Role of Apolipoprotein D and its homologs, in normal and pathological aging, in *Drosophila melanogaster*

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Julien A. Muffat

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I would like to dedicate this manuscript to my family back in France. While I have enjoyed every minute of my time working on this project, I have missed you terribly over the years. I would not be here today if my parents, Annie and Pierre, had not always encouraged my curiosity, and supported me in all my endeavors, with their ups and downs. My grandparents, Mammina and Gigi, Mami and Papi, are certainly responsible for instilling in all of us this taste for excellence, and this conviction that a good education is a key to happiness. My brothers, Jerome and Romain, have provided the healthy emulation needed to achieve success, academic or otherwise. A special note to my dear uncle Paul, whose own career in science is a true inspiration, and who, more than anyone, has shaped my drive to understand the natural world and its wonders. And of course, thanks to everyone else, who have always made family gatherings so pleasant and exciting; I only wish I could see you more often. Finally, in my adoptive land, I would not have even functioned properly without my dear Meghan's love and support. Thank you all so much, I love you dearly.

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Abstract

The free radical theory of aging is probably the most enduring one to date. It stipulates that, in the process of normal cellular function, in particular due to oxygen-based respiration, reactive oxygen species are formed. These constantly put a strain on the cell, damaging lipids in the membranes, causing protein aggregation and loss-of-function, or mutations in the genome. Over time, this accumulated damage overcomes the repair potential of a given cell, and scaled up to an entire organism, results in the deterioration seen in normal aging. Under these assumptions, age-related pathologies are only an acceleration of the process in a given tissue, leading to the emergence of the pathology over the noise of normal aging. In the past decade, invertebrates such as *Drosophila melanogaster* and *C. elegans* have provided invaluable insight into these processes and the canonical pathways that regulate them. This success is owed in part to the power of the genetic tools available, to their relatively short lifespans, and to the wide arrays of phenotypes that can be studied. We set out to study a particular protein, Glaz, whose overexpression enables fruitflies to live 30% longer than normal. This protein was identified as a hit from a screen looking at an accelerated aging paradigm, placing fruitflies in 100% oxygen. While an interesting protein in its own right, it was through its homology with mammalian Apolipoprotein D (ApoD), that our interest was truly piqued. ApoD and its homologs turn out to be fascinating proteins, upregulated by various stresses and in age-related diseases such as cancers and Alzheimer's. We showed, in our model organism of choice, that this upregulation is part of a beneficial stress response. Understanding and harnessing its functions can only help provide therapeutic approaches for a wide range of disorders.

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Summary

The process of aging is understood to be a general deterioration over time of the cells, tissues, and organs of an organism, ultimately reducing normal function and increasing the probability of mortality. Aging is a complex process that involves a multitude of both environmental and genetic factors that ultimately results in the determination of an organism's lifespan. However, there is some hope in the fight against aging: slight alterations in many of these factors can result in significant increases in both mean and maximum lifespan. It has been known for some time that fruit flies can be selectively bred for extended lifespan. While the results from these experiments are certainly encouraging they fail in one key aspect, they deal with a complex set of mutations in multiple genes making it difficult to identify the part of the aging process that has been altered. A more rewarding avenue of exploration is the characterization of single gene mutants that appear to regulate lifespan. Already many single gene mutations have been identified within a variety of model organisms, which include but are not limited to yeast, *C. elegans*, *Drosophila*, and mice. Within our model

organism of choice, *D. melanogaster*, the discovery of these lifespan-extending single-gene mutants has been a recent advance with the first such mutant, *methuselah (mth)* being identified in 1998 in the Benzer lab.

The connection between aging and free radicals has been around for a long time, with the original “free radical theory” of aging being proposed by Denham Harman in the 1950s. At present, it is generally understood that as a by-product of normal metabolic activities certain reactive oxygen species (ROS) are produced and then damage a variety of cellular macromolecules including nucleic acids, proteins, and lipids. The accumulation of this damage over the lifetime of the organism is then thought to give rise to some of what we understand to be the aging phenotype. More evidence for this has been found from long-lived mutants such as *mth* and *daf-2* (in nematodes). In particular, it has been shown that *mth* exhibits an increased resistance to dietary paraquat, which produces superoxide anions (O^{2-}). Long-lived *daf-2* mutants are similarly resistant to hydrogen peroxide and have additionally been shown to upregulate the catalase transcript and SOD-3, both of which are enzymes used

in converting ROS to water. Moreover, in the case of long-lived *p66*^{shc} mice it has been shown that cultured fibroblast cells from the tail exhibit an increased survivorship when exposed to hydrogen peroxide.

What makes *Drosophila* useful as a model system for studying aging is in large part what makes it useful as a model genetic organism: a short life cycle (~14 days) and a low cost of husbandry. The most important features of *D. melanogaster* in studying aging are the multitude of molecular and genetic tools available to study the particular genes of interest. The first of these are the large number of well-characterized P-element insertion collections that are widely available. These provide a vast number of different mutant lines that can be screened in a straightforward manner for desired characteristics. Many of the current fruit fly aging mutants have in fact been identified from these collections, including *mth*. Additionally, many of these collections have been generated with P-elements that contain UAS (upstream activating sequences) that can be coupled to lines that express the GAL4 protein in certain tissue specific manors. GAL4 binds to the UAS regions of the P-element and is able to drive the expression of genes downstream of the insertion site. This

provides a unique ability to look at the effects of under-expression and tissue specific overexpression in the same mutant lines.

Toward a genetic dissection of the oxygen defense pathway, a genetic screen of approximately 1000 UAS-containing P-element insertion lines on chromosome 2 of *D. melanogaster* was undertaken. The flies were incubated for their entire lifespan in 100% O_2. We identified lines that displayed a significantly altered sensitivity (shorter or longer lifespans) to oxidative stress. From this, 20 lines were identified as exhibiting hypersensitivity to hyperoxia. The focus of the project has been to characterize these mutants as a method of identifying genes involved in stress resistance, as we hypothesize that such genes are likely to be involved in aging. We started this project, focusing on one of these genes, *Glial lazarillo,* which exhibited the stronger overexpression phenotypes. *Glial Lazarillo* is a homolog of human Apolipoprotein D. In the following chapter 1, the reader will find a review of the current state of knowledge on ApoD. In chapter 2, we describe the original results of the screen, and the characterization of *GLaz* overexpressors. In chapter 3, we went on to study the regulation of *GLaz* expression in normal flies, as well as the

effect of *Glaz* expression in a more reductionist cell culture system. In this chapter, we also describe the study of flies overexpressing human ApoD, and the resulting increase in longevity and stress resistance. Finally, in chapter 4, we report the effects of gain and loss of function of the other *Drosophila* homolog of ApoD, *Neural Lazarillo,* and tie them to other canonical pathways known to control aging and stress resistance, the insulin signaling pathway, and the JNK pathway.

Chapter 1

Apolipoprotein D: An Overview of its Role in Aging and Age-Related Diseases

"*It's got to be doing something important!*"-Seymour Benzer, reflecting on the observation that ApoD mRNA levels increase 500-fold following neuronal crush injury. Seymour Benzer's curiosity was legendary and seemingly limitless (Anderson and Brenner 2008; Dudai 2008; Greenspan 2008; Jan and Jan 2008; Tanouye 2008). Towards the end of his life, one of the (many) questions that kept him awake at night concerned the emerging role of Apolipoprotein D (ApoD) in aging and neurological disease. In this review, we will discuss the clinical and biochemical data on ApoD, and the input from the recent genetic studies in model systems, including those from the Benzer lab.

If the current literature is any indication, ApoD is getting more and more interest as a marker of disease. In fact, it was recently shown to be the most upregulated gene, commonly conserved between mice, monkeys and humans, as they age (Loerch, Lu et al. 2008). While the picture painted by Loerch et al. also higlights the great differences in aging transcriptomes, it is striking to find that ApoD, which has not diverged that much from ancestral bacterial homologs, is elevated in old and sick animals, and that data

accumulates showing that this response is mostly beneficial. Our own work extends such data to *Drosophila* homologs of ApoD, and demonstrates, along with data obtained in transgenic mice, that increased human ApoD is beneficial to an aging or ailing organism. These provide tantalizing foundation for the further study of ApoD's regulation and action, with regards to therapeutic potential.

Molecular Biology of ApoD

Human ApoD was first formally identified in plasma high density lipopoproteins (HDL) in 1973 (McConathy and Alaupovic, 1973). The cDNA is 855 bp long (Drayna, Fielding et al. 1986), processed from a gene region with 5 exons spanning 20 kbp, typical of the superfamily (Sanchez, Ganfornina et al. 2003). *In silico* promoter analysis (Lambert, Provost et al. 1993) identified multiple steroid response elements (RE) upstream of ApoD. Further analysis in cell culture (Do Carmo, Seguin et al. 2002) showed the gene was also under the primary control of serum-responsive elements during cellular growth arrest, and has response elements for oestrogen, glucocorticoids, progesterone, and vitamins A and D.

In humans, the protein is widely expressed, hinting at fundamental cellular functions. Other apolipoproteins are mainly produced by the liver, while ApoD's main sites of expression are the brain and the testes. It is expected to be transported through the bloodstream, while a different pool resides in the cerebrospinal fluid (CSF). In the central nervous system, it seems to be mainly expressed by glial cells (both astrocytes and oligodendrocytes) and their precursors (Hu, Ong et al. 2001), but can also be expressed by neurons in pathological situations. In the peripheral nervous system, it is primarily secreted by endoneurial fibroblasts (Boyles, Notterpek et al. 1990).

The protein itself is predicted to be small (18 kD), soluble and secreted. It has no homology to other apolipoproteins (such as the well-known ApoE). Early studies showed diverse levels of glycosylation, sometimes specific to expression site (as in axillary secretions, (Zeng, Spielman et al. 1996)). Based on its primary sequence, it was expected to be a member of the lipocalin family of proteins. These are cup-shaped molecules, presenting 8 beta-pleated sheets arranged in a calyx structure, capable of binding a single hydrophobic ligand in the pocket they form. Such a predictive

structure was originally proposed upon alignment with bilin binding protein (BBP), and recently confirmed by the 1.8 Å structure of a hydrophilized form of recombinant ApoD (Nasreen, Vogt et al. 2006); (Eichinger, Nasreen et al. 2007). These studies highlighted the presence of hydrophobic residues outside of the binding pocket, which may allow the molecule to interact with membranes or HDL, and favor ligand exchange, while the base of the calyx could provide a docking site for an unidentified cellular receptor.

