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Short Communication

Extension of Lifespan and Amelioration of Alzheimer's Disease Phenotypes by Genetic Manipulation of Mitochondrial NAD^+/NADH Ratio

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ABSTRACT: Aging remains the most significant risk factor for common neurodegenerative diseases including Alzheimer's disease (AD). According to the geroscience hypothesis, aging is malleable and that by targeting basic aging physiology, we can alleviate many of the age-related chronic diseases. The common mechanisms driving aging and age-related diseases remain poorly defined. Mitochondrial dysfunction is recognized as a fundamental hallmark of aging, and recent studies implicate mitochondrial reverse electron transport (RET) as a driver of aging. The key outcomes of RET, increased ROS and decreased NAD^+/NADH ratio, have both been associated with aging and age-related disease, but the causal relationship remains uncertain. Here we applied causal metabolism to test the role of mitochondrial NAD^+/NADH in aging and AD, using *Drosophila* as a model system. By using a mitochondrial targeted version of *Lactobacillus brevis* NADH oxidase (LbNox) to boost mitochondrial NAD^+/NADH ratio independent of the energy state of the cell, we found that increasing mitochondrial NAD^+/NADH ratio in neuronal or muscle tissues is sufficient to extend lifespan. Moreover, boosting mitochondrial NAD^+/NADH ratio is beneficial in two independent models of AD, rescuing the proteostasis failure, locomotor and cognitive deficits, and lifespan shortening in these models. Our results identify altered mitochondrial NAD^+/NADH ratio as a major contributor to the biological effects of RET on aging and age-related diseases and a potential therapeutic target.

Keywords: Reverse electron transport, NAD^+/NADH ratio, ROS, LbNox, Aging, Alzheimer's disease

INTRODUCTION

Aging is a progressive decline of physiological function, accompanied by the onset of age-related chronic diseases, that lead to eventual organismal death. Of all age-related diseases, Alzheimer's disease (AD) is the definitive representative: The risk doubles every five years after age 65, reaching a staggering 30-50% prevalence in those over 85 [1]. Despite intensive efforts and the recent approval of antibody-based drugs, effective treatment for AD is urgently needed. The geroscience hypothesis posits that physiological aging is the major driver of age-related chronic diseases, aging is malleable, and that by targeting fundamental mechanisms of aging, we can alleviate multiple age-related diseases simultaneously [2-4]. Identifying the molecular and cellular mechanisms commonly involved in aging and age-related diseases thus holds tremendous promise for therapeutic intervention.

Mitochondrial dysfunction is a key hallmark or pillar of aging [5, 6]. It is intimately connected to and maybe causal for other hallmarks of aging [7]. The fundamental mitochondrial process that drives aging remains to be defined. Recent studies implicate mitochondrial reverse electron transport (RET) as a fundamental player in aging [8, 9]. RET, opposite of the forward electron transport (FET) that produces ATP during oxidative phosphorylation (OxPhos), is a process by which electrons flow backward along the mitochondrial electron transport chain (ETC) from $CoQH_2$ to the NAD^+/NADH binding site of mitochondrial complex I, when FET is

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inefficient and thermodynamic forces driving RET (e.g., $CoQH_2$ and mitochondrial membrane potential) are high [10]. This leads to excessive reactive oxygen species (ROS) formation and conversion of NAD^+ to NADH, thus lowering NAD^+/NADH ratio [11]. First observed in the 1960s [12], RET was regarded as a mere *in vitro* phenomenon until it was observed *in vivo* during cardiac ischemia-reperfusion injury [13]. RET is activated during normal aging [9], in models of Notch-induced brain tumors [14], and in neurodegenerative disease settings [15, 16]. While RET is considered a major source of mitochondrial ROS [17] and RET-ROS is heavily studied as the causative agent of RET related pathologies [13, 16], the role of RET-ROS and mitochondrial ROS in aging remains controversial [8, 9, 18]. RET is also a main determinant of mitochondrial and cellular NAD^+/NADH ratio, which mediates much of the biological effects of RET [9, 14, 15]. However, the direct role of NAD^+/NADH ratio in aging and age-related diseases has not been directly tested.

