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Improvement of mitochondrial function decreases cytosolic mitochondrial DNA and ameliorates inflammaging

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Roberta Alessi

2023

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2023

ABSTRACT OF THE THESIS

Improvement of mitochondrial function decreases cytosolic mitochondrial DNA and ameliorates inflammaging

by

Roberta Alessi

Master of Science in Physiological Science

University of California, Los Angeles, 2023

Professor David William Walker, Chair

Mitochondrial dysfunction, a hallmark of aging and degenerative diseases, increases the production of mitochondrial Reactive Oxygen Spices and decreases the production of ATP. Damage mitochondria can result in the release of mitochondrial DNA (mtDNA) into the cytosol, where it acts as a damage-associated molecular pattern (DAMP) and triggers the activation of the immune response. Our study demonstrates that as flies age, there is an accumulation of cytosolic mtDNA. We found that improving mitochondrial function through the overexpression of Parkin during adulthood and DRP1 from midlife onwards effectively reduces the build-up of cytosolic mtDNA in both heads and thoraces of aged flies. Moreover, we observed a significant decrease in the expression of antimicrobial peptides (AMPs) and stress immune effectors. These findings highlight the potential of mitophagy-promoting interventions to mitigate cytosolic mtDNA accumulation and dampen the immune response activation in aging organisms.

The thesis of Roberta Alessi is approved.

Xia Yang

Claudio Javier Villanueva

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David William Walker, Committee Chair

University of California, Los Angeles

2023

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1. Introduction

1.1 Mitochondria and Aging

Aging is a process characterized by the time-dependent and progressive decline of vital functions and physiological integrity resulting in altered cellular homeostasis in every organism [1]. This physiological deterioration constitutes the primary threat for chronic diseases such as cardiovascular diseases, cancer, metabolic syndrome, and age-related diseases. A number of causes can be attributed to this event, including genetic, physical, and environmental patterns. At the biological level, aging results from the impact of the progressive accumulation of a wide variety of molecular and cellular damage over time [2]. Several diseases associated with aging such as autoimmune diseases show an increase in cellular hyperactivity and increased inflammatory phenotype. Among other characteristics, aging organisms generally show an increase in oxidative stress and a decrease in mitochondrial function. The mitochondrion, the small bioenergetic apparatus, is not only regarded as the powerhouse of the cell and disposed to produce ATP but has been proposed to be responsible for degeneration and aging. As the organism age, the efficacy of the respiratory chain tends to diminish with consequences that lead to reduced ATP production and mitochondrial failure, and consequent cellular damage [3].

Mitochondrial dysfunction constitutes one of the hallmarks of aging and is characterized by irregular mitochondrial morphology, insufficient ATP production, accumulation of mitochondrial DNA (mtDNA) mutations, increased production of mitochondrial Reactive Oxygen Species (mtROS) which are by-products of energy metabolism, and the consequent of oxidative damage [4]. The mitochondrial theory of aging is established on the fact that mtDNA has a higher tendency to undergo mutations compared to nuclear DNA and provides less effective mechanisms of repair. In particular, these mutations are 15 times greater than the nuclear DNA [5] and are due to the proximity to the ROS-producing complexes of the

respiratory chain and to the absence of histones. Such mutations can alter the expression of the components of the oxidative phosphorylation (OXPHO) complexes leading to mitochondrial dysfunction and accelerated ROS production [6]. A study conducted in a mouse model carrying mtDNA mutations in the γ-polymerase emphasized the considerable likelihood of mtDNA mutation during aging. These mice exhibited impaired mechanisms for mtDNA proofreading during replication leading to the emergence of a significant number of new mutations and the onset of premature aging phenotypes [7]. Based on the evidence of the vicious cycle, mtDNA mutations accumulate rapidly causing an increase in the generation of mtROS. The study that involved the mtDNA mutator mice also revealed that accumulation of mtDNA mutations across the lifespan, without any notable alteration in ROS production compared to wild-type animals, reduces the mice lifespan [7]. As a result, this observation greatly challenges the vicious cycle hypothesis which proposes that mtDNA mutation and impaired oxidative phosphorylation rather than mtROS production, are the primary cause of premature aging in mtDNA mutator mice.

Dysfunctional mitochondria may also directly impact molecular signaling and inter-organellar crosstalk becoming a threat to the cell. It has been proposed to play a critical role in determining lifespan [8]. Mitochondrial dysfunction, as well as impaired mitochondrial biogenesis, and mitochondrial DNA mutations, have been shown to be detrimental leading to cellular damage and eventually cell death. Interventions that improve mitochondrial function can extend the lifespan of various organisms including fruit flies [9] and worms [10]. Interestingly, low concentrations of ROS have been shown to extend the lifespan in *C. elegans* while high concentrations have the opposite effect [11]. A surprising increase in lifespan was observed in *C.elegans* carrying a mutation in mitochondrial respiration under high levels of ROS [11]. In many species including mice, worms and *Drosophila melanogaster*, a modest decrease in mitochondrial respiration has been demonstrated to increase lifespan, indicating that the

extension of longevity by moderate mitochondrial respiration inhibition is conserved throughout evolution. The role of aging in mitochondria is complex and more studies need to be addressed to fully understand the role of mitochondria in aging and age-related diseases.

1.2 Mitochondria quality control

Evolutionally, mitochondria are derived from proteobacteria which lived in symbiosis within the eukaryotic cell. Part of their genome was transferred to the eukaryotic nucleus while the rest of it was retained in the mitochondria for the synthesis of proteins involved in mitochondrial function [12]. The mitochondria have their own DNA and only encode 1% of their ”proteome”, while the majority of mitochondrial proteins are expressed by nuclear DNA and delivered to the mitochondria through cytoplasm trafficking. These proteins are involved in many functions including ATP synthesis, oxidative phosphorylation, metabolism, β-oxidation, regulation of calcium homeostasis, thermogenesis, ROS production, and other functions involved in mitochondrial maintenance and turnover [13]. Mitochondrial quality control is a feature that refers to the mechanisms and processes that sustain mitochondria and maintain their integrity and function. It involves a range of molecular pathways, including mitochondrial biogenesis, mitochondrial fission and fusion, and protein degradation to maintain cell homeostasis [14]. Considering the myriad of processes performed by the mitochondria the proper functioning and control of these pathways are essential for maintaining cellular homeostasis. The disruption in these controlled pathways has been reported to determine a decline in mitochondrial function and cellular homeostasis and the onset of several pathologies including cancer, neurodegenerative diseases, and cardiomyopathies. Therefore, to face this problem, mitochondria have evolved stress response networks that detect and respond to impaired mitochondrial activity to ensure proper function [15]. Such mechanisms involve the sensing of misfolded proteins, mtDNA mutations, metabolic disturbance, and oxidative

stress. There are two pathways involved in mitochondrial quality control: the regulation at the molecular level that involves the mitochondrion-nuclear communication and the regulation at the organelle level including mitochondrial dynamics and mitophagy. In this thesis, I will focus mainly on the control mechanisms within the mitochondria and their benefits in preventing aging.

1.3 Mitochondrial Dynamics: fusion and fission

Mitochondria are dynamic organelles that have the ability to change in size, shape, and number through the process named fusion and fission to adapt to stress and meet cellular requirements. [16]. However, in case of extreme and prolonged stress, cells can remove mitochondria through a process called mitophagy. Cellular and organismal health relies on the tight regulation of mitophagy and a disruption of these pathways also due to genetic mutations leads to aging and the onset of age-related diseases. [17,18]. Mitochondrial dynamics such as fission and fusion, are two components of the mitochondrial stress response. Through these two processes, mitochondria can contribute to homeostasis and survival under stress conditions. In response to high or prolonged stress mitochondria undergo fission while in response to mild or low stress fusion is favored [19]. Some studies have reported that mitochondria can alter their morphology based on the type of stressors: starvation induces fusion, while hypoglycemia and reduced OXPHOS induce fission [20, 21].

Mitochondrial fusion involves the process of maintaining mtDNA integrity, regulating apoptosis and calcium signaling as well as mitochondrial respiration membrane potential maintenance. This process is regulated both at the level of the inner mitochondrial membrane (IMM) guided by the protein Optic Atrophy (OPA1) and at the outer mitochondrial membrane (OMM) where the GTPase Mitofusin (MFN1) and Mitofusin 2 (MFN2) are involved. Some evidence suggests the essential role of fusion in embryo development [22]. Another study has

shown that mitochondrial fusion protects against neurodegeneration in the cerebellum [23]. In another study, MFN2-deficient mice showed a decrease in physical activity, and a lack of MFN2 in dopaminergic neurons is associated with mitochondrial fragmentation and reduced motility [24]. OPA1 mediates mitochondrial fission in the inner membrane and there are many isoforms produced. The regulation of OPA1 is important to maintain mitochondrial function and mutations in its gene cause Behr syndrome [25].

On the other hand, mitochondrial fission is regulated by the GTPase Dynamin-Related Protein 1 (DRP1) found in the cytosol (Figure 1) and the receptors Mitochondrial Fission Factor (MFF) and mitochondrial Fission Protein 1 (FIS1). DRP1 is recruited on the outer mitochondrial membrane following its phosphorylation by a Cyclic AMP-dependent Protein Kinase (PKA) and it combines with other DRP1 proteins forming a multimer. The complex then undergoes a conformational change and is hydrolyzed by the GTP which leads to constriction of the mitochondrial membranes and induces division [26, 27].

DPR1 is crucial for the segregation and degradation of damaged mitochondria and dysregulation in this process is linked to cardiomyopathy and heart failure [28]. Mitochondrial fission is also important for development since DRP1 knockout is embryonic lethal [29] confirming the relevance of mitochondrial dynamics during development. Several pathologies such as pancreatic cancer [30], lung cancer [31], melanoma [32], and ovarian cancer [33] are associated with DRP1, suggesting that tumor growth requires DRP1. In addition, imbalances in mitochondrial fusion and fission with decreased MFN1 and MFN2 expression and increased DRP1 expression are associated with Alzheimer's (AD) and Huntington's disease (HD) [34, 35]. Moreover, one week of DRP1 overexpression in middle-aged flies preserves mitochondrial respiratory function and prolongs a healthy lifespan improving the overall quality of mitochondria [36]. According to these findings, proper mitochondrial function is crucial for maintaining cellular homeostasis.

