Activation of autophagy attenuates motor deficits and extends lifespan in a *C. elegans* model of ALS

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Abstract

Mutations in Cu/Zn–superoxide dismutase 1 (SOD1) are linked to amyotrophic lateral sclerosis (ALS). Using a line of ALS-related mutant human SOD1 (hSOD1) transgenic *Caenorhabditis elegans,* we determined the effects of metformin on the progression of ALS-like pathological abnormalities. We found that metformin significantly extended the lifespan, improved motor performance, and enhanced antioxidant activity of mutant worms. We further showed that metformin enhanced expression of *lgg-1, daf-16, skn-1* and other genes known to regulate autophagy, longevity and oxidative stress in hSOD1 transgenic worms. Accordingly, overexpression of *lgg-1* or *daf-16* attenuated the aging and pathological abnormalities of mutant human SOD1 worms, while genetic deletion of *lgg-1* or *daf-16* abolished the beneficial effects of metformin. Collectively, we demonstrate that metformin protects against mutant SOD1-induced cytotoxicity in part through enhancement of autophagy and extends lifespan through daf-16 pathway. Our findings suggest that metformin could be further explored as a potential therapeutic agent in treating ALS.

Keywords

Metformin; Lifespan; Neuroprotection; ALS; Autophagy

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common degenerative motor neuron disease [1], Most ALS cases occur sporadically, while approximately 10% are familial [2]. One of the most prevalent familial forms of the disease involves mutations of Cu/Zn–superoxide dismutase 1 (*SOD1*) [3]. Since the discovery of *SOD1* G93A missense mutation in 1993 [4], more than 185 different ALS-related *SOD1* mutations have been identified [3], The disease causal effect of SOD1 mutations is attributed to the degeneration of motor neurons [5], Despite decades of research, the pathogenesis of ALS and the mechanism by which SOD1 mediates its toxic effects in motor neurons are not fully understood. Studies on neuronal dysfunction have primarily relied on models overexpressing human SOD1 protein and have supported a role for SOD1 dysfunction in ALS pathogenesis [6], However, these findings have limited translational value in terms of extending the lifespan of ALS patients and the development of effective therapeutics. Although the degeneration of motor neurons in ALS is mainly attributable to the missense mutation in *SOD1* and aggregation of SOD1 protein, the specific molecular events and pathways involved have yet to be fully elucidated. As such, there is a need for a more suitable model to investigate the etiopathogenesis of ALS and evaluate the efficacy of potential drugs for its treatment. Although the clinical course is variable, ALS patients generally have short lifespan. The molecular basis for short lifespan and whether it is mediated by pathways known to regulate longevity are open questions. It was reported that a prolonged lifespan depends on the FOXO transcription factor DAF-16 [7] and may be related to autophagy and various aspects of cellular and organismal aging. Furthermore, autophagy has been directly linked to neurodegeneration and ageing by an extensive body of research [8], However, our understanding of the mechanistic link between autophagy and lifespan is limited, particularly with regard to neurodegenerative diseases.

Metformin (metformin hydrochloride) is the most widely prescribed biguanide drug worldwide for the treatment of type II diabetes and metabolic syndromes [9]. Previous research has shown that metformin is associated with a reduced risk of cancer and cardiovascular disease, raising the possibility that it could have beneficial effects in other age-related diseases [10]. Metformin is thought to act by activating the 5′ adenosine monophosphate-activated protein kinase (AMPK) pathway or by inhibiting the mammalian target of rapamycin (mTOR) pathway [11], while some studies have indicated that it also activates the lysosomal degradation pathway [12]. Metformin was reported to slow aging in *C. elegans* by altering microbial folate and methionine metabolism [13] and prolong lifespan through mitohormesis by modulating peroxiredoxin 2 (PRDX-2) [9]. In addition, it was reported that metformin-bacterial interactions engaged lipid metabolism in *C. elegans* to extend lifespan [14], Moreover, a recent study has shown that metformin could inhibit RAN translation through PKR pathway [15]. However, whether and how metformin exerts these effects in the context of neurodegenerative diseases and the associated mechanisms are not known.

Transgenic *Caenorhabditis elegans* (*C. elegans*) models have been established for a number of neurodegenerative diseases, such as Alzheimer's disease (AD) [16], Parkinson's disease (PD) [17], and ALS [18]. It is also a useful tool for evaluating the toxicity of mutant SOD1 in motor neurons and for exploring the mechanisms by which SOD1 mutation

affects longevity and other physiologic functions in ALS. SOD1 overexpression models support a role for SOD1-mediated toxicity in the pathogenesis of ALS [19]. In our previous work, we found that hSOD1 aggregates and forms inclusions in the motor neurons of *C. elegans* [20]. The hSOD1-induced death of motor neurons is independent of the Cell death protein 4 (CED-4)/CED-3 apoptosis pathway [20]. Recent studies in ALS models have demonstrated that mutant SOD1 not only induces toxicity in motor neurons but also reduces longevity, possibly due to mutant SOD1 aggregation [21,22]. Verapamil was shown to inhibit the aggregation of mutant SOD1, promote motor neuron survival, and extend lifespan in SOD1^{G93A} mice by inducing the activation of autophagy [23]; meanwhile, shatavarin IV prevented reactive oxygen species-induced oxidative damage in *C. elegans* and prolonged lifespan by downregulating the expression of the Leucine-rich repeats, Ras-like domain, kinase 1 *(lrk-1)* gene [24], These findings provide evidences that mutant SOD1 causes motor neuron damage and short lifespan through complex mechanisms. However, the specific mechanisms underlying these effects remain unknown.

