

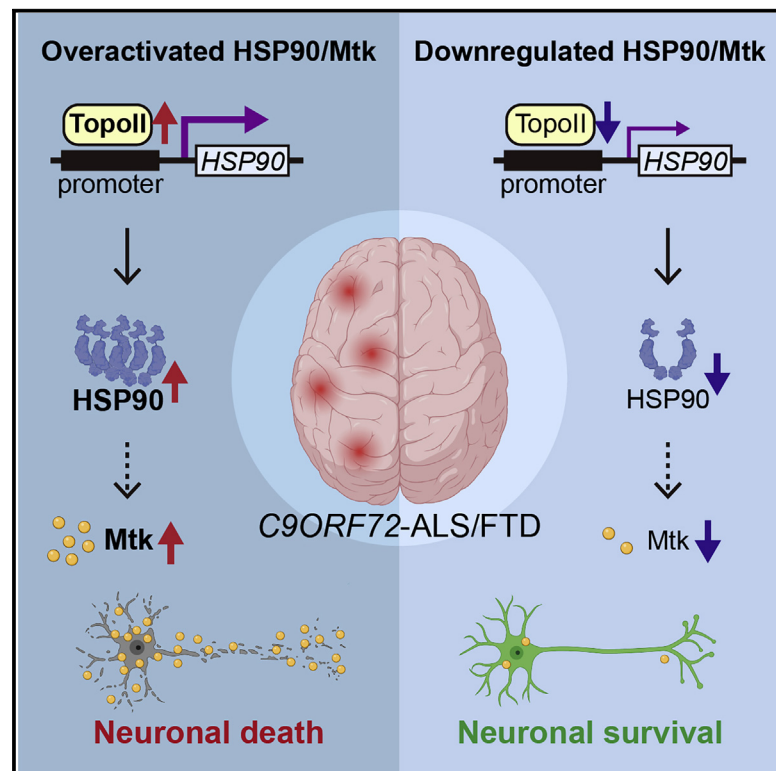
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Downregulation of Hsp90 and the antimicrobial peptide Mtk suppresses poly(GR)-induced neurotoxicity in C9ORF72-ALS/FTD

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Downregulation of Hsp90 and the antimicrobial peptide Mtk suppresses poly(GR)-induced neurotoxicity in *C9ORF72*-ALS/FTD

Graphical abstract



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In brief

Using fruit flies and neurons derived from stem cells of patients with ALS/FTD as their experimental systems, Lee et al. found that the knockdown of three proteins that likely function in a related pathway partially suppresses the neurotoxicity of poly(GR), a pathological protein in ALS/FTD.

Highlights

- Many antimicrobial peptides and heat shock proteins are activated in poly(GR) flies
- Knockdown of Mtk partially rescues poly(GR)-induced toxicity in *Drosophila* neurons
- Hsp90 knockdown suppresses poly(GR) toxicity in flies and death of patient neurons
- Hsp90 and Mtk are regulated by Topoll, another suppressor of poly(GR) toxicity

Report

Downregulation of Hsp90 and the antimicrobial peptide Mtk suppresses poly(GR)-induced neurotoxicity in C9ORF72-ALS/FTD

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SUMMARY

GGGGCC repeat expansion in the C9ORF72 gene is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Repeat RNAs can be translated into dipeptide repeat proteins, including poly(GR), whose mechanisms of action remain largely unknown. In an RNA-seq analysis of poly(GR) toxicity in *Drosophila*, we found that several antimicrobial peptide genes, such as *metchnikowin* (*Mtk*), and heat shock protein (*Hsp*) genes are activated. *Mtk* knockdown in the fly eye or in all neurons suppresses poly(GR) neurotoxicity. These findings suggest a cell-autonomous role of *Mtk* in neurodegeneration. *Hsp90* knockdown partially rescues both poly(GR) toxicity in flies and neurodegeneration in C9ORF72 motor neurons derived from induced pluripotent stem cells (iPSCs). Topoisomerase II (TopoII) regulates poly(GR)-induced upregulation of *Hsp90* and *Mtk*. TopoII knockdown also suppresses poly(GR) toxicity in *Drosophila* and improves survival of C9ORF72 iPSC-derived motor neurons. These results suggest potential novel therapeutic targets for C9ORF72-ALS/FTD.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are major devastating neurodegenerative diseases. ALS and FTD are considered to be part of a spectrum disorder because of their shared genetic causes, the most common being a GGGGCC (G_4C_2) repeat expansion in the first intron of C9ORF72.^{1,2} Repeat RNAs and adjacent intronic sequences transcribed from both sense and antisense directions can be translated into abnormal proteins containing different dipeptide repeats (DPRs), such as poly(GR) and poly(GA).^{3–5} In C9ORF72 patient brains, the pattern of poly(GR) expression appears to correlate with neurodegeneration,^{6–8} suggesting a central role for poly(GR) in the molecular pathogenesis of C9ORF72-ALS/FTD.

G_4C_2 repeats and DPR proteins cause cellular toxicity in numerous cellular and animal models through impacting multiple molecular pathways.^{9–11} For instance, nucleocytoplasmic transport is disrupted by both toxic G_4C_2 -repeat-containing RNA and

arginine-containing DPR proteins.^{12–15} Poly(GR) and poly(PR) bind to motor complexes and microtubules, resulting in compromised axonal transport.¹⁶ These DPR proteins also inhibit global protein synthesis by binding to ribosomes.^{17–20} Another important pathway disrupted by arginine-containing DPR proteins is DNA damage. Control human neurons ectopically expressing poly(GR) or aged C9ORF72 patient neurons differentiated from induced pluripotent stem cell (iPSC) lines show increases in DNA damage and in the levels of both total and phosphorylated p53.^{18,21} Importantly, knocking down p53 dramatically suppresses poly(GR) or poly(PR) toxicity in *Drosophila*¹⁸ or cultured mammalian neurons.²¹ Moreover, downregulation of *Ku80*, a key DNA damage repair gene upstream of p53 activation, also rescues poly(GR) toxicity in *Drosophila* and neurodegeneration in C9ORF72 iPSC-derived motor neurons.²² Despite this progress, it remains to be determined what other suppressor genes and parallel molecular pathways contribute to poly(GR) toxicity.

In this study, we set out to perform an RNA sequencing (RNA-seq) analysis of poly(GR) toxicity in *Drosophila* and identified the

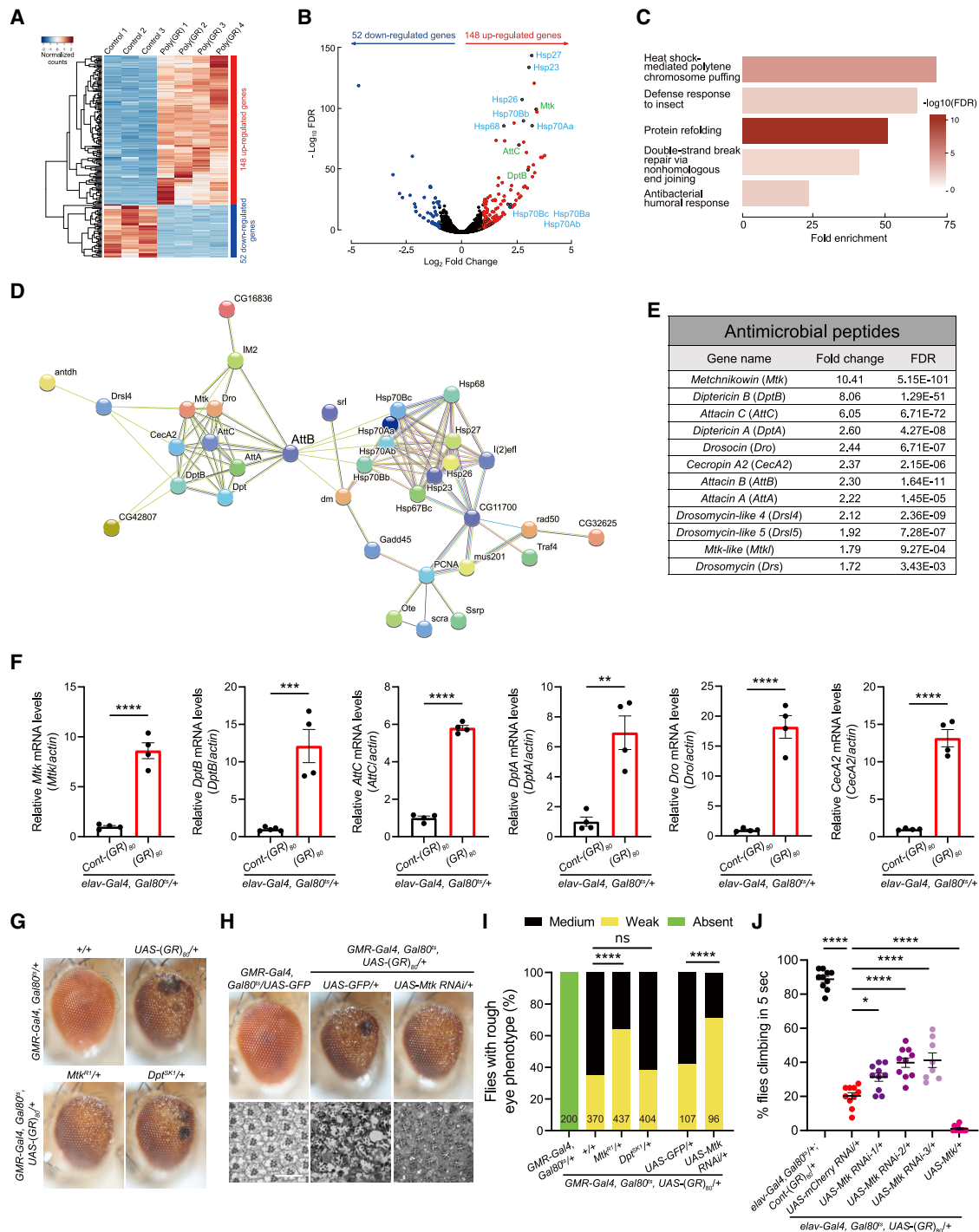


Figure 1. RNA-seq analysis identifies overactivated AMP gene *Mtk* as a contributing factor to poly(GR) toxicity in *Drosophila*

(A) Hierarchical clustering heatmap showing the relative expression of differentially expressed genes in the heads of 3-week-old flies expressing *Cont-(GR)₈₀* or *(GR)₈₀* driven by *elav-Gal4*. *UAS-Cont-(GR)₈₀* expresses *(GR)₈₀* mRNA, but the start codon AUG is changed into the stop codon UAA. Each sample is from an independent cross, n = 3 and 4 independent crosses.