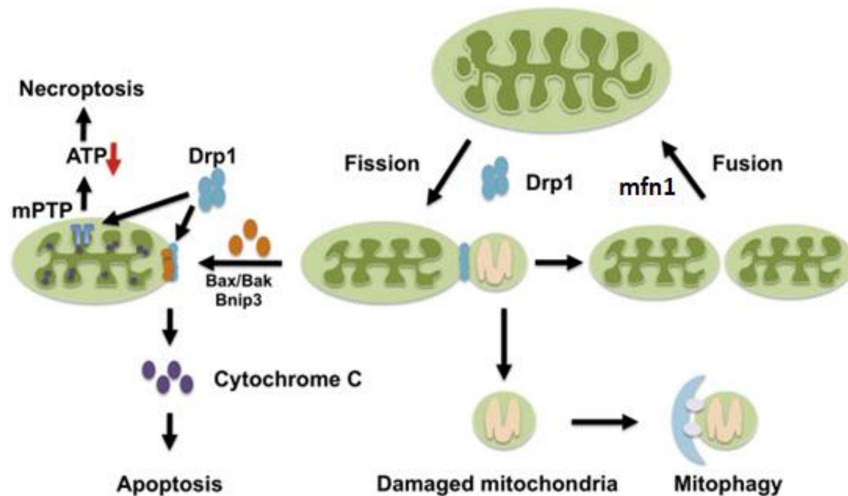

Figure 1: Role of DRP1 in mitochondrial fission and mitophagy. When dysfunctional mitochondria accumulate within the cell ATP production drops and dynamin-related protein 1 (DRP1) is recruited. DRP1 promotes mitochondrial fission and delivers damaged mitochondria to mitophagy degradation. By contrast, mitofusin 1 (mfn1) promotes mitochondrial fusion escaping from mitophagy (Mingming Tong et al. JMCC, 2020).

1.4 Mitophagy

Damaged mitochondria are eliminated by an autophagy process called mitophagy [37]. Through this process, cells can degrade mitochondria that are dysfunctional through the acquisition of these organelles within the autophagosome and delivered to the lysosome for digestion [38]. The term mitophagy was introduced by Lemaster's team after discovering mitochondria inside the lysosome of rat hepatocytes during starvation [39]. This discovery led to the question of how cells detect damaged mitochondria and initiate mitophagy. This process involves the recognition of the damaged mitochondria by the autophagy machinery. Two different pathways have been described to recruit the autophagy machinery to damage mitochondria: the PINK1/Parkin pathway and the Nix/BNIP3 pathway [40].

In healthy mitochondria, PINK1 (PTEN-induced putative kinase 1) is rapidly degraded in the cytosol, while Parkin, an E3 ubiquitin kinase is kept in an inactive state. However, when mitochondria become damaged, and lose their mitochondrial membrane potential PINK1

accumulates on its surface and recruits Parkin, which ubiquitinates proteins in the mitochondrial outer membrane. Ubiquitination of outer mitochondria proteins results in the recruitment of mitophagy receptors, including the autophagy receptor P62. P62 has a LIR domain (LC3 Interacting Region) that recruits LC3, ATG8 in *Drosophila melanogaster*, to the mitochondria and creates the autophagosome. This double membrane organelle interacts with the lysosome to generate an acidic organelle termed mitolysosome where the dysfunctional mitochondria are degraded (Figure 2) [40].

Mutations in PINK1 have been linked to Hereditary Early Onset Parkinson's disease and mutation in Parkin occurs in Autosomal recessive juvenile Parkinsonism [41, 42]. PINK1 and Parkin are known to maintain mitochondrial function in brain and muscle tissues in fruit flies and mutation in their genes leads to neurodegeneration [43]. Additionally in mice deleting Parkin has been shown to cause the loss of dopaminergic neurons which is a feature of Parkinson's disease [44]. The fact that dysfunctional mitochondria accumulate in neurodegenerative diseases highlights the significance of mitophagy and the role of the PINK1/parkin pathway in age-related diseases [41, 42, 43, 45].

The Nix/BNIP3 pathway activates the mitophagy pathway in many conditions such as hypoxia, oxidative and genotoxic stress. Nix and BNIP3 (BCL2-interacting protein 3) are two related proteins located in the outer mitochondrial membrane involved in the recognition of damaged mitochondria. When mitochondria become dysfunctional, Nix and BNIP3 interact through a LIR domain with LC3 which allows the formation of the autophagosome which fuses with the lysosome to create the mitolysosome. Neuronal induction of BNIP3 induces mitophagy and has beneficial effects on the lifespan and healthspan of fruit flies [46].

Mitophagy decline has been observed in several tissues in aging mice. A decrease in mitophagy was observed in the hippocampus of aged mice compared to younger mice [47]. Similarly, a loss of Parkin causes a massive loss of dopaminergic neurons suggesting that Parkin prevents

neuronal deterioration [48]. Several interventions have been shown to activate mitophagy. These include caloric restriction, exercise, nutrient deprivation, and some pharmacological agents such as resveratrol and rapamycin. There is increasing evidence that inducing mitophagy mitigates the effects of aging and prolongs the lifespan of different model organisms. In *C.elegans* mitophagy is necessary for lifespan extension in long-lived mutants [49]. In the fruit fly, ubiquitous Parkin overexpression improves mitochondrial function, reduces proteotoxicity, and extends lifespan [9]. It is important to note that mitophagy is regulated by multiple signaling pathways and more research is needed to fully understand the mechanisms involved in mitophagy and how it can be effectively targeted for therapeutic purposes.

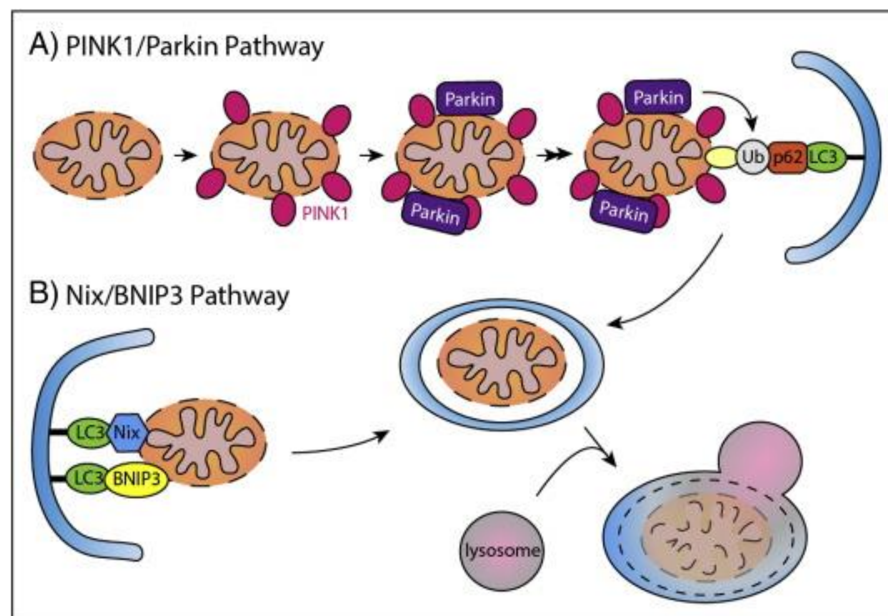

Figure 2: PINK1/Parkin and Nix/BNIP3 mediated mitophagy. **A)** PINK1/parkin pathway: Mitochondrial dysfunction is characterized by a reduction in mitochondrial membrane potential. PINK1 is constitutively degraded under steady-state but in damaged mitochondria, it accumulates in the outer mitochondrial surface and, when activated, recruits Parkin. Parkin leads to the polyubiquitination of proteins designed for proteasomal degradation. **B)** Nix/BINIP3 pathway: Nix and BNIP3 act as autophagy receptors and bind to LC3 on the autophagosome. Both pathways lead to mitochondrial sequestration within the autophagosome, fusion with the lysosome, and further degradation of the organelle (Babette C. Hammerling and Asa B. Gustafsson, 2014).

1.5 Mitochondrial DNA and the immune response

The mitochondrion is a self-replicating organelle containing its own double-stranded DNA. In recent years, the role of mtDNA in regulating the immune response has gained significant attention. Mitochondrial DNA shares several characteristics with bacterial DNA such as containing a high number of unmethylated CpG islands. Because of these similarities, mtDNA can be recognized as a PAMP by the innate immune system. In normal states, mtDNA is usually contained within the double membrane in the matrix of the mitochondria. However, in pathological or stress conditions, it can be released into the cytoplasm [50].

Mitochondrial dysfunction and the increase in oxidative stress may affect mitochondrial membrane permeability in response to stress rendering this organelle vulnerable to releasing its own internal components such as mtDNA. It has been reported that the accretion of mitochondrial damage and dysfunction as well as different stressors lead to the release of mtDNA and accumulation into the cytosol. This cytosolic mtDNA acts as a trigger for the activation of the immune response [50, 51, 52]. Cytosolic mtDNA, activates the innate immune response through the activation of the cGAS-STING pathway resulting in the production of inflammatory effectors (Figure 3). Cytosolic mtDNA can induce other innate immune signaling involving NLRP3 and the toll-like receptor 9 (TLR9) which triggers a downstream cascade that stimulates the expression of effector molecules including antimicrobial genes and inflammatory cytokines [51, 52, 53]. Another study has demonstrated the activation of the immune response in both *Prkn* $^{-/-}$ and *Pink1* $^{-/-}$, and that this activation is ameliorated in STING knockdown mice [54].

A study demonstrated that increased mutation rates and high levels in mtDNA related to age in PINK1 and Parkin-deficient mice lead to activation of cGAS sting and consequent inflammatory phenotype mediated by type 1 interferon in the brain with degeneration of dopaminergic neurons [53].

In *Drosophila melanogaster*, the homolog of STING (dSTING) is required for the defense of RNA viruses and bacterial DNA. dSTING similar to its mammal homolog also interacts with the cyclic dinucleotide 2’,3’ cGAMP. The recognition of this intermediate leaves the activation of the *Drosophila melanogaster* immune deficiency (IMD) pathway that induces the phosphorylation and cleavage of the transcription factor Relish. Active Relish translocates to the nucleus where it induces the activation of several immune response genes including antimicrobial peptides (AMPs) [55].

