset S1), consistent with its role as a transcription factor. Functional enrichment analysis of DEAF1-bound genes revealed significant associations with pathways involved in

translation and mitophagy (Fig. 4*B*). Importantly, DEAF1 bound the promoter region of *mTOR* (Fig. 4*C*), where a specific DEAF1 motif (5′-CTTCCG-3′) was identified (14) (Fig. 4*D*). This interaction was validated by ChIP-qPCR, which showed a marked enrichment of DEAF1 at the *mTOR* promoter compared to controls (Fig. 4*E*).

To assess the functional impact of DEAF1 on mTOR transcription, we modulated *Deaf1* expression in C2C12 myotubes. *Deaf1* knockdown reduced *mTOR* mRNA levels, whereas *Deaf1* overexpression elevated them (Fig. 4 *F* and *G*). Luciferase assays using the *mTOR* promoter demonstrated that DEAF1 enhances transcriptional



Relative level	p-mTOR/ Tubulin	mTOR/ Tubulin	p-mTOR/ mTOR
shLuc	1.00	1.00	1.00
shDeaf1#1	0.44	0.55	0.80
shDeaf1#2	0.48	0.60	0.80

Relative level	p-mTOR/ Tubulin	mTOR/ Tubulin	p-mTOR/ mTOR
Control	1.00	1.00	1.00
Deaf1	8.51	6.10	1.39

Fig. 4. DEAF1 directly activates mTOR and mTORC1. (*A*) DEAF1 binding sites across the mouse genome. (*B*) Gene ontology (GO) enrichment analysis identifies that DEAF1 binds in the proximity of genes involved in regulation of translation and mitophagy. (*C* and *D*) ChIP-seq and genome browser tracks reveal DEAF1 binding at the *mTOR* promoter. Arrows indicate the ATG start codon. (*E*) ChIP-qPCR confirms DEAF1-*mTOR* promoter interaction. (*F–H*) DEAF1 increases *mTOR* mRNA and promoter activity; Depletion of the DEAF1 binding motif abolishes transcriptional activation. (*I*) DEAF1 elevates mTOR protein and mTORC1 signaling.

activity, which was abolished by deletion of the DEAF1 motif (mTOR1-ΔCTTCCG motif) (Fig. 4 *D* and *H*). These findings demonstrate that DEAF1 acts as a positive transcriptional regulator of *mTOR*.

These findings were further validated by western blot analysis. C2C12 myocytes expressing sh*Deaf1* exhibited reduced mTOR protein and decreased phosphorylation of mTOR and S6, whereas *Deaf1*-overexpressing cells showed the opposite effects (Fig. 4*I*), demonstrating that changes in mTOR mRNA levels translate into altered protein abundance and overall mTORC1 activity. Importantly, no changes were observed in Raptor or Rictor protein levels, or in total and phosphorylated AKT, in C2C12 myocytes expressing either *Deaf1* or sh*Deaf1* (*SI Appendix*, Fig. S4*A*), indicating that DEAF1 specifically regulates *mTOR* transcription and mTORC1 signaling, rather than broadly affecting other core mTOR complex components. Notably, this regulatory role of DEAF1 was not restricted to myocytes: similar effects were seen in L cells (fibroblasts) and LL2 cells (Lewis Lung Carcinoma), where Deaf1 depletion consistently reduced *mTOR* expression, while *Deaf1* overexpression increased it (*SI Appendix*, Fig. S4 *B* and *C*). Together, these findings establish DEAF1 as a general and conserved regulator of *mTOR* expression across diverse cell types.

FOXO1/3 Inhibits DEAF1 to Suppress mTORC1-Regulated Proteostasis and Senescence. FOXO transcription factors, known as "longevity genes", regulate protein homeostasis and stress responses (15). Our previous work showed that FOXO1 and FOXO3 repress *Deaf1* transcription by binding its promoter (14). Given that FOXO1 and FOXO3 are also known to inhibit mTORC1 under stress (28), we hypothesized that they act through DEAF1 to modulate mTORC1 activity.