MATERIALS AND METHODS

Drosophila genetics

The *UAS-mito-LbNox* transgene was generated at *BestGene* Inc (Chino Hills, CA, USA) using the PhiC31 integrase-mediated site-specific transgenesis. Lifespan, behavioral analysis, immunofluorescence, NAD^+/NADH and ROS measurements in Drosophila were performed essentially as described [9]. See Supplementary Materials for detailed methods of *Drosophila* genetics and other methods used in this study.

RESULTS AND DISCUSSION

We used the water-forming *Lactobacillus brevis* NADH oxidase (LbNox) as a genetic tool to convert NADH to NAD^+ and therefore boost NAD^+/NADH ratio [19] without altering the NAD(H) pool size in *Drosophila*, a well-established model system for studying aging and neurodegenerative diseases. To mimic the effect of RET on mitochondrial NAD^+/NADH ratio, we used a mitochondrial-targeted LbNox (mito-LbNox). Given the importance of mitochondrial function to the neuromuscular system, we used the *UAS-Gal4* system to express mito-LbNox in neuronal (*elav-Gal4>mito-LbNox*) or muscle (*Mhc-Gal4>mito-LbNox*) tissues. We first used muscle tissues to assess the effect of mito-LbNox on mitochondrial and cellular NAD^+/NADH ratio. We found that when purified mitochondria from young (7 days) or aged (40 days) muscle were respiring under RET condition (fueled with succinate as the substrate), control mitochondria exhibited age-related decrease of NAD^+/NADH ratio. This was blocked by mito-LbNox (Fig. 1A). When mitochondria were respiring under FET condition (fueled with the TCA cycle substrates malate and glutamate that feed into complex I), no obvious age effect was observed in control or mito-LbNox expressing samples (Fig. 1B). When tissue lysates were used to measure NAD^+/NADH ratio, we also observed an age-related decline, and this effect was also effectively rescued by mito-LbNox (Fig. 1C), consistent with mitochondrial NAD^+/NADH being a major determinant of cellular NAD^+/NADH ratio. Interestingly, mito-LbNox also significantly decreased ROS level in aged flies (Fig. 1D). This suggests an intimate connection between mitochondrial NAD^+/NADH and cellular ROS. The ROS decrease by mito-LbNox could be mediated by the increased NAD^+/NADH ratio that leads to decreased electron leakage along the ETC, or increased antioxidant enzyme activity through activation of the NAD^+-dependent Sirtuin signaling.

We next examined the effect of mito-LbNox on aging. We found that both neuronal and muscle expression of mito-LbNox resulted in extended lifespan (Fig. 1E, F). This effect was observed in both males and females. Aged flies exhibited impaired locomotor activity in the climbing assay. This was also significantly rescued by mito-LbNox, suggesting that boosting NAD^+/NADH ratio could extend healthspan (Fig. 1G). Proteostasis failure is a major hallmark of aging. We found that the accumulation of protein aggregates positive for ubiquitin and the autophagy receptor p62 in aged muscle (Fig. 1H, J) and neuronal (Fig. 1I, J) tissues was effectively rescued by mito-LbNox, suggesting mito-LbNox influences healthspan and lifespan by impinging on major hallmarks of aging.

