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Metformin suppresses progression of muscle aging *via* activation of the AMP kinase-mediated pathways in *Drosophila* adults

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Abstract. – OBJECTIVE: Metformin, a medicine used for the treatment of type 2 diabetes, was previously reported to suppress age-dependent hyperproliferation of intestinal stem cells in *Drosophila*. Here, we aimed to investigate its anti-aging effects on other tissues, such as adult muscle and elucidate the mechanisms underlying the anti-ageing effect.

MATERIALS AND METHODS: To evaluate the anti-muscle ageing effect of Metformin, we visualized ubiquitinated protein aggregates accumulated in adult muscle as the flies age by immunostaining and measured the total pixel size of the aggregates. We altered gene expression in the muscle by induction of dsRNA against the relevant mRNAs or mRNAs encoding the constitutively active mutant proteins using the Gal4/UAS system. We determined the mRNA levels by quantitative Real Time-Polymerase Chain Reaction (QRT-PCR).

RESULTS: Continuous metformin feeding significantly extended the lifespan of *Drosophila* adults. Furthermore, the feeding suppressed the aging-dependent accumulation of ubiquitinated aggregates in adult muscle. To delineate the mechanism through which metformin influences the muscle aging phenotype, we induced the constitutively active AMPK specifically in the muscles and found that the activation of the AMPK-mediated pathway was sufficient for the anti-aging effect of Metformin. Furthermore, the AMPK-mediated downregulation of Tor-mediated pathways, subsequent induction of an eIF-4E inhibitor were involved in the effect. These genetic data suggested that the metformin effect is related to the partial suppression of protein synthesis in ribosomes. Furthermore, metformin stimulated autophagy induction in adult muscles.

CONCLUSIONS: Our results suggest that metformin can be regarded as an anti-aging compound in *Drosophila* muscle. The stimulation of autophagy was also involved in the anti-ageing effect, which delayed the progression of muscle aging in *Drosophila* adults.

Key Words:
Metformin, *Drosophila*, Anti-aging, Muscle, AMPK, Tor.

Introduction

Metformin is a medicine used for the treatment of type 2 diabetes. It is a biguanide with an anti-hyperglycemic effect, which functions by decreasing glucose production in the liver and increasing the insulin sensitivity of tissues[1]. Primarily, metformin inhibits enzymes of the mitochondrial respiratory complex I[2], increases the AMP/ATP ratio in cells, and subsequently activates the AMP-activated protein kinase (AMPK)[3]. The activated AMPK phosphorylates glucose transporter type 4 (GLUT4), which then transports glucose from the blood to cells, thereby playing a crucial role in regulating the blood glucose level in patients with diabetes[4,5]. AMPK also phosphorylates mTOR (mammalian/mechanistic target of rapamycin) to suppress the activity of the complexes mTORC1 and mTORC2 containing mTOR[6]. mTOR is a serine/threonine kinase that mainly regulates cell growth, proliferation, and survival, protein synthesis, autophagy, and transcription. mTOR also functions as a tyrosine protein kinase that promotes the activation of insulin receptors and insulin-like growth factor 1 (IGF-1) receptors as mTORC2[6]. Under a better nutrient condition, the Akt/Protein kinase B (Akt) in the insulin signaling pathway phosphorylates Forkhead box, sub-group O (FOXO) to inhibit a nuclear accumulation of the transcription factor. As the Akt-mediated signaling is suppressed in the presence of various cellular stresses, FOXO changes its intra-cellular localization from cytoplasm to nucleus, and it induces expression of several target genes, such as Eukaryotic initiation factor 4E-binding protein 1 (4E-BP) gene[7,8]. The 4E-BP binds to Eukaryotic initiation factor 4E (eIF-4E) in an essential protein complex for translation initiation, and this inhibits the interaction of the eIF-4E with the cap structure of mRNAs. The 4E-BP activity is shown to be

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regulated by multiple protein kinases[9,10]. The active mTOR phosphorylates the protein and the phosphorylation causes its release from the eIF4E to allow cap-dependent translation to proceed. In addition, the active mTORC1 also phosphorylates ribosomal S6 kinase (S6K) to activate it in the response to upstream stimuli. The activated S6K phosphorylates ribosome S6 subunit to stimulate protein synthesis at ribosome[11].

In addition to these biochemical aspects of the AMPK-mTOR pathway, genetic studies on AMPK function in various model organisms revealed that the AMPK activity plays a role in the mechanism underlying lifespan extension associated with calorie restriction[12,13]. Adult stage-onset depletion of AMPK in muscle shortened lifespan of *Drosophila* adults[14]. Conversely, overexpression of AMPK in adult fat body or adult muscle extended the lifespan[15]. The protein kinase can slow tissue aging and prolongs the lifespan in non-cell-autonomous manner[16]. Inhibition of Tor activity using rapamycin or genetic inactivation also extends the lifespan of *Drosophila*[17,18].

Several lines of genetic evidence[19-23] have been reported that mutations of genes for Insulin/IGF signaling pathways extend in *Drosophila*, worms and mammalian models. These results also allowed us to expect that metformin which ameliorates diabetic conditions may have an effect that retards ageing progression of living organisms. Recent studies have shown that metformin can delay aging and extend healthy lifespan *in vivo*, specifically in nematodes[23,24] and some rodent strains[21,25]. Inconsistently, a previous *Drosophila* study[26] reported that metformin treatment at a lower concentration was insufficient to extend in the wild-type adults. The administration at higher concentration than 10 mM rather provided a deleterious effect that shortened the lifespan. In contrast, Na et al[23,27] reported that the medicine showed an anti-aging effect that suppressed aging-dependent phenotypes in the *Drosophila* midgut, and that the effect resulted from the downregulation of the Akt/Tor pathway. However, it was uncertain whether the medicine enabled the retardation of aging phenotypes in tissues other than the midgut. Even if such effects were exerted, it was not certain whether the mechanism underlying the effect of metformin was the same in other tissues. Identification of novel factors involved in this pathway may provide evidence supporting the potential use of metformin as anti-aging medicine.

D. melanogaster is considered an ideal genetic model, owing to its high fecundity and short life cycle[28-31]. Furthermore, advancements in genetic techniques have facilitated experimental investigation in *Drosophila*. In addition, *Drosophila* has played a crucial role in drug discovery[30,32] and has been widely used as an experimental model for studies on aging.

As *Drosophila* adults age, they display impaired locomotor activity. One can quantitate the activity by climbing assay and flight assay[33-35]. The flies also show accumulation of abnormal protein aggregates containing ubiquitinated protein in the muscle[33]. The accumulation of poly-ubiquitinated protein aggregates reflects age-dependent impairment of protein homeostasis during muscle aging[36,37]. The loss of dopaminergic neurons in the brain has also been observed with aging[34,38]. Furthermore, as the flies aged, the occurrence of intestinal stem cells increased in intestinal epithelial cells[39,40]. Previously, Oka et al[33] demonstrated that adults with tissue-specific depletion of *Sod1* and its hypomorphic mutant, $Sod1^{n1}$, exhibited not only a shortened lifespan but also accelerated aging-related phenotypes earlier during the adult stage[29]. We have established an experimental system that allowed us to downregulate *Sod1*, which encodes the Cu/Zn superoxide dismutase that eliminates the superoxide anions among other reactive oxygen species (ROS)[32,33,41]. Therefore, in addition to mouse and rat models, the *Drosophila* mutant also represents a good senescence-accelerated model for the rapid evaluation of the effects of anti-ageing substances that suppress aging phenotypes[29,33]. Using the *Drosophila* aging-accelerated model, Le et al[29] succeeded to elucidate the anti-aging effects of another chemical called sesamin, a major lignan constituent of sesame seeds. Sesamin possesses various health benefits; it improved aging-related phenotypes in the muscle, brain, and midgut of normal flies, as well as the senescence-accelerated models ($Sod1^{n1}$ mutant and *Sod1*-depleted flies). More recently, the same research group demonstrated that the administration of the chemical can induce the Nrf2-dependent transcription in several types of neurons in *Drosophila* adult brains[42].

The organization and metabolism of *Drosophila* muscles are similar to those of the mammalian skeletal muscle. Indeed, many features of the muscles of aged mammals and *Drosophila* are overlapping[43]. Therefore, adult *Drosophila* muscle can be considered as a useful model system, particularly in investigations regarding

muscle aging and evaluation of drugs that delay the aging phenotype and reduce oxidative stress damage[43,44]. In *Drosophila* muscle, abnormal ubiquitinated protein aggregates (Ub) accumulate with aging[29,37]. Thus, the extent of aging can be assessed by evaluating the Ub aggregates that reflect age-dependent impairment of protein homeostasis during muscle ageing[29,36,37]. Autophagy is also required for maintenance of muscle integrity and protein homeostasis[45]. It can eliminate damaged proteins and/or proteins that no longer use from a cell for maintain energy metabolism in whole body. A previous study[37] demonstrated that overexpression of FOXO or 4E-BP in fly's muscle restored age-related decline of autophagy and extended their lifespan. Furthermore, muscle specific depletion of *Autophagy-related gene 7* (*Atg7*) resulted in acceleration of muscle atrophy in mice[46]. When nutrient and growth-related signaling pathways, as well as anti-aging pathways are inhibited, lifespan could extend *via* the induction of autophagy in worms, *Drosophil*a and mice[47]. These studies suggest that autophagy is strongly related to with tissue and organismal ageing. In addition, association of AMPK with *Autophagy-related gene 1* (*Atg1*) can induce autophagy *via* inactivation of mTORC1 in mammalian cells[48]. In *Drosophila* adult fat body, starvation-induced activation of TOR is sufficient to suppress autophagy induction[49]. Thus, it is conceivable that genetic modification of autophagy induction resulted in a delay of muscle ageing in *Drosophila* and mammals.

In this study, we aimed to assess the anti-aging effect of metformin on the *Drosophila* muscle. As we demonstrated the effect on the adult muscle, we next aimed at indentifying the signaling pathway associated with the anti-aging effect of metformin and unravel the mechanism of metformin's action in anti-aging *via* genetic analyses. Our findings regarding its anti-aging effects in muscle will contribute to understanding the anti-aging effect of metformin and other antioxidants suspected of possessing similar properties. Finally, we argue here that the genetic methods using *Drosophila* are useful for studying the mechanism of action of various drugs.

Materials and Methods

Fly Stocks

w^{1118} was used as a normal control stock. The following lines were obtained from the Bloomington *Drosophila* Stock Center: *P{tubP-GAL80^{ts}};P{GAL4-Mef2.R}R1* (described as *Mef2-Gal4^{ts}*) (#67063) as Gal4 driver stocks. For ectopic expression and depletion of target genes in adult muscle, we used the GAL4/UAS system. To restrict the Gal4 activation in adult stage, the stock carrying a temperature-sensitive mutant of the *Gal80* gene, which encodes an inhibitor of the Gal4 protein together with *Mef2-Gal4*, was used. The mutant protein was specifically inactivated at adult stage by transferring the flies at a non-permissive temperature, 28°C. *P{UAS-AMPKa^{T184D}}* (*UAS-AMPKa^{T184D}*) (#32110), *P{UAS-S6K^{STDETE}}* (*UAS-S6K-^{CA}*) (#6941), *P{UAS-Tor^{TED}}* (*UAS-Tor^{TED}*) (#7013), *P{UAS-eIF-4E1}* (*UAS-eIF-4E1*) (#8650). Other *RNAi* stocks were obtained as follows: *P{TRiP.GL00029}attP2* (*UAS-AMPK RNAi*)[50] (NIG, Mishima, Japan) and *P{GD11647}* (*UAS-Atg6 RNAi*)[51] (#v22122, VDRC, Vienna, Austria). We used *P{UAS-Sod1RNAi^{F103}}* (*UAS-Sod1RNAi*) to stimulate oxidative stress accumulation in muscle as shown previously[29,33]. This allowed us to observe the muscle ageing phenotype from younger adult stage. As controls for Gal4/UAS experiments, we used F1 progenies from outcross between w^{1118} and *Mef2-Gal4^{ts}* (*Mef2^{ts}* > +).

Fly stocks were maintained on standard cornmeal food at 25°C[33]. For adult-specific overexpression of full length cDNAs or that of dsRNAs against certain mRNAs for depletion experiments, adult flies were raised at 28°C, unless noted. The fly food consisted of 7.2 g agar, 100 g glucose, 40 g dried yeast, 40 g cornmeal, 5 mL 10% parahydroxybenzonate, 5 mL propionic acid, and 1.0 L water. The standard cornmeal medium was used for stock maintenance and crossing experiments.

Lifespan Assay

The lifespan extension effect of metformin was examined using the normal control stock and our *Drosophila* senescence-accelerated model harboring muscle-specific depletion of *Sod1* (*Mef2^{ts}>Sod1RNAi^{F103}*)[29,33]. To avoid influence of oogenesis to their longevity and accidental death during oviposition[29,30,32,33,42], we used adult males rather than females. Flies were maintained in vials at a density of 20 flies per vial on *Drosophila* instant medium (Formula 4-24, Blue, Carolina Biological Supply Co., Burlington, NC, USA). Metformin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled

water, and the stock solution was diluted with distilled water to achieve final concentrations of 5 mM in the instant medium. As controls, distilled water alone was added to the instant food at the corresponding concentrations. The flies were transferred to culture tubes with fresh diet every 3 days. The acquired survival data were analyzed using the GraphPad Prism software (La Jolla, CA, USA) and a Kaplan-Meier survival plot was constructed.

Feeding Assay

Metformin was added to the instant medium at concentrations described previously. For experiments to observe the ubiquitinated protein aggregates in the indirect flight muscles after drug treatment, male flies were transferred to food within 24 h after eclosion and reared at 28°C or 25°C. The flies were transferred to culture tubes containing metformin-supplemented fresh diet after every 3 days.

Immunofluorescence

For muscle immunohistochemistry, indirect flight muscles were collected from the thoraces of male flies in relaxing buffer (0.1 M KCl, 20 mM Tris-HCl, pH 7.2, 1 mM $MgCl_2$, 1 mM EDTA) and immersed in 4% paraformaldehyde for 30 min. The fixed samples were washed with Phosphate Buffered Saline-Triton X-100 (PBST) and blocked with 10% normal goat serum for 30 min at room temperature. For detection of the ubiquitinated protein aggregates that accumulated with age, the mouse antibody that recognizes both mono- and poly-ubiquitinated proteins (FK2, Enzo Life Science, Inc. Farmingdale, NY, USA) was used at the dilution of 1:300. The samples were incubated with the primary antibody overnight at 4°C. After washing with PBST, the samples were incubated for 2 h with Alexa Fluor 647-conjugated secondary antibodies at the dilution of 1:400. Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 647-conjugated anti-mouse IgG were subsequently used as the secondary antibodies for the anti-mitochondrial complex V subunit.

For visualization of myofibrils in the muscles, we incubated the fixed muscle samples with Alexa Fluor 488-conjugated-phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) at the dilution of 1:100 at room temperature, washed in PBST, and mounted with VectaShield (Vector Laboratories, Burlingame, CA, USA). All muscle samples were examined using a laser scanning confocal microscope (FV10i, Olympus, Tokyo, Japan). Brightness and contrast of entire images were adjusted using the FV10i software.

Quantitation of the Ubiquitinated Protein Aggregates in the Area of Interest

Image J (NIH, MH, USA) was used to determine the number of ubiquitinated aggregates or the mCherry-tagged Atg8-positive foci. A protein aggregate of size 10 pixels or more was considered a unit, and the number of units per confocal microscope view field (4.0×10^{-2} mm^2) was counted. Statistical analyses were performed after capturing the images. *p*-values were calculated to indicate the significant difference using the Student's *t*-test and log-rank test of Excel (Microsoft, Redmond, IL, USA).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR analysis was performed to determine the expression level of target genes in adult muscles. Total RNA was extracted from adult thoraces using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from the total RNA using a PrimerScript II High Fidelity RT-PCR kit (TaKaRa, Kusatsu, Shiga, Japan) with random primers. qRT-PCR was performed using FastStart Essential DNA Green master mix (Roche, Mannheim, Germany) and a LightCycler Nano (Roche, Basel, Switzerland). *RP49* was used as a normalization reference[33]. Relative mRNA levels were quantified using the LightCycler Nano software version 1.0 (Roche, Basel, Switzerland). Each sample was used in duplicate for the PCR. The ΔΔCt method was used to determine the differences between target gene expression relative to *RP49* (reference gene) expression. The qPCR primers used in this study were as follows: RP49Fw: $^{5'}$TTCCTGGTGCACAACGTG$^{3'}$, RP49Rv: $^{5'}$TCTCCTTGCGCTTCTTGG$^{3'}$, 4E-BPFw: $^{5'}$TCCTGGAGGCACCAAACTTATC$^{3'}$, 4E-BPRv: $^{5'}$GGAGCCACGGAGATTCTTCA$^{3'}$.

Statistical Analysis

A survival curve was plotted following Kaplan-Meier survival estimation and analyzed using the log-rank test between the metformin-treated group and the appropriate control group. The Student's *t*-test was used for comparing two groups. One-way analysis of variance (ANOVA)

with post-hoc Tukey's test was applied to assess the differences in more than two groups. Two-way ANOVA with post-hoc Student's *t*-test was performed to compare the mean differences between groups that were split into two independent variables. The statistical analysis was performed using GraphPad Prism (Version 9, GraphPad Software, San Diego, CA, USA). Data were considered significant at *p*-values < 0.05.

Results

Metformin Feeding Extended the Lifespan of Drosophila adults

Metformin suppressed the aging phenotype of hyper-proliferation of intestinal stem cells in the adult midguts of aging flies[23]. To further determine whether this anti-aging effect of the medicine appeared in other tissues, we initially investigated whether metformin administration extended the lifespan of adult flies. In our previous study[33], we demonstrated that metformin at 5 mM has anti-ageing effect that suppress age-dependent hyper-proliferation phenotype of stem cells in adult intestines. Thus, we determined the survival rates of normal control flies (w^{1118}) raised on fly diet supplemented with 5 mM metformin under 25°C (Figure 1). The lifespan of flies fed metformin (pink curve) extended significantly, compared to that of the control without metformin feeding (gray curve) ($p < 0.01$, log-rank test, n ≥ 105). Flies fed metformin required 63 days, to show 50% lethality, compared to 54 days for the control flies reared without metformin (Figure 1). To further confirm the result in a shorter period, we examined the lifespan-extension effect using the aging-accelerated model flies (*Mef2*^{ts}>*Sod1RNAi*^{F103})[33]. To restrict the Gal4 activation at adult stage, we used the *Mef2*^{ts}*-Gal4*, carrying both a temperature-sensitive mutant of the *Gal80* gene and *Mef2-Gal4*. We determined the survival rates under more severe depletion condition (raised under 28°C, at which Gal4 transcription factor acted more efficiently) (Figure 1). The results showed that the lifespan of flies fed 5 mM metformin extended significantly but not that of flies fed the medicine at 10mM, compared to that of flies fed the fly diet (****$p < 0.0001$, log-rank test, n > 100). Flies fed 5- and 10-mM metformin required 40 and 36 days, respectively, to show 50% lethality, compared to 36 days for the control. Feeding with 5 mM metformin was more effective in extending the adult lifespan than with 10 mM metformin. Therefore, we fed flies 5 mM metformin in subsequent experiments to further investigate the anti-aging effects of metformin.

Metformin Suppressed the Age-Dependent Accumulation of Ubiquitinated Protein Aggregates in the Adult Indirect Flight Muscles

As metformin enabled to extend the lifespan of adult flies, we next investigated whether metformin affected the aging-related pheno-

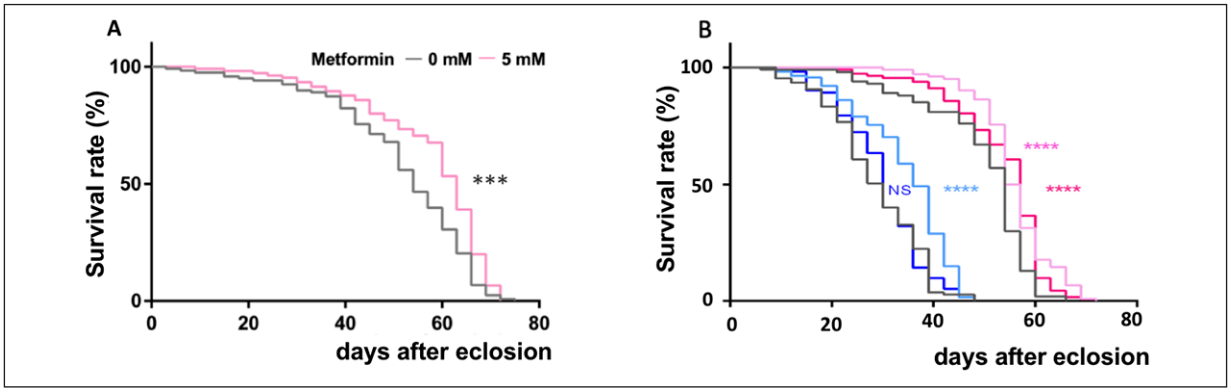

Figure 1. Metformin extends lifespan in the *Drosophila* adult flies. **A,** Survival rates of adult flies collected within 24 hours after eclosion were examined every three days. The flies were fed with fly diet supplemented with 5 mM metformin (n = 105 flies, pink curve) and without metformin (controls) (n =118 flies, gray curve). **B,** Survival rates of adult males (*Mef2*>*Sod1RNAi*^{F103}) fed the diet with 5, 10 mM metformin. Light pink (5 mM metformin), magenta (10 mM metformin) or without the drug (gray lines) represent lifespan curves of adults raised at 25°C. Light blue (5 mM metformin), blue (10 mM metformin) and gray (control) lines represent lifespan curves of adults raised at 28°C. Lifespan curves were plotted using Kaplan-Meier survival analysis. A log-rank test was performed for each pair of adults fed with the control diet and the diet supplemented with 5 mM metformin. (***$p < 0.001$).

type that appeared in normal adult muscle. We continuously fed the adults with fly diet, or a diet containing 5 mM metformin for 5, 20, 30, 40 and 50 days. Immunostaining of indirect flight muscles from the flies was performed to score ubiquitinated protein aggregates accumulated in the muscles (Figure 2a-j). The abnormal aggregates in the muscles of adults fed 5 mM metformin continuously for 5 days to 40 days significantly decreased in number, compared to that in the controls of the same age without the feeding ($p < 0.05$, Student's *t*-test, n > 10). The average number of the aggregates in the adults fed the drug for 50 days also decreased, although there was no statistical significance (Figure 2k). Based on these results, we concluded that metformin feeding suppressed the progression of the aging-related phenotype in adult muscle.

AMPK Activation was indispensable for the Metformin Effects that Suppressed Accumulation of Ubiquitinate Protein Aggregates in the Adult Muscle

It is known that metformin can activate AMPK. Therefore, we investigated whether the metformin-mediated suppression effect on aging-related phenotype in the muscles involved the local activation of AMPK in adult flies. We utilized the Gal80^{ts}/Gal4 system that enabled adult stage-specific depletion of the *AMPK* mRNA in the adult muscle (*Mef2^{ts}>AMPKRNAi*) and compared the muscle aging phenotype of adults treated with or without metformin (Figure 3a-e). The control flies (*Mef2^{ts}>+*) showed a suppression of the Ub aggregates by 5 mM metformin for 20 days ($p < 0.05$, Figure 3b, f, the third and fourth bars from the left in Figure 3g-i). In contrast, we scored 86.10 units of Ub aggregates on average in a confocal microscope field (4.0×10^{-2} mm^2) of the muscles from 20-day-old *Mef2^{ts}>AMPKRNAi* adults without metformin feeding. We also scored 90.86 units of aggregates on average in the adults of the same age fed 5 mM metformin. We did not observe significant differences between flies with or without metformin feeding ($p > 0.05$, Student's *t*-test, n > 20, two bars on the right in Figure 3i). Therefore, we concluded that AMPK is required for the suppression effect of metformin on the aging-related phenotype in the *Drosophila* muscle.

Next, we investigated whether activation of AMPK by metformin is sufficient for the suppression of the muscle aging phenotype in *Drosophila* adults. We induced muscle-specific expression of a constitutively active form of AMPK (AMPK^{T184D}) in adult male flies (*Mef2^{ts}>AMPKα^{T184D}*). Subsequently, we assessed the

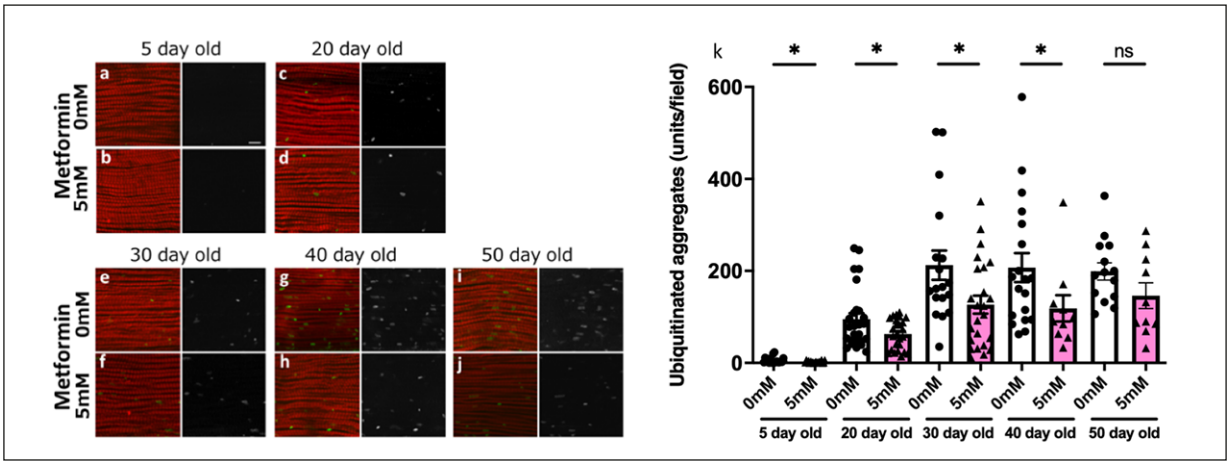

Figure 2. Ubiquitinated protein aggregates in the indirect flight muscles of adults continuously fed on metformin. **a-j,** Immunostaining of the indirect flight muscles with an antibody against mono-and poly-Ub. The muscles were collected from control adults continuously fed on metformin after eclosion; Anti-Ub immunostaining (green in a-j, white in a'-j') and phalloidin staining of F-actin (red in a-j). **a, b,** The flies were raised for 5 days, (**c, d**) 20 days, (**e, f**) 30 days, (**g, h**) 40 days and (**i, j**) 50 days on the fly diet (**a, c, e, g, i**), or the diet supplemented with 5 mM metformin (**b, d, f, h, j**). Scale bar represents 10 μm. **k,** The average number of ubiquitinated aggregates per single confocal optic fields (4.0×10^{-2} mm^2) in control (open bars), 5 mM metformin fed flies (pink bars) are presented on the y-axis. Data are shown as mean ± SE. A two-way ANOVA with post hoc Student's *t*-test was applied to compare the mean differences (*$p < 0.05$, ns: not significant, Student's *t*-test), 5 days ($n \geq 18$), 20 days ($n \geq 15$), 30 days ($n \geq 18$), 40 days ($n \geq 10$), and 50 days ($n \geq 10$).

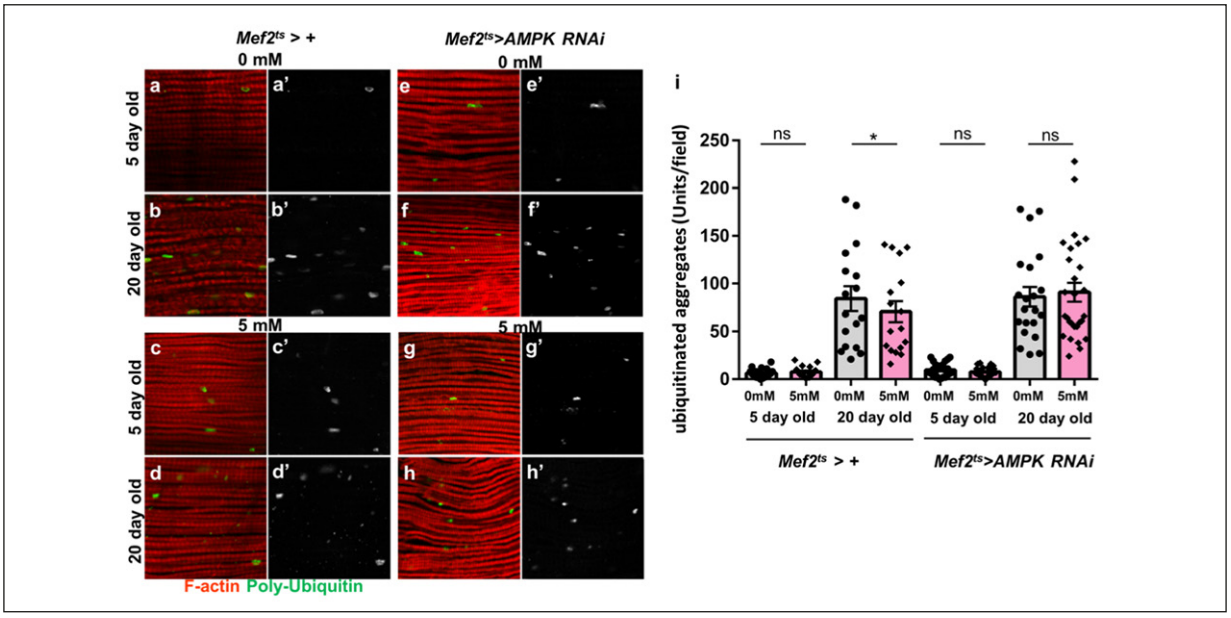

Figure 3. The depletion of the AMPK mRNA by expression of the dsRNA against the mRNA in the adult muscles canceled the suppression effect of metformin on the aging-related phenotype. **a-h,** Anti-Ub immunostaining of indirect flight muscles from males having muscle-specific depletion of AMPK (*Mef2^{ts}>AMPKRNAi*) after eclosion. Anti-Ub immunostaining (green in a-h, white in a'-h') and phalloidin staining of F-actin (red in a-h). These individuals were raised at 19ºC to avoid developmental defects due to the hyper AMPK activation. The adult males were collected within 24 h after eclosion and raised at 28ºC to induce depletion of the *AMPK* mRNA for 5 days (**a, c, e, g**), and 20 days (**b, d, f, h**). Scale bar represents 10 µm. **i,** The average numbers of ubiquitinated aggregates per single confocal optic fields (4.0×10^{-2} mm^2) in control flies (gray bars) and flies fed 5 mM metformin (pink bars) are shown on the y-axis. Data are shown as mean ± SE. A two-way ANOVA with post-hoc Student's *t*-test was applied to compare the mean differences. (ns; not significant, *$p > 0.05$, n > 20).

survival rate of adult flies with a muscle-specific expression of AMPK^{T184D} every 24 h (Figure 4a). Results showed that compared to that of the control, the lifespan of the flies expressing the activated form of AMPK in the adult muscle increased significantly ($p < 0.01$, log-rank test, n > 85). In addition, we compared the number of ubiquitinated protein aggregates in flies expressing the activated form of AMPK in adult muscles to that in the control males of the same age (*Mef2^{ts}* > +) (Figure 4b-f). The number of ubiquitinated protein aggregates in 5-day-old flies did not vary significantly between controls and flies expressing AMPK^{T184D} ($p < 0.01$, Student's *t*-test, n > 20). In contrast, compared to that in controls, the number decreased significantly in the muscle of 20-day-old flies with a continuous expression of AMPK^{T184D} (*Mef2^{ts}* > +). These results suggested that the AMPK activation in the adult muscles was sufficient for the suppression of the muscle aging phenotype. Therefore, we concluded that the suppression of the muscle aging phenotype by metformin was associated with AMPK activation.

Tor-Mediated Pathway was Involved in the Suppression Effect of Metformin on the Aging-elated Phenotype in Adult Muscles

We obtained genetic evidence possibly suggesting that the activation of AMPK may play an important role in the anti-aging effect of metformin on the adult muscle. To further confirm this observation, we next investigated whether the anti-aging effect of metformin involved Tor, which is known to be negatively regulated by AMPK. Thus, we investigated whether the suppression of Tor activity mimicked the anti-aging effect of metformin. First, we induced expression of wild-type Tor (Tor^{WT}) in adult muscle (*Mef2^{ts}>Tor^{WT}*). Without metformin feeding, the flies accumulated almost same numbers of Ubiquitylated aggregates on average as control flies did (*Mef2^{ts}* > +) (Figure 5a, c, g). The administration neither showed any suppression effect in *Mef2^{ts}* > *Tor^{WT}* flies, in contrast to the effect shown in control flies (*Mef2^{ts}* > +). ($n > 0.05$, Student's *t*-test, n > 20) (Figure 5a-d). Ectopic expression of wild-type Tor did not provide effects on the muscle

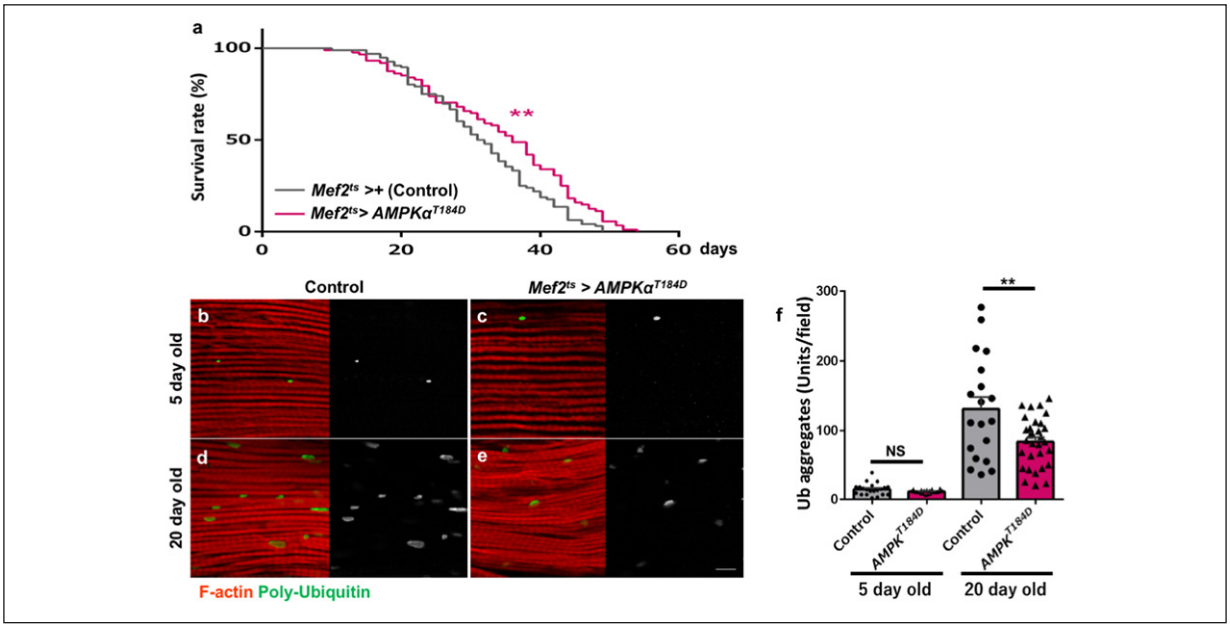

Figure 4. Lifespan extension and suppression of the muscle aging phenotype in the indirect flight muscles in adults having a muscle-specific expression of a constitutively active form of AMPK. **a,** Lifespan curves of adult males having an adult muscle-specific expression of AMPK^{T184D} (*Mef2^{ts}>AMPKα^{T184D}*). The adult males raised at 19ºC were collected within a day after eclosion to provide for the feeding experiments. Since Gal4 is not active at 19ºC, the flies collected had not expressed the constitutively active AMP before eclosion. Then, the eclosed flies were continuously maintained at 28ºC to stimulate overexpression of the mutant AMPK. A pink curve (*Mef2^{ts}>AMPKα^{T184D}*) and gray curve (control flies, *Mef2^{ts}>+*) represent the lifespan curves of those adults. Curves were plotted using Kaplan-Meier survival analysis. A log-rank test was performed for each pair of adults fed with the control diet and adults fed with the metformin diet (**$p < 0.01$, log-rank test, $n > 85$). **b-e,** Immunostaining of indirect flight muscles collected from control adult males (**b, d**), males continuously expressing AMPK^{T184D} after eclosion (**c, e**). Anti-Ub immunostaining (green in b-e, white in b'-e') and phalloidin staining of F-actin (red in b-e). The adult males collected within 24 h after eclosion were raised for 5 days (**b, c**) and 20 days (**d, e**). Scale bar represents 10 μm. **f,** The average number of ubiquitinated aggregates per single confocal optic fields (4.0×10^{-2} mm^2) in control flies (gray bars), flies having the AMPK^{T184D} expression (pink bars) are shown on the y-axis. Data are shown as mean ± SE. At least 10 images of muscle samples prepared from at least 10 flies were observed. A two-way ANOVA with post-hoc Student's *t*-test was applied to compare the mean differences. (**$p < 0.01$, $n > 20$).

ageing phenotype (Figure 5g). Next, we induced ectopic expression of a dominant-negative form of Tor (Tor^{TED}) in adult muscles to downregulate Tor activity (*Mef2^{ts} > Tor^{TED}*). We fed the adults with the fly diet or diet supplemented with 5 mM metformin. Anti-Ub-immunostaining of muscles suggested that the degree of muscle aging was significantly suppressed by the downregulation of Tor. The metformin feeding no longer suppressed the muscle ageing phenotype in flies expressing a dominant negative form of Tor ($p < 0.0001$, Student's *t*-test, $n \geq 19$) (Figure 5e-g). Therefore, we concluded that the muscle anti-aging effect was abrogated upon Tor activation. These pieces of genetic evidence suggested that the downregulation of Tor activity was involved in the muscle anti-aging effect of metformin. We interpreted that the suppression of Tor activity was necessary and sufficient for metformin's anti-aging effect.

Translation Initiation Factor eIF-4E and Ribosomal Protein S6 Kinase were Involved in the Suppression Effect of Metformin on the Aging-related Phenotype in Adult Muscles

As described above, we obtained genetic evidence showing that the downregulation of Tor, one of the key regulators of protein synthesis, was involved in the suppression effect of metformin in adult muscle. Considering that 4E-BP/Thor and eIF-4E are known downstream factors of Tor, we next investigated whether the mRNA level of Thor, encoding 4E-BP, was affected after metformin feeding. We isolated total mRNA from adults in the muscles. Initially, we confirmed that the level of 4E-BP mRNA increased by two times in adult muscles expressing a dominant-negative form of Tor (*Mef2^{ts} > Tor^{TED}*) for 20 days after eclosion (Figure 6). As

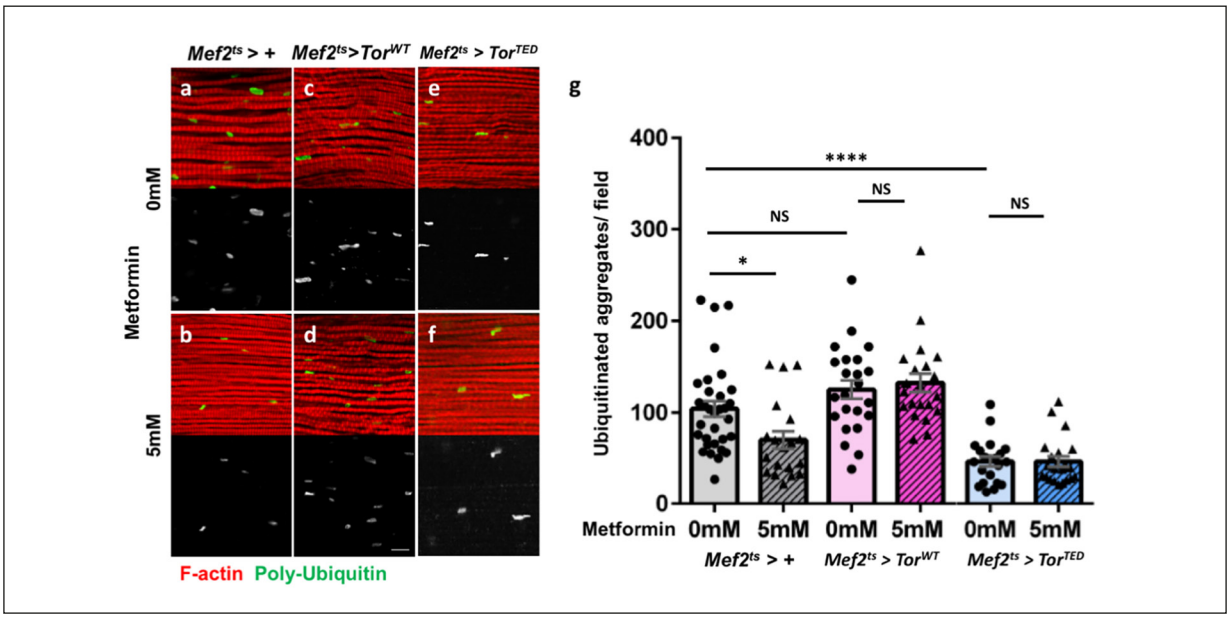

Figure 5. The Tor-mediated pathway is involved in the anti-aging effect of metformin on the adult muscle. **a-d,** Immunostaining of indirect flight muscles prepared from control adult males (*Mef2*^{ts} > +) or adults expressing a dominant-negative form of Tor (*Mef2*^{ts} > *Tor*^{TED}) for 20 days after eclosion. Muscles were prepared from adults fed on a fly diet without metformin (**a, c, e**) and those fed on a fly diet with 5 mM metformin (**b, d, f**). Anti-Ub immunostaining (green in a-f, white in a'-f') and phalloidin staining of F-actin (red in a-f). The adult males were collected within 24 h after eclosion. Scale bar represents 10 μm. **g,** The average of the number of the ubiquitinated aggregates per single confocal optic fields (4.0 × 10^{-2} mm^2) in flies fed without or with 5 mM metformin are shown on the y-axis. Data are shown as mean ± SE. A two-way ANOVA with post-hoc Student's *t*-test was applied to compare the mean differences. (*$p < 0.05$, **** $p < 0.0001$, NS: not significant, Student's *t*-test, n ≥ 19).

we were able to confirm that the 4E-BP mRNA level shifted in response to the downregulation of Tor, we next quantified the mRNA level in metformin-fed flies. Compared to that of the adults fed the diet without metformin, the 4E-BP mRNA level increased 1.43-fold in adults fed 5 mM metformin for 5 days. Similarly, compared to that in adults not treated with metformin, the level increased 1.57-fold in adults fed 5 mM metformin for 20 days. Compared to that in flies without metformin diet, feeding with metformin significantly increased the mRNA level of 4E-BP in the indirect flight muscle.

Furthermore, we investigated whether the overexpression of eIF-4E, which is an essential factor for the initiation of protein syn-

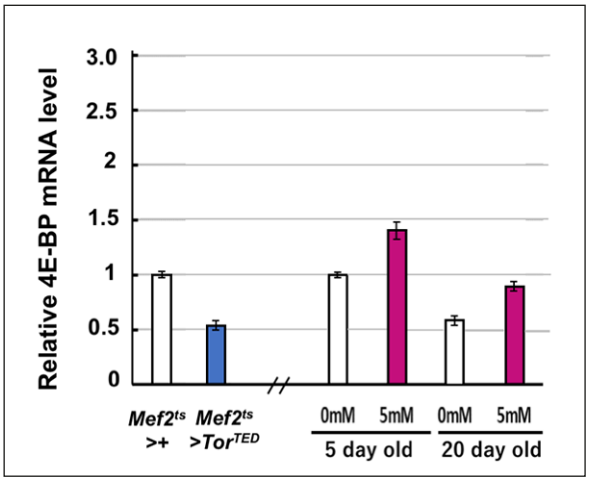

Figure 6. Quantitation of mRNA levels of the 4E-BP gene in the adults fed with or without metformin. Total RNAs from adults fed on the diet with or without 5 mM metformin for 5 days and 20 days after eclosion (right four bars). The 4E-BP mRNA level of adults. Note that the 4E-BP mRNA level increased by double in the adult muscle expressing a dominant-negative form of Tor (*Mef2ts > TorTED*) for 20 days (Left two bars). The mRNA levels of the 4E-BP gene in adults fed metformin. Relative mRNA levels of the gene in adults fed with 5 mM metformin (red bars), and those in adults fed with the diet without the medicine (control, white bars) were calculated using real-time-qRT-PCR analysis. Statistical analysis was performed using Student's *t*-test. **$p < 0.01$; ***$p < 0.001$.

thesis, negates the anti-aging effect of metformin. Tor phosphorylates the eIF-4E binding protein, 4E-BP, in *Drosophila*[52]. Eventually, this represses the eIF-4E activity in the ribosome. Hence, we expected that overexpression of eIF-4E might result in the production of free molecules of the initiation factor (free from its inhibitor). We generated adults overexpressing wild-type eIF-4E in adult muscles and analyzed whether metformin feeding affected the muscle aging phenotype. Toward this, we quantified the number of ubiquitinated aggregates in the muscles of flies fed with or without metformin. In contrast to the control flies in which metformin suppressed the ubiquitinated aggregates ($p < 0.05$, n > 20, Figure 7a, b, and left two bars in g), we did not observe any statistically significant differences in the average number of aggregates in the muscles of flies with muscle-specific expression of eIF-4E (*Mef2*^{ts} > *elF-4E*) ($p > 0.05$, student *t*-test, n > 20) (Figure 7a-d, light orange and orange bars in **g**). As eIF-4E is negatively regulated by Tor, these genetic data suggested that the downregulation of eIF-4E expression plays a critical role in the anti-aging effect of metformin.

Ribosome S6 kinase (S6K) is also known to be another target of Tor, which positively regulates the activity of S6 kinase to promote protein synthesis. Hence, we further examined whether the overexpression of the constitutively active form of S6 kinase, S6K^{CA} can replace the suppression effect of metformin on the aging-related phenotype in muscle. We induced ectopic expression of the mutant form, specifically in adult muscles (*Mef2*^{ts} > *S6K*^{CA}). The accumulation of the ubiquitinated aggregates was suppressed in adult muscle having ectopic expres-

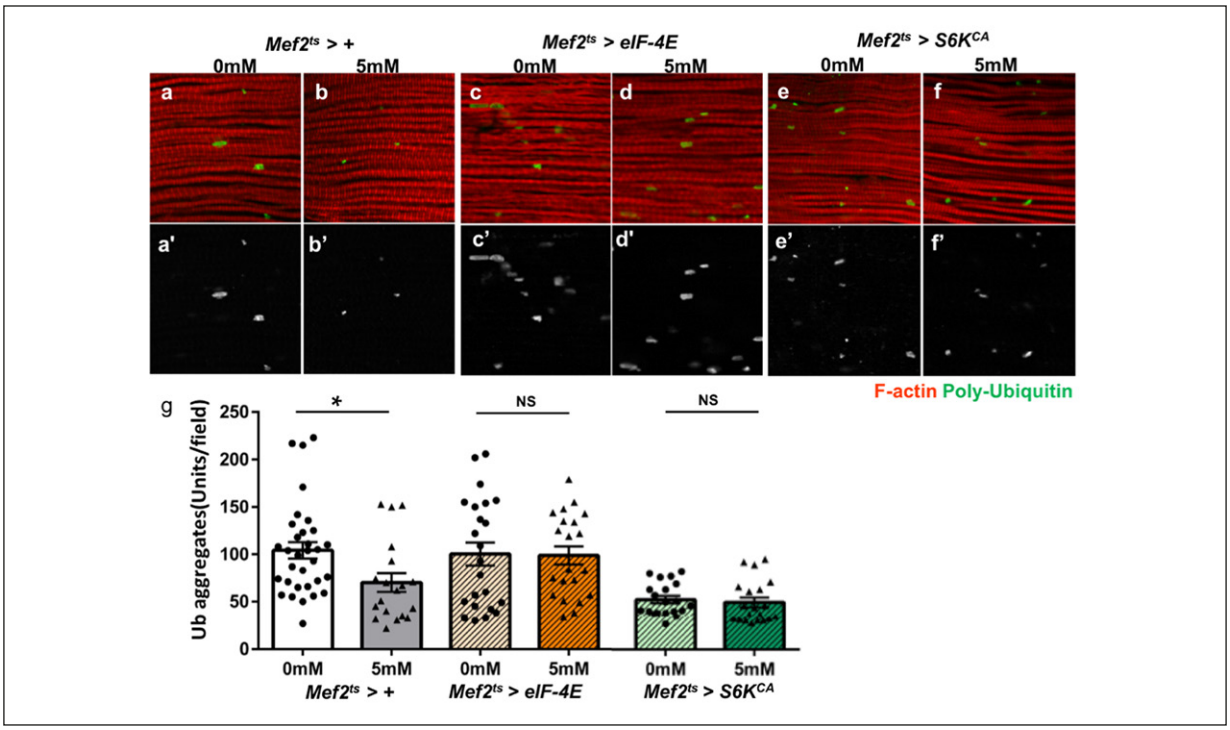

Figure 7. Loss of metformin's anti-muscle aging effect in adults having ectopic over-expression of eIF-4E and those having a muscle-specific expression of a constitutively active form of S6 kinase in their muscle. **a-f,** Anti-Ub immunostaining of the indirect flight muscles prepared from control adult males (**a, b**), those having a muscle-specific expression of the translational initiation factor, eIF-4E (*Mef2*^{ts} > *eIF-4E*) (**c, d**), those expressing a constitutively active form of S6 kinase (*Mef2*^{ts} > *S6K*^{CA}) (**e, f**) for 20 days after eclosion. Muscle prepared from the adults fed on the fly diet without metformin (**a, c, e**), fed on the fly diet with 5 mM metformin (**b, d, f**). Anti-Ub immunostaining (green in **a-f**, white in **a'-f'**) and phalloidin staining for F-actin visualization (red in **a-f**). Scale bar represents 10 μm. Scale bar represents 10 μm. **g,** The average of the number of the ubiquitinated aggregates per single confocal optic fields (4.0×10^{-2} mm^2) in control flies without the medicine feeding (white, light orange, light green bars), flies fed 5 mM metformin (light gray, orange and green bars) are shown on the y-axis. Data are shown as mean ± SE. A two-way ANOVA with post-hoc Student's *t*-test was applied to compare the mean differences. (NS; not significant, *$p > 0.05$, Student's *t*-test, n > 20).

sion of $S6K^{CA}$ even without metformin feeding (Figure 7e-g). In flies overexpressing $S6K^{CA}$, we did not observe any statistical differences in the average numbers between control flies (*Mef2^{ts}* > +) or those of the *Mef2^{ts}* > *S6K^{CA}* flies between with and without metformin administration ($p > 0.05$, Student's *t*-test n > 20) (right two bars in Figure 7g). These genetic data suggested that the upregulation of the S6K acting downstream of Tor invalidated the suppression effect of metformin. In conclusion, AMPK, as well as factors involved in the transmission of the AMPK-mediated signal, Tor, eIF-4E and S6K are also involved in the suppression effect of metformin on the aging-related phenotype in muscle.

Administration of Metformin Induced Autophagy in Adult Muscles

Next, we investigated whether the induction of autophagy in muscle was involved in the suppression of the metformin-induced muscle aging phenotype. Autophagy acts as an intracellular clearance system to eliminate large cellular components, including damaged organelles and proteins that accumulate with aging. As described previously, AMPK can be activated by metformin, which then negatively regulates Tor. Both proteins are required for the effect of metformin on aging. Tor negatively regulates autophagy *via* suppression of autophagy initiation factor, Atg1[53]. Based on previous results and the findings of this study, we hypothesized that metformin also stimulates autophagy by suppressing Tor. To test the hypothesis, we investigated whether autophagy induction is involved in the suppression effect of metformin on the ageing-related phenotype in muscle. To quantify autophagy induction, we examined the accumulation of a known autophagy marker, Atg8[54]. We induced muscle-specific expression of mCherry-Atg8a in adult males, which accumulated in autolysosomes. We fed adult flies control diet or diet supplemented with 5 mM metformin and observed the mCherry fluorescence in adult muscles (*Mef2^{ts}* > *mCherry-Atg8a*) (Figure 8). We quantified the Atg8 area in the direct flight muscles of 7-day-old and 21-day-old flies (Figure 8a-d). The Atg8 fluorescent area increased significantly in the muscles of 7-day- and 21-day-old adult flies fed metformin (**** $p < 0.0001$, Student's *t*-test, n > 20) (Figure 8e). On the basis of these results, we concluded that the metformin feeding can promote the autophagy induction in adult muscle.

The Suppression Effect of Metformin on the Ageing-Related Impairment of Protein Homeostasis in Muscle Required an Essential Component for Autophagy Induction

At the end, we investigated whether metformin-induced autophagy was involved in the effect of the medicine in adult muscle. We depleted of *Atg6*, which is one of the autophagosome components essential for autophagy induction in the adult muscle (*Mef2^{ts}* > *Atg6RNAi*). In the 20-day-old flies, we scored the ubiquitinated protein aggregates accumulated in the indirect flight muscles (Figure 8f-h). By examination of the muscles in the flies having adult- stage-specific depletion of *Atg6,* fed on the fly diet with metformin for 20 days after eclosion, we scored significantly more aggregates on average (99.8 units/single confocal optic fields, n > 20), compared to the aggregates in control muscle (*Mef2^{ts}* > +) (94.3/single confocal optic fields, n > 20) ($p < 0.05$). Inhibition of autophagy induction by *Atg6* depletion abrogated the effect of metformin on age-related impairment of protein homeostasis in adult muscle. Hence, we concluded that the autophagy induction played an important role in the metformin's effect that suppressed age-related impairment of protein homeostasis during muscle ageing in *Drosophila* adults.

Discussion

Metformin suppresses the age-dependent hyperproliferation of intestinal stem cells in *Drosophila* adults[23]. However, its anti-aging effects on other tissues had not been clarified yet. In this study, we first demonstrated that continuous feeding of 5 mM metformin extended the lifespan of *Drosophila* adults. A previous study[26] reported that metformin treatment at lower concentrations was insufficient for the lifespan extension of wild-type flies. For our lifespan assay of *Drosophila* adults fed with metformin, we used male flies of *w* control strain. We fed the flies the instant *Drosophila* food supplemented with metformin to avoid thermal inactivation of the medicines during preparing the standard cornmeal food by boiling the ingredients. Initially, using the aging-accelerated model flies (*Mef2^{ts}*>*Sod1*RNAi flies) to save time, we showed that metformin at 5 mM, but not 10mM, was effective for *Drosophila* lifespan extension. Although we have not performed the lifespan assays of *w* flies at other concentrations, Slack

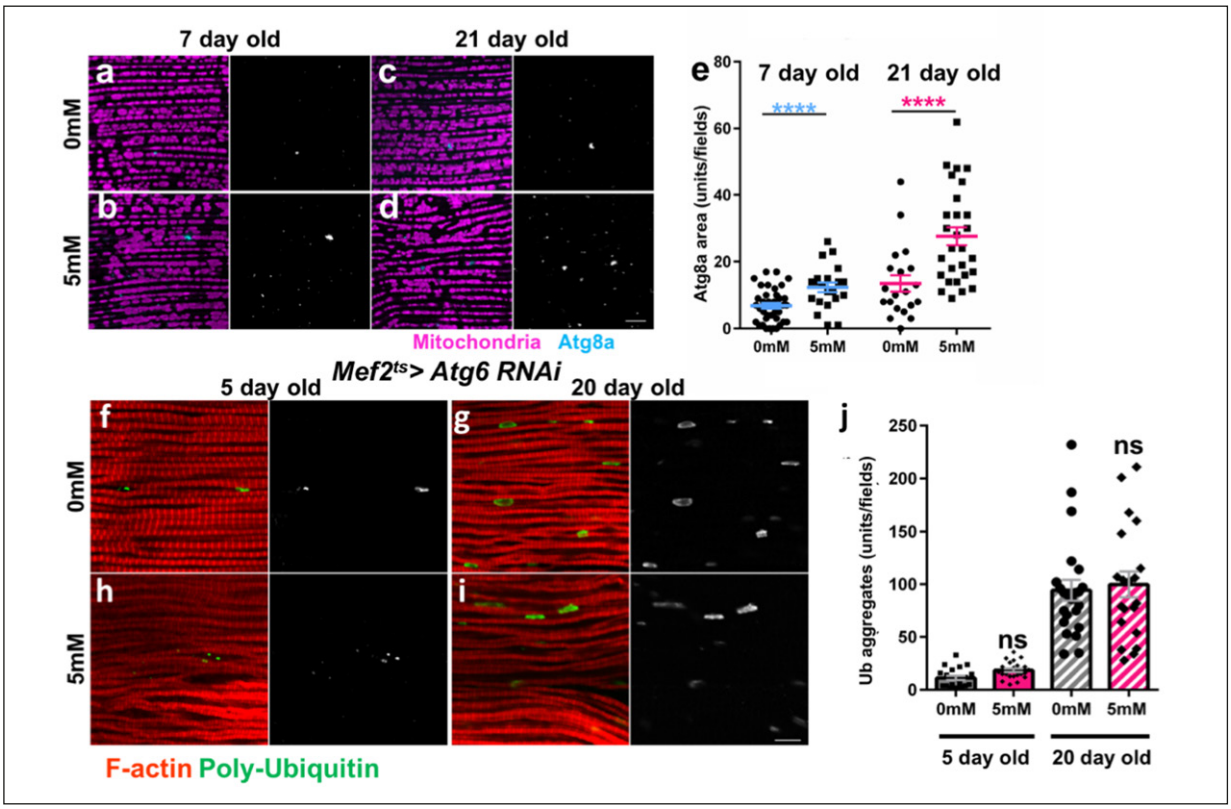

Figure 8. The autophagosome area in the indirect flight muscle significantly increased after the metformin feeding. **a-d,** Immunostaining of indirect flight muscles prepared from adult males expressing a mCherry-tagged Atg8a with the antibody that recognized mitochondria (*Mef2 ^{ts} > mCherry-Atg8a*). The adults fed on the fly diet with or without 5 mM metformin for 7, or 21 days after eclosion. The left panels in a-d represented merged fluorescence images of mCherry-Atg8 (light blue in **a-d**, white in **a'-d'**) and anti-mitochondrial immunostaining (magenta in **a-d**). The adult males collected within 24 h after eclosion, raised on the diet for 7 days or 21 days. Scale bar represents 10 µm. **e,** The average area of mCherry-Atg8 fluorescence per single confocal optic fields (4.0×10^{-2} mm^2) are represented on the y-axis. Blue bar; 7-day-old flies, pink bars; 21-day-old flies. Data are shown as mean ± SE. Two-way ANOVA with post-hoc Student's *t*-test were applied to compare the mean differences. (****$p > 0.0001$, Student's *t*-test, n > 20). **f, g,** Immunostaining of indirect flight muscles dissected from adult males depleted Atg6 (*Mef2^{ts}>Atg6RNAi*) after eclosion. Anti-poly-Ub immunostaining (green) and phalloidin-staining of F-actin (red). The adult males were collected within 24 h after eclosion, raised on the diet supplemented with 5 mM metformin (**h, j**) or without (**f, g**) for 5 days (**f, h**), and 20 days (**g, i**). Scale bar represents 10 µm. **j,** The average of the number of the ubiquitinated aggregates per single confocal optic fields (4.0×10^{-2} mm^2) in control (gray bars), 5 mM metformin (pink bars) fed flies are shown on the y-axis. Data are shown as mean ± SE. Two-way ANOVA with post-hoc Student's *t*-test were applied to compare the mean differences. (NS; not significant, $p > 0.05$, n > 20).

and colleagues had reported that metformin treatment at 1 to 5 mM concentration failed to extend the lifespan of flies from another wild-type stock called *Dahomey*[26]. The authors fed the flies the standard sugar/yeast/agar medium. The administration at higher concentrations than 10mM rather shortened the lifespan. The authors confirmed that the metformin administration at every concentration for 7 days induced self-phosphorylation of AMPK in females, but not in males at 5 mM concentration. However, it is not easy to directly compare our results with the previous one, as the lifespan assays in each study were performed under the different conditions (using different diets and different *Drosophila* strains). Three other papers have already reported that metformin administration suppressed the age-related hyper-proliferation of midgut stem cells and DNA damage accumulation in *Drosophila* gut epithelial cells of flies fed 5 mM metformin[23,29,55]. In addition, the lifespan extension effect of metformin has been reported from other organisms, mice, rats and nematode[56-59]. Therefore, in addition to the metformin's effect appeared in adult gut, our current evidences that metformin suppressed

age-dependent accumulation of ubiquitinated aggregates in adult muscle also support our results regarding the lifespan extension effect of the medicine.

Next, we found that metformin feeding suppressed the aging-dependent accumulation of aggregates containing damaged proteins in the adult muscle. To delineate the mechanism via which metformin delayed the *Drosophila* muscle aging-related phenotype, we induced the constitutively active AMPK in adult muscles, and found that the activation was sufficient for the suppression effect on the aging-related phenotype in muscle. We showed that the ubiquitinated protein aggregates in muscle of 20-day old *Mef2^{ts}>AMPK RNAi* flies did not change in the absence of metformin, compared to the control adults (*Mef2^{ts}>+*) of the same age. These genetic data are consistent that AMPK is required for the metformin's effect that suppresses accumulation of ubiquitinated protein aggregates. We also found that the aggregates decreased in amount in the muscle expressing the dominant negative TOR (*Mef2^{ts}>Tor^{TED}*), but not in the muscle harboring mere overexpression of the wild-type gene. From these genetic evidences, we consider that down-regulation of TOR signaling, which reproduces metformin's effect, is involves in the suppression effect during muscle ageing. We obtained genetic evidences suggesting that AMPK-mediated down-regulation of Tor pathways, and subsequent induction by an eIF-4E inhibitor were also required for the effect that suppressed the aging-related phenotype. Furthermore, we showed that metformin feeding stimulated autophagy induction in adult muscle, and that the suppression effect in muscle was involved in activation of the AMPK-mediated signaling pathway and suppression of elF-4E.

Many basic and clinical studies have revealed that metformin exerts an anti-hyperglycemic effect on type 2 diabetes, as well as other age-related diseases, both in humans and other model organisms[60-62]. The medicine suppressed the aging phenotypes that appeared in intestinal stem cells in *Drosophila*[23,29]. As flies age, the stem cells in the gut epithelia increase in number to replace the damaged epithelial cells. The aging phenotype in the tissue is attributed to the accumulation of DNA damage associated with increased activity of Akt owing to aging and/or oxidative stress. Previous studies have shown that metformin suppresses the aging phenotype in intestines via reduced Akt activity in adults[23]. In this study, we analyzed the protein aggregates containing ubiquitinated proteins, which accumulated as the flies aged[33,37,43]. We obtained genetic evidences suggesting that the suppression effect of metformin in muscles was associated with the activation of AMPK, and thereby inactivation of Tor-mediated pathways. Our results were consistent with that of similar studies on *Drosophila* or other model organisms. For example, suppression of the Tor-mediated signaling pathway via genetic modification extended the lifespan of invertebrates, such as *Caenorhabditis elegans* and *Drosophila*[17,63]. In addition, the administration of rapamycin, a Tor inhibitor, also extended the lifespan of various organisms such as yeast, nematodes, flies, and mice[21,64-66]. AMPK activation *via* metformin inhibits Tor activity *in vitro* as well as in mammalian cultured cells[67]. These pieces of evidence made us speculate that Tor-mediated pathways, which are conserved among species, play a central role in organism longevity. Hence, we speculated that the suppression effect of metformin on the muscle aging-related phenotype might involve the down-regulation of Tor and/or downstream pathway(s) activated by Tor.

Previous studies[37] have reported that muscle-specific activation of 4E-BP in *Drosophila* leads to lifespan extension. We also observed that metformin increased the mRNA level of 4E-BP, suggesting that muscle-specific upregulation of 4E-BP may have inhibited translation initiation owing to impaired recruitment of the ribosomal subunit to mRNAs[68]. Consistently, FOXO1 regulates the expression of 4E-BP1 and inhibits mTOR signaling in mammalian skeletal muscle[69]. In *Drosophila* adult muscle, FOXO is also required for 4E-BP transcription, and FOXO/4E-BP signaling plays a key role in both organismal and muscle aging[37]. Whether FOXO is involved in the metformin-induced increase in mRNA levels of 4E-BP warrants further investigation. Furthermore, we showed that the ectopic overexpression of eIF-4E, which is a target of 4E-BP, abolished the metformin effect. These results suggested that the downregulation of eIF-4E by the 4E-BP binding may play a crucial role in lifespan extension and suppression of muscle ageing in *Drosophila* owing to metformin.

Tor can activate the ribosomal S6 kinase associated with ribosome biogenesis under normal conditions[70]. Subsequently, the S6 kinase phosphorylates components of the ribosome small subunit were involved in the translation machinery. The kinase activates the transla-

tion initiation factor 4B in mammalian cells[71]. However, expression of constitutively active S6 kinase conversely suppressed the ubiquitinated protein aggregate accumulation. Our genetic result that the expression of the hyper-active kinase cancelled the metformin's effect suggests that the downregulation of S6 kinase is also involved in the suppression effect of the medicine. Before conclusion, the hypothesis warrants further investigation. As the results suggest the involvement of the Tor-mediated pathways in the suppression effect of metformin, we speculated that partial inhibition of protein synthesis by metformin might contribute to the suppression of the muscle aging phenotypes (Figure 9).

It is also possible to consider another model where metformin promotes autophagy induction *via* activation of AMPK and inhibition of Tor activity, which is responsible for the anti-aging effects of the medicine in *Drosophila* adult muscle (Figure 9). The autophagy in muscles can eliminate damaged proteins, which accumulated as the flies aged. Tor is known to function as a central growth regulator that modulates signaling involved in cell proliferation and differentiation[8]. The kinase is also involved in repression

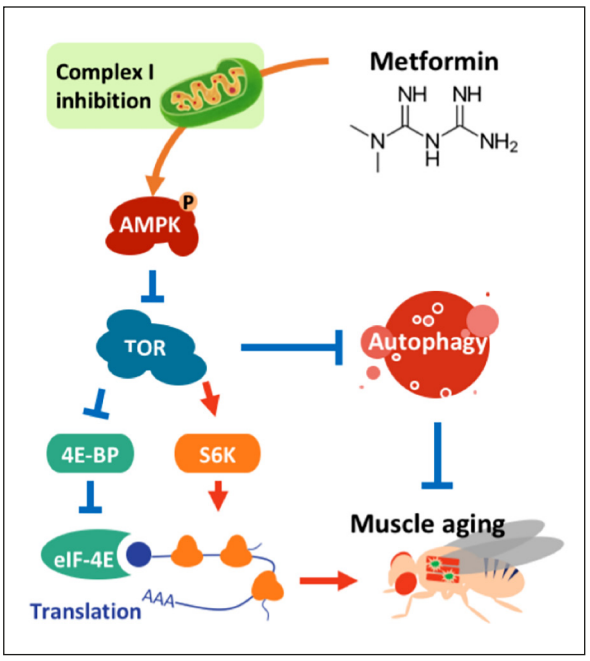

Figure 9. Metformin suppresses the age-related impairment of protein homeostasis during muscle aging through suppression of Tor-mediated signaling that regulates protein translation in the ribosome and/or stimulates autophagy induction in *Drosophila* adult.

of autophagy, depending on the Atg1 activity[53]. Autophagy is a cell clearance system responsible for removal of abnormal proteins and damaged organelles[46]. Therefore, it is possible to consider that metformin induced autophagy *via* suppression of Tor, leading to the suppression of the muscle ageing. Previous reports described that activation of mTORC1 was observed in skeletal muscle from aged mice[72]. As mice age, activity of autophagic proteolysis decreased, and thereby the amount of abnormal proteins increased[73].

In addition, the muscle-specific disruption of *Atg5* or *Atg7* gene in mice increased in amount of ubiquitinated proteins in muscle[46,74]. In summary, it was possible to estimate that the suppression effect of metformin in muscle was brought about by chronic suppression of Tor activity, and resultant activation of autophagy induction. In this study, we showed that metformin promotes autophagy induction in the adult muscle. In addition, our data that muscle-specific overexpression of constitutively active form of AMPK regenerated the suppression effect of metformin allowed us to consider that the medicine stimulates autophagy induction *via* AMPK activation (thereby downregulation of Tor). Furthermore, our muscle-specific *Atg6* depletion experiments suggests that the autophagy induction is involved in metformin-induced suppression of muscle ageing. The finding was consistent with the previous results that *Atg6*-depletion in the *Drosophila* intestine epithelial cells invalidated the metformin effect to suppress hyper-proliferation of the stem cells[27]. Another evidence that the feeding of rapamycin suppressed muscle ageing in *Drosophila via* autophagy is also consistent with our result[18]. This is consistent with previous reports[27,75] showing that muscle-specific overexpression of *Atg8* is sufficient for lifespan extension of *Drosophila* adults. On the basis of those results, we speculate that metformin may promote the autophagy induction as a consequence of AMPK activation, and thereby inhibition of Tor activity. In addition to a partial suppression of protein synthesis as discussed above, the autophagy induction is another possible process that is related to anti-ageing effects of the medicine in *Drosophila* adult muscle (Figure 9). Our findings improve our understanding of the anti-aging effect of metformin and other antioxidants presumed to possess similar properties. Previous studies[23,27] reported that metformin shows an anti-aging effect in suppressing age-dependent hyper-proliferation

of intestine stem cells through modification of Akt dependent on DNA damage accumulation. Combined with our current conclusion, we infer that metformin suppresses the progression of aging phenotypes through at least three different mechanisms in *Drosophila*. We propose that genetic methods using *Drosophila* are useful for studying the mechanism of action of various drugs. However, it should be investigated whether the results we obtained with *Drosophila* can be extended to mammalian model organisms. If confirmed, metformin will be a promising medicine for retarding the appearance of aging phenomena in multiple tissues.

Conclusions

We evaluated the anti-aging effects of metformin using the *Drosophila* adults. Continuous feeding of the medicine can extend the lifespan of *Drosophila* adults. The feeding also suppressed the accumulation of ubiquitinated protein aggregates generated in adult muscle as a consequence of age-dependent impairment of proteostasis. Metformin activates AMPK *via* the Mitochondrial Respiratory Complex I and increases of the AMP/ATP ratio in cells. Our data suggest that the activation of the AMPK-mediated pathway, thereby the downregulation of Tor-mediated pathways, and subsequent induction of an eIF-4E inhibitor were involved in the effect. The stimulation of autophagy is also involved in the anti-ageing effect. Metformin can be regarded as an anti-aging compound, which delayed the progression of muscle aging in *Drosophila* adults.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Conceptualization, Y.H.I. and S.S.; methodology, S.S. and L.T.D.; formal analysis, S.S., H.N., M.O., N.K., and L.T.D.; investigation, S.S., H.N., M.O., N.K., and L.T.D.; data curation, Y.H.I.; writing—original draft preparation, S.S.; writing—review and editing, Y.H I.; supervision, Y.H.I.; funding acquisition, Y.H.I. All authors have read and agreed to the published version of the manuscript.

Ethics Committee Approval

Not applicable.

Informed Consent

Not applicable.

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