To test this hypothesis, we induced expressions of constitutively active *FOXO1 (FOXO1-ADA)* or *FOXO3 (FOXO3-AAA)* in C2C12 myotubes. Both *FOXO1-ADA* and *FOXO3-AAA* reduced mTORC1 activity, as evidenced by decreased pS6K and pS6, detected via immunofluorescence and immunoblotting (*SI Appendix*, Fig. S5 *A* and *B*). Coexpression of *Deaf1* partially restored pS6K and pS6 levels that were reduced by FOXO1 or FOXO3 activation, suggesting that DEAF1 mediates FOXO-dependent inhibition of mTORC1 (*SI Appendix*, Fig. S5 *A* and *B*). Conversely, depletion of *FOXO1* or *FOXO3* resulted in increased pS6K and pS6 levels (*SI Appendix*, Fig. S5 *A* and *B*), accompanied by the accumulation of protein aggregates and LC3 puncta, as well as the upregulation of p16 and p21, key markers of cellular senescence (*SI Appendix*, Fig. S5 *C–E* and S6). These findings establish a regulatory axis wherein FOXO1 and FOXO3 inhibit *Deaf1* transcription to suppress mTORC1 activity and mitigate mTORC1-mediated muscle atrophy during aging. This axis links FOXO signaling to the regulation of proteostasis and the prevention of senescence through DEAF1.

Increases in *mTOR* mRNA Levels by the FOXO1/3–DEAF1 Axis in Aging Muscles. Although mTORC1 activation is commonly observed in aging muscles and chronic activation promotes muscle atrophy (29), the mechanisms driving this hyperactivation remain unclear. Our *Drosophila* and mammalian data suggest that mTORC1 activation arises from FOXO1/3–DEAF1-mediated transcriptional upregulation of *mTOR* (Figs. 1 and 2 and *SI Appendix*, Fig. S5). To validate this in vivo, we examined *mTOR* expression in aging muscles. Consistent with our hypothesis, qPCR analysis showed significantly elevated *mTOR* mRNA levels in aged tibialis anterior (TA) and gastrocnemius (GAS) muscles compared to young muscles (Fig. 5*A*). Publicly available SarcoAtlas data similarly revealed increased *mTOR* expression in aged GAS muscles (*SI Appendix*, Fig. S7 *A* and *B*) (30). Correspondingly, old muscles exhibited higher pS6K and pS6 levels, while pAKT remained unchanged, indicating that mTORC1 hyperactivation stems from *mTOR* transcriptional upregulation rather than upstream AKT signaling (*SI Appendix*, Fig. S7*C*).

To test whether modulation of the FOXO1/3–DEAF1 axis can restore *mTOR* regulation during aging, we either transduced skeletal muscles with adeno-associated virus serotype 9 (AAV9) vectors or performed intramuscular injections of the FOXO activator LOM612 in aged muscles (Fig. 5*B* and *SI Appendix*, Fig. S7 *D–E*). LOM612 promoted nuclear translocation of FOXO1/3 and significantly reduced *mTOR* mRNA levels (*SI Appendix*, Fig. S7 *F* and *G*). Similarly, AAV9-mediated expression of constitutively active FOXO3 (*FOXO3-AAA*) or *Deaf1* knockdown (AAV9-sh*Deaf1*) decreased *mTOR* transcripts (Fig. 5*C*). Furthermore, the *FOXO3-AAA*–induced reduction of *mTOR* transcripts was reversed by *Deaf1* overexpression, indicating that DEAF1 acts downstream of FOXOs to mediate their transcriptional control of *mTOR* (Fig. 5*C*). Together, these findings identify the FOXO1/3–DEAF1 axis as a key driver of age-related *mTOR* upregulation and mTORC1 overactivation, linking disrupted FOXO signaling to muscle decline during aging.

