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Modulation of protease expression by the transcription factor Ptx1/PITX regulates protein quality control during aging

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SUMMARY

Protein quality control is important for healthy aging and is dysregulated in age-related diseases. The autophagy-lysosome and ubiquitin-proteasome are key for proteostasis, but it remains largely unknown whether other proteolytic systems also contribute to maintain proteostasis during aging. Here, we find that expression of proteolytic enzymes (proteases/peptidases) distinct from the autophagy-lysosome and ubiquitin-proteasome systems declines during skeletal muscle aging in *Drosophila*. Age-dependent protease downregulation undermines proteostasis, as demonstrated by the increase in detergent-insoluble poly-ubiquitinated proteins and pathogenic huntingtin-polyQ levels in response to protease knockdown. Computational analyses identify the transcription factor Ptx1 (homologous to human PITX1/2/3) as a regulator of protease expression. Consistent with this model, Ptx1 protein levels increase with aging, and Ptx1 RNAi counteracts the age-associated downregulation of protease expression. Moreover, Ptx1 RNAi improves muscle protein quality control in a protease-dependent manner and extends lifespan. These findings indicate that proteases and their transcriptional modulator Ptx1 ensure proteostasis during aging.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

J.J. did most of the experiments, with help from M.C., F.A.G., and M.R-M.; J.J. and F.D. analyzed most of the data; A.S. analyzed confocal microscopy images; D.F., B.X., and Y.F. analyzed RNA-seq data and did computational analyses. F.D. supervised the project and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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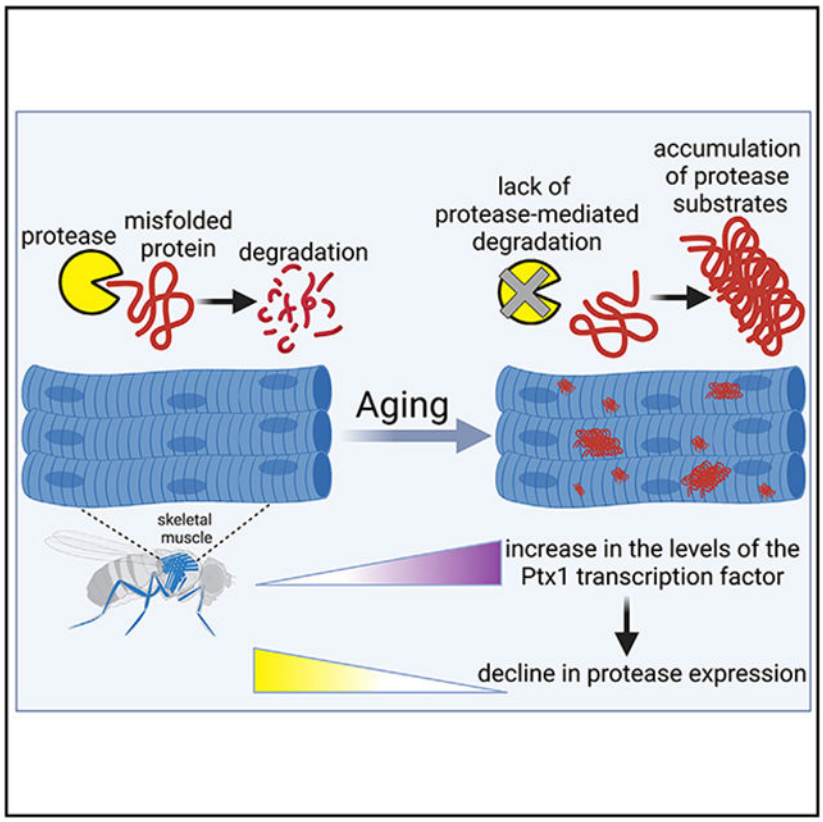

In brief

Jiao et al. find that protease expression declines during skeletal muscle aging in *Drosophila* and that this undermines proteostasis. They identify the transcription factor Ptx1/PITX as a repressor of protease expression and show that Ptx1/PITX knockdown improves muscle protein quality control during aging in a protease-dependent manner and extends lifespan.

INTRODUCTION

Collapse of protein homeostasis (proteostasis) is a major hallmark of aging in all organisms,[1] and it is causally involved in the pathogenesis of many age-related diseases such as Alzheimer, Huntington, and other neurodegenerative diseases.[2,3] The maintenance of protein quality relies on the concerted action of chaperones and protein degradation,[4] which is carried out by the autophagy/lysosome and ubiquitin/proteasome systems.[5] It is largely unknown whether other proteolytic systems contribute to degrade dysfunctional proteins and maintain protein quality control during aging.

Proteases and peptidases are a large class of proteolytic enzymes with diverse substrate specificity and intracellular localization.[6-8] Despite the involvement of proteases in some age-related processes,[9-12] there is limited understanding of the role that proteases play in protein quality control during aging.[8] Apart from mitochondrial proteases, which have long been known to maintain mitochondrial proteostasis and function,[9] less is known on whether proteases that localize to other cellular compartments also contribute to protein

quality control during aging. In support of this hypothesis, some cytoplasmic proteases are induced in response to and partially compensate for proteasome dysfunction.[13-16] Moreover, puromycin-sensitive aminopeptidase (Psa) promotes the degradation of pathogenic huntingtin in cooperation with autophagy[11] and the proteasome.[17] In addition to the degradation of pathogenic proteins such as huntingtin-polyQ, proteases have also been found to degrade misfolding-prone proteins (Highroad and Jonah65A-IV proteases[16,18]) and the heterogeneous pool of poly-ubiquitinated proteins that accumulate in cells exposed to proteasome inhibitors (TPPII protease[13,15]). However, it remains largely undetermined whether proteases can target misfolded and/or poly-ubiquitinated proteins in aging tissues, which have reduced capacity for protein turnover.

Skeletal muscle is one of the tissues with the highest challenges to protein quality control due to contraction-associated mechanical protein unfolding and oxidative damage.[19-23] In both *Drosophila* and mammals,[24] skeletal muscle aging is characterized by a prominent loss of protein quality control,[20,21,25-28] as indicated by the progressive accumulation of poly-ubiquitinated protein aggregates with aging and by the age-related increase in ubiquitinated proteins detected in detergent-insoluble fractions (Figure 1A). Although dysfunction of the autophagy-lysosome and ubiquitin-proteasome systems has been shown to contribute to loss of proteostasis in muscle with aging,[20,29-31] it is largely unknown whether other proteolytic systems contribute to the derangement of muscle proteostasis.

Here, we report that protein quality control is ensured during aging by proteases/peptidases that prevent the accumulation of poly-ubiquitinated and aggregation-prone proteins. Computational analyses indicate that the transcription factor Ptx1 is a regulator of protease expression and, consistent with this model, we find that Ptx1 protein levels increase during aging concomitant to downregulation of protease expression. Remarkably, Ptx1 RNAi impedes the age-dependent downregulation of protease expression and preserves muscle protein quality control during aging in a protease-dependent manner. Altogether, these findings indicate that Ptx1 and proteases/peptidases have important roles in maintaining proteostasis during aging.

RESULTS

Decline in protease/peptidase expression occurs during skeletal muscle aging

As previously described,[20,29,30,33-37] skeletal muscle aging is characterized by a progressive decline of protein quality control in *Drosophila* (Figure 1A). To determine unexplored mechanisms that govern proteostasis during muscle aging, RNA sequencing (RNA-seq) was used to examine the gene expression changes that occur in *Drosophila* skeletal muscles with aging.[32] Analysis of gene ontology categories revealed that there is increased expression of proteasomal components and chaperones in old versus young (Figure 1B), presumably as part of an age-induced adaptive stress response.[38] However, a gene cluster consisting of peptidases and proteases is the most downregulated with aging (Figure 1C). Analysis of age-associated mRNA changes in protease levels, as annotated in the MEROPS database,[7] further indicates that there are many proteases that are age-downregulated, whereas fewer have increased expression with aging (Figure 1C). Specifically, out of 532 proteases annotated in MEROPS, 341 were found to be expressed in skeletal muscle and 198

were significantly regulated by aging ($p < 0.05$). For example, the proteases *CG16749, CG17571, CG17633, CG31233, CG31343, CG4 563, CG6733, CG8560, CG8774, iotaTry*, and *Jon99Ci* are significantly downregulated (Figure S1).

Analysis of protease gene families[7] revealed that there is an overall decline in the average gene expression of aspartic peptidases, metallopeptidases, and serine peptidases in old versus young skeletal muscle (Figure 1D). Conversely, there is overall increased expression with aging of cysteine peptidases (Figure 1D), which include calpains that have been previously implicated in protein degradation and muscle mass loss in aged mammals.[39,40] Further analysis of a set of 72 proteases more stringently regulated by aging ($p < 0.05$ and Log2R > 1 and <−1) revealed a similar distribution. Altogether, these findings suggest that decreased expression of proteases in skeletal muscle may contribute to the decline in protein quality control during aging.