(B) Volcano plot depicting the $-\log_{10}$ p value vs. \log_2 fold change of genes between controls and poly(GR). Genes with FDR < 0.05 and \log_2 FC > 1 are shown in red. Genes with FDR < 0.05 and \log_2 FC < -1 are shown in blue. Genes of particular interest are labeled.

(C) Top enriched gene ontology terms of biological processes for upregulated, differentially expressed genes in poly(GR)-expressing flies compared with the control.

(D) Protein-protein interactions for upregulated genes identified through the STRING database (<https://string-db.org/>). Only proteins with connections are shown.

(legend continued on next page)

antimicrobial peptide (*AMP*) gene *metchnikowin* (*Mtk*) as a novel contributing factor to poly(GR) toxicity in *Drosophila*. We further showed that the knockdown of Hsp90 decreased Mtk levels, and genetic or antisense oligonucleotide (ASO) knockdown of Hsp90 and its transcriptional regulator topoisomerase II rescued neurodegeneration in both fly and iPSC-derived human neuron models of C9ORF72-ALS/FTD.

RESULTS

Overactivation of the *AMP* gene *Mtk* contributes to poly(GR)-induced neurotoxicity in *Drosophila*

Drosophila models have been widely used to understand pathogenic mechanisms of various neurodegenerative diseases.²³ To further understand the molecular mechanisms of poly(GR)-induced neurodegeneration, we performed an RNA-seq analysis in *Drosophila* brains expressing poly(GR) in the neurons. In this experiment, 80 copies of GR (GR₈₀) with a FLAG tag at the N terminus were expressed from (GGXCGX)₈₀ sequence using the *UAS-Gal4* system. GR₈₀ was expressed in all neurons only at the adult stage; after 1–3 days, adult male flies were shifted from low temperature (18°C) to high temperature (25°C).²⁴ In this fly model, the temperature-sensitive yeast transcription suppressor Gal80 (*Gal80^{ts}*) was able to inhibit the pan-neuronal *elav-Gal4*-mediated transcription at 18°C but not at 25°C. *UAS-Cont-(GR)₈₀* contains (GGXCGX)₈₀ DNA sequence, but the start codon ATG is changed into the stop codon TAG, which serves as the negative control.²⁵ Total RNAs from heads of poly(GR)-expressing flies of four independent crosses were isolated and compared with three groups of control flies (Figure 1A). We identified 52 significantly downregulated genes and 148 upregulated genes in poly(GR)-expressing flies (Figures 1A and 1B). Gene ontology analysis showed dysregulation of genes in several major molecular pathways, including *Ku80* in the nonhomologous end-joining DNA repair pathway (Figures 1C and S1), consistent with our earlier findings in poly(GR)-expressing flies and in C9ORF72 patient neurons.^{22,24} Biological processes enriched in upregulated genes included heat-shock-mediated polytene chromosome puffing, defense response to insect, protein refolding, and antibacterial humoral response (Figures 1C and S1). A STRING, a proteomic database of known and predicted protein-protein interactions and association networks,²⁶ analysis showed highly interconnected clusters of proteins involved in the stress response and innate immune pathways (Figure 1D).

A number of *AMP* genes are significantly upregulated in poly(GR)-expressing flies, such as *Mtk* and *Diptericin B* (*DptB*) (Figures 1E and S1). To verify the results from RNA-seq, we validated each of the top 6 *AMP* genes using real-time quantitative PCR in flies obtained from a new genetic cross and showed that, indeed, these *AMP* genes were upregulated in flies expressing poly(GR) (Figure 1F). *AMP* genes are key players in preventing pathogen-induced infections and are upregulated during innate immune responses to infection in animals, including humans^{27,28}; however, the roles of *AMP* genes in animal models of ALS/FTD or other neurodegenerative diseases are poorly understood. To examine whether overactivation of some *AMP* genes contributes to poly(GR) toxicity in flies, we expressed poly(GR) in fly eyes under the control of *GMR-Gal4* at 23°C and also *Gal80^{ts}* to ensure a low level of poly(GR) expression, and a modest retinal degeneration phenotype was induced.²² This phenotype was suppressed by the loss of one allele of *Mtk* but not *DptA/B* (Figures 1G and 1I). The loss of function of mutant alleles of *Mtk* and *DptB* was confirmed by real-time quantitative PCR analysis (Figures S2A and S2B). *Mtk* reduction by RNAi also suppressed poly(GR) toxicity, as evidenced by both the external eye morphology (Figures 1H and 1I) and internal structures of the ommatidium (Figure 1H). Moreover, partial knockdown of *Mtk* using three different RNAi lines in fly neurons also partially rescued the locomotor defect caused by poly(GR) toxicity in a climbing assay, and, conversely, *Mtk* overexpression in neurons enhanced the locomotor defect (Figure 1J), indicating a cell-autonomous function for *Mtk* in neurodegeneration. The knockdown efficiency of *Mtk* RNAi lines was confirmed by real-time quantitative PCR analysis (Figure S2C). Taken together, overactivation of the *AMP* gene *Mtk* contributes to poly(GR) neurotoxicity in a cell-autonomous manner in this *Drosophila* model of C9ORF72-ALS/FTD.

Activation of *Mtk* is mediated by Hsp90, and partial knockdown of Hsp90 suppresses poly(GR) toxicity in *Drosophila* and neurodegeneration in C9ORF72 iPSC-derived motor neurons

Our RNA-seq analysis revealed that genes encoding many heat shock proteins (HSPs) are also upregulated in poly(GR)-expressing neurons (Figures 1B and 2A). To investigate the involvement of different HSPs in poly(GR) toxicity, we used two independent RNAi lines for each gene and found that partial knockdown of *Hsp23*, *Hsp26*, and *Hsp27* enhanced

(E) Table showing upregulated antimicrobial peptide (*AMP*) genes in a poly(GR)-expressing fly model.

(F) Validation of RNA-seq data of *Mtk*, *DptB*, *AttC*, *DptA*, *Dro*, and *CecA2* gene expression by real-time quantitative PCR in the head of 3-week-old male flies expressing poly(GR) under *elav-GAL4*. The *p* value was determined by two-tailed Student's *t* test. *n* = 4, each from an independent genetic cross and mRNA measurement.

(G) Representative images of adult eye phenotypes of different genotypes showing partial inhibition of poly(GR) toxicity by decreasing Mtk activity but not *DptA/B*. Flies were crossed to a *w¹¹¹⁸* control strain.

(H) External and internal eye phenotypes of poly(GR) flies are partially blocked by Mtk depletion. Toluidine blue staining of eye cross-sections shows the structure of rhabdomeres. *UAS-GFP* was used as control for genetic crosses with other *UAS* elements. Scale bars, 25 μm.

(I) Quantification of the genetic effects of *Mtk* and *DptA/B* on the rough eye phenotype caused by poly(GR). The number of flies of each genotype is shown in each column. The *p* value was determined by chi-squared test.

(J) Control and pan-neuronal poly(GR)-expressing male flies, with or without *Mtk* modulation, were analyzed by negative geotaxis climbing assay. For each genotype, the climbing assay was performed in at least eight cohorts, consisting of 14–21 flies per vial grown at 27°C for 18 days (*n* = 10, 10, 10, 10, 8, and 10 vials, respectively, Tukey-Kramer test). All data values are mean ± SEM. *****p* < 0.0001, ****p* < 0.001, ***p* < 0.01, **p* < 0.05; ns, not significant.

See also Figure S1.