In Vivo Modulation of FOXO–DEAF1 Signaling Reverses Muscle Atrophy During Aging. To further investigate the role of FOXO–DEAF1 signaling, we injected LOM612 into TA and GAS muscles of aged mice to reduce *Deaf1* expression (*SI Appendix*, Fig. S7*E*). LOM612-mediated *Deaf1* suppression decreased *mTOR* levels and mTORC1 activity, inhibited protein synthesis, and enhanced autophagy, as evidenced by increased LC3 puncta formation in aging muscles (*SI Appendix*, Fig. S7 *H–K*). Additionally, reduced *Deaf1* expression suppressed age-related cellular senescence, indicated by lower p16 and p21 levels and decreased SPiDER-β-gal activity (*SI Appendix*, Fig. S7 *L* and *M*). These results suggest that FOXO activation reduces *Deaf1* expression, improving protein homeostasis and mitigating senescence in aging muscles.

Consistently, AAV9-mediated expression of *FOXO3-AAA* or *Deaf1* knockdown not only lowered *Deaf1* levels in muscles but also decreased *mTOR* levels, inhibited protein synthesis, increased autophagy, and suppressed senescence (Fig. 5 *D–J*). Co-expression of *Deaf1* reversed the *FOXO3-AAA*-induced effects, confirming that Deaf1 functions downstream of FOXOs in this pathway (Fig. 5 *D–J*). Collectively, these results establish the FOXO-DEAF1 signaling pathway as a key regulator of muscle homeostasis and senescence in aging muscles.

Exercise Activates FOXOs to Suppress DEAF1 and Improve Aging Muscle Defects. Regular exercise is a well-established intervention for maintaining muscle mass and function, making it a key strategy to combat sarcopenia (4). Studies in *Drosophila* have shown that exercise activates muscle FOXO, mitigating age-related decline (31, 32). Supporting these findings, recent evidence shows that *FOXO1* and *FOXO3* gene expression is upregulated following acute bouts of exercise in humans and rodents (33–35). Furthermore, exercise has been shown to inhibit both *mTOR* expression and phosphorylated mTOR levels (36). These findings suggest that exercise may regulate the FOXO–DEAF1–mTORC1 axis to preserve muscle homeostasis during aging.

To test this, we examined whether exercise-activated FOXOs suppress *mTOR* expression via *Deaf1* downregulation. Exhaustive treadmill running and resistance wheel exercise in aged mice markedly reduced *mTOR* mRNA and protein levels and mTORC1 activity compared to sedentary controls (Fig. 6 *A–E* and *SI Appendix*, Fig. S8 *A–C*). Exercise also decreased protein synthesis, increased autophagy, and suppressed senescence, indicating a reduction in mTORC1 activity and reversal of muscle aging (Fig. 6 *F–J* and *SI Appendix*, Fig. S8