We next tested whether restoring NAD^+/NADH ratio by mito-LbNox could rescue phenotypes of age-related diseases as would be predicted by the geroscience hypothesis. Overexpression of the C-terminal region (C99) of the AD-associated amyloid precursor protein (APP) in neuromuscular tissues has been an effective way to model AD [20]. Consistent with previous findings, RET is activated in transgenic flies expressing C99 in the muscle, resulting in reduced NAD^+/NADH ratio in total lysates or purified mitochondria respiring under RET condition. This was fully rescued by mito-LbNox (Fig. 2A). Furthermore, the elevated ROS level in C99 flies was also significantly attenuated by mito-LbNox (Fig. 2B), as was the C99 induced shortened lifespan (Fig. 2C). This was correlated with the reduction of ubiquitin- and p62-positive protein aggregates (Fig. 2D, G). Interestingly, mito-LbNox expression also removed the stalled and CAT-tailed, ribosome-associated quality control (RQC) products of C99 (Fig. 2E), which have the propensity to aggregate and were previously shown to be causal of the

proteostasis failure in AD models [20]. The effect of mito-LbNox on RQC could be mediated by the NAD⁺-dependent Sirtuin signaling or mitochondrial ROS signaling pathways.

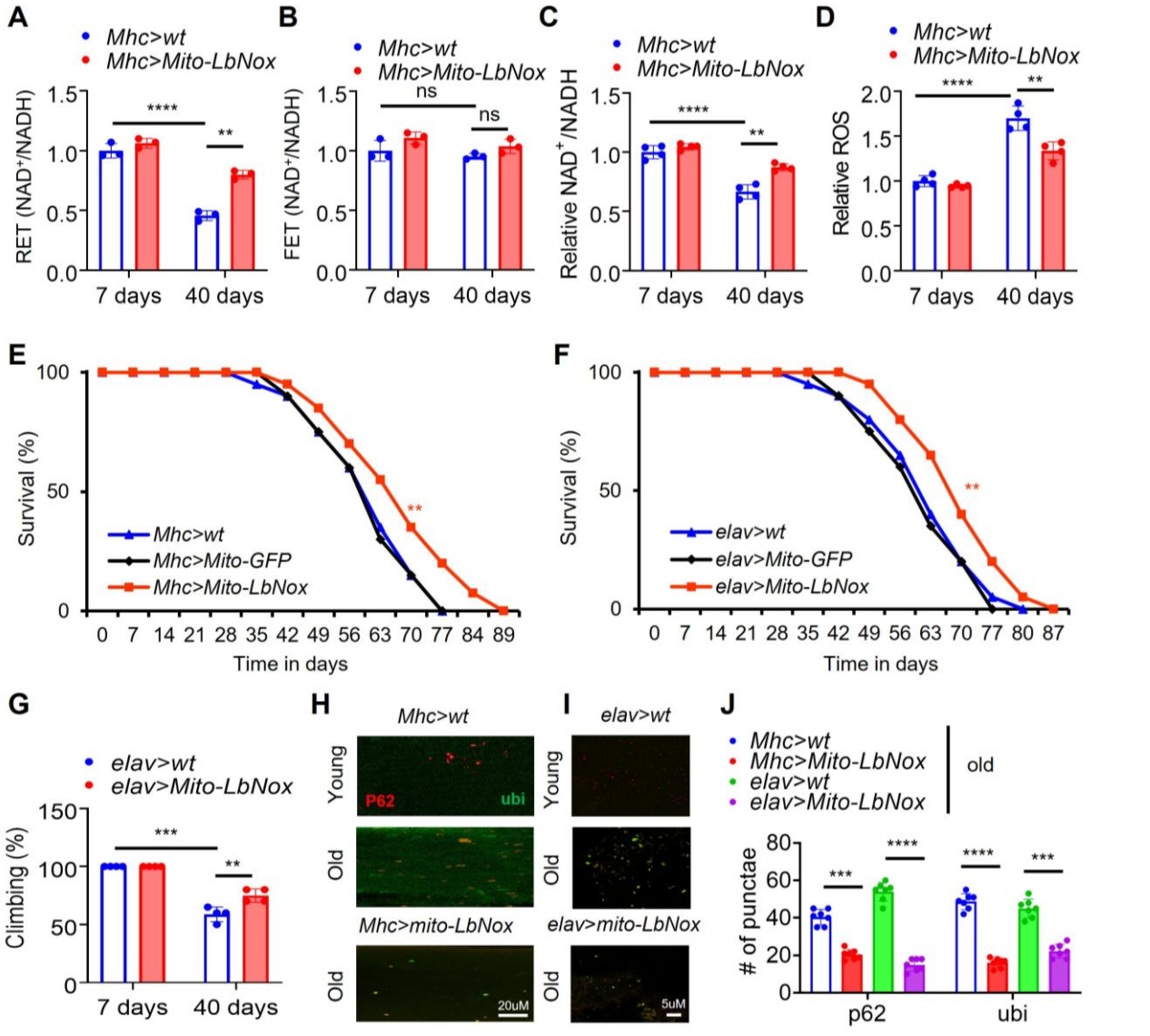

Figure 1. Increasing mitochondrial NAD⁺/NADH with mito-LbNox extends lifespan and healthspan in wildtype *Drosophila*. (**A, B**) Measurement of NAD⁺/NADH ratio in purified mitochondria from young and aged *Mhc-Gal4>wt* control and *Mhc-Gal4>mito-LbNox* transgenic flies respiring under RET (**A**) or FET (**B**) conditions. (**C, D**) Measurement of NAD⁺/NADH ratio (**C**) or ROS level (**D**) in tissue samples from young and aged *Mhc-Gal4>wt* control and *Mhc-Gal4>mito-LbNox* transgenic flies. (**E, F**) Survival curves of control flies and transgenic