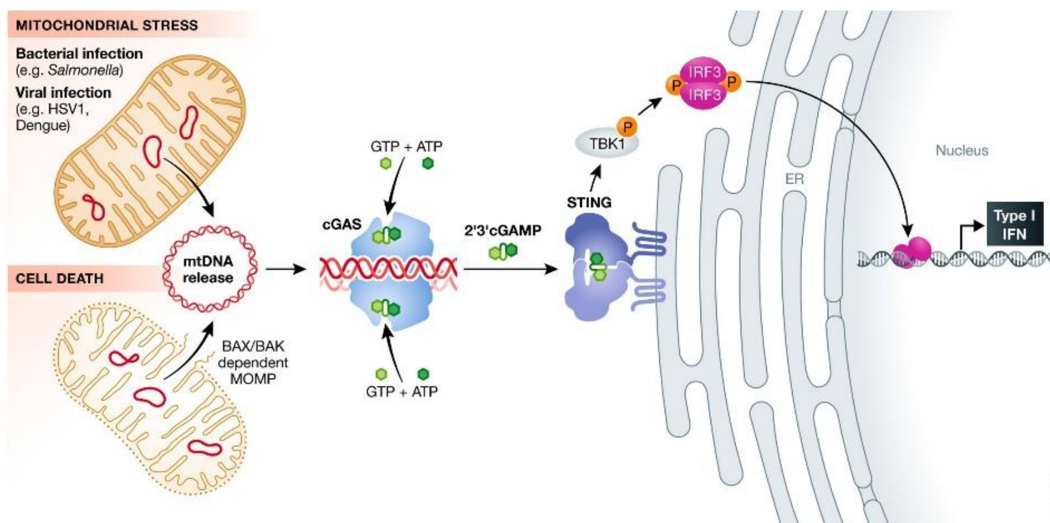

Figure 3: mtDNA-dependent activation of cGAS-STING signaling. Various mitochondrial stresses including bacterial and viral infection can lead to mtDNA release. Alternatively, activation of BAX and BAK leads to outer mitochondrial membrane permeabilization pore (MOMP) and mtDNA release. Once in the cytosol, mtDNA can bind to the DNA sensing protein cGAS that catalyzes the synthesis of the secondary messenger 2’3’cyclic GMP-AMP from ATP and GTP. cGAMP binds to the adaptor molecule STING on the endoplasmic reticulum (ER) leading to the activation of the kinase TBK1. Once active, TBK1 phosphorylates the transcription factor IRF3 initiating a type 1 interferon response (Joel S Riley, Stephen WG Tait EMBO Reports 2020).

In *Drosophila melanogaster*, there are two classical immunity pathways: the Immune Deficiency (IMD) and the Toll pathway. The immune deficiency pathway is a major innate immune response pathway in fruit flies. It is involved in defending the organism against bacterial and fungal infections, as well as in regulating the homeostasis of the gut microbiota. The IMD pathway is activated when microbial components such as peptidoglycan (PGN) from

bacterial cell walls are detected by transmembrane receptors on the surface of immune cells, called hemocytes. This recognition leads to a signaling cascade that ultimately activates transcription factors, including Relish, which drives the expression of antimicrobial peptides and other immune effector molecules [55]. Major antimicrobial peptides produced by the activation of the IMD pathway include Attacin A which is active against Gram-negative bacteria and has been shown to be important for the defense against bacterial infections and Diptericin, another well-characterized peptide that fights against bacterial infection. The Toll pathway is another major innate immune response pathway in fruit flies and it is involved in defending the organism against both bacterial and fungal infections, as well as in regulating development and morphogenesis. This pathway is activated when microbial components such as fungi or bacteria are recognized by transmembrane receptors on the surface of immune cells, called hemocytes. This recognition leads to a signaling cascade that ultimately activates a transcription factor called Dorsal, which drives the expression of antimicrobial peptides such as Drosocin, Drosomycin, and Defensin active against gram-positive bacteria and fungi. In addition to its role in immunity, the Toll pathway also plays a crucial role in embryonic patterning and morphogenesis. During embryonic development, the pathway is activated by a maternal protein called Spatzle, which is released upon the activation of another signaling pathway called the Spatzle-processing enzyme cascade [55]. Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR) that play a critical role in the innate immune response of mammals, including humans. TLRs are expressed in various cell types, including immune cells such as macrophages, dendritic cells, and B cells, as well as non-immune cells such as epithelial cells. TLRs also play an important role in the adaptive immune response, as they can activate antigen-presenting cells such as dendritic cells and B cells to present antigen to T cells, leading to the activation of a specific adaptive immune response. Dysregulation of TLR signaling has been implicated in a variety of diseases, including inflammatory and

autoimmune disorders, cancer, and infectious diseases. Therefore, understanding the function and regulation of TLRs is important for the development of novel therapies for these diseases. According to another study [56], the activation of the immune response in *Drosophila melanogaster* is age-dependent and NF-κB-dependent AMP gene expression in the brain correlates with progressive and severe neurodegeneration, locomotor defects, and reduced lifespan. By contrast, the downregulation of the IMD/ NF-κB Relish immune signaling in glia correlates with improved survival in fruit flies. This study demonstrates the strong relationship between high levels of AMPs such as Drosocin, Diptericin, and Attacin that contribute to neurodegeneration and the loss of intracellular negative IMD regulation correlates with age-dependent neurodegeneration.

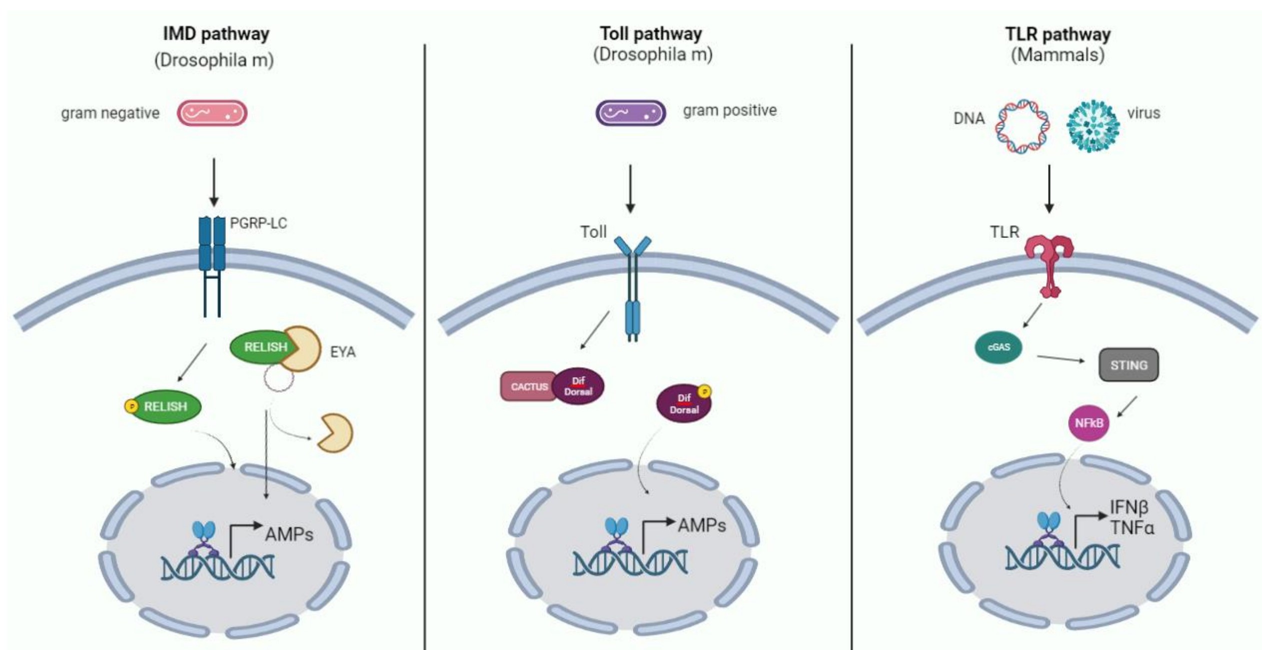

Figure 4: Immune response pathways in *Drosophila melanogaster* and mammals. Two classical signaling pathways control inducible immune responses in D. melanogaster: the Toll pathway and the immune deficiency (Imd) pathway, while the TLR is stimulated in mammals. Both the Imd pathway and Toll control the systemic production of antimicrobial peptides (AMPs). The Imd pathway is activated by gram-negative bacteria and the activation stimulates the phosphorylation of Relish that drives the nuclear expression of antimicrobial peptides (AMPs). The Toll pathway instead is mainly active in the fat body and becomes stimulated by gram-positive bacteria. When the Toll pathway is activated, the downstream cascade includes the phosphorylation of dif and dorsal that translocate within the nucleus leading to the expression of antimicrobial peptides (AMPs). Mammalian TLRs are activated by bacterial, viral DNA. Depicted are TLR9-dependent signal transduction events until the expression of proinflammatory genes (created with Biorender).

Likewise, bacteria, microorganisms and fungi, free circulating mitochondrial DNA may act as a modulator of the immune response leading to the production of proinflammatory factors. One study found that mtDNA is released from mitochondria upon infection with bacterial pathogens and that this mtDNA can activate the immune response by stimulating the production of antimicrobial peptides (AMPs) through the IMD pathway [51, 52]. The depletion of mtDNA or inhibition of mitochondrial respiration decreased the production of AMPs and increased susceptibility to bacterial infection. Another study found that the eya gene in *Drosophila melanogaster* for eye development contains a threonine phosphatase domain that has been linked to playing a role in the activation of the inflammatory response against DNA [57]. This study demonstrated that the threonine phosphatase domain of EYA is responsible for immunity through the association with IKKβ and Relish to induce IMD pathway activation and AMP production and this is triggered by undigested DNA in a DNAseII deficient model. Increasing evidence has shown that hypomethylated circulating free DNA, in particular hypomethylated cf-mtDNA, acts as a powerful proinflammatory DAMP. A study conducted on patients affected by sickle cell disease (SCD) found that the levels of cell-free mtDNA (cf-mtDNA) disproportionately unmethylated correlate with increased immune response and inflammation acting as a DAMP [58]. This study has demonstrated that the increased levels of cell-free DNA (cfDNA) in individuals with SCD actively contributed to pathological inflammation. Specifically, among the elevated cfDNA levels, patients with SCD exhibit a notably higher ratio of cell-free mtDNA to cell-free nuclear DNA when compared to healthy individuals serving as controls. Findings demonstrate that cell-free mitochondrial DNA (cf-mtDNA) derived from red blood cells (RBCs) of individuals with sickle cell disease (SCD) acts as endogenous ligands for DNA-sensing pattern recognition receptors. Specifically, cf-mtDNA functions as a proinflammatory damage-associated molecular pattern (DAMP) through the

cGAS-STING-TBK1 pathway in neutrophils. This mechanism potentially contributes to the persistent chronic inflammatory state observed in SCD patients.