In addition, the insulin-IGF1 signaling pathway is known to be involved in the determination of lifespan. The daf-16 signaling pathway, a homologous to the insulin/IGF-1 signaling network in humans, is critical in endocrine processes that regulate longevity, metabolism, stress resistance, and development [7]. In this study, we generated and characterized a strain of transgenic *C. elegans* that specifically overexpresses ALS-related SOD1 G93A mutation in motor neurons to systematically investigate the molecular pathogenesis of ALS and evaluate the therapeutic potential of metformin for ALS treatment. We found that metformin had beneficial effects on the lifespan, motor behavior, and oxidative stress response of mutant worms by stimulating autophagy and daf-16 pathways.

2. Materials and methods

2.1. Worm strains and culture conditions

Worms were grown on nematode growth medium (NGM) plates seeded with *E. coli* strain OP50. The N2 (Bristol) strain was used as nontransgenic control worms. *Daf-16* (m26) and DA2123(adIs2122, lgg-1p::GFP::lgg-1+rol-6) strains were provided by the Caenorhabditis Genetics Center (CGC), which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). The unc-25 promoter was amplified using KOD-Plus Neo DNA polymerase from N2 worm genomic DNA. The unc-25 promoter was inserted into the Hind III and Bam HI sites of pPD95.77 to generate pPD95.77-unc-25::GFP. The plasmids of pPD95.77-unc-25::hSOD1^{G93A}::GFP were made using the same method. The following crossing strains were used in this study: hSOD1^{G93A}::*lgg-1*::GFP; hSOD1^{G93A}::*daf-16*::GFP; and *lgg-1*::*daf-16*::GFP. All experiments were performed at 20°C according to standard *C. elegans* culture techniques [25].

2.2. Determination of optimal metformin concentration

Metformin (Solarbio, Beijing, China) was dissolved in sterile water to prepare a 1 M stock solution, which was added to OP50 culture solution (OD 0.3–0.5) to prepare working solutions at concentrations of 25, 50, and 100 mM. OP50 without metformin was used as a

control. The working solutions were coated onto the surface of NGM plates. Synchronized L1 larval stage worms were transferred to fresh NGM plates with or without the drug and grown at 20°C for 3 days until they reached the adult stage. The worms were washed and transferred to fresh regular NGM plates and grown at 20°C.

2.3. RNA interference

RNAi experiments were performed as previously described [26]. Briefly, freshly streaked single colonies of HT115 bacteria containing either the empty L4440 vector or hSOD1 RNAi plasmid were grown overnight at 37°C in Luria-Bertani medium supplemented with tetracycline (50 μg/ml). The next day, freshly grown bacteria were seeded onto NGM plates supplemented with tetracycline (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM). Synchronized L1 larval stage worms were washed from regular NGM plates, transferred to RNAi plates, and grown at 20°C for 3 days until the adult stage. The worms were then washed and transferred to fresh regular NGM plates and grown at 20°C.

2.4. Lifespan assay

Lifespan experiments were performed as previously described [20], with minor modifications. Briefly, synchronized L4 stage worms (n ≈ 100) were placed on NGM plates, and the following day was defined as day 1. The viability of each worm was scored every day thereafter; those that did not respond to external mechanical stimuli or swallow were judged to be dead. Events in which worms crawled off the plate were not recorded. All worms were transferred to new NGM plates every other day until death. The survival curves were analyzed by GraphPad Prism software (GraphPad, La Jolla, CA, USA).

2.5. Behavioral assay

To evaluate locomotion of worms, we monitored their crawling behavior using previously described methods [27]. Worms from different experimental conditions were individually carried out immediately after each worm had been transferred to a fresh NGM plate, and their crawling tracks were monitored for 30 s. A video was collected, and the ratio of the movement distance to the body length was measured by Image J software. A total of 30 worms per experimental group were monitored.

2.6. Stress response assay

To assess resistance to oxidative stress, we transferred adult worms with or without drug treatment or RNAi to fresh NGM plates containing paraquat (3 mM, Solarbio, Beijing, China) at 20°C. The number of dead worms was recorded every hour. Worms without treatment served as the control. The survival curves were analyzed by GraphPad Prism software.

2.7. Quantitative real-time polymerase chain reaction (RT-PCR)

Gene expression levels in worms were quantified by RT-PCR [28]. Total mRNA was extracted from each worm groups using TRIzol reagent (Takara Biomedical Technology, Beijing, China) and reverse-transcribed using the TransScript cDNA Synthesis SuperMix kit (TransGen Biotech Co, Beijing, China). RT-PCR was performed on a 7500 Dx PCR

system (Life Technologies, Carlsbad, CA, USA) with a mixture of TransStart Top Green qPCR SuperMix kit (TRAN), 10 ng cDNA, and 0.4 μM each primer in a final volume of 20 μl. The relative expression level of target genes was normalized to that of actin (*act-1*) and quantified using Applied Biosystems 7500 software v2.0.5 (Life Technologies). Three repeats were performed per condition. The primers used for RT-PCR are shown in Table 1.

2.8 Fluorescence microscopy

Worms were washed off NGM plates and immobilized in 5 mM sodium azide (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in M9 buffer on glass slides. Images were collected using an A1R multiphoton confocal microscope (Nikon Instruments, Shanghai, China). At least 20 worms per strain were examined.

2.9. Western blotting

To analyze protein expression, adult worms were washed 4 times with M9 buffer and packed worms were lysed by sonication on ice in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) containing a 1% protease inhibitor cocktail. The lysates were centrifuged at 13,000×g for 15 min at 4°C using a Sorvall Legend Micro 17R centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was collected and protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Beyotime). The remaining supernatant was combined with $5 \times$ sodium dodecyl sulfate (SDS; Beyotime) sample buffer and boiled for 5 min at 95°C. Equal amounts of total protein (40–60 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a 0.45-μm pore size polyvinylidene difluoride membrane (Millipore, Kankakee, MA, USA). After blocking with 5% skimmed milk for 1 h at room temperature, the membrane was incubated overnight (16–18 h) at 4°C with primary antibody, then washed with Tris-buffered saline containing Tween-20 and incubated with secondary antibody. Protein bands were visualized using an enhanced chemiluminescence detection kit (Advansta, San Jose, CA, USA) and signal intensity was normalized to that of actin (clone C4; Millipore, Darmstadt, Germany) using the FluorChem Q system (Protein Simple, San Jose, CA, USA). The experiment was repeated 3 times. The following antibodies were used: anti-SOD1 (Abeam, Cambridge, MA, USA), anti-actin (Millipore, Temecula, CA, USA), goat anti-mouse IgG H&L (Proteintech, Rosemont, II, USA), and goat anti-rabbit IgG H&L (Proteintech).

