

Ginsenoside Re Targets Drp1 Leu94 across Species to Restore Mitochondrial Balance through Atg1 ULK1 Mediated Fission and Mitophagy Coupling in Age Related Degeneration

João Silva^{1*}, Pedro Costa¹, Ana Beatriz²

¹ Department of Pharmacognosy and Mitochondrial Pharmacology, Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

² Department of Phytochemistry and Aging Research, Faculty of Pharmaceutical Sciences, University of Campinas, Campinas, Brazil.

*E-mail ✉ joao.silva@ufrj.br

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ABSTRACT

Aging exerts a major influence on the brain and acts as the leading contributor to neurodegenerative conditions, primarily via pathways involving mitochondrial impairment. Although this connection holds substantial clinical importance, the underlying molecular processes are not fully understood, and there is an immediate demand for safe and potent treatments. In this work, we explored the neuroprotective capabilities of ginseng by screening blood-brain barrier-crossing saponins for superior protective effects and pinpointed ginsenoside Re (Re) as the main mitochondrially directed neuroprotective component. Administering Re during midlife, coinciding with the natural phase of mitochondrial hyperfusion, effectively countered age-associated degenerative changes in *Drosophila*. Re treatment reduced the loss of dopaminergic neurons, lessened muscle deterioration, enhanced cognitive and motor performance, and prolonged healthspan. Mechanistic investigations showed that Re binds directly to Drp1 from various species at the highly conserved L94 site, promoting strong phosphorylation at S616. This modification facilitates Drp1 movement to mitochondria, rebalancing fission and fusion dynamics. Additionally, Re coordinated fission with mitophagy via the Drp1-Atg1/ULK1 pathway, supporting autophagosome formation and the efficient removal of impaired organelles. This combined action boosted cellular energy production and slowed functional deterioration. Removing the L94 residue in Drp1 through genetic means eliminated Re's protective outcomes, while studies in mice verified that healthspan benefits depended on functional Drp1-L94. Importantly, Re retained its neuroprotective effects in human iPSC-derived dopaminergic neurons and a *Drosophila* Parkinson's disease model, highlighting the evolutionary conservation of the Drp1-mitophagy mechanism. These results position Re as a geroprotective agent that acts on the conserved Drp1-L94 residue to reinstate mitochondrial balance. By linking fission to Atg1-driven mitophagy specifically during the critical midlife hyperfusion period, Re postpones neurodegeneration and offers a molecular foundation for therapies aimed at age-related deterioration.

Keywords: Ginsenoside Re, Brain aging, Mitochondrial dynamics, Mitophagy axis, Drp1 mutants

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Introduction

Healthy aging involves building and sustaining the capabilities required for a fulfilling life in later years and serves as a fundamental approach to managing worldwide demographic shifts toward older populations [1, 2]. However, progress is impeded by the increasing prevalence of age-related illnesses affecting multiple organs, with neurodegenerative disorders posing a particular concern. The brain is especially susceptible to aging effects, showing early deterioration in functions such as cognition and motor control—key features of conditions like Alzheimer's disease (AD) and Parkinson's disease (PD) [3-7]. These disorders frequently co-occur or are

interconnected, representing a serious risk to elderly health. According to World Health Organization estimates, the global population affected by neurodegenerative diseases stood at 55 million in 2022 and is expected to rise to 139 million by 2050 [8]. This trend presents a critical challenge for healthcare systems in mitigating brain aging-related neurodegenerative risks and extending healthspan.

Declines in brain performance can start around age 30, often appearing before changes in other organs [9, 10]. Regions like the ventral tegmental area and substantia nigra pars compacta, which contain abundant dopaminergic (DA) neurons, are among the earliest impacted by aging [11]. These neurons play essential roles in integrating cognitive and motor functions through neural networks, yet their elevated energy requirements make them highly vulnerable to mitochondrial impairments, a core element of the aging process [12, 13]. Mitochondria are dynamic structures that continuously divide and merge. During the final stages of fission, they generate daughter mitochondria with differing membrane potentials: those with higher potential typically proceed to fusion, while others are directed toward autophagy for turnover. Aging interferes with this balance, resulting in the buildup of defective mitochondria—a common pathological feature in AD and PD [14, 15]. Studies indicate that Dynamin-related protein 1 (Drp1) promotes mitochondrial fission through phosphorylation at Serine 616 (S616), which can correct defects in PTEN-induced kinase 1 (PINK1) neurons and improve hippocampal learning and memory in mouse models [16]. Likewise, restoring mitochondrial dynamics in aging satellite cells (via enhanced fission or suppressed fusion) and stimulating mitochondrial autophagy has been shown to rejuvenate regenerative capacity and address sarcopenia [17]. Although compounds such as the mitochondrial fusion promoter M1 and VLX600 offer potential for modulating mitochondrial balance [18, 19], challenges including off-target actions and unclear safety limit their clinical use, highlighting the necessity for safer options.

Natural compounds have historically provided promising alternatives or adjuncts for managing aging and associated conditions [20–22], especially those phytochemicals capable of crossing the blood-brain barrier (BBB). *Panax ginseng* is a traditional medicinal plant noted in ancient texts like Shennong's Classic of Materia Medica for its reputed longevity-promoting, cognition-enhancing, and life-extending qualities [23, 24]. Contemporary research identifies ginsenosides as the primary active constituents responsible for ginseng's anti-aging benefits. Several ginsenosides, including Rg1, Re, Rb1, and Rc, demonstrate the ability to penetrate the BBB and influence central nervous system (CNS) functions [25]. For instance, ginsenoside Rg1 (Rg1) engages the AMPK/Drp1 pathway to normalize mitochondrial fission-fusion balance in AD models, thereby improving hippocampal function [26]. Ginsenoside Re counters rotenone-triggered oxidative damage in PD *Drosophila* models by activating both PI3K/AKT and ERK signaling, thereby protecting DA neurons and maintaining motor abilities [27]. Ginsenoside Rb1 (Rb1) supports PINK1/Parkin-dependent mitophagy, which decreases ROS levels and slows brain aging [28]. The ideal timing for ginseng-based interventions to counteract aging requires further clarification. Overall, these observations point to ginsenosides as strong candidates for delaying aging and preventing related diseases. Nevertheless, most prior studies have used induced aging models that do not completely mirror the complex, progressive nature of natural aging, leaving the precise mechanisms and best intervention strategies unresolved.

The present research utilized naturally aging *Drosophila* and mouse models to thoroughly examine the timing of ginseng-based interventions for lifespan and healthspan extension, along with their capacity to alleviate age-related degenerative traits [29, 30]. Through affinity ultrafiltration combined with Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), we screened neuroactive saponins and identified Re as a major active molecule. We show that Re slows dopaminergic neuron degeneration, tissue pathology, and cognitive-motor impairments by modulating Drp1-controlled mitochondrial equilibrium and Atg1-mediated autophagy. By examining the Drp1-Atg1 signaling pathway, we clarified how Re organizes the coupling of mitochondrial fission and autophagy. Structural and genetic analyses of Drp1 and Atg1 demonstrated that Re directly interacts with Drp1 proteins across species, specifically at the conserved Leucine 94 (L94) residue, leading to S616 phosphorylation. This modification increases Drp1 localization to mitochondria and initiates a series of events: (1) mitochondrial fission, (2) recognition of damaged organelles by autophagosomes, and (3) effective elimination of compromised mitochondria.

Taken together, these results reveal a new pathway by which Re maintains mitochondrial protein homeostasis, thereby reducing age-related degeneration and promoting longer healthspan. This study supplies strong support for Re's clinical promise and creates a theoretical foundation for targeted therapies addressing mitochondrial dynamics in disorders linked to aging.

Materials and Methods

Chemicals and reagents

Total ginseng root saponins (TGGR) were acquired from Shanghai Yuanye Biotechnology Co., Ltd. (product code S25997, Shanghai, China; purity 80.6%). Pure ginsenoside Re (CAS No. 52286-59-6, purity 98%), ginsenoside Rg1 (CAS No. 41753-43-9, purity 98%), and ginsenoside Rd (CAS No. 52705-93-8, purity 98%) were obtained from Lemeitian Biotechnology Co., Ltd. (Chengdu, China). Acetylcholinesterase (AChE, catalog C3389-10KU), monoamine oxidase B (MAO-B, catalog M7441), and DMEM culture medium (catalog D5796) came from Sigma Co., Ltd. (Shanghai, China). The N-methyl-D-aspartate (NMDA) receptor preparation (catalog H00002902-Q01) was supplied by Abnova Co., Ltd. (Taiwan, China). Dopamine (DA) and ATP ELISA assay kits (catalogs YX-040100F and YX-012016F) were purchased from Shanghai Preferred Biotechnology Co., Ltd. (Shanghai, China). Additional reagents including BCA protein quantification kit (P0010S), ECL detection kit (P0018S), Lentivirus Packaging Vectors Set A (L0002S), and Lipo8000 transfection reagent (C0533) were sourced from Beyotime Biotechnology (Shanghai, China). *Drosophila* Schneider's insect medium (catalog 21720024T), TMRM mitochondrial membrane potential kit (M20036), MitoSOX Red reagent (M36008), N2/B27 neural differentiation medium (A3653401), and B27 supplement minus AA (A3582801) were provided by Thermo Fisher Scientific (Massachusetts, USA). Antibodies such as anti-tyrosine hydroxylase (TH, 1253414), anti-mouse IgG conjugated to Alexa 488 (A28175), and goat anti-mouse Alexa 647 (A21235) were obtained from Invitrogen (California, USA). Other primary antibodies including anti-ATP5a (14748), anti-P62 (178400), anti-dsDNA (27156), anti-NDUFS3 (14711), anti-Mfn2 (ab56889), anti-Hsp90 (EPR3953), anti-GABARAP/GABARAPL1/GABARAPL2 (ab109364), and anti-UQCRC2 (203832) were from Abcam (Cambridge, UK). From Cell Signaling Technology (Massachusetts, USA) we obtained anti-FK2 (3936T), anti-Atg8a/LC3 (E1J4E), anti-Drp1 (8570S), anti-phospho-Drp1 Ser616 (3455S), anti-ULK1 (8054T), anti-OPA1 (D6U6N), Phospho-(Ser) PKC substrate antibody (2261S), and anti-rabbit IgG Alexa 647 (4414S). Anti- β -actin (AP0060), anti-Fis1 (BS7881), and anti-COX-4 (P13073) were purchased from BioWorld Technology (Wuhan, China). Purified recombinant Drp1 protein (Ag24263) and goat anti-rabbit Alexa 488 secondary antibody (RGAR002) came from Proteintech (Wuhan, China). Actin-Tracker Red (catalog 112-585-003) was supplied by Jackson ImmunoResearch (California, USA). VDAC1 antibody (MEXHY-P80369), along with small molecules DAPT (HY-13027), SAG (HY-12848), Noggin (HY-P70558), CHIR-99021 (SML1046), and Y-27632 ROCK inhibitor were acquired from Merck (Darmstadt, Germany). FGF8b (FGB-H5115) and GDNF (GDF-H5118) were obtained from ACRO Biosystems (Beijing, China).

Ethics statement

All procedures involving live animals strictly followed the ARRIVE reporting guidelines, the UK Animals (Scientific Procedures) Act 1986, and the WMA principles regarding animal use in scientific research. The experimental protocols received formal approval from the Institutional Animal Care and Use Committee of Changchun University of Chinese Medicine (Jilin, China; approval No. 2025379) to guarantee proper animal handling, welfare, and reliable scientific reporting.

Affinity ultrafiltration

Affinity ultrafiltration screening was carried out based on the standard procedure reported earlier [31]. TGGR powder was accurately weighed and dissolved in phosphate-buffered saline (PBS) to generate a 10 mg/mL working solution. Target proteins (AChE, MAO-B, and NMDA receptor) were individually prepared at 1 mg/mL in PBS. Equal volumes (200 μ L each) of the TGGR solution and each target protein solution were combined and incubated at 37°C for 60 minutes in a water bath. Following incubation, the mixtures were centrifuged at 1,000 $\times$ g for 20 minutes and the pellets were washed five times using fresh PBS. The retained complexes were loaded onto 2 mL ultrafiltration devices. Bound compounds were then eluted by successive washes with 200 μ L, 100 μ L, and 50 μ L of 50% methanol in water, with each wash subjected to 15-minute centrifugation. All eluates were pooled, evaporated to dryness under a nitrogen stream at 40°C, and the residue was redissolved in 50 μ L methanol. After a final centrifugation at 1,000 $\times$ g for 10 minutes, the clear supernatant was collected for downstream analysis.

UPLC-Q-TOF-MS analysis

UPLC-Q-TOF-MS analysis was performed using a ZORBAX Eclipse Plus C18 analytical column (Agilent Technologies, China). Samples (10 μ L) were injected at a flow rate of 0.3 mL/min. The mobile phase comprised 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient conditions were: 15–30% B from 0 to 10 min, held at 30% B for 10–25 min, 30–50% B for 25–35 min, 50–70% B for 35–45 min, 70–90% B for 45–50 min, returned to 15% B over 50–55 min, and held at 15% B until 60 min. Electrospray ionization served as the ion source, with data acquired in both positive and negative ion modes. TOF-MS scan range was set to 100–2000 Da and MS/MS to 50–2000 Da. Key parameters included GS1 and GS2 at 60 psi, curtain gas at 35 psi, source temperature 500°C, ion spray voltage 5000 V, declustering potential 100 V, collision energy 10 V for precursors and 45 V for fragments, and collision energy spread of 15 V. The binding rate of individual compounds was computed as binding degree (%) = $(A_a / A) \times 100$, where A is the chromatographic peak area of the compound in TGGR alone and A_a is the peak area recovered from the target-bound fraction.

AChE and MAO-B inhibitory activity

Inhibition of acetylcholinesterase was determined using *Electrophorus electricus* AChE (Type VI-S) with 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 0.5 mM acetylthiocholine iodide as substrate, according to established protocols [32, 33]. MAO-B inhibitory activity was evaluated with benzylamine (0.3 mM) as substrate following previously published methods [34]. Substrate concentration was maintained at approximately $1.9 \times K_m$ ($K_m = 0.16$ mM). Percent inhibition was calculated using the equation: inhibition (%) = $[(A_b - A_i) / A_b] \times 100$, where A_b denotes absorbance of the untreated control and A_i represents absorbance in the presence of the test sample.

NMDA receptor inhibitory activity

Electrophysiological evaluation of NMDA receptor inhibition was conducted via whole-cell patch-clamp technique on HEK293T cells heterologously expressing GluN1-4a/GluN2A subunits, as outlined in earlier publications [35, 36]. Cells were bathed in extracellular solution composed of (mM): 160 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 0.2 EDTA, 0.7 $CaCl_2$ (pH 7.3, adjusted with NaOH). Borosilicate glass pipettes (3–6 M Ω) pulled with a P-1000 micropipette puller were filled with internal solution containing (mM): 125 gluconic acid, 15 CsCl, 5 EGTA, 10 HEPES, 3 $MgCl_2$, 0.5 $CaCl_2$, 2 Mg-ATP (pH 7.2 with CsOH). Recordings were performed at room temperature across holding potentials from -80 mV to $+60$ mV in 20 mV increments. Currents were elicited by rapid perfusion of 1 mM glutamate in the continuous presence of 100 μ M glycine. Data were processed with Clampfit 10.2 software (Molecular Devices). The degree of inhibition was expressed as the percentage of glutamate-evoked current remaining after compound application relative to the steady-state current before inhibition.

Experimental animals

Drp1 L94A knock-in (KI) mice were created using CRISPR/Cas9 genome editing technology at the Shanghai Model Organisms Center. In brief, the guide RNA sequence (GAAGGCGGAATAGCAGTGCTTGG) was chosen based on on-target and off-target prediction scores from the CRISPR design tool. CRISPR RNA and trans-activating crRNA were combined in 100 μ L of nuclease-free duplex buffer to reach a final concentration of 1 mM each. The mixture was heated to 95°C for 5 minutes and then cooled gradually to room temperature. Ribonucleoprotein complexes were formed by incubating the guide RNA duplex with Cas9 protein at 37°C, followed by addition of single-stranded DNA donor oligonucleotides carrying the Drp1 L94A mutation. The injection mixture was introduced into the cytoplasm of fertilized C57BL/6 eggs using piezo-driven microinjection. All experimental mice were female, with body weights ranging from 25 to 30 g at the start of experiments. They were housed in specific pathogen-free facilities under a 12-hour light/12-hour dark cycle, maintained at 22–24°C with 50–60% relative humidity, and provided unlimited access to standard chow and water. Anesthesia was performed using 2% isoflurane.

Canton-S, da-Gal4, and PINK1B9 [w(*) Pink1 (B9)] mutant strains were kindly provided by Dr. Yufeng Yang (Institute of Life Sciences, Fuzhou University, Fuzhou, China). The following *Drosophila* lines were obtained from the Bloomington *Drosophila* Stock Center: Mef2-Gal4 (BDSC_50742), TH-Gal4 (BDSC_51982), UAS-Drp1 (BDSC_51647), UAS-Drp1 RNAi (BDSC_67160), UAS-Marf (BDSC_67157), UAS-Marf RNAi (BDSC_67158), UAS-Atg1 RNAi (BDSC_44034), and UAS-Atg5 RNAi (BDSC_27551). Tissue-specific transgene expression in adult flies was achieved using the Gal4/UAS binary system following standard crossing schemes. hDrp1 S616A transgenic flies were generated according to the previously established protocol [37]. Drp1 L94A mutant flies in the Canton-S background were produced via CRISPR/Cas9 editing. Target sequences

were inserted into the pU6-BbsI-chiRNA plasmid. Suitable target sites near the Drp1 locus were identified using the fly-CRISPR Optimal Target Finder online tool. The donor plasmid and pU6-BbsI-chiRNA constructs were co-injected into Canton-S embryos. The Drp1 L94A donor template was amplified from the original Drp1 donor using PCR and circularized via seamless cloning. Amplification primers were: forward 5'-TGCAGGACGTCTTCAACACCGT-3' and reverse 5'-TGTTGATGTAGGCCAGCTCGA-3'. Flies were raised at 25°C with 60% relative humidity under a 12-hour light/12-hour dark cycle. All experimental flies were female and maintained on standard cornmeal-molasses food containing corn flour (62.5 g/L), soybean flour (10.7 g/L), yeast (21.4 g/L), sucrose (17.8 g/L), glucose (17.8 g/L), agar (6.8 g/L), methylparaben (1.4 g/L), and propionic acid (7 mL/L). Flies were anesthetized using CO₂.

Re, Rg1, and Rd treatment

Dosages were selected based on earlier reports and appropriate interspecies conversion factors [38]. Mice received oral administration of Re at 10 mg/kg body weight, beginning at 8 months of age and continuing every 3 days for a total duration of 10 months.

For *Drosophila* experiments, Re, Rg1, and Rd were incorporated directly into the sugar-yeast agar (SYA) medium. Tested concentrations were: Re (0.15, 0.25, 0.3, 0.4, 0.5, 0.6, and 0.8 mg/mL), Rg1 (0.125, 0.25, 0.3, 0.4, 0.5, 0.6, and 0.8 mg/mL), and Rd (0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 mg/mL). For lifelong treatment, 2-day-old adult flies were transferred to drug-supplemented medium and maintained there until death. Early-life intervention involved exposure from day 2 to day 22. Mid-life intervention started at day 22 and continued until day 42. Late-life intervention began at day 42 and lasted until the end of life. Ginsenosides were added and thoroughly mixed into the molten fly food to achieve uniform distribution. Drug-containing vials were replaced every other day. Fresh preparations were made weekly and stored at 4°C in the dark to preserve compound stability [39].

Analysis of lifespan

For mice, survival monitoring during the first year was conducted every 3 months (n = 30 per group). In the second year, checks occurred monthly, shifting to weekly observations after 2 years of age. Survival curves were generated using GraphPad Prism 9 software based on recorded mortality.

For flies, 100 newly eclosed (2-day-old) females per group were collected and placed on fresh medium, which was replaced daily. Deaths were recorded daily until all individuals had died. Survival curves were plotted using GraphPad Prism 9.

2.10. Morris water maze (MWM) analysis

The apparatus consisted of a circular tank 180 cm in diameter and 60 cm in height. One day prior to training, mice were allowed a 60-second habituation session in the pool. Over the subsequent 4 days, animals were trained to locate a hidden platform submerged 1 cm below the water surface; escape latency was recorded for each trial. Mice that did not locate the platform within 60 seconds were gently guided to it and allowed to remain there for 30 seconds. On the final day, a 60-second probe trial was performed with the platform removed. The percentage of time spent in the target quadrant and the number of platform crossings were recorded and analyzed using video tracking software.

Open field test (OFT) analysis

The open field apparatus measured 40 cm × 40 cm × 40 cm, with a defined central zone of 20 cm × 20 cm. Experiments were conducted in a quiet environment starting at 9:30 a.m. using an automated video tracking system. Mice were placed individually in the center of the arena, and activity was recorded for 5 minutes. Locomotor parameters were subsequently analyzed from the recorded video data.

Analysis of climbing ability

Flies were briefly anesthetized and transferred into vertical climbing tubes (25 cm length, 1.6 cm diameter). After a 10-minute acclimation period at 25°C, flies were tapped to the bottom of the tubes. The position of each fly within 9 equal divisions (total height 18 cm) was scored after 5 seconds. Climbing performance was quantified by multiplying the score of each section by the number of flies in that section and summing the values [40].

Assays of locomotor activity

Individual flies were placed into glass tubes (5 × 65 mm) containing fresh medium and inserted into a DAM2 Drosophila Activity Monitor system (TriKinetics, USA). Activity data were collected and processed using DAM System 310 software. Beam crossings were recorded every 5 minutes under a 12-hour light/12-hour dark cycle for 24 hours. Circadian activity patterns were visualized using MATLAB software.

Analysis of learning and memory abilities

Learning and memory performance was assessed using a T-maze apparatus according to a previously validated protocol [41]. Groups of 30 flies were loaded into training tubes lined with mineral water-soaked cotton. Odors 3-octanol (OCT) and 4-methylcyclohexanol (MCH) were diluted to 1% in paraffin oil and delivered via volatile containers. Training tubes were coated with filter paper soaked in 1.5 M sucrose solution and air-dried. Each conditioning session started with 90 seconds of clean air, followed by 120 seconds of one odor, a 30-second clean air interval, 120 seconds of the second odor, and a final 30-second clean air period. Sucrose reward was paired with one of the two odors. After training, flies were given 2 minutes to choose between the two arms of the T-maze (each scented with OCT or MCH) starting 30 seconds post-conditioning. The Performance Index (PI) was calculated as the difference between the number of flies choosing the rewarded odor arm and those choosing the unrewarded arm, divided by the total number of flies.

Blinding and reproducibility

Behavioral tests and their subsequent data evaluations were carried out by experimenters kept unaware of the specific group allocations. An uninvolved colleague generated random identification codes for each fly cohort prior to any experimentation. These coded designations were employed consistently for recording observations, capturing outputs from automated systems, and conducting statistical tests. Group identities remained concealed until every statistical analysis had been finalized.

To promote consistency across experiments, all fly behavioral procedures adhered to uniform, well-defined protocols. Both treated and control fly populations were cultured at the same time by identical staff under matching environmental parameters and using the same nutritional medium. Testing occurred specifically on post-eclosion day 42, within a fixed 2–6 hour interval after the onset of the light period, inside a controlled, isolated facility free from interruptions. Flies received fresh housing tubes and a brief acclimation period immediately before each test. Regular calibration of all automated devices helped sustain performance reliability. Results were derived from no fewer than 6 separate biological replicates, each involving 60 individual flies. Survival data underwent Log-rank testing, while multi-group comparisons relied on ANOVA.

Analysis of starvation, heat, and oxidative stress

Flies (n = 50 per group, aged 22 days) were maintained on standard SYA food, either containing Re or without it, until reaching 42 days of age. They were subsequently shifted to SYA medium supplemented with 1% agar to assess starvation resistance, or to medium containing 5% (w/v) H₂O₂ to evaluate oxidative stress tolerance. Heat stress was applied by exposing flies to a 37°C chamber. Dead flies were counted at regular 2-hour intervals. Corresponding survival plots were prepared in GraphPad Prism 9.