RNAi for age-downregulated proteases compromises muscle protein quality control during aging

To test the function of age-downregulated proteases, we used the UAS/Gal4 system and the skeletal muscle driver *Mhc-Gal4* to knockdown from young age the levels of proteases that are age-downregulated. Specifically, we targeted 35 age-downregulated proteases (for which RNAi stocks were available) out of the 72 proteases that are stringently regulated by aging ($p < 0.05$ and Log2R > 1 and <−1; Figure 1D). For these experiments, we examined by western blot the levels of poly-ubiquitinated proteins in detergent-soluble and -insoluble fractions of skeletal muscle (Figure S2) from flies at 5 weeks of age, a time point at which there are moderate defects in proteostasis (Figure 1A) and hence interventions that worsen proteostasis can be scored easily.

In many cases, RNAi for individual proteases induced only minor changes in poly-ubiquitinated proteins found in detergent-insoluble fractions (Figures 2A and 2B), which correspond to misfolded poly-ubiquitinated proteins stored in protein aggregates,[20,41] suggesting that there might be redundancy in maintaining proteostasis among age-downregulated proteases and/or insufficient RNAi-mediated knockdown. However, 31 out of 121 RNAi interventions targeting age-downregulated proteases displayed a substantial increase (>50%) in the levels of detergent-insoluble poly-ubiquitinated proteins and/or Ref(2)P/p62 (Figures 2A-2C). Overall, these findings suggest that many age-downregulated proteases are necessary to preserve muscle proteostasis.

To further examine the role of age-downregulated proteases in protein quality control, confocal microscopy with anti-ubiquitin and anti-Ref(2)P/p62 antibodies was used to visualize protein aggregates in skeletal muscles of 5-week-old flies (Figure 2D). As expected based on the biochemical analysis with western blots for detergent-soluble and -insoluble fractions (Figures 2A-2C and S2), overall similar findings were obtained via confocal microscopy. Specifically, RNAi for proteases that scored in western blots (Figures 2A-2C) led to an increase in the number and total levels of poly-ubiquitin protein aggregates also when analyzed by confocal microscopy (Figures 2D and 2E). Altogether, these findings indicate that decreased expression of some proteases during aging contributes to age-related decline in protein quality.

To determine whether these proteases could be acting via autophagy, we examined the levels Atg8/LC3-I and LC3-II: lipidation of LC3-I leads to the formation of LC3-II, which is a key component of autophagosomal membranes and hence it is indicative of autophagosome formation.[42-46] Interestingly, RNAi for a few proteases led to changes in the total levels of LC3 and/or in the LC3-II to LC3-I ratio (Figure S2), which can indicate induction of autophagy or conversely a block in the autophagic flux.[42-46] However, many proteases did not affect Atg8/LC3-I and LC3-II levels (Figure S2), suggesting that RNAi for many age-regulated proteases might not affect autophagy.

Knockdown of age-downregulated proteases increases pathogenic huntingtin aggregates in the retina during aging

To further test the role of age-downregulated proteases, we next examined their capacity to degrade a model aggregation-prone protein, pathogenic huntingtin. For these experiments, we examined *Drosophila* retinas that express GFP-tagged huntingtin-polyQ72 because this system allows for the analysis of huntingtin-polyQ pathogenicity without compromising organismal survival.[47] Specifically, we examined whether RNAi for proteases that are age-downregulated can increase or decrease the amount of huntingtin-polyQ72-GFP aggregates. As expected based on a previous study,[11] RNAi for the puromycin-sensitive aminopeptidase Psa and RNAi for the ubiquitin-conjugating enzyme Ubc6 led to an increase in the total amount of protein aggregates, compared with control RNAi interventions such as cherry^{RNAi} (Figures 3A and S3). Conversely, RNAi for Pdk1 and Akt1 reduced the amount of protein aggregates, consistent with the previously reported improvement in proteostasis upon reduced Insulin receptor/Akt signaling.[2] Interestingly, RNAi for many age-downregulated proteases led to an overall increase in the amount of huntingtin-polyQ72-GFP aggregates, indicating that their adequate expression is needed to preserve proteostasis (Figures 3A, 3B, and S3).

We further tested these findings by probing the levels of huntingtin-polyQ72-GFP by western blot (Figure S4). Consistent with the analysis of fluorescent protein aggregates (Figures 3A, 3B, and S3), RNAi for age-downregulated proteases led to an increase in the levels of huntingtin-polyQ72-GFP (Figures 3C and 3D). This change was particularly prominent for polyQ proteins detected in the stacking gel (Figure 3D), which consists of urea-resistant aggregates that are not resolved by SDS-PAGE.[47] Altogether, quantification of western blots indicates that RNAi for many age-downregulated proteases leads to an increase in huntingtin-polyQ protein aggregates (Figures 3C and 3D), as also observed by microscopy (Figures 3A, 3B, and S3).

The protein levels of the transcription factor Ptx1 increase during aging, and Ptx1 knockdown promotes the expression of age-downregulated proteases

Having determined an important role for age-downregulated proteases in protein quality control, we next examined the mechanisms underlying their age-related transcriptional down-regulation. To this purpose, the proximal promoter regions of age-downregulated proteases were examined via computational analyses to identify enrichment for transcription factor binding sites.

This analysis pinpointed to possible roles of the homeodomain transcription factor Ptx1 (Figure 4A), homologous to human PITX1/PITX2/PITX3 transcription factors, which have been implicated in the development of many tissues.[48-51] Specifically, PITX1 binding sites ranked second (after TATA box sequences) among motifs enriched in the promoter regions of age-downregulated proteases (Figures 4A and S5).

Because this computational survey suggests that Ptx1 may regulate protease gene expression during aging, we next tested whether Ptx1 protein levels are age-regulated. For these studies, we utilized a previously generated antibody that was used to characterize Ptx1 protein expression during *Drosophila* development.[52] Western blot analysis with this antibody indicates that Ptx1 protein levels increase during aging (Figure 4B). We also confirm that this antibody recognizes a specific band that corresponds to the molecular weight of Ptx1 (~62 kDa) and also find that Ptx1 RNAi prevents its age-dependent upregulation (Figure 4C). Altogether, these findings suggest that Ptx1 is a repressor of protease gene expression and that its age-dependent upregulation contributes to the decline in protease mRNA levels that occurs with aging.

To test this hypothesis and determine whether Ptx1 indeed regulates protease gene expression, we examined the transcriptomes of muscles with $Ptx1^{RNAi}$ and control $white^{RNAi}$ from 1-week-old flies. Interestingly, peptidases/proteases were the top GO term category of genes upregulated by Ptx1 RNAi (Figure 4D). These findings indicate that Ptx1 normally limits protease expression. To further probe the interaction among Ptx1, protease gene expression, and aging, we cross-compared the age- and $Ptx1^{RNAi}$-induced gene expression changes. Interestingly, many age-downregulated proteases were upregulated by $Ptx1^{RNAi}$ but not by a control GFP^{RNAi} (Figure 4E). Together, these findings indicate that Ptx1 RNAi regulates protease gene expression in a manner opposite to aging.

Knockdown of Ptx1 promotes protein quality control during aging

Having determined that the transcription factor Ptx1 promotes the expression of proteases downregulated by aging (Figures 1 and 4) and necessary for protein quality control (Figures 2 and 3), we next examined whether $Ptx1^{RNAi}$ improves proteostasis during aging. Consistent with its capacity to promote the expression of age-downregulated proteases (Figure 4), confocal microscopy indicated that $Ptx1^{RNAi}$ reduces the size and total area of poly-ubiquitin protein aggregates that accumulate with aging, compared with control $white^{RNAi}$ (Figures 5A-5C). Similarly, western blot analysis indicated that $Ptx1^{RNAi}$ reduces the levels of poly-ubiquitinated proteins that accumulate in detergent-insoluble fractions during aging (Figures 5D and 5F), whereas there are limited changes in detergent-soluble fractions (Figure 5E). Quantitation of western blot data for multiple biological replicates (Figure 5G) further confirms that aging significantly increases detergent-insoluble levels of poly-ubiquitinated proteins and Ref(2)P/p62 in control muscles, whereas such increase is reduced by Ptx1 RNAi (Figure 5G). Altogether, these findings indicate that knockdown of Ptx1 improves muscle protein quality control during aging.

Analysis of LC3 (Atg8) indicates that there is an increase in LC3-II levels with aging (Figure 5E), possibly indicative of an age-related decline in the autophagic flux and of a consequent reduction in the lysosomal degradation of LC3-II. However, these age-related

defects in autophagy occur similarly in response to Ptx1^{RNAi} and control white^{RNAi} (Figure 5E), indicating that they are unlikely responsible for the difference in the abundance of poly-ubiquitin protein aggregates in Ptx1^{RNAi} versus control white^{RNAi}.

Ptx1 RNAi promotes protein quality control via upregulation of the betaTrypsin protease

To examine whether the preservation of protein quality in response to Ptx1^{RNAi} depends on proteases, we tested the interaction between Ptx1 and betaTrypsin (betaTry), a protease downregulated by aging (Figures 1C and 1D), necessary for the degradation of detergent-insoluble poly-ubiquitinated proteins (Figures 2A and 2B) and of pathogenic huntingtin (Figure 3), and with a putative Ptx1 binding site in its promoter (CACGT TCACGTA; 2R:11344587).

In comparison with the reduction of *betaTry* expression during aging (6 weeks versus 1 week of age) in the wild-type B3 strain, *betaTry* expression is higher and/or does not decline with aging in the long-lived O1 and O3 *Drosophila* strains (Figure 6A), which have negligible senescence compared with the parental B3 strain.[32,53] Moreover, Ptx1 RNAi increases *betaTry* expression, compared with controls (Figure 6B).