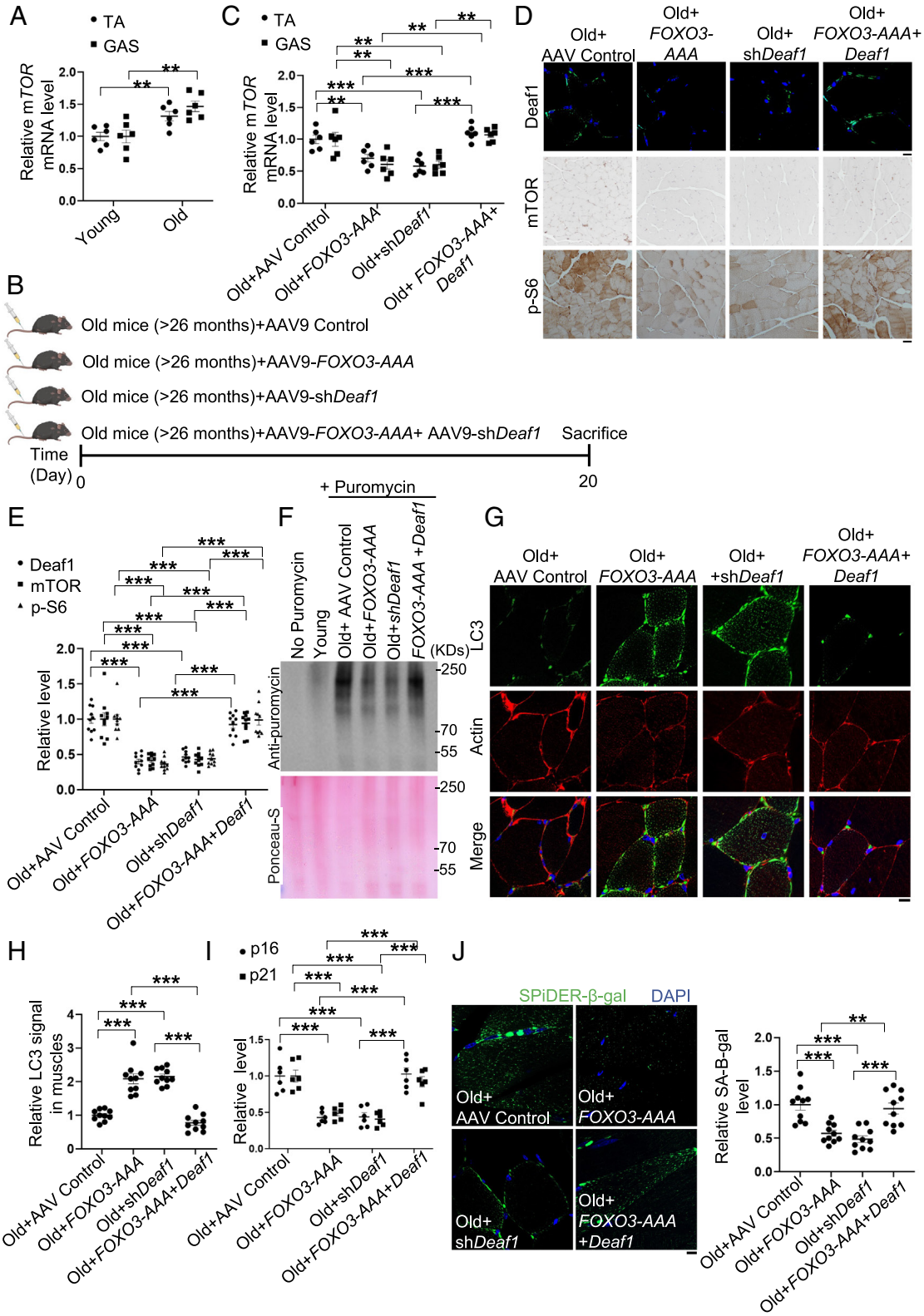


Fig. 5. Modulating FOXO–DEAF1 signaling reverses aging-related muscle defects. (*A*) *mTOR* expression is elevated in aged TA and GAS muscles. (*B–E*) FOXO activation or *Deaf1* knockdown suppresses *mTOR* expression and mTORC1 activity in old muscles, shown by qPCR (*C*), immunostaining/IHC (*D*), and quantification (*E*). [Scale bar, 10 μm (DEAF1), 100 μm (mTOR, pS6).] (*F–J*) FOXO–DEAF1 regulates protein synthesis, autophagy, and senescence in aged GAS muscles, assessed by puromycin incorporation (*F*), LC3/phalloidin staining (*G* and *H*), and SPiDER-βGal/qPCR (*I* and *J*). (Scale bar, 10 μm.)

D–J). Notably, *Deaf1* expression was significantly decreased in exercised aged muscles (Fig. 6 *D–E* and *SI Appendix*, Fig. S8 *D–E*). Inhibition of FOXO activity using AS1842856, AAV9-mediated knockdown of *FOXO1* or *FOXO3*, or *Deaf1* overexpression reversed these benefits—restoring *Deaf1* expression and abolishing exercise-induced changes in mTOR signaling, proteostasis, and senescence (Fig. 6 *D–J* and *SI Appendix*, Fig. S8). These findings show that FOXO-mediated downregulation of *Deaf1* is indispensable for the ability of exercise to suppress *mTOR* signaling, contributing to improved muscle proteostasis and delayed aging.

