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Investigating Peroxiredoxin Impact on the Intersect of Constitutive AMP Production and Aging in *Drosophila*

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ABSTRACT

The Orr-Radyuk lab is interested in understanding the link between aging and genetic influences controlling the cell's redox state, as determined by enzymes involved in reducing and oxidizing (redox) reactions. They have observed that *Drosophila* normally exhibit a shift towards a pro-oxidizing cellular environment and spikes in antimicrobial peptide (AMP) levels, independent of infections, when they reach old age. Additionally, peroxiredoxins (PRXs), a family of thiol-dependent peroxidases, have been shown to impact lifespan, and regulate the same pro-oxidizing shift seen in advanced age. Beyond their peroxidase functions, PRXs can also interact with signaling pathways related to immunity. Previous data shows that PRXs influence the IMD inflammatory

pathway, resulting in an age associated increase in AMP expression, independent of infection. Specifically, a reduction of mitochondrial PRXs (dPrx3 and dPrx5) causes a spike in AMPs during old age and rapid aging leading to early death. This report shows a reduction of ER localized Prx4 in addition to a reduction of Prx3/Prx5 mitigated the age associated AMP expression, but did not alter the rapid aging phenotype. Thus, a PRX associated redox signal seems to require dPrx 4 to be transferred from the mitochondria to the ER and finally the nucleus to drive AMP expression. Further studies are needed to determine if dPrx4 remains in the ER lumen and interacts with unfolded-protein-response (UPR) membrane proteins, or if dPrx4 leaves the ER under conditions of cellular

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oxidative stress to interact directly with IMD pathway enzymes.

1. BACKGROUND INFORMATION

One of the key interests of lab group directed by Dr. Svetlana Radyuk and Dr. William Orr is characterizing the genetic influences and signaling pathways that regulate the cell's redox state, leading to changes in immune activation and contributing to the aging process. Understanding the relationship between inflammatory responses, redox state, and aging can have important implications for lifespan extension with decreased morbidity. The question of age-associated morbidity becomes ever more prevalent as the elderly population increases. Indeed, the WHO predicts that the number of people above the age of 60 will overcome the number of children aged 5 years and younger for first time in human history by the year 2020 [15]. This trend is expected to continue; by 2050 the CDC predicts the average lifespan in America will have increased by 10 years [2]. The proposed increase in maximum lifespan may be partially attributed to biomedical interventions.

Although many forms of age interventions have been proposed (caloric

restriction, telomerase activity alteration, resveratrol supplementation etc), the Orr-Radyuk lab focuses on modulating the reduction-oxidation (redox) reactions involved in aging. Throughout the organism's life, the mitochondria generate reactive oxygen species (ROS), like the superoxide radical (O_2^-), as a side product from the oxidation of acetyl CoA along the electron transport chain. O_2^- radicals are then rapidly converted into hydrogen peroxide (H_2O_2), which is less damaging to the cellular genome and cellular organelles (Harman, 1956). Still, if too much oxidative stress builds up, it can shift the cell's redox state. In the Radyuk-Orr lab, redox state can be measured using high pressure liquid chromatography (HPLC) to assess the ratio of reduced glutathione to oxidized glutathione, expressed as GSH:GSSG [10]. Glutathione is a key cellular antioxidant.

The redox signaling hypothesis suggests that age induced fluctuations in ROS levels cause oxidative stress, which can act as an intracellular signal to trigger certain pathways converging on target genes that cause functional damage, apoptosis (programed cell death), and constitutive inflammation [5]. One such family of inflammatory genes thought to be associated with inappropriate redox signaling is a

derivative of the humoral immune response; antimicrobial peptides (AMP). AMPs are expressed in part by the NF- κ B transcription factor [5]. The NF- κ B transcription factor has been implicated in regulating lingering inflammation across many age-related chronic diseases of many different tissues [1, Franceschi & Campisi, 2014]. Thus, it's possible that AMPs, when hyper-expressed, have some sort of maladaptive effect.