To test whether betaTry contributes to preserve proteostasis in response to Ptx1 RNAi, we next examined the area and number of poly-ubiquitin protein aggregates in skeletal muscles at 5 weeks of age. As expected based on its modulation by aging and Ptx1 (Figures 6A and 6B), immunostaining and confocal microscopy revealed that betaTry^{RNAi} impedes the preservation of proteostasis due to Ptx1^{RNAi}, in comparison with control white^{RNAi}. Specifically, the area and number of protein aggregates found in the muscle with Ptx1^{RNAi} + betaTry^{RNAi} is significantly higher compared with that of Ptx1^{RNAl} + white^{RNAl} (Figures 6C-6E).

Similar to confocal microscopy, western blots of detergent-soluble and -insoluble fractions at 5 weeks of age revealed that Ptx1^{RNAi} + betaTry^{RNAi} increases detergent-insoluble Ref(2)P/p62 and poly-ubiquitinated proteins, compared with control Ptx1^{RNAi} + control white^{RNAi} (Figure 6F). Quantitation of additional biological replicates confirmed that Ptx1 RNAi reduces the age-related accumulation of detergent-insoluble poly-ubiquitinated proteins and Ref(2)P/p62 in a betaTry-dependent manner (Figures 6G and 6H). Altogether, these findings indicate that the betaTry protease contributes to preserve proteostasis during aging in response to Ptx1 knockdown.

Proteases modulated by aging and Ptx1 may impact protein quality control independently or in cooperation with the proteasome and/or the autophagy-lysosome system of protein degradation.[8] To determine whether betaTrypsin regulates the autophagic flux, we have analyzed skeletal muscles that express GFP-mcherry-Atg8a concomitantly to betaTry RNAi and control white RNAi. GFP-mcherry-Atg8a marks autophagosomes (defined by the concomitant green and red fluorescence of GFP and mcherry) and autolysosomes (defined only by the red fluorescence of mcherry) originating from the fusion of autophagosomes to lysosomes, which results in quenching of the GFP (but not mcherry) fluorescence due to the acidic lysosomal environment.[45,46,54] Analysis of the autophagic flux with the

GFP-mcherry-Atg8a reporter revealed no significant difference (Figure S6), suggesting that betaTrypsin regulates proteostasis independently from autophagy.

Drug-induced muscle-specific Ptx1 knockdown promotes protein quality control and extends lifespan

Skeletal muscle proteostasis has been previously shown to be an important determinant of lifespan and systemic aging in *Drosophila*[20,55] and higher organisms.[56-58] On this basis, we next assessed the physiological outcome of drug-induced muscle-specific expression of Ptx1^{RNAi}, obtained by using the *Act88F-GeneSwitch-Gal4* driver.[59,60] As expected, drug-induced Ptx1 knockdown significantly reduced *Ptx1* mRNA levels compared with controls (Figure 7A) and had little effect on protein quality control in skeletal muscles of young flies (Figure 7B), where proteostasis is optimal (Figure 1A). However, drug-induced Ptx1 knockdown significantly reduced the age-related accumulation of poly-ubiquitinated proteins found in detergent-insoluble fractions from skeletal muscle of old flies (Figure 7C). Quantitation of additional biological replicates confirmed that drug-induced Ptx1 knockdown impedes the accumulation of detergent-insoluble poly-ubiquitinated proteins and Ref(2)P/p62 during aging (Figures 7D and S7). Altogether, these findings indicate that drug-induced Ptx1 knockdown preserves protein quality control during skeletal muscle aging.

To probe the physiological relevance of muscle protein quality control mediated by Ptx1^{RNAi}, we next examined its effect on lifespan. Lifespan was significantly extended by drug-induced expression of two distinct Ptx1^{RNAi} transgenes, compared with the corresponding uninduced controls (Figures 7E-7G). Such longevity was due to Ptx1^{RNAi} and not to the RU486 drug itself, as demonstrated by the fact that drug-induced expression of a control vermillion^{RNAi} transgene did not extend lifespan (Figure 7E). Lifespan extension was also obtained via the muscle-specific expression of Ptx1 RNAi with the constitutive *Mhc-Gal4* driver, compared with control white RNAi (Figure 7H). Altogether, these findings indicate that preservation of muscle protein quality by Ptx1 knockdown extends lifespan.

DISCUSSION

Decline in protein quality control contributes to the pathogenesis of many age-related diseases and it is a defining feature of aging in all tissues, including muscle.[21] Although skeletal muscle proteostasis prominently declines during aging, the mechanisms involved are only in part known and include age-related dysfunction in the autophagy-lysosome and ubiquitin-proteasome systems of protein degradation.[21,24] In addition to these well-studied proteolytic systems, the human genome encodes for >600 proteases/peptidases with predicted diverse cellular localization but with largely unstudied functions.[8,61,62]

Here, we have examined the role of age-downregulated proteases/peptidases in muscle protein quality control in *Drosophila*. Overall, this study indicates that preservation of muscle protein quality is controlled during aging by proteases and their transcriptional modulator Ptx1, and that muscle proteostasis has important systemic consequences on lifespan. Specifically, we have found that age-related loss of proteostasis is defined by decreased expression of proteases (Figure 1), many of which are necessary to prevent the

age-related accumulation of poly-ubiquitinated and aggregation-prone proteins (Figures 2 and 3). These findings indicate that, in addition to the ubiquitin-proteasome and autophagy-lysosome systems, proteostasis is ensured during aging by many proteases that reduce the levels of poly-ubiquitinated and aggregation-prone proteins. Some of these proteases may preserve protein quality control during aging by digesting misfolded proteins, as previously shown for the Highroad and Jonah65A-IV proteases,[16,18] or by degrading the poly-ubiquitinated proteins that accumulate during aging, as previously found for the TPPII protease in cells treated with proteasome inhibitors.[13,15] However, proteases may promote protein quality control also by acting in cooperation and/or via the autophagy-lysosome and ubiquitin-proteasome systems of protein degradation, as previously shown for the Psa protease,[11,17] and consistent with evidence for cross-talk between proteolytic systems.[4] Altogether, our findings suggest that proteases are an important component of the network that maintains protein quality control during aging.

Several transcription factors likely contribute to modulate protease/peptidase expression during aging and age-related diseases. In this study, we have found a key role for the transcription factor Ptx1 in *Drosophila*, which is homologous to human PITX1, PITX2, and PITX3. Specifically, Ptx1 protein levels increase during aging (Figure 4B), coincident with protease downregulation, and Ptx1 RNAi promotes the expression of age-downregulated proteases (Figures 4D and 4E). Although we have found a role for the protease betaTry in maintaining proteostasis downstream of Ptx1 RNAi (Figures 5 and 6), it is likely that other proteases also contribute to these effects considering that Ptx1 knockdown promotes the expression of several proteases that are otherwise downregulated during aging (Figures 4D and 4E).

Our RNA-seq analysis did not identify any changes indicative of autophagic activation by Ptx1 RNAi (Figure 4D). Moreover, we have not found any substantial changes in LC3-I and LC3-II levels in response to Ptx1 RNAi compared with control RNAi (Figure 5E). Therefore, these findings suggest that Ptx1 regulates proteostasis independently from autophagy. However, Ptx1 RNAi reduced the age-related accumulation of detergent-insoluble p62 (*Drosophila* Ref(2)P), which is a ubiquitin-binding protein that associates with poly-ubiquitin protein aggregates and that is co-degraded with such aggregates by the autophagy/lysosome system.[29,44-46] On this basis, reduction in insoluble Ref(2)P/p62 may result from lower levels of p62 binding partners (insoluble poly-ubiquitinated proteins, i.e., aggregates) because of their degradation by proteases induced by Ptx1 RNAi. Alternatively, this finding may indicate that Ptx1 RNAi promotes the autophagic removal of p62/poly-ubiquitin protein aggregates via protease-dependent or independent mechanisms. Therefore, although we have no evidence for autophagic induction, it is possible that Ptx1 RNAi and Ptx1-regulated proteases may preserve proteostasis at least in part via the autophagy-lysosome system.

In addition to determining a role for the transcription factor Ptx1 in muscle proteostasis during aging, our study also indicates that the muscle-specific modulation of Ptx1 regulates lifespan, reinforcing the notion that preservation of muscle protein quality control is key for longevity.[56,57] Together, these findings highlight a novel function of PITX transcription factors in protein quality control and aging.

Traditionally, PITX transcription factors have been identified as key regulators of many developmental processes in mammals and *Drosophila*,[48,63-66] including myogenesis.[49,67-69] For instance, PITXs regulate the terminal differentiation of progenitor cells and the establishment and maintenance of cell type-specific features.[70-74] Interestingly, it was also reported that PITX1 can work as transcriptional repressor of virus-induced Interferon A promoters[75] and thus work analogously as observed here in the repression of target gene expression. Moreover, loss of PITX transcription factors is causally associated with developmental defects in humans, including Liebenberg syndrome, congenital clubfoot, and facioscapulohumeral muscular dystrophy-1 (PITX1[76-78]), Axenfeld-Rieger syndrome type 1 (PITX2[79]), and cataract 11 with microphthalmia and neurodevelopmental abnormalities (PITX3[80]).