Both the crystallization studies and earlier affinity assays (Morais Cabral, Atkins et al. 1995);(Vogt and Skerra 2001), point to arachidonic acid (AA) and progesterone (PG) as the best putative physiological ligands for ApoD. Cholesterol itself was originally thought to be a primary candidate, and the jury is still out on its affinity for ApoD under physiological conditions. Finally, cysteine residues may allow homodimerization or the covalent linking of ApoD with partner proteins such as apolipoprotein AII.

From these biochemical characterizations and the lack of data on physiological ligands, a complicated picture for ApoD biology emerges. The protein is expressed in many different tissues, with different glycosylation profiles. It can bind with micromolar affinity

several ligands and interact with membranes and circulating lipid particles. Its expression appears to be under the control of a complex array of regulatory elements. ApoD is realistically a protein with multiple ligands and interacting partners, through which it can exert multiple different functions in different tissues. It is in this context, a veritable *terra incognita* of actual functions, that our lab became interested in the study of ApoD and its homologs. For years, the Benzer lab was interested in the genetics of aging, and more specifically pathological processes related to neurodegeneration. Many studies have, in fact, identified ApoD as a good marker of several pathological processes, especially in the nervous system.

ApoD and Psychiatric Disorders

ApoD is associated with psychiatric disorders such as schizophrenia and bipolar disorder. Loci neighboring the human chromosomal locus for ApoD have been linked with these disorders (Camp and Farnham 2001); (Thomas, Dean et al. 2003). Circulating ApoD is elevated in the serum of schizophrenic patients receiving no medication, and in the brains of treated patients (Thomas, Dean et al.

2001); (Mahadik, Khan et al. 2002). Similarly, a 2-fold increase in ApoD can be found in the prefrontal cortex of patients with either schizophrenia or bipolar disorder, but a concurrent 2-fold increase in the parietal cortex appears to be specific to patients with bipolar disorder (Thomas, Dean et al. 2003). It is possible that elevation of ApoD in these specific regions is related to the membrane pathology observed in these disorders. Indeed, lipid homeostasis has been implicated in the pathogenesis of schizoaffective diseases, with evidence for AA and phospholipase A2 (PLA2) dysfunctions (Yao JK, 2000). It is therefore interesting to find that high ApoD levels correlate with membrane AA in erythrocytes of schizophrenic patients (Yao, Thomas et al. 2005). Moreover, in human cells in culture, transfected apoD results in higher membrane-bound AA content, and retention when AA secretion is stimulated by PLA2 activation. Also, exogenous ApoD can prevent AA uptake by the cells (Thomas, George et al. 2003), exemplifying a scavenger role for ApoD against free AA.

Interestingly, atypical antipsychotic drugs such as clozapine, which work best in treating these psychiatric disorders, further elevate the levels of ApoD. Conversely, typical antipsychotic drugs, such as haloperidol, downregulate expression of ApoD. Haloperidol is known

to eventually cause cell damage and tardive dyskinesia in patients. Free arachidonic acid is the starting point for the synthesis of prostaglandins, and an inflammation cascade that can lead to further neuronal damage. One may therefore suggest that ApoD elevation in these disorders, and a further boost by drugs, can act to prevent deleterious events, stabilizing membrane bound AA.

ApoD in Neurological Disorders and Injury to the Nervous System

ApoD has also been described in association with various neurological disorders, many of them age related. Oxidative stress can be considered an underlying common denominator in most of these pathologies. For example, an imbalance in reactive oxygen species (ROS) production and clearance in the dopaminergic neurons of the substantia nigra (SN) is often considered to be a cause of Parkinson's disease (PD). In fact, many genes linked with familial Parkinson's disease appear to be implicated in mitochondrial function, and dopamine metabolism itself can produce free radicals. Recently, it was reported that high levels of ApoD could be found in

the SN of patients with PD (Ordonez C, 2006). In this case, the neurons that are the target of the pathology do not express ApoD, but glial cells surrounding them display an increased immunoreactivity.

ApoD is elevated in normal aging brains (Kalman, McConathy et al. 2000); (Thomas, Laws et al. 2003), but more strongly so in patients with Alzheimer's disease (2–fold elevation over age-matched controls). In these patients, expression can be seen immunohistochemically in oligodendrocytes and astrocytes, but also in neurons affected by neurofibrillary tangles, and in the vicinity of amyloid plaques. We and others have observed an increase in cortical and hippocampal ApoD immunoreactivity with advanced AD stages. It is worth noting that a particular allele of ApoD (intron 1) was enriched in AD patients, in a small African-American sample (Desai, Hendrie et al. 2003), while another allele (-352G SNP) was enriched in a Finnish population with early onset AD (Helisalmi, Hiltunen et al. 2004). This association with AD may be linked to cholesterol homeostasis theories of AD etiology. By controlling membrane composition, and perhaps binding cholesterol itself, ApoD may play a part in initiation of the amyloid cascade.

Since ApoD is a lipid carrier, it is particularly interesting to observe its upregulation in disorders of the myelin sheath. Plasma and CSF ApoD levels are significantly elevated in multiple sclerosis and other inflammatory diseases of the central nervous system (Reindl, Knipping et al. 2001). As mentioned in our introduction, one of the most striking increases is observed in regenerating and remyelinating sciatic nerve, in the rat. After 3 weeks post-crush injury, ApoD increases 500-fold at the site of the lesion, and remains elevated while attempts at regeneration take course (Boyles, Notterpek et al. 1990).

More recently, in a rat model of stroke, ApoD was found to increase up to a week after reperfusion, in the penumbra of the injury (Rickhag, Wieloch et al. 2006; Rickhag, Deierborg et al. 2008). It is important to note that ApoD protein levels, but not its mRNA, increased in the dying neurons. On the contrary, in the zone bordering the infarct, oligodendrocytes were shown to upregulate ApoD expression, thereby suggesting secretion and recruitment in the dying areas.

In all those cases, and particularly in cases of acute insult, inflammation, oxidative stress and cell death are intertwined. As a

general rule, dying neurons and glia release an enormous amount of free cholesterol and arachidonic acid in the vicinity of the lesion. To prevent further damage, these lipids have to be cleared by surrounding glial cells. ApoD could provide a means to recycle these molecules, while making them available again to any subsequent regenerative process. Other apolipoproteins such as ApoE and ApoA-I have been studied in this context, and are thought to be necessary for regenerative lipid redistribution. Such data is currently lacking for ApoD. ApoD is significantly elevated in ApoE knock-out mice (Terrisse, Seguin et al. 1999), and it is conceivable that this represents a compensatory mechanism for membrane remodeling in these mutant mice.

ApoD and Cancer

A quick look at the literature on ApoD will turn up many references to cancer (Hunter, Young et al. 2002; Hunter, Weiss et al. 2005; Hunter, Varma et al. 2005; Doane, Danso et al. 2006; Soiland, Skaland et al. 2008), another age-related pathology. It is worth remembering that ApoD was identified early on as the main protein of breast cyst fluid,

with a 1000-fold higher concentration than in plasma, along with very high concentrations of progesterone. Such cysts may be related to tumor formation, and to the presence of lower-grade tumors.

Primarily, studies have observed the upregulation or downregulation of ApoD in tumor or stromal cells. They often consider ApoD as a biomarker, with potential prognostic value. As a general rule, high levels of ApoD in the tumor cells have been linked to low-grade tumors, and low proliferation. In cell culture, ApoD overexpression promotes quiescence. However, the picture is complicated since growth arrest triggered by stress (e.g., serum withdrawal), can itself upregulate ApoD (Do Carmo, Levros et al. 2007). High expression of ApoD in the stroma surrounding a tumor may, in turn, be the mark of an invasive tumor, with poor prognosis. Speculatively, such an upregulation of ApoD in the stroma of an otherwise ApoD negative tumor is a paracrine attempt at limiting tumor proliferation. An ApoD positive tumor has its own autocrine supply of ApoD, capable of limiting its growth. High levels of ApoD may not simply promote quiescence, but cell senescence. A senescent stroma is more likely to allow invasion and potential migration by tumor cells out of the primary site. Since it has been

shown in culture that ApoD expression can promote motility (Leung, Lawrie et al. 2004), it is also possible that, in some cases, high stromal ApoD would result in a particularly aggressive tumor, with greater metastatic potential. These observations may be tied mechanistically with the affinity of ApoD for AA and PG, and to the regulation of ApoD expression by steroid hormones.

For example MAPK (ERK1/2) cannot translocate to the nucleus in the presence of ApoD (Sarjeant, Lawrie et al. 2003). The targets of MAPK would otherwise favor proliferation, in response to estrogen receptor (ER) activation, through its fast-acting pathway. Estrogens, acting through their slow-acting genomic pathways, can also downregulate ApoD expression, thereby promoting cell proliferation effects. In this context, a proper balance of ApoD, either expressed or taken up from the milieu, may have strong repercussion on tumor development.

In addition the binding of ApoD to AA would prevent synthesis of prostaglandins and leukotrienes through the cyclooxygenase pathway (COX-2). These proinflammatory molecules are known activators of cell proliferation, inhibitors of apoptosis (Taketo and

Sonoshita 2002), and can even promote angiogenesis. ApoD elevation could act as a scavenger of AA, thereby decreasing tumor aggressiveness.

As mentioned earlier, PG is the second best candidate ligand for ApoD. It is therefore very possible that cytoplasmic ApoD would act as a sink for progesterone, either on its way to catabolism, or to control the kinetics of its transcriptional action. The picture is particularly complex, since, as with all steroid hormones, there are two competing pathways. Moreover the PG receptor (PR) comes in two flavors with opposite transcriptional effects, and opposite effects on cell proliferation. The net effect of ApoD presence on a functional PR response will depend on the isoform expressed in the particular cell of interest.