Overall, these findings suggest that mtDNA plays a critical role in innate immunity both in *Drosophila melanogaster* and in mammals by triggering the expression of immune response genes and contributing to the age-dependent inflammatory phenotype referred as inflammaging. However, further research is needed to fully understand the mechanisms underlying the relationship between mtDNA and the immune response in fruit flies and other organisms.

2. Research goals and hypothesis

Two questions still remain poorly understood: how does aging affect immunity, and how does immunity affect aging? Aging contributes to the deterioration of the immune system (immuno-senescence) and predisposes the organism to infections. The main objective of this research thesis was to study the interplay between mitochondrial dynamics and the accumulation of cytosolic mtDNA, and the role this cytosolic mtDNA plays in the activation of the immune response in aged flies. In order to study the relationship between mitochondrial function and the immune response my research project pursued these specific aims as follow:

Aim 1: Analyzing the accumulation of cytosolic mtDNA in aged flies and in DRP1 and Parkin overexpressing flies.

Hypothesis: It is thought that mtDNA is released under stress conditions. We propose that mitochondrial DNA accumulates in aged flies and that improving mitochondrial function could ameliorate the accumulation of cytosolic mtDNA. To study this hypothesis, I analyzed the accumulation of cytosolic mtDNA in young and middle-aged flies in wild-type conditions and in the overexpression of DRP1 and Parkin that improve mitophagy.

Aim 2: Analyzing the immune response in flies overexpressing DRP1 and Parkin.

Hypothesis: Several studies have demonstrated that cytosolic DNA activates the immune response. Here we are interested in understanding if the removal of dysfunctional mitochondria by mitophagy that dampens the accumulation of cytosolic mtDNA could ameliorate the activation of the immune response in aged flies. Here we propose that the overexpression of DRP1 and Parkin genes could reduce the activation of the immune response in aged flies.

3. Materials and methods

3.1 Fly strains and media

Briefly, I use the Gene Switch system to activate the expression of different genes in a tissue-time-dependent manner [59]. Using this system eliminates all the genetic differences since control and experimental flies share the same genetic background and they only differ in the presence of the inducing agent (RU486) or diluent (ethanol) (Figure 5). The fly lines used in this study are daughterless GeneSwitch (daGS) provided by H. Tricoire (Universite´ Paris Diderot–Paris7, Paris, France), UAS-Drp1-HA provided by J. Chung (Korea Advanced Institute of Science and Technology, Republic of Korea), and UAS-parkin provided by L. Pallanck (University of Washington, Seattle, WA). Flies were reared in vials containing cornmeal medium (1% agar, 3% yeast, 1.9% sucrose, 3.8% dextrose, 9.1% cornmeal, 1.1% acid mix, and 1.5% methylparaben, all concentrations given in wt/vol).

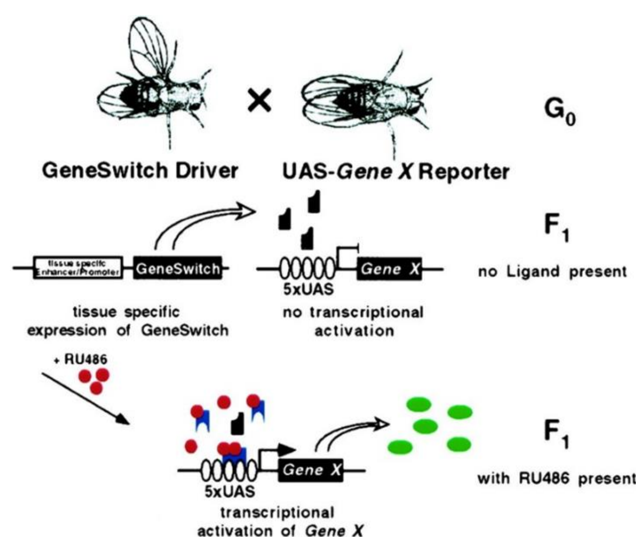

Figure 5: The GeneSwitch/UAS expression system. Driver lines expressing the transcriptional activator GeneSwitch are crossed to UAS-reporter lines (Upstream Activating Sequence) with genomic inserts of a target gene fused to five GAL4-binding sites arrayed in tandem. In the absence of an activator, the GeneSwitch protein is expressed in target tissues but remains transcriptionally silent (black); Gene X cannot be expressed. With the induction of RU486 (red), the GeneSwitch protein becomes transcriptionally active (blue), mediating the expression of gene X (green) in only those tissues expressing GeneSwitch (Thomas Osterwalder, Kenneth S. Yoon, Benjamin H. White, and Haig Keshishian, PNAS, 2001).

3.2 Lifespan analysis

For the lifespan analysis, female flies were taken under nitrogen-induced anesthesia and sorted at a density of 30 female flies per vial with the appropriate food. Flies were kept in the incubator at 25°C, at the humified condition with a 12h on/off light cycle. RU486 concentration and administration time depend on fly genotype. Flies were flipped every 2-3 days and deaths were scored.

3.3 RNA extraction, cDNA synthesis, and quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's protocols. Samples were treated with DNAse, and then cDNA synthesis was carried out using the First Strand cDNA Synthesis Kit from Fermentas. PCR was performed with Power SYBR Green master mix (Applied Biosystems) on a BioRad Real-Time PCR system. Cycling conditions were as follows: 95 °C for 10 min; 95 °C for 15 s then 60 °C for 60 s, cycled 40 times.

3.4 Cytosolic mtDNA/nuclei isolation

25 heads and 25 thoraces were collected and homogenized in cold mitochondrial isolation medium (MIM) (250mM sucrose, 10mM Tris-HCl (pH 7.4), 0.15 mM MgCl2) and filtered in 64 μm nylon mesh, spin for 5 seconds and centrifuged at 600g at 4°C for 10 minutes. Supernatant and pellet were separately collected to obtain the nuclear DNA and cytosolic fraction. Pellet, the nuclear fraction was resuspended in 100μl of nuclear isolation buffer (10 mM HEPES-KOH, pH 7.5; 2.5 mM MgCl2; 10 mM KCl) and incubated with proteinase K for 15 min at RT. The supernatant containing mitochondria and cytosolic fraction were spun at 15000g for 15 min. at 4C. Supernatant was transferred to a new vial and incubated with

proteinase K for 15 minutes at RT. Afterward, proteinase K was inactivated for 10 minutes at 95°C. One volume of phenol:chloroform:]isoamyl alcohol (25:24:1) was added to both fractions and centrifuged at 16000g for 15 min. at RT. Once centrifuged, the upper phase, the aqueous phase was transferred to a new tube and added 1µl of Glycogen (20 µg/µl), 0.5 volumes of 7.5 M NH_4OAc and 2.5 volumes (sample + NH_4OAc) of 100 % ethanol. Samples were placed overnight at -20°C to let the DNA precipitate. The following day, samples were centrifuged at 4°C for 30 minutes at 16,000g to precipitate the DNA. The supernatant was removed and the pellet was washed twice with ethanol 70%. Supernatant was discarded and the DNA was dried for 5 minutes at RT. Finally, DNA was resuspended in TE buffer (EDTA 1 mM & Tris-Cl 10 mM ph 8) by pipetting up and down 30–40 times and stored at -80°C.

3.5 Total DNA isolation

For the total DNA isolation, 15 thoraces or heads were ground and homogenized in 100µl total DNA isolation medium (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% Triton X-100 and 10µg/ml proteinase K) and incubated for 1h at 37°C. In the following step, the samples were incubated at 95°C for 10 min. to inactivate proteinase and then centrifuged at 4°C 600g for 5 minutes. The supernatant containing the total DNA was taken and one volume of phenol:chloroform: isoamyl alcohol (25:24:1) was added to the fraction and centrifuged at 16000g for 15 min. at room temperature. Once centrifuged, the upper phase, the aqueous phase was transferred to a new Eppendorf and added 1ul of Glycogen (20 µg/µl), 0.5 volumes of 7.5 M NH_4OAc and 2.5 volumes (sample + NH_4OAc) of 100 % ethanol. Samples were placed overnight at -20°C to let the DNA precipitate. The following day, samples were centrifuged at 4°C for 30 minutes at 16,000g to precipitate the DNA. The supernatant was removed and the pellet was washed with ethanol 70% and centrifuged twice for 2 minutes at 16,000g at 4°C. the supernatant was discarded and the DNA was dried for 5 minutes at RT. Finally, the DNA was

resuspended in TE buffer (EDTA 1 mM & Tris-Cl 10 mM ph 8) by pipetting up and down 30–40 times and stored at -80°.

3.6 Western Blot

Whole flies (5 flies per sample, 5 replicates) were homogenized in Lysis Buffer (PBS 1X, Protease Inhibitors 1X, NuPAGE LDS Sample Buffer 1X, and DTT (Dithitreitol 0.05M). Samples from whole flies were separated by SDS-PAGE gels and proteins were transferred to Nitrocellulose membranes. Samples were collected and lysates were separated by SDS-PAGE using standard procedures. Membranes were probed with antisera against anti-actin peroxidase-conjugated 1:15000 (A3854, Sigma) and rabbit anti-HA 1:1000 (3724S Cell signaling).