A prospective organelle for the origin of this so-called age related redox signal from increased H_2O_2 seems to be the mitochondria, although the mitochondria don't necessarily have to be damaged to initiate cellular functional decline as the old free radical theory to aging would suggest [6, 7]. It is also known that enzymes called peroxiredoxins (PRXs) have peroxidase activity to reduce H_2O_2 into water by oxidizing its sulfhydryl (SH) group on a cysteine residue present in the active site [3]. PRXs can also exchange the oxidative state of its own reactive cysteine, to SH group on reactive cysteines of other enzymes within the cell who are normally insensitive to local H_2O_2 levels [8]. This provides a mechanism for redox-responsive signaling to propagate from PRXs to other cellular enzymes. Due to highly conserved sequences and functions

between mammalian PRXs and their *Drosophila* orthologues (*Drosophila* peroxiredoxins or dPrx), the *Drosophila* model is an important tool used by the Orr-Radyuk lab to elucidate the role peroxiredoxins play in modulating this redox signal. Additionally, because flies have no adaptive immune response and do not produce antibodies, the entirety of their humoral immunity is mediated by a diverse set of AMPs [11].

For this study, three specific dPrxs were of interest. The first two, dPrx3 and dPrx5 are found in the mitochondria, and third, dPrx4, is found in the endoplasmic reticulum [11, 5]. The ER and the mitochondria are both locations of high oxidative stress. Previous studies in the Orr-Radyuk lab have shown that aged wild type (WT) flies show increased expression in many different AMP genes, compared to younger flies. Additionally, by knocking out or overexpressing different dPrxs, there is a significant impact on lifespan, redox state, and AMP expression. For instance, when dPrx3 is under expressed using RNAi in a dPrx5 null mutant background, maximum lifespan was drastically cut down to around 15-18 days as opposed to around 70 days in WT flies [11]. These flies, referred to henceforth as double mutant (DM) flies,

were also far less resistant to external oxidative stress, experimentally introduced from hydrogen peroxide added into their food [11]. This lack of resistance to external oxidative stress in DM flies paralleled an internal shift to a more pro oxidative redox state, as determined by decreased levels of oxidized protein SH groups and decreased GSH:GSSG ratios compared to age matched controls [11]. It's important to note that when GSH and GSSG were measured separately GSH levels remained constant while GSSG significantly increased, specifically indicating the pro oxidative shift. However, measures of mitochondrial damage including mitochondrial Complex I and Complex II activity screening, membrane potential integrity, and NAD⁺/NADH levels all indicated no mitochondrial damage in DM flies [11]. Therefore, the pro-oxidative shift initiated by a lack of dPrx3/dPrx5 function in the mitochondria is not related to mitochondrial organelle dysfunction, but instead is related to a specific loss of peroxiredoxin function. This pro-oxidative shift, and early death phenotype was accompanied by higher levels of apoptosis revealed by TUNNEL staining, especially in the gut, where the fat body cells reside [11]. Fat body cells in

Drosophila are the tissues most involved with immune stimulation.

When dPrx4 was overexpressed, it reduced lifespan down to around 21 days, similar but not quite as dramatic as the DM fly lifespan. At the same time, RNAi knockdowns of dPrx4 resulted in reduced resistance to oxidative stress, despite not having any impact on maximum lifespan [8]. This reduced lifespan in the dPrx4 overexpressing flies was accompanied by immune activation, seen in increased levels of key AMPs such as dipterocins (Dipt) and attacins (Att) at 10 days of age [14]. This shows evidence of increased inflammation at the 50% lifespan mark. However, this spike in AMPs was not observed if dPrx4 was overexpressed in a Relish null background. Relish, a cytosolic protein, is the NF- κ B transcription factor homologue in *Drosophila*. Also, a spike in AMPs was observed in response to induced