Very recently, an interesting cell culture study showed conclusively that ApoD is under the control of p53 family members (Sasaki, Negishi et al. 2009). P53 is probably the most well-studied tumor suppressor, and is found to be mutated or inactive in up to 70% of all cancers. In many ways, p53 may constitute a nexus between cancer and aging (Serrano and Blasco 2007; Campisi 2008). An overactive p53 may result in accelerated aging, through depletion of

renewal potential for a given tissue. A mildly overactive p53 pathway could have beneficial effects, promoting tissue quiescence and repair. An inactive p53 pathway would, in turn, promote tumor formation. The study of its close homologs p63 and p73 has provided some intriguing observations. Most notably, it appears that there may be some level of functional redundancy, as p73 has recently been characterized as tumor suppressor as well (Rosenbluth and Pietenpol 2008). Some genes may respond to all p53 family members, while others may be specific to either p53, p63 or p73. In any case, Sasaki et al. demonstrate that the ApoD promoter can bind p63 and p73, and that p73 represents the functional activator. They observe that several phenotypes, relevant to cancer formation and cell senescence, are under the control of p73 through transactivation of ApoD. Indeed, in their system, proliferation and differentiation induced by p73 in osteosarcoma and astrocytoma cell lines, necessitates ApoD. Even DNA damage induced by cisplatin, which upregulates ApoD, appears to do so through p73 activation. Their result on the medulloblastoma cell line (SH-SY5Y), and how ApoD can drive neuronal differentiation, is particularly interesting, as it may

provide some information on the role of ApoD in brain maintenance and repair.

Modeling the role of ApoD in Longevity and Stress Resistance

Our own interest in ApoD began when we performed a genetic screen to identify genes that protect the fruit fly *Drosophila* against oxidative stress and aging. As is often the case, there were a relatively large number (~20) of 'hits' from the screen (Walker, Hajek et al. 2006; Walker, Muffat et al. 2006). One of these 'hits', Glial Lazarillo (*GLaz*), was a fly homolog of human ApoD. We remember vividly showing our mentor, Seymour Benzer, the very active flies that were overexpressing the ApoD homolog next to the very sickly control flies. From that day on, we would discuss ApoD on an almost daily basis until the end of Seymour's life.

To validate the protective effects of *Glaz*, we generated independent transgenic lines carrying the GLaz cDNA under control of the UAS/GAL4 system (Brand and Perrimon 1993). Upon doing so, we discovered that overexpression of *Glaz* protected against a range of extrinsic stressors and extended the lifespan of normal flies

by ~30% (Walker, Muffat et al. 2006). An independent loss-of-function study revealed that *GLaz* mutants are sensitive to both oxidative and starvation stress (Sanchez, Lopez-Arias et al. 2006). Interestingly, a GLaz-GFP reporter line was expressed in glial cells in the adult fly brain. This expression pattern is consistent with the developmental expression pattern of *GLaz* in *Drosophila* (Sanchez, Ganfornina et al. 2000), and with the mainly glial expression pattern of vertebrate ApoD (Sanchez, Ganfornina et al. 2002; Navarro, Del Valle et al. 2004; Ganfornina, Sanchez et al. 2005). In addition, *GLaz* gene activity was found to protect against neuronal apoptosis as a function of both age and extrinsic oxidative stress (Sanchez, Lopez-Arias et al. 2006). At a physiological level, *GLaz* mutant flies display decreased fat content (Sanchez, Lopez-Arias et al. 2006), highlighting a putative role for GLaz in lipid metabolism.

Recently, two additional studies have reported characterizing the function of ApoD orthologs in mammals (Ganfornina, Do Carmo et al. 2008) and also in plants (Charron, Ouellet et al. 2008). An ApoD knockout mouse (ApoD-KO) was generated by replacing the wild-type ApoD gene with a copy interrupted by the insertion of the neomycin resistance gene, which rendered a transcription null

mutant. Interestingly, ApoD-KO mice display reduced locomoter and exploratory behavior as well as deficits in learning (Ganfornina, Do Carmo et al. 2008). Remarkably, murine ApoD appears to protect against oxidative stress also. ApoD null mice display reduced tolerance to the ROS generator paraquat. This result was observed in two different genetic backgrounds, strongly indicating that loss of ApoD gene function is responsible for the phenotype. Bioinformatic studies have revealed that plants also possess lipocalins, which were classified as temperature-induced lipocalins (TILs), including the ApoD ortholog *AtTIL* identified in *Arabidopsis thaliana* (Charron, Ouellet et al. 2005). *AtTIL* knockout plants are acutely sensitive to oxidative stress, cold and light (Charron, Ouellet et al. 2008). Whereas, overexpression of the normal gene confers resistance to extrinsic stress.

The upregulation of human ApoD (hApoD) in various diseases involving chronic stress predicts that experimentally induced stress may regulate the expression of ApoD orthologs in model systems. To gain insight into this question, we examined the regulation of *GLaz* (fly ortholog of ApoD) as a function of extrinsic stress. Quantitative

real-time PCR (qRT-PCR) revealed that *GLaz* mRNA levels were dramatically increased in response to dietary paraquat, hyperoxia or heat stress (Muffat, Walker et al. 2008). A careful study in mice has revealed the temporal and spatial expression of ApoD in response to oxidative stress (Ganfornina, Do Carmo et al. 2008). An acute up-regulation of ApoD in mouse brain was observed 3 hours after exposure to paraquat, and the expression returns to baseline by 24 hours. No up-regulation was observed in the lung or liver. In plants, *AtTIL* is induced by both thermal and water stress (Frenette Charron, Breton et al. 2002). Understanding the relationship between different stressors and ApoD induction may shed light on the mechanisms underlying neurological disease. Toward this goal, a recent study examined the response of ApoD to a range of stressors in cell culture (Do Carmo, Levros et al. 2007). Interestingly, stresses that cause an extended growth arrest, such as UV light or hydrogen peroxide, increase ApoD expression. At the same time, lipopolysaccharide (LPS) a proinflammatory agonist was able to induce ApoD expression, perhaps hinting at a feedback inhibitory loop. This study also addressed the subcellular localization of ApoD under normal and stressful conditions. Interestingly, under normal conditions ApoD is

mainly perinuclear but it accumulates in the cytoplasm and nucleus under stressful conditions. In fact, exogenous ApoD from the medium can be taken up by cells and translocated in this way, making the search for the receptor(s) involved in this trafficking a high priority in ApoD research. These studies in plants, insects and mammals strongly support a conserved function for these lipocalins, responding to and protecting against extrinsic and intrinsic stress.

Manipulating Human ApoD (hApoD) Gene Function *In Vivo*

As outlined above, ApoD homologs in model systems appear to show conservation of both regulation and function. But what about the human gene itself? To examine the role of hApoD on longevity and stress resistance directly, we generated two independent transgenic fly lines carrying the hApoD cDNA under control of the UAS/GAL4 system (Brand and Perrimon 1993). Using this system, we have shown that overexpression of hApoD in flies protects against oxidative stress and extends lifespan under normal conditions (Muffat, Walker et al. 2008). To examine the role of hApoD in mice, a transgenic animal (HApoD-Tg) overexpressing hApoD under the control of a neuronal promoter was generated (Ganfornina, Do Carmo et al. 2008). Indeed, HApoD-Tg mice display improved survival following exposure to two different concentrations of paraquat. More recently, Do Carmo et al. showed conclusively that ApoD overexpression is sufficient to temper inflammation observed in coronavirus-induced encephalitis (Do Carmo, Jacomy et al. 2008). The study of mutant mice in similar conditions would greatly inform our current understanding of some of ApoD's physiological functions.

Conclusion

Such results highlight the exciting studies that can stem from animal models, whether using ApoD homologs or human ApoD transgenics. It is easy to see how further studies of these fly and mouse strains can inform the processes in which ApoD is now thought to play an important role, which we highlighted in this review. We note that whether we consider cancers, neuronal disorders, or wound healing, there is an underlying age-dependency. In part, the involvement of ApoD in these disorders could be related to a direct scavenging activity against free-radical damage. Studies in both mice and fruitflies have shown ApoD's ability to decrease lipid peroxides in membranes. In mice, it is quite possible that this effect is exerted on inflammatory pathways, or more directly on clearance of peroxidized arachidonic acid. In any case, the community working on this protein and its homologs has only started to scratch the surface of ApoD's mechanisms of action. Over the years, ApoD has been studied from widely different angles, which are today primed to coalesce, and provide a significant new understanding of its various roles. The recent elucidation of its crystal structure can only benefit biochemical

studies of ligands and interacting partners, and provides educated targets for mutational analysis. We believe ApoD constitutes an important therapeutic target for several devastating diseases. Harnessing its beneficial powers will rely on accurate understanding of its regulation and functions. If direct ligand interactions are involved in these therapeutic approaches, it is important to note that ApoD will benefit from work to generate anticalins, engineered lipocalins, with customized affinities for lipidic ligands of choice (Vogt and Skerra 2004).

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Chapter 2

Overexpression of a *Drosophila* Homolog of Apolipoprotein D Leads to Increased Stress Resistance and Extended Lifespan

Summary

An increase in Apolipoprotein D (ApoD) expression has been reported in a number of neurological disorders, including Alzheimer's disease, schizophrenia and stroke, as well as in the aging brain (Rassart, Bedirian et al. 2000). However, whether ApoD is toxic or part of a defense strategy is unknown.

In a screen to identify genes that protect *Drosophila* against acute oxidative stress, we isolated a fly homolog of ApoD, *Glial Lazarillo* (*GLaz*). We generated independent transgenic lines, and found that overexpression of *GLaz* resulted in an increase in resistance to hyperoxia (100% O_2) as well as a 29% extension of lifespan under normoxia. These flies also displayed marked improvements in climbing ability and locomotion activity, after sub-lethal exposure to hyperoxia. Overexpression of *Glaz* also increased resistance to starvation, without altering lipid or protein content. To determine whether *GLaz* might be important in protection against reperfusion injury, we subjected the flies to hypoxia, followed by recovery under normoxia. Overexpression of *GLaz* was protective

against behavioral deficits caused in normal flies by this ischemia/ reperfusion paradigm.