In our study, we have examined the function of the single *Drosophila* PITX family member Ptx1 in adulthood. Because some genetic redundancy likely exists between PITX family members (PITX1, PITX2, PITX3) in mammals, our study in *Drosophila* has potentially uncovered a general function of PITX transcription factors in protein quality control that may have been masked by genetic redundancy between PITX family members in mammals. Therefore, based on our findings that *Drosophila* Ptx1 regulates protein quality control, we propose that PITX transcription factors may similarly regulate proteostasis in humans and contribute to the pathogenesis of age-related conditions characterized by deranged proteostasis. Moreover, we propose that modulation of protease expression may contribute to the developmental processes controlled by PITX via their impact on proteostasis and possibly also via the proteolytic processing of developmental signaling factors.

In summary, these findings provide new insight into protein quality control and how it is deranged by aging. We propose that acting on age-downregulated proteases may represent an underexplored strategy for restoring and maintaining protein quality control during aging and for preventing the onset and progression of age-related diseases that arise from defective proteostasis.

Limitations of the study

It remains undetermined whether proteases can play similar roles in maintaining muscle protein quality control during aging in higher organisms as we have observed in *Drosophila*, and whether human PITX1/2/3 transcription factors that are homologous to *Drosophila* Ptx1 similarly regulate protease gene expression and proteostasis during aging.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fabio Demontis (fabio.demontis@stjude.org).

Materials availability—There are no restrictions to the availability of tools generated in this study.

Data and code availability

- All data supporting the findings of this study are available within the paper and the Supplemental information, including Figures S1-S7 and Table S1. The RNA-seq data discussed in this publication has been deposited in the NCBI's Gene Expression Omnibus and is accessible through GEO series accession numbers GEO: GSE156346 and GEO: GSE129922.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

***Drosophila* husbandry—**Flies were kept (~25 flies/tube) at 25°C, 60% humidity, and a 12h/12h light-dark cycle in tubes containing cornmeal/soy flour/yeast fly food, and aged to the age indicated in the manuscript, figures, and figure legends at 25°C. The fly food was changed regularly every 2–3 days. All experiments were done with male flies. Fly stocks were obtained from the Bloomington Drosophila Stock Center (BDSC) and the Vienna Drosophila Resource Center (VDRC). The fly stock used in Figures 2, 3, S2 and S3 are indicated in the figure panels (VDRC stocks are denoted by a "v" whereas other stocks are from the Bloomington stock center). The RNA-seq in Figure 4 was done with Ptx1 RNAi stock #58124 and with control white RNAi (#33623) and GFP RNAi (#41552) stocks.

For Figures 5, 6 and 7, additional Ptx1 RNAi stocks were also used (#57494 and #v107785). Epistasis experiments in Figure 6 were done with the following stocks: Ptx1 RNAi (#58124), betaTry RNAi (#v41238), white RNAi (#33623), and GFP RNAi (#41552). Lifespan in Figure 7H was done with #57494 and #v107785. *Mhc-Gal4* and *Act88F-GS-Gal4* were previously validated as skeletal muscle-specific Gal4 drivers.[20,59,60,81] For drug-induced transgene expression, 200 μM RU486 (dissolved in ethanol) was supplemented to the food from week 1 onward and compared to ethanol-alone controls. In all cases, transgene expression was confirmed by qRT-PCR.

In experiments with constitutive Gal4 drivers (*Mhc-Gal4*) 1-week-old flies were utilized as young controls whereas 5-weeks and 8/10-weeks-old flies were examined as old-age time points. There are strong defects in proteostasis at the 8/10-weeks timepoints (Figure 1A) and therefore this is an ideal setting for testing whether interventions (such as Ptx1 RNAi) can rescue defects in proteostasis. On the other hand, there are only partial defects in proteostasis at the 5-weeks time point: therefore this is an ideal setting for testing whether interventions (such as protease RNAi) can worsen age-related defects in proteostasis. In experiments with the GeneSwitch system (*Act88F-GS-Gal4*), 2 weeks were utilized as young time point because transgene expression is induced by RU486 feeding, which is provided after 1 week from eclosion to avoid potential effects of RU486 on postnatal development.

METHOD DETAILS

Whole-mount immunostaining of *Drosophila* skeletal muscle—The immunostaining of flight skeletal muscle was done as previously described.[20,82,83] In brief, thoraces were dissected, fixed for 30 min in PBS with 4% paraformaldehyde and 0.1% Triton X-100 at room temperature, washed >3 times in PBS with 0.1% Triton X-100 at room temperature, and immunostained overnight at 4°C with rabbit anti-poly-ubiquitin (FK2; Enzo Life Sciences #BML-PW8810-0100) and anti-Ref(2)P/p62 antibodies (Abcam #178840). After washes with PBS with 0.1% Triton X-100, the samples were incubated with secondary antibodies and Alexa 635-phalloidin for 2 h at room temperature, washed and mounted in antifade medium. Subsequently, the samples were imaged on a Nikon C2 confocal microscope. For the analysis of the autophagic flux with GFP-mcherry-Atg8a, flies were dissected in ice-cold PBS, fixed in 4% PFA for 20–30 min, and washed 3 times with PBS for 5 min each. Muscles were incubated overnight with Alexa 635-phalloidin in PBS (1:100). Tissues were mounted in anti-fade and imaged immediately. The scheme was made with BioRender.

Confocal image analysis—Machine learning methods implemented in FIJI[84,85] were used to train three classifiers to segment protein aggregates imaged in the Ref(2)P/p62 and poly-ubiquitin channels, and regions representing muscle fibers in the phalloidin (F-actin) channel. Segmented muscle fiber images were used as masks to remove fluorescent signals outside the muscle fiber regions. Subsequently, the number and total area occupied by protein aggregates were determined and normalized by the total area of muscle tissue in the same image.

Analysis of pathogenic huntingtin aggregation—Pathogenic huntingtin-polyQ72-GFP protein aggregates[47] were imaged with an epifluorescence ZEISS SteREO Discovery.V12 microscope with consistent exposure time and settings. The acquired gray scale images were then analyzed in an automated manner by using Cell Profiler 3.0.0 (cellprofiler.org) to determine the number and/or total area of protein aggregates (Huntingtin-polyQ72-GFP speckles) normalized by the retinal tissue area. This analysis was done with male flies after aging at 25°C for 30 days (the same conditions were applied to all the samples and the respective controls in any given experiment). The raw values for each set of flies were normalized by the corresponding raw values of the negative control RNAi intervention from the same fly stock collection. Specifically, all values obtained from Bloomington RNAi stocks were normalized to $mCherry^{RNAi}$ (#BL35785) whereas the values of VDRC RNAi stocks were normalized by the raw values of vermillion RNAi, v^{RNAi} (#v107798).

Western blots for detergent-soluble and -insoluble fractions—Western blots for detergent-soluble and insoluble fractions were done as before.[20,32,86] In brief, thoraces were dissected from 20 male flies/sample and homogenized in ice-cold PBS with 1% Triton X-100 containing protease and phosphatase inhibitors. Homogenates were centrifuged at 14,000 rpm at 4°C and supernatants collected (Triton X-100 soluble fraction). The remaining pellet was washed in ice-cold PBS with 1% Triton X-100. The pellet was then resuspended in RIPA buffer containing 8M urea and 5% SDS, centrifuged at 14,000 rpm

at 4°C. The supernatants (Triton X-100 insoluble fraction) were collected and analyzed on 4–20% SDSPAGE with anti-ubiquitin (Cell Signaling Technologies P4D1, #3936) and anti-Ref(2)P/p62 (Abcam #178840) antibodies.

Western blots—For the Western blot analysis of flies that express Hungtingtin-polyQ72-GFP, 30-day-old flies (n = 5/sample) were homogenized in RIPA buffer containing 8M urea and 5% SDS, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected and analyzed on 4–20% SDS-PAGE with anti-GFP antibodies (Cell Signaling Technologies D5.1, #2956). Anti-myosin antibodies (Babraham, #BT-GB-147p) were used as loading controls.