Discussion

mTORC1 hyperactivation is a hallmark of age-related muscle deterioration, yet the mechanisms underlying this dysregulation have remained unclear. Here, we identify DEAF1, a FOXO-regulated transcription factor, as a key upstream activator of mTORC1 signaling in aging muscle. Increased *Deaf1* levels in aged muscle promote *mTOR* transcription, leading to heightened mTORC1 activity, excessive protein synthesis, impaired autophagy, and muscle senescence. Conversely, exercise suppresses *Deaf1* expression via

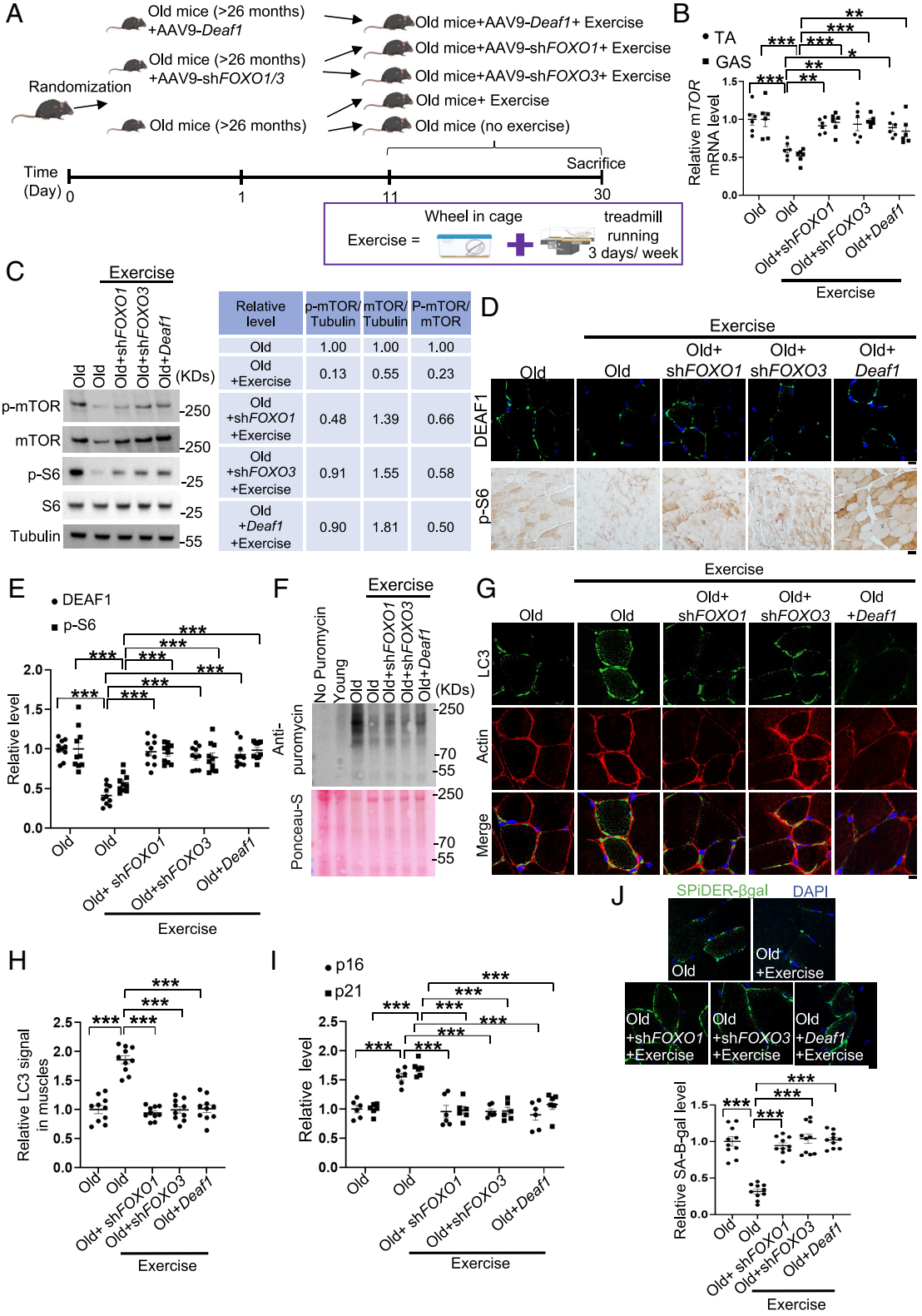


Fig. 6. Long-term exercise activates FOXOs to decrease *Deaf1* expression and improve aging muscle defects. (*A–E*) Exercise reduces mTOR and mTORC1 activity via the FOXO–DEAF1 axis. Old mice with AAV9-sh*FOXO1/3*, AAV9-*Deaf1*, or exercise (*A*) were analyzed by qPCR (*B*), Western blot (*C*), and DEAF1/pS6 immunostaining (*D* and *E*). [Scale bar, 10 μm (DEAF1), 100 μm (pS6).] (*F–J*) Exercise preserves proteostasis and limits senescence, assessed by puromycin incorporation (*F*), LC3/phalloidin staining (*G* and *H*), and SPiDER-βGal/qPCR (*I* and *J*). (Scale bar, 10 μm.)

FOXO activation, normalizing mTORC1 activity, restoring proteostasis, and mitigating muscle senescence. These findings reveal exercise as a physiological modulator of DEAF1 and highlight the FOXO–DEAF1–mTORC1 pathway as a therapeutic target for sarcopenia (*SI Appendix*, Fig. S9).

Age-Dependent Role of DEAF1 in Muscle. DEAF1 shares functional parallels with mTORC1, regulating protein synthesis, autophagy, and metabolism (3, 10). Our ChIP-seq data show that DEAF1 binds directly to the *mTOR* promoter, enhancing its transcription. However, an intriguing aspect of our findings is that *Deaf1* overexpression in *Drosophila* muscle primarily affects aging muscle, while young muscle remains resistant to *Deaf1*-induced phenotypes. This observation raises the fundamental question: What age-related changes contribute to this differential response?