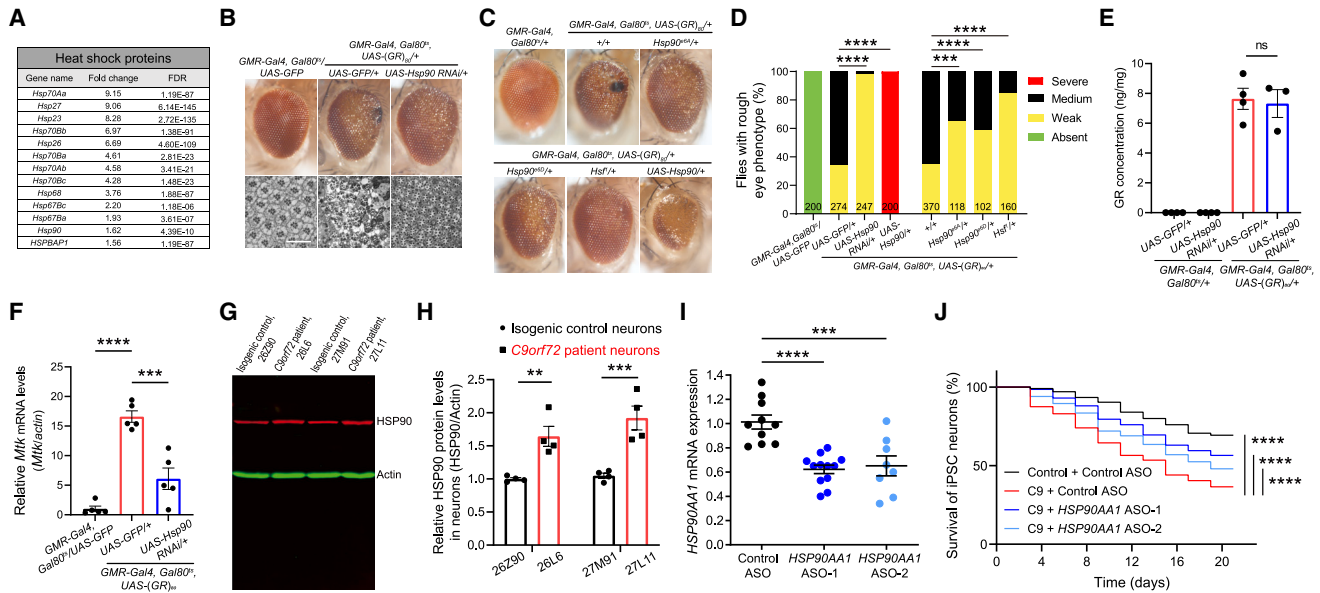


Figure 2. The AMP gene *Mtk* is activated by Hsp90, and inhibition of Hsp90 alleviates neurodegeneration in poly(GR)-expressing flies and C9ORF72 iPSC-derived motor neurons

(A) Table showing upregulated HSPs in a pan-neuronal poly(GR)-expressing fly model.

(B) External and internal eye phenotypes of poly(GR) flies are dramatically rescued by *Hsp90* knockdown. Sectioned adult eyes stained with toluidine blue. *UAS-GFP* was used as control for genetic crosses with other *UAS* elements. Scale bars, 25 μ m.

(C) Representative images of adult eye phenotypes in flies of different genotypes showing suppression of poly(GR) toxicity by genetic reduction or overexpression of *Hsp90* activity. Flies were crossed to a *w¹¹¹⁸* control strain.

(D) Quantification of the suppressor effects of *Hsp90* on the eye degeneration phenotype caused by poly(GR). The number of flies of each genotype is presented in each column. The *p* value was determined by chi-square test.

(E) *Hsp90* knockdown does not change poly(GR) protein levels in the fly head. One-way ANOVA with Tukey post hoc test for multiple comparisons.

(F) Elevated *Mtk* expression level is dramatically attenuated by *Hsp90* knockdown in poly(GR)-expressing flies. One-way ANOVA with Tukey post hoc test for multiple comparisons. *UAS-GFP* was used as control for genetic crosses with other *UAS* elements.

(G and H) Western blot analysis (G) and quantification (H) of HSP90 level in 3-month-old control and C9ORF72 iPSC-derived motor neurons. *n* = 4, two-way ANOVA and Bonferroni post hoc test.

(I) Quantification of the efficiency of HSP90AA1 ASOs knockdown in iPSC-derived motor neurons. One-way ANOVA with Dunnett post hoc test for multiple comparisons.

(J) Decreased survival of C9ORF72-ALS patient iPSC-derived motor neurons upon withdrawal of neurotrophic factors is partially rescued by HSP90AA1 ASO treatment. Three biologically independent iPSC lines were differentiated (*n* = 100 neurons per condition). All motor neuron survival experiments were analyzed by two-sided log-rank test, and statistical significance was calculated from the entire survival time course. All data values are mean \pm SEM. *****p* < 0.0001, ****p* < 0.001, ***p* < 0.01, **p* < 0.05; ns, not significant.

See also Figure S2.

poly(GR) toxicity in the fly eye (Figures S2D and S2E). The RNAi knockdown efficiency for different *Hsp* genes was confirmed by real-time quantitative PCR analysis (Figures S2F–S2J). In contrast, downregulation of *Hsp90* activity by a *Hsp90* RNAi greatly suppressed poly(GR) toxicity, as quantified by the severity of rough eye phenotype (Figures 2B and 2D). The abnormal internal structure of the ommatidium caused by poly(GR) toxicity was also partially rescued by reducing *Hsp90* activity (Figure 2B). To confirm this finding, we used two different genetic mutant alleles of *Hsp90* (*Hsp90^{e6A}* and *Hsp90^{e6D}*) and found that partial loss of *Hsp90* function indeed rescued poly(GR) toxicity (Figures 2C and 2D). Conversely, *Hsp90* overexpression greatly enhanced poly(GR) toxicity (Figures 2C and 2D). *Hsp90*, whose expression is controlled at the transcriptional level by the evolutionarily conserved heat shock transcription factor (Hsf),²⁹ is the most abundant constitutively expressed stress protein in the cells and accounts for 1%–2% of

total cellular proteins under physiological conditions.^{30,31} Poly(GR) toxicity was also greatly reduced in mutant flies heterozygous for *Hsf* (Figures 2C and 2D). These results are consistent with a previous report demonstrating that the upregulation of heat shock genes by Hsf contributes to poly(GR) toxicity in fly models.³² *Hsp90* downregulation does not affect the expression level of poly(GR) protein (Figure 2E), as measured by a Meso Scale Discovery assay,^{22,33} indicating that the suppressor effect of *Hsp90* downregulation on poly(GR) toxicity is not simply due to lower poly(GR) level; rather, it affects a pathway downstream of poly(GR) expression. Due to both *Hsp90* and *Mtk* being suppressors of poly(GR) toxicity, we examined whether *Hsp90* acts upstream of *Mtk*. Indeed, *Hsp90* knockdown attenuated the increased *Mtk* expression in poly(GR)-expressing fly heads (Figure 2F). However, *Hsp90* overexpression by *GMR-Gal4* does not increase *Mtk* level in wild-type flies (Figure S2K), suggesting regulation of *Mtk*

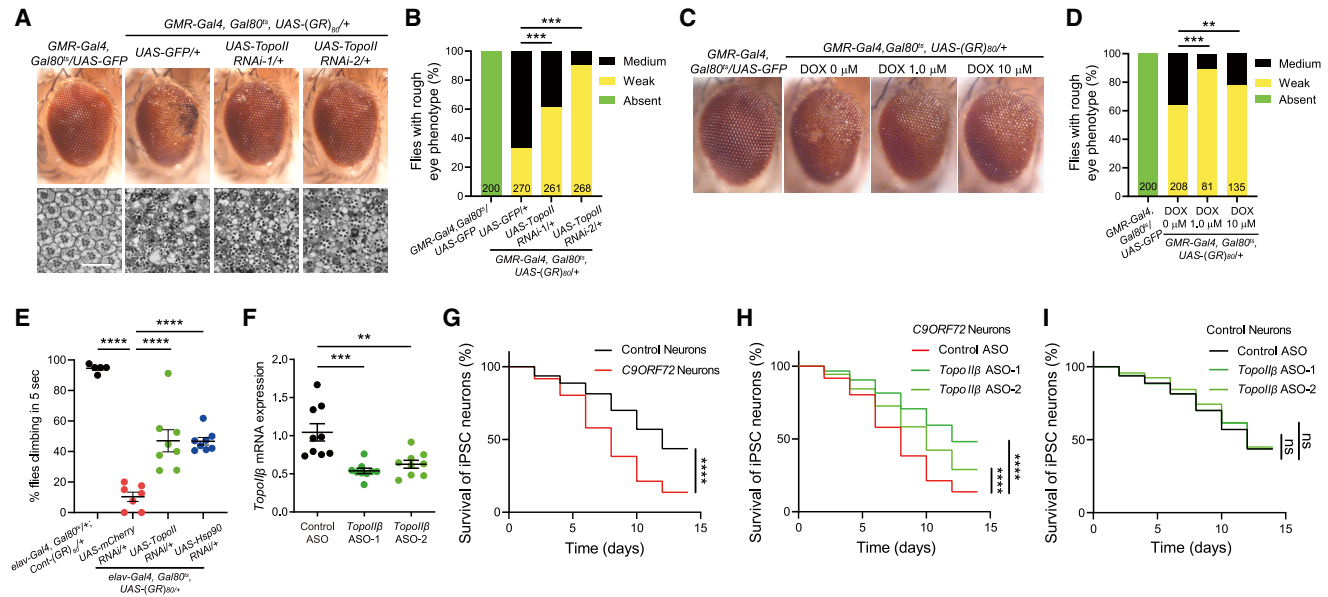


Figure 3. Partial loss of TopoII activity suppresses poly(GR) toxicity in flies and neurodegeneration in C9ORF72 iPSC-derived motor neurons (A) Genetic knockdown of *TopoII* strongly suppresses poly(GR) toxicity in *Drosophila* photoreceptor neurons. Representative images of adult external and internal eye phenotypes of flies with different genotype. *UAS-GFP* was used as control for genetic crosses with other *UAS* elements. Scale bars, 25 μm. (B) Quantification of the effects of *TopoII* knockdown on poly(GR)-induced external eye degeneration phenotype. The number of flies of each genotype is presented in each column. The p value was determined by chi-square test. (C) Doxorubicin suppresses poly(GR)-induced toxicity in *Drosophila* eye. Representative images of eye phenotypes of control and (GR)₃₀ flies fed vehicle or doxorubicin. (D) Quantification of the eye degeneration phenotype. The number of flies of each genotype is shown in each column. The p value was determined by chi-square test. (E) Pan-neuronal downregulation of *TopoII* or *Hsp90* rescues the locomotor defect of (GR)₃₀ flies in negative geotaxis climbing assay. For each genotype, climbing assay was performed for least five cohorts consisting of 16–20 flies grown at 27°C for 3 weeks (n = 5, 7, 8, and 8, Tukey-Kramer test). *UAS-mCherry RNAi* was used as control for genetic crosses with other *UAS-RNAi* elements. (F) Quantification of the efficiency of *TopoIIβ* ASOs knockdown in iPSC-derived motor neurons. One-way ANOVA with Dunnett post hoc test for multiple comparisons. (G) Decreased survival of C9ORF72 patient motor neurons. Three control and three C9ORF72 iPSC lines were differentiated (n = 100 neurons per line). (H) Decreased survival of C9ORF72 motor neurons was significantly increased by *TopoIIβ* ASOs treatment. Three C9ORF72 iPSC lines were differentiated (n = 100 neurons per line). (I) Survival of control iPSC-derived motor neurons treated with *TopoIIβ* ASOs or control ASO. Three iPSC lines were differentiated (n = 100 neurons per line). All motor neuron survival experiments were analyzed by two-sided log-rank test, and statistical significance was calculated from the entire survival time course. All data values are mean ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05; ns, not significant. See also Figure S3.

expression by elevated Hsp90 under pathological conditions may be indirect.