Detection of compound eye degeneration

Heads from adult flies were harvested and stored frozen at –80°C for 1 hour ahead of examination. Surrounding non-eye tissues were removed by microdissection, after which compound eye morphology was visualized and photographed under a Zoom-stereomicroscope (OPTEC, China) equipped with a 200 µm scale bar.

Micro computed tomography (Micro CT)

Thoracic muscle samples were first fixed for 3 hours in 4% paraformaldehyde, followed by immersion for 12 hours at ambient temperature in PBS solution containing 1% iodine and 2% potassium iodide. Individual thoraces were secured onto a precision positioning stage using Vaseline, with wings serving as anatomical landmarks for proper alignment. Scanning was performed on a PerkinElmer micro CT system (USA) set to 90 kV and 88 µA, using high-resolution mode with a 36 µm isotropic voxel size; each scan required about 20 minutes. Muscle tissue cross-sectional areas were then quantified from the reconstructed images via ImageJ analysis software.

Transmission electron microscopy (TEM)

Sample preparation for ultrastructural examination largely followed the approach detailed in reference [42], incorporating several minor adaptations. Dissected mouse brain and muscle tissues, together with fly dorsal longitudinal flight muscles (DLMs), underwent primary fixation in 2% glutaraldehyde. Subsequent processing involved resin infiltration with Eponate 12, embedding, and overnight polymerization. Semithin sections of roughly 1.5 μm thickness were produced using an ultramicrotome and briefly stained with toluidine blue to select appropriate regions for further study. Final ultrathin sections were viewed on a JEOL 100CX transmission electron microscope (Japan) operated at 60 kV. Mitochondrial lengths were measured quantitatively from consistently captured electron micrographs using ImageJ software.

ELISA

Tissues from fly brains, fly muscles, and mouse striatal regions were homogenized in physiological saline (1 mg tissue per 900 μL) with a mechanical grinder, then centrifuged (600 g, 10 min, 4°C). Supernatants were retained for quantification of dopamine (DA) and ATP levels according to the supplied ELISA kit protocols. Absorbance readings at 450 nm were obtained on an Infinite 200 Pro plate reader (Tecan, China), with final concentrations reported as pg per mL of protein.

Protein measurements

Total protein extraction from flies was performed via mechanical grinding as described earlier (section 2.14). BSA calibration standards were generated across a concentration range of 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL. For each sample, 2.0 μL of extract was diluted with 18.0 μL PBS and combined with 200 μL of BCA reagent in multi-well plates. Following a 20-minute incubation at 37°C, absorbance was recorded at 562 nm using an Infinite 200 Pro microplate reader (Tecan), allowing calculation of protein concentrations against the standard curve.

Analysis of mitochondrial respiratory chain complex activity

Mitochondrial fractions were obtained using a dedicated isolation kit. Complex I enzymatic function was determined by monitoring absorbance decline at 600 nm in a reaction mix that included 200 μM NADH, 240 μM KCN, 60 μM DCIP, 70 μM decacyclobenzoquinone, 25 μM antimycin A, and 2 μM rotenone. Complex II activity was similarly assayed at 600 nm in buffer supplemented with 2 mM malate, 10 mM glutamate, and 5 mM ADP. Complex III measurements at 550 nm utilized a medium containing 200 μM coenzyme Q10, 50 μM cytochrome c, 100 mM potassium phosphate, 250 μM EDTA, and 1 mM KCN, performed both with and without 2.5 mg/mL antimycin A. Complex IV activity was tracked as the rate of reduced cytochrome c oxidation at 550 nm, employing 5 μg of mitochondrial protein in buffer composed of 10 mM Tris-HCl, 120 mM KCl, 12.5 mM sucrose, and 110 nM reduced cytochrome c. All recorded activities were adjusted according to total mitochondrial protein levels.

TMRE staining analysis

After anesthesia, fly hemi-thoraces and brains were isolated in chilled DSM buffer. The dissected samples were exposed to 100 nM TMRE in DSM for 12 minutes at room temperature. Control tissues received a 10-minute pre-incubation with 20 μM FCCP prior to TMRE exposure. Post-incubation imaging was conducted on a Leica SP8 confocal microscope (Germany).

MitoSox staining analysis

Hemi-thoraces and brains from anesthetized flies were dissected in cold DSM. Hemi-thoraces were then stained for 12 minutes at room temperature in DSM containing 5 μM MitoSOX Red, followed by two brief 30-second rinses in fresh DSM before confocal imaging.

Immunofluorescence (IF) staining

Fly brain and muscle tissues were fixed for 30 minutes in 4% paraformaldehyde and blocked for 90 minutes with 5% goat serum at room temperature. Overnight incubation at 4°C was performed using primary antibodies (1:200) targeting TH, ATP5a, FK2, P62, Atg8a/LC3, and dsDNA. The following day, fluorescent secondary antibodies were applied for 2 hours at 25°C. Muscle preparations additionally received Actin-Tracker Red-Rhodamine for a further 30 minutes. DAPI nuclear counterstaining lasted 10 minutes in the dark at room temperature. Mounted specimens (using ProLong Antifade) were examined with a Leica SP8 confocal microscope (Wetzlar, Germany). TH-immunoreactive PPL1 neurons were enumerated per brain hemisphere across 30 flies per batch and 6

independent biological repeats. Mitochondrial and protein aggregate areas were measured in ImageJ, with aggregate signals normalized relative to DAPI area.

For mouse samples, paraformaldehyde-fixed brain and muscle tissues were sectioned after paraffin embedding. Sections were blocked with 3% BSA (30 min, room temperature) and incubated overnight at 4°C with anti-Atg8a/LC3 (1:200). Fluorescent secondary detection was performed for 1 hour at room temperature, followed by DAPI staining and imaging on a Leica SP8 confocal system.

Immunohistochemistry

Deparaffinized and rehydrated brain sections underwent peroxidase quenching with 3% H₂O₂ for 15 minutes. After antigen retrieval and blocking (5% BSA in PBST), anti-TH antibody (1:200) was applied overnight at 4°C. HRP-linked secondary antibody incubation lasted 1 hour at room temperature, and staining was visualized with DAB reagent for approximately 5 minutes. Sections were then counterstained, dehydrated, mounted, and photographed using a Zoom-stereomicroscope.

Western blot

Mitochondria were isolated via a commercial kit. Protein lysates from brain, muscle, whole-cell, and mitochondrial samples were prepared in RIPA buffer plus 1% protease inhibitors. Concentrations were quantified by enhanced BCA assay. Equal amounts of protein were denatured by boiling in sample buffer (5 min), resolved on 10–15% SDS-PAGE gels, and electrotransferred to PVDF membranes. Membranes were blocked for 2 hours with 5% non-fat dry milk and incubated overnight at 4°C with primary antibodies (1:1000) directed against P62, Drp1, phospho-Drp1 (Ser 616), ULK1 (cross-reactive with *Drosophila* Atg1), NDUFS3, SDHA, UQCRC2, COX-4, ATP5a, Fis1, Mfn2, OPA1, Atg8a I/II, Hsp90, β -actin, and VDAC1. HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit) were applied for 1 hour at room temperature, and bands were detected via ECL chemiluminescence. Densitometric quantification of band intensities was performed in ImageJ, using β -actin or VDAC1 for normalization.

Co-immunoprecipitation (Co-IP)

Co-IP experiments utilized a commercial immunoprecipitation kit (P2179M, Beyotime) and followed the supplier's recommended steps. Extracted proteins were incubated overnight at 4°C together with either phospho-(Ser) PKC substrate antibody or control IgG, followed by precipitation using protein A+G agarose beads for 1 hour at room temperature. The resulting precipitates were rinsed thoroughly and then boiled for 5 minutes. Western blotting of these samples was subsequently conducted using specific anti-Hsp90 antibodies.

Oxidative phosphorylation (OXPHOS) capacity and electron transport system (ETS) capacity detection

Mitochondrial respiratory function was assessed in permeabilized tissues according to the established protocol described previously [42]. Measurements were carried out at 25°C with high-resolution respirometry (Oxygraph-2k, Oroboros, Austria) in oxygen-saturated medium (550–350 nmol/mL O₂) [43]. Fly thoraces were permeabilized using 50 μ g/mL saponin in ice-cold BIOPS buffer, rinsed in MiR05 respiration medium, weighed, and placed into the instrument chambers (two thoraces per chamber). Fly heads were similarly permeabilized with 25 μ g/mL saponin in BIOPS, rinsed quickly, and loaded into chambers (ten heads per chamber).

A substrate-uncoupler-inhibitor titration (SUIT) protocol with sequential additions of saturating substrates was used to evaluate OXPHOS and ETS capacities. For complex I-supported respiration, the following were added: 5 mM pyruvate, 5 mM proline, 2 mM malate, 10 mM glutamate, and 2.5 mM ADP (for thoraces) or 5 mM pyruvate, 5 mM proline, 2 mM malate, and 2.5 mM ADP (for heads). Maximal convergent flux through complexes I+II (OXPHOS capacity) was triggered by supplementation with 15 mM glycerol-3-phosphate and 10 mM succinate. ETS capacity was determined through progressive titration of 0.5 μ M FCCP to reach peak uncoupled respiration. Rotenone (0.5 μ M) was then introduced to inhibit complex I and isolate its contribution. Finally, non-mitochondrial oxygen consumption was recorded after adding 2.5 μ M antimycin A. All respiration rates were normalized to tissue wet weight and corrected by subtracting residual non-mitochondrial activity.

Cell culture and treatment

HEK 293T cells were cultured in DMEM containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, maintained at 37°C in a humidified 5% CO₂ atmosphere. Cultures were routinely passaged upon reaching 90% confluence every three days. Transfection procedures followed the established method

previously reported [44]. Drp1-targeted siRNA was obtained from Santa Cruz (Texas, USA). Expression plasmids encoding *Drosophila* Drp1 (wild-type, L94A, and H95A variants) and mouse Drp1 (wild-type and L94A) were generated by Saixin (Jilin, China). Lentiviral packaging and transfection reagents (Lipo 8000 TM kit) were used per manufacturer guidelines. In brief, 100 nM siRNA or 2 µg plasmid DNA was complexed with Lipo 8000 in Opti-MEM before addition to the cells.

Human induced pluripotent stem cells (iPSCs) were sourced from Procell Life Science & Technology Co., Ltd. Differentiation into dopaminergic neurons was performed using a previously optimized protocol [45]. iPSC colonies were dissociated with Accutase for 20 min at 37°C and seeded at high density onto Geltrex-coated plates in N2/B27 neural induction medium supplemented with 200 ng/mL Noggin and 10 µM SB-431542. SAG (1 µM) was introduced on day 1, and CHIR-99021 (3 µM) was added on day 3. Gradual medium transition from DMEM/F12 + N2 to Neurobasal + B27 began on day 4 (75:25), continuing through day 5 (50:50), day 6 (25:75), day 7 (5:95), and day 8 (0:100). On day 6, neural progenitors were passaged onto plates coated with 50 µg/mL poly-D-lysine and 10 µg/mL laminin in a mixed DMEM/F12/Neurobasal medium containing N2, B27, and growth factors (200 ng/mL Noggin, 10 µM SB-431542, 1 µM SAG, 3 µM CHIR-99021, and 10 µM ROCK inhibitor). FGF8b (100 ng/mL) was supplemented on days 9–11. From day 13 onward, cells were maintained in neuronal differentiation medium supplemented with 20 ng/mL BDNF, 20 ng/mL GDNF, 2.5 µM DAPT, 500 µM cAMP, 1 ng/mL TGF-β, and 200 µM ascorbic acid. Upon completion of differentiation, medium was refreshed with 2 mL of neuronal differentiation medium. Mature neurons were then treated for 24 h with aging-inducing compounds O151 (10 µM) [46] and Re (3 µM).

Cell viability by cell counting kit-8 (CCK-8) assay

Cells were seeded at a density of 2.0×10^5 cells/mL. Re stock solution (1 mg/mL in PBS) was diluted in complete medium to final concentrations of 0.5, 1, 3, 8, 15, 30, 50, 70, 90, or 100 µM. After treatment, culture medium was removed and 100 µL of fresh CCK-8 working solution was added per well. Plates were incubated at 37°C for 0.5–4 h protected from light, after which absorbance was recorded at 450 nm.

Cell thermal shift assay (CETSA)

Transfected HEK 293T cells or fly tissue lysates were incubated for 2 h in serum-free medium containing either 0.1% DMSO vehicle or 3 µM Re. Cells were harvested, aliquoted into 0.2 mL PCR tubes, and heated for 3 min across a temperature gradient from 48°C to 72°C in a thermal cycler. RIPA lysis buffer was added to each aliquot, followed by vigorous mixing and centrifugation at 1000 g for 20 min at 4°C to clear insoluble material. Supernatants were then processed according to standard Western blot procedures.

Molecular docking analysis

Docking simulations between Re and Drp1 were executed using AutoDock 4.2 software, with resulting poses visualized in PyMOL. Binding free energies were refined via MM/GBSA calculations for each docking conformation. Key interacting residues were determined through per-residue energy decomposition analysis.

Surface plasmon resonance (SPR)

SPR experiments were conducted on a Biacore 8K system (Cytiva, Sweden). Drp1 protein (0.5 mg/mL, pH 4.5) was covalently immobilized onto a Series S sensor chip using standard amine-coupling chemistry. PBS buffer containing 5% DMSO served as the running buffer during immobilization. Serial dilutions of Re prepared in running buffer were injected at seven different concentrations over the sensor surface at a flow rate of 65 µL/min with a 60 s association phase at 25°C. Reference-subtracted sensorgrams were generated, and data were fitted to an appropriate kinetic model using Biacore 8K evaluation software to determine the equilibrium dissociation constant (K_d).

Microscale thermophoresis (MST)

Re was serially diluted to concentrations ranging from 50 mM to 24.4 µM and incubated with 80 nM fluorescently labeled purified Drp1 protein for 15 min in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). Mixtures were loaded into NanoTemper glass capillaries, and thermophoresis measurements were performed at 80% LED power and 80% MST power. K_d values were derived from duplicate experiments using the mass action law model within NanoTemper analysis software.

Statistical analysis

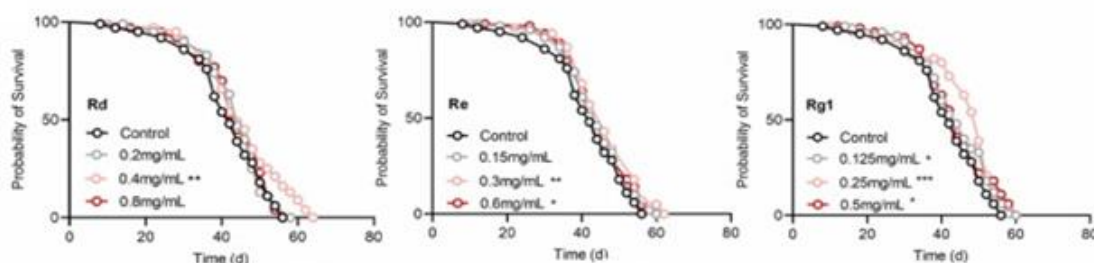
Quantitative data were processed and graphed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) and presented as mean $\pm$ SD. Survival curves were compared via Log-rank (Mantel-Cox) test. Other datasets were evaluated using Student's t-test, one-way ANOVA, or two-way ANOVA as appropriate, with results expressed as mean $\pm$ SD. Statistical significance was denoted as follows (unless stated otherwise): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; # $p < 0.01$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$. Schematic illustrations were created with BioRender 2023 software (BioRender, CA, USA) and include appropriate copyright licensing.

Results and Discussion

Identification of neuroactive ginsenosides via affinity ultrafiltration-UPLC-QTOF-MS

Age-related cognitive decline and impaired motor function are strongly associated with diminished cholinergic signaling, disrupted dopamine homeostasis, and reduced synaptic plasticity in the aging brain [47-49]. Leveraging this knowledge, AChE, MAO-B, and NMDA receptor were selected as primary screening targets to identify bioactive components within ginseng saponins capable of modulating neurodegenerative pathways. Affinity ultrafiltration coupled with UPLC-Q-TOF-MS profiling identified 14, 7, and 13 distinct monomers from total ginseng glycosides (TGGR) that interacted with AChE, MAO-B, and NMDA receptors, respectively. Binding affinity quantification highlighted Rd, Re, and Rg1 as standout candidates showing superior target engagement compared to other saponins: Rd exhibited binding percentages of 7.56% (AChE), 63.25% (MAO-B), and 76.37% (NMDA); Re showed 40.00%, 81.32%, and 68.79%; while Rg1 displayed 44.74%, 58.57%, and 61.84%. Follow-up functional inhibition assays validated their activity. All three compounds demonstrated effective target suppression. Re exhibited strong inhibition of AChE ($IC_{50} = 31.42 \mu M$) and NMDA receptor ($IC_{50} = 26.42 \mu M$). Rg1 proved most potent against MAO-B ($IC_{50} = 25.37 \mu M$). Rd displayed balanced, broad inhibitory effects across all three targets, consistent with its high binding profiles. These results confirmed that Rd, Re, and Rg1 can meaningfully influence critical neuronal signaling cascades, positioning them as strong leads for deeper investigation.

Using these integrated screening outcomes, the neuroprotective potential of ginsenosides Rd, Re, and Rg1 was further examined in a naturally aging *Drosophila* model, focusing on lifespan extension and mitigation of age-associated neurodegenerative features. When administered at their respective optimal doses (Rd 0.4 mg/mL, Re 0.3 mg/mL, Rg1 0.25 mg/mL), each ginsenoside significantly extended fly lifespan (**Figure 1a**), although differences among the three compounds were not statistically significant (**Figure 1b**). In 42-day-old aged flies, clear neurodegenerative deficits were evident relative to young (7-day-old) controls, including a 40% reduction in learning index (**Figure 1c**), 23% decrease in climbing performance (**Figure 1d**), and 28% drop in spontaneous locomotion (**Figures 1e, 1g**). While lifespan benefits were comparable, Re demonstrated superior rescue of behavioral impairments at 0.5 mg/mL (the concentration aligned with optimal behavioral outcomes). Compared with aged controls, Re treatment improved learning index by 55% ($p < 0.0001$), climbing ability by 23% ($p < 0.05$), and locomotor activity by 33% ($p < 0.0001$), outperforming both Rd and Rg1 (**Figures 1c, 1g**). Consequently, subsequent mechanistic investigations centered primarily on Re.



a)

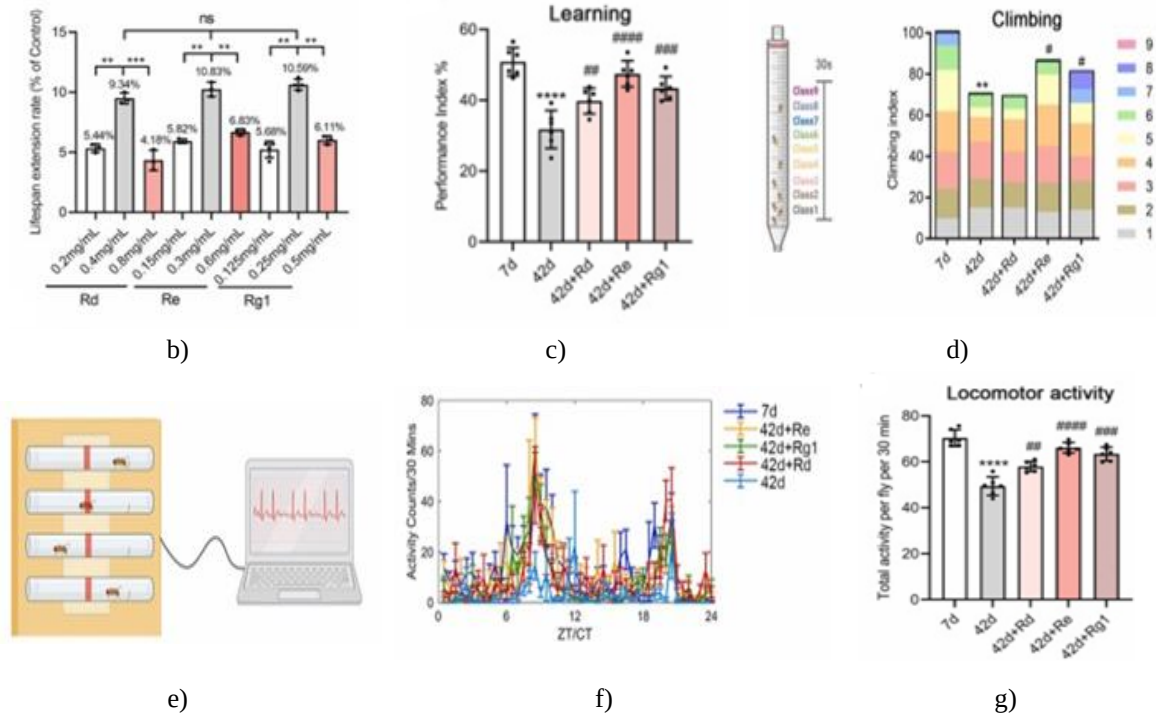
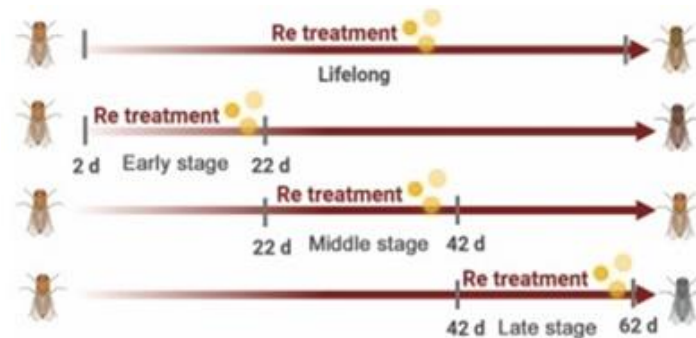


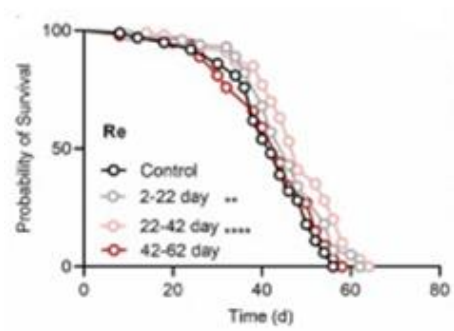
Figure 1. Screening of neural activity of different ginsenosides. (a) Survival curves for flies receiving various ginsenoside treatments, with $n = 100$ flies per group. (b) Quantitative comparison of mean lifespan extension percentages across the different ginsenosides. (c) Assessment of learning performance, (d) negative geotaxis climbing ability, and (e-g) spontaneous locomotor activity following treatment with the ginsenosides. Each experiment included $n = 6$ independent replicates with 60 flies per replicate. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus 7-day-old flies or untreated controls; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ versus 42-day-old aged flies. Analyses were performed using Log-rank test for survival data and one-way ANOVA for behavioral measures.

Midlife intervention represents the optimal window for Re to ameliorate age-related degeneration

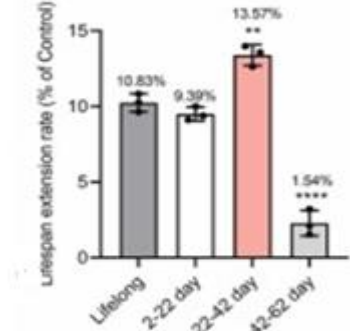
Because continuous, lifelong drug administration is rarely feasible in clinical settings, determining the most effective timing for treatment initiation is essential for maximizing healthspan benefits. Previous work from our group established that administering total ginseng glycosides (TGGR) during midlife effectively extends healthy lifespan and preserves intestinal function in *Drosophila* [50, 51]. Extending this concept, we investigated how the timing of Re supplementation influences outcomes when applied during early adulthood (days 2–22), midlife (days 22–42), or late life (days 42–62) (**Figure 2a**). Midlife Re treatment produced the strongest lifespan-prolonging effect, elevating the average extension rate from 10.83% (lifelong treatment) to 13.57% (**Figures 2b, 2c**). Intervention restricted to early adulthood also yielded a significant increase in longevity, though the mean extension rate reached only 9.39%, which was lower than continuous lifelong exposure. In contrast, supplementation confined to late life stages failed to produce any meaningful improvement in overall lifespan.