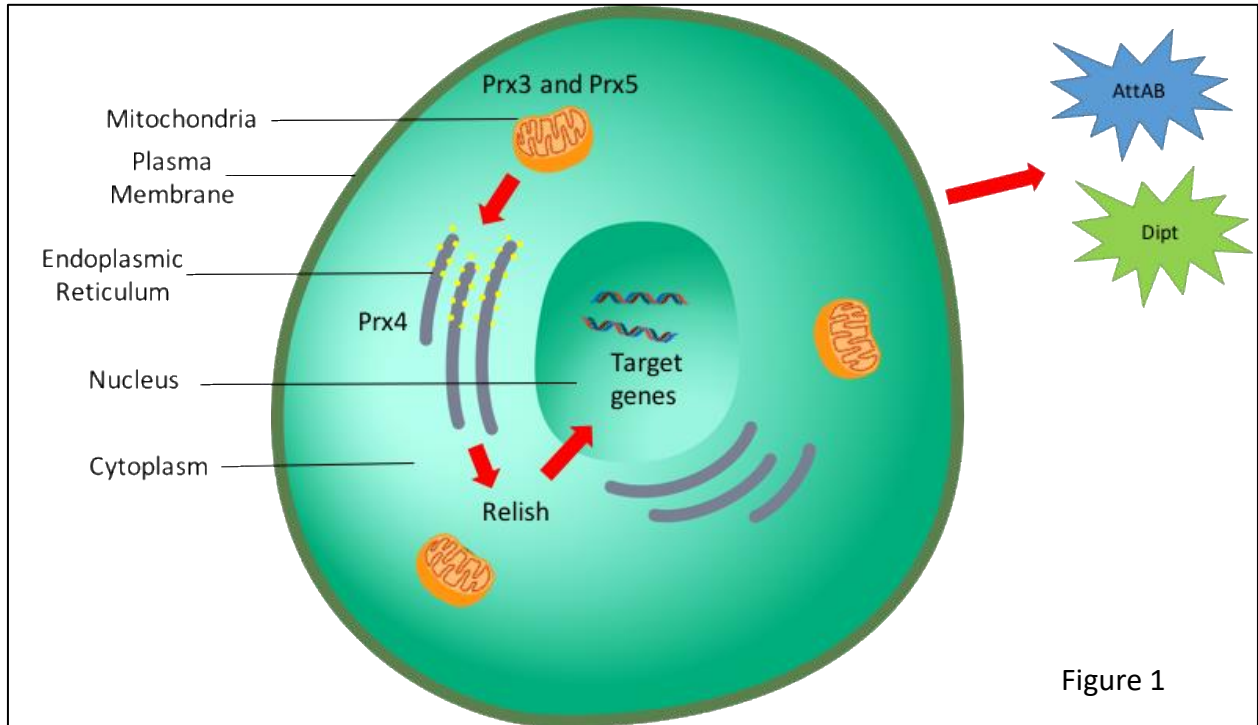


Figure 1

oxidativestress from paraquat (an oxidant that generates superoxide radicals), and was almost twice as high as the increase in AMPs in response to septic infection with *E. coli* [14]. However, if dPrx4 was under-expressed, the fly still showed an AMP response to infection, but did not show an AMP response to paraquat. Therefore, this dPrx4 mediated spike in immune activation at the midlife time point requires NF-KB activation, and is associated with early death similar to the DM flies. Finally, the cell signaling pathway linking dPrx4 to Relish activation of AMPs is a specific response to increased oxidative stress in the form of H_2O_2 . It was previously mentioned that the DM flies lacking both mitochondrial dPrxs

lead to a pro-oxidative state in the fly. This could potentially trigger dPrx4 to influence Relish downstream, and thus drive up AMP levels.

Given these results, a theoretical redox-signaling pathway inside cells was proposed, as illustrated by Figure 1. First, as a fly ages, the mitochondrial dPrxs become overwhelmed by the progressive buildup of H_2O_2 , shifting the cell to a pro-oxidative state. This pro-oxidative state can be mimicked in flies lacking mitochondrial dPrxs altogether, resulting in the rapid aging/early death phenotype. From the mitochondria, this oxidative stress signal enters into the ER, to interact with dPrx4 through a process called mitochondrial-ER

cross talk. The literature has previously shown the exchange of molecules between the two organelles contributes to Ca^{2+} homeostasis, and involves mitochondrial membrane fusion with the ER [14]. Potentially, once dPrx4 becomes activated by the oxidative stress signal, dPrx4 transduces the signal through other mediating proteins, either inside the ER or within the cytosol, so it can eventually converge on Relish. Relish drives increased expression of AMP genes in the nucleus. In short, this peroxiredoxin-mediated redox pathway is a possible link between inflammation and aging is. It begins in the mitochondria, travels to the ER by way of mitochondrial-ER crosstalk, then goes to the cytosol, and finally ends up in the nucleus via Relish. Loss of normal dPrx3 and dPrx5 function is involved in beginning the redox signal, but the only way to get the signal to Relish is through dPrx4.