Ptx1 protein levels were detected in thoraces, which consist primarily of skeletal muscle, with anti-Ptx1 antibodies (1:1000) raised against *Drosophila* Ptx1.[52]

qRT-PCR—qRT-PCR was performed as previously described.[55,87-90] Total RNA was extracted with the TRIzol reagent (Life Technologies) from *Drosophila* thoraces, consisting primarily of skeletal muscle, from >20 male flies/replicate, followed by reverse transcription with the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed with SYBR Green and a CFX96 apparatus (Bio-Rad). Three biological replicates were used for each genotype and time point; alpha-Tubulin at 84B was used as a normalization reference. The comparative C_T method was used for relative quantitation of mRNA levels. The qRT-PCR oligos used are the following:

Ptx1: 5′-ACCGGCGATTTCCGTGATAG-3′ and 5′-ATCCCGACTCCGGTTCGATA-3′

betaTry: 5′-GATCCATCTACTCCGCCAGG-3′ and 5′-TTGGCGTTGTAACCCTCGTG-3′

Tub84B: 5′-GTTTGTCAAGCCTCATAGCCG-3′ and 5′-GGAAGTGTTTCACACGCGAC-3′

RNA-sequencing—Total RNA was extracted from *Drosophila* thoraces (which consist primarily of skeletal muscle) of 1-week-old male flies. Three biological replicates were prepared for RNA-seq with the TruSeq stranded mRNA library preparation kit (Illumina) and sequenced on the Illumina HiSeq 4000 platform. Around 100 million reads were obtained for each sample. FASTQ sequences derived from mRNA paired-end 100-bp sequences were mapped to the *Drosophila melanogaster* genome (BDGP5) with the STAR aligner (version 2.5.3a;[91]). Transcript level data were counted using HTSeq (version 0.6.1p1;[92]) based on the BDGP5 GTF release 75. The TMM method[93] was used to calculate the normalization factors. Then linear modeling was carried out on the $\log_2$(CPM) (count per million) values where the mean-variance relationship is accommodated using precision weights calculated by the voom function[94] of the limma package in R 3.2.3.[95] A *q* value (FDR) was calculated for multiple comparison adjustments of RNA-seq data. The lmFit, eBayes, and contrasts.fit functions from the limma package were used for the linear modeling. GO term analysis was done with DAVID.[96] The RNA-seq used in this study have been deposited at the Gene Expression Omnibus with accession numbers GEO: GSE129922 (age-induced transcriptional changes in skeletal muscle) and GEO: GSE156346

(transcriptional changes induced by Ptx1 RNAi in skeletal muscle). In Figures 1B and 1C, the RNA-seq was done with the B3 wild-type strain at 1 week of age (young) and 6 weeks of age (old) and previously reported.[32]

Computational analyses of proteases and peptidases—*Drosophila* protease and peptidase annotation was based on the MEROPS database.[7,97]

Computational prediction of transcription factors that regulate age-downregulated proteases—Based on gene annotation download from UCSC Genome Browser (dm6), proximal promoter (TSS+/−100bp) sequences were extracted for downregulated proteases using fastaFromBed (BEDTools version 2.24.0),.[98] Subsequently, findMotifsGenome.pl (default parameters, homer version 4.10;[99]) were used to find motifs enriched against 50,000 random background regions matched for GC-content distribution.

QUANTIFICATION AND STATISTICAL ANALYSES

All experiments were performed with biological triplicates unless otherwise indicated. The unpaired two-tailed Student's t-test was used to compare the means of two independent groups to each other. A one-way ANOVA with Tukey's post hoc test was used for multiple comparisons of more than two groups of normally distributed data. Survival data was analyzed with OASIS 2[100] by using log rank tests. The "n" for each experiment can be found in the figures and/or figure legends and represents independently generated samples, including individual flies for lifespan assays, and batches of flies or fly thoraces for other assays. Bar graphs represent the mean ± SEM or ±SD as indicated in the figure legend. Throughout the figures, asterisks indicate a significant p value (*$p < 0.05$). Statistical analyses were done with Excel and GraphPad Prism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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Highlights

- Age-dependent downregulation of protease expression impairs muscle proteostasis
- The protein levels of the transcription factor Ptx1 increase with aging in muscle
- Ptx1 knockdown increases the expression of age-downregulated proteases
- Ptx1 RNAi improves muscle proteostasis via proteases and extends lifespan

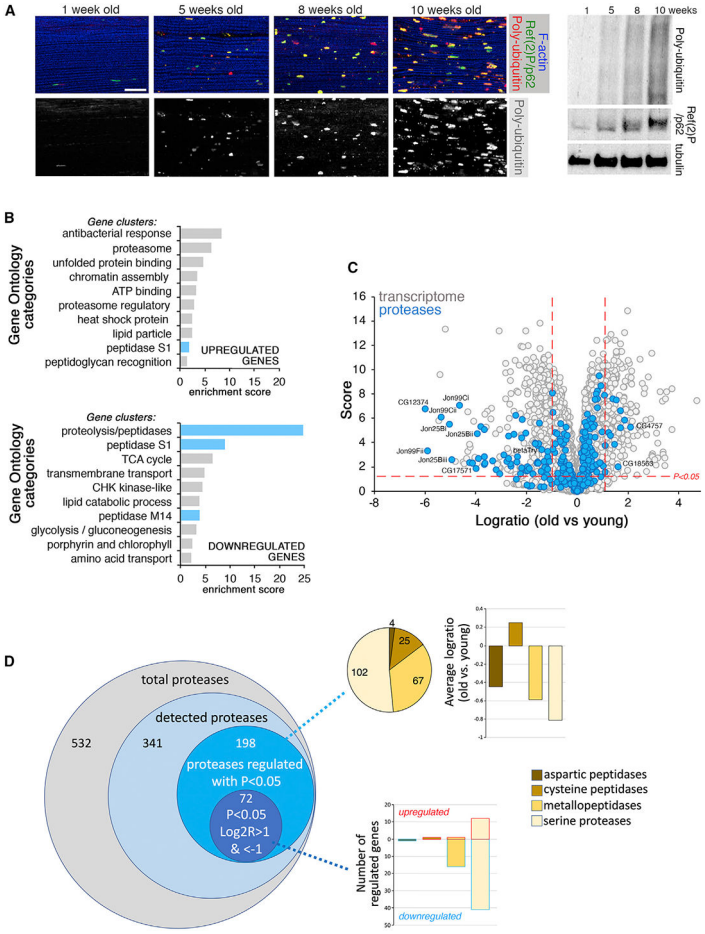

Figure 1. Loss of protein quality control during skeletal muscle aging is defined by a decline in protease/peptidase expression

(A) As previously reported, immunostaining and western blot analyses of detergent-insoluble fractions from skeletal muscle from *Drosophila* at 1, 5, 8, and 10 weeks of age identify a progressive age-related accumulation of poly-ubiquitinated proteins. The scale bar is 20 μm.

(B) RNA sequencing of skeletal muscles from 6-week-old (old) versus 1-week-old (young) wild-type B3 flies[32] identifies categories of age-regulated genes. The expression of proteases and peptidases declines with aging.

(C) Age-induced transcriptional changes (gray) include proteases (blue; from the MEROPS database). Overall, proteases are primarily downregulated by aging in skeletal muscle. The y axis reports the score, which corresponds to −log10 (p value), whereas the x axis reports the log ratio of old versus young mRNA changes (n = 3).

(D) Out of 532 proteases annotated in the MEROPS database, 341 were found to be expressed in skeletal muscle and 198 were significantly regulated by aging ($p < 0.05$): these consisted primarily of serine peptidases (102) and metallopeptidases (67), whereas fewer cysteine peptidases (25) and aspartic peptidases (4) were age-regulated. Analysis of the average log ratio in old versus young indicates that these peptidase classes are on average downregulated by aging whereas cysteine proteases are upregulated. Further analysis of a set of 72 proteases more stringently regulated by aging ($p < 0.05$ and Log2R > 1 and <−1)

revealed a similar distribution. A subset of age-downregulated proteases out of these 72 proteases was used for subsequent analyses in Figures 2 and 3. See also Figure S1 and Table S1.

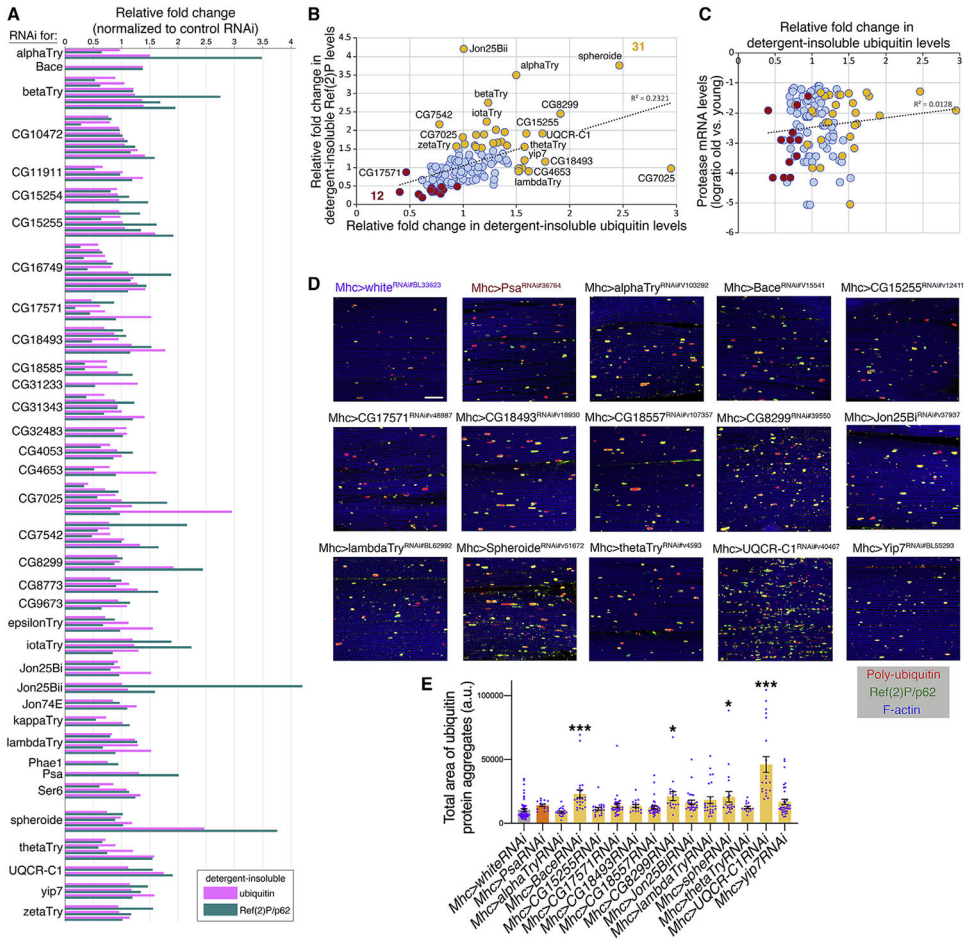

Figure 2. RNAi for age-downregulated proteases compromises muscle protein quality control

(A) Summary of western blot analyses of detergent-insoluble fractions from skeletal muscle from *Drosophila* at 5 weeks of age indicates that RNAi for several age-downregulated proteases compromises protein quality control, as indicated by higher age-related accumulation of insoluble poly-ubiquitinated proteins (pink) and Ref(2)P/p62 (green), normalized to control RNAi. A total of 121 RNAi lines have been screened and these target 35 age-downregulated proteases from the set of 72 proteases stringently regulated by aging (identified in Figure 1). The results obtained from multiple RNAi lines are shown next to the corresponding protease gene name. Psa RNAi is a positive control. See also Figure S2 and Table S1.