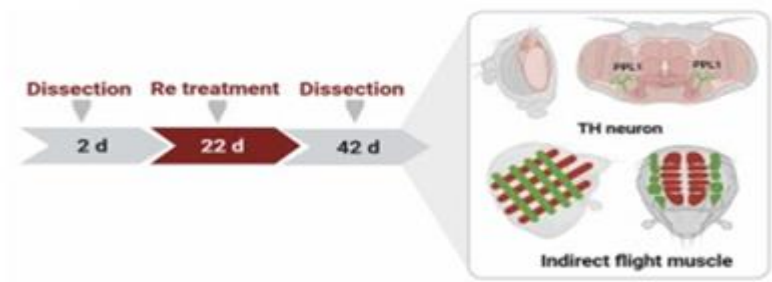
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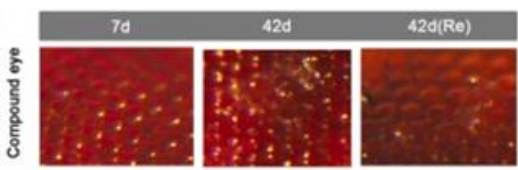
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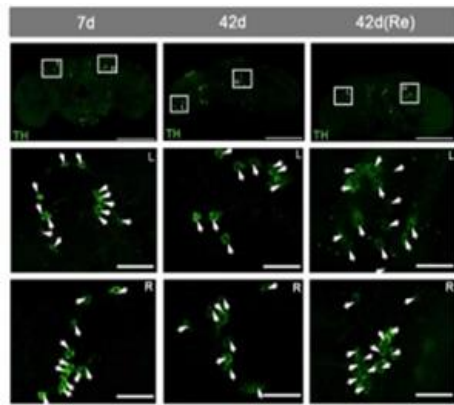
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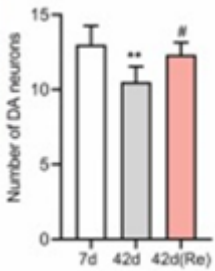
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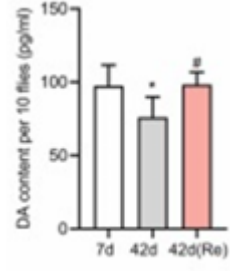
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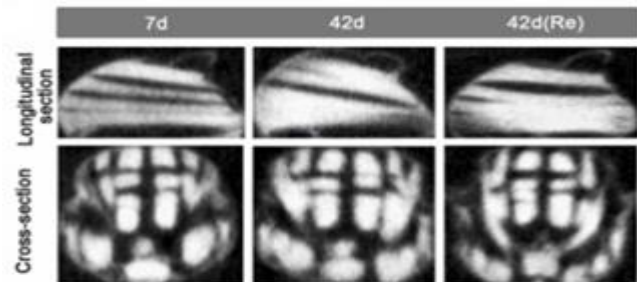
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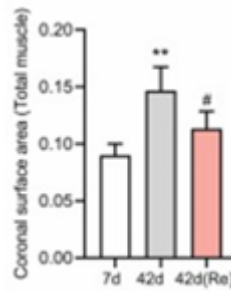
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Figure 2. Midlife Re intervention delays age-related pathology and extends healthspan. (a) Overview diagram illustrating the timing of Re administration during different life stages. (b-c) Lifespan curves and quantitative comparison of survival extension following early, midlife, late, and continuous Re treatments (n = 100 flies per group). (d) Timeline and anatomical overview of midlife Re supplementation. (e) Representative images of compound eyes from 7-day-old young flies, 42-day-old aged controls, and 42-day old flies treated with Re during midlife. (f) Immunofluorescence staining and (g) quantification of TH-positive PPL1 dopaminergic neurons in each brain hemisphere (green: anti-TH; scale bars = 128 μ m and 25 μ m), n = 6 per group. (h) Measurement of dopamine (DA) concentration in fly brain extracts by ELISA, n = 6 per group. (i) Typical cross-sectional micro-CT images and (j) quantitative analysis of dorsal longitudinal muscles (DLMs) in 7-day-old, 42-day-old control, and midlife Re-treated 42-day-old flies. Statistical notations: *p < 0.05, **p < 0.01, ****p < 0.0001 versus 7-day-old flies or untreated controls; #p < 0.05 versus 42-day-old aged flies. Analyses employed Log-rank test for survival and one-way ANOVA for other parameters.

To better understand the protective actions of midlife Re administration, we conducted detailed evaluations of degenerative changes in the compound eyes, central nervous system, and flight muscles of aging flies (**Figure 2d**). The compound eye serves as a reliable, non-invasive indicator of broader tissue deterioration and aging progression [52]. In 42-day-old flies, pronounced structural abnormalities appeared, including irregular pigment accumulation and disrupted photoreceptor organization, resulting in a 37% increase in surface roughness. Midlife Re treatment substantially alleviated these defects, decreasing the affected area by 58% and recovering structural organization to approximately 72% of levels observed in young flies (**Figure 2e**).

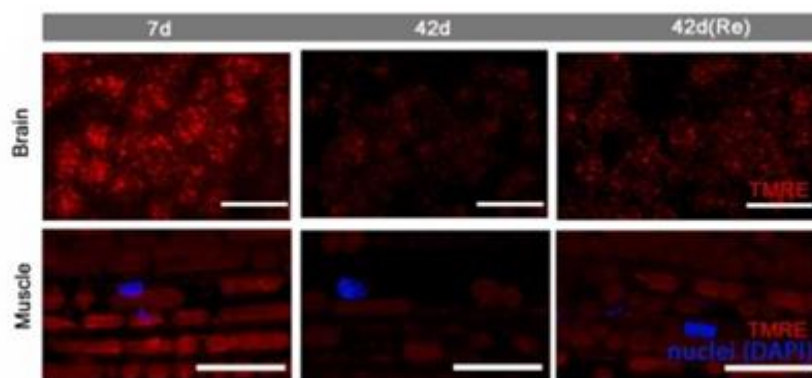
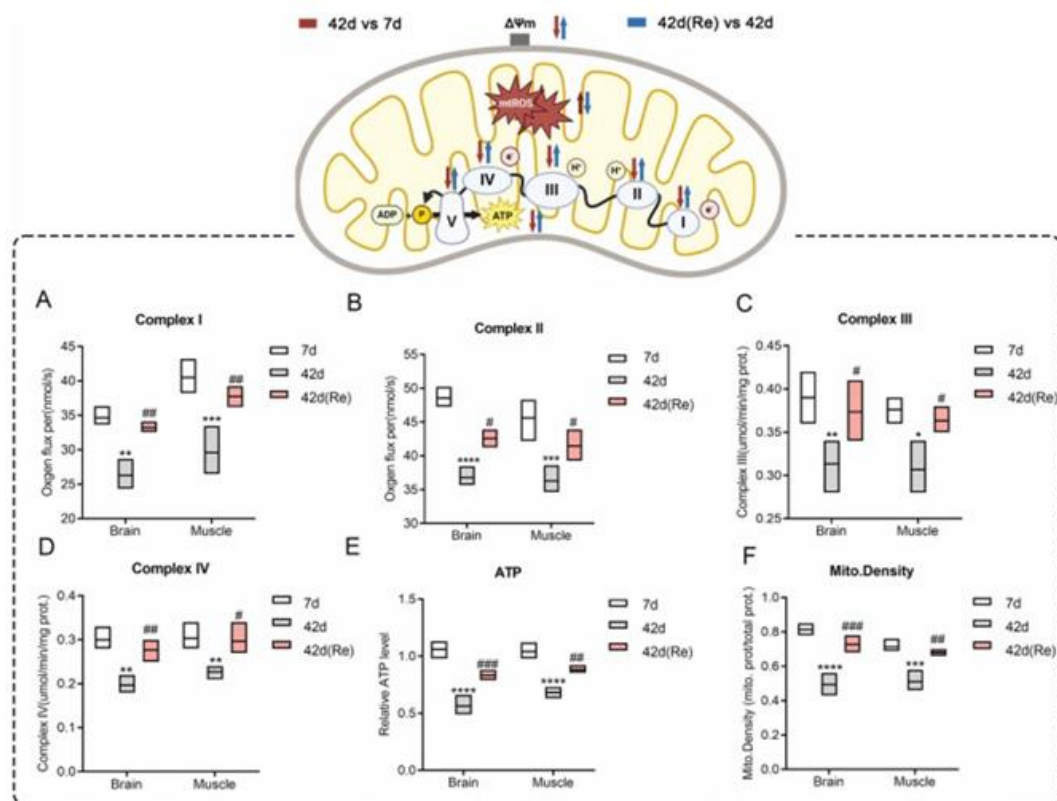
Dopaminergic neurons project to multiple regions of the mushroom body and play pivotal roles in coordinating learning, memory formation, motor control, and precise muscle regulation [53]. Dopamine itself functions as a key neurotransmitter modulating diverse physiological and behavioral processes [54]. With advancing age, there was a notable reduction in DA neuron numbers (from 15–12 to 8–11 cells per hemisphere); (**Figures 2f, 2g**) along with decreased DA content (**Figure 2h**). Re supplementation during midlife effectively preserved neuron counts (restored to 10–13 cells) and brought DA levels back toward youthful values.

In parallel, aging caused a dramatic 63% enlargement of DLM cross-sectional area due to myofibrillar disorganization and fusion (**Figures 2i, 2j**). Midlife Re intervention strongly suppressed this pathological expansion, limiting it to less than 22% and thereby protecting muscle fiber integrity. Collectively, these observations demonstrate that midlife represents the most advantageous period for Re to counteract multiple facets of age-related decline by simultaneously preserving neuronal populations, restoring neurotransmitter balance, and maintaining muscle architecture.

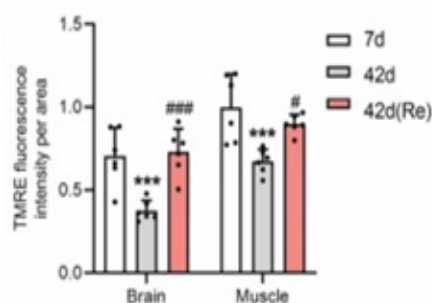
Midlife Re intervention restores mitochondrial function and redox homeostasis

Mitochondrial impairment represents a fundamental feature of the aging process, with neural and skeletal muscle tissues showing heightened susceptibility to progressive mitochondrial deterioration [55]. To determine how Re influences mitochondrial integrity, we examined brain and dorsal longitudinal muscle (DLM) tissues from flies that received midlife treatment. Compared with young (7-day-old) controls, 42-day-old aged flies displayed significantly lowered activities of respiratory chain Complexes I through IV (p < 0.01); (**Figures 3a, 3d**) and substantially reduced ATP generation (p < 0.0001); (**Figure 3e**). Midlife Re administration successfully recovered both ATP production and Complex I-IV enzymatic functions to levels approaching those of young flies.

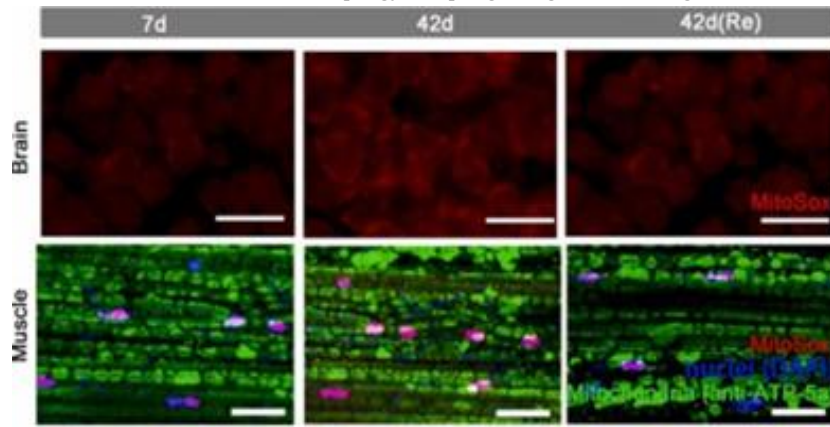
Mitochondrial abundance, an indicator of biogenesis, declined markedly during aging but was effectively restored following Re treatment (**Figure 3f**) [56]. In line with compromised bioenergetics, mitochondrial membrane potential (MMP) — evaluated by TMRE staining — showed a sharp decrease in aged tissues. Re supplementation reversed this loss, as demonstrated by enhanced red fluorescence signals (**Figures 3g, 3h**).



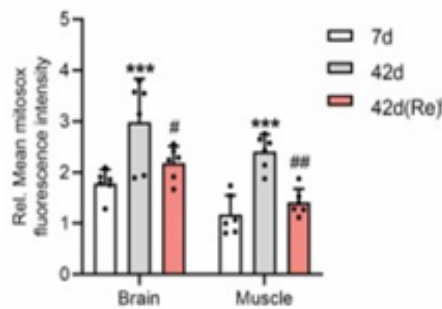
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Figure 3. Re rescues mitochondrial dysfunction and attenuates oxidative stress.

(a-d) Enzymatic activities of mitochondrial respiratory chain Complexes I-IV, (e) relative ATP levels, and (f) mitochondrial density in brains and dorsal longitudinal muscles (DLMs) of 7-day-old young flies, 42-day-old aged controls, and 42-day-old flies receiving midlife Re intervention. $n = 6$ per group. (g) Representative immunofluorescence images of TMRE staining (red channel) indicating mitochondrial membrane potential in brain and DLM tissues, with DAPI nuclear counterstaining (blue channel); scale bars = 5 μm and 10 μm . (h) Quantitative analysis of mitochondrial membrane potential (MMP) based on TMRE fluorescence intensity from panel G, $n = 6$ per group. (i) Immunofluorescence images showing mitochondrial marker ATP5a (green), mitochondrial superoxide levels via MitoSOX (red), and nuclei with DAPI (blue) in brain and DLM tissues from the three experimental groups; scale bars = 5 μm and 10 μm . (j) Quantification of mitochondrial superoxide radical levels corresponding to panel I, $n = 6$ per group. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus 7-day-old flies; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus 42-day-old aged flies (two-way ANOVA).

Aging-related mitochondrial impairment was closely linked to elevated reactive oxygen species (ROS) buildup. Compared with young (7-day-old) flies, 42-day-old individuals displayed markedly higher mitochondrial ROS production. Midlife Re supplementation significantly lowered these ROS levels relative to aged controls (**Figures 3i, 3j**). Notably, Re treatment improved fly survival under hydrogen peroxide (H_2O_2)-induced oxidative challenge by 29.96%, yet it provided no protection against starvation or thermal stress. This selective benefit highlights Re's specific action on oxidative stress response pathways. Taken together, these results suggest that Re's ability to sustain mitochondrial functional balance constitutes a central mechanism underlying its neuroprotective properties.

Re reconstitutes mitochondrial dynamics and ultrastructural morphology

Mitochondria exist as highly dynamic structures that constantly undergo fusion and fission events, which are crucial for preserving their proper function and quality control [57, 58]. To explore Re's influence on these processes, we combined immunofluorescence staining with transmission electron microscopy (TEM). In midlife (around day 22), mitochondria in dopaminergic neurons (**Figures 4a, 4b**) and DLMs (**Figures 4c, 4f**) already displayed early signs of swelling and lengthening. By day 42, these alterations intensified, resulting in

predominantly elongated organelles with lengths predominantly in the 3–5 μm range — a morphology indicative of excessive fusion and reduced fission activity. Midlife Re intervention effectively counteracted these changes, decreasing the occurrence of swollen or overly elongated mitochondria and restoring a more compact, rounded appearance with median lengths of 2–3 μm , similar to patterns seen in young flies. These observations demonstrate that Re helps maintain mitochondrial structural integrity by rebalancing fusion-fission dynamics and preventing the morphological abnormalities that accompany aging.

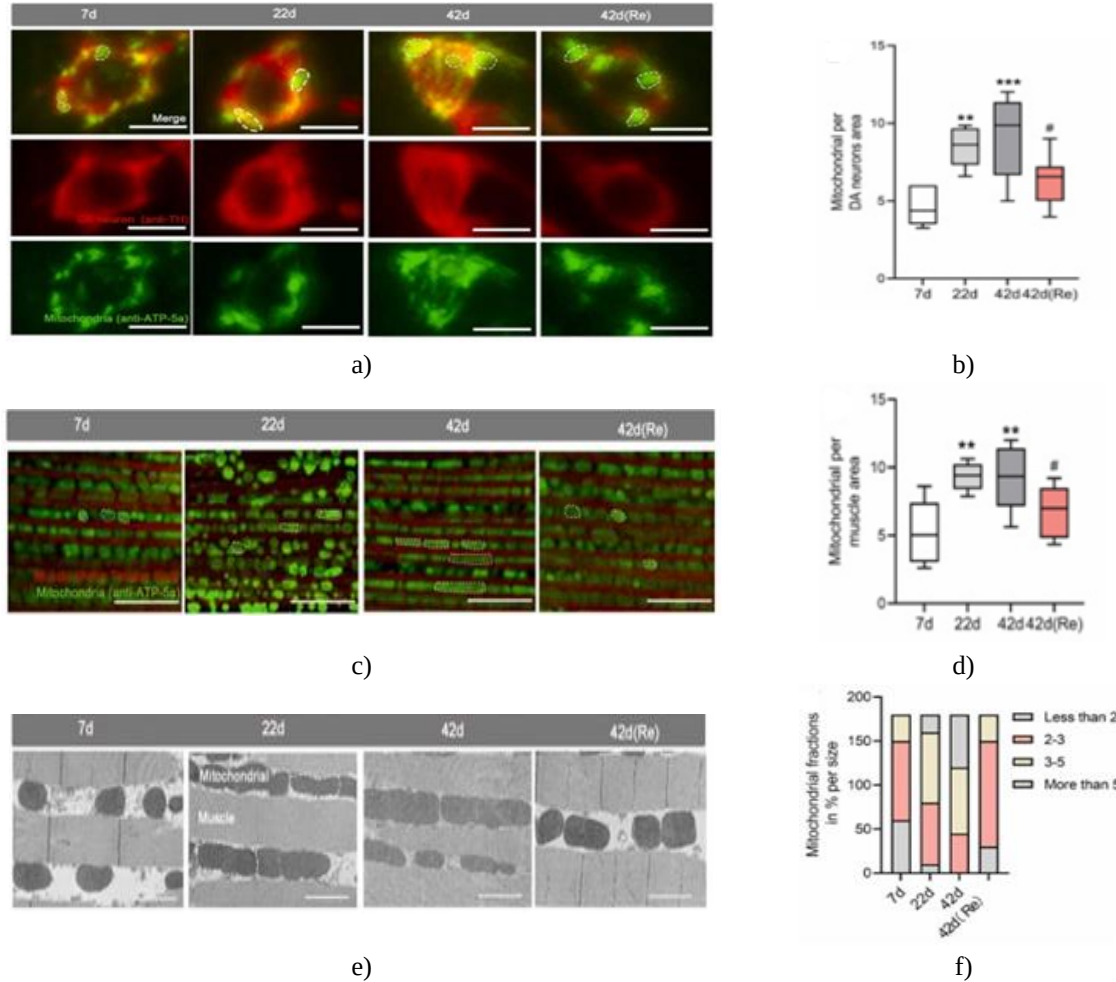
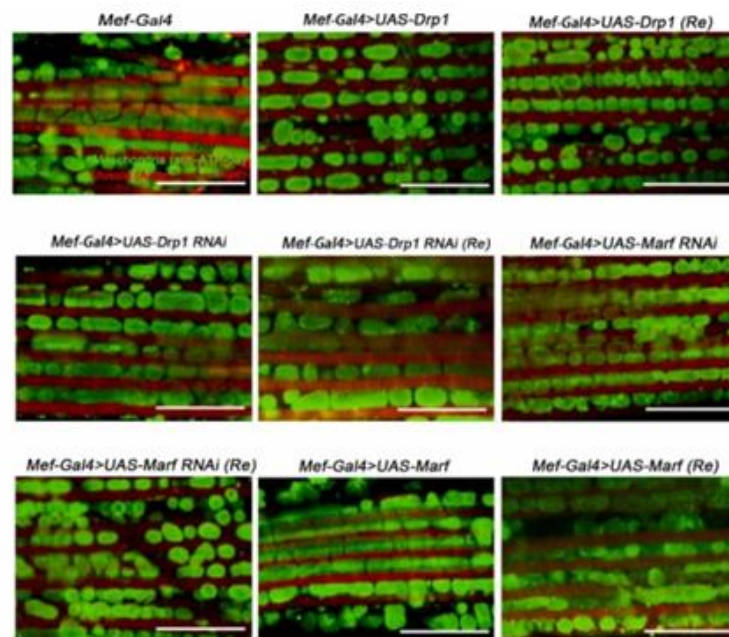
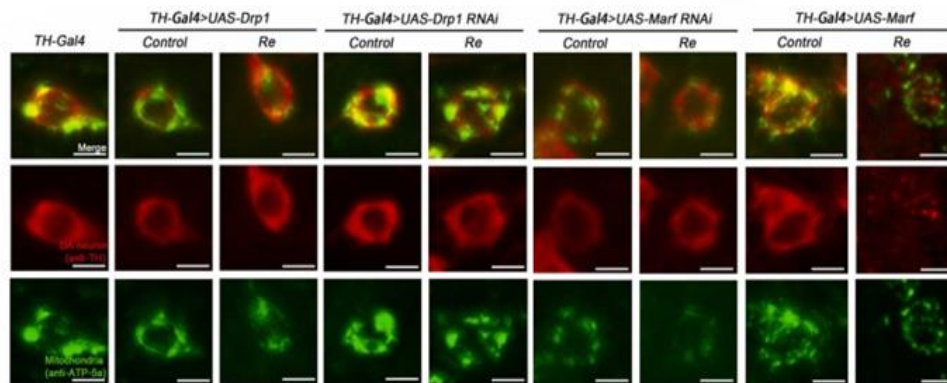
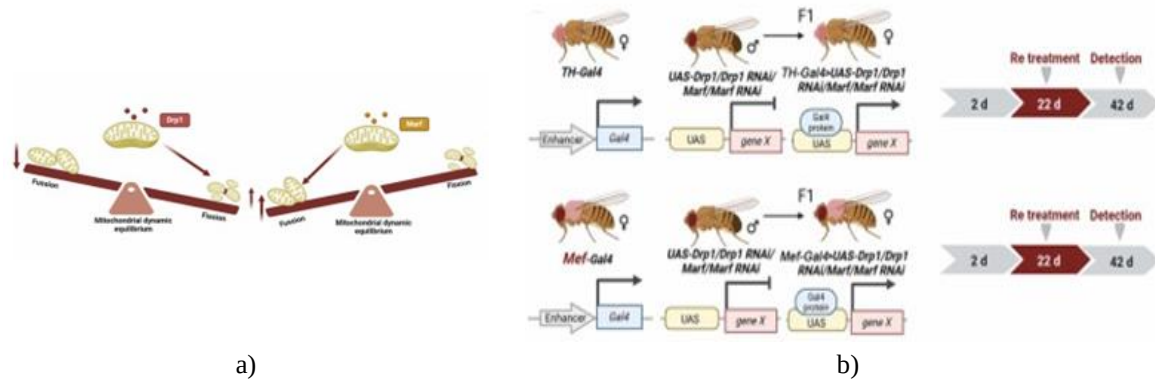


Figure 4. Re restores mitochondrial dynamic equilibrium and ultrastructure. (a) Immunofluorescence images of PPL1 dopaminergic neurons in the brain from 7-day-old, 22-day-old, 42-day-old control, and midlife Re-treated 42-day-old flies. Mitochondria are labeled in green (anti-ATP5a) and dopaminergic neurons in red (anti-TH); scale bar = 10 μm . (b) Quantitative measurement of mitochondrial size in DA neurons from panel A, $n = 6$ per group. (c) Immunofluorescence staining of DLMs in 7-day-old, 22-day-old, 42-day-old control, and Re-treated 42-day-old flies showing mitochondria (green, anti-ATP5a) and muscle fibers (red, Actin tracker); scale bar = 10 μm . (d) Quantification of mitochondrial size in DLMs corresponding to panel C, $n = 6$ per group. (e) Transmission electron micrographs of DLMs from the same experimental groups, highlighting mitochondrial and myofibrillar structures; scale bar = 2 μm . (f) Quantitative analysis of relative mitochondrial size in DLMs from the TEM images in panel E. Statistical significance: ** $p < 0.01$, *** $p < 0.001$ versus 7-day-old flies; # $p < 0.05$ versus 42-day-old aged flies (one-way ANOVA).