One possibility is that the regulatory landscape of mTORC1 undergoes a significant shift with aging. In young muscle, mTORC1 activity is tightly regulated through a dynamic balance between anabolic and catabolic signals, ensuring proper protein synthesis while maintaining cellular homeostasis. With aging, this balance is disrupted—autophagic capacity declines, impairing the muscle's ability to clear damaged proteins and organelles. Simultaneously, oxidative stress accumulates due to mitochondrial dysfunction and reduced antioxidant defenses, creating a more vulnerable environment where mTORC1 overactivation becomes harmful (18). In this context, DEAF1-driven enhancement of mTORC1 signaling may push aged muscle beyond a critical threshold, leading to excessive protein synthesis, impaired proteostasis, and cellular senescence. Increased *Deaf1* expression in aged muscle may further amplify *mTOR* transcription and exacerbate mTORC1 hyperactivation. Compounding this, FOXO transcription factors—which normally act as negative regulators of DEAF1—are subject to age-related repression, permitting unchecked DEAF1 activity. Together, these factors suggest that the impact of DEAF1 on mTORC1 signaling is highly context-dependent, with aging muscle becoming increasingly vulnerable to its dysregulation.

Our ChIP-seq also identified 38 DEAF1 target genes linked to mitophagy regulation, consistent with FOXO's known role in controlling autophagy and mitochondrial quality (37). While direct repression of mitophagy by DEAF1 remains to be proven, its enrichment near mitophagy-related genes supports a model in which DEAF1 disrupts mitochondrial turnover, contributing to autophagy impairment and senescence.

DEAF1-Driven *mTOR* Upregulation and mTORC1 Hyperactivation. mTOR is a nutrient- and growth-responsive kinase that integrates anabolic and catabolic cues (3, 38). While mTORC1 activity is classically regulated post-translationally, recent studies indicate that *mTOR* transcriptional control represents an additional regulatory layer. As the core kinase of mTORC1, mTOR abundance directly influences complex formation and activity, where even moderate increases in *mTOR* transcription can drive mTORC1 hyperactivation (39, 40). Studies suggest that multiple transcriptional activators elevate *mTOR* mRNA levels and enhance mTOR phosphorylation, leading to sustained mTORC1 activation (39, 40). Moreover, dysregulated *mTOR* expression is implicated in various human diseases, underscoring its critical role in cellular homeostasis and pathology (39).