To examine whether HSP90 is involved in the pathogenesis of C9ORF72-ALS/FTD in a human neuron model, we differentiated two C9ORF72 patient iPSC lines and their isogenic control lines, which had CRISPR-Cas9-mediated deletion of expanded G₄C₂ repeats, into motor neurons.²² Consistent with our fly data, HSP90 protein expression level was increased in 3-month-old C9ORF72 iPSC-derived motor neurons (Figures 2G and 2H). We then investigated neuronal survival using the culture system as previously reported.³⁴ We tested two different ASOs to reduce HSP90AA1 expression, and saw about 40% reduction (Figure 2I). After the withdrawal of neurotrophic factors, cell death was greater in C9ORF72-ALS patient motor neurons than in controls (3 donors per genotype, Figure 2J), as reported.³⁴ Both HSP90AA1-targeted ASOs

significantly rescued the neurodegeneration phenotype of C9ORF72-ALS/FTD patient motor neurons (Figure 2J), suggesting that HSP90AA1 is also a novel genetic modifier of C9ORF72-related neurotoxicity in human patient neurons.

TopoII is also a genetic modifier of poly(GR) toxicity in Drosophila and C9ORF72 iPSC-derived motor neurons

During our studies of Mtk and Hsp90, we also found that *TopoII* knockdown by two different RNAi lines partially rescued poly(GR) toxicity in the adult fly eye, as revealed by external eye morphology (Figures 3A and 3B) or internal structure of the ommatidium (Figure 3A). *TopoII* RNAi lines were validated by real-time quantitative PCR analysis (Figure S2L). TopoII is an enzyme that generates a transient double-strand DNA break during replication and transcription.³⁵ To further confirm this finding, we found that doxorubicin, a TopoII-specific inhibitor

used as an anticancer drug,³⁶ did not affect the eye morphology of control flies (data not shown) but partially suppressed the eye degeneration phenotype when fed to poly(GR)-expressing flies in food at a concentration of 1 or 10 μ M (Figures 3C and 3D). Moreover, neuron-specific knockdown of *Topoll* or *Hsp90* partially rescued locomotor defects of flies with neuron-specific expression of poly(GR) (Figure 3E), indicating a cell-autonomous role for both *Topoll* and *Hsp90* in promoting poly(GR)-induced neurodegeneration. Previously, we reported that downregulation of the DNA damage repair gene *Ku80*, or the DNA damage response gene *p53*, attenuates poly(GR) toxicity in flies and partially rescues neurodegeneration in C9ORF72 patient neurons.^{18,22} Here, we found that downregulation of *Topoll* does not affect poly(GR)-induced increases in the expression of *Ku80*, *p53*, or other DNA damage response genes (Figures S3A–S3D). These results further support the notion that *Topoll* is part of a novel parallel pathway independent of DNA damage repair pathways mediating poly(GR) toxicity in *Drosophila* (Figure S3E).

Mammalian cells have two isoforms of DNA Topoll: α and β . Unlike *Topoll* α , *Topoll* β is highly expressed in mammalian postmitotic neurons and has a key role in the transcriptional regulation of neuronal immediate-early genes and neuronal survival.^{37,38} To examine whether *Topoll* β modifies C9ORF72-related neurodegeneration in human ALS/FTD patient neurons, we used ASOs to partially knockdown *Topoll* β expression in control human motor neurons (Figure 3F). As reported before,³⁴ C9ORF72-ALS patient motor neurons were more vulnerable than control neurons upon withdrawal of neurotrophic factors (Figure 3G). Interestingly, partial downregulation of *Topoll* β by two different ASOs significantly increased the survival of C9ORF72-ALS patient motor neurons (Figure 3H) but had no effect in controls (3 donors per genotype, Figure 3I). Thus, upregulation of *Topoll* β also contributes to neurodegeneration in C9ORF72 patient neurons.

Upregulation of Mtk and Hsp90 in poly(GR)-expressing flies is controlled by Topoll

It is not known how Mtk and Hsp90 expression levels are upregulated during poly(GR)-induced neurodegeneration. We found that the level of *Topoll* was increased in poly(GR)-expressing flies. This observation was made at the mRNA level by real-time quantitative PCR analysis (Figure 4A), protein levels as measured by western blot analysis (Figures 4B and 4C), and immunostaining (Figure S4A). In C9ORF72 iPSC-derived motor neurons, *Topoll* β protein levels were elevated (Figures 4D and 4E), similar to those in our *Drosophila* model (Figures 4B and 4C).

Since *Topoll* is also a genetic modifier of poly(GR) toxicity, we investigated whether *Topoll* regulates Mtk activation in poly(GR)-expressing flies. To this end, we used two different *Topoll*-specific RNAi lines to decrease *Topoll* mRNA expression level in poly(GR)-expressing flies to the level seen in control flies (Figure 4F). This manipulation greatly attenuated the increased expression of the AMP gene *Mtk* (Figure 4G), but not of *DptB* (Figure 4H). The effects of *Topoll* downregulation on Mtk expression were not due to changes in poly(GR) level, as poly(GR) mRNA and protein levels were not regulated by *Topoll* (Figures 4I and 4J). Thus, upregulation of the AMP gene *Mtk* in

response to poly(GR) toxicity is mediated in part through transcriptional regulation by *Topoll*. Poly(GR) expression increased *Hsp90* mRNA expression and the partial knockdown of *Topoll* by two different RNAi lines restored the *Hsp90* mRNA level to that of control flies (Figure 4K) but did not affect other heat shock pathway genes such as *Hsp27* (Figure 4L), *Hsp23* (Figure S4B), *Hsp68* (Figure S4C), and *Hsf* (Figure S4D). Moreover, we performed a chromatin immunoprecipitation (ChIP) experiment in HEK293T cells and demonstrated that *Topoll* β directly binds to the promoter region of the *HSP90AA1* gene (Figures 4M and 4N). Thus, poly(GR)-induced overactivation of Hsp90 is mediated by the transcriptional regulation of *Topoll*, revealing a direct regulatory link between *Topoll* and Hsp90 in C9ORF72-related neurodegeneration.

DISCUSSION

Using *Drosophila* as an experimental system, we identified the AMP gene *Mtk* as a novel genetic modifier of poly(GR) toxicity in C9ORF72-related neurodegeneration. The Mtk level was greatly elevated in poly(GR)-expressing flies, and downregulation of Mtk suppressed poly(GR) toxicity, highlighting the role of overactivated Mtk in poly(GR)-induced neurotoxicity. In our study, neuron-specific knockdown of *Mtk* could partially rescue poly(GR)-induced climbing defects, while *Mtk* overexpression in neurons had the opposite effect (Figure 1J), indicating that Mtk has a cell-autonomous role in promoting neurodegeneration. Whether overactivation of Mtk in glial cells also contributes to poly(GR)-induced neurotoxicity remains to be investigated. Mtk is one of innate immunity-related AMPs that protect insects against invading bacteria, fungi, and other pathogens.²⁸ Different AMPs have remarkable specificity targeting diverse pathogens;³⁹ however, their endogenous functions in the nervous system during development or aging are poorly understood. Some AMPs are involved in dendrite degeneration during aging and infection in *C. elegans*⁴⁰ and in neuronal cell loss after traumatic brain injury in *Drosophila*.⁴¹ Our findings here provide the first example that activation of the AMP gene, *Mtk*, has a cell-autonomous role in promoting neurodegeneration caused by FTD/ALS-related disease proteins. AMP genes are present in mammals, but their encoded amino acid sequences differ from those in insects.²⁸ In addition, mammals have evolved highly complex innate immune systems.⁴² It remains to be determined which specific AMPs and related molecular pathways in human neurons or glial cells contribute to C9ORF72-related neurodegeneration. Moreover, loss of C9ORF72 protein function may alter immune cell function.⁴³ It will be interesting to investigate how poly(GR) and the partial loss of C9ORF72 synergize to dysregulate the AMP gene pathway in neurons and glial cells.