4. Results

4.1 Cytosolic mtDNA accumulates in aged flies

Previous studies have shown that dysfunctional mitochondria release mtDNA into the cytosol and activate the immune response through the cGAS/STING pathway [60]. A decline in mitochondrial function has been reported in aging organisms such as fruit flies, worms, and rodents as well as in neurodegenerative diseases. To better understand if cytosolic mtDNA accumulates in aged organisms we have analyzed the levels of cytosolic mtDNA in *daGS>DRP1* and *daGS>Park* flies. qPCR analysis showed that levels of cytosolic mtDNA increase in aged wild-type flies (Figures 6 and 8).

4.2 Ubiquitous Parkin induction decreases cytosolic mtDNA accumulation in aged flies

A previous study has shown that Parkin overexpression improves mitochondrial function and extends *Drosophila melanogaster* lifespan [9]. To test if Parkin overexpression reduces cytosolic mtDNA, we analyzed the levels of the mtDNA genes Cytochrome C Oxidase Subunit I (COI) and mitochondrial NADH-ubiquinone oxidoreductase chain 2 (mt-ND2) in the cytoplasm of aged Parkin overexpressing flies. To do so, we used the ubiquitous driver daughterless Gene-Switch. Using this system removes the differences between different genetic backgrounds as all the flies share the same genetic background and only differ with the presence of the RU486 inducer and ethanol as diluent. qPCR analysis in heads and thoraces shows that the levels of the COI in the cytoplasm of *daGS>Parkin* flies increase with age (Figure 6 compares day 10 uninduced with day 30 uninduced). However, middle-aged flies with high levels of Parkin (figure 7) have less levels of cytosolic mtDNA in heads and thoraces than control flies (Figure 6, compares day 10 uninduced with day 10 induced and day 30 uninduced with day 30 induced). To discard the possibility that the reduction in cytosolic mtDNA accumulation in *daGS>Park* overexpressing flies is due to a decrease in mitochondrial

content we analyzed the levels of total mtDNA in these conditions. As shown in figure 11, Parkin overexpression does not reduce the total mtDNA levels in heads or thoraces. Collectively, these data demonstrate that the induction of mitophagy through Parkin overexpression reduces the accumulation of mtDNA in the cytoplasm in aged flies.

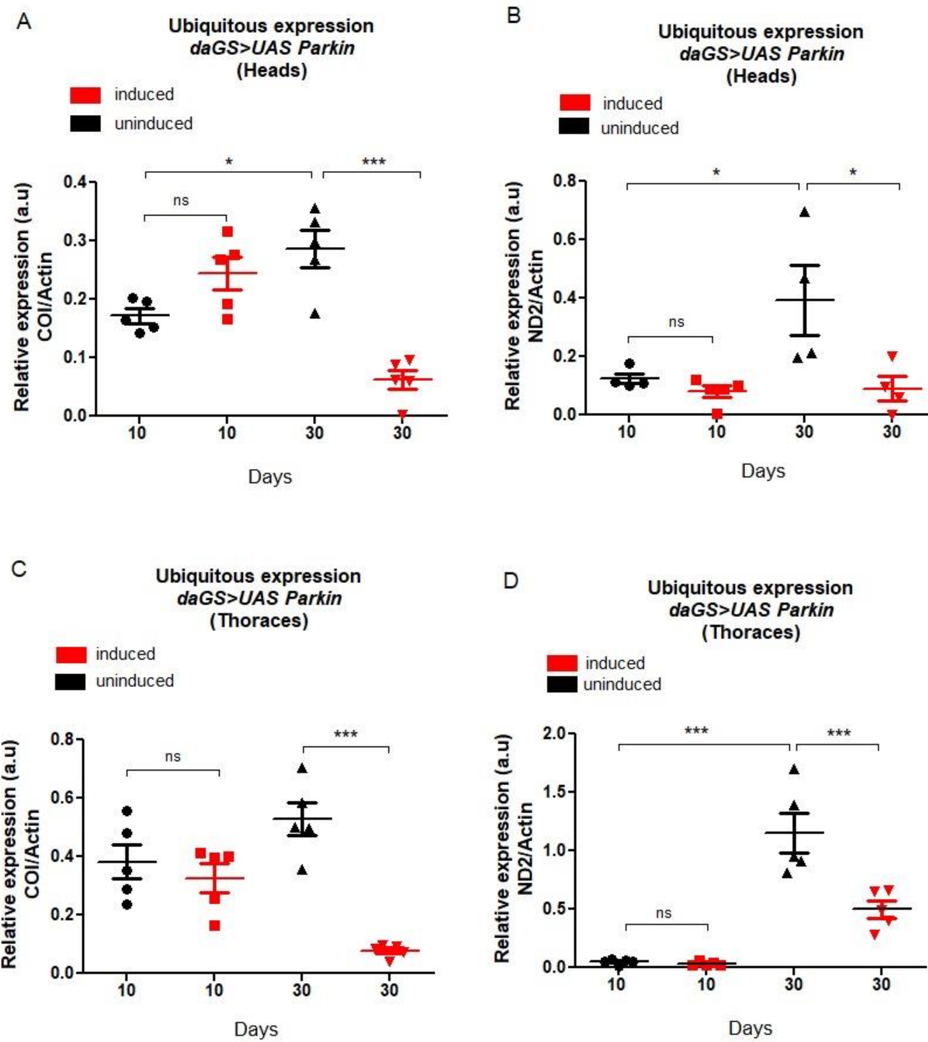

Figure 6: Parkin overexpression reduces the levels of cytosolic mtDNA in the heads and thoraces of aged flies. A) Cytosolic mtDNA levels in heads of daGS>UAS Parkin female flies at day 10 and 30 with and without RU486 induction; (one-way ANOVA/Bonferroni's multiple-comparisons test. * $p<0.05$ ***$p<0.001$ n.s. non-significant); **B)** Cytosolic mtDNA levels in heads of daGS>UAS Parkin female flies at day 10 and 30 with and without RU486 induction; (one way ANOVA/Bonferroni's multiple-comparisons test.*$p<0.05$); **C)** Cytosolic mtDNA levels in thoraces of daGS>UAS Parkin female flies at day 10 and 30 with and without RU486 induction; (one-way ANOVA/Bonferroni's multiple-comparisons test.***$p<0.001$).**D)** Cytosolic mtDNA levels in thoraces of daGS>UAS Parkin female flies at day 10 and 30 with and without RU486 induction; (one-way ANOVA/Bonferroni's multiple-comparisons test. ***$p<0.001$). RU486 was provided in the media at a concentration of 5 μg/mL Error bars represent SEM. n=5 replicates with 25 heads or thoraces, respectively, per replicate.

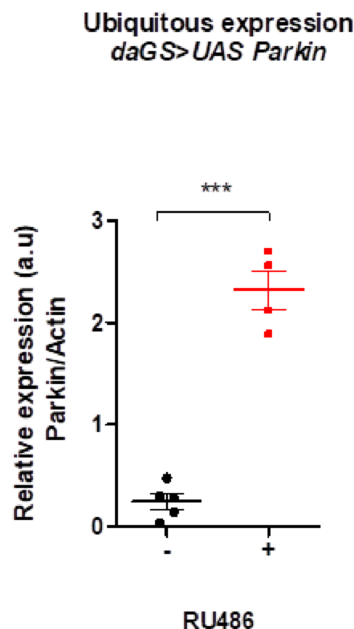

Figure 7: Parkin overexpression analysis. Ubiquitous expression of Parkin. Relative expression of Parkin at day 17 under the induction with RU0 and RU5 (unpaired t-test ***$p<0.001$); RU486 was provided in the media at a concentration of 5 µg/mL. Error bars represent SEM. n>4 replicates with 5 flies per replicate.

4.3 Midlife mitochondrial fission induction reduces the accumulation of cytosolic mtDNA in middle-aged flies

A recent study showed that DRP1 up-regulation is important to extend lifespan and decrease mtDNA in aged flies [36]. To investigate whether the overexpression of DRP1 involved in mitochondrial fission reduces the accumulation of cytosolic mtDNA in aged flies, we analyzed the levels of cytosolic mtDNA in midlife DRP1 overexpressing flies. Here, we observed an increase in cytosolic mtDNA levels throughout *Drosophila melanogaster* lifespan. Interestingly, short-term, midlife DPR1 induction reduces the accumulation of cytosolic mtDNA in aged flies in heads and thoraces (Figure 8). We can observe a relevant increase in mtDNA throughout aging in brain and muscle tissues suggesting that with age mitochondrial dysfunction leads to the leakage of its genetic material into the cytosol. Once mitochondrial fission is induced from day 30 onward with the overexpression of DRP1, the accumulation of

the mtDNA is strongly reduced in aged flies compared to the control group. Prior these analyses, DRP1 overexpression was confirmed by western blot (figures 9).