Therefore, in the present study, it was hypothesized that if the theoretical redox signal pathway is true, then DM flies also lacking dPrx4 would not show hyperactivation of AMPs compared to regular DM and control flies containing dPrx4. This hypothesis was tested using a triple mutant (TM) fly, which had reduced dPrx5, dPrx4, and dPrx3. I hypothesized

further that due to a decrease in AMP expression, TM flies' lifespan would be partially rescued in comparison to the DM flies from the relief in inflammation.

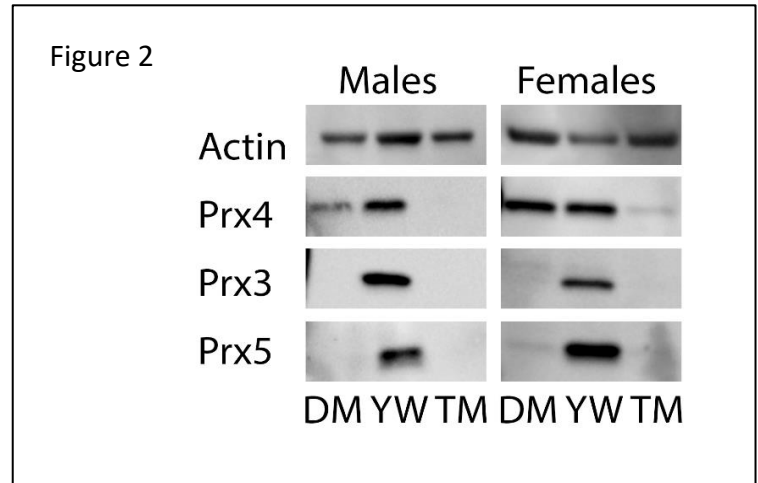
2. METHODS

To develop the TM fly, DM flies, which had null dPrx5 and a dPrx3 RNAi knock down construct on the third chromosome, were bred with flies that had an RNAi knockdown construct for dPrx4 on the second chromosome. The RNAi system eliminated 90% of the peroxiredoxin protein expression once flies containing it were crossed with flies possessing the global high level Da-GAL4 (Da) driver [11]. After developing the TM fly stock on a cornmeal-sucrose food source at 25 degrees Celsius, the genetic background was verified with polyacrylamide gel electrophoresis and western blotting on samples of the TM stock, DM stock and yellow white (YW) wild type stock. The samples were prepared from 10 flies, frozen in liquid nitrogen, stored at -80 degrees Celsius, and homogenized in lysis buffer to prevent premature protein degradation. The gel was then transferred to a nitrocellulose

membrane and stained with antibodies against dPrx3, dPrx4, dPrx5, and Actin. Since Actin is abundant in all animal tissues, it was used as a positive control to confirm loading accuracy.

The next step was to determine the maximum lifespan of the TM flies in comparison to the DM flies. To accomplish this, the flies were collected in tubes 1-2 days post hatching, then reared on the cornmeal-sucrose food source at 25 degrees Celsius until death, with fresh food provided each day. The survivorship graph for the flies, seen in Figure 3, show four different strains of flies compared; the Da>TM and Da>DM are the DM and TM flies that have the Da drivers to activate the RNAi constructs. The DM/+ and TM/+ are merely the constructs without the drivers meaning they have nearly wild type genetic backgrounds (the TM/+ and DM/+ flies still have one null copy of dPrx5 and thus are not completely WT).

The final step was to measure the levels of AMPs in the TM and DM flies. The AMPs measured included dipterin (Dipt) and attacins AB (AttAB). These and many other AMPs become constitutively expressed in flies at a time point close to old age death. The AMP levels were studied at 3 different age points (young, middle, and old)



based on percent death of a cohort of 50 flies. Specifically, the AMP mRNA levels were measured using polymerase chain reaction (PCR) assays. The mRNA transcripts transiently exist within the cellular cytoplasm before being translated into the protein primary structures by ribosomes, so higher levels of mRNA indicate higher levels of functional protein. Before the mRNA could be measured, it had to be extracted from samples of 10 flies frozen in liquid nitrogen, using Trizol. Then, the RNA samples had to be converted into cDNA, and diluted in nuclease free water. The expression levels of Dipt and AttAB in samples, displayed in Figure 3, are relative to levels of the housekeeping gene RP-49. RP-49 has stable levels of expression and should not be altered as a result of genetic mutations. By comparing the levels of the AMP mRNA to RP-49 mRNA, differences in individual expression levels of genes

based on random variation can be accounted for.