flies expressing mito-GFP or mito-LbNox in the muscle (**E**) or neurons (**F**). (**G**) Assessment of the climbing ability of young and aged *elav-Gal4>wt* control and *elav-Gal4>mito-LbNox* transgenic flies. (**H-J**) Immunostaining (**H, I**) and quantification (**J**) of p62 and ubiquitin positive protein aggregates in the muscle (**H**) or brain (**I**) of aged control or mito-LbNox transgenic flies. All data are presented as mean ± SEM. Statistical significance was determined using two-way ANOVA followed by Sidak's post hoc test for multiple comparisons (**A**, **B**, **C**, **D**, **G**, **J**) or Kaplan–Meier survival analysis (**E**, **F**). Each group consisted of 10 flies for NAD⁺/NADH, ROS and climbing assays, and 20 flies for survival assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. For NAD⁺/NADH, ROS, lifespan, and climbing assays, n=4 biological replicates. For *in vitro* experiments and confocal imaging, n=3–5 biological replicates.

Neuronal expression of C99 also led to the accumulation of ubiquitin- and p62-positive aggregates in the brain (Fig. 2F, G). This was correlated with compromised locomotor activity in the climbing assay (Fig. 2H) and learning and memory deficit in the aversive taste memory assay (Fig. 2I) and the olfactory learning and memory assay (Fig. 2J), an assay commonly used for cognitive assessment in fly AD model studies [21, 22],

and shortened lifespan (Fig. 2K). All these phenotypes were rescued by mito-LbNox (Fig. 2F-K). In another AD fly model [23] featuring neuronal co-expression of APP and b-secretase (BACE), the enzyme involved in the cleavage of APP to generate C99, mito-LbNox also rescued the learning and memory deficit (Fig. 2L). Co-expression of APP and b-secretase (BACE) in neurons also resulted in a shortened lifespan, which was rescued by mito-LbNox as well (Fig. 2M).