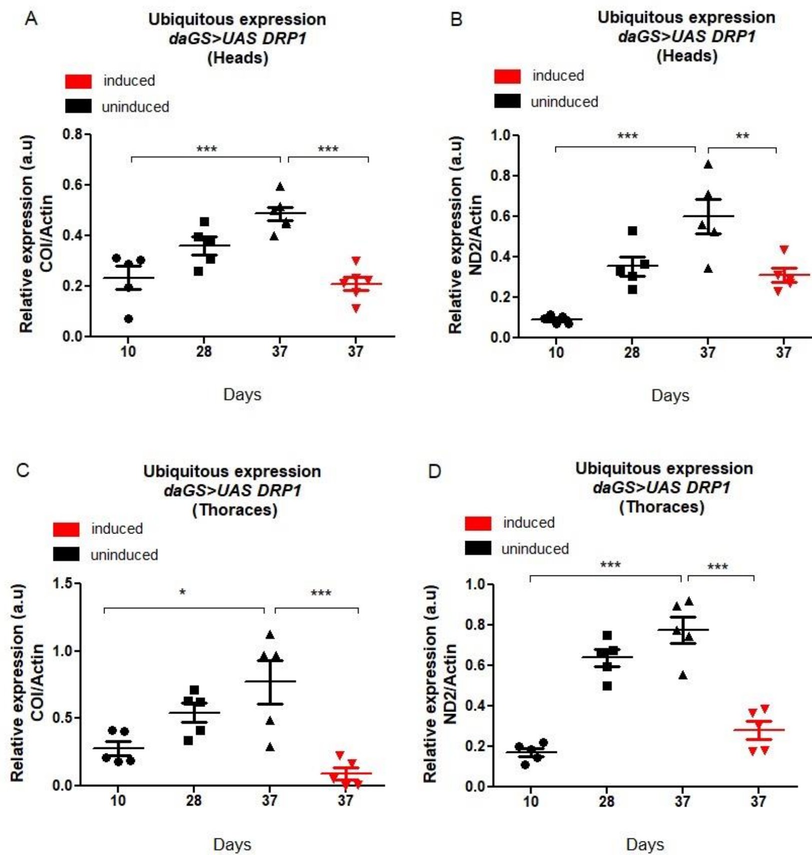

Figure 8: One week of DRP1 induction contributes to reducing the levels of cytosolic mtDNA in the heads and thoraces of aged flies. A) qPCR analysis of relative levels of cytosolic mtDNA in heads of daGS>UAS DRP1 female flies at day 10, day 28, and day 37 with and without RU486. (one way ANOVA/Bonferroni's multiple-comparisons test. ***p<0.001); **B**) qPCR analysis of relative levels of cytosolic mtDNA in heads of daGS>UAS DRP1 female flies at day 10, day 28, and 37 with and without RU486 (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01). **C)** qPCR analysis of relative levels of cytosolic mtDNA in thoraces of daGS>UAS DRP1 female flies at day 10, day 28, and 37 with and without RU486 (one way ANOVA/Bonferroni's multiple-comparisons test.***p<0.001) **D)** qPCR analysis of relative levels of cytosolic mtDNA in thoraces of daGS>UAS DRP1 female flies at day 10, day 28, and 37 with and without RU486 (one way ANOVA/Bonferroni's multiple-comparisons test. ***p<0.001. RU486 was provided in the media at a concentration of 25 µg/mL Error bars represent SEM. n=5 replicates were tested with 25 thoraces or heads or thoraces, respectively.

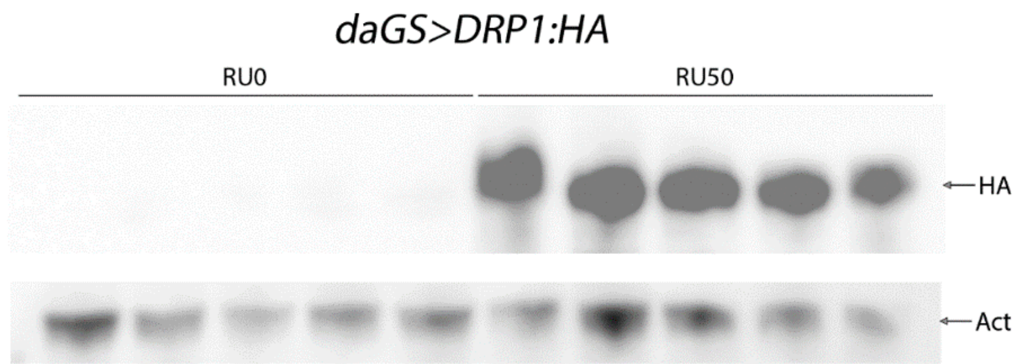

Figure 9: Western blot analysis for DRP1 expression. Western blot detection of Drp1:HA in daGS>UAS DRP1:HA female flies at day 15 with and without RU486.. Actin was used as a loading control. RU486 was provided in the media at a concentration of 50 µg/mL.

Analyzing the effects of DRP1 overexpression on total mtDNA levels in *Drosophila melanogaster*, we found that there was no significant increase in total mtDNA levels in the heads of DRP1 overexpressing flies (Figure 10). However, in the thoraces of these flies, there was a relevant increase in mtDNA levels with age, particularly in the group overexpressing DRP1.

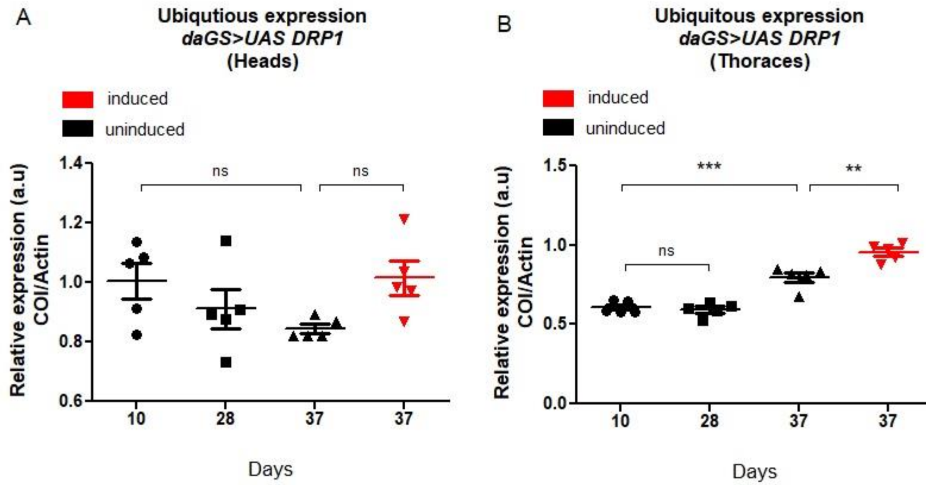

Figure 10: **Total mtDNA levels in DRP1 overexpressing flies. A)** qPCR analysis of relative levels of total DNA in heads of daGS>UAS DRP1 female flies at day 10, 28, and 37 with and without RU486. (one way ANOVA/Bonferroni's multiple-comparisons test. p >0.05 n.s); **B**) qPCR analysis of relative levels of total DNA in thoraces of daGS>UAS DRP1 female flies at day 10, day 28, and 37 with and without RU486 (one way ANOVA/Bonferroni's multiple-comparisons test.**p<0.010. ***p<0.001). RU486 was provided in the media at a concentration of 25 µg/mL Error bars represent SEM. n=5 replicates with 15 heads or thoraces respectively, per replicate.

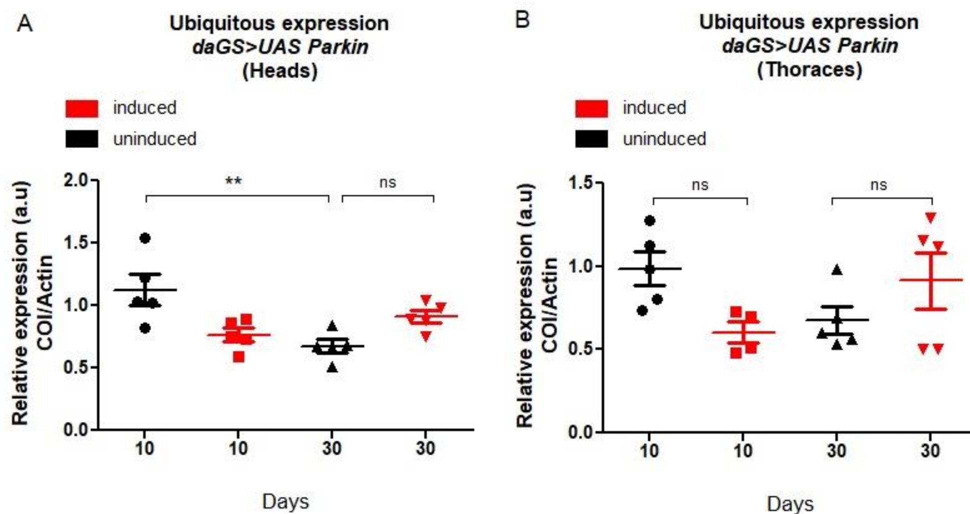

Figure 11: Total mtDNA levels in Parkin overexpressing flies. A) qPCR analysis of relative levels of total DNA in heads of daGS>UAS Parkin female flies at day 10, and day 30 with and without RU486. (one way ANOVA/Bonferroni's multiple-comparisons test. p >0.05 n.s, **p<0.01); **B**) qPCR analysis of relative levels of total DNA in thoraces of daGS>UAS Parkin female flies at day 10 and day 30 with and without RU486 (one way ANOVA/Bonferroni's multiple-comparisons test. p>0.05 n.s). RU486 was provided in the media at a concentration of 5 µg/mL Error bars represent SEM. n= 5 replicates with 15 heads or thoraces respectively, per replicate.

4.4 Induction of Parkin ameliorates inflammation in *Drosophila melanogaster* brain and muscles

In a previous study, the chronic activation of the immune response occurred with aging [61]. In our study, we observed that aging in flies is associated with the accumulation of cytosolic mtDNA, but this accumulation was significantly reduced by overexpression of the protein Parkin. In mammals, there are several pieces of evidence that cytosolic mtDNA can activate the immune response. We wonder if the reduction of cytosolic mtDNA in these transgenic flies ameliorates the activation of the immune response in aged flies. To do so, we analyzed by qPCR the expression levels of different AMPs and stress-induced humoral factors. Specifically, we measured the expression levels of the genes Attacin A, Drosocin, and Diptericin, Turandot A, and Turandot Z, in flies with constitutively induced Parkin expression. Our findings revealed that the levels of AMPs and stress immune effectors tend to increase with age in both heads and thoraces. We found that there was no significant decrease in the levels of AMPs on day 10 after Parkin induction with RU486. Remarkably, at day 30, the levels of these AMPs were significantly decreased in flies with high levels of Parkin. Interestingly, we also found that mitophagy reduced the expression of the stress-induced humoral factors Turandot A and Turandot Z in both brains and muscles. Taken together, our results suggest that cytosolic mtDNA accumulation during aging may play a role in the activation of the immune response, but the induction of Parkin-mediated mitophagy can help to mitigate this response and improve the overall immune function in mid-aged flies.