As a transcriptional activator, DEAF1 enhances *mTOR* mRNA expression, increasing mTOR protein levels. Given that mTOR abundance is a key determinant of mTORC1 activity, its elevated expression may promote complex formation and sustain signaling (39, 40). Prolonged mTORC1 hyperactivation suppresses autophagy, as mTORC1 phosphorylates key autophagy-related proteins, inhibiting autophagosome formation (3). In aged muscle, where autophagic clearance is already compromised, excessive mTORC1 activity accelerates the accumulation of damaged organelles and misfolded proteins, exacerbating cellular stress and muscle degeneration. This self-perpetuating cycle of mTORC1 hyperactivation reinforces proteostasis dysfunction and accelerates muscle decline. Thus, DEAF1-driven mTOR upregulation transforms mTORC1 from a dynamic signaling hub into a persistently active complex that drives age-related muscle decline. Targeting DEAF1 or its downstream effectors may therefore offer a strategy to restore proteostatic balance and mitigate sarcopenia.

Exercise, FOXO, DEAF1, and mTORC1: A Balancing Act in Muscle Aging. Exercise exerts dual effects on mTORC1 depending on duration and intensity. Acute or resistance exercise transiently stimulates mTORC1 to promote muscle protein synthesis (41, 42), whereas chronic exercise suppresses mTORC1 via AMPK activation (35, 43), and FOXO-mediated repression of *Deaf1*. Since DEAF1 enhances *mTOR* transcription, its inhibition by FOXO serves as a key regulatory checkpoint, maintaining mTORC1 balance to support anabolism while preserving proteostasis and preventing muscle degeneration.

FOXO transcription factors are conserved regulators of oxidative stress resistance, longevity, and suppression of senescence (44). While their protective role has been primarily attributed to the induction of antioxidant genes (44), our findings reveal an additional mechanism: FOXO restrains mTORC1 by repressing DEAF1, reducing *mTOR* transcription and preventing hyperactivation. Given that sustained mTORC1 signaling promotes oxidative stress and muscle aging, FOXO-mediated suppression of DEAF1 represents a crucial homeostatic control.

Context-Dependent Roles of FOXO in Muscle Aging. FOXO factors are traditionally associated with muscle atrophy through activation of proteolytic and autophagy–lysosome pathways. However, their role in aging muscle appears highly context-dependent. In young muscle, FOXO activation drives catabolic programs that promote atrophy, whereas in aged muscle, FOXO supports beneficial processes such as autophagy, mitochondrial quality control, and stem cell maintenance (31, 45, 46). Consistent with this view, our data indicate that FOXO activation preserves muscle integrity during aging by repressing DEAF1 and preventing mTORC1 overactivation.

In contrast, Penniman et al. reported that FOXO inhibition improved muscle function in aged mice (47). These divergent outcomes may reflect differences in age-related physiology, compensatory mechanisms in knockout models, or variations in the extent and duration of FOXO modulation. Collectively, the evidence suggests that FOXO's effects are finely tuned by context rather than inherently beneficial or detrimental. Precise modulation of FOXO signaling—balancing its catabolic and protective functions—will be key to harnessing its therapeutic potential in muscle aging.

Materials and Methods

***Drosophila* Husbandry, Genetic crosses, Climbing Assay and Drug Screenings.** *Drosophila* were maintained at 25 °C under 60% humidity with a 12-h light/dark cycle and fed standard cornmeal food as described in the *SI Appendix*. Details of fly stocks, genetic crosses, aging induction, and drug screening procedures are provided in the *SI Appendix*.

Cell Culture and Miscellaneous Treatments. C2C12, HEK293T, LL/2, and L cells were obtained from Prof. David Virshup (CSCB Department, Duke-NUS Medical School, Singapore). Details of cell maintenance, differentiation protocols, and drug treatments are provided in the *SI Appendix*.

Plasmid Constructs, Transfection, and Virus Production. Plasmids were constructed for gene overexpression or knockdown. Transfections, lentivirus and AAV production, and subsequent cell or mouse infections were performed as described. Detailed protocols are provided in the *SI Appendix*.