Rescue of mitochondrial and neurodegenerative phenotypes by Re requires Drp1

To pinpoint the key molecular mediators through which Re influences mitochondrial shape (**Figure 5a**), we utilized tissue-specific Gal4 driver lines — da-Gal4 for ubiquitous expression, TH-Gal4 for dopaminergic neurons, and Mef-Gal4 for muscle tissue — to either overexpress or silence Drp1 (a key fission protein) and Marf (a primary fusion regulator). These genetic manipulations were combined with Re treatment (**Figure 5b**). Re worked cooperatively with Drp1 overexpression to promote mitochondrial fragmentation, boosting the proportion

of small, fragmented mitochondria in both DA neurons and muscle by 52–58% while decreasing enlarged organelles by 28%. In contrast, reducing Drp1 expression completely prevented Re from correcting age-induced mitochondrial swelling, demonstrating that Re’s beneficial effects depend on Drp1 function (**Figures 5c, 5f**). Manipulating Marf levels (either through overexpression or RNAi) did not interfere with Re’s capacity to decrease mitochondrial cross-sectional area (reductions of 22–49%), suggesting that Re primarily acts via the fission pathway rather than fusion mechanisms (**Figures 5c, 5f**). Overall, these genetic interaction studies identify Drp1 as the essential downstream target mediating Re’s ability to remodel mitochondrial morphology and alleviate age-related cellular decline.



d)

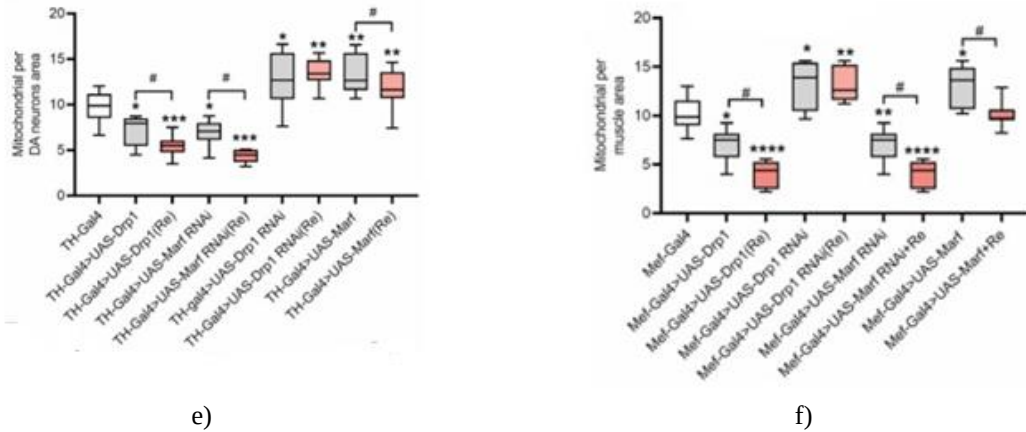
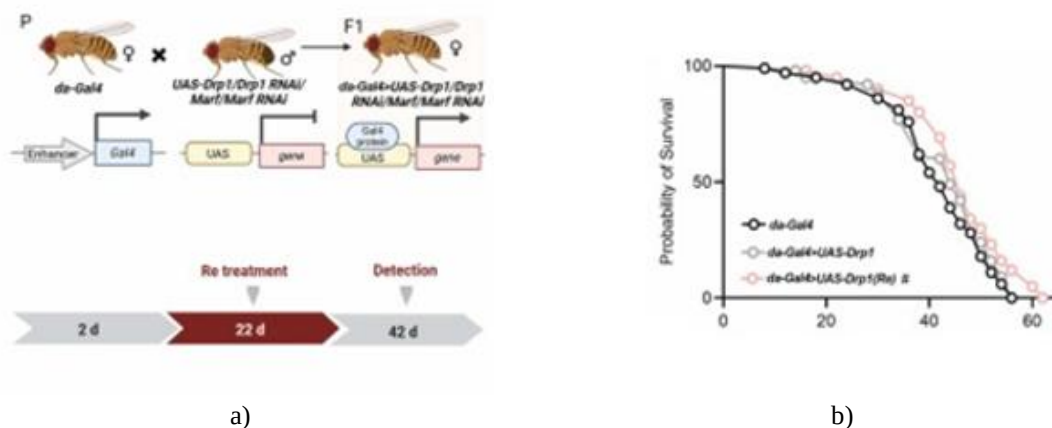


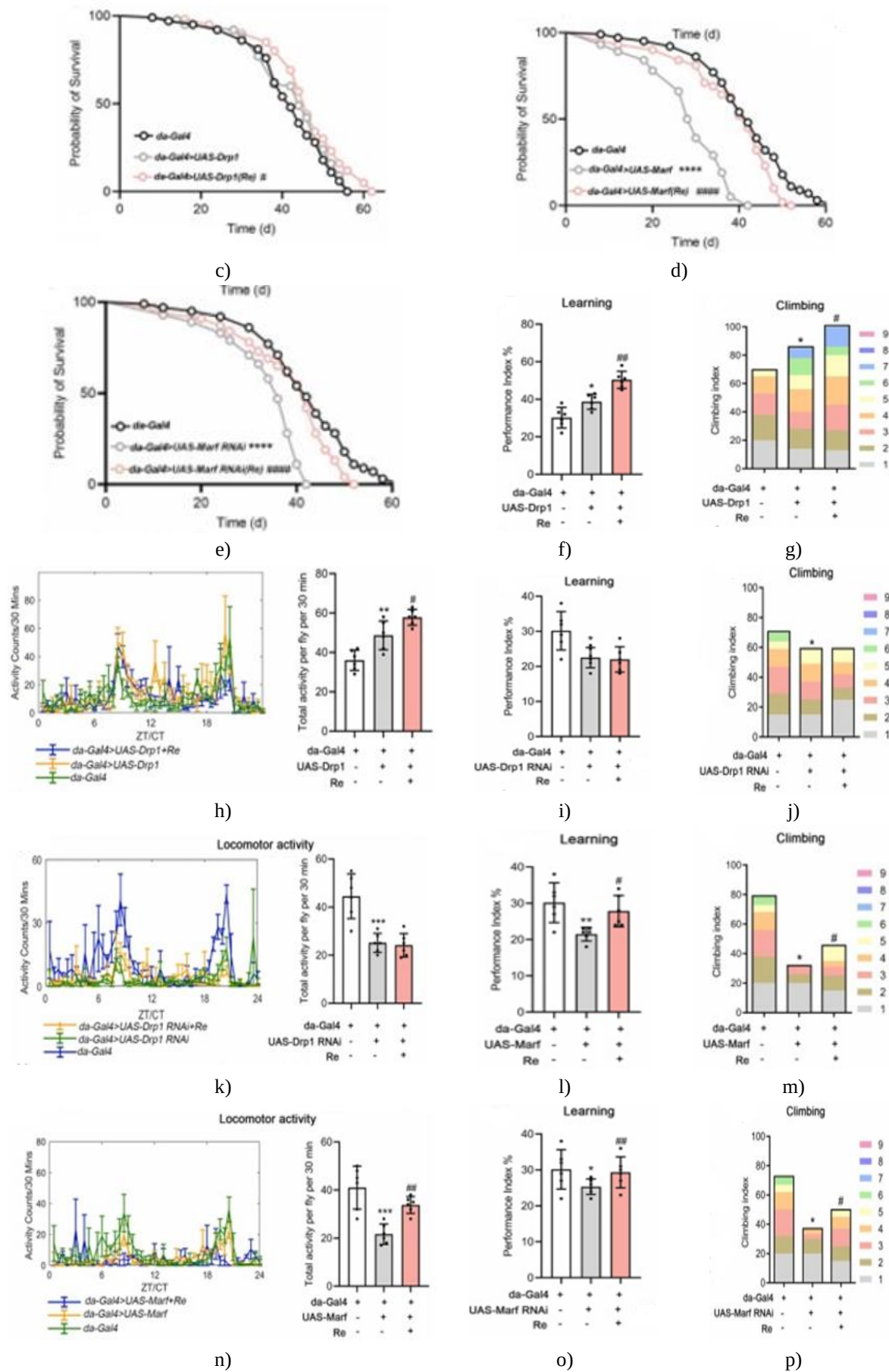
Figure 5. Drp1-dependent maintenance of mitochondrial homeostasis by Re. (a) Diagram illustrating the balance between mitochondrial fusion and fission. (b) Schematic representation of the genetic crosses used to generate flies with tissue-specific manipulation of Drp1 and Marf, including TH-Gal4 > UAS-Drp1, TH-Gal4 > UAS-Drp1 RNAi, TH-Gal4 > UAS-Marf, TH-Gal4 > UAS-Marf RNAi, Mef-Gal4 > UAS-Drp1, Mef-Gal4 > UAS-Drp1 RNAi, Mef-Gal4 > UAS-Marf, and Mef-Gal4 > UAS-Marf RNAi lines, along with the timing of Re treatment. (c) Immunofluorescence staining of PPL1 dopaminergic neurons in the brain (green: anti-ATP5a for mitochondria; red: anti-TH for DA neurons); scale bar = 10 μ m. (d) Immunofluorescence images of DLMs (green: anti-ATP5a for mitochondria; red: Actin tracker for muscle fibers); scale bar = 10 μ m. (e) Quantification of mitochondrial size in DA neurons from panel C, n = 6 per group. (f) Quantification of mitochondrial size in DLMs from panel D, n = 6 per group. Statistical notations: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus TH-Gal4 and Mef-Gal4 control flies; #p < 0.05 (one-way ANOVA).

Additional experiments revealed that altering Drp1 expression (**Figure 6a**) did not substantially alter baseline lifespan (**Figures 6b, 6c**). However, Re supplementation produced an extra 7.2% lifespan extension in flies overexpressing Drp1 ($p < 0.05$); (**Figure 6b**), whereas it failed to provide any benefit in flies with Drp1 knockdown (**Figure 6c**). Both Marf overexpression and RNAi shortened median lifespan to approximately 42 days (compared to 62 days in wild-type controls). Re treatment partially rescued this reduction, extending survival to 52 days ($p < 0.0001$); (**Figures 6d, 6e**).

Regarding behavioral performance, Drp1 overexpression alone improved learning index by 28%, climbing ability by 11%, and spontaneous locomotor activity by 35%. These gains were further augmented by Re co-treatment (additional improvements of 12–30%); (**Figures 6f, 6g, 6h**). In contrast, Drp1 RNAi caused significant declines (14–43%) in these behavioral parameters, and Re was unable to reverse the deficits (**Figures 6i–6k**).

Interestingly, although Marf manipulation (either overexpression or knockdown) also impaired function (reductions of 16–47%), Re administration still produced clear improvements in learning index (**Figures 6l, 6o**), climbing performance (**Figures 6m, 6p**), and locomotor activity (**Figures 6n, 6q**), restoring values to 82–97% of control levels. These findings collectively indicate that Re sustains mitochondrial balance and counters age-related structural and functional deterioration primarily through a Drp1-dependent mechanism.





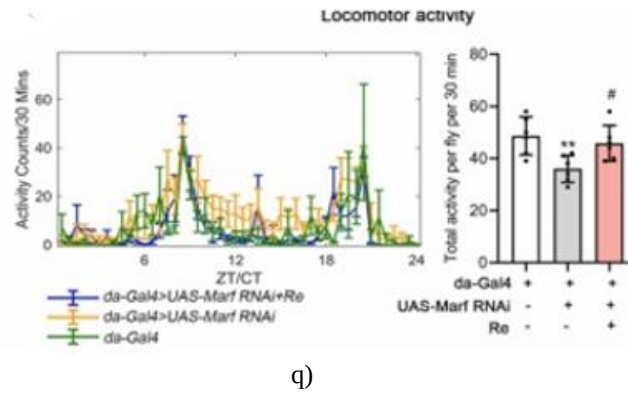
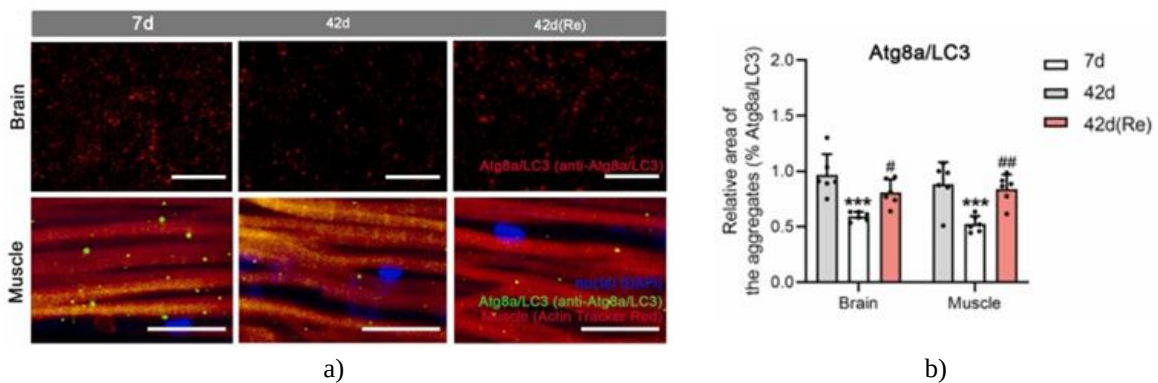


Figure 6. Re intervention enhances cognitive-motor performance and extends lifespan in a Drp1-dependent manner. (a) Overview diagram depicting the generation of flies carrying da-Gal4-driven UAS-Drp1, UAS-Drp1 RNAi, UAS-Marf, and UAS-Marf RNAi constructs, together with the schedule for Re administration. Survival curves showing the effects of Re in (b) da-Gal4 > UAS-Drp1 flies, (c) da-Gal4 > UAS-Drp1 RNAi flies, (d) da-Gal4 > UAS-Marf flies, and (e) da-Gal4 > UAS-Marf RNAi flies (n = 100 flies per group). Learning performance in (f) da-Gal4 > UAS-Drp1, (i) da-Gal4 > UAS-Drp1 RNAi, (l) da-Gal4 > UAS-Marf, and (o) da-Gal4 > UAS-Marf RNAi backgrounds with or without Re treatment (n = 6 independent replicates, 60 flies each). Climbing ability in (g) da-Gal4 > UAS-Drp1, (j) da-Gal4 > UAS-Drp1 RNAi, (m) da-Gal4 > UAS-Marf, and (p) da-Gal4 > UAS-Marf RNAi flies with or without Re (n = 6 replicates, 60 flies per replicate). Spontaneous locomotor activity in (h) da-Gal4 > UAS-Drp1, (k) da-Gal4 > UAS-Drp1 RNAi, (n) da-Gal4 > UAS-Marf, and (q) da-Gal4 > UAS-Marf RNAi flies with or without Re supplementation (n = 6 replicates, 60 flies per replicate). Statistical notations: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to da-Gal4 controls; #p < 0.05, ##p < 0.01 compared to the respective Drp1 or Marf manipulated groups without Re. Survival was evaluated by Log-rank test; behavioral assays by one-way ANOVA.

Re enhances mitophagy and attenuates dysfunctional mitochondrial accumulation

The selective degradation of defective mitochondria through mitophagy is essential for cellular quality control, yet this process becomes markedly impaired as organisms age [59, 60]. To assess whether Re influences mitophagic activity, we examined multiple indicators of autophagy in brain and DLM tissues from 42-day-old flies. Immunostaining demonstrated that Re supplementation during midlife produced a clear rise in Atg8a/LC3 signal (1.36-fold higher intensity); (**Figures 7a, 7b**), reflecting improved autophagic capacity. Aged control flies accumulated higher amounts of the adaptor protein P62 (1.7-fold increase); (**Figures 7c, 7d**), misplaced mitochondrial DNA fragments (1.69-fold increase); (**Figures 7e, 7f**), and ubiquitinated protein aggregates (**Figures 7g, 7h**). Treatment with Re substantially decreased these accumulations — by 42%, 33%, and 41%, respectively. These changes support the conclusion that Re facilitates the removal of compromised mitochondria via enhanced mitophagy and helps restore proper protein homeostasis by reducing the buildup of damaged cellular components.



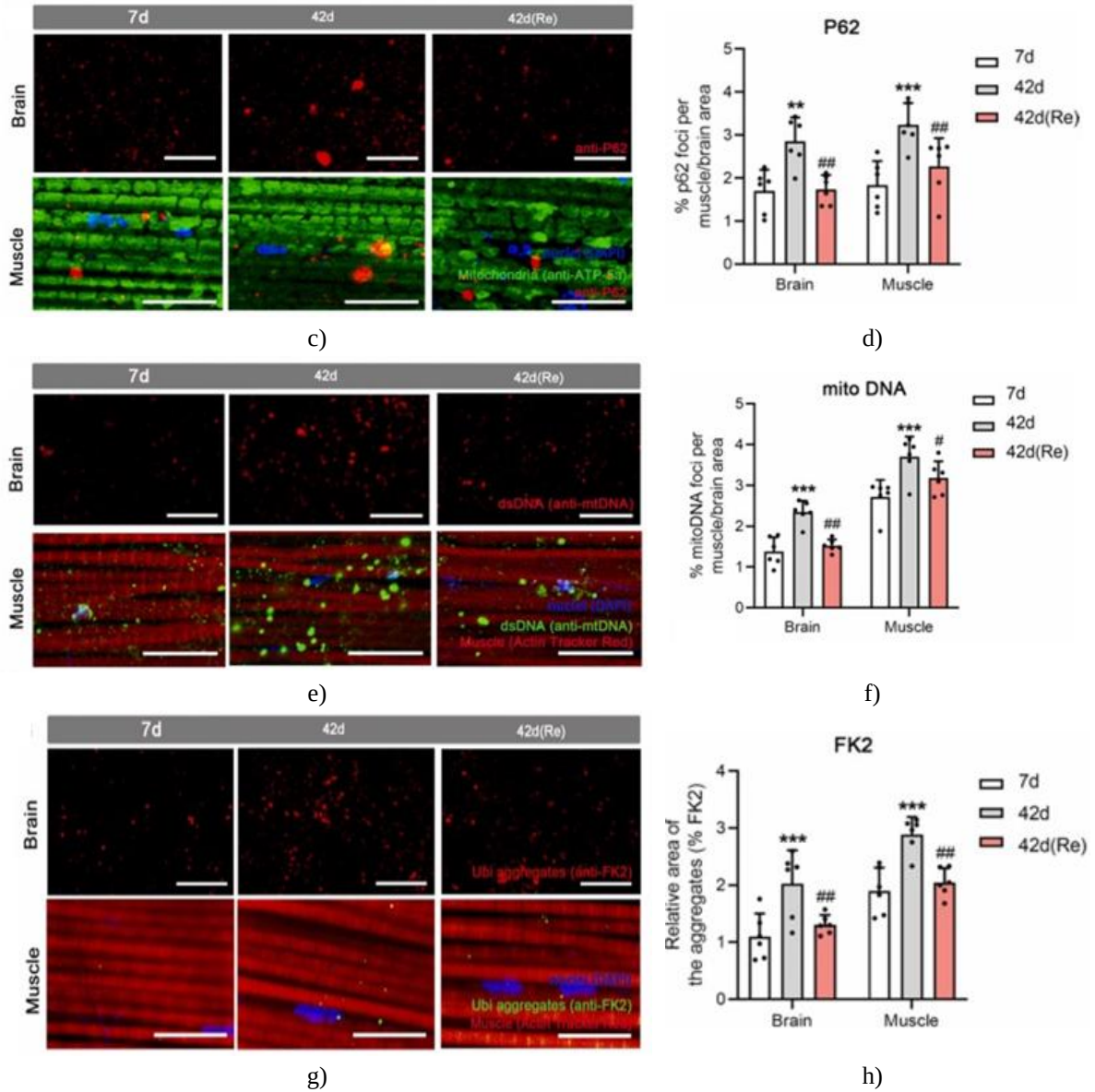
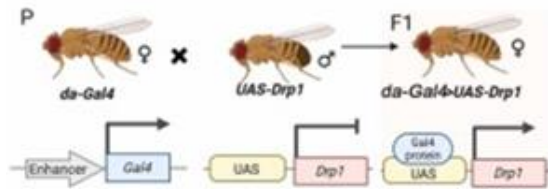


Figure 7. Re mitigates accumulation of dysfunctional mitochondria. (a) Representative immunofluorescence images of brain and DLM tissues from 7-day-old young flies, 42-day-old aged controls, and midlife Re-treated 42-day-old aged flies. Atg8a/LC3 is shown in red (brains) or green (DLMs), with Actin tracker (red) and DAPI (blue) in muscle samples; scale bars = 10 μ m (brain) and 5 μ m (DLM). (b) Quantitative analysis of Atg8a/LC3 intensity in brain and DLM tissues from panel A. (c) Immunostaining for mitochondria (green, anti-ATP5a), P62 (red, anti-P62), and nuclei (blue, DAPI) in brain and DLM samples across the three groups; scale bars = 5 μ m and 10 μ m. (d) Quantification of P62-positive foci in brains and DLMs corresponding to panel C. (e) Detection of mitochondrial DNA (red in brain, green in DLM via anti-dsDNA), muscle fibers (red, Actin tracker in DLM), and nuclei (blue) by immunofluorescence; scale bars = 5 μ m and 10 μ m. (f) Quantitative measurement of mitochondrial DNA signals in brain and DLM tissues from panel E. (g) Immunofluorescence visualization of polyubiquitinated protein aggregates (red in brain, green in DLM), muscle fibers (red, Actin tracker), and nuclei (blue) in the experimental groups; scale bars = 5 μ m and 10 μ m. (h) Quantification of polyubiquitin aggregate levels in brains and DLMs from panel G. n = 6 per group. Statistical significance: **p < 0.01, ***p < 0.001 versus 7-day-old flies; #p < 0.05, ##p < 0.01 versus 42-day-old aged flies (two-way ANOVA).

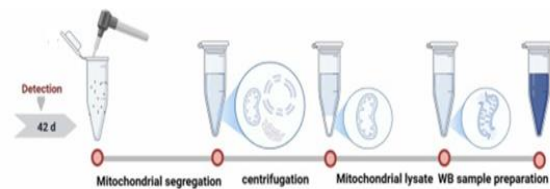
Drp1-dependent mitochondrial fission mediates Re-induced mitophagy

The coordinated interplay between mitochondrial dynamics and autophagy plays a central role in preserving mitochondrial quality. Fragmentation through fission helps segregate damaged mitochondrial portions, facilitating their removal by autophagic machinery [61]. To explore this relationship, we examined the impact of

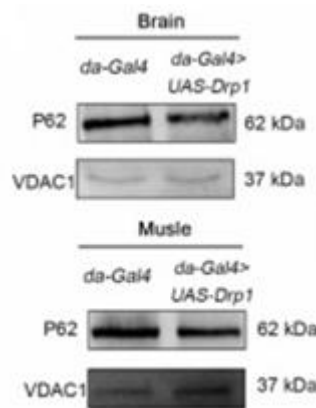
Drp1 overexpression on both mitochondrial morphology and autophagy markers (**Figures 8a, 8b**). Drp1 upregulation promoted greater mitochondrial fragmentation (31% rise in the fragmentation index; **Figures 8c, 8d**) and led to a substantial drop in P62 co-localization with mitochondria (54% reduction; **Figures 8i, 8j**). It also lowered the levels of oxidative phosphorylation complexes I-V (19–24% decrease; **Figures 8e, 8f**), consistent with enhanced clearance of compromised components. Additionally, Drp1 overexpression boosted Atg8a/LC3 levels (1.3-fold increase; **Figures 8g, 8h**) and decreased extramitochondrial DNA accumulation (45% reduction; **Figures 8k, 8l**). These observations indicate that Drp1-driven fission facilitates mitophagic removal of defective mitochondria, thereby supporting Re's protective mechanism.