This and the accompanying paper by Sanchez et al. are the first to manipulate the levels of an ApoD homolog in a model organism. Our data suggest that human ApoD may play a protective role, and thus may constitute a therapeutic target to counteract certain neurological diseases.

Results:

Overexpression of *GLaz* confers resistance to hyperoxia, as well as lifespan extension in normoxia.

Oxidative stress has been widely implicated as an underlying mechanism in the pathology of neurodegenerative disorders, including Alzheimer's disease (AD) and schizophrenia (Yao, Reddy et al. 2001; Andersen 2004), as well as in the injury resulting from stroke (Chan 2005). In addition, an overwhelming body of evidence supports the idea that reactive oxygen species (ROS) are a major cause of aging (Harman 1957; Balaban, Nemoto et al. 2005). As the

formation of ROS is a function of ambient oxygen concentration (Turrens 2003), exposure to hyperoxia (100% O_2) offers an attractive model for physiological studies of oxidative stress.

We therefore performed a screen to identify genes that confer increased resistance to hyperoxia. This and all the subsequent experiments in this report were performed with male flies only. We drove high-level expression of various genes by crossing Enhancer Promoter (EP) element lines (Rorth, Szabo et al. 1998) having known insertion sites, with a driver line providing a ubiquitous GAL4 source, namely *daughterless* (*da*-GAL4). One of the lines, *EP(2)2383*, carrying an insertion on the second chromosome, displayed a striking GAL4-dependent phenotype and was chosen for further study. Flies carrying both *EP(2)2383* and *da-GAL4* are much more resistant to hyperoxia (Figure 1A). The insertion in *EP(2)2383* is 1.5 kb upstream of *Glial Lazarillo* (*GLaz).* RT-PCR confirmed that the *EP(2)2383* insertion causes a GAL4-dependent upregulation of the *GLaz* transcript (Figure S1). A microarray showed a 7-fold upregulation of the *GLaz* transcript, while nearby genes (*NrkI, TppII, SptI* and *CG17724*) in the genomic region were not affected (data not shown).

Glial Lazarillo (*Glaz*) is a *Drosophila* homolog of human Apolipoprotein D (ApoD), with 40% identity and 80% similarity to the human protein. Both are predicted to be very similar to the ancestral proteins that gave rise to the lipocalin family (Ganfornina, Gutierrez et al. 2000; Gutierrez, Ganfornina et al. 2000; Sanchez, Ganfornina et al. 2000). ApoD is a member of the lipocalin family, cup-shaped soluble molecules able to carry hydrophobic ligands in a 1:1 molar ratio. The physiological ligand for ApoD is undetermined, although, based on binding studies, arachidonic acid and steroids are the best candidates (Vogt and Skerra 2001). In Alzheimer's disease, both mRNA and protein levels of ApoD are dramatically increased (350% in the cerebro-spinal fluid), and the protein is recruited to the amyloid plaques (Terrisse, Poirier et al. 1998; Kalman, McConathy et al. 2000; Navarro, Del Valle et al. 2003). Following crush injury of the rat sciatic nerve, regeneration is accompanied by a 500-fold upregulation of ApoD at the site of the lesion, for several weeks after the injury (Boyles, Notterpek et al. 1990; Spreyer, Schaal et al. 1990). Neuronal degeneration induced by kainic acid triggers an upregulation of ApoD in the affected area (Ong, He et al. 1997). An increase in ApoD expression has been detected in a murine model of Niemann-Pick

disease type C (NPC), an inherited lysosomal cholesterol disorder (Suresh, Yan et al. 1998). Elevated ApoD expression has been reported in schizophrenia and bipolar disorder (Thomas, Dean et al. 2001; Sutcliffe and Thomas 2002; Thomas, Copolov et al. 2003). ApoD levels have also been reported to increase with aging, in both humans and mice (Kalman, McConathy et al. 2000; Lee, Weindruch et al. 2000).

In general, there is a strong correlation between resistance to oxidative stress and increased longevity (Larsen 1993; Sun and Tower 1999; Melov, Ravenscroft et al. 2000; Sun, Folk et al. 2002; Schriner, Linford et al. 2005). Therefore, we measured the lifespan under normoxia of flies carrying EP(2)2383 and *da*-GAL4. Overexpression of *GLaz* led to an increase in mean lifespan at 29°C of 18% ($p<0.001$, Figure 1B).

Tissue-specific overexpression of *GLaz.*

To study the overexpression further, and confirm that overexpressing *GLaz* was sufficient to cause the lifespan extension, we generated independent transgenic lines carrying the *Glaz* cDNA

under GAL4 regulation. The complete *Glaz* cDNA was cloned in the pUAST vector, thereby placing the insert under the control of UAS sequences within the boundaries of a P-element. Two independent transformants were generated (*UAS-GLaz1*, and *UAS-GLaz2*), both on the second chromosome. RT-PCR confirmed that both lines displayed GAL4-dependent upregulation of the *Glaz* transcript (Figure S2). Based on its relatively higher level of inducibilty, *UAS-GLaz2* was selected for further studies.

To address whether specific tissues are responsible for the lifespan extension, *UAS-GLaz2* and *EP(2)2383* were crossed with GAL4 drivers having preferential patterns of expression in various tissues. Since GAL4 generally yields stronger effects at 29°C (Seroude, Brummel et al. 2002), we chose to perform these experiments at that temperature. The control used was w^{1118}, the host strain in which both UAS-*Glaz* contructs were generated. The results are outlined in Table 1. Best results were obtained with relatively weak nervous system expression, combined with some expression in thoracic or abdominal muscles (*PO163*-GAL4, $GAL4^{109(2)80}$). Drivers with expression primarily in the fat or in the muscles (*DJ634*, *24B*) caused extension of lifespan, as compared to

the control crosses to w^{1118}. *GMR-GAL4*, also induced a robust extension of lifespan. Although it is commonly described as an eye-specific driver, we note that it also drives strong expression of a reporter in the salivary gland. When either the EP line or the cDNA transgene was driven by the strong nervous system drivers (*D42-GAL4*, *Elav-GAL4*), there was no beneficial effect. No lifespan extension was observed with *Actin-GAL4* or *Hsp70-GAL4* when driving the cDNA transgene, although *Actin-GAL4* did give a notable extension of lifespan when driving the EP line. These results do not clearly identify a specific tissue as responsible for the phenotypic effects.

One of the drivers, $GAL4^{109(2)80}$, resulted in a particularly strong extension of lifespan with both the EP and the cDNA line. $GAL4^{109(2)80}$ is expressed especially in cells in the thoracic ganglion and the brain, and in the vertical flight muscles of the thorax (Figure 2A). While this pattern is maintained throughout adulthood (L. Seroude, personal communication), during development it is expressed in only a small subset of sensory neurons (Tracey, Wilson et al. 2003). Flies carrying $GAL4^{109(2)80}$ and *EP(2)2383* displayed a 29% increase in

mean lifespan compared with the driver crossed to w^{1118} (Figure 2B) while driving *UAS-GLaz2* results in a 23% increase (Figure 2C).

Flies carrying both $GAL4^{109(2)80}$ and *UAS-GLaz2* displayed a 12% increase in survival under hyperoxia (Figure 2D) compared to the driver control. To test whether the overexpressor flies also had improved vigor associated with their hyperoxia resistance, we performed behavioral assays. After 4 days in hyperoxia both vertical and horizontal locomotor performances were much better in flies overexpressing *GLaz,* as compared to controls (Figure 3A and 3B). The same genotypes, when maintained under normoxia, showed no differences.

Flies overexpressing *Glaz* are more resistant to starvation.

Resistance to multiple extrinsic stressors is a hallmark of several long-lived mutants (Larsen 1993; Lithgow, White et al. 1995; Murakami and Johnson 1996; Lin, Seroude et al. 1998; Wang, Kazemi-Esfarjani et al. 2004). We tested flies overexpressing *GLaz* for resistance to wet or dry starvation. Overexpression of *Glaz* led to a 60% increase in lifespan under wet starvation (Figure 4A) and a

30% increase under dry starvation (Figure 4B). Given the predicted nature of the protein as a secreted lipid carrier, we checked whether the flies overexpressing *GLaz* were larger, or displayed higher lipid or protein content. There was no significant difference in dry weight (Figure S3A), protein content (Figure S3B), or fat content (Figure S3C) between flies overexpressing *GLaz*, compared to the driver crossed to w^{1118}.

GLaz overexpression protects against behavioral deficits caused by hypoxia

ApoD is upregulated in a mouse model of stroke (Trieu and Uckun 2000). Therefore, we tested the ability of flies that overexpress *Glaz* to withstand periods of oxygen deprivation, followed by recovery under normoxia. The flies were subjected to hypoxia in 100% N_2 for 30 minutes, then transferred back to a normoxic environment. After awakening, flies over-expressing *Glaz* were much more active than control flies. In our assay of vertical locomotion performance, one hour after their recovery, 75% of the *GLaz* overexpressors climbed to the top one third of the vial, while only 10-15% of control flies

managed to do the same (Figure 4C). This difference was still evident 2 days later. Before hypoxia exposure, no differences in climbing ability was observed.

Discussion

We report that overexpression of *GLaz,* using independent EP insertions or transgenic lines, can result in increased resistance to stress, and extension of lifespan. These results are consistent with ApoD being part of a damage-control system. The mechanisms involved in these effects remain to be determined: GLAZ, and perhaps APOD, may transport lipophilic molecules to and from cells, thus helping to repair damaged membranes, or clear cholesterol and fatty acids released by dying cells. In mammals, the binding affinity for arachidonic acid may point to a role in the control of inflammation. They may also quench deleterious molecules such as lipid peroxides, or scavenge other free radicals. In the accompanying paper, Sanchez et al. observe a depletion of lipid stores in *GLaz* deficient mutants. GLAZ may work as part of a shuttle system, used to maintain stores

at a normal level, while providing support to other tissues in situations of stress.