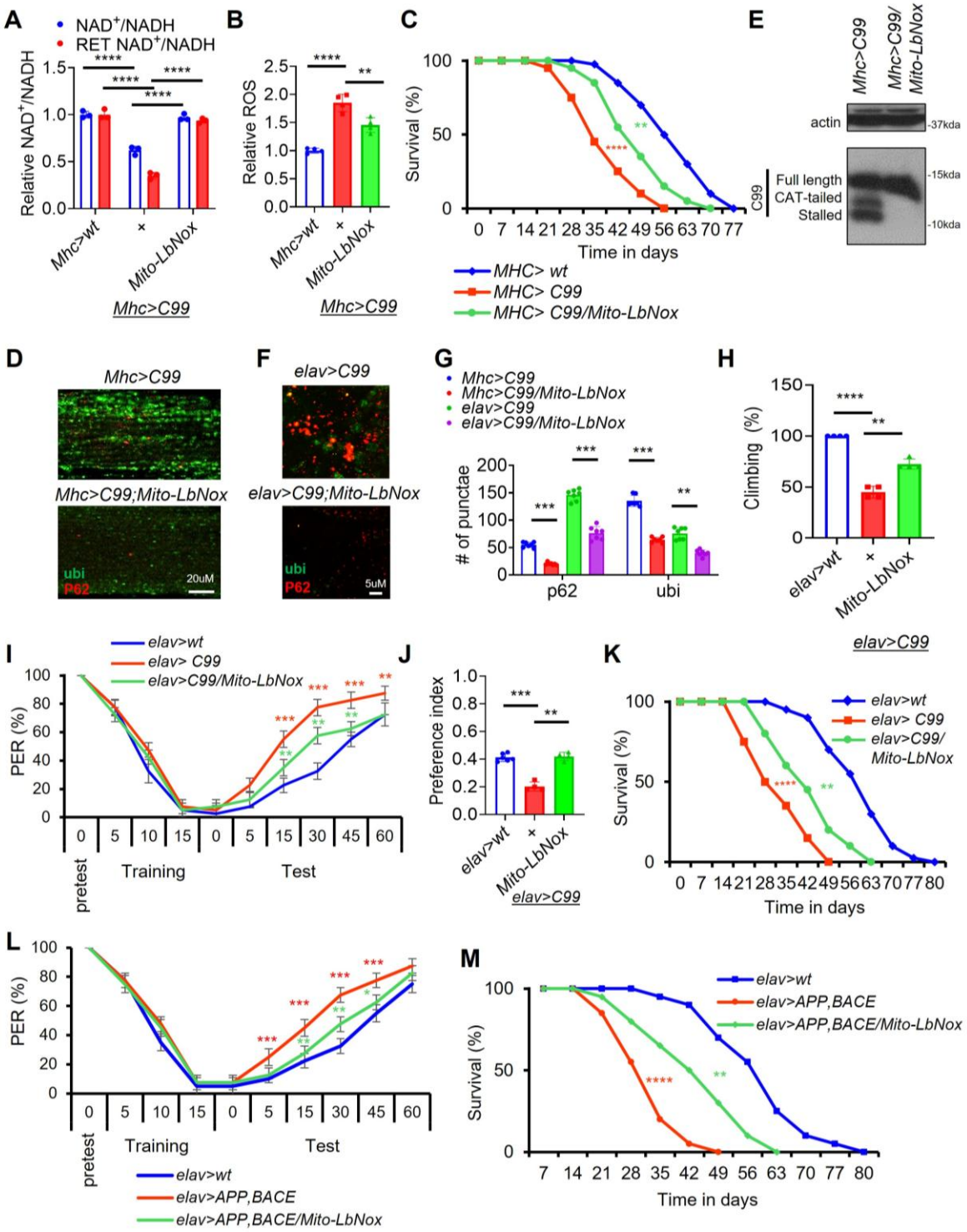

Figure 2. Increasing mitochondrial NAD$^+$/NADH ratio with mito-LbNox protects against Alzheimer's disease in *Drosophila* models. (**A**) Measurement of NAD$^+$/NADH ratio in tissue samples or RET-induced NAD$^+$/NADH in purified mitochondria from *Mhc>wt* control, *Mhc>C99* transgenic flies, or *Mhc>C99* transgenic flies co-expressing mito-LbNox. (**B**) Measurement of ROS levels in muscle tissues of *Mhc>wt* control, *Mhc>C99* transgenic flies, or *Mhc>C99* transgenic flies co-expressing mito-LbNox. (**C**) Survival curves of control and *Mhc>C99* transgenic flies with or without muscle-specific expression of mito-LbNox. (**D**) Immunostaining of p62- and ubiquitin-positive protein aggregates in the muscle of C99 transgenic flies with or without mito-LbNox co-expression. (**E**) Western blot analysis showing the effect of mito-LbNox in removing the aberrant CAT-tailed and stalled translation products of C99. The protein levels were normalized with actin as the loading control. (**F**) Immunostaining of p62- and ubiquitin-positive protein aggregates in the brain of C99 transgenic flies with or without mito-LbNox co-expression. (**G**) Quantification of p62- and ubiquitin-positive protein aggregates shown in **D** and **F**. (**H**) Climbing assay showing the effect of mito-LbNox on motor function in neuronal C99 transgenic flies. (**I**) Percentage of proboscis extension response (PER) indicating improved learning and memory in the aversive taste memory assay in neuronal C99 transgenic flies co-expressing mito-LbNox. (**J**) Preference of flies to neutral odor over electric shock-associated odor in the olfactory learning and memory assay in neuronal C99 transgenic flies co-expressing mito-LbNox. (**K**) Survival curve of control and neuronal C99 transgenic flies with or without mito-LbNox co-expression. (**L**) Effect of mito-LbNox on learning and memory in the aversive taste memory assay in neuronal APP; BACE transgenic flies. (**M**) Survival curves of control and neuronal APP; BACE transgenic flies with or without the co-expression of mito-LbNox. All data are presented as mean ± SEM. Statistical significance was determined using two-way ANOVA followed by Sidak's post hoc test for multiple comparisons (**A**, **G**), one-way