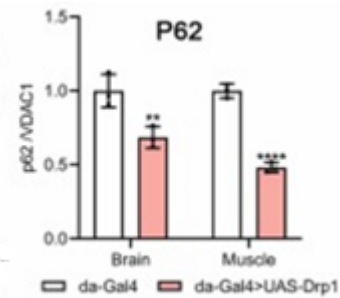
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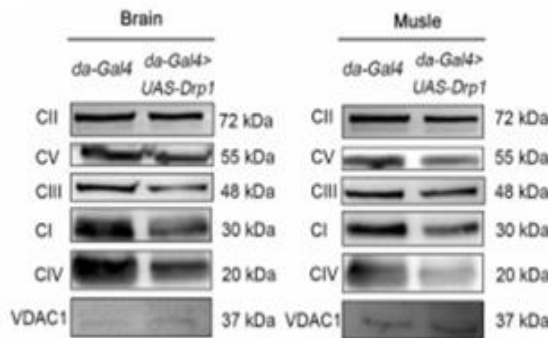
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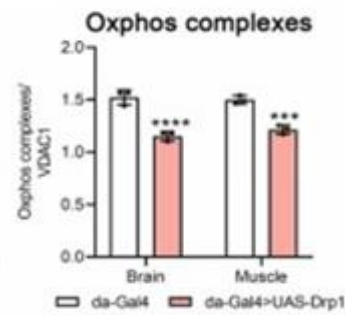
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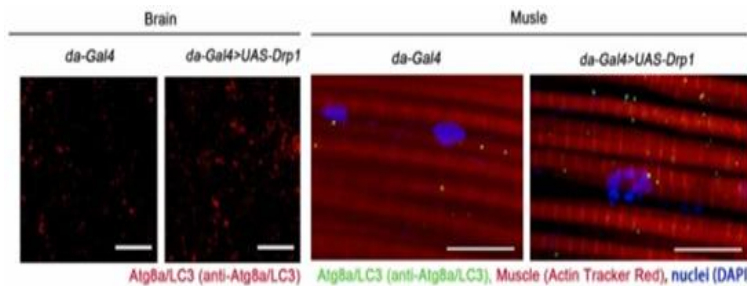
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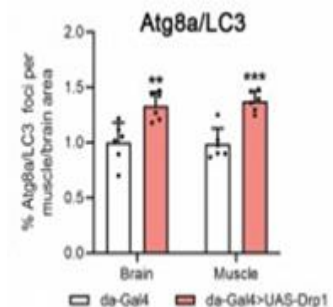
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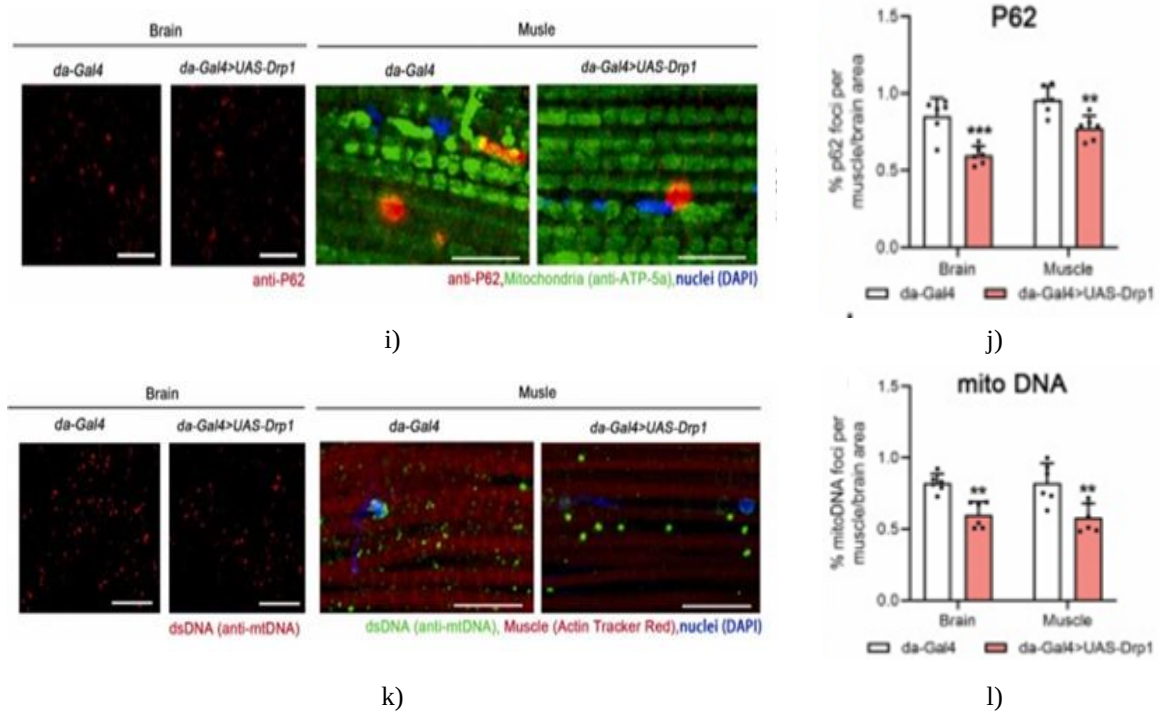
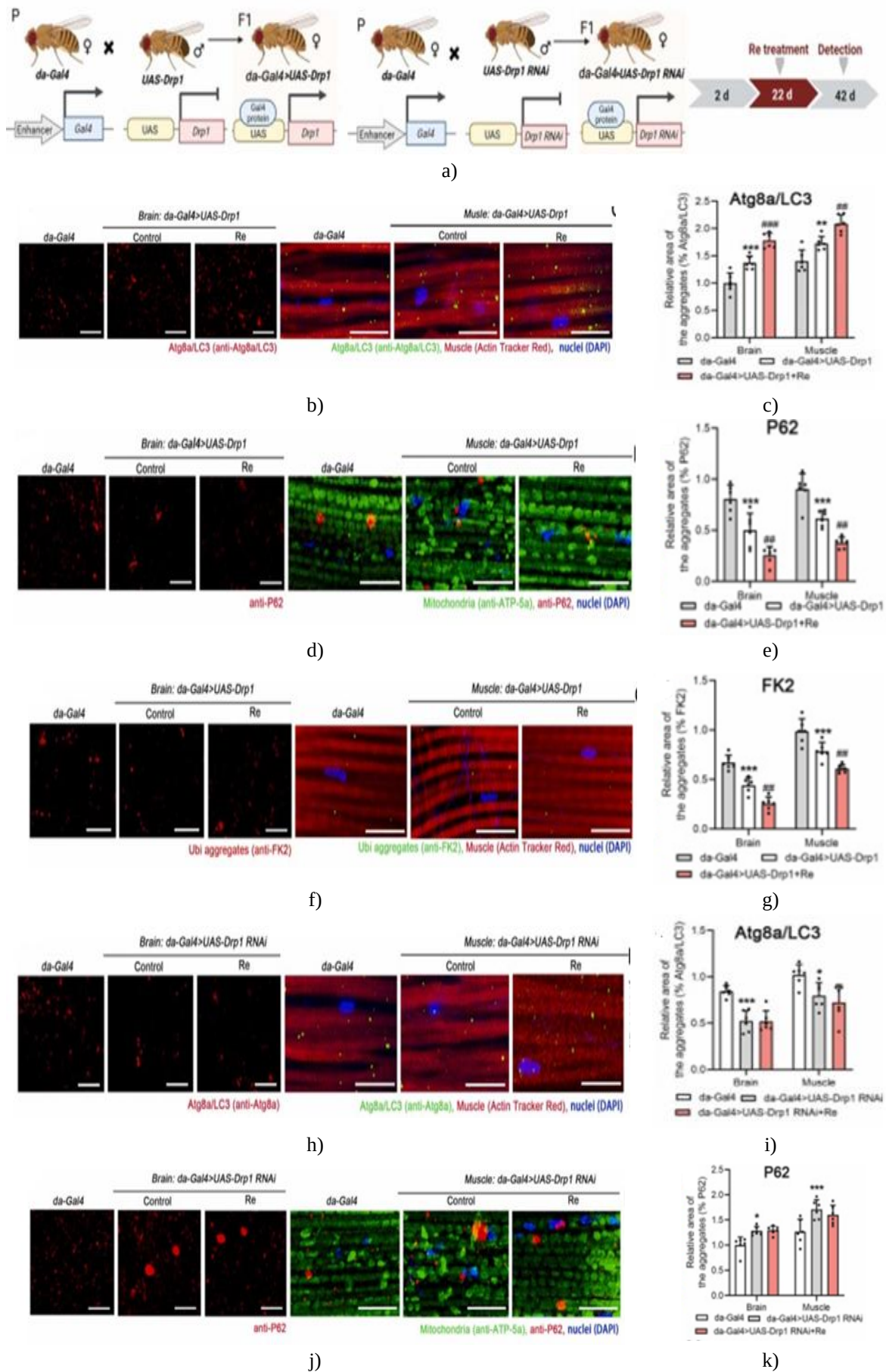


Figure 8. Drp1 promotes mitophagic clearance of damaged organelles. (a) Schematic illustration of the genetic construction and experimental timeline for *da-Gal4 > UAS-Drp1* flies. (b) Overview of the procedure for isolating mitochondrial fractions from fly tissues. (c) Western blot analysis of P62 protein levels in purified mitochondria from *da-Gal4* control and *da-Gal4 > UAS-Drp1* flies (n = 3 independent replicates). (d) Densitometric quantification of mitochondrial P62 bands from the blots in panel C. (e) Immunoblotting of mitochondrial respiratory chain complex subunits in thoracic samples from *da-Gal4* and *da-Gal4 > UAS-Drp1* flies (n = 3 replicates). (f) Quantitative densitometry of the OXPHOS complex proteins shown in panel E. (g) Immunofluorescence staining for Atg8a/LC3 in brain (red) and DLM tissues (green), with Actin tracker (red) and DAPI (blue) in muscle; scale bars = 5 μ m and 10 μ m (n = 6 per group). (h) Quantification of Atg8a/LC3 puncta co-localized with mitochondria from panel G. (i) Double labeling of mitochondria (green, anti-ATP5a) and P62 (red, anti-P62) with nuclear DAPI (blue) in brain and DLM samples; scale bars = 5 μ m and 10 μ m (n = 6 per group). (j) Measurement of P62-positive foci per unit area corresponding to panel I. (k) Immunostaining for mitochondrial DNA (red in brain, green in DLM via anti-dsDNA), muscle fibers (red, Actin tracker), and nuclei (blue); scale bars = 5 μ m and 10 μ m (n = 6 per group). (l) Quantitative assessment of mitochondrial DNA signals in brain and DLM tissues from panel K. Statistical significance: **p < 0.01, ***p < 0.001, ****p < 0.0001 versus *da-Gal4* controls (two-way ANOVA).

To further define the pathway by which Re controls mitochondrial autophagy, we examined its effects in flies with genetically altered Drp1 expression (**Figure 9a**). When Drp1 was overexpressed, Re produced additional synergistic improvements in autophagy: Atg8a/LC3 protein levels rose by 1.3-fold (**Figures 9b, 9c**), mitochondria-associated P62 decreased by 52% (**Figures 9d, 9e**), and ubiquitinated protein aggregates were reduced by 33% (**Figures 9f, 9g**). These changes indicate that Re boosts autophagic removal efficiency by strengthening Drp1 activity.

On the other hand, knocking down Drp1 completely blocked the autophagic response: Atg8a/LC3 expression dropped by 48% (**Figures 9h, 9i**), P62 co-localization with mitochondria increased 1.3-fold (**Figures 9j, 9k**), protein aggregate buildup rose 1.8-fold (**Figures 9l, 9m**), and Re lost all ability to provide rescue. These results demonstrate that Re strengthens mitophagy specifically by promoting Drp1-mediated fission. The complete reliance on functional Drp1 highlights a precise, targeted mechanism for addressing mitochondrial decline during aging.



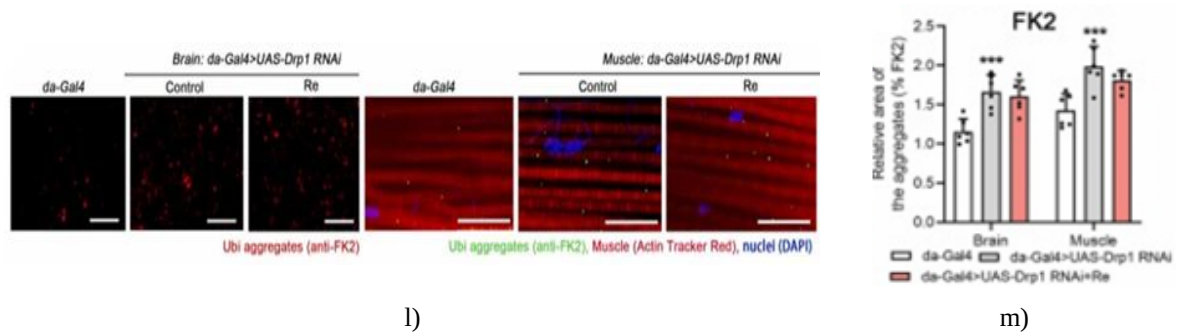
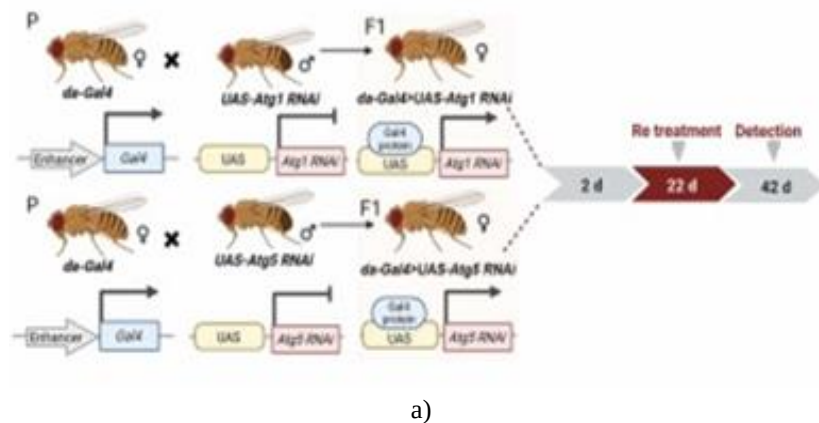


Figure 9. Re orchestrates Drp1-dependent phagocytic activation. (a) Schematic summary of the genetic lines and treatment regimens for da-Gal4 > UAS-Drp1 and da-Gal4 > UAS-Drp1 RNAi flies. (b) Immunofluorescence images of brain and DLM tissues from da-Gal4 controls and da-Gal4 > UAS-Drp1 flies, with or without Re treatment. Atg8a/LC3 labeling (red in brain; green in DLM), Actin tracker (red in DLM), and DAPI (blue); scale bars = 5 μ m and 10 μ m. (c) Quantitative analysis of Atg8a/LC3 signals from panel B. (d) Staining for mitochondria (green, anti-ATP5a), P62 (red, anti-P62), and nuclei (blue) in brain and DLM samples from the same groups; scale bars = 5 μ m and 10 μ m. (e) Measurement of P62 accumulation corresponding to panel D. (f) Visualization of polyubiquitinated protein aggregates (red in brain, green in DLM), muscle fibers (red, Actin tracker), and nuclei (blue) by immunofluorescence; scale bars = 5 μ m and 10 μ m. (g) Quantification of polyubiquitin aggregates from panel F. (h) Atg8a/LC3 immunostaining (red in brain, green in DLM) with Actin tracker (red) and DAPI (blue) in da-Gal4 and da-Gal4 > UAS-Drp1 RNAi flies $\pm$ Re; scale bars = 5 μ m and 10 μ m. (i) Quantification of Atg8a/LC3 intensity from panel H. (j) Immunodetection of mitochondria (green, anti-ATP5a) and P62 (red) with nuclear counterstain in the Drp1 knockdown background $\pm$ Re; scale bars = 5 μ m and 10 μ m. (k) Quantitative evaluation of P62 signals from panel J. (l) Staining of polyubiquitinated aggregates (red in brain, green in DLM), muscle (red, Actin tracker), and nuclei (blue) in da-Gal4 and Drp1 RNAi flies with or without Re; scale bars = 5 μ m and 10 μ m. (m) Quantification of polyubiquitin aggregates shown in panel L. n = 6 per group. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus da-Gal4 controls; ###p < 0.01, ####p < 0.001 versus da-Gal4 > UAS-Drp1 or da-Gal4 > UAS-Drp1 RNAi groups (two-way ANOVA).

Re activates the Drp1-Atg1 signaling axis to sustain mitochondrial homeostasis

Initiation and expansion of autophagosomes are two essential phases in autophagy, primarily regulated by Atg1 (the *Drosophila* homolog of mammalian ULK1) and Atg5, respectively [62, 63]. To determine whether Re-stimulated mitophagy relies on these key components, we conducted targeted genetic silencing experiments (**Figure 10a**). Re treatment successfully prolonged lifespan in both control flies and those with Atg5 knockdown. However, the lifespan-extending benefit was entirely abolished in Atg1-deficient flies (**Figures 10b, 10c**). This demonstrates that Re's ability to promote longevity critically depends on Atg1 function, while remaining largely independent of Atg5.



a)

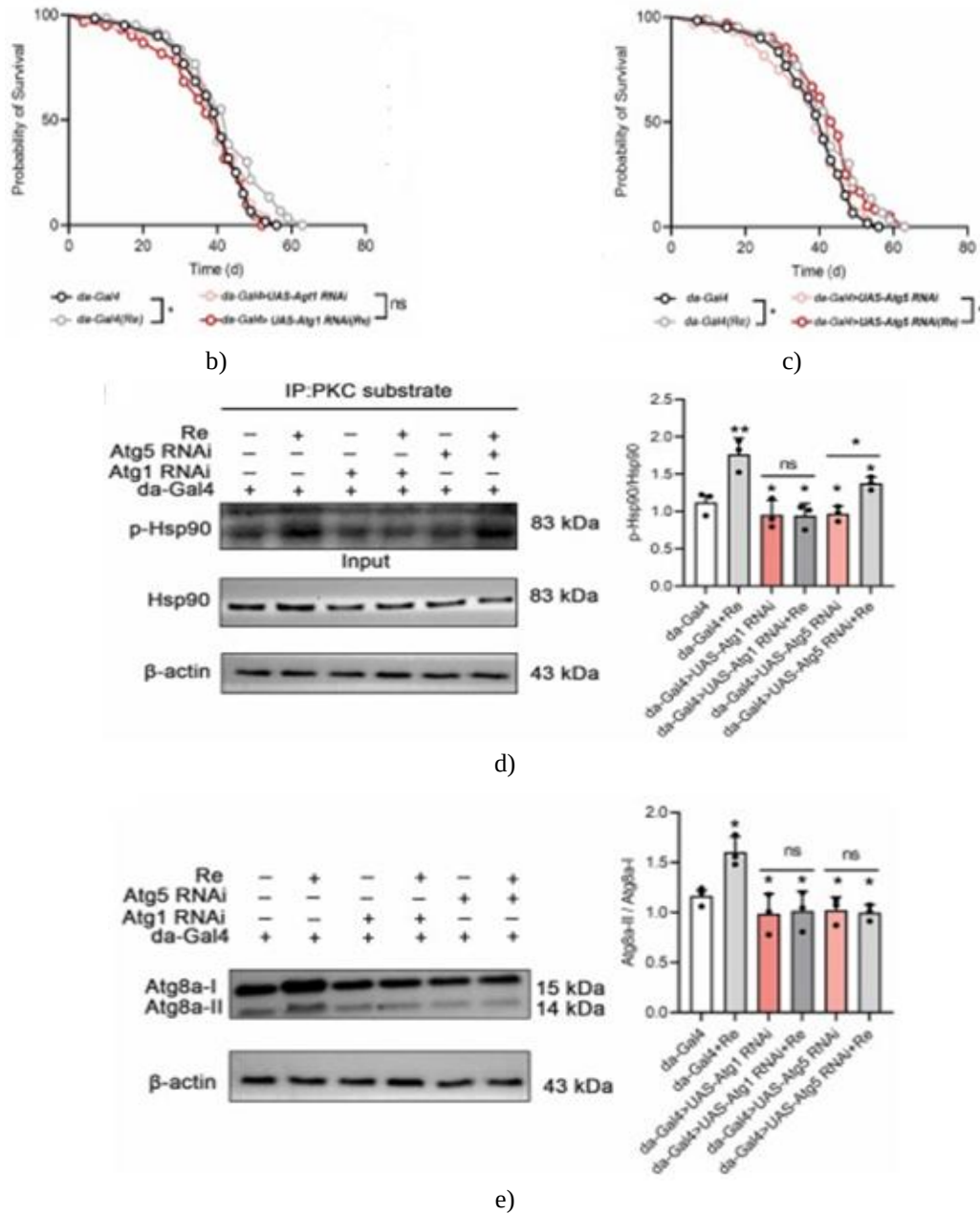
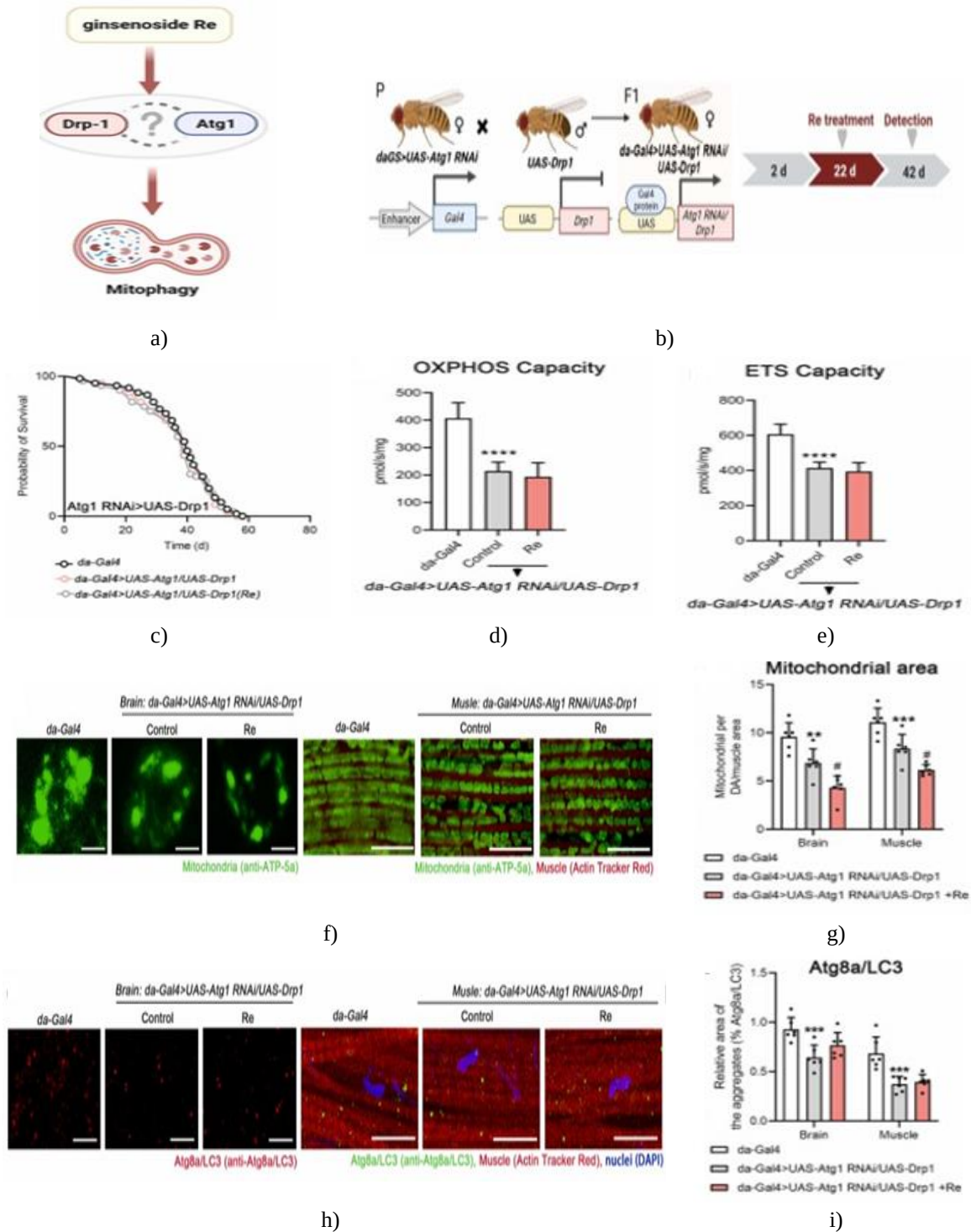


Figure 10. The longevity extending effect of Re is strictly dependent on Atg1-mediated autophagy initiation, but autophagy completion still requires Atg5. (a) Diagram outlining the generation of *da-Gal4 > UAS-Atg1 RNAi* and *da-Gal4 > UAS-Atg5 RNAi* transgenic lines along with the corresponding Re administration schedule. Survival curves of (b) *da-Gal4 > UAS-Atg1 RNAi* and (c) *da-Gal4 > UAS-Atg5 RNAi* flies in the presence or absence of Re treatment (n = 100 flies per experimental group). Western blot results showing (d) phosphorylated Hsp90 and (e) lipidated Atg8a levels in tissues from *da-Gal4* control flies, Atg1 knockdown flies, and Atg5 knockdown flies treated with or without Re (n = 3 biological replicates). Statistical notations: *p < 0.05, **p < 0.01 versus *da-Gal4* controls. Log-rank test was used for survival analysis and one-way ANOVA for protein quantification.