In identifying upstream regulators of the AMP gene, *Mtk*, we found that downregulation of Hsp90 also suppressed poly(GR)-induced neurotoxicity without affecting the poly(GR) level in *Drosophila*. This suppressor effect correlates with attenuated poly(GR)-induced Mtk activation. However, the molecular link between Hsp90 and Mtk is unclear, which is a limitation of our study. It is possible that under pathological conditions, elevated Hsp90 may participate in the regulatory circuit that activates *Mtk* expression. Indeed, some Hsp90-ligand complexes

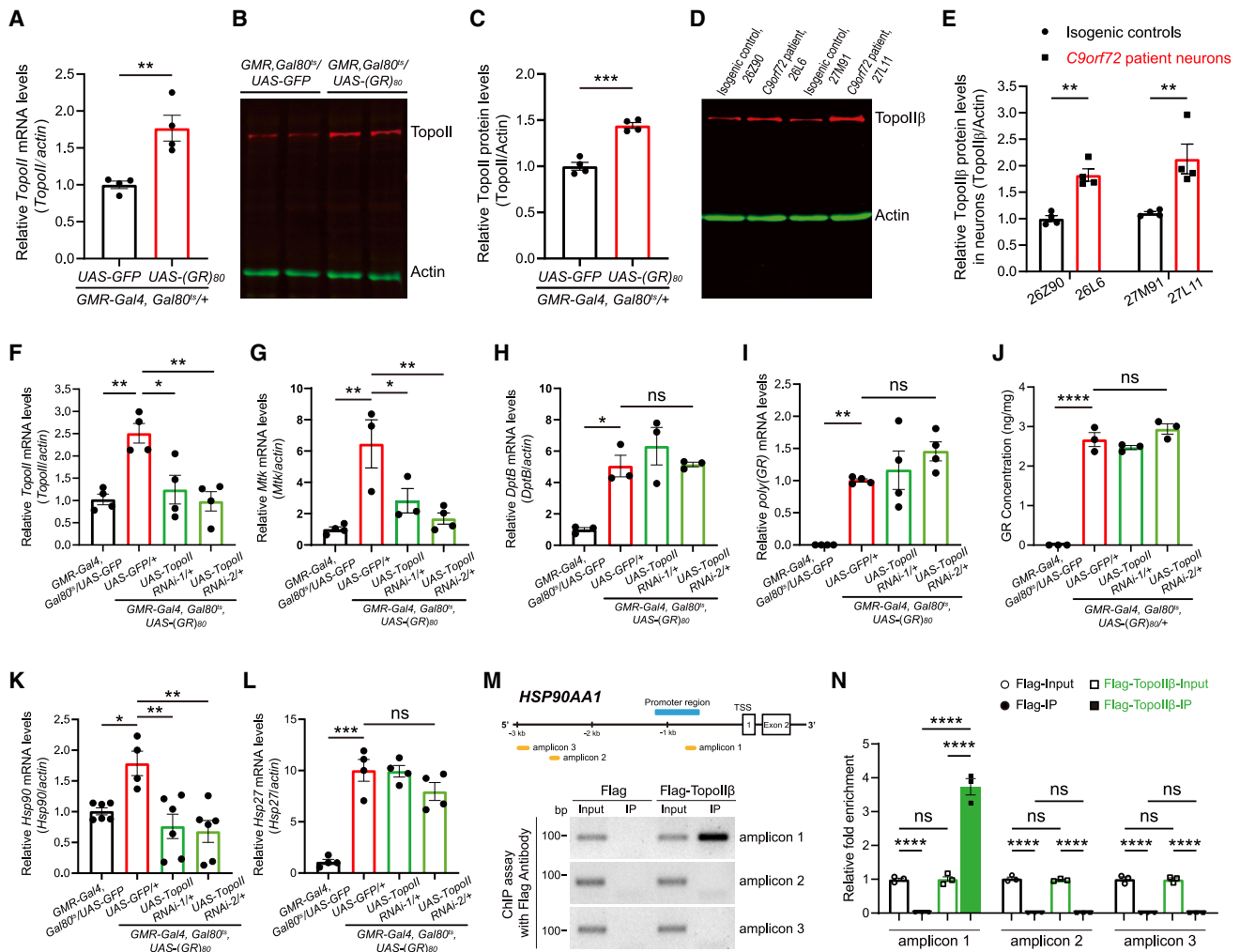


Figure 4. Upregulation of *Mtk* and *Hsp90* in poly(GR)-expressing flies is controlled by TopoII

(A) Real-time quantitative PCR analysis of *TopoII* expression in 10-day-old fly heads expressing poly(GR) under *GMR-Gal4* driver. $n = 4$, the p value was determined by two-tailed Student's t test.

(B and C) Western blot analysis (B) and quantification (C) of TopoII protein level in 10-day-old control and poly(GR)-expressing flies. *UAS-GFP* was used as control for genetic crosses with other *UAS* elements. $n = 4$, the p value was determined by two-tailed Student's t test.

(D and E) Western blot analysis (D) and quantification (E) of TopoII β protein level in 3-month-old control and *C9ORF72* iPS-derived motor neurons. $n = 4$, two-way ANOVA and Bonferroni post hoc test for multiple comparisons.

(F–H) Real-time quantitative PCR analysis of *TopoII* (F), *Mtk* (G), and *DptB* (H) mRNA levels in the heads of control and (*GR*)₈₀ flies, with or without *TopoII* knockdown. *UAS-GFP* was used as control for genetic crosses with other *UAS* elements. $n \geq 3$, one-way ANOVA with Tukey post hoc test for multiple comparisons.

(I and J) *TopoII* knockdown does not change poly(GR) mRNA level (I) and protein levels (J) in the fly head. One-way ANOVA with Tukey post hoc test for multiple comparisons.

(K and L) Real-time quantitative PCR analysis of *Hsp90* (K) and *Hsp27* (L) mRNA levels in the heads of control and (*GR*)₈₀ flies, with or without *TopoII* knockdown. *UAS-GFP* was used as control for genetic crosses with other *UAS* elements. $n \geq 4$, one-way ANOVA with Tukey post hoc test for multiple comparisons.

(M) TopoII β ChIP assay of the human *HSP90AA1* gene. Schematic diagram of the human *HSP90AA1* promoter and amplicons used in the ChIP assay (upper). ChIP-qPCR showing TopoII β binding to the indicated sites in HEK293T cells (bottom).

(N) Quantification of TopoII β binding to regions 1 to 3. One-way ANOVA with Tukey post hoc test for multiple comparisons. All data values are mean \pm SEM. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; ns, not significant.

See also Figure S4.

can activate innate immunity in certain cell types.^{44,45} Alternatively, downregulation of Hsp90 may indirectly affect Mtk activation through attenuated poly(GR) toxicity. It remains to be determined how Hsp90 in poly(GR)-expressing cells activates Mtk or other genes in the innate immunity pathway. More impor-

tantly, the knockdown of *HSP90AA1* expression also partially rescued neurodegeneration in *C9ORF72* iPS-derived motor neurons, revealing an unexpected role for HSP90 in promoting *C9ORF72*-related neurodegeneration. Our results are consistent with the work by Mordes et al.,³² who reported that HSF1, the

master transcription factor that controls the expression of many HSPs, is upregulated in both poly(GR)-expressing flies and C9ORF72-ALS/FTD patient brains and contributes to poly(GR) toxicity.³² However, their study did not specifically examine the role of Hsp90; thus, our work extended their findings and pinpointed Hsp90, the most abundant constitutively expressed stress protein in the cell,^{30,31} as a key player in the upregulation of AMPs and, potentially, the activation of other innate immune responses that further exacerbate neurodegenerative processes. Moreover, our ChIP analysis indicates that HSP90AA1 expression is directly regulated by Topoll β , and downregulation of Topoll β also partially suppresses neurodegeneration in C9ORF72 iPSC-derived motor neurons. Thus, the Topoll-regulated Hsp90 and some AMPs are novel modifier genes in C9ORF72-ALS/FTD-related neurodegeneration, and the downregulation of these genes represents a potential therapeutic avenue.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Project initiation, F.-B.G. and S.L.; conceptualization, F.-B.G., S.L., and N.S.; investigations, S.L., Y.-W.J., G.K., Y.Y.-A., G.R.L., B.R.-J., M.S., and M.P.; formal analysis, S.L., J.M., and Z.W.; funding acquisition, F.-B.G., J.K.I., and Z.W.; supervision, F.-B.G.; writing – original draft, F.-B.G., S.L., G.R.L., and J.M.; writing – review & editing, F.-B.G. and S.L.