We screened multiple drivers, with multiple responder lines, and could not point to a single tissue as a necessary or sufficient site of upregulation to produce increased longevity. The amount of overexpression of such a secreted protein may matter more than its site of production. Furthermore, it must be borne in mind that the expression patterns of many drivers change during development and with age, and that *GLaz* is likely to be secreted. Interestingly, overexpression with strong neuronal drivers did not extend adult lifespan. In the accompanying paper, Sanchez et al. report that normal adult expression of *GLaz* is primarily in glial cells, and to some extent in the cardia and hemocytes. In embryos, the transcript is present in the nervous system (Sanchez, Ganfornina et al. 2000), and the developing gut. In the embryonic nervous system, *Glaz* is expressed in the longitudinal glia. These cells are known to be crucial for axon guidance and neuronal trophic support, and can be considered homologous to oligodendrocytes of vertebrates. Originally, the grasshopper homolog, *Lazarillo,* was found to be important for axon guidance (Sanchez, Ganfornina et al. 2000). One

of our most effective drivers, *GAL4*$^{109(2)80}$, is expressed in adult vertical flight muscles, and, to a lesser extent, in the adult nervous system, but has a very restricted expression pattern during development. It is therefore possible that strong early overexpression in neurons has adverse effects that mask the benefits of GLAZ in the adult, where it may circulate in the hemolymph to reach its target tissues.

Flies overexpressing *Glaz* fared much better in behavioral tests after hyperoxia exposure. We have recently shown that the flight muscle is one of the earliest tissues to suffer damage under hyperoxia, and that old flies also display some of the alterations seen under hyperoxic stress (Walker and Benzer 2004). It is conceivable that overexpression of *GLaz* in the flight muscles (using *GAL4*$^{109(2)80}$) is beneficial either by improving nutrient exchange, or by delaying cellular damage. In the mouse, ApoD is upregulated in response to cerebral stroke (Trieu and Uckun 2000). Some of the common therapies for victims of such damage aim at lowering the overall metabolism of the affected area, to decrease the oxidative burden on cells. We show that *GLaz* overexpression can protect against behavioral decline induced by exposure to hypoxia. *GLaz*

overexpression might make the flies more resistant to the cellular insult of the stresses studied here, and hastening the recovery.

In recent years, *Drosophila* has become a powerful model for studying neurodegenerative disorders (Muqit and Feany 2002; Bilen and Bonini 2005). Understanding how lipocalins regulate lifespan, or rescue neurological disorders in *Drosophila* may help to understand the role of ApoD in the human brain, and suggest ways of manipulating its expression and function, providing clues to potential therapeutic routes.

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Figures and Legends:

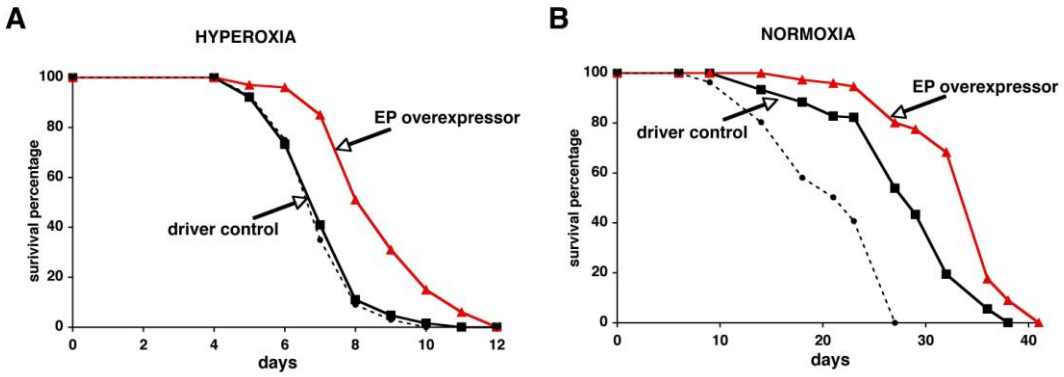


Figure 1*:* Increase in hyperoxia resistance and extension of lifespan by overexpression of *GLaz*.

A: Survival under hyperoxia (100% O_2). *da*-GAL4 (ubiquitous GAL4 driver) is used to activate the UAS sequences in *EP(2)2383*. Each graph represents a trial with at least 100 flies, typical of several repeated experiments. The EP overexpressor (w^{1118}; *EP(2)2383/+; da-GAL4/+,* red curve) lives 20% longer ($p<0.001$) than the driver control (w^{1118}; *da-GAL4/+,* black curve). The dotted line represents

the EP alone (*w*^{1118}*; EP2383*/+). **B :** At 29ºC, under normoxia, the EP overexpressor lives 18% longer than driver control.

DRIVER		control (*w*^{1118})	*GLaz* overexpression (*EP(2)2383*)		*GLaz* overexpression (*UAS-GLaz2*)	
		Mean ± SEM	Mean ± SEM	% diff	Mean ± SEM	% diff
CNS/muscle	*PO163*	25.6 ±0.6	30.4 ±0.6	**18%, p<0.001**	27.8 ±0.8	**8%, p<0.001**
	GAL4$^{109(2)80}$	25.3 ±0.6	32.5 ±0.5	**29%, p<0.001**	31.0 ±0.5	**23%,p<0.001**
Fat/muscle	*DJ634*	22.8 ±0.6	26.6 ±0.6	**17%, p<0.001**	26.5 ±0.6	**16%, p<0.001**
	24B	23.2 ±0.5	25.5 ±0.6	**10%, p<0.001**	29.7 ±0.7	**28%, p<0.001**
Eye/Salivary gland	*GMR*	25.4 ±0.6	31.0 ±0.3	**22%, p<0.001**	31.5 ±0.4	**23%, p<0.001**
Ubiquitous	*Actin*	25.6 ±0.8	30.9 ±0.5	**21%, p<0.001**	26.6 ±0.5	4%, n.s.
	Hsp70	30.3 ±0.5	30.3 ±0.5	0%, n.s.	29.9 ±0.5	-1%, n.s.
Neuronal	*Elav*	31.0 ±0.6	31.4 ±0.7	1%, n.s.	29.3 ±0.6	-5%, n.s.
	D42	20.2 ±0.5	19.1 ±0.5	-5%, n.s.	21.1 ±0.8	4%, n.s.

TABLE 1

Table 1: Lifespan analysis of tissue-specific GAL4 driver lines.

The different drivers are grouped by expression patterns. The first column shows the mean lifespan +/-SEM of each driver alone (crossed to w^{1118}) as controls. The second column shows the mean lifespan +/-SEM of the various drivers crossed to *EP(2)2383*, and the

percentage difference with the driver control (n.s. = not significant). The third column displays the same information for each driver crossed to the transgenic cDNA insertion line *UAS-GLaz*^{2}. Drivers that did not have a strong expression during development all gave good extensions of lifespan. The same drivers that extended lifespan of the EP line also increased lifespan of the cDNA transgenic line, with the exception of Actin-GAL4.

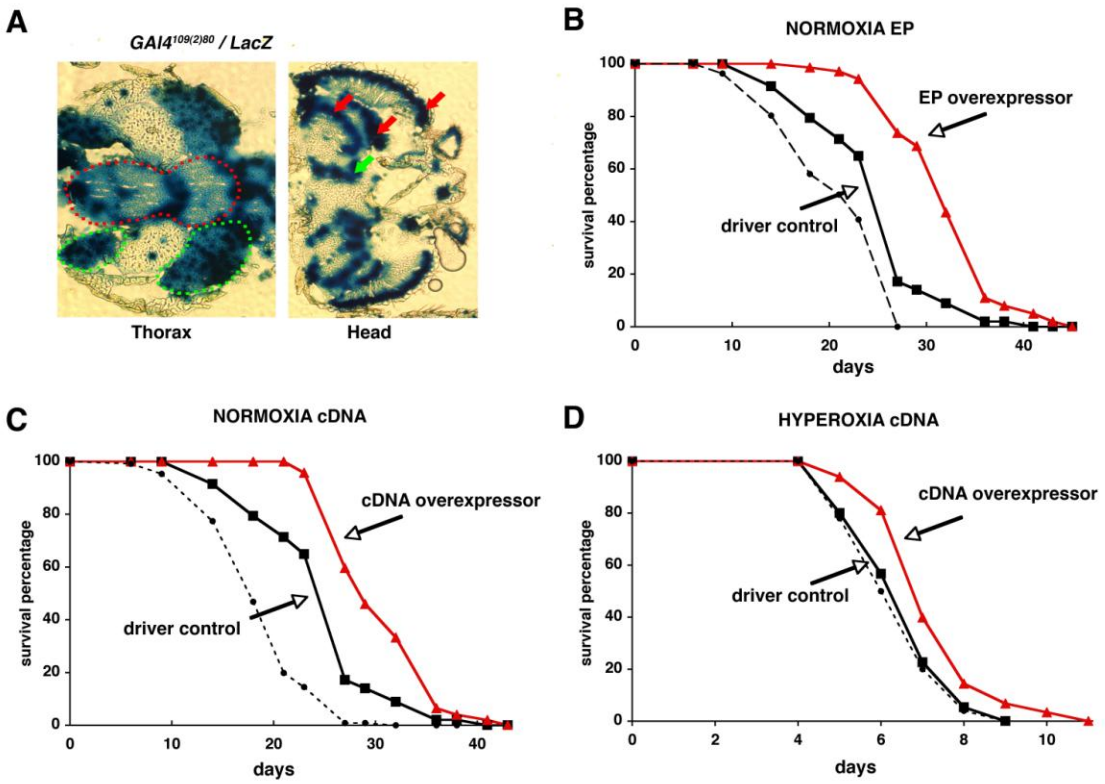


Figure 2: **Protection against hyperoxia and extension of lifespan by overexpression of GLaz using *GAL4*$^{109(2)80}$ as the driver**