2.10. Statistical analysis

Data were analyzed using Prism software. Differences between groups were evaluated with the Student's *t*-test (2-tailed; 2-sample equal variance): ****$P < 0.001$; ***$P < 0.005$; **$P < 0.001$; *$P < 0.01$. Lifespan was compared between groups by Kaplan-Meier survival analysis and with the log-rank test. Other data were evaluated by one-way analysis of variance followed by the Tukey post hoc test. $P<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Overexpression of mutant hSOD1 induces motor neuron degeneration and shortens the lifespan of transgenic worms

To examine the effect of ALS-associated human mutant SOD1 in motor neurons, we generated transgenic *C. elegans* overexpressing ALS-linked G93A mutant hSOD1 (hSOD1^{G93A}) in motor neurons. In these worms, hSOD1 protein was tagged with green fluorescent protein (GFP). We examined the expression of hSOD1 mRNA and protein in transgenic worms and found expression of hSOD1^{G93A} mRNA and almost 8-fold increase of protein levels against native worm SOD1 protein, respectively (Fig. S1A–B). The hSOD1^{G93A} mutant worms displayed uncoordinated movements starting at age of 3 days and showed markedly reduced locomotor activity at age of 7 days (Fig. S1C). In correlation with the motor deficits, we observed substantial loss of motor neuron axons in hSOD1^{G93A} transgenic worms at age of 7 days (Fig. S1D). We then examined the morphology of remaining motor neurons and found motor neuron in the hSOD1^{G93A} transgenic worms were completely broken down (Fig. S1E). Therefore, we generated a line of motor neuron-specific hSOD1^{G93A} transgenic worms that recapitulate ALS-like motor neurodegeneration.

We next assessed the lifespan of hSOD1^{G93A} transgenic *C. elegans* by recording the survival rate every day from L4 stage until death. The survival analysis showed that hSOD1^{G93A} transgenic worms had a significantly shorter lifespan than controls, with median survival reduced from 17 to 10 days (Fig. S1F). Thus, in addition to inducing motor neuron degeneration, the hSOD1^{G93A} mutation decreases the longevity of worms.

As *C. elegans* are well suited to the study of stress response [29], we also investigated the effects of hSOD1^{G93A} mutation on the oxidative stress responses in transgenic worms. As expected, hSOD1^{G93A} worms exposed to paraquat had a shortened lifespan compared to their control counterparts under the same stress (Fig. S1G). Together, these findings indicate that hSOD1^{G93A} mutation decreases longevity and enhances the stress response.

3.2. Metformin extends lifespan and alleviates motor dysfunction in hSOD1 mutant worms

To assess the therapeutic potential of metformin for the treatment of ALS, we mixed metformin with OP50 and fed the mixture to hSOD1^{G93A} transgenic worms. The optimal concentration of metformin was determined to be 50 mM (Fig. S2). The hSOD1 RNA interference (RNAi) served as a positive control. Compared to the hSOD1^{G93A} group, hSOD1 mRNA and protein levels were significantly reduced in worms fed metformin (Fig. 1A–B). Metformin also attenuated the hSOD1^{G93A}-induced degeneration of motor neuron axons, indicating that it has a neuroprotective effect (Fig. 1C). Moreover, compared to untreated hSOD1^{G93A} worms, those fed metformin showed partly restored motor neuron morphology (Fig. 1D). Similarly, hSOD1 knockout by RNAi partly rescued the morphologic defects in motor neurons caused by hSOD1^{G93A} mutation. We also compared lifespan in different groups to determine whether metformin has beneficial effects on the longevity of ALS model worms. Metformin treatment significant prolonged the lifespan of hSOD1^{G93A} worms, with median survival increased from 10 to 13 days (Fig. 1E). Metformin also

improved the locomotion of hSOD1^{G93A} worms (Fig. 1F), and extended the lifespan of hSOD1^{G93A} worms under the oxidative stress (Fig. 1G). These results demonstrate that metformin can improve the lifespan, motor activity, motor neuron survival, and stress response of hSOD1^{G93A} worms.

3.3. Metformin increases the expression of daf-16 and lgg-1 genes in hSOD1 mutant worms

It is known that *daf-2* modulates the aging process via insulin/insulin-like growth factor (IGF)-1 signaling [7]. *Daf-16* and *age-1* are downstream proteins of *daf-2* involved in regulating the lifespan of C. *elegans* [30]. *Lgg-1* and *beclin-1* homolog *(.bec-1)* genes play critical roles in autophagy that regulates the lifespan of C. *elegans* [31,32], Furthermore, *skn-1* regulates lifespan in C. *elegans* by conferring protection against stress [33], In the present work, we investigated the effects of hSOD1^{G93A} mutation and metformin on the expression of genes associated with autophagy, aging, and the stress response, with hSOD1 RNAi serving as a positive control. Compared to the control worms, the relative expressions of *lgg-1, bec-1, daf-16* and *skn-1* were slightly decreased while those of *daf-2* and *age-1* were significantly increased in hSOD1^{G93A} worms, indicating that autophagy and oxidative stress genes were partly decreased and longevity genes were significantly reduced (Fig. 2A). In hSOD1^{G93A} worms fed metformin, the levels of *lgg-1, bec-1* and *skn-1* were significantly increased, indicating that autophagy was activated and antioxidative response was intensified. We also tested the relative mRNA expressions of *skn-1* and *bec-1* in DA2123 worms, and found that *lgg-1* increased the mRNA expression of *skn-1* and *bec-1* in worms, verifying the conclusion that activation of autophagy can improve the antioxidant capacity by increasing *skn-1* in worms (Fig. 2B). In addition, the level of *daf-16* was notably increased while those of *daf-2* and *age-1* showed a marked decrease, indicating that daf-16 pathway was activated and longevity genes were significantly increased. Thus, metformin can enhance the expression of genes associated with longevity and the stress response, possibly through activation of genes in the autophagy and daf-16 pathway such as *lgg-1* and *daf-16.*

3.4. Overexpression of daf-16 and lgg-1 extends lifespan and oxidative stress in hSOD1^{G93A} worms

To investigate the roles of *lgg-1* and *daf-16* in ALS, we used hSOD1^{G93A}::*daf-*16 and hSOD1 ^{G93A}::*lgg-1* worms, and also generated the *lgg-1::daf-16* double transgenic strain to examine the interaction between the two genes. The survival curves showed that the lifespan of hSOD1^{G93A}::*daf-16* worms was prolonged compared to hSOD1^{G93A} worms, with an increase in median survival from 10 to 13 days (Fig. 3A). This suggests that activation of the daf-16 signaling pathway is involved in the extension of lifespan in ALS. We next examined whether changes in autophagy contributed to the reduction in longevity caused by hSOD1 mutation. The lifespan of hSOD1^{G93A}::*lgg-1* worms was extended relative to that of hSOD1^{G93A} worms, with median survival increased from 10 to 13 days (Fig. 3B), implying that activation of *lgg-1* promotes longevity in hSOD1 mutant worms by inducing the degradation of hSOD1 and thereby alleviating hSOD1 aggregation-induced neurotoxicity. In addition, while *lgg-1* and *daf-16* both prolonged lifespan, there was no additive effect when both genes were simultaneously overexpressed (Fig. 3C). We speculate that the two

genes are involved in distinct signaling pathways and thus interact separately with hSOD1 to increase longevity.

We also examined the number and morphology of motor neurons, the hSOD1 protein level, and locomotor behavior and stress response in hSOD1^{G93A}::*daf-16* and hSOD1^{G93A}::*lgg-1* worms. Overexpression of *daf-16* in hSOD1^{G93A} worms did not abrogate the toxic effects of hSOD1 mutation on motor neurons, while the number of neurons was slightly increased in hSOD1^{G93A}::*lgg-1* worms (Fig. 3D). Furthermore, compared to hSOD1^{G93A} worms, motor neurons in hSOD1^{G93A}::*lgg-1* worms were morphologically normal (Fig. 3E). The results of the western blot analysis indicated that overexpression of *daf-16* in hSOD1 mutants did not induce the degradation of hSOD1 protein, whereas *lgg-1* overexpression decreased hSOD1 protein level (Fig. 3F). Moreover, hSOD1^{G93A}::*lgg-1* but not hSOD1^{G93A}::*daf-16* worms showed clear improvements in locomotion (Fig. 3G). Overexpression of both *daf-16* and *lgg-1* significantly enhanced the oxidative stress response in ALS model worms (Fig. 3H and I). Thus, *lgg-1* overexpression induces the degeneration of hSOD1 protein and alleviates the toxic effects of hSOD1 mutation; however, *daf-16* overexpression did not reduce the level of hSOD1 protein, implying that it prolongs lifespan in ALS through a different mechanism.

3.5. Genetic knockout of daf-16 and lgg-1 abrogates the beneficial effects of metformin

To investigate whether the beneficial effect of metformin on the longevity of ALS model worms involves *daf-16* and *lgg-1,* we generated the genetic knockout lines hSOD1^{G93A}::*daf-16*(RNAi) and hSOD1^{G93A}::*lgg-1*(RNAi). We first examined the survival curves of worms treated with metformin and found that lifespan was not prolonged (Fig. 4A–B), suggesting that the effect of metformin on the lifespan of ALS model worms is mediated by *daf-16* and *lgg-1*. We also examined the number and morphology of motor neurons in the two strains after treatment with metformin. The number of motor neurons in hSOD1^{G93A}::*lgg-1*(RNAi) worms fed metformin was slightly increased but neuronal morphology remained abnormal (Fig. 4C–D). In *lgg-1*-deficient worms, neither the number nor the morphology of motor neurons was restored by metformin. Moreover, metformin did not improve stress tolerance in hSOD1^{G93A}::*daf-16*(RNAi) and hSOD1^{G93A}::*lgg-16*(RNAi) worms (Fig. 4E–F) and had no effect on their locomotion (Fig. 4G). Finally, we examined the expression of hSOD1 protein in the two strains by treatment with metformin and found that compared to hSOD1^{G93A} worms, the decrease in hSOD1 protein levels observed in the absence of *daf-16* and *lgg-1* genes was abrogated (Fig. 4H). Taken together, the above results demonstrate that metformin extends lifespan and alleviates motor deficits in a *C. elegans* model of ALS by activating autophagy and the daf-16 pathway.

4. Conclusions

In summary, we developed a ALS model by generating a transgenic *C. elegans* strain that specifically overexpress human SOD1 G93A mutation in motor neurons to study the molecular pathogenesis and evaluate the therapeutic effects of metformin on ALS. hSOD1 mutant worms had a shorter lifespan, and displayed locomotor defects and paralysis in the adult stage, which was accompanied by a reduction in antioxidant activity. We found

that treatment with metformin restored lifespan and locomotion and improved the stress response capacity of hSOD1^{G93A} worms, providing evidence for the therapeutic potential of metformin in the treatment of ALS. We also found that treatment with metformin induced the upregulation of genes associated with autophagy. This suggests that metformin can activate the autophagy pathway by which the levels of hSOD1 mRNA is decreased, and promote the degradation of hSOD1 aggregates in motor neurons, thus abrogate their toxicity in ALS. We demonstrated that metformin or hSOD1 RNAi can upregulate *daf-16* and downregulate *daf-2* in hSOD1^{G93A} worms; metformin can also reverse the negative effect of hSOD1 mutation on lifespan and protect against oxidative stress. These findings imply that the ability of metformin to reverse the decline in lifespan caused by hSOD1 mutation in the *C. elegans* ALS model involves the activation of the daf-16 pathway. Collectively, our data provide strong evidence for the regulatory effects of autophagy and the daf-16 signaling pathway in ALS (Fig. 5) and suggest that metformin may be an effective drug for delaying age-related ALS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations:

SOD1	superoxide dismutase 1
C. elegans	Caenorhabditis elegans
ALS	amyotrophic lateral sclerosis
AMPK	adenosine monophosphate-activated protein kinase
mTOR	mammalian target of rapamycin
PRDX-2	peroxiredoxin 2
AD	Alzheimer's disease
PD	Parkinson's disease
lrk-1	Leucine-rich repeats, Ras-like domain, kinase 1
NGM	nematode growth medium
IPTG	isopropyl-β-D-thiogalactopyranoside
IGF	insulin/insulin-like growth factor

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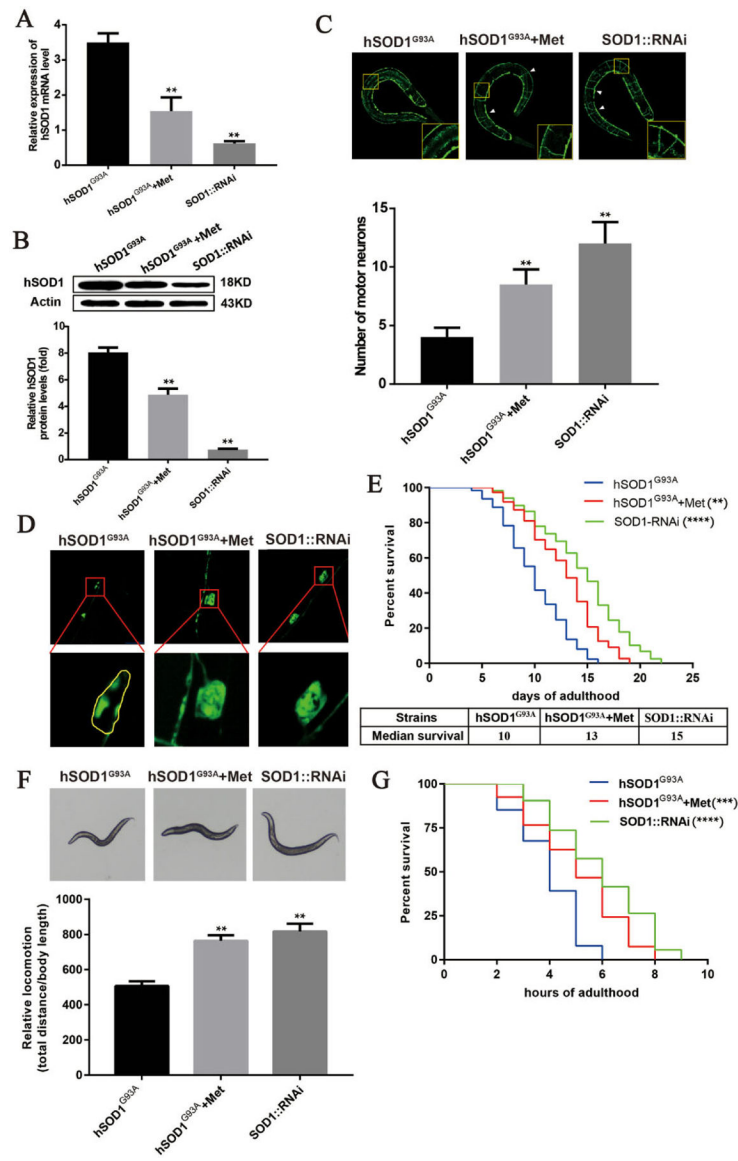
Fig. 1.
Metformin extends lifespan and alleviates locomotor deficits and motor neuron degeneration in hSOD1^{G93A} mutant *C. elegans*. (**A**) Relative expression of hSOD1 mRNA in hSOD1^{G93A} transgenic worms treated with metformin or hSOD1 RNAi. (**B**) Western blot analysis of hSOD1 protein level in extracts from transgenic strains. hSOD1 expression was lower in hSOD1^{G93A} worms fed metformin than in untreated mutant worms. (**C, D**) Motor neuron survival and morphology in transgenic worms. Metformin prevented motor neuron degeneration (**C**) and restored neuronal morphology (**D**) in hSOD1^{G93A} worms. (**E**) Suivival analysis. Median suivival of hSOD1^{G93A} worms was increased by metformin treatment (13 days vs 10 days without treatment). (**F**) Locomotion was partly restored in hSOD1^{G93A} worms treated with metformin. (**G**) Oxidative stress response in transgenic worms treated with metformin. Metformin prolonged the lifespan of hSOD1^{G93A} transgenic

worms exposed to paraquat. **P<0.001, ***P<0.0005, ****p<0.0001 (N ~ 100 worms per condition from 3 independent experiments).
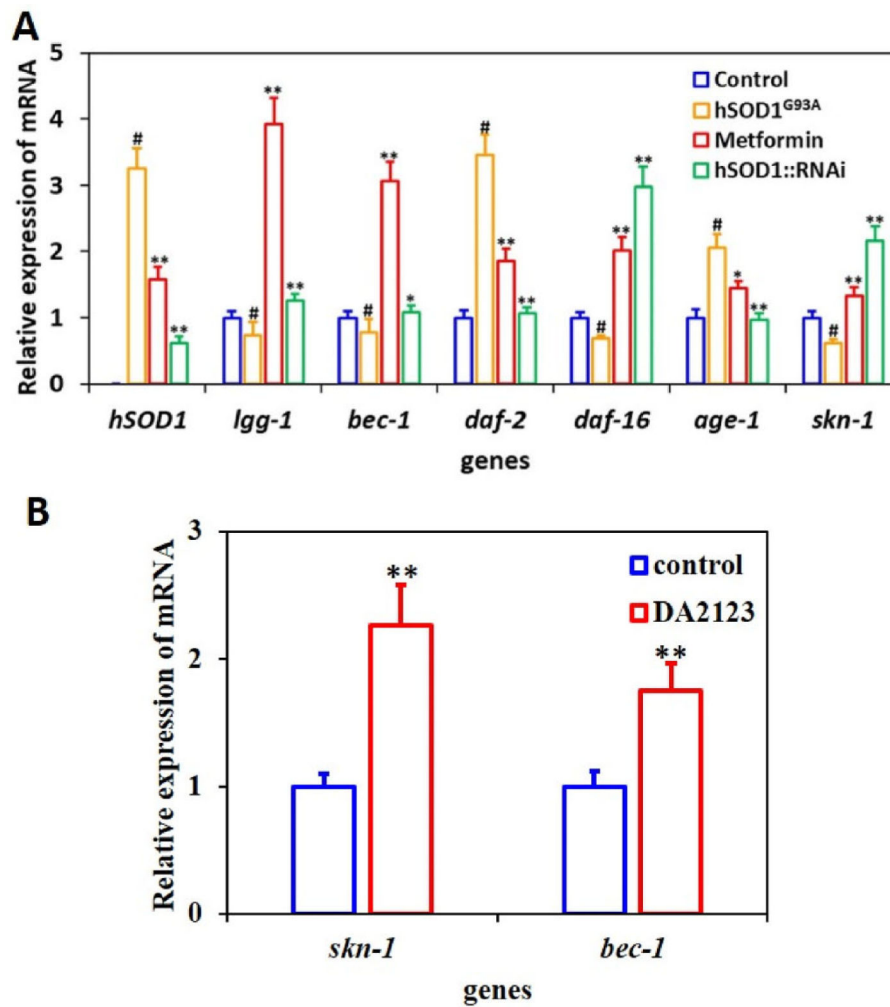
Fig. 2.
Metformin alters the expression of genes related to autophagy, aging, and stress response in a *C. elegans* model of ALS. (A) Quantification of gene expression in hS0Dl^{G93A} transgenic worms fed metformin or treated with RNAi, hSOD1^{G93A} worms without treatment served as the control. Metformin reduced *hSOD1* expression in hSOD1^{G93A} worms and increased Lisal of genes related to autophagy, lifespan, and stress response. (**B**) Quantification of gene expression in DA2123 worms. $^{\#}$P<0.01 (*vs.* control), **P<0.001,*P<0.01 (*vs.* hSOD1^{G93A}). N ~ 1000 worms per condition front 3 independent experiments.
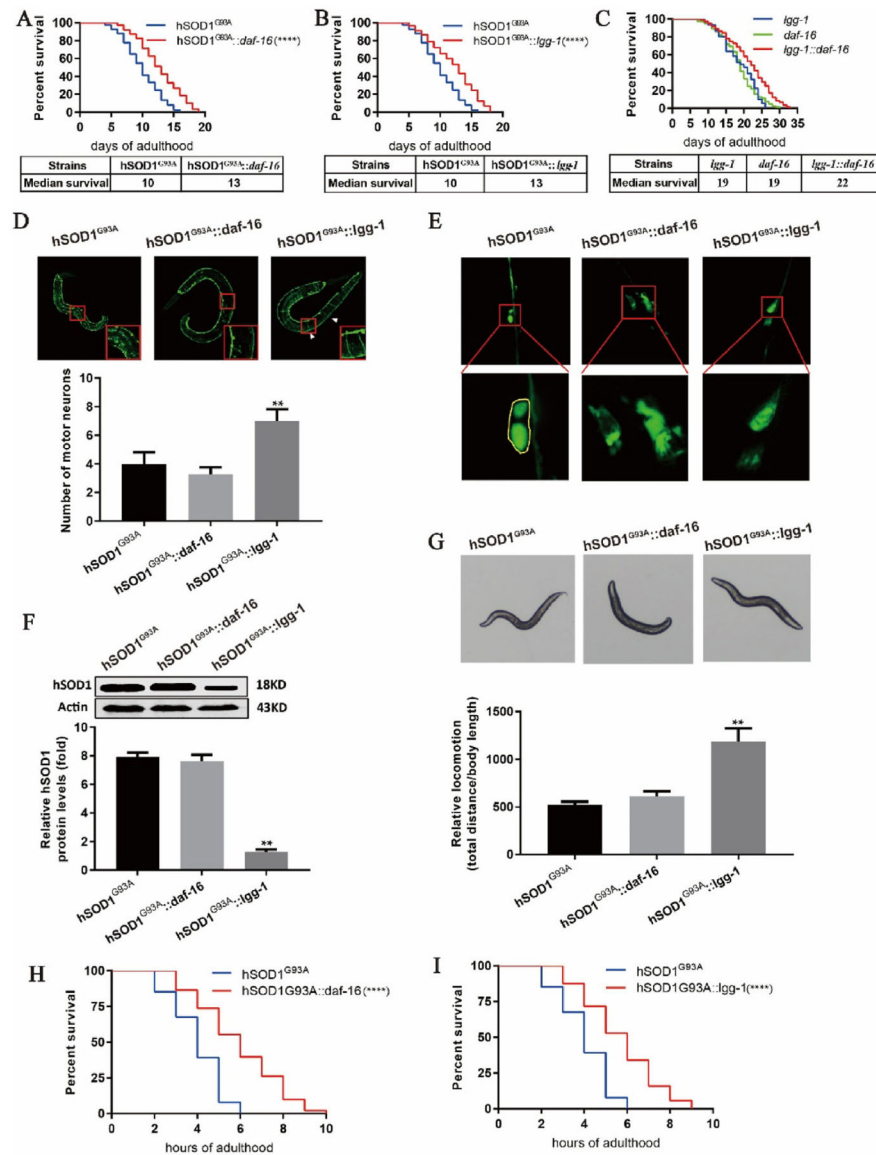
Fig. 3.
Overexpression of *daf-16* and *lgg-1* extends lifespan, alleviates locomotor deficits, and improves stress response in hSOD1^{G93A} worms. (**A-C**) Survival curves for hSOD1^{G93A}::*daf-16*, hSOD1^{G93A}::*lgg-1*, and *lgg-1::daf-16* worms. (**D, E**) Motor neuron survival and morphology in transgenic worms. Overexpression of *lgg-1* but not daf-16 protected against hSOD1^{G93A}-induced neurotoxicity (**D**) and restored motor neuron morphology (**E**) in hSOD1^{G93A} worms. (**F**) Western blot analysis of protein extracts from hSOD1^{G93A} transgenic strains. *Lgg-1* overexpression reduced the level of hSOD1 protein in hSOD1^{G93A} worms, whereas *daf-16* overexpression had no effect. (**G**) Locomotor behavior in hSOD1 transgenic worms overexpressing *daf-16* or *lgg-1.* Locomotion was restored in hSOD1^{G93A}*::lgg-1* worms, and to a lesser extent in hSOD1^{G93A}::*daf-16* worms. (**H, I**) Oxidative stress response in hSOD1^{G93A} transgenic worms, hSOD1^{G93A} worms over expressing *lgg-1* and exposed to paraquat showed increased longevity compared to

hSOD1^{G93A} worms. Overexpression of *daf-16* had no obvious effect on hSOD1 protein degradation, but partly restored the oxidative stress response that was diminished by hSOD1^{G93A} mutation. ****P<0.0001, **P <0.001 (N ~ 100 worms per condition from 3 independent experiments).
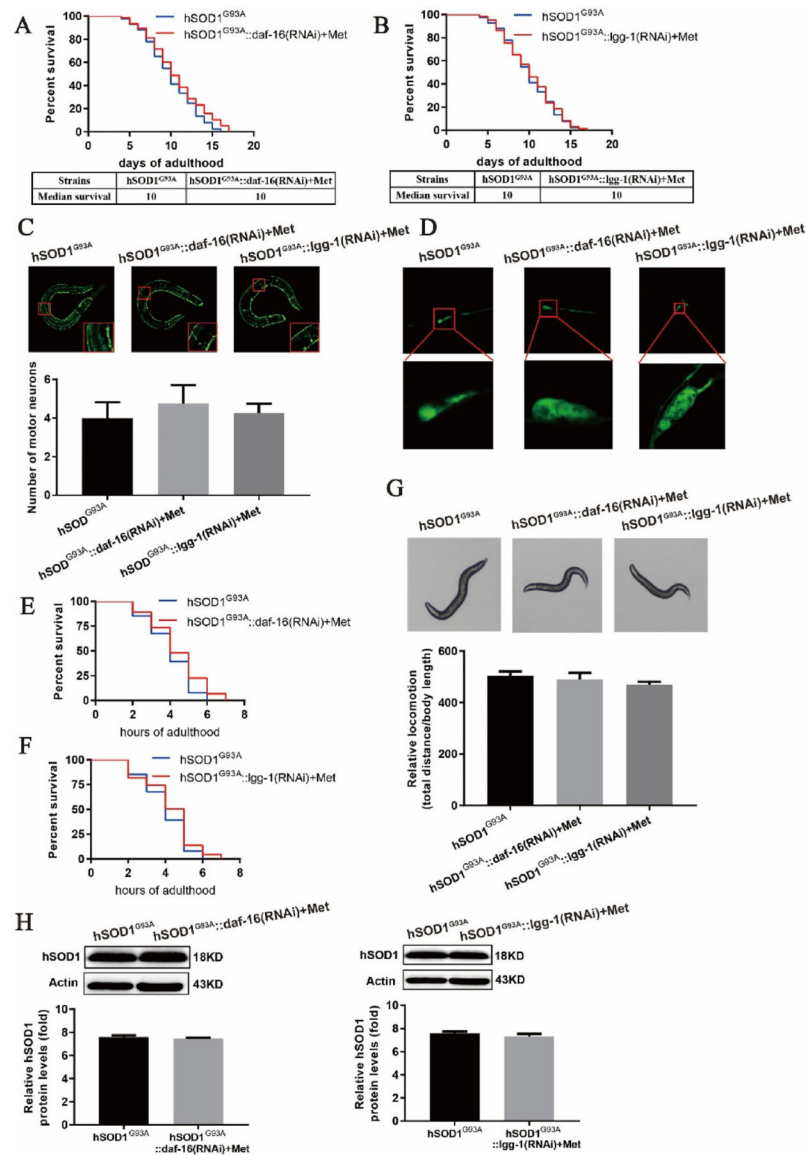
Fig. 4.

Metformin has no significant effect in *daf-16-* and *lgg-1*-deficient hSOD1 mutant *C. elegcuis.* (**A, B**) Survival curves for hSOD1[G93A] worms lacking *daf-16* or *lgg-1.* Metformin did not prolong the lifespan of hSOD1[G93A] worms lacking the *daf-16* or *lgg-1* gene. (**C, D**) Motor neuron survival and morphology in *daf-16* and *lgg-1* knockout worms treated with metformin. Compared to hSOD1[G93A] worms, metformin did not alleviate the neurotoxicity (**C**) or defects in neuronal morphology (**D**) caused by hSOD1 mutation in hSOD1[G93A] worms lacking *daf-16* and *lgg-1.* (**E, F**) Oxidative stress response in hSOD1[G93A] mutant *daf-16* and *lgg-1* knockout worms treated with metformin. Metformin did not prolong the lifespan of hSOD1[G93A] worms lacking the *daf-16* or *lgg-1* gene and exposed to paraquat. (**G**) Locomotor behavior in hSOD1[G93A] worms lacking *daf-16* or *lgg-1.* Metformin had no significant effect on the locomotion of hSOD1[G93A] mutant *daf-16* or *lgg-1* knockout worms. (**H**) Western blot analysis of protein extracts from transgenic strains. hSOD1[G93A] protein

level in hSOD1 mutant worms was not reduced by metformin treatment in the absence of the *daf-16* or *lgg-1* gene. N ~ 100 worms per condition from 3 independent experiments.

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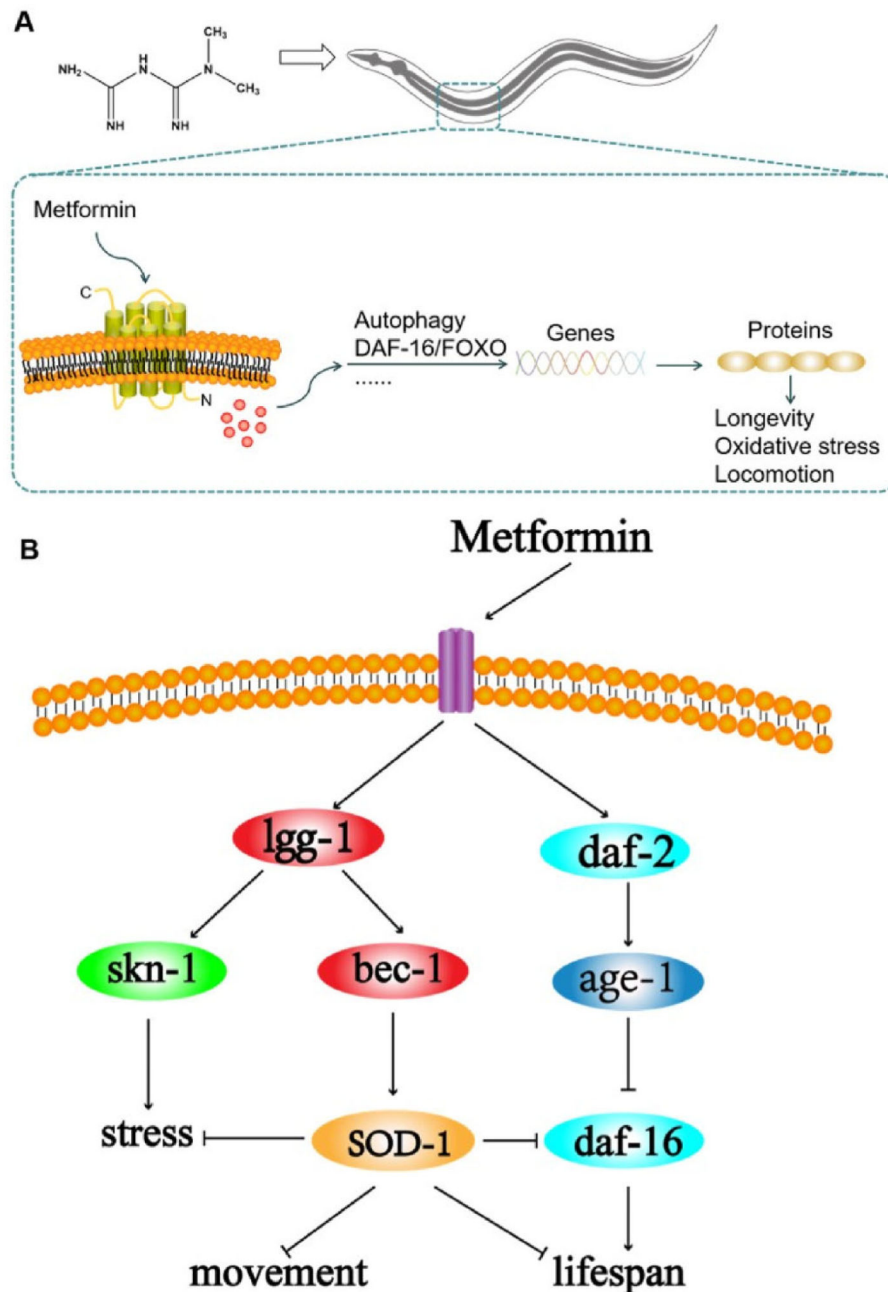Author Manuscript

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Fig. 5.
Schematic for the metformin extends lifespan and ameliorates motor deficits in an ALS *C. elegans* model. (**A**) The addition of metformin activates the autophagy and daf-16 pathways in *C. elegans* and upregulates autophagy and longevity genes *in vivo,* such as *lgg-1* and *daf-16.* Increased autophagic activity promotes the degradation of toxic substances, enhances stress response and promotes longevity. (**B**) Schematic of interactions between the *sod-1, lgg-1* and *daf-16* genes in ALS *C. elegans.*

Table 1

Primer sequences of RT-PCR.

Primers	Forward (5'-3')	Reverse (5'-3')
act-1	GCTGGACGTGATCTTACTGATTACC	GTAGCAGAGGTTGTCGTTGATGTC
hsod-1	GGTGGGCCAAAGGATGAAGAG	GGACAAGCCAAACGACTTGC
lgg-1	CCGCAGAAAGTACCCAGACC	CTGGACGAAGTTGGATGCGT
bec-1	CCGATGAGGAGAAGGAGC	AACCCAAATGTGGAAGCA
daf-16	GCGAATCGGTTCCAGCAATTCCAA	ATGCACGGACAGTGTTCAAGTGGT
daf-2	GGCCGATGGACGTTATTTTG	TTCCACAGTGAAGAAGCCTGG
age-1	CGGAAAGAGCAAACTTGGGATC	GGTAGGCTTCGACGCATAAGG
skn-1	AGTGTCGGCGTTCCAGATTTC	GTCGACGAATCTTGCGAATCA