DECLARATION OF INTERESTS

F.-B.G. has an active research agreement with, and receives funding from, Stealth BioTherapeutics. Z.W. co-founded Rgenta Therapeutics, and she serves as a scientific advisor for the company and is a member of its board. J.I. is a co-founder of AcuraStem, Inc. and Modulo Bio, serves on the scientific advisory boards of AcuraStem, Spinogenix, Synapticure, and Vesalius Therapeutics, and is employed at BioMarin Pharmaceutical.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper received support from a program designed to increase minority representation in their field of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Hsp90	BD Biosciences	Cat#610419; RRID:AB_397799
Mouse anti-β actin	Santa Cruz Biotechnology	Cat#sc-47778; RRID:AB_626632
Mouse anti-β actin	DSHB	Cat#jla20; RRID:AB_528068
Rabbit anti-TOP2B	Novus	Cat#NBP1-89527; RRID:AB_11035093
Rabbit anti-Topoll	Buchenau et al., 1993 ⁴⁶	N/A
IRDye 680RD goat anti-rabbit secondary antibody	LI-COR Biosciences	Cat#926-68071; RRID:AB_10956166
IRDye 800CW donkey anti-mouse secondary antibody	LI-COR Biosciences	Cat#926-32212; RRID:AB_621847
Chemicals, peptides, and recombinant proteins		
Doxorubicin hydrochloride	Sigma-Aldrich	Cat#D1515; CAS: 25316-40-9
Critical commercial assays		
miRNeasy Kit	Qiagen	Cat#74106
TaqMan Reverse Transcription Kit	Thermo Fisher Scientific	Cat#N8080234
SYBR Select Master Mix	Thermo Fisher Scientific	Cat#4472918
ChIP kit	Abcam	Cat#ab500
Experimental models: Cell lines		
Human: HEK293T cells	ATCC	Cat#CRL-11268
Human: Isogenic control line 26Z90	Lopez-Gonzalez et al. ²²	N/A
Human: Isogenic control line 27M91	Lopez-Gonzalez et al. ²²	N/A
Human: C9ORF72 carrier 26L6	Almeida et al., 2013 ⁴⁷	N/A
Human: C9ORF72 carrier 27L11	Almeida et al., 2013 ⁴⁷	N/A
Human: control line 1 (CTRL-1)	NINDS Biorepository at the Coriell Institute	Cat#ND03231
Human: control line 2 (CTRL-2)	NINDS Biorepository at the Coriell Institute	Cat#ND03719
Human: control line 3 (CTRL-3)	NINDS Biorepository at the Coriell Institute	Cat#ND05280
Human: C9ORF72-ALS line 1 (C9 ALS-1)	NINDS Biorepository at the Coriell Institute	Cat#ND06769
Human: C9ORF72-ALS line 2 (C9 ALS-2)	NINDS Biorepository at the Coriell Institute	Cat#ND10689
Human: C9ORF72-ALS line 3 (C9 ALS-3)	NINDS Biorepository at the Coriell Institute	Cat#ND12099
Experimental models: Organisms/strains		
<i>D. melanogaster</i> : GMR-Gal4: <i>w</i> [1118]; <i>P</i> {GMR-GAL4. <i>w</i> [-]}2/ <i>CyO</i>	Bloomington Drosophila Stock Center	BDSC:9146; FlyBase: FBti0072862
<i>D. melanogaster</i> : elav-Gal4: <i>P</i> { <i>w</i> [+ <i>mC</i>]=GAL4- <i>elav</i> .L}2/ <i>CyO</i>	Bloomington Drosophila Stock Center	BDSC:8765; FlyBase: FBti0072909
<i>D. melanogaster</i> : Tub-Gal80 ^{ts} : <i>w</i> [*]; <i>P</i> { <i>w</i> [+ <i>mC</i>]= <i>tubP</i> -GAL80[<i>ts</i>]20; <i>TM2/TM6B</i> , <i>Tb</i> [1]}	Bloomington Drosophila Stock Center	BDSC:7019; FlyBase: FBti0027796
<i>D. melanogaster</i> : UAS-GFP	Yang et al. ²⁵	N/A
<i>D. melanogaster</i> : UAS- <i>mCherry</i> -RNAi: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; <i>P</i> { <i>VALIUM20</i> - <i>mCherry</i> } <i>attP2</i>	Bloomington Drosophila Stock Center	BDSC:35785; FlyBase: FBti0143385
<i>D. melanogaster</i> : <i>w</i> ¹¹¹⁸	Bloomington Drosophila Stock Center	BDSC:3605; FlyBase: FBal0018186
<i>D. melanogaster</i> : UAS- <i>Mtk</i> -RNAi-1: <i>w</i> [1118]; <i>P</i> {GD3790}v8792/ <i>TM3</i>	Vienna Drosophila Resource Center	VDRC:8792; FlyBase: FBst0471250
<i>D. melanogaster</i> : UAS- <i>Mtk</i> -RNAi-2: <i>P</i> {KK113189}VIE-260B	Vienna Drosophila Resource Center	VDRC:109740; FlyBase: FBst0481400
<i>D. melanogaster</i> : UAS- <i>Mtk</i> -RNAi-3: <i>y</i> [1] <i>v</i> [1]; <i>P</i> { <i>TRiP</i> .HM05032}attP2/ <i>TM3</i> , <i>Sb</i> [1]}	Bloomington Drosophila Stock Center	BDSC:28546; FlyBase: FBst0028546

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster</i> : UAS-Hsp23-RNAi-1: <i>y</i> [1] <i>v</i> [1]; P{TRiP.HMS06055}attP40	Bloomington Drosophila Stock Center	BDSC:82961; FlyBase: FBti0201590
<i>D. melanogaster</i> : UAS-Hsp23-RNAi-2: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; P{TRiP.HMS02745}attP40	Bloomington Drosophila Stock Center	BDSC:44029; FlyBase: FBti0158629
<i>D. melanogaster</i> : UAS-Hsp26-RNAi-1: P{KK106347}VIE-260B	Vienna Drosophila Resource Center	VDRC: 100955; FlyBase: FBst0472828
<i>D. melanogaster</i> : UAS-Hsp26-RNAi-2: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; P{TRiP.GL00329}attP2	Bloomington Drosophila Stock Center	BDSC:35408; FlyBase: FBti0144411
<i>D. melanogaster</i> : UAS-Hsp27-RNAi-1: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; P{TRiP.HMS00807}attP2	Bloomington Drosophila Stock Center	BDSC:33007; FlyBase: FBti0140519
<i>D. melanogaster</i> : UAS-Hsp27-RNAi-2: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; P{TRiP.HMS00867}attP2	Bloomington Drosophila Stock Center	BDSC:33922; FlyBase: FBti0140576
<i>D. melanogaster</i> : UAS-Hsp70-RNAi: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; P{TRiP.GLV21036}attP2	Bloomington Drosophila Stock Center	BDSC:35671; FlyBase: FBti0144632
<i>D. melanogaster</i> : UAS-Hsp90-RNAi: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; P{TRiP.HMS00899}attP2	Bloomington Drosophila Stock Center	BDSC:33947; FlyBase: FBti0140605
<i>D. melanogaster</i> : UAS-Hsp90: <i>y</i> [1] <i>w</i> [*]; PBac{ <i>y</i> + <i>mDint2</i> } <i>w</i> [+ <i>mC</i>]=UAS-Hsp83.Z}VK00037	Bloomington Drosophila Stock Center	BDSC:58469; FlyBase: FBti0163127
<i>D. melanogaster</i> : Hsp90 ^{e6A} : <i>w</i> [*]; Hsp83[e6A]/TM6B, <i>Tb</i> [1]	Bloomington Drosophila Stock Center	BDSC:36576; FlyBase: FBal0029644
<i>D. melanogaster</i> : Hsp90 ^{e6D} : <i>w</i> [*]; Hsp83[e6D]/TM3, <i>Sb</i> [1]	Bloomington Drosophila Stock Center	BDSC:5696; FlyBase: FBal0029645
<i>D. melanogaster</i> : Hsf ¹ : <i>net</i> [1] <i>cn</i> [1] Hsf[1]/CyO	Bloomington Drosophila Stock Center	BDSC:5491; FlyBase: FBal0044857
<i>D. melanogaster</i> : UAS-TopoII-RNAi-1: <i>y</i> [1] <i>v</i> [1]; P{TRiP.JF01300}attP2	Bloomington Drosophila Stock Center	BDSC:31342; FlyBase: FBti0130747
<i>D. melanogaster</i> : UAS-TopoII-RNAi-2: <i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; P{TRiP.GL00338}attP2	Bloomington Drosophila Stock Center	BDSC:35416; FlyBase: FBti0144419
<i>D. melanogaster</i> : Dpt ^{SK1}	Hanson et al. ³⁹	N/A
<i>D. melanogaster</i> : Mtk ^{R1}	Hanson et al. ³⁹	N/A
<i>D. melanogaster</i> : UAS-Mtk	Cao et al., 2013 ⁴⁸	N/A
<i>D. melanogaster</i> : GMR-Gal4, Tub-GAL80 ^{ts} , UAS-(GR) ₈₀ /CyO	Lopez-Gonzalez et al. ²²	N/A
<i>D. melanogaster</i> : <i>elav</i> -Gal4, Tub-Gal80 ^{ts} /CyO; UAS-Control-(GR) ₈₀ /TM6B, <i>Tb</i>	Yuva-Aydemir et al. ²⁴	N/A
<i>D. melanogaster</i> : <i>elav</i> -Gal4, Tub-Gal80 ^{ts} , UAS-(GR) ₈₀ /CyO	Yuva-Aydemir et al. ²⁴	N/A
Oligonucleotides		
See Table S2 for Oligonucleotides	This paper	N/A
Recombinant DNA		
Plasmid: p3xFLAG-CMV TM -7.1	Sigma-Aldrich	Cat#E7533
Plasmid: pCMV-3xFLAG-TopoIIβ	This paper	N/A
Hb9::RFP+ lentiviral vector	Addgene	Cat#37081
Software and algorithms		
FIJI	NIH, USA	https://imagej.net/Fiji
Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
Zen	Zeiss	N/A
ImageStudio	Li-Cor	https://www.licor.com/bio/image-studio-lite/
Other		
Biorender for model preparations	Biorender	https://biorender.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fen-Biao Gao (fen-biao.gao@umassmed.edu).

Materials availability

Requests for resources and additional information should be directed to and will be fulfilled by the [lead contact](#). Reagents generated in this study will be made available on request, but we may require a completed Materials Transfer Agreement if there is potential for commercial application.