A: X-GAL staining of a horizontal cryosection showing the adult expression pattern of the driver, using nuclear-LacZ under UAS control as a reporter. In the thorax, *GAL4*$^{109(2)80}$ is expressed in the two vertical flight muscles (green dotted line), and in cells of the ventral ganglion (red dotted line). In the head, the driver is expressed predominantly in the retina and optic lobes (red arrows), and central brain areas (green arrow). **B:** EP line lifespan extension under normoxia, at 29ºC. *GLaz* EP overexpressors (*w*^{1118}; *GAL4*$^{109(2)80}$/*EP(2)2383*, red curve) live 23% longer ($p<0.001$) than driver control (*w*^{1118}; *GAL4*$^{109(2)80}$/+, black curve). The dotted line represents the EP alone (*w*^{1118}; *EP(2)2383*/+). **C:** *GLaz* cDNA transgenic lifespan extension under normoxia, at 29ºC. *GLaz* cDNA overexpressors (*w*^{1118}; *GAL4*$^{109(2)80}$/*UAS-GLaz*^{2}, red curve) live 23% longer ($p<0.001$) than driver control (*w*^{1118}; *GAL4*$^{109(2)80}$/+, black curve). The dotted line represents the transgene alone (*w*^{1118}; *UAS-GLaz*^{2}/+). **D:** GAL4-dependent lifespan extension under hyperoxia in the transgenic flies. GLaz cDNA overexpressors live 12% longer ($p<0.001$) than the driver control. Each graph represents a trial with at least 100 flies, typical of several repeated experiments.

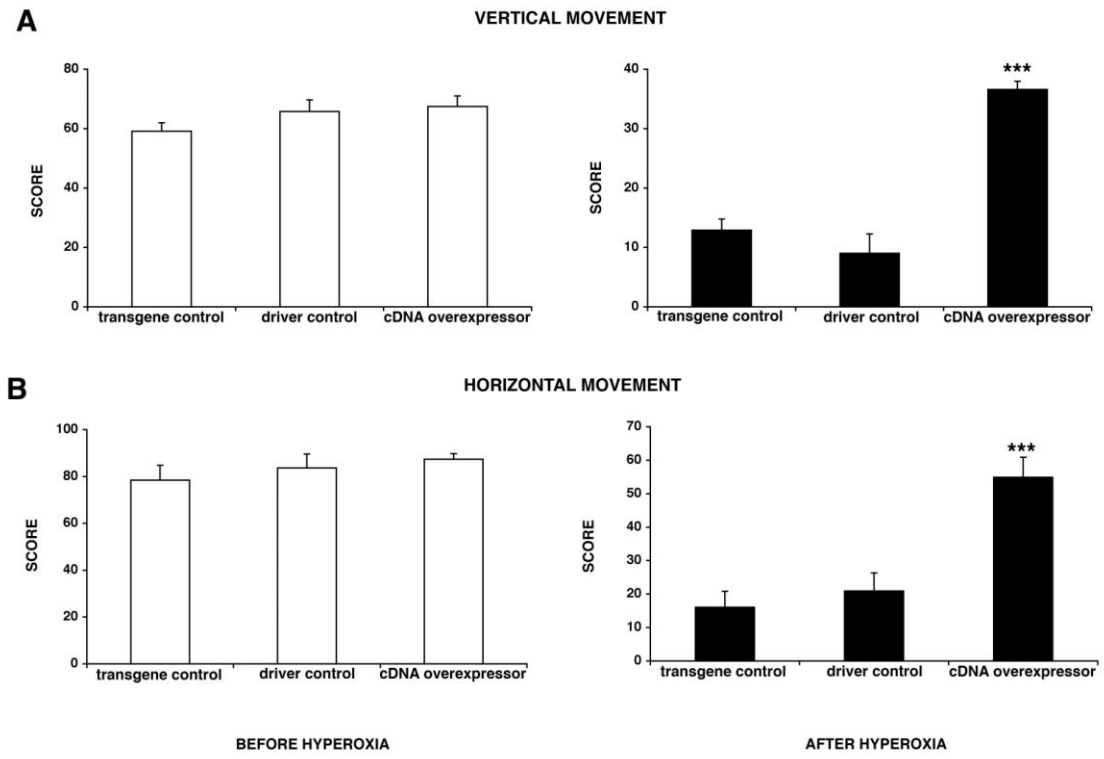


Figure 3: Overexpression of *Glaz* protects against hyperoxia-induced behavioral decline.

A: Assay of vertical locomotion performance before (white bars) and after 4 days under hyperoxia (black bars). After hyperoxia, flies overexpressing GLaz (w^{1118}; $GAL4^{109(2)80}$/*UAS-GLaz2*) climbed significantly better than either control (w^{1118}; $GAL4^{109(2)80}$/+ or w^{1118}; *UAS-GLaz2/+,* Student's t test, $p<0.001$). **B :** Assay of horizontal locomotion performance before (white bars) and after 4 days under

hyperoxia (black bars). Flies overexpressing *GLaz* (*w*^{1118}; *GAL4*$^{109(2)80}$/*UAS-GLaz2*) performed significantly better than controls (*w*^{1118}; *GAL4*$^{109(2)80}$/+ or *w*^{1118}; *UAS-GLaz2/+,* Student's t test, p<0.001).

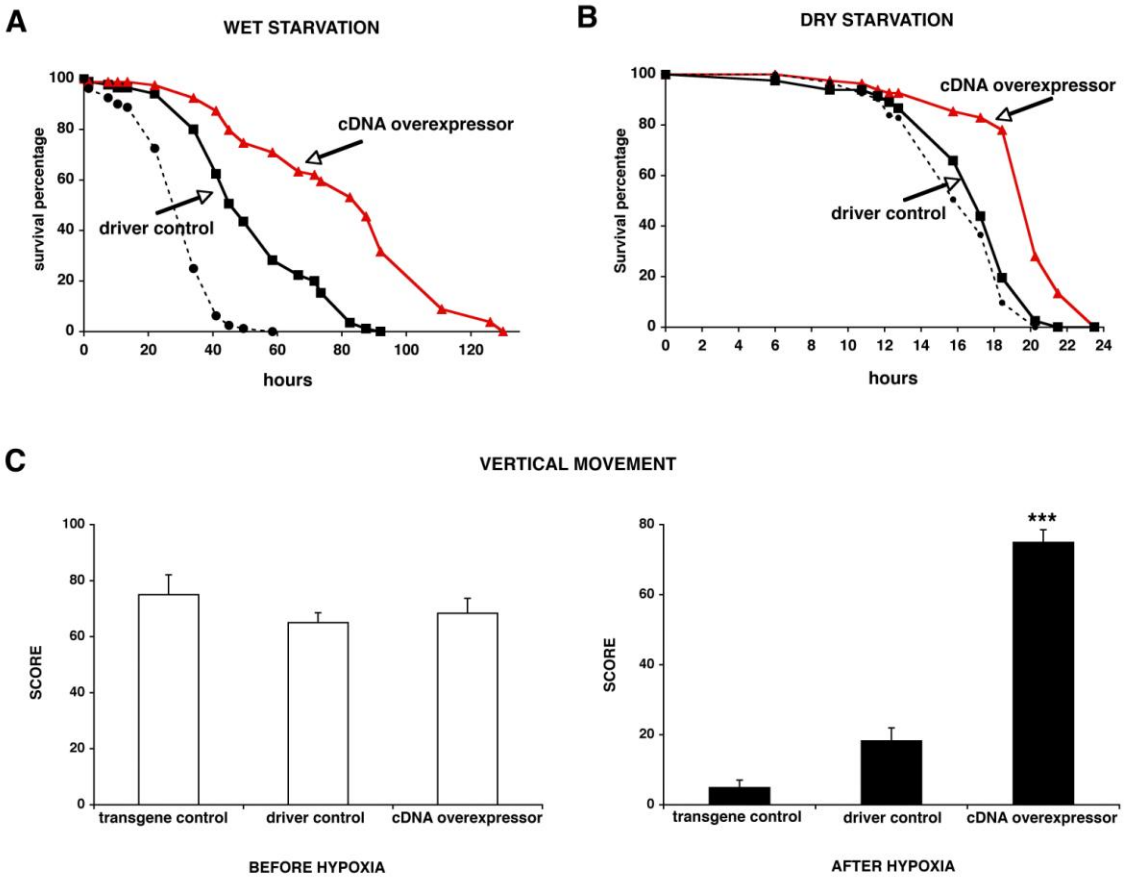


Figure 4: Overexpression of *Glaz* increases resistance to multiple extrinsic stresses

A : Wet starvation. Flies over-expressing GLaz (*w*^{1118}; *GAL4*$^{109(2)80}$/*UAS-GLaz2*, red curve) lived 60% longer than driver controls (*w*^{1118}; *GAL4*$^{109(2)80}$/+, p<0.001). **B :** Dry starvation (desiccation). Flies over-expressing *GLaz* (*w*^{1118}; *GAL4*$^{109(2)80}$/*UAS-GLaz2*) lived 30% longer than driver controls (*w*^{1118}; *GAL4*$^{109(2)80}$/+, p<0.001). Dotted line represent the transgene alone (*w*^{1118}; *UAS-GLaz2/+).* Each graph represents a trial with at least 100 flies, typical of several repeated experiments. **C:** Hypoxia. Assay of vertical locomotion performance after 30 minutes under hypoxia, followed by one hour recovery under normoxia. White bars represent performance before hypoxia exposure, with no significant difference between genotypes. Black bars represent performance after hypoxia exposure and recovery: Flies over-expressing GLaz (*w*^{1118}; GAL4$^{109(2)80}$/*UAS-GLaz2*) climbed significantly better (Student's t test, p<0.001) than either controls (*w*^{1118}; *GAL4*$^{109(2)80}$/+ or *w*^{1118}; *UAS-GLaz2/+)*

Supplementary figures:

Figure S1 : RT-PCR showing a GAL4-dependent increase in *GLaz* transcript using the EP line.