To clarify the reason behind this differing reliance, we evaluated key indicators associated with the start and extension phases of autophagy [64, 65]. Re treatment in normal flies raised Hsp90 phosphorylation levels by 36% and boosted Atg8a lipidation by 23%. Silencing Atg1 prevented Re from activating either of these events. On the other hand, loss of Atg5 eliminated the enhancement of Atg8a lipidation without interfering with Re's effect on Hsp90 phosphorylation (**Figures 10d, 10e**). These observations establish that Re stimulates the initial stage of autophagy exclusively via Atg1, whereas progression through the elongation phase necessitates Atg5. The fact that Re could still promote longevity in Atg5-deficient animals suggests that initiation driven by Atg1 is the

critical step for lifespan extension, possibly engaging backup mechanisms that bypass the need for full autophagosome maturation.

Next, we investigated the interplay between Drp1 and Atg1 in this regulatory network (**Figure 11a**). Knocking down Atg1 in flies that overexpress Drp1 (**Figure 11b**) completely eliminated the lifespan-prolonging action of Re (**Figure 11c**). It also resulted in pronounced mitochondrial impairment, reflected by a 47% decrease in OXPHOS function and a 31% reduction in ETS capacity (**Figures 11d, 11e**). Mitophagy was likewise severely disrupted, showing a 47% drop in Atg8a/LC3 together with a 1.4-fold buildup of ubiquitinated protein aggregates (**Figures 11h, 11k**). Re proved unable to reverse any of these defects, highlighting the indispensable function of Atg1 downstream of Drp1 in executing mitophagy.



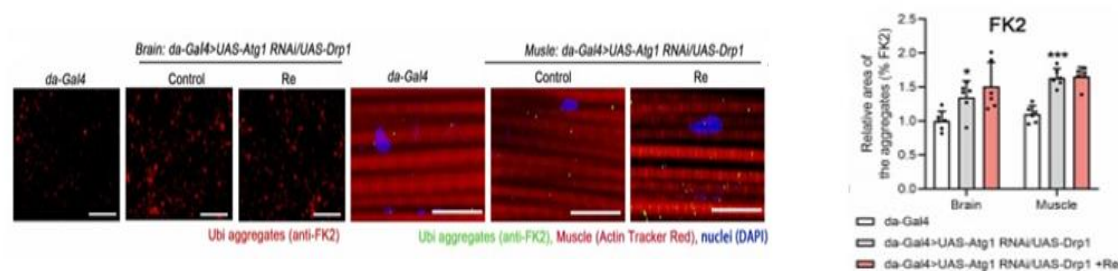
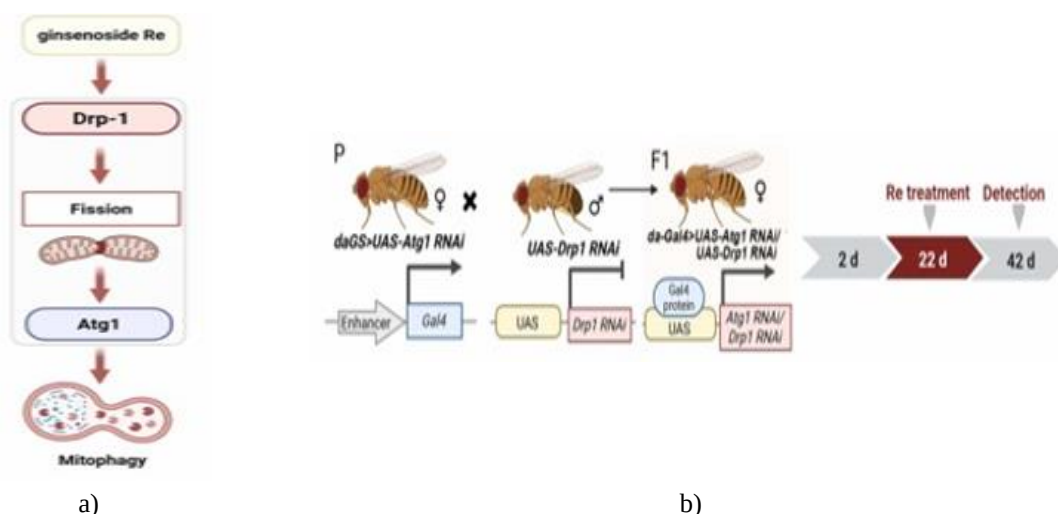


Figure 11. Augmentation of Atg1-dependent mitophagy via Re-induced Drp1 modulation. (a) Pathway illustration summarizing how Re controls autophagy at the molecular level. (b) Diagram detailing the creation of da-Gal4 > UAS-Atg1 RNAi/UAS-Drp1 transgenic flies and the timing of Re exposure. (c) Lifespan curves comparing da-Gal4 control animals to da-Gal4 > UAS-Atg1 RNAi/UAS-Drp1 flies in the presence or absence of Re (n = 100 flies per group). (d-e) Assessment of OXPHOS and ETS respiratory capacities in da-Gal4 > UAS-Atg1 RNAi/UAS-Drp1 flies treated with or without Re (n = 6 per group). (f) Confocal images of PPL1 DA neurons and DLM tissues from control and double-manipulated flies $\pm$ Re, labeled for mitochondria (green, anti-ATP5a) and muscle structure (red, Actin tracker); scale bars = 5 μ m and 10 μ m. (g) Analysis of average mitochondrial dimensions in the tissues shown in panel F (n = 6 per group). (h) Immunofluorescence detection of Atg8a/LC3 (red in brain, green in DLM), Actin tracker (red in DLM), and DAPI nuclei (blue) across experimental conditions; scale bars = 5 μ m and 10 μ m. (i) Measurement of Atg8a/LC3 intensity from the images in panel H (n = 6 per group). (j) Visualization of polyubiquitinated protein aggregates (red in brain, green in DLM), muscle fibers (red, Actin tracker), and nuclei (blue); scale bars = 5 μ m and 10 μ m. (k) Quantification of polyubiquitin aggregate abundance corresponding to panel J (n = 6 per group). Statistical notations: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus da-Gal4 controls; #p < 0.05 versus untreated da-Gal4 > UAS-Atg1 RNAi/UAS-Drp1 flies. Survival data were analyzed with the Log-rank test, while other comparisons employed one-way or two-way ANOVA.

To strengthen evidence for their functional linkage, double knockdown flies lacking both Atg1 and Drp1 were produced. These insects suffered major deficits in mitochondrial energy production, displaying 45% lower OXPHOS activity and 30% reduced ETS capacity. Mitophagy was also inactivated, indicated by a 28% decrease in Atg8a/LC3 (**Figures 12d, 12e, 12h, 12k**). Because Re supplementation could not improve any of these abnormalities, the results confirm that Drp1 and Atg1 act sequentially within the same pathway, with Atg1 functioning as a required effector positioned downstream of Drp1.



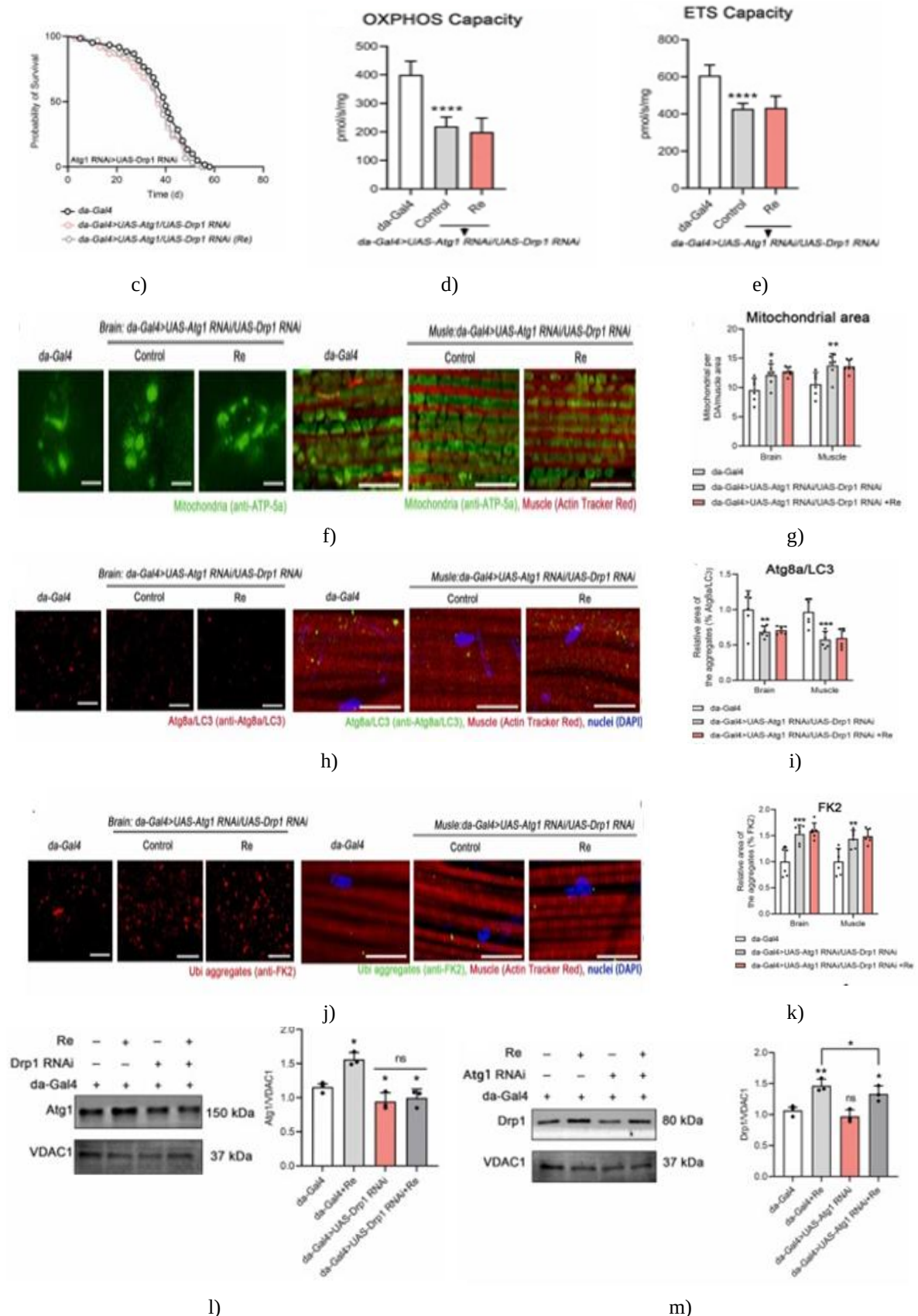


Figure 12. Drp1/Atg1 dual ablation abolishes Re-regulated mitochondrial quality control. (a) Diagram outlining the molecular steps through which Re influences autophagy. (b) Overview of the genetic engineering and Re treatment timeline for da-Gal4 > UAS-Atg1 RNAi/UAS-Drp1 RNAi flies. (c) Lifespan curves for da-Gal4 wild-type flies versus da-Gal4 > UAS-Atg1 RNAi/UAS-Drp1 RNAi double knockdown animals, with or without Re (n = 100 flies per group). (d-e) Evaluation of OXPHOS and ETS respiratory

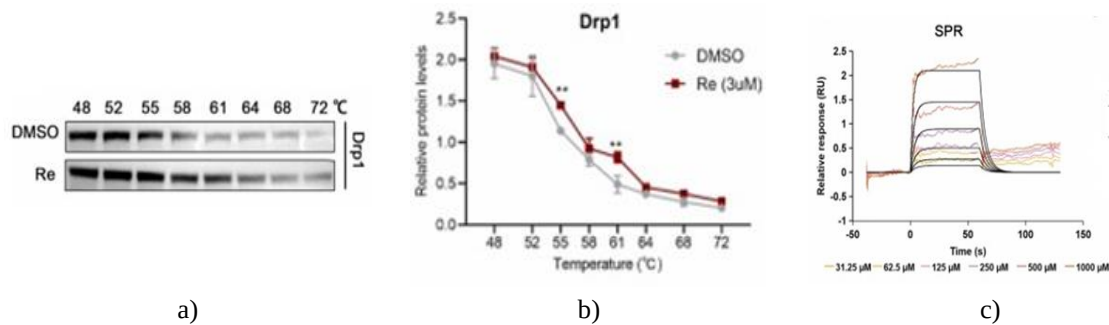
functions in the double knockdown flies treated $\pm$ Re (n = 6 per group). (f) Confocal microscopy images of PPL1 region DA neurons and DLMs in control and double knockdown flies $\pm$ Re. Mitochondria visualized in green (anti-ATP5a) and muscle in red (Actin tracker); scale bars = 5 μ m and 10 μ m. (g) Measurement of mitochondrial size in tissues from panel F (n = 6 per group). (h) Immunofluorescence for Atg8a/LC3 (red in brain, green in DLM), Actin tracker (red in DLM muscle), and DAPI nuclei (blue); scale bars = 5 μ m and 10 μ m. (i) Quantitative assessment of Atg8a/LC3 from panel H (n = 6 per group). (j) Detection of polyubiquitinated aggregates (red in brain, green in DLM), muscle fibers (red, Actin tracker), and nuclei (blue); scale bars = 5 μ m and 10 μ m. (k) Quantification of polyubiquitin aggregate abundance from panel J (n = 6 per group). (l) Immunoblotting of Atg1 protein abundance in da-Gal4 and da-Gal4 > UAS-Drp1 RNAi flies $\pm$ Re (n = 3 replicates). (m) Immunoblotting of Drp1 protein abundance in da-Gal4 and da-Gal4 > UAS-Atg1 RNAi flies $\pm$ Re (n = 3 replicates). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus da-Gal4 controls. Survival comparisons used Log-rank test; remaining analyses used one-way and two-way ANOVA.

Mitochondrial shape analysis further confirmed the hierarchical organization of this pathway. Atg1 knockdown in a Drp1 overexpression background did not interfere with fragmentation (**Figures 11f, 11g**), allowing Re to successfully restore normal mitochondrial dimensions (35% decrease in area). In sharp contrast, combined loss of Drp1 and Atg1 triggered pronounced hyperfusion and organelle elongation (1.28-fold increase), a phenotype entirely unresponsive to Re (**Figures 12f, 12g**). This places Drp1 upstream, where its fission activity is a prerequisite for Atg1 to initiate autophagic elimination of damaged mitochondria.

Reciprocal localization studies revealed mutual dependence between the two proteins. Drp1 knockdown strongly reduced Atg1's presence at mitochondria, and Re treatment failed to restore proper localization (**Figure 12l**). Atg1 depletion, meanwhile, decreased mitochondrial Drp1 levels by 18%. Re was still able to raise Drp1 recruitment by 28% without Atg1, yet this increase remained substantially lower than the 36% enhancement seen in normal flies (**Figure 12m**). The data are consistent with a bidirectional Drp1-Atg1 module in which Drp1 primarily recruits Atg1, and Atg1 reinforces Drp1 mitochondrial association via positive feedback to optimize mitophagic efficiency.

Cross-species structural binding of Re with conserved Drp1-L94 modulates GTPase activity

To define the biochemical interaction between Re and Drp1 and evaluate its conservation across species, binding was examined using the human version of the protein. Treatment with 3 μ M Re optimally improved survival of O151-stressed senescent HEK 293T cells by 21%. Thermal stability assays (CETSA) in these cells showed that Re increased Drp1's resistance to heat-induced denaturation, consistent with direct engagement (**Figures 13a, 13b**). Parallel *in vivo* experiments in *Drosophila* confirmed similar stabilization of Drp1 by Re [66]. Surface plasmon resonance and microscale thermophoresis measurements verified saturable, dose-dependent binding, yielding a K_d of 820 μ M (**Figures 13c, 13d**). Selectivity was confirmed as Re did not increase thermal stability of other mitochondrial dynamics regulators including Fis1, OPA1, and Mfn2.



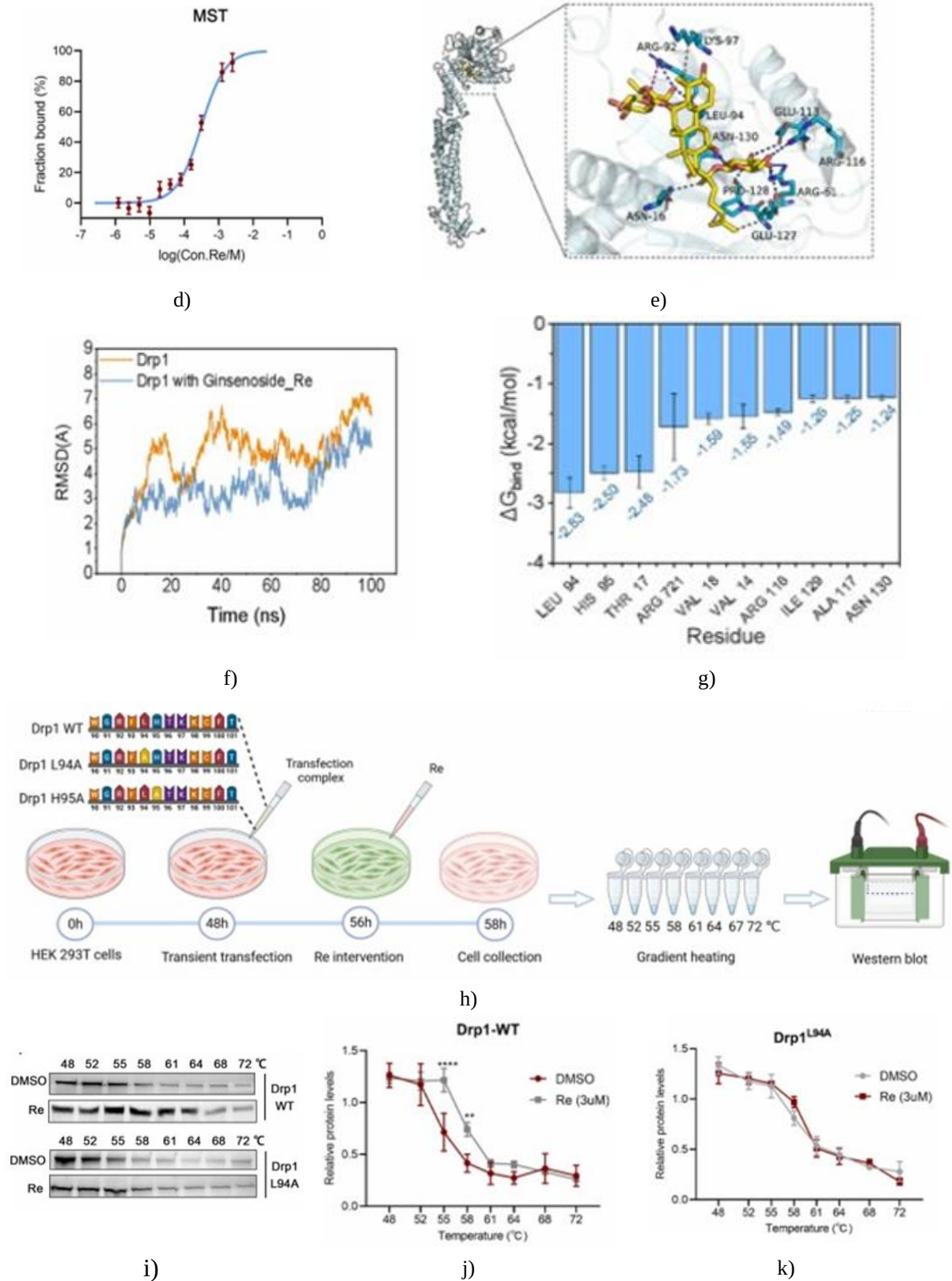


Figure 13. Re specifically binds to the Drp1 L94 site and forms a stable complex. (a) CETSA results demonstrating that Re increases Drp1 resistance to thermal denaturation across a range of temperatures in HEK 293T cells. (b) Melting curves generated using GraphPad Prism software from the CETSA experiment. (c) SPR sensorgrams showing the binding kinetics and affinity of Re to purified Drp1 protein, with a calculated K_d value of 820 μM . (d) MST analysis performed by incubating labeled Drp1 with a titration series of Re concentrations. (e) Three-dimensional structural model illustrating Re docked into the binding pocket at the N-terminal region of Drp1. Two-dimensional interaction diagram highlighting contacts with

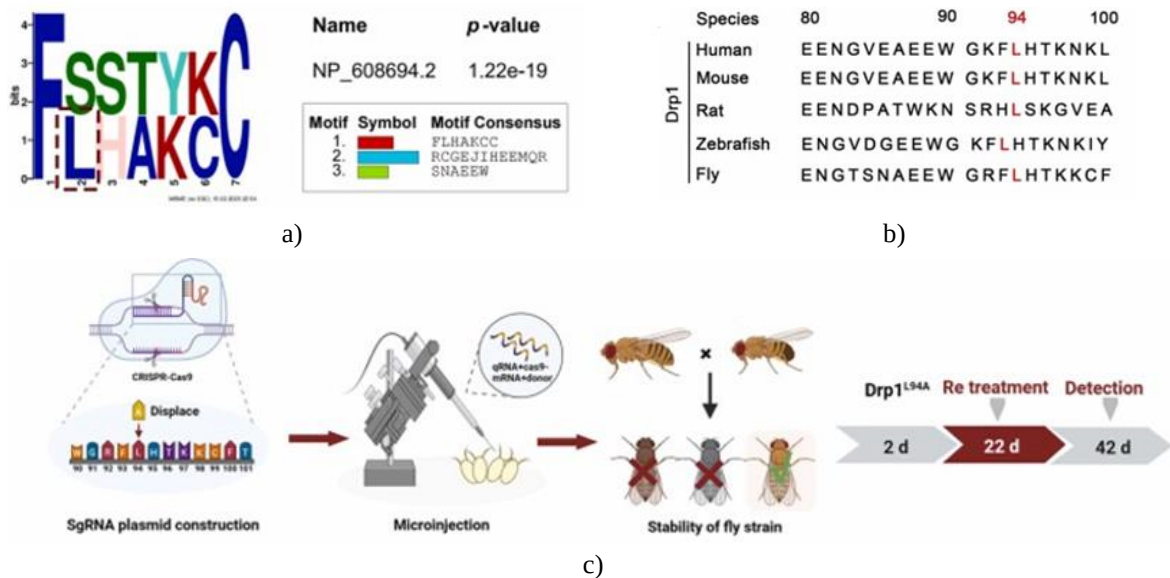
multiple amino acid residues. (f) Plot of root mean square deviation (RMSD) over the course of molecular dynamics simulation for the Drp1-Re complex. (g) Box plot displaying per-residue binding energy contributions for the top 11 interacting amino acids. (h) Schematic of the experimental design for generating and testing Drp1 wild-type and L94A mutant constructs in cells, including the CETSA workflow. (i) CETSA thermal shift profiles for Drp1-WT and Drp1-L94A mutants following treatment with 3 μ M Re. (j) Quantitative analysis of thermal stability curves for Drp1-WT. (k) Quantitative analysis of thermal stability curves for Drp1-L94A. n = 3 replicates per condition. Statistical significance: **p < 0.01, ****p < 0.0001 versus DMSO control (two-way ANOVA).