Data and code availability

All data are available in the main text or supplementary materials and will be shared by the [lead contact](#) upon request. 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 strains and genetics

Flies were maintained at 25°C on a standard yeast diet unless otherwise stated. *GMR-Gal4* (BL9146), *elav-Gal4* (BL8765), *Tub-Gal80^{ts}* (BL7019), *UAS-GFP*, *UAS-mCherry RNAi* (BL35785) *w¹¹¹⁸* (BL3605), *UAS-Mtk RNAi-1* (v8792), *UAS-Mtk RNAi-2* (v109740), *UAS-Mtk RNAi-3* (BL28546), *UAS-Hsp23 RNAi-1* (BL82961), *UAS-Hsp23 RNAi-2* (BL44029), *UAS-Hsp26 RNAi-1* (v100955), *UAS-Hsp26 RNAi-2* (BL35408), *UAS-Hsp27 RNAi-1* (BL33007), *UAS-Hsp27 RNAi-2* (BL33922), *UAS-Hsp70 RNAi* (BL35671), *UAS-Hsp90 RNAi* (BL33947), *UAS-Hsp90* (BL58469), *Hsp90^{e6A}* (BL36576), *Hsp90^{e6D}* (BL5696), *Hsf¹* (BL5491), *UAS-Topoll RNAi-1* (BL31342), *UAS-Topoll RNAi-2* (BL35416) mutant and RNAi lines were from the Bloomington *Drosophila* Stock Center and the Vienna *Drosophila* Resource Center. *Dpt^{SK1}* and *Mtk^{R1}* mutant lines were kindly provided by Dr. Bruno Lemaitre (Ecole Polytechnique of Lausanne, Switzerland). The *Dpt^{SK1}* line includes two deletions removing both *DptA* and *DptB* genes. *UAS-Mtk* line was gift from by Dr. Stanislava Chtarbanova (University of Alabama). *GMR-Gal4*, *Tub-GAL80^{ts}*, *UAS-(GR)₈₀/CyO* and *elav-Gal4*, *Tub-Gal80^{ts}/CyO*; *UAS-Control-(GR)₈₀/TM6B*, *Tb*, and *elav-Gal4*, *Tub-Gal80^{ts}*, *UAS-(GR)₈₀/CyO* lines were generated in our laboratory.^{22,24} All the fly genotypes in this study can be found in [Table S1](#).

Human iPSC culture

We used previously published iPSC lines from two C9ORF72 carriers (26L6 and 27L11) and their corresponding isogenic control lines (26Z90 and 27M91) with CRISPR/Cas9-mediated deletion of expanded G₄C₂ repeats.²² The use of these human iPSC lines is approved by UMass Chan Medical School Institutional Biosafety Committee (Docket # I-435-20). Briefly, iPSCs were plated and expanded in mTeSR1 medium (mTeSRTM 1 5X Supplement diluted 1:4 in mTeSRTM Basal Medium, Stem Cell Technologies) on coated 6-well plates with Matrigel (Cat#354230, Corning) diluted 1:100 in Knockout DMEM/F-12 (Cat#12660012, Gibco/Thermo Fisher Scientific). The mTeSR1 medium was replaced every day. When cells reached 70–80% confluency, they were washed with DPBS and dissociated with Accutase (Cat#SCR005, Millipore) diluted 1:2 in DPBS. After washing with DPBS, cells were scraped with a cell lifter in mTeSR1 medium and seeded into Matrigel-coated plates at the desired number. The mTeSR1 medium was replaced every day and differentiated cells/colonies were removed if necessary.

Motor neuron differentiation for western blot

Human iPSCs were differentiated into motor neurons with some modifications as described.²² Briefly, iPSCs were expanded in Matrigel-coated wells and at 60% confluency were split with Accutase (Cat#SCR005, Millipore) into Matrigel-coated wells; 24 h after plating, the culture medium was replaced with neuroepithelial progenitor (NEP) induction medium, including Knockout DMEM/F12 (Cat#12660012, Gibco), Neurobasal medium (Cat#21103049, Gibco) at 1:1, 0.5X N2 supplement (Cat#17502-048, Gibco), 0.5X B27 supplement (Cat#17504044, Gibco), 0.1 mM Ascorbic acid (Cat#A4403, Sigma-Aldrich), 1X Glutamax (Cat#35050061, Thermo Fisher Scientific), 3 μM CHIR99021 (Cat#72054, Stem Cell Technologies), 2 μM DMH1 (Cat#4126, Tocris Bioscience), and 2 μM SB431542 (Cat#04-0010-10, Stemgent), for 6 days. NEPs were dissociated with Accutase and split 1:6 into Matrigel-coated wells. NEPs were cultured in motor neuron progenitor (MNP) induction medium (the same NEP medium as described above with 0.1 μM retinoic acid (Cat#R2625, Sigma-Aldrich) and 0.5 μM Purmorphamine (Cat#540220, Millipore)) for 6 days. MNPs were dissociated with Accutase and split into culture dishes (Corning) to generate suspension cultures. The neurospheres were cultured in motor neuron differentiation medium (the same NEP medium as described above with 0.5 μM retinoic acid and 0.1 μM Purmorphamine) for 6 days. Lastly, the neurospheres were dissociated into single cells, plated on Matrigel-coated plates in motor neuron media, including Neurobasal medium, 0.5X B27 supplement, 0.1 mM Ascorbic acid, 1X Glutamax, 0.1 μM Compound E (Cat#73954, Stem Cell Technologies), 0.26 μg/ml cAMP, 1 μg/ml Laminin (Cat#L2020, Sigma-Aldrich), 10 ng/ml GDNF (Cat#PHC7041, Thermo Fisher Scientific), and 10 ng/ml BDNF, for up to 3 months. The culture medium was partially replaced twice a week.

Generating induced motor neurons for survival

Induced motor neurons (iMNs) were generated as described.³⁴ Cells were purchased from Coriell Institute. The control lines were ND03231 (CTRL-1), ND03719 (CTRL-2), and ND05280 (CTRL-3). The C9ORF72-ALS lines were ND06769 (C9 ALS-1), ND10689 (C9 ALS-2), and ND12099 (C9 ALS-3). Briefly, human secondary fibroblasts were transduced with a cocktail of transcription factors (*Ascl1*, *Brn2*, *Isl1*, *Lhx3*, *Ngn2*, *NeuroD1*, and *Myt1l*) using retrovirus. The next day, Hb9::RFP⁺ lentivirus was added to the fibroblast cultures. On day 4, primary glia isolated from male and female ICR mouse pups (P2-P3) were added to the cultures in glia medium. To induce the formation of neurons, the medium was replaced the following day with N3 medium consisting of DMEM/F-12 (Life Technologies), 2% fetal bovine serum, B27 and N2 supplements (Life Technologies), 1% penicillin/streptomycin, 7.5 μ M Rep Sox (Selleck), and growth factors (GDNF, BDNF, CNTF, and FGF, 10 ng/ml each, R&D Systems). The culture medium was fully replenished every 2 days, until 14 days after transduction.

METHOD DETAILS

RNA-seq

Total RNA from heads of 3-week-old control and (GR)₈₀ male flies aged at 25 °C was isolated with the Qiagen miRNeasy Mini Kit (Cat#217004, Qiagen). RNA-seq libraries were prepared with the Ovation Universal RNA-seq system and *Drosophila* rRNA depletion module according to the manufacturer's protocol (Cat#0343, Nugen). cDNA was fragmented with an ultrasonicator (Cat#E220, Covaris). Library size distribution was assessed with an Agilent Bioanalyzer. Libraries were sequenced with an Illumina HiSeq Platform PE 150.

RNA-seq analysis

Sequenced reads were trimmed for Illumina adaptors and low-quality bases with Trimmomatic (v0.39)⁴⁹ and aligned to the dm6 genome with HISAT2 (v2.2.1).⁵⁰ Read counts per gene were calculated with HTSeq⁵¹ and FlyBase gene annotations (dme-l-all-r6.34.mod.gtf).⁵² Differential gene expression was analyzed with DESeq2. Gene Ontology analysis was done with PantherDB,⁵³ and protein-protein network analysis was done with STRING.²⁶ Relevant code for this analysis can be found at <https://github.com/weng-lab/Fly-ALS-Project>.

RNA extraction and real-time quantitative PCR

Fly heads were collected and frozen in liquid nitrogen. Total RNA was extracted with QIAzol (Cat#79306, Qiagen) and the RNeasy Mini Kit (Cat#74106, Qiagen), and 1 μ g of RNA of each sample was then reverse transcribed to cDNA with the TaqMan Reverse Transcription Kit (Cat#N8080234, Thermo Fisher Scientific). Real-time quantitative PCR was done with SYBR Select Master Mix (Cat#4472918, Thermo Fisher Scientific) on a QuantStudio 3 System. Ct values for each gene were normalized to internal controls as indicated. Relative mRNA expression was calculated with the 'delta-delta Ct' method.

Total RNA from iPSC-derived motor neurons was extracted with the RNeasy Plus Mini Kit (Qiagen) 72 h after ASO administration and reverse transcribed with an Oligo-dT and a Protoscript II First Strand Synthesis Kit (NEB). RNA integrity was assessed with a NanoDrop 1000 (Thermo). Real-time quantitative PCR was performed with an Applied Biosystems ViiA 7 Real-Time PCR System (Life Technologies) and iTaq Universal SYBR Green Supermix (Bio-Rad). Gene expression levels were normalized to the mRNA levels of *GAPDH* or *HPRT*. All the primer information is in Table S2.

Quantification of *Drosophila* external eye phenotype

All flies for eye phenotype analysis were crossed and raised at 23 °C to avoid lethality. Flies were collected and aged for ~14 days to see degenerative eye phenotype. Eye phenotypes of 14-day-old adult flies were immobilized by freezing at -20 °C for 4 hr, and then imaged under a dissecting microscope (Nikon, SMZ 1500). To quantify the phenotypical differences between adult eyes in each genotype, we use an eye phenotype grading scale as a relative output of how much a gene or treatment modifies the ommatidia while toxic poly(GR) is expressed in the eye. Phenotype categories correspond to the levels of eye degeneration observed and are classified into the following 4 groups. (1) Absent: wild-type, driver-only transgenic flies were used as the baseline to establish this phenotype. All ommatidia are completely normal, well organized, have normal pigment, and show no disruptions. (2) Weak: ommatidia are slightly abnormal in size and arrangement of cells, there is a partial loss of pigment, but no necrosis (black tissue) is observed. (3) Medium: there is considerable disarrangement to the ommatidia and their size, more cells have loss of pigment, and there is obvious necrosis in the eye. (4) Severe: substantial necrosis and loss of ommatidia in the eye, leading to a noticeably smaller eye size. Each group of flies was quantified by category, and the data were analyzed by chi-square test.

Drosophila eye internal section with TB staining

Flies were reared at 23 °C and aged for ~10 days and then were anesthetized with CO₂. Fly heads were hemisected, and fixed overnight at 4 °C in 0.05 M Sodium Cacodylate buffer containing 2.5% Glutaraldehyde. The samples were then washed with the buffer solution three times, and fixed again with 2% Osmium tetroxide for 2 hr. After rinse with D.W, the samples were then dehydrated through an ethanol series (10%, 30%, 50%, 70%, 85%, 95%, and 100%), and the head tissues were incubated in 100% propylene oxide for 1 h at room temperature. The samples were transferred to 1:1 ratio of Poly/Bed and propylene oxide at room temperature for

overnight, and then embedded in Poly/Bed 812 resin. Eye cross-sections were cut at 1 μ m thickness and stained with 1% toluidine blue (TB) for confocal microscopy in bright-field (Carl Zeiss, LSM800).

Negative geotaxis climbing assay

All flies were crossed and raised at 18°C until eclosion to avoid developmental defects. Twenty male flies were collected and maintained at 27°C for 3 weeks. For climbing assay, aged flies were placed in a 10 cm long plastic vial and incubated for 3 h at room temperature for environmental adaptation. Using negative geotaxis in *Drosophila*, we tapped flies to the bottom of the vial and counted the number of flies that climbed 3 cm in 5 sec. The assay was repeated twice for each vial. For each genotype, at least total of 100 flies were analyzed.

Poly(GR) measurement

RIPA buffer for soluble- and urea buffer for insoluble-poly(GR) levels in flies were measured with a Meso Scale Discovery (MSD)-based immunoassay and custom-made polyclonal anti-poly(GR) antibodies as described²⁴. Briefly, 30–40 frozen aged fly heads were lysed, and diluted to the same concentration, and tested in duplicate wells. Response values upon electrochemical stimulation were acquired with a plate reader (QuickPlex SQ120, Meso Scale Discovery). All response signals were background corrected and interpolated against standard curve using (GR)₈ peptide and presented as concentration in ng/mg units.

Western blot analysis

Motor neurons were briefly washed with PBS and homogenized in RIPA buffer (Thermo Scientific, Cat#89901) containing protease and phosphatase inhibitor Cocktail (Cat#5872, Cell Signaling) and the lysates were centrifuged at 14,000 \times g for 15 min at 4°C to remove the debris. The supernatant was transferred, and then quantified with a Pierce BCA protein assay (Thermo Scientific, Cat#23227). The lysates were mixed with 4 \times Laemmli Sample Buffer (Bio-Rad, Cat#1610747) with beta-mercaptoethanol (Cat#M3148, Sigma-Aldrich) and incubated at 95 °C for 10 min.

Adult fly heads were homogenized in 2 \times Laemmli buffer (Cat#1610737, Bio-Rad) containing beta-mercaptoethanol (Cat#M3148, Sigma-Aldrich) and protease and phosphatase inhibitor Cocktail (Cat#5872, Cell Signaling). Lysates were incubated at 95 °C for 5 min and centrifuged for 5 min at 16,000 \times g to remove the debris. Proper amounts of lysates were separated by SDS-PAGE and transferred to PVDF membranes. Then they were immunoblotted with rabbit anti-Topoll β (1:2,000; Cat#NBP1-89527, Novus), mouse anti-HSP90 (1:2,000; Cat#610419, BD Biosciences), mouse anti β -actin (1: 2,000, Cat#sc-47778, Santa Cruz Biotechnology), rabbit anti-Topoll (1:5,000; a gift from Dr. Donna Arndt-Jovin, Max Planck Institute for Biophysical Chemistry), or mouse anti-actin (1: 2,000; JLA20, Developmental Studies Hybridoma Bank) overnight at 4 °C. After washing with 1 \times TBST three times, the membranes were incubated with IRDye 680RD goat anti-rabbit (Cat#926-68071, LI-COR Biosciences) or IRDye 800CW donkey anti-mouse (Cat#926-32212, LI-COR Biosciences) secondary antibodies. All blocking and antibody dilutions were done with Odyssey Blocking Buffer (Cat#927-40010, LI-COR Biosciences). The images were acquired with Odyssey Imaging System (LI-COR Biosciences).

Drug administration for *Drosophila*

For drug treatment experiments, fly embryos were raised in plastic vials with instant medium (Cat#173202, Carolina Biological Supply) containing vehicle or 1.0 or 10 μ M doxorubicin (Cat#D1515, Sigma-Aldrich) dissolved in water. Flies were grown on food containing vehicle or doxorubicin at 23 °C and collected for analysis at 3 days of age.

Immunohistochemistry

Third instar larval brains were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 4 min, and washed three times with PBS containing 0.1% Triton X-100 (PBST). Larval tissues were blocked with 2% normal goat serum in PBST and incubated with rabbit anti-Topoll (1:1000; a gift from Dr. Donna Arndt-Jovin, Max Planck Institute for Biophysical Chemistry) primary antibody for overnight at 4 °C. The samples were washed three times with PBST and incubated with donkey anti-rabbit Alexa Fluor 568 secondary antibody (Cat#A10042, Thermo Scientific) and Hoechst 33258 (1:1000; Cat#H3569, Fisher Scientific) for 1 h at room temperature. After three washes with PBST, larval brains were dissected and mounted with Vectashield mounting medium (Vector Laboratories). The images were acquired with a laser-scanning confocal microscope (Carl Zeiss, LSM 800).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using ChIP Kit (Cat#ab500, Abcam) following the manufacturer's protocol with some modifications.⁵⁴ Briefly, 3xFlag or 3xFlag-Topoll β expressing HEK293T cells were treated with the fresh fixing buffer (1.1% paraformaldehyde in PBS) at room temperature for 10 min for the crosslinking of DNA and proteins. The reaction was stopped with the glycine buffer provided in the kit, and cells were pelleted by centrifugation at 500 \times g for 5 min at 4°C. Cells were then lysed and the chromatin was sheared by sonication to about 200–1,000 base pairs in length at 30% amplitude for 15 min with 10 sec pulse on and 50 sec pulse off time using Fisherbrand™ Model 120 Sonic Dismembrator (Cat#FB120110, Fisher Scientific). The DNA fragments were then immunoprecipitated with antibodies against Flag (Cat#M8823, Sigma-Aldrich) for 2 hr and were purified with DNA purifying slurry provided in the kit. The immunoprecipitated DNA fragments and input DNA were amplified by PCR using specific primers targeting the amplicons of *HSP90AA1* DNA sequence. PCR products were analyzed on a 2% agarose gel. The following primers were used for amplifying

the amplicon 1 (forward: 5'-gttcgggaggcttctggaaa-3' and reverse primer: 5'-gggacgctgaagcaactga-3'), amplicon 2 (forward: 5'-aatggcagaaactgcggtag-3' and reverse primer: 5'-ctttgcctgttccttctctg-3'), and amplicon 3 (forward: 5'-acattaaagatggggcctga-3' and reverse primer: 5'-ctttgtttggggacctca-3'). All the experiments were repeated at least three times.

Neuron survival assay and ASO treatment

Neuronal survival was assayed starting on Day 14, when the iMNs were maintained in N3 medium without neurotrophic factors or RepSox. Baseline images were taken using the Molecular Devices Image Express prior to the addition of antisense oligonucleotide (ASO)s. The following day iMNs were treated (a single time) with 9 μ M of negative control ASO, *HSP90AA1* ASOs, or *Topoll β* ASOs for 72 h. The ASOs were synthesized by Integrated DNA Technologies which contained phosphorothioate bonds and the modified base 2'-O-methoxy-ethyl for increased stability and binding affinity to the mRNA target of interest. For longitudinal tracking of Hb9::RFP iMNs, the plates were imaged every 2 days, and iMNs were manually counted with SVCell 3.0 (DRVision Technologies). Neurons were scored as dead when their somas were no longer visible.

ASOs used for iMN survival experiment

For *HSP90AA1* experiments, the negative control ASO was mG*mC*mG*mA*mC*T*A*T*A*C*G*C*G*C*A*mA*mU*mA*mU*mG, *HSP90AA1* ASO-1 was mU*mU*mU*mU*mC*T*C*A*G*C*T*C*A*mU*mC*mA*mU*mC and *HSP90AA1* ASO-2 was mC*mC*mU*mC*mA*G*C*C*A*G*A*G*A*T*T*mA*mG*mU* mC*mU. For *Topoll β* experiments, the *Topoll β* ASO-1 was eA*eG*eC*eA*eG*G*T*C*T*G*T*A*G*T*T*eT*eG*eT*eA*eA and ASO-2 was eG*eA*eA*eT*eG*T*C*T*T*G*C*A*C*A*G*eT*eT*eC*eA. * = phosphorothioate linkage, m = 2'-O-methyl ribose, e = 2'-O-methoxyethyl ribose.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Data were analyzed and graphs were made with Prism 9 (GraphPad). For comparisons of different treatment groups or genotypes, two-tailed unpaired *t* tests were used for two groups and a one-way ANOVA followed by a Tukey, Bonferroni or Dunnett post hoc analysis was used for more than two groups. Categorical data were analyzed by chi-square test. Statistical analysis of iMN survival experiments was conducted a two-sided log-rank test. Data are presented as mean \pm SEM. *p* < 0.05 was considered statistically significant. Statistical analysis of RNA-seq data and Gene Ontology is described in RNA Seq and Gene Ontology analysis above.