Immunofluorescence, Histology, Immunohistochemistry, SPiDER-βGal, and ProteoStat Staining. For Drosophila muscle staining, thoraces were dissected, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, 15710), and bisected along the axial plane (48). For cell staining, cells were fixed with 4% paraformaldehyde or ice-cold methanol (Merck, 106009) and blocked with 5% goat serum. Mouse muscle tissues were fixed in 10% formalin (VWR, VWRQ11699404), dehydrated, paraffin-embedded, sectioned at 5 μm, deparaffinized, rehydrated, and subjected to antigen retrieval in citric acid buffer. Detailed protocols for subsequent steps are provided in the *SI Appendix*.

Surface Sensing of Translation (SUnSET) Assay and Western Blot. For the Drosophila SUnSET assay, thoraces were dissected and incubated in puromycin-containing Schneider's medium for 30 min, then lysed in RIPA buffer (BioBasic, RB4475) supplemented with protease inhibitor cocktail (MedChemExpress, HY-K0010). For mouse experiments, puromycin (40 nmol/g body weight; InvivoGen, ant-pr-1) was administered intraperitoneally 30 min before sacrifice, followed by gastrocnemius muscle collection and lysis in RIPA buffer with protease inhibitors. Cultured cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (PhosSTOP, Roche, PHOSS-RO). Protein quantification and Western blot procedures are described in the *SI Appendix*.

RNA Isolation and Quantitative RT-PCR. Cultured cells or tissues were lysed with TRIzol reagent (Invitrogen, 15596-018) and RNA was extracted as described previously (49). RNA was reverse transcribed to cDNA with PureNA First strand cDNA synthesis Kit (Research Instruments, KR01-100). qRT-PCR was performed using Taq Pro Universal SYBR qPCR Master Mix (Vazyme Q717-02) according to the manufacturer's instructions, with details in *SI Appendix*.

Chromatin Immunoprecipitation (ChIP), Sequencing, and ChIP-qPCR. ChIP was performed in C2C12 myocytes using the Simple ChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology, 9005) according to the manufacturer's instructions. ChIP-seq and ChIP-qPCR were conducted as previously described (14, 50). Detailed protocols are provided in the *SI Appendix*.

Mouse Husbandry and Exercise Regimen. Mice were housed in individually ventilated cages under 12-h light/dark cycles with ad libitum access to food and water. All experiments were conducted at Duke-NUS Medical School, Singapore, in accordance with IACUC-approved protocols (2021/SHS/1695). Six male mice per group were used. Mice underwent voluntary wheel running and treadmill exercise; detailed protocols are provided in the *SI Appendix*.

Miscellaneous Mouse Experiments. Old (>26 mo) C57BL/6 mice received intramuscular injections of 10 μL of 2 μM LOM612, 10 μL of 50 μM AS1842856, or DMSO into the gastrocnemius every two days. After 20 d, mice were sacrificed, and hindlimb muscles and plasma were collected for analysis.

AAV9-Mediated Gene Delivery. AAV9 vectors were administered to mice in conjunction with exercise interventions. Detailed procedures are provided in the *SI Appendix*.

Statistical Analysis. Data are presented as mean ± SEM. Differences between two groups were analyzed by unpaired Student's *t* test, and among multiple groups by one-way ANOVA with Tukey's post-hoc test. Analyses were performed using GraphPad Prism. Significance was defined as *P*-values were less than *0.05, **0.01, ***0.001, and ****0.0001.

Data, Materials, and Software Availability. ChIP-seq data has been submitted to GEO (GSE293804) (51). Code to recreate the reported ChIP-seq analysis are available from https://github.com/harmstonlab/Choy_et_al_deaf1 (52).

ACKNOWLEDGMENTS. This work was supported by Singapore Ministry of Education (2022-MOET1-0004, FY2025-MOET1-0004), Diana Koh Innovative Cancer Research Award (Duke-NUS-DKICRA/2024/0001), National Academy of Medicine (MOH-001189-00), and National Medical Research Council (MOH-001208-00, MOH-001885-00, MOH-001831-00). Q.G. and G.K.P. were supported by the Khoo Postdoctoral Fellowship (Duke-NUS-KPFA/2025/0078; Duke-NUS-KPFA/2024/0075).

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