To confirm the relevance of the predicted binding site in the living organism used throughout the study, molecular docking was performed on *Drosophila* Drp1. Simulations revealed that Re is anchored by eight hydrogen bonds and multiple van der Waals contacts involving residues such as Arginine 116, Glutamate 113, and L94 (**Figure 13e**). Molecular dynamics trajectories indicated that Re binding stabilizes the GTPase domain of Drp1 (**Figure 13f**), while energy decomposition pinpointed L94 and Histidine 95 (H95) as major contributors to the overall binding free energy (**Figure 13g**).

Experimental validation involved creating Drp1-L94A and Drp1-H95A mutants and testing Re interaction (**Figure 13h**). CETSA assays confirmed strong thermal stabilization of wild-type Drp1 by Re (**Figures 13i, 13j**), whereas the L94A mutation completely abolished this protective effect (**Figures 13i, 13k**). The H95A mutant showed only intermediate stabilization, confirming L94 as the dominant binding residue.

Re promotes Drp1 S616 phosphorylation via L94 engagement to mitigate degenerative pathology

Sequence alignment across species demonstrated that the FLHAKCC motif harboring the L94 residue in Drp1 isoform A is highly conserved (**Figures 14a, 14b**), suggesting an important functional role. To test the significance of this site for Re's protective activity, a Drp1L94A point mutation was introduced using CRISPR-Cas9 technology (**Figure 14c**). In wild-type flies, Re administration elevated phosphorylation of Drp1 at serine 616 — a modification critical for promoting mitochondrial fission — by 1.3-fold (**Figure 14d**) [37]. In Drp1L94A mutants, baseline S616 phosphorylation was already 51% lower, and Re treatment was unable to increase it (**Figure 14d**). These findings establish that the L94 residue is essential for Re to enhance Drp1 S616 phosphorylation.



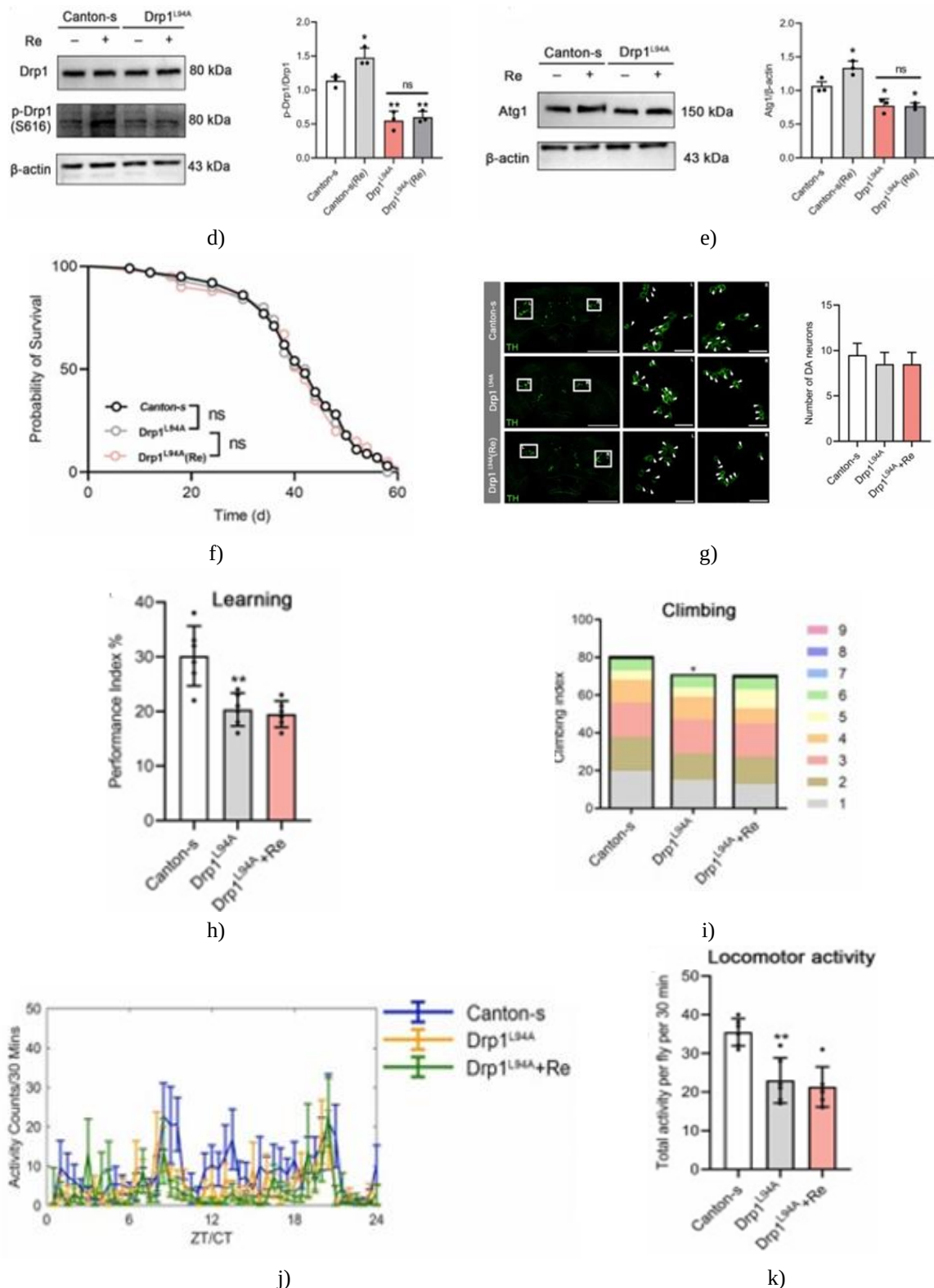


Figure 14. Drp1-L94 phosphorylation drives Re-induced mitophagy and healthspan extension. (A) MEME motif analysis highlighting the conserved region in Drp1 isoform A across multiple species. (B) Cross-species sequence alignment showing the position corresponding to L94 in Drp1. (C) Diagram illustrating the CRISPR-based generation of Drp1^{L94A} mutant flies and the schedule for Re administration. (D) Immunoblotting results for phosphorylated Drp1 at Ser616 in wild-type versus Drp1^{L94A} mutant backgrounds (n = 3 replicates). (E) Western blot analysis of Atg1 expression levels in wild-type and Drp1^{L94A} flies (n = 3 replicates). (F) Lifespan curves for Drp1^{L94A} mutant flies treated with or without Re (n = 100 flies per group). (G) Counts of TH-immunoreactive PPL1 neurons per brain hemisphere (green,

anti-TH antibody; scale bars = 128 μm and 25 μm), $n = 3$ replicates. (H) Learning index, (I) negative geotaxis climbing performance, and (J-K) spontaneous locomotor activity measured in wild-type and mutant flies $\pm$ Re treatment ($n = 6$ replicates with 60 flies each). Statistical significance: * $p < 0.05$, ** $p < 0.01$ versus Canton-S control flies. Log-rank test for survival and one-way ANOVA for behavioral parameters.

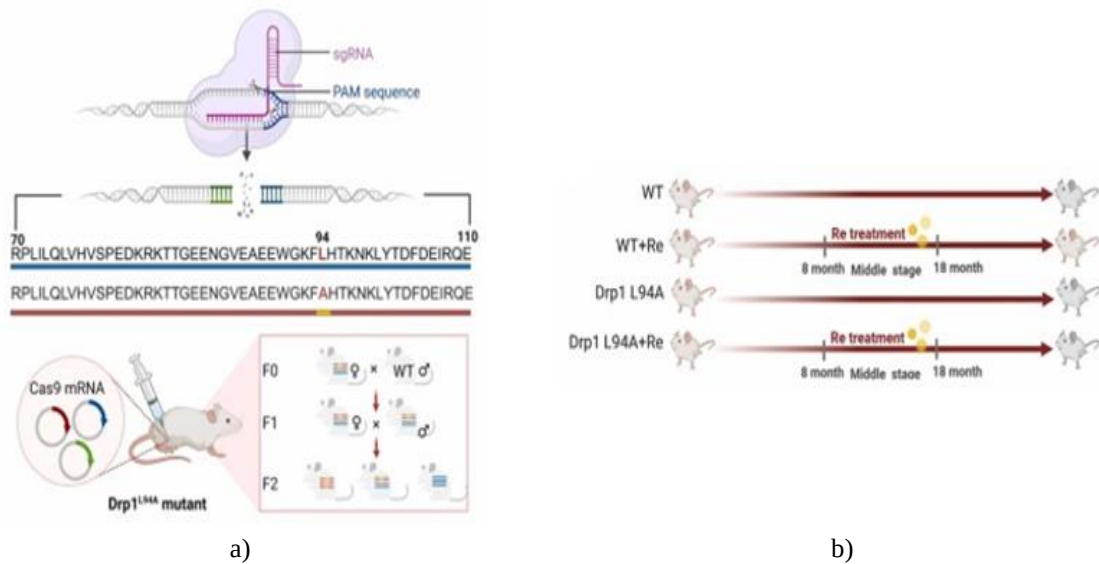
We then examined whether phosphorylation at S616 is directly required for Re's effects on mitochondrial quality control. Using an established phospho-defective hDrp1S616A *Drosophila* line [37], we observed a 23% rise in the fraction of enlarged mitochondria along with suppressed autophagy, indicated by 19% lower Atg1 protein, 25% reduced Hsp90 phosphorylation, and compromised flux. Re was able to promote fission and autophagy initiation in normal flies, but these responses were lost in the S616A mutant, proving that S616 phosphorylation is a necessary step in Re-mediated mitophagy.

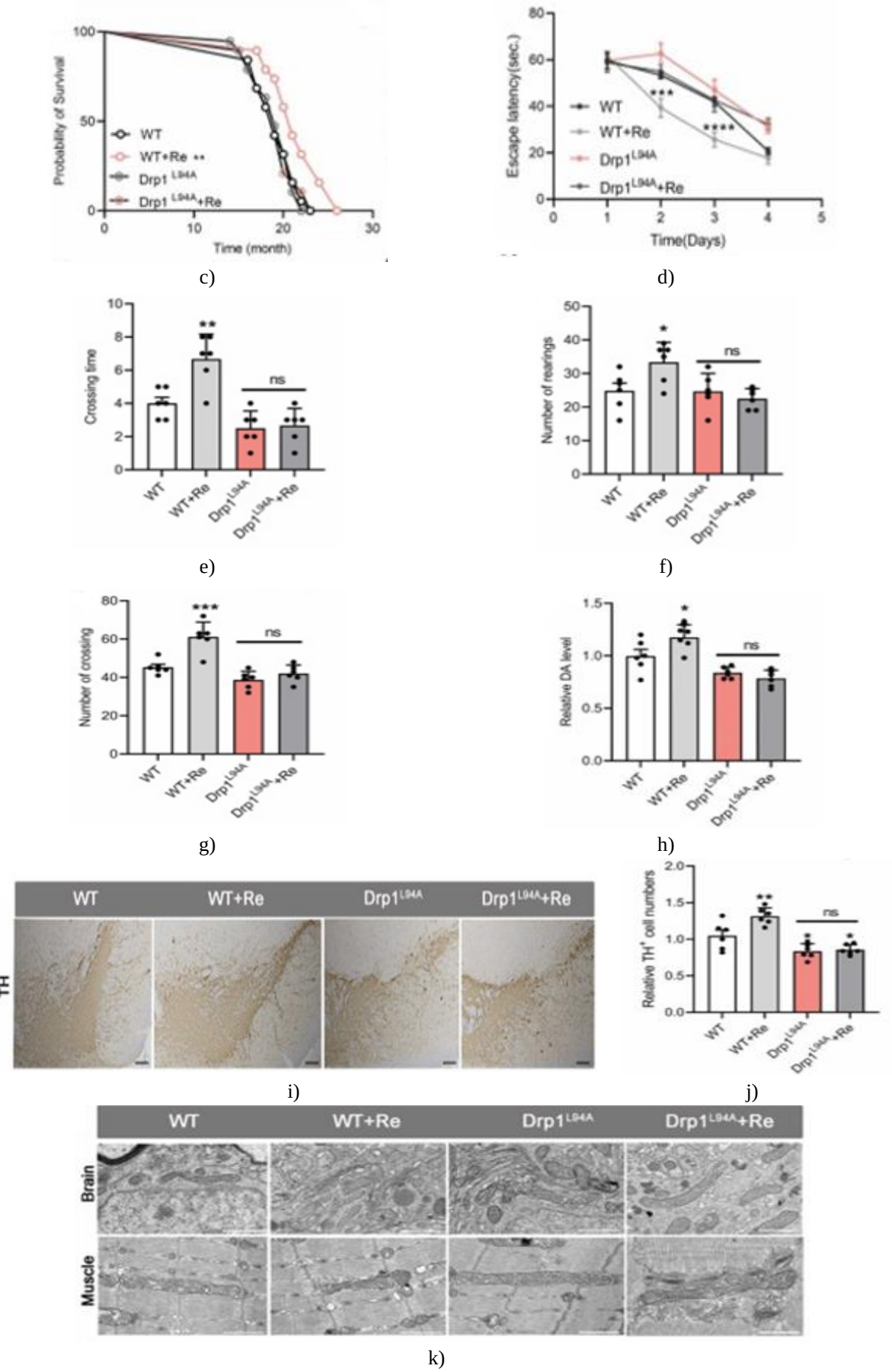
Further tests addressed whether the L94 site is needed for autophagy initiation. Drp1L94A mutants exhibited a 46% reduction in Atg1 protein that could not be rescued by Re (**Figure 14e**). Behaviorally, the mutation led to shorter lifespan (**Figure 14f**), accelerated dopaminergic neuron loss (dropping from 12–14 to 8–10 cells per hemisphere); (**Figure 14g**), and clear functional impairments — 32% worse learning, 11% reduced climbing, and 35% lower spontaneous activity (**Figures 14h, 14k**). Re treatment provided no improvement in any of these parameters in the mutant background.

In summary, Re engages the conserved L94 residue on Drp1 to increase S616 phosphorylation. This modification activates mitochondrial fission and launches an Atg1-dependent mitophagy cascade that is indispensable for protecting neurons and extending healthy lifespan.

Conserved efficacy of Re in delaying mammalian age-related degeneration targeting Drp1-L94 in mammals

To test evolutionary conservation of this mechanism in mammals, we studied Re's interaction with mouse Drp1 and developed Drp1L94A knock-in mice (**Figure 15a**). Computational simulations predicted that Re binding restricts structural flexibility and stabilizes murine Drp1 conformation. CETSA experiments confirmed that Re significantly raised the thermal stability of wild-type mouse Drp1 but had no impact on the L94A mutant protein, verifying that L94 is the key interaction site in mammals.





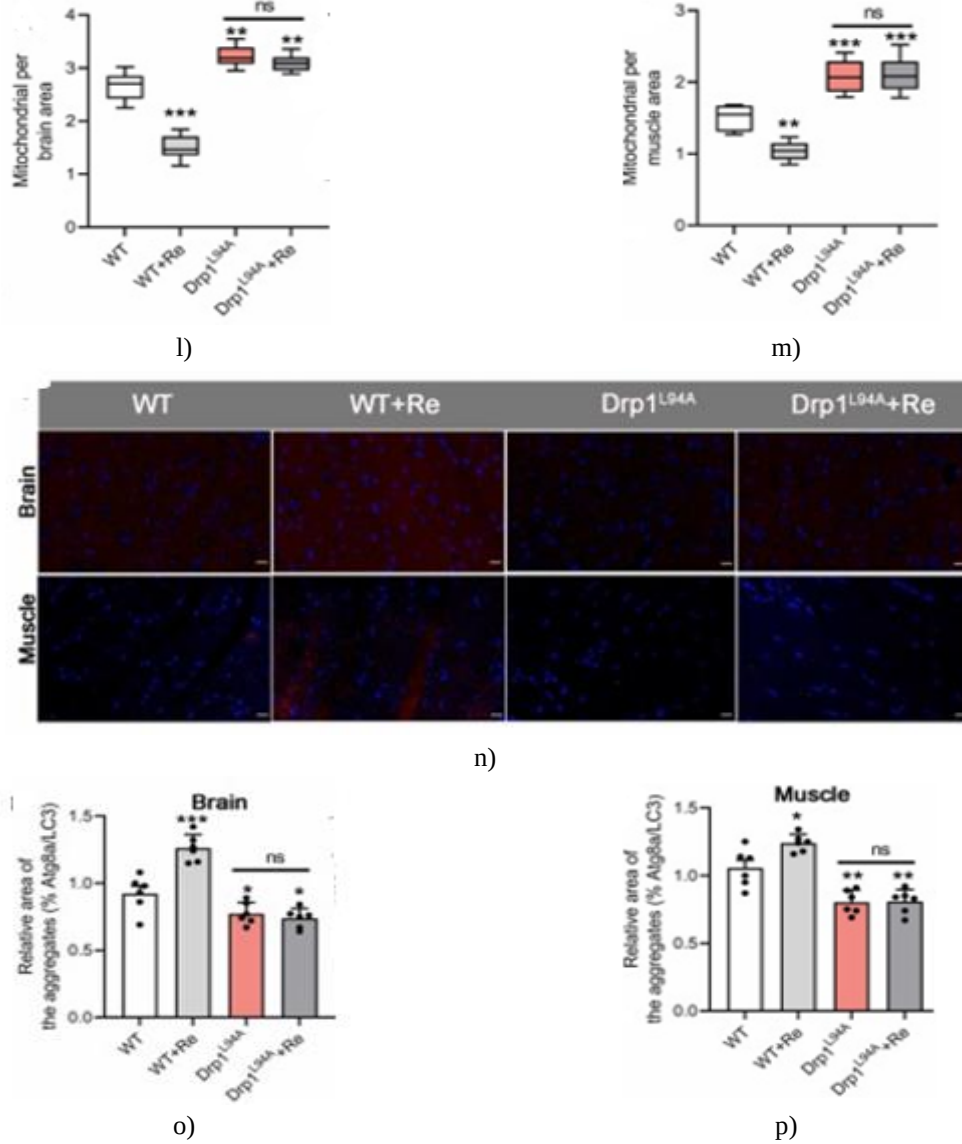


Figure 15. Re enhances cognitive-motor function and prolongs healthspan in mice by directly binding to Drp1 L94 site. (a) Diagram illustrating the strategy for generating Drp1^{L94A} knock-in mice. (b) Experimental timeline for Re administration. (c) Survival curves comparing wild-type and Drp1^{L94A} mice treated with or without Re (n = 30 animals per group). (d-e) Performance metrics in the Morris water maze test, including escape latency (D) and number of platform crossings (e) for WT and mutant mice ± Re (n = 6 per group). (f-g) Exploratory behavior in the open field test, measured by rearing events (f) and line crossings (g) (n = 6 per group). (h) Dopamine concentration in brain extracts from WT and Drp1^{L94A} mice ± Re (n = 6 per group). (i) Typical TH immunohistochemistry images and (j) quantification of TH-positive neurons in the substantia nigra (scale bar = 200 μm), n = 6 per group. (k) Electron micrographs of mitochondria in brain and skeletal muscle from the four experimental groups (scale bar = 1 μm). (l-m) Analysis of mitochondrial size in brain (l) and muscle (m) tissues from the TEM images. (n) Immunofluorescence for Atg8a/LC3 (red) with DAPI nuclear stain (blue) in brain and muscle sections (scale bar = 20 μm). (O-P) Quantitative measurement of Atg8a/LC3 signal intensity in brain (o) and muscle (p) (n = 6 per group). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus wild-type controls. Log-rank test for survival; one-way and two-way ANOVA for other endpoints.

Evaluation of the L94A mutation revealed clear functional consequences. Although lifespan remained comparable (**Figure 15c**), mutant mice showed pronounced deficits in cognition and movement. Morris water maze testing indicated a 50% longer escape latency and fewer platform crossings (reduced from 4 to 2.5); (**Figures 15d, 15e**). In the open field, exploratory activity decreased markedly, with 26% fewer rearings and 36.78% fewer crossings (**Figures 15f, 15g**). These behavioral changes were linked to a 28.7% reduction in dopaminergic neurons and

lower dopamine levels (**Figures 15h, 15j**). Midlife Re treatment (months 8–18); (**Figure 15b**) in wild-type animals extended healthspan parameters by 12.15% (**Figure 15c**), improved platform crossings to 6, increased rearings by 34.22%, and raised crossings by 34.92% (**Figures 15d, 15g**). Dopaminergic neuron counts and dopamine content also recovered by 17.7% (**Figures 15h, 15j**). None of these restorative effects appeared in Drp1L94A mice, confirming the critical importance of the L94 residue for Re activity.

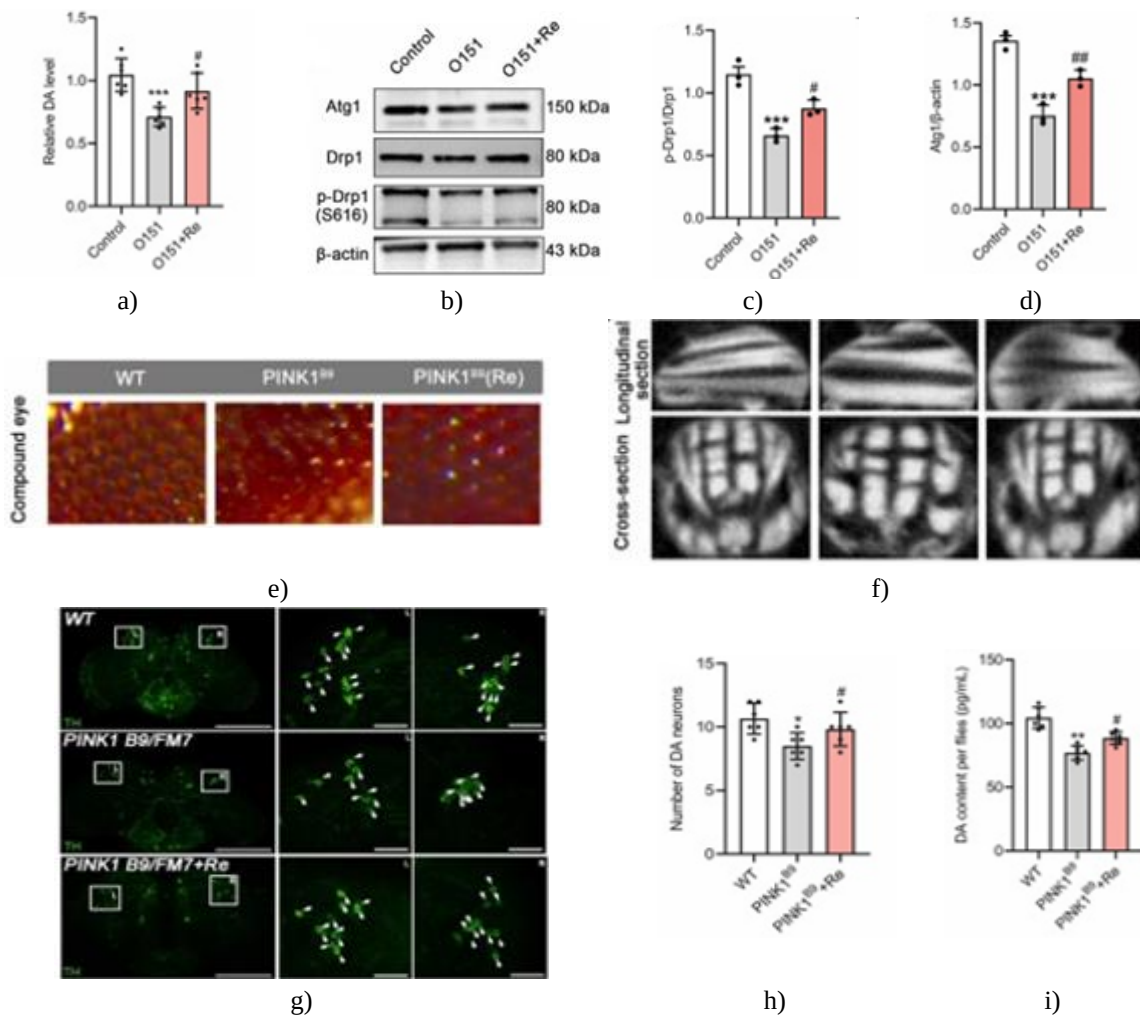
Further analysis focused on mitochondrial maintenance. In normal mice, Re boosted Drp1 S616 phosphorylation, favored fission (37% shorter mitochondria), and elevated autophagy markers (24% higher Atg8a/LC3); (**Figures 15k, 15p**). The L94A mutation blocked Re's ability to increase phosphorylation, normalize mitochondrial size (**Figures 15k, 15m**), or enhance autophagic activity (**Figures 15n, 15p**).