(B) Plotting of detergent-insoluble Ref(2)P/p62 versus poly-ubiquitinated protein levels highlights the RNAi interventions that target age-downregulated proteases and that worsen protein quality control (31; yellow), whereas fewer RNAi interventions improve proteostasis (12; red): these hits were selected based on a >50% change in detergent-insoluble Ref(2)P/p62 and/or poly-ubiquitinated protein levels. Overall, these findings suggest that proteases that are age-downregulated contribute to preserve muscle proteostasis. There is a correlation value of 0.23 between detergent-insoluble Ref(2)P/p62 and poly-ubiquitinated protein levels.

(C) Cross-comparison of RNAi-induced changes in detergent-insoluble poly-ubiquitinated protein levels and age-related transcriptional changes in the expression of these proteases. All these RNAi interventions target age-downregulated proteases ($p < 0.05$ and $Log2R < -1$) but the extent of age-downregulation does not correlate with the impact on proteostasis. (D) Immunostaining of skeletal muscle from *Drosophila* at 5 weeks of age indicates that RNAi for age-downregulated proteases compromises protein quality control. The scale bar is 20 μm. The quantitation of the total area of poly-ubiquitin protein aggregates is shown in (E), with SD, $n \geq 14$, and p values indicated (one-way ANOVA), $^{*}p < 0.05$ and $^{***}p < 0.001$.

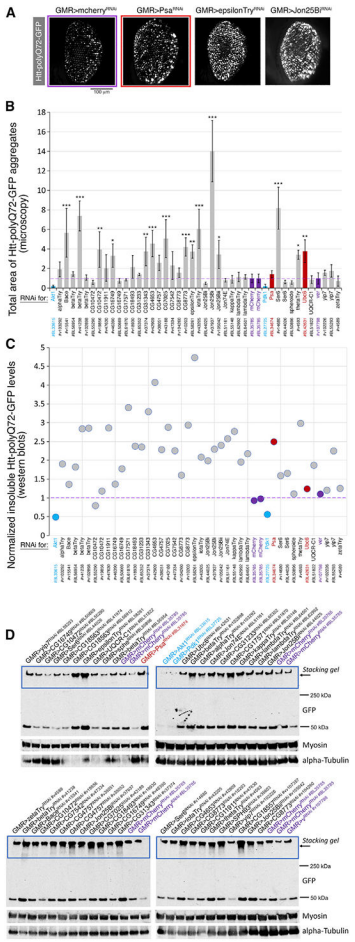

Figure 3. RNAi for age-downregulated proteases increases aggregates of pathogenic huntingtin-polyQ in the retina

(A) Pathogenic GFP-tagged huntingtin-polyQ (Htt-polyQ72-GFP) driven in the retina with *GMR-Gal4* leads to GFP-fluorescent Htt protein aggregates at 30 days of age. Compared with negative controls (mCherry^{RNAi}; purple), RNAi for many age-downregulated proteases (such as epsilonTry and Jon25Bi) significantly increases the amount of Htt-polyQ72-GFP aggregates, similar to what is observed with RNAi for the Psa protease (positive controls; red). Scale bar, 100 μm.

(B) Quantitation of the total area of Htt-polyQ72-GFP aggregates. Relative fold changes compared with control RNAi interventions are shown; n = 5, SD, and p values (one-way ANOVA) are indicated, with *p < 0.05, **p < 0.01, ***p < 0.001, compared with mcherry^{RNAi}. Other positive controls include Ubc6^{RNAi} (red), which increases Htt-polyQ72-GFP aggregates, and Akt^{RNAi} and Pdk1^{RNAi} (cyan), which reduce Htt-polyQ72-GFP aggregates.

(C) Quantitation of the detergent-insoluble levels of Htt-polyQ72-GFP aggregates detected by western blot in (D): these western blot analyses with anti-GFP antibodies identify Htt-polyQ72-GFP monomers (~50 kDa) and aggregates in the stacking gel, which are quantified in (C); n = 5 flies of the same genotypes as in (A) and (B). Cross-comparison of microscopy (B) and western blot results (C) indicates that RNAi for many age-downregulated

proteases increases insoluble Htt-polyQ72-GFP protein aggregates compared to control RNAi interventions. See also Figures S3, S4 and Table S1.

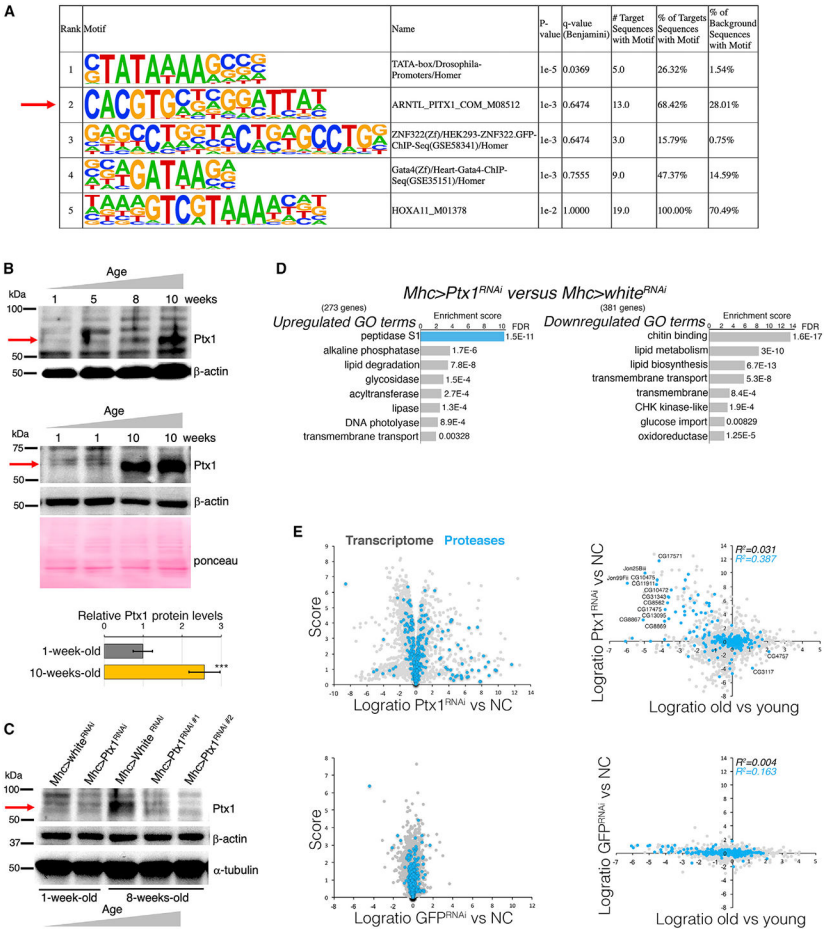

Figure 4. Protein levels of the transcription factor Ptx1 increase with aging, and Ptx1 knockdown promotes the expression of age-downregulated proteases

(A) Computational analysis of age-downregulated proteases identifies a significant enrichment for PITX1 motifs. See also Figure S5.

(B and C) (B) The protein levels of the transcription factor Ptx1 (~62 kDa) increase with aging (n = 5, SD, two-tailed unpaired Student's t test) and this can be prevented by Ptx1 knockdown (C).

(D) RNA sequencing identifies categories of genes regulated by Ptx1 RNAi in *Drosophila* muscle, including proteases/peptidases, which are upregulated.

(E) Comparison of RNA-seq data (n = 3) from muscle with Ptx1 RNAi (normalized to control white RNAi, NC) from 1-week-old flies, and from old versus young muscle indicates that Ptx1 RNAi induces gene expression changes opposite to those induced by aging. In particular, age-downregulated proteases (blue) are conversely regulated by Ptx1 RNAi but not by a control GFP RNAi.