3. RESULTS

Figure 2, a representation of the western blot results, indicates that the TM flies did in fact possess the correct genetic background. A dark band means that the sample contains the protein of interest, whereas the absence of a band means the opposite. The DM flies do not show bands for dPrx3 or dPrx5 but do show bands for dPrx4. The TM flies show no bands for any dPrxs, and the YW flies show bands for all three dPrxs. Additionally, all samples

showed bands for Actin, meaning the western blot data is reliable.

Figure 3 illustrates that the difference in lifespan between the DM and TM flies is very small, and displays sexual dimorphism. Both TM and DM flies expressed the rapid death phenotype, with most males dying around day 15-16 and most females dying around day 13. The DM male flies exhibited a slightly higher, yet statistically significant, maximum lifespan compared to the TM male flies. But, DM female flies had a slightly lower maximum lifespan compared to the TM female flies (also statistically significant). Therefore, the proposed secondary hypothesis that the TM

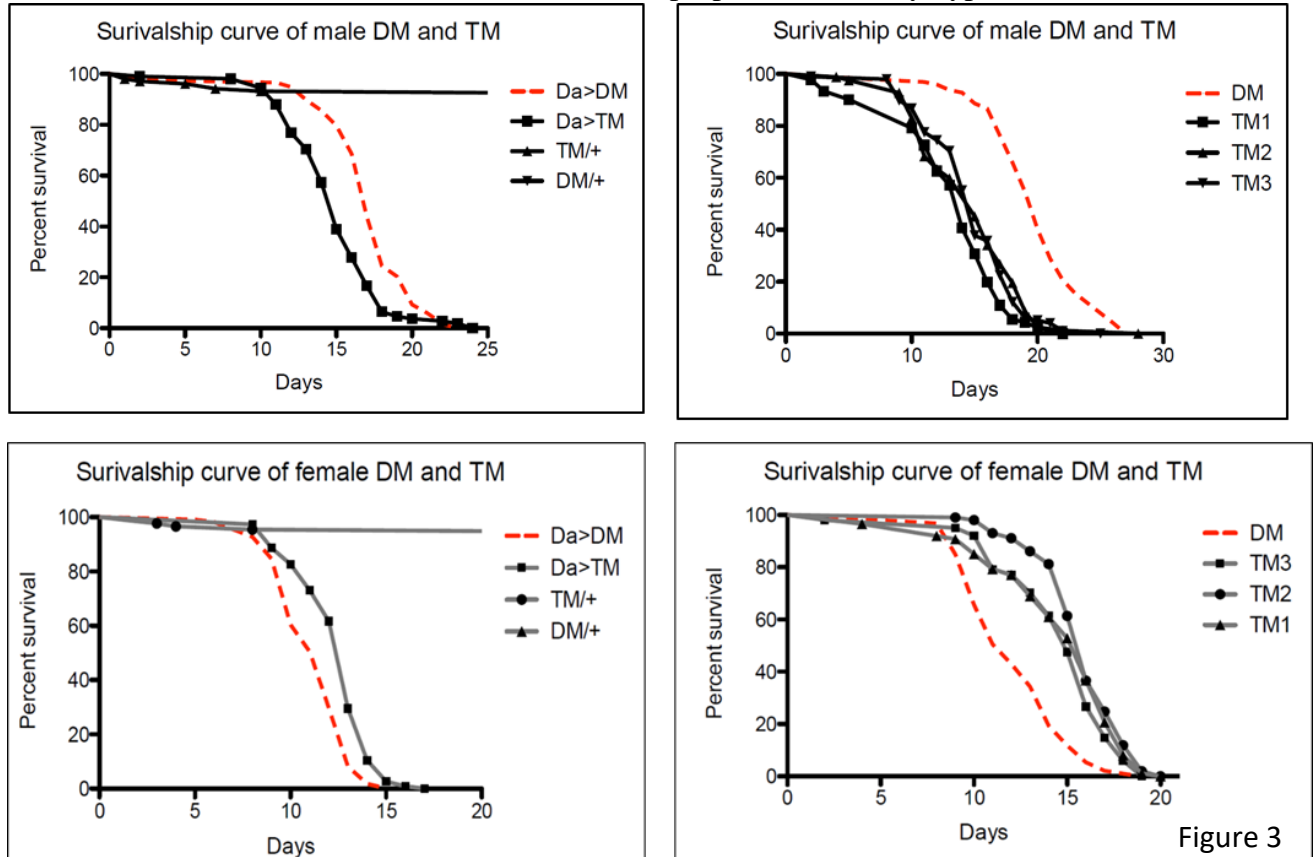


Figure 3

flies would live longer than the DM flies was rejected, at least for the males. This could indicate that the altered cellular redox state, induced by the knock down of the mitochondria peroxiredoxins, activates other lethal intracellular pathways besides the immune pathways that upregulate the AMPs, contributing to the early death phenotype. The excessive oxidative stress could also be inherently toxic to the flies.

Figure 4 represents the average AMP expression levels of three biological repeats for the males, and two biological repeats for the females. It shows four different lines of flies; the Da are Da driver flies without any

RNAi constructs to activate (making them essentially wild type except they contain one *dprx5* null gene), the C flies are the TM constructs with both RNAi constructs but no drivers (making them essentially wild type as well, still with one *dprx5* null copy), and the DM and TM flies have RNAi constructs and the Da drivers. The AMPs were measured at three time points for each fly strain relative to the maximum lifespan of each strain. The three measurements corresponded to three ages; an early age at around 15% max lifespan, a middle age at around 50% maximum lifespan, and an old age at around 70% maximum lifespan. This