A : Marked increase in the amount of *GLaz* mRNA in w^{1118}; *EP(2)2383/+; da-GAL4/+* compared to the w^{1118}; *da-GAL4/+* and w^{1118}; *EP(2)2383/+* controls , consistent with GAL4 activation of UAS in *EP(2)2383*, enhancing *Glaz* trancription. Amounts of total RNA reverse transcribed were normalized. An RNA control was included to ensure equal loading after PCR (200 bp loading control) according to the Retroscript kit's instructions.

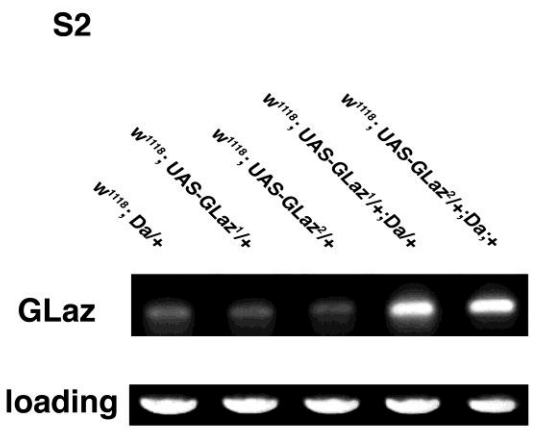
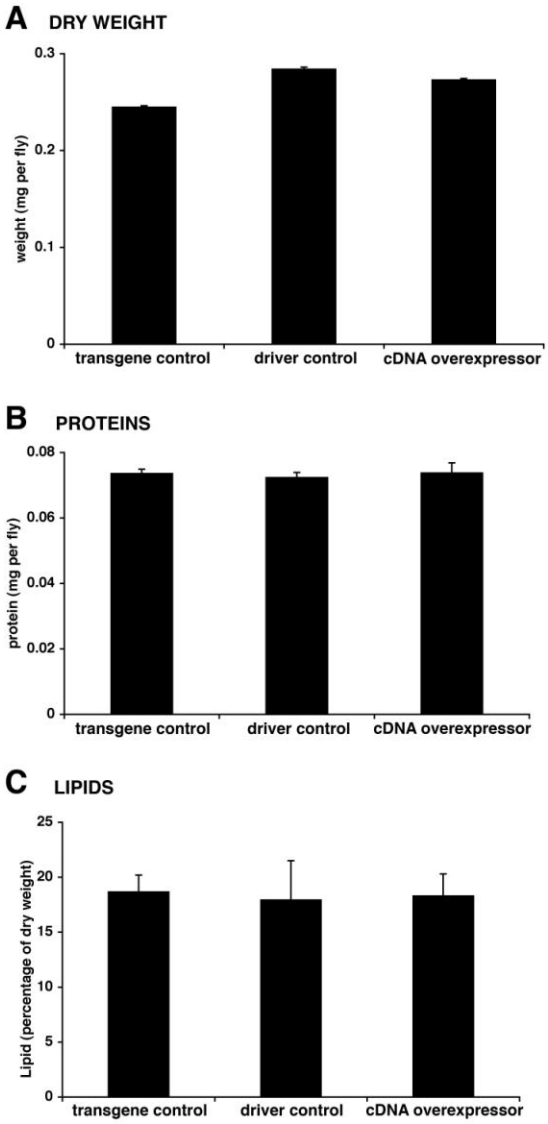


Figure S2: RT-PCR showing a GAL4-dependent increase in *GLaz* transcript using the cDNA transgenic lines.

Transgenic cDNA insertion lines *UAS-GLaz*^{1} and *UAS-GLaz*^{2}. Both *w*^{1118}; *UAS-GLaz*^{1}/+; *da-GAL4*/+ and *w*^{1118}; *UAS-GLaz*^{2}/+; *da-GAL4*/+ display increases in the amount of *GLaz* transcript compared to controls. A 200 bp rp49 RNA was also reverse transcribed and amplified to ensure that the reactions were properly normalized.



S3

Figure S3 : Overexpression of *Glaz* does not alter weight, protein or lipid content.

A : comparison between flies over-expressing Glaz (*w*^{1118}; *GAL4*$^{109(2)80}$/*UAS-GLaz2*) and controls (*w*^{1118}; *GAL4*$^{109(2)80}$/+ or *w*^{1118}; *UAS-GLaz2*/+) in terms of **A:** dry weight (in mg/fly), **B:** protein content (in mg/fly) and **C:** lipid content (percentage of dry weight).

MATERIALS AND METHODS

Drosophila **strains**: *Da-Gal4, 24B-GAL4, GMR-GAL4, PO163-GAL4, Actin-GAL4, D42-GAL4, Elav-GAL4* were all obtained from the Drosophila stock center (Bloomington, IN). *DJ634* was isolated and characterized by L. Seroude in our laboratory (Seroude, Brummel et al. 2002). We thank Lily and Yuh-Nung Jan for providing us with $GAL4^{109(2)80}$. The *Drosophila* strain $white^{1118}$ was used in all control crosses and as the background for generation of transgenic lines. Male flies were used throughout the study. We screened a publicly available collection of insertion lines containing enhancer-promoter transposable elements (Rorth collection of EP lines), out of which *EP(2)2383* was selected for this study.

Lifespans: All crosses were performed at 18ºC to minimize the effects of GAL4 during development. For each lifespan experiment (all at 29ºC), at least 100 2-4 days old males, were separated from females on a Peltier plate maintained at 4ºC to avoid possible negative effects of CO_2 anesthesia on lifespan. 20-30 flies were put in a single vial, transferred every 3-4 days to fresh standard food vials,

and the number of dead flies recorded each time. Survival curves were analyzed using the Graphpad Prism 4 software, yielding p values for a logrank test.

Exposure to hyperoxia: Adult males (2–4 days old) in shell vials (20–30 flies per vial) containing standard food were maintained in a Plexiglas enclosure of 28 x 28 x 24 inches at room temperature (22–24°C). Oxygen (100%) was passed through the box at a constant rate of 300 ml/min.

Starvation: For each lifespan experiment, at least 100 2-4 days old males were separated from females as previously described. 20-30 flies were put in a single vial, transferred every day to fresh vials, and the number of dead flies recorded approximately every 2 hours. For dry starvation, the vials contained 5 g of silica gel (desiccant), while for wet starvation the vials contained 1% agar, providing water throughout the experiment, but no food source. Each experiment was repeated at least twice.

Generation of transgenic flies: an EST carrying the full *GLaz* cDNA (GH09946) was procured from the DGRC (Indiana, US) and partially digested with EcoRI and XhoI. The cDNA fragment was then subcloned into the expression vector pUASt, putting the *GLaz* cDNA 3' of a series of five UAS repeats. The purified pUASt-*GLaz* vector was subsequently injected into w^{1118} embryos, and 2 single-insertion lines were selected. Both mapped to the 2nd chromosome.

XGAL staining: 7-day-old adult males were cryosectioned, fixed for 20 min in 1% glutaraldehyde in PBS (pH 7.2), reacted with 0.1% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and incubated at 37°C for 4 hours. The reaction was stopped by rinsing twice with PBS before mounting in 70% glycerol/PBS.

Dry weight measurement: Flies were raised on standard food for 7 days at room temperature, then desiccated overnight in a vacuum oven at 80°C. Flies were weighed in three groups of 40, and the weights are reported as mg dry weight per fly. Each experiment was repeated at least twice.

Protein content: 40 7-day old males were homogenized in a Potter apparatus, in a detergent buffer (10mM Tris, 2mM EDTA, 150mM NaCl, 0.15% NP40, protease inhibitors). After a 10 minute spin at 13,000 rpm, the supernatant was assayed using the BCA (Pierce, IL) assay according to manufacturer's instructions. Amounts are reported as mg protein per fly. Each experiment was done in triplicate, and repeated at least twice.

Lipid content: 40 7-day old males were desiccated and weighed as previously described. They were then submerged in 5 mL of ether for 24 hours to dissolve lipids. The ether was removed and the remaining tissue desiccated and weighed again. The weight difference corresponds to the lipid fraction, reported as percentage of dry weight. Each experiment was done in triplicate, and repeated at least twice.

Behavioral assays before and after hyperoxia: All assays were performed at room temperature. Vertical locomotion performance was measured by putting 40 flies in a vial, tapping them to the bottom, and measuring the percentage of flies that reached the top third of the vial

after 10 seconds. Horizontal locomotion performance was measured by putting 20 flies in a narrow tube covered in black tape, tapping them to one end, with a fiber-optic light at the other end. After 1 minute, the number of flies having traveled more than 2 inches away from the origin was counted and expressed as a percentage of the total number. All assays were done in triplicate, and the experiment was repeated at least twice.

RT-PCR: Total RNA was extracted from 40 flies of the various genotypes using Trizol reagent (Invitrogen, CA). RNA concentration was measured using a Nanodrop spectrophotometer, and sample concentrations were normalized. The Retroscript kit (Ambion, TX) was used according to the manufacturers instruction, using the Oligo-dT primers provided in the kit. The cDNA was then amplified by PCR using two primers specific for *GLaz*, giving a single 600 bp amplicon (F: 5'-ATGATGAGTGGCCAGCCACTT-3'; R: 5'-GTCGCGGAGCCCCGTAA-3').

Hypoxia paradigm: At room temperature, 7-day old adult males were placed in 100% N_2 for 30 minutes, then brought back to

normoxia and allowed to recover for 20 minutes. All flies were awake by 20 minutes. 1 hour later, vertical locomotion performance was scored, as previously described.

Statistical analyses: All survival curves were analyzed using the Prism4 software (Graphpad), and we report mean lifespan +/- SEM. The *p* value given is the result of a logrank test on the Kaplan-Meier data. For all other assays (histogram charts), error bars represent SEM, and the *p* values are the result of the Student t test.