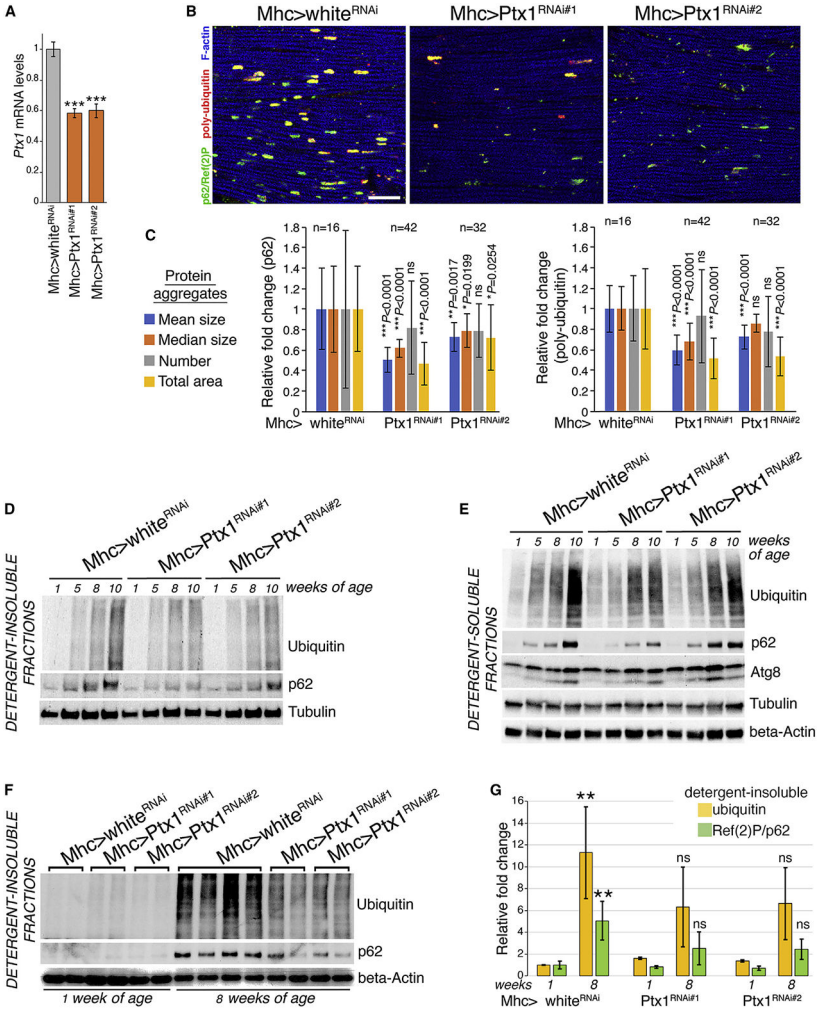

Figure 5. RNAi for the transcription factor Ptx1 promotes protein quality control during aging

(A) qRT-PCR confirms that Ptx1 RNAi driven by *Mhc-Gal4* reduces *Ptx1* mRNA levels in muscle (n = 3; SEM; ***p < 0.001, one-way ANOVA).

(B) Immunostaining of *Drosophila* skeletal muscle at 10 weeks of age indicates that Ptx1 RNAi preserves protein quality control, as indicated by lower age-related accumulation of Ref(2)P/p62-positive aggregates of poly-ubiquitinated proteins, compared with negative controls (white RNAi). Scale bar, 20 μm.

(C) Quantitation of the total area of poly-ubiquitin and p62-positive protein aggregates examined in (B), with SD, n, and p values indicated (one-way ANOVA).

(D and E) Western blot analysis of detergent-insoluble (D) and -soluble (E) fractions from skeletal muscle from *Drosophila* at 1, 5, 8, and 10 weeks of age indicates that Ptx1 RNAi improves protein quality control during aging, as indicated by lower age-related accumulation of insoluble Ref(2)P/p62 and poly-ubiquitinated proteins compared with negative controls (white RNAi).

(F) Additional biological replicates confirm that Ptx1 RNAi reduces the age-related accumulation of poly-ubiquitinated proteins and Ref(2)P/p62 in the detergent-insoluble fraction.

(G) Quantitation of western blot data indicates that aging significantly increases detergent-insoluble levels of poly-ubiquitinated proteins (yellow) and Ref(2)P/p62 (green) in control muscles, whereas such increase is reduced in muscles with Ptx1 RNAi. The statistical analysis refers to the comparison of the 8-week versus the 1-week time point for each genotype; ≥3, SD, **$p < 0.01$, ns = not significant (two-tailed unpaired Student's t test).

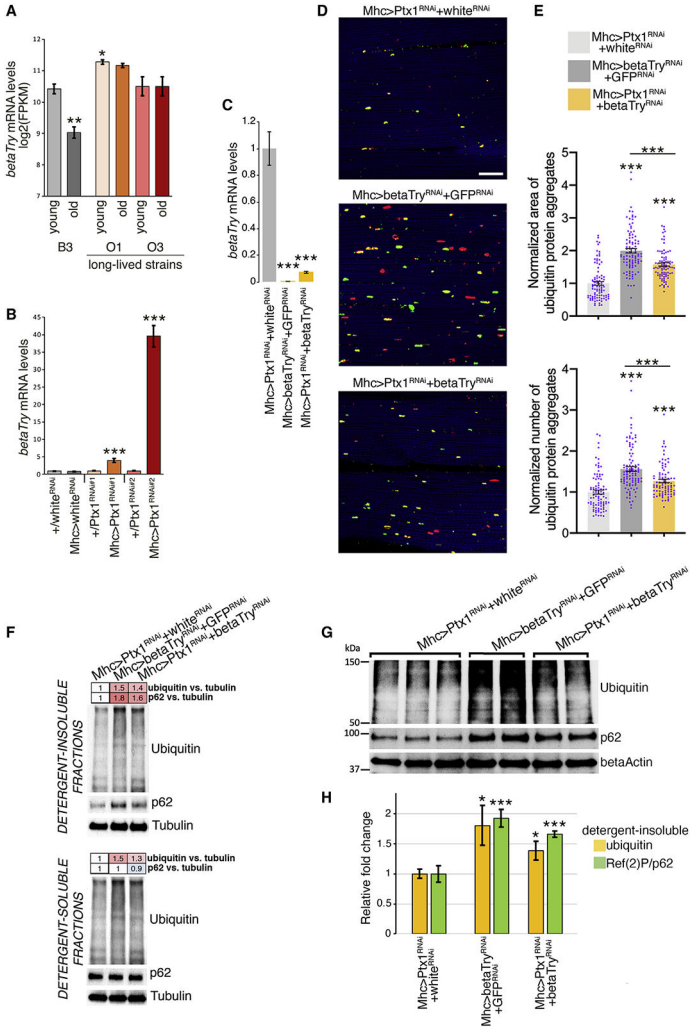

Figure 6. Knockdown of Ptx1 preserves proteostasis during aging via the protease betaTrypsin

(A) The mRNA levels of *betaTry* decline in skeletal muscle during aging (6-week-old versus 1-week-old) in the control B3 strain but not in the long-lived O1 and O3 *Drosophila* strains (n = 3 with SD and *p < 0.05, **p < 0.01 obtained by one-way ANOVA).

(B) qRT-PCR indicates that RNAi for Ptx1 increases *betaTry* mRNA levels, compared with control white RNAi and transgene alone controls (n = 3; SEM; ***p < 0.001, two-tailed unpaired Student's t test).

(C) qRT-PCR indicates that *betaTry* mRNA levels decline upon betaTry RNAi (n = 4; SEM; ***p < 0.001, one-way ANOVA).

(D and E) (D) Immunostaining of *Drosophila* skeletal muscle at 5 weeks of age indicates that $Ptx1^{RNAi}$ preserves protein quality control at least in part via betaTrypsin, as indicated by the quantitation of the area and number of protein aggregates (E): there is a higher age-related accumulation of insoluble Ref(2)P/p62 and poly-ubiquitinated proteins in *Mhc > $Ptx1^{RNAi}$ + betaTry^{RNAi}* compared with *Mhc > $Ptx1^{RNAi}$ + white^{RNAi}*; SD, n, and p values are indicated (one-way ANOVA). Scale bar, 20 μm

(F) Western blot analysis of detergent-insoluble and -soluble fractions from skeletal muscle from *Drosophila* at 5 weeks of age indicates that Ptx1 RNAi improves protein quality

control during aging in a betaTry-dependent manner, as indicated by a higher age-related accumulation of insoluble Ref(2)P/p62 and poly-ubiquitinated proteins in *Mhc > Ptx1^{RNAi} + betaTry^{RNAi}* compared with *Mhc > Ptx1^{RNAi} + white^{RNAi}*. The same number of UAS transgenes is used in these comparisons to avoid Gal4 titration effects.

(G) Additional biological replicates confirm that Ptx1 RNAi reduces the age-related accumulation of detergent-insoluble poly-ubiquitinated proteins and Ref(2)P/p62 in a betaTry-dependent manner.

(H) Quantitation of western blot data indicates that betaTry RNAi increases detergent-insoluble levels of poly-ubiquitinated proteins (yellow) and Ref(2)P/p62 (green) in muscles with Ptx1 knockdown (Ptx1^{RNAi} + betaTry^{RNAi}), compared with control Ptx1^{RNAi} + white^{RNAi}; n ≥ 3, SD, ns = not significant (two-tailed unpaired Student's t test). See also Figure S6.