In conclusion, Re improves neurological performance and extends healthspan in mammals via a preserved mechanism that depends on binding to Drp1 at L94. This interaction facilitates S616 phosphorylation, drives fission, and triggers downstream autophagy to clear damaged mitochondria.

Re rescues age-related pathology in neuronal and PD Drosophila models by upregulating mitochondrial fission and autophagy

To thoroughly assess Re's disease-modifying potential, we utilized both a human iPSC-derived dopaminergic neuron culture system [45] and a Drosophila model of Parkinson's disease (PINK1B9) that mirrors important aspects of human neurodegeneration.

Dopaminergic neurons differentiated from human iPSCs were exposed to the senescence inducer O151 (10 μ M). This treatment caused a 38% drop in dopamine content, which Re supplementation largely prevented (**Figure 16a**). At the molecular level, Re increased Drp1 S616 phosphorylation and raised Atg1 protein expression (**Figures 16b, 16d**). These changes indicate that Re exerts neuroprotection by strengthening mitochondrial fission and autophagy processes.



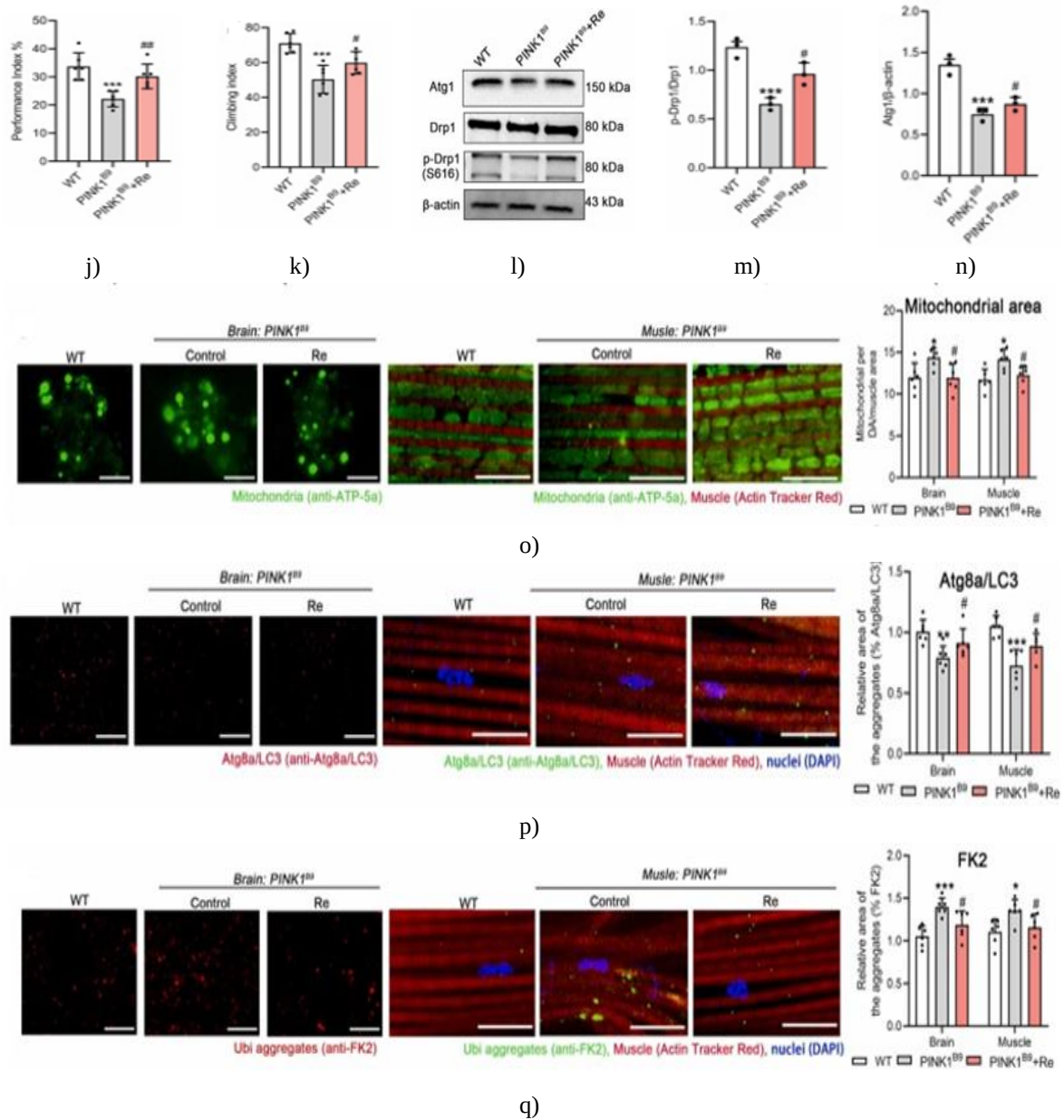


Figure 16. Re rescues age-related pathology in neuronal and PD Drosophila models by upregulating mitochondrial fission and autophagy. (a) Dopamine concentration measured by ELISA in human iPSC-derived dopaminergic neurons (n = 6 replicates). (b) Western blot analysis of Drp1 Ser616 phosphorylation and Atg1 protein levels in iPSC-derived DA neurons (n = 3 replicates). (c) Densitometric quantification of Drp1 Ser616 phosphorylation from panel B. (d) Densitometric quantification of Atg1 levels from panel B. (e) Representative images of compound eye morphology in wild-type, PINK1B9 mutant, and Re-treated PINK1B9 flies. (f) Micro-CT cross-sectional images of dorsal longitudinal muscles in WT, PINK1B9, and Re-treated PINK1B9 flies. (g) Immunofluorescence staining and (h) quantification of TH-positive PPL1 neurons per brain hemisphere (green, anti-TH; scale bars = 128 μ m and 25 μ m), n = 6 per group. (i) Dopamine content in fly brain extracts measured by ELISA (n = 6 per group). (j) Learning performance and (k) climbing ability in WT, PINK1B9, and Re-treated PINK1B9 flies (n = 6 replicates, 60 flies each). (l) Western blot analysis of Drp1 Ser616 phosphorylation and Atg1 levels in WT, PINK1B9, and Re-treated PINK1B9 flies (n = 3 replicates). (m) Densitometry of Drp1 Ser616 phosphorylation from panel L. (n) Densitometry of Atg1 levels from panel L. (o) Immunofluorescence of mitochondria (green, anti-ATP5a) in brain DA neurons and DLMs (red, Actin tracker) from the experimental groups (scale bars = 5 μ m and 10 μ m), n = 6 per group. (p) Immunostaining for Atg8a/LC3 (red in brain, green in DLM), Actin tracker (red in DLM), and DAPI (blue) (scale bars = 5 μ m and 10 μ m), n = 6 per group. (q) Staining of polyubiquitinated protein aggregates (red in brain, green in DLM), muscle fibers (red, Actin tracker), and nuclei (blue) (scale

bars = 5 μ m and 10 μ m), n = 6 per group. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 versus WT or control; #p < 0.05, ##p < 0.01 versus PINK1B9 or O151-treated groups (one-way and two-way ANOVA).

We further tested Re in the PINK1B9 *Drosophila* model, a widely used PD model featuring progressive dopaminergic neuron loss and motor impairment [67, 68]. Relative to wild-type controls, PINK1B9 mutants displayed pronounced degeneration of compound eyes and flight muscles (**Figures 16e, 16f**), substantial reduction in DA neuron numbers, lowered dopamine levels (**Figures 16g, 16i**), and deficits in learning and climbing performance (**Figures 16j, 16k**). Re treatment markedly ameliorated these abnormalities. It improved eye and muscle structural integrity, restored DA neuron counts to 10–12 per hemisphere, increased brain dopamine by 16%, and enhanced learning and motor functions by 31% and 18%, respectively (**Figures 16e, 16k**). Mechanistic studies revealed that PINK1B9 mutants had decreased Drp1 Ser616 phosphorylation, accumulation of swollen mitochondria (**Figures 16l, 16m, 16o**), reduced Atg1 and Atg8a/LC3 expression, and elevated protein aggregates, reflecting defective autophagy (**Figures 16l, 16n, 16p, 16q**). Re administration corrected these alterations by restoring Drp1 phosphorylation, normalizing mitochondrial morphology, and boosting autophagic clearance (**Figure 16l, 16q**).

Taken together, these experiments show that Re effectively counters age- and disease-related neurodegeneration in both human neuronal cultures and *Drosophila* PD models. It does so by activating Drp1-mediated mitochondrial fission and enhancing autophagic flux, positioning Re as a promising candidate for treating degenerative conditions.

Mitochondrial impairment plays a central role in driving age-related tissue degeneration, making restoration of mitochondrial balance a promising therapeutic objective [69, 70]. Our work demonstrates that administering Re during midlife effectively mitigates mitochondrial abnormalities, alleviates cognitive and motor decline, and extends healthy lifespan by coupling Drp1-dependent fission with mitophagy. At the molecular level, Re directly interacts with the evolutionarily conserved L94 residue on Drp1, promoting its phosphorylation at S616. This post-translational modification facilitates Drp1 recruitment to mitochondrial membranes, triggering fission and activating an Atg1-dependent mitophagic program that eliminates damaged organelles, reduces proteotoxic stress, and safeguards neuronal health.

Ginseng has a long-standing reputation for anti-aging effects and a strong safety record, supported by traditional use and contemporary clinical data [71, 72]. Modern trials further indicate that ginseng supplementation may help maintain cognitive abilities in older adults, with sustained intake associated with better scores on CERAD and MMSE assessments [73]. Nevertheless, challenges remain, including uncertainty regarding the most active constituents and incomplete understanding of their molecular targets, which has slowed progress in developing ginseng-derived therapies for age-related disorders. Through systematic screening, we identified ginsenosides Rd, Re, and Rg1 as multi-target compounds with strong affinity for AChE, MAO-B, and NMDA receptor — three proteins centrally involved in the pathology of Alzheimer's and Parkinson's diseases [5-7]. In addition to their well-known signaling roles, these targets influence mitochondrial function by dysregulating Drp1: AChE promotes A β aggregation [74, 75] and downstream Drp1 activation [76-78]; elevated MAO-B generates H₂O₂ and ROS that oxidatively modify Drp1 and induce pathological fission [79, 80]; and overstimulated NMDA receptors cause calcium overload [81] that leads to CaMKII-dependent Drp1 S616 phosphorylation [82, 83]. The ability of these ginsenosides to engage multiple relevant targets, together with their documented blood-brain barrier permeability [25, 84] and excellent safety profile (including minimal toxicity at high doses), makes them attractive candidates for restoring mitochondrial homeostasis in the central nervous system [25, 84].

A notable observation from our side-by-side comparison was that, despite comparable effects on lifespan extension, Re stood out in its capacity to reverse age-associated cognitive and motor impairments. This distinction may stem from differences in pharmacokinetics. Re exhibits lower overall exposure and a shorter half-life compared with Rg1 and Rd [85-87], potentially allowing more targeted action in neural and muscular tissues [88-90]. In contrast, the greater systemic availability and longer circulation time of Rg1 and Rd may suit them better for broader anti-oxidative and anti-inflammatory effects [91, 92]. This division of labor among ginsenosides illustrates nature's strategy for addressing the multifaceted nature of aging.

The non-linear nature of aging progression highlights the critical role of intervention timing [93]. Growing data suggest that therapies started in middle age—when molecular damage is mostly reversible—frequently produce better results than treatments applied throughout life [94]. This concept is illustrated by ginseng studies, in which

administering TGGR during midlife boosted lifespan extension from 11.49% to 15.24% compared with continuous lifelong treatment [50]. Our results follow this pattern, showing that Re achieves its strongest healthspan-prolonging benefits when given in midlife.

At the cellular level, Re provides notable neuroprotection by safeguarding dopaminergic neuron integrity, which is essential for cognitive and motor abilities. In our experiments, Re recovered DA neuron numbers and neurotransmitter concentrations in aged *Drosophila*, changes that corresponded with enhanced learning scores and better locomotor activity. The compound eye, recognized as a reliable indicator of whole-body aging [95], additionally validated Re's broad anti-aging impact through decreased neurodegeneration and better-preserved ultrastructure. At the same time, Re reduced age-associated muscle deterioration by reversing the hypertrophic fragmentation and disorganized fiber patterns seen in the indirect flight muscles of older flies. These observations support accumulating research emphasizing that mitochondrial balance in skeletal muscle is vital for neuromuscular function and for postponing sarcopenia [96]. Collectively, these data reinforce the idea that Re slows degenerative conditions linked to aging and offer a foundation for future mechanistic investigations.

The brain and muscle tissues are especially susceptible to mitochondrial impairment because of their elevated energy demands. As organisms age, electron transport chain performance drops, upsetting redox balance, elevating ROS generation, lowering ATP output, intensifying oxidative injury, and undermining tissue health [97]. Our work demonstrates that midlife Re treatment interrupts this harmful sequence by maintaining mitochondrial membrane potential (MMP), lowering mitochondrial ROS via enhanced oxidative phosphorylation (OXPHOS), and boosting ATP production. Importantly, Re restores mitochondrial network adaptability by readjusting the opposing processes of fission and fusion to achieve a balanced state that supports organelle quality control [98-100].

Mitochondrial dynamics during aging typically shift from initial hyperfusion to eventual fragmentation. Our findings of pronounced mitochondrial fusion in middle-aged flies are consistent with recent studies indicating that a measured increase in fission can promote healthspan [43]. To pinpoint Re's primary mitochondrial target, we conducted a focused genetic screen using the UAS/Gal4 system. Neuronal expression was controlled via TH-Gal4 for DA neuron clusters (such as PAM, PPL1, and PPM1/2/3 [101]), while Mef2-Gal4 drove muscle-specific expression [102]. Particular attention was given to the PPL1 cluster because of its well-documented importance in long-term memory and motor function [103-106]. This emphasis was supported by initial observations showing Re's stronger protective influence on PPL1 neuron viability and activity relative to other neuron groups. These strategies uniformly pointed to Drp1 as the main mediator through which Re encourages appropriate mitochondrial fission in both neuronal and muscle contexts. This key function of Drp1 was confirmed in whole-body experiments with the *da*-Gal4 driver, confirming its essential role in Re's homeostatic benefits [107]. The high degree of selectivity was further verified by CETSA, which revealed Re's specific stabilization of Drp1 among various fission and fusion proteins, pointing to a targeted action with low risk of off-target effects.

Re activates the autophagy system via a unique route focused on Atg1. Our results indicate that Re enhances autophagic initiation (through elevated Hsp90 phosphorylation [64]) and supports autophagosome expansion (via increased Atg8a lipidation [65]). Although both Atg1 and Atg5 appear involved, Re's lifespan-extending activity depends critically on Atg1 yet remains largely unaffected by Atg5 reduction. This observation implies that Re's Atg1-driven pathway may rely on residual Atg5 activity, compensatory pathways, or alternative quality-control systems. Based on these results, we propose a self-regulating quality-control cycle driven by coordinated Drp1 and Atg1 activity. We describe this interaction as "spatial coupling," reflecting their functional interdependence rather than direct physical linkage, as shown by disrupted mitochondrial localization of each protein upon knockdown of the other. A plausible and increasingly evidenced model involves Drp1 recruitment that strengthens mitochondria-endoplasmic reticulum contact sites (MERCs), thereby drawing in Atg1 [108, 109]. The resulting localized autophagy components then reinforce the site and further recruit Drp1, establishing a positive feedback loop [110, 111]. This framework accounts for their functional partnership without physical binding, and the exact signaling details will be the subject of upcoming studies.

A notable feature of Re is its selective interaction with the autophagy pathway, setting it apart from other known Drp1 modulators. Most previously reported small-molecule Drp1 regulators, such as Mdivi-1 and related division inhibitors, act via direct GTPase blockade, while peptide inhibitors like P110 interfere with Drp1-Fis1 protein interactions [112-114]. These approaches cause broad, sustained suppression of mitochondrial fission. However, prolonged global inhibition carries substantial drawbacks. First, Drp1-dependent fission supports numerous essential cellular processes, including energy production, cell division, and programmed cell death; its total

blockade disturbs mitochondrial network balance and impairs cell function [115, 116]. Second, aging involves a time-dependent shift in mitochondrial dynamics—from early hyperfusion to later hyperfragmentation—requiring adaptive, physiology-compatible strategies rather than indiscriminate long-term shutdown. Finally, synthetic Drp1 inhibitors like Mdivi-1 frequently exhibit off-target effects and toxicity, including poor stability in vivo, limited precision [117, 118], and unintended inhibition of mitochondrial complex I [119, 120], which can worsen oxidative stress and induce apoptosis [119, 120]. Such toxicities in neural and cardiac tissues have notably restricted the clinical development of Mdivi-1 [120-122].

In contrast, Re offers a more refined regulatory approach (**Figure 17**). (1) Allosteric and site-specific modulation: Re binds selectively to the L94 site on Drp1 and enhances S616 phosphorylation via allosteric activation rather than broadly inhibiting Drp1 activity. This strategy improves Drp1's functional precision while preserving its normal fission capabilities, thereby avoiding major disruption to physiological mitochondrial dynamics. (2) Self-limiting quality-control loop: Re simultaneously initiates Drp1-mediated fragmentation of damaged mitochondria and stimulates Atg1-dependent autophagy, generating a coupled “fission–autophagy” positive feedback mechanism. This loop enables prompt clearance of P62-positive aggregates, restores mitochondrial network balance, and curbs pathological hyper-fission (for instance, in A β -associated neurodegeneration). The result is a bidirectional, self-regulating process. (3) Temporal precision: Re demonstrates effectiveness beginning in the hyperfusion phase of aging, supporting midlife intervention as a viable strategy. Moreover, as a natural component of *Panax ginseng*, Re benefits from a well-documented safety history and synergistic effects observed in preclinical and human research [123]. Through its smart, gentle, and homeostatic mode of action, Re provides a superior safety profile for extended use compared with synthetic inhibitors. Relative to other natural mitochondrial modulators such as resveratrol and metformin—which mainly act via AMPK stimulation or antioxidant properties [124-126]—Re stands out by directly engaging the mitochondrial fission apparatus with fine spatiotemporal control. This precise Drp1 targeting, together with its established safety as a ginseng-derived compound, makes Re a strong prospect for sustained therapy against age-related mitochondrial decline.

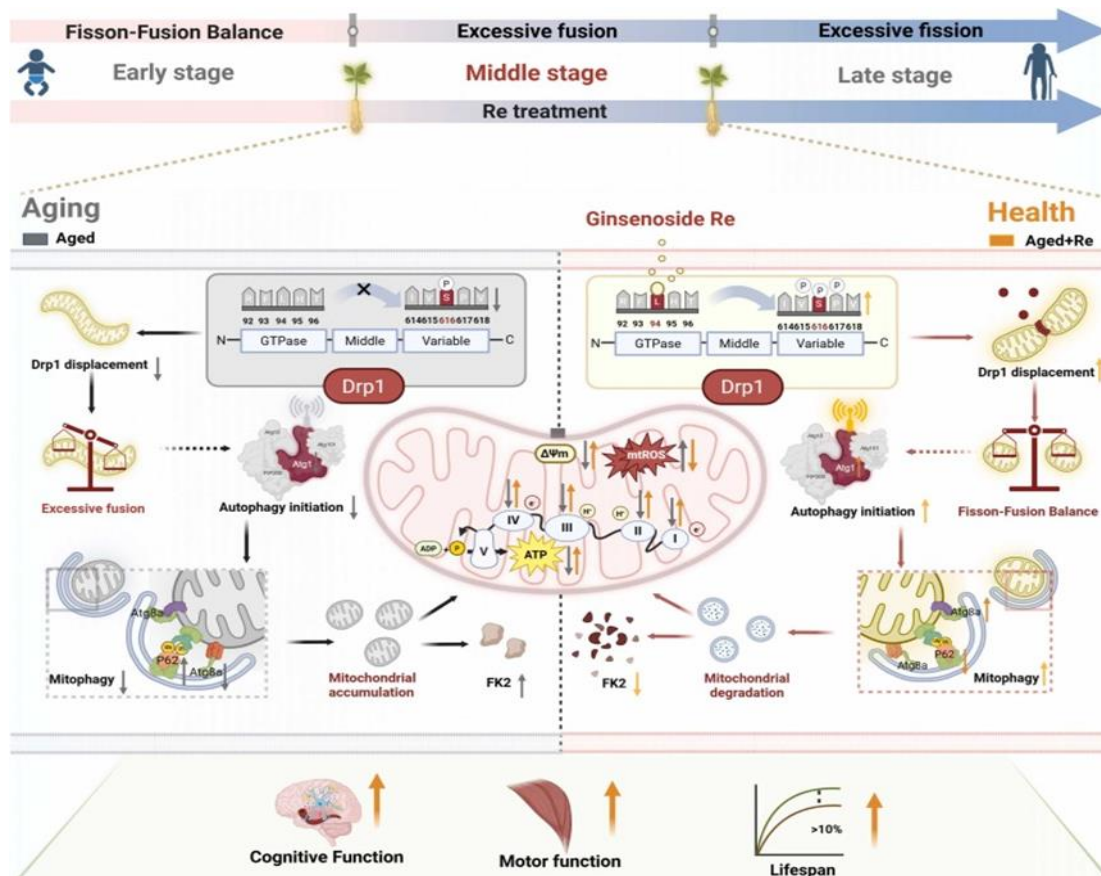


Figure 17. Schematic overview of how midlife Re treatment mitigates age-related decline through the restoration of mitochondrial balance.

The therapeutic promise of Re is strengthened by the strong evolutionary preservation of its mode of action. The L94 binding site on Drp1 is highly conserved among species, and our binding experiments verified direct interaction between Re and Drp1 in human, mouse, and *Drosophila* models. Importantly, Drp1L94A mutant mice display age-associated neurological deficits similar to those seen in flies and fail to respond to Re, further confirming the mechanism's fundamental conservation. Re also exerts clear neuroprotective actions in both human neuronal cultures and Parkinson's disease *Drosophila* models via its integrated fission-mitophagy pathway, underscoring its promise as a potential treatment for human neurodegenerative diseases.

It is important to acknowledge that the broader applicability of our results has some constraints, notably the absence of analysis regarding possible sex-specific effects. Emerging clinical data reveal marked sexual dimorphism in the development and course of neurodegenerative diseases. For example, women diagnosed with Alzheimer's disease often show more pronounced tau accumulation and faster cognitive deterioration, while men have approximately 1.5 times higher rates of Parkinson's disease [127, 128]. These variations are believed to arise from differences in sex hormone levels and genetic influences. Although our investigation, consistent with much of the existing literature, did not thoroughly examine sex as a biological factor, upcoming studies will directly investigate whether Re's protective benefits vary between males and females. Such insights could guide the development of more individualized treatment approaches.

Additionally, multiple steps are still required to translate Re's potential into clinical use. To move from mechanistic understanding to practical application, we intend to conduct detailed pharmacokinetic analyses and Good Laboratory Practice (GLP)-standard toxicology assessments in rodents and non-human primates. At the same time, structure-activity relationship (SAR) investigations will aim to improve Re's drug-like characteristics, including better bioavailability and overall therapeutic performance. These initiatives will be essential for progressing Re through the development pipeline and determining its readiness for human clinical studies.

Conclusion

Overall, our work indicates that initiating Re treatment during midlife can slow age-related deterioration by reinstating mitochondrial equilibrium. In human cells, *Drosophila*, mice, and neuronal disease models, Re interacts with the evolutionarily preserved L94 site on Drp1, leading to specific phosphorylation at S616 and subsequent GTPase-driven mitochondrial fission. This process launches a self-regulating quality control loop in which Atg1-dependent mitophagy eliminates impaired organelles, thereby maintaining the health of neurons and muscle tissue. Notably, Re's selective stimulation of the Drp1-Atg1 pathway effectively prevents the loss of DA neurons, corrects cognitive and motor impairments, and promotes healthspan benefits that are eliminated in Drp1L94A mutants. By coordinating mitochondrial dynamics with autophagic clearance, Re moves beyond traditional fission blockers to deliver a refined, non-disruptive strategy for supporting healthy aging.

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Ethics Statement: None

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