ANOVA followed by Tukey's post hoc test for multiple comparisons (**B**, **H**, **J**), Kaplan–Meier survival analysis (**C**, **K**, **M**), or group analysis using multiple *t* test with Sidak-Bonferroni corrections (**I**, **L**). Each group consisted of 10-12 flies for NAD$^+$/NADH, ROS, climbing activity, and learning and memory assays, 50-60 flies for olfactory learning and memory assays, and 20 flies for survival assays. *$p < 0.05$; **p < 0.01; ***$p < 0.001$; ****p < 0.0001. For NAD$^+$/NADH, ROS, survival, learning and memory, and climbing assays, n=4 biological replicates. For *in vitro* experiments, western blot, and confocal imaging, n=3–5 biological replicates.

In summary, restoring mitochondrial NAD$^+$/NADH ratio by the expression of mito-LbNox extended the healthspan and lifespan of normally aging flies and AD model flies and alleviated the proteostasis failure associated with aging and AD. Mito-LbNox also rescued the cognitive and behavioral deficits in AD fly models. Our study provides direct support for the geroscience hypothesis. These findings suggest that genetic or pharmacological manipulation of mitochondrial NAD$^+$/NADH ratio may represent a rational strategy to promote organismal resilience during aging. It is worth noting that NAD$^+$-boosting interventions in mammalian systems, such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) supplementation, have been shown to offer certain healthspan benefits, although challenges remain [24, 25]. Moreover, NAD$^+$-boosting interventions increase both NAD$^+$ and NADH pools and they may not restore NAD$^+$/NADH ratio as effectively as mito-LbNox, which changes NAD$^+$/NADH ratio without affecting the total NAD(H) pool. Further validation in higher organisms is required to validate the translational relevance of manipulating mitochondrial NAD$^+$/NADH ratio. If conserved, it could represent a potential strategy to improve healthspan in normal individuals and a therapeutic avenue for AD and possibly other age-related diseases featuring RET activation.

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Conflict of interest

The authors declare that they have no competing interests.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0011.

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