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Chapter 3

Human ApoD, an apolipoprotein upregulated in Alzheimer's and Parkinson's diseases, extends lifespan and increases stress resistance in *Drosophila*

Apolipoprotein D (ApoD) expression increases in several neurological disorders, and in spinal cord injury. We provide the first report of a physiological role for human ApoD (hApoD): flies overexpressing hApoD are long-lived, and protected against stress conditions associated with aging and neurodegeneration, including hyperoxia, dietary paraquat, and heat stress. We show that the fly ortholog, *GLaz*, is strongly upregulated in response to these extrinsic stresses, and also can protect *in vitro* cultured cells in situations modeling Alzheimer's disease (AD) and Parkinson's disease (PD). In adult flies, hApoD overexpression reduces age-associated lipid peroxide accumulation, suggesting a proximal mechanism of action.

In two accompanying papers (see appendix), Ganfornina et al. present similar results in mice, while Charron et al. show that a plant ortholog of ApoD has similar effects. These data suggest that ApoD and its orthologs play an evolutionarily-conserved role in response to stress, possibly managing or preventing lipid peroxidation.

ApoD is a small, soluble lipid carrier found in most human tissues, but especially in glia of the nervous system (Provost, Weech et al. 1990; Provost, Villeneuve et al. 1991; Weech, Provost et al. 1991; Rassart, Bedirian et al. 2000). The protein is highly conserved among mammals, and close homologs can also be found in plants and bacteria, implying an important basic function. ApoD is elevated in many pathological situations, including Alzheimer;s disease (AD), Parkinson's Disease(PD), stroke, schizophrenia, and bipolar disorder (Terrisse, Poirier et al. 1998; Kalman, McConathy et al. 2000; Thomas, Dean et al. 2001; Sutcliffe and Thomas 2002; Navarro, Del Valle et al. 2003; Thomas, Copolov et al. 2003; Rickhag, Deierborg et al. 2007). In AD, it can be found in amyloid plaques, within the brains of patients (Desai, Ikonomovic et al. 2005). It is upregulated 500-fold at the site of sciatic nerve crush injury in the rat (Boyles, Notterpek et al. 1990; Boyles, Notterpek et al. 1990). *In vitro* evidence indicates that it can carry membrane lipids, such as arachidonic acid and sterols (Morais Cabral, Atkins et al. 1995; Vogt and Skerra 2001; Nasreen, Vogt et al. 2006; Eichinger, Nasreen et al. 2007) and may be involved in clearance and/or repair of damaged membranes, perhaps quenching harmful material released by neurons and glial

cells in response to damage, or recruiting lipids to expanding membranes. In the etiology of many of the disorders in which ApoD is elevated, oxidative stress is thought to play an important part (Andersen 2004; Wallace 2005). For example, in AD, β-amyloid (Aβ42) is known to produce free radicals and hydrogen peroxide, which can in turn damage surrounding cells (Butterfield 2002), causing an accumulation of lipid peroxides and protein carbonylation adducts. Recently, mitochondrial oxidative stress has also been implicated in the genesis of hyperphosphorylated tau, another canonical feature of AD (Melov, Adlard et al. 2007).

In an unbiased screen to identify genes that protect *Drosophila* against hyperoxia (100% O_2), we discovered that transgenic overexpression of a fly ortholog of ApoD, *Glial Lazarillo* (*Glaz*), could protect against a range of extrinsic stressors, and extend lifespan (Walker, Muffat et al. 2006). The relatively high level of homology between hApoD and GLaz led us to propose GLaz as a fly model of ApoD (Gutierrez, Ganfornina et al. 2000; Sanchez, Ganfornina et al. 2003)(see Figure S1). We asked whether this effect of *GLaz* overexpression could provide insight into the role of ApoD upregulation in neurological disorders, since, to date, no correlation

had been established between high ApoD levels and protection against degeneration. In the first part of this report, we demonstrate, in our transgenic system, that human ApoD (hApoD) can itself protect *Drosophila* under stress conditions relevant to pathological processes, and extend lifespan. Overexpression of hApoD also reduces the age-related accumulation of lipid peroxides.

We show that, in adult flies, extrinsic stressors also induce *GLaz* expression, reinforcing the notion that *GLaz*, the fly ortholog of ApoD, is also part of a canonical stress response. Moreover, *GLaz* overexpression in *Drosophila* S2 cell cultures can protect against xxx Aβ42-induced cytotoxicity, and against paraquat, suggesting that the elevation of ApoD in AD or PD may play a role in salvaging neurons under oxidative stress. This study and the accompanying report by Ganfornina et al. in the mouse are the first to look at the targeted effects of overexpressed hApoD on stress resistance and longevity in model organisms. Note that a third accompanying paper by Charron et al. describes a similar role for a plant ortholog of ApoD.

RESULTS

Generation of hApoD transgenic flies.

We generated two independent lines carrying hApoD cDNA under control of GAL4 binding sites (UAS), using a gateway-modified pUASt expression vector. In the line UAS-*hApoD1,* hApoD cDNA is fused with Venus Fluorescent Protein (VFP) cDNA at its C-terminus, while in *UAS-hApoD2*, hApoD cDNA is fused with a C-terminal HA (influenza hemagglutinin) tag. Both insertions map to the 3rd chromosome. In both lines, there is GAL4-dependent increase in the amount of hApoD mRNA (Fig. 1*A*), when driven by the ubiquitous driver *Daughterless-GAL4* (*Da*). This mRNA was not detected in control flies (w^{1118}, not shown), but there was some weak transcription in heterozygous flies lacking the driver. Figure 1*B* displays the green fluorescence (at 485nm) induced by *Da* on *UAS-hApoD1* (lower panel), confirming that the C-terminus fusion allows proper folding of VFP. *Da/+* flies are not fluorescent (data not shown) while *UAS-hApoD1/+* flies have a low background level of fluorescence, consistent with the leaky transcription detected by RT-

PCR. Figure 1*C* shows *Da/UAS-hApoD2* overexpressing the hApoD-HA fusion. Staining with an anti-HA primary antibody, followed by a FITC-conjugated secondary antibody (lower panel) reveals expression of the HA tag in a GAL4-dependent fashion. Although we know from the RT-PCR result that some hApoD-HA is transcribed, no fluorescent signal was observed in *UAS-hApoD2/+* flies (upper panel), probably because of the lower detection sensitivity of this protocol.

hApoD overexpression extends normal lifespan in *Drosophila*.

In humans, hApoD is known to be upregulated with age, possibly in response to accumulated damage (Kalman, McConathy et al. 2000). We used the ubiquitous *Da* tostronglydrive expression of *UAS-hApoD* transgenes in the whole organism. Using the fact that GAL4 expression is reduced at lower temperatures (Duffy 2002), expression was minimized during early development by raising the larvae and pupae at 18°C, on standard food (Lewis 1960). Crosses of w^{1118} to either transgene or driver were used as genetic controls and also raised at 18°C, on standard food. For all genotypes, we raised adult

flies on standard food, at 25°C, and followed their survival . Flies overexpressing hApoD (*Da/UAS-hApoD1*) had 40% longer median lifespan (Fig. 2*A*) than the longest-lived control (*UAS-hApoD1/w*^{1118}). For *UAS-hApoD2*, the median lifespan was increased by 41% (Fig. 2*B*). In each case maximum lifespan was also improved.

Overexpression of hApoD protects *Drosophila* against various extrinsic stresses.

We next exposed the hApoD transgenic flies and their genetic controls to 100% oxygen for their entire adult lives, in the paradigm originally used to isolate *GLaz* as a stress-resistance gene. As previously shown, such an exposure, for normal flies, results in mitochondrial damage consistent with elevated oxidative stress, as well as apoptotic muscle degeneration and death within a week (Walker and Benzer 2004). This treatment can be considered to be a model of accelerated pathological aging. Flies overexpressing *UAS-hApoD1* (*Da/UAS-hApoD1*) throughout adult life lived on average 36% longer than the longest-lived control (*UAS-hApoD1/*+), under hyperoxia (Fig. 3*A*). *Da/UAS-hApoD2* flies lived 25% longer than

UAS-hApoD2/+ flies (Fig. 3*B*) under the same conditions. Maximum lifespans were also improved significantly.

Next, we assessed whether hApoD overexpression increases resistance to dietary paraquat, which is thought to target complex I of the mitochondrial respiratory chain, thereby producing deleterious reactive oxygen species. Paraquat has been used to model PD(Peng, Stevenson et al. 2005). When hApoD was overexpressed in flies exposed to 20mM paraquat (see Methods), there was a 40% longer median survival on paraquat in *Da/UAS-hApoD1* than in their control counterparts (Fig. S2*A*). Similarly *Da/UAS-hApoD2* were 38% longer-lived, on average, than either of their genetic controls (Fig. S2*B*), although maximum lifespans in the population were not affected.

Heat stress was tested by exposing the flies, in standard food vials, to 37°C. *Da/UAS-hApoD1* flies had a median lifespan 35% longer than *UAS-hApoD1/+* controls (Fig. S3*A*), and *Da/UAS-hApoD2* lived on average 40% longer than *UAS-hApoD2/+* controls at 37°C (Fig.S3*)*. In this case, maximum lifespan also increased. All the control fly genotypes were dead within 30 hours, while hApoD overexpressors survived to the 38-hour mark

hApoD overexpression reduces accumulation of lipid peroxides in old flies.

Lipid peroxides are formed when free radicals such as superoxide produced by mitochondria, react with membrane and storage lipids (Halliwell and Chirico 1993). These lipid peroxides alter the normal properties of the membrane, and impair cellular function. Their accumulation is a measure of oxidative stress (Toroser, Orr et al. 2007), and we observed that lipid peroxides normally accumulate in wild-type Canton-S (CS) flies over the course of their life (data not shown). Here we report that hApoD appears to function, at least in part, by constraining lipid peroxides. While control flies display a significantly increased lipid peroxide burden between day 4 and day 40 of adult life, flies overexpressing hApoD1 or hApoD2 clearly accumulate less, maintaining the levels of younger flies (Fig. 4). These data do not address whether the reduction is at the level of generation of lipid peroxides or on their clearance.

Upregulation of the fly ortholog of hApoD, GLaz, by extrinsic stress.

Given the upregulation of hApoD in various diseases involving chronic stress, as in the degeneration seen in stroke, AD or