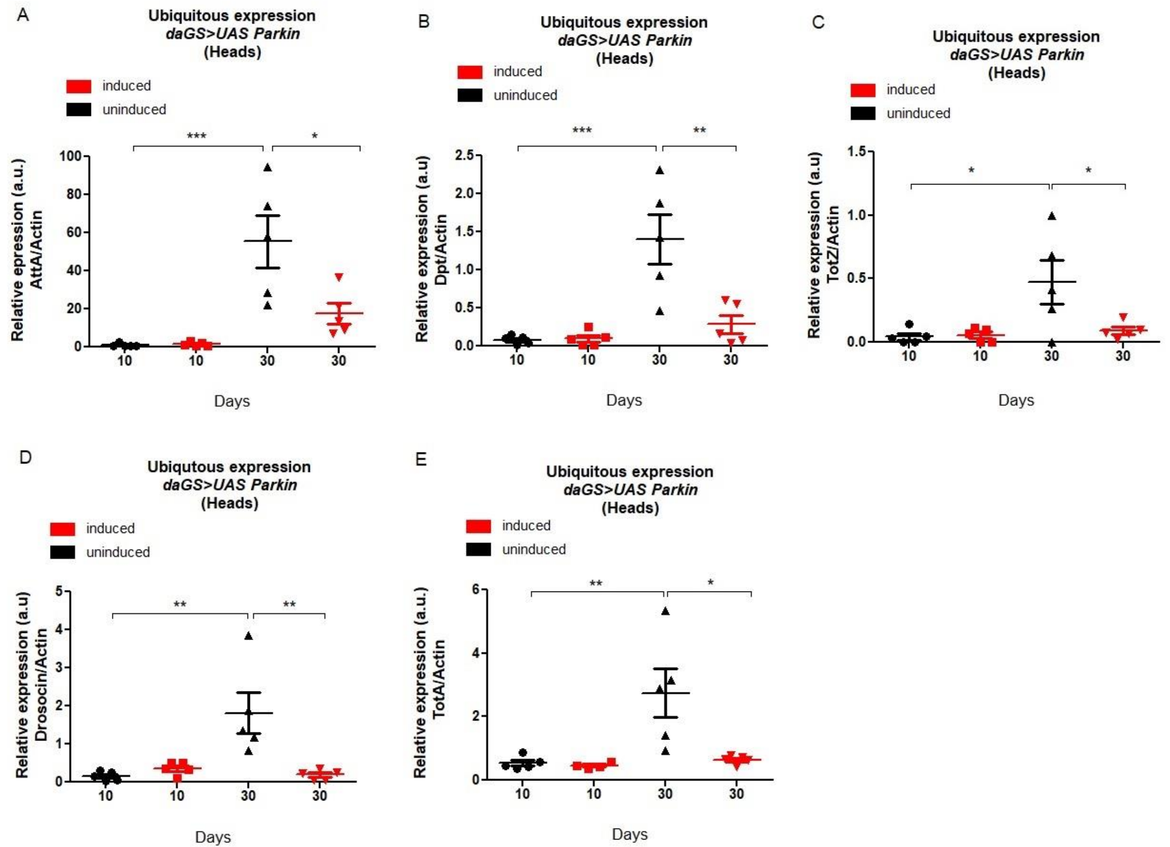

Figure 12: Constitutive Parkin induction reduces inflammation in the heads of aged flies. **A)** qPCR analysis of relative levels of Attacin A in heads of daGS>UAS Parkin at day 10 and 30 with and without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. *p<0.05, ***p<0.001); **B)** qPCR analysis of relative levels of Diptericin in heads of daGS>UAS Parkin at day 10 and 30 with or without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01, ***p<0.001); **C)** qPCR analysis of relative Turandot Z levels in thoraces of daGS> UAS parkin at day 10 and 30 with our without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. *p<0.05 **p<0.01); **D)** qPCR analysis of relative Drosocin levels in thoraces of daGS>UAS Parkin at day 10 and 30 with or without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01). **E)** qPCR analysis of relative Turandot A levels in heads of daGS>UAS Parkin at days 10 and 30 with or without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. *p<0.05, **p<0.01)**.** RU486 was provided in the media at a concentration of 5 µg/mL. Error bars represent SEM. n= 5 replicates with 5 heads or thoraces, respectively, per replicate.

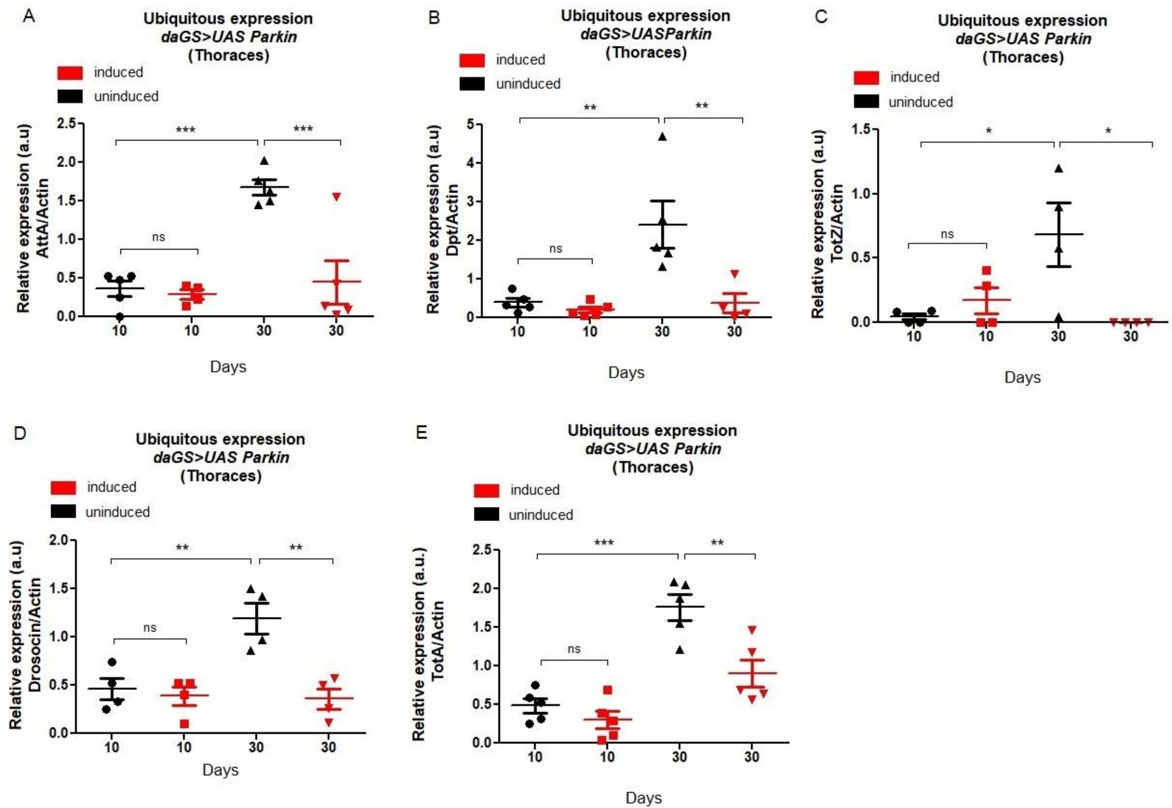

Figure 13: Constitutive Parkin induction reduces inflammation in the thoraces of aged flies. A) qPCR analysis of relative levels of Attacin A of daGS>UAS Parkin at day 10 and day 30 with and without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. ***p<0.001); **B)** qPCR analysis of relative levels of Diptericin of daGS>UAS Parkin at day 10 and 30 with or without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01); **C)** qPCR analysis of relative levels of TotZ of daGS> UAS parkin at day 10 and 30 with our without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test **p<0.01); **D)** qPCR relative levels of Drosocin levels in thoraces of daGS>UAS Parkin at day 10 and 30 with or without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01) **E)** qPCR relative levels of Turandot A levels of daGS>UAS Parkin at day 10 and 30 with or without RU induction from development (one way ANOVA/Bonferroni's multiple-comparisons test. *p<0.05). RU486 was provided in the media at a concentration of 5 µg/mL. Error bars represent SEM. n=5 replicates with 5 flies per replicate.

4.5 DRP1 overexpression reduces the levels of AMPs in heads and thoraces

Several studies have suggested that mitophagy plays a role in regulating the immune response and inflammation. In particular, mitophagy has been implicated in the regulation of the production of cytokines and chemokines that contribute to the inflammatory response [62]. Here, we wonder if inducing mitochondrial fission, which improved mitochondrial function and induces mitophagy could ameliorate the activation of the immune response in aged flies. First, we have analyzed the levels of different AMPs and stress immune factors in aged *daGS>DRP1* flies. Figures 14 and 15 show how these genes are upregulated in aged flies. Interestingly, overexpression of DRP1 for one week from day 30 onward reduces the levels of the AMPs Attacin A, Diptericin, and Drosocin and also the stress-immune effector Turandot A in both heads and thoraces. These findings suggest that inducing mitochondrial fission and improving mitochondrial function reduced the expression of AMPs in both heads and thoraces of aged flies.