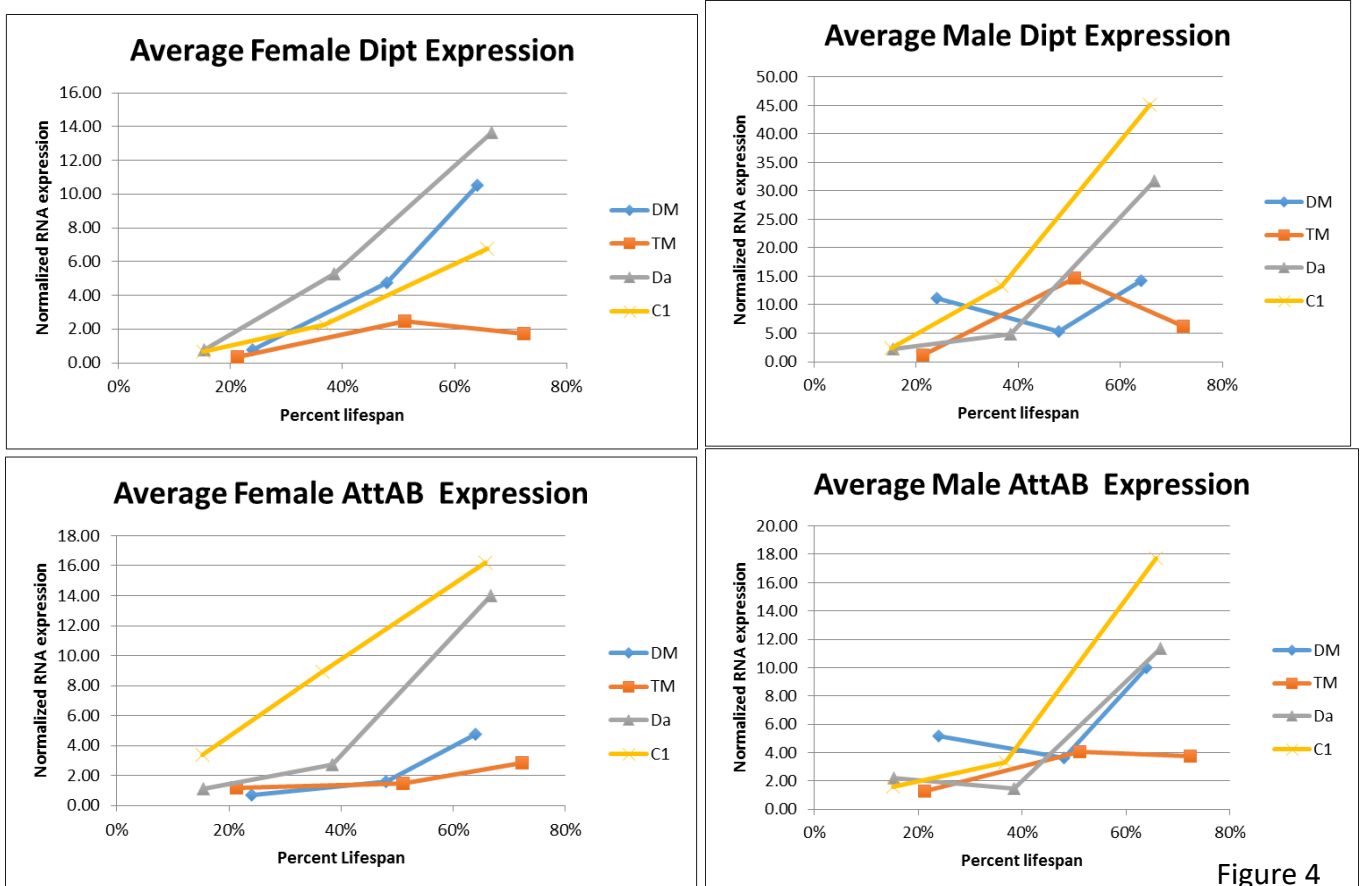


Figure 4

was done so that C and Da flies, which both have a wild type lifespan, could be compared with DM and TM flies who are of the same "physiological age," despite being frozen in liquid nitrogen at different chronological time points. For example, early age for DM and TM males was around 3 days, whereas the early age was around 10 days for the C and Da flies. But, these times corresponded to similar ages relative to their maximum lifespans. In the first two ages, the levels of AMPs showed moderate differences with the Da and C flies having higher levels compared to the DM and TM flies. This was probably because the AMPs are not activated unless the flies are in old age, giving the oxidative stress time to accumulate. At the third age point, closest to death, the C, Da, and DM fly lines showed drastically increased Dipt and AttAB levels. The TM flies maintained relatively low AMP activation in comparison. Interestingly enough, the levels of Dipt and AttAB actually slightly decrease in the male TM old age group. The female average data was similar to the male average data for Dipt, but did not show the same large difference in the old age point between DM and TM flies for AttAB expression. Still, taken together the results validates the first hypothesis; the TM flies lack dPrx4 which was predicted to be

crucial for the immune signal to be transduced from the mitochondria through the ER to the transcription factor Relish leading to AMP over expression.

4. DISCUSSION

These results raise the mechanistic question as to the role dPrx4 plays in transducing the redox signal. Does dPrx4 leave the ER under high oxidative stress to interact with other cytosolic proteins of the immune deficiency (IMD) pathway, which eventually converges on Relish [4]? Or, does dPrx4 remain inside the ER lumen and interact with membrane proteins like ATF6, that are involved in the unfolded protein response (UPR), which can also activate Relish [5]? These questions are currently being addressed through epistatic genetic analysis. First mutants with knockouts in enzymes along the IMD pathway upstream from Relish, such as TAK1, will be generated in a dPrx4 overexpression background. At the same time, mutants with knockouts of ER membrane proteins like ATF6, will be generated in a dPrx4 overexpression background. This will reveal which pathway (or if both) is being used to drive up AMP levels in old age through the Relish transcription factor. If the null mutants, representing elements of either the

IMD or ER UPR pathways, in the dPrx4 overexpression background fails to show AMP expression, then dPrx4 probably interacts with the associated pathways to transduce the redox signal. Additionally, cellular fractionation experiments will be used to separate out cytosolic and ER cellular fractions from DM flies at an old age time point to determine if dPrx4 is released into the cytosol in response to the mitochondrial oxidative stress generated from the loss of dPrx3/dPrx5 function. Whole fly samples and hemolymph samples will also be compared to the cytosolic and ER fractions. If dPrx4 is found in the cytosol and not in the ER in DM flies, it would lend support to dPrx4 interacting with the cytosolic proteins in the IMD pathway.

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