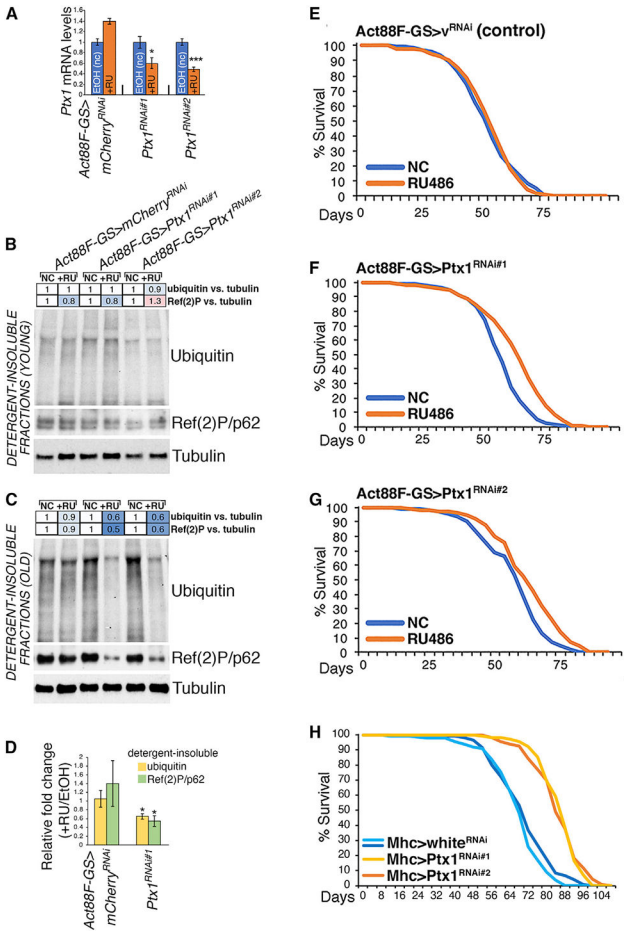

Figure 7. Drug-induced muscle-specific Ptx1 knockdown promotes protein quality control and extends lifespan

(A) qRT-PCR confirms that *Ptx1* mRNA levels decline as expected in response to drug-induced expression of Ptx1 RNAi with the GeneSwitch system (n = 3; SEM; *p < 0.05, ***p < 0.001, two-tailed unpaired Student's t test).

(B and C) Western blot analysis of detergent-insoluble fractions from skeletal muscle with drug-induced expression of Ptx1 RNAi and control mCherry RNAi, obtained from *Drosophila* at 2 weeks (B; young) and 6 weeks of age (C; old) after treatment with RU486 for 1 and 5 weeks, respectively. Induced (+RU; 200 μM RU486 in ethanol) and uninduced controls (NC; ethanol alone) are shown. These analyses indicate that Ptx1 RNAi improves protein quality control during aging, as indicated by the lower age-related accumulation of detergent-insoluble Ref(2)P/p62 and poly-ubiquitinated proteins compared with uninduced controls and mCherry RNAi. See also Figure S7.

(D) Quantitation of western blot data indicates that drug-induced expression of Ptx1 RNAi reduces detergent-insoluble levels of poly-ubiquitinated proteins (yellow) and Ref(2)P/p62 (green) found in old muscles, compared with controls; n ≥ 3 with SD and *p < 0.05 indicated (two-tailed unpaired Student's t test).

(E–G) Drug-induced expression of Ptx1 RNAi in indirect flight muscle by using the GeneSwitch system and the RU486 drug significantly extends lifespan, whereas expression of control transgenic RNAi for vermillion (*v*) does not.

(E) *Act88F-GS > v*$^{RNAi\ \#v3349}$ (RU486-treated versus control ethanol-treated): age in days at 50% and 90% of mortality, 53 versus 53 days, and 67 versus 67 days; n = 351 and n = 335; p value (log rank test) = 0.9757.

(F) *Act88F-GS > Ptx1*$^{RNAi\ \#BL57494}$ (RU486 versus control): age in days at 50% and 90% of mortality, 64 versus 57 days, and 78 versus 71 days; n = 325 and n = 333; $p < 0.001$ (log rank test).

(G) *Act88F-GS > Ptx1*$^{RNAi\ \#v107785}$ (RU486 versus control): age in days at 50% and 90% of mortality, 64 versus 60 days, and 78 versus 71 days; n = 185 and n = 193; $p < 0.001$ (log rank test).

(H) Lifespan extension is also obtained with Ptx1 RNAi driven by the constitutive *Mhc-Gal4* driver, compared with control white RNAi; n = 120 and n = 135 for *Mhc > white*^{RNAi}, and n = 115 and n = 165 for *Mhc > Ptx1*$^{RNAi\ \#57494}$ and *Mhc > Ptx1*$^{RNAi\ \#v107785}$, respectively; $p < 0.001$ (log rank test). See also Figure S7.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-poly-ubiquitin (FK2)	Enzo Life Sciences	BML-PW8810-0100
Rabbit anti-Ref(2)P/p62	Abcam	ab178840
Mouse anti-ubiquitin (P4D1)	Santa Cruz	Sc-8017; RRID:AB_628423
Rabbit anti-alpha-tubulin (11H10)	Cell Signaling Technologies	2125; RRID:AB_2619646
Rabbit anti-GFP antibodies	Cell Signaling Technologies	2956; RRID:AB_1196615
Anti-myosin antibodies	Babraham Institute	BT-GB-147p
Rabbit anti-Atg8/GABARAP	Abcam	ab109364; RRID:AB_10861928
Rabbit anti-beta-actin	Cell Signaling Technologies	8457; RRID:AB_10950489
Rabbit anti-Ptx1	(Vorbruggen et al.)[52]	N/A
AlexaFluor555 anti-mouse	Life Technologies	A28180; RRID:AB_2536164
AlexaFluor488 anti-rabbit	Life Technologies	A11034; RRID:AB_2576217
AlexaFluor488 anti-mouse	Life Technologies	A11001; RRID:AB_2534069
AlexaFluor555 anti-rabbit	Life Technologies	A21428; RRID:AB_2535849
Anti-mouse IgG, HRP-linked	Cell Signaling Technologies	7076; RRID:AB_330924
Anti-rabbit IgG, HRP-linked	Cell Signaling Technologies	7074; RRID:AB_2099233
Chemicals, peptides, and recombinant proteins		
AlexaFluor635 Phalloidin	Life Technologies	A22284
DAPI	Roche	10236276001
Trizol	Ambion	15596018
IQ Sybr Green supermix	Bio-Rad	170-8885
6-well plates	Corning	REF3516
96-well PCR plates	Biorad	HSP9601
Transparent 96-well plates	Corning	REF3599
PBS	Gibco	10010023
Blue loading buffer pack	Cell Signaling	7722
Precision Plus protein standard	Bio-Rad	1610374
4–20% Mini-PROTEAN TGX pre-cast gels	Bio-Rad	4561096
Immobilon-P PVDF membrane	Millipore	IPVH00010
Ponceau S	Sigma-Aldrich	P7170-1L
16% Paraformaldehyde	Electron Microscopy Sciences	15710
Critical commercial assays		
Pierce BCA protein assay kit	Thermo Scientific	23225
iScript reverse transcriptase	Bio-Rad	1708840
Deposited data		
RNA-seq of transcriptional changes induced by Ptx1 RNAi in skeletal muscle	This study	GEO: GSE156346
RNA-seq of age-induced transcriptional changes in skeletal muscle	(Hunt et al.)[32]	GEO: GSE129922
Experimental models: Organisms/strains		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Drosophila: w^{1118}; Mhc-Gal4	(Demontis et al.; Schuster et al.)[20,81]	N/A
Drosophila: Act88F-GS-Gal4	(Robles-Murguia et al.)[59,60]	N/A
Drosophila: w^{1118} (+)	Demontis Lab collection	N/A
Drosophila: UAS-cherry RNAi	Bloomington stock center	#35785
Drosophila: UAS-white RNAi	Bloomington stock center	#33623
Drosophila: UAS-vermillionRNAi	VDRC	#v107798
Drosophila: UAS-GFP RNAi	Bloomington stock center	#41552
Drosophila: UAS-Ptx1 RNAi	Bloomington stock center	#58124
Drosophila: UAS-Ptx1 RNAi	Bloomington stock center	#57494
Drosophila: UAS-Ptx1 RNAi	VDRC	#v107785
Drosophila: UAS-betaTry RNAi	VDRC	#v41238
Drosophila: UAS-GFP-mcherry-Atg8a	Bloomington stock center	#37749
Drosophila: GMR-Gal4, UAS-Htt-Q72-GFP	(Zhang et al.)[47]	N/A
Oligonucleotides		
Primers: *Ptx1* Forward: ACCGGCGATTTCCGTGATAG	This study	N/A
Primers: *Ptx1* Reverse: ATCCCGACTCCGGTTCGATA	This study	N/A
Primers: *betaTry* Forward: GATCCATCTACTCCGCCAGG	This study	N/A
Primers: *betaTry* Reverse: TTGGCGTTGTAACCCTCGTG	This study	N/A
Primers: *Tub84B* Forward: GTTTGTCAAGCCTCATAGCCG	This study	N/A
Primers: *Tub84B* Reverse: GGAAGTGTTTCACACGCGAC	This study	N/A
Software and algorithms		
GraphPad Prism	Graphpad	https://www.graphpad.com/
Photoshop CSX	Adobe	https://www.adobe.com/products/photoshop.html
ImageJ	NIH	https://imagej.nih.gov/ij/
BioRender	BioRender	https://biorender.com