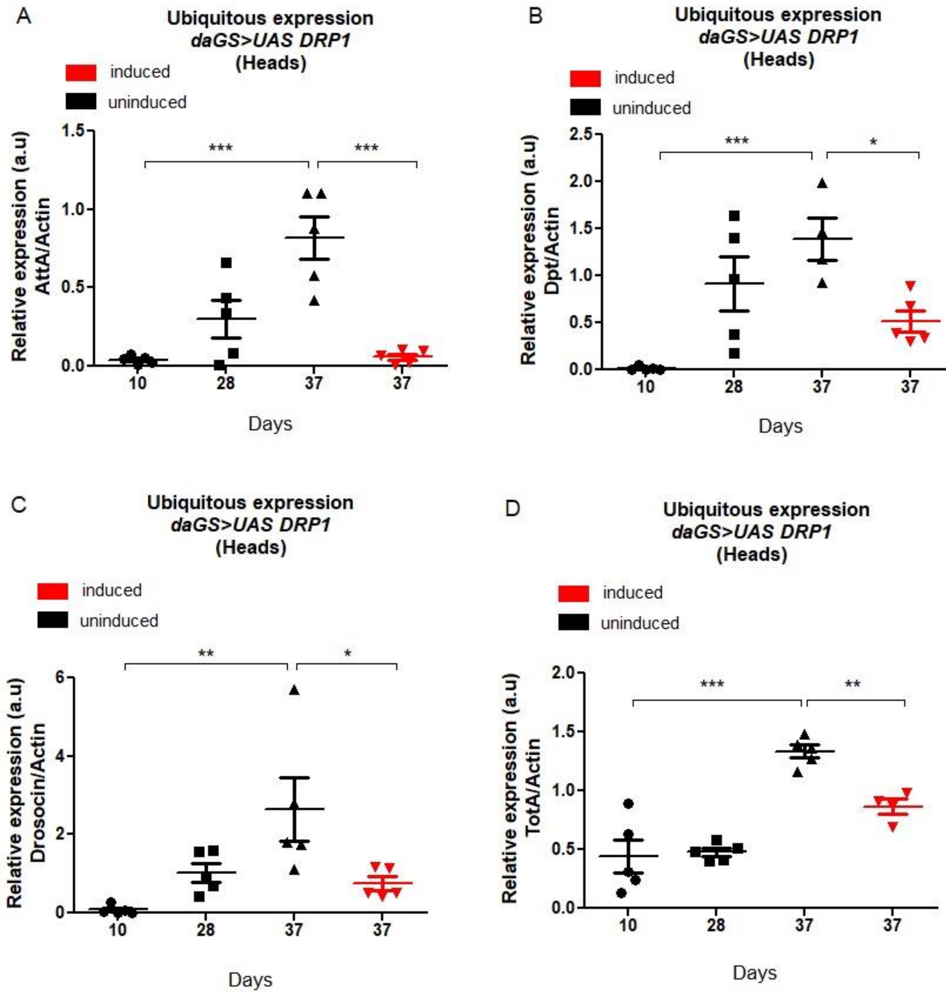

Figure 14: Midlife DRP1 induction reduces the activation of the immune response in the heads of aged flies. A) qPCR analysis of relative levels of Attacin A in the heads of *daGS>UAS DRP1* at day 10 and 28, prior to RU486 induction and after RU induction at day 37 after RU induction from day 30 onward (one way ANOVA/Bonferroni's multiple-comparisons test. ***p<0.001*)*; **B)** qPCR analysis of relative levels of Diptericin in the heads of *daGS>UAS DRP1* at day 10 and 28 prior to RU486 induction and at day 37 after RU induction from day 30 onward (one way ANOVA/Bonferroni's multiple-comparisons test. *p<0.05); **C)** qPCR analysis of relative levels of Drosocin in thoraces of *daGS> UAS DRP1* at day 10 and day 28 prior RU486 induction and at day 37 after RU induction from day 30 onward (one way ANOVA/Bonferroni's multiple-comparisons test. *p<0.05); **D)** qPCR analysis of relative levels of Turandot A of *daGS>UAS DRP1* at day 28 prior RU486 induction and at day 37 after RU induction from day 30 onwards (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01). RU486 was provided in the media at a concentration of 25 µg/mL Error bars represent SEM. n=5 replicates with 5 heads or thoraces respectively, per replicate.

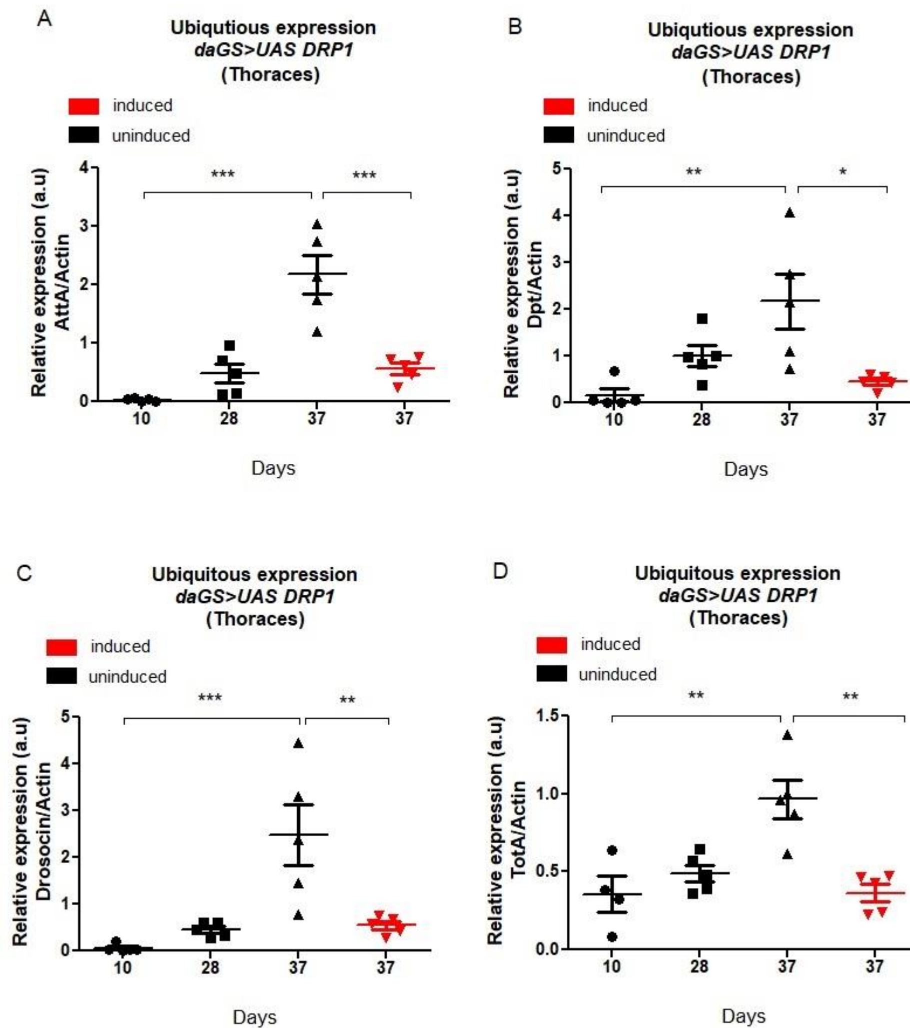

Figure 15: Midlife DRP1 induction reduces the activation of the immune response in the thoraces of aged flies. A) qPCR analysis of relative levels of Attacin A in the heads of *daGS>UAS DRP1* at day 10 and 28, prior to RU486 induction and after RU induction at day 37 after RU induction from day 30 onward (one way ANOVA/Bonferroni's multiple-comparisons test. ***p<0.001*)*; **B)** qPCR analysis of relative levels of Diptericin in the heads of *daGS>UAS DRP1* at day 10 and 28 prior to RU486 induction and at day 37 after RU induction from day 30 onward (one way ANOVA/Bonferroni's multiple-comparisons test. *p<0.05); **C)** qPCR analysis of relative levels of Drosocin of *daGS> UAS DRP1* at day 10 and day 28 prior RU486 induction and at day 37 after RU induction from day 30 onward (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01); **D)** qPCR analysis of relative levels of Turandot A of *daGS>UAS DRP1* at day 28 prior RU486 induction and at day 37 after RU induction from day 30 onwards (one way ANOVA/Bonferroni's multiple-comparisons test. **p<0.01). RU486 was provided in the media at a concentration of 25 µg/mL Error bars represent SEM. n=5 replicates with 5 heads or thoraces respectively, per replicate.

5. Discussion

The purpose of this study was to determine the interplay between mitochondrial dysfunction, the accumulation of the cytosolic mtDNA, and the immune response activation during aging in *Drosophila melanogaster*. Mitochondrial dysfunction is one of the hallmarks observed during aging and this impaired function correlates to increased ROS, mtDNA mutations, and decrease in mitochondrial membrane potential. In pathological conditions with impaired mitochondrial function, it has been reported that mtDNA is released into the cytosol. In this context, we have used *Drosophila melanogaster* to address the question if mtDNA accumulates in the cytosol of aged. We show that in wild type conditions cytoslic mtDNA increases through the lifespan of fruit flies. Cytosolic mtDNA accumulation correlates with a decline in mitochondrial function, in this context, we wonder if improving mitochondrial function could decline the presence of mtDNA in the cytosol of aged flies. In this study, we show how improving mitochondrial function reduces the accumulation of cytosolic mtDNA. This reduction in cytosolic mtDNA is not due to a decrease in total DNA. To date, there is increasing evidence that mitophagy is significantly impaired in several human pathologies including age-related diseases such as neurodegenerative diseases and cancer [63]. Previous studies have shown the beneficial effects of mitophagy in enhancing mitochondrial function, improving proteostasis, and extending lifespan in *Drosophila melanogaster* [9]. Age-related changes in mitochondrial dynamics, including alterations in fusion and fission processes and impaired mitophagy can affect mitochondrial quality control and lead to dysfunctional mitochondria and the onset of age-related diseases [64]. Some studies have reported the interplay between the accumulation of the cytosolic mtDNA and the activation of the inflammatory response in mammals and fruit flies [56]. The irreversible damage to the inner and outer mitochondrial membrane eventually causes the leakage of mtDNA into the cytoplasm. DNA material in the cytosol acts as a potential DAMP triggering the activation of the immune response genes [65].

Some studies have reported that free circulating mtDNA correlates to inflammation and activation of the immune response through the stimulation of different immunity pathways such as the IMD and Toll pathways in *Drosophila melanogaster* and the cGAS-STING pathway in mammals. The activation of these pathways in flies and mammals leaves to the activation of AMPs and pro-inflammatory cytokines, respectively. AMPs accumulate in aged flies [66]. In this study, we found that the levels of AMPs and stress immune effectors increase with age. However, when inducing mitophagy through the overexpression of Parkin or DRP1, there is a significant reduction in the levels of AMPs and stress immune factors in both heads and thoraces. However, the contribution of cytosolic mtDNA to slow aging in fruit flies remains unknown as well as the relationship between cytosolic mtDNA and the activation of the immune response in aged organisms. Previous studies have indicated that EYA plays a role in the inflammatory response by recognizing cytosolic mtDNA [57]. Consequently, investigating the impact of *eya* gene knockout on age flies would be of great interest to understand the role of cytosolic DNA in the activation of the immune response in aged organisms. Two questions still remain poorly understood: how does aging affect immunity, and how does immunity affect aging? Aging contributes to the deterioration of the immune system (immuno-senescence) and predisposes the organism to infections [67]. Furthermore, hyperactivation of the immune system can accelerate degenerative processes contributing to "inflammaging" and this results in the onset of chronic diseases. Further studies need to evaluate and verify the pathways to understand the role of cytosolic mtDNA in the activation of the immune response in aged organism and how this could affect the progression of age-related diseases. Our findings here help to better understand the relationship between mitochondrial function, immune response activation, and aging. Hence, improving mitochondrial function by pharmacological means in aged organisms or in patients with age-

related diseases could be an effective therapeutic treatment to prevent the development of age-related diseases or ameliorate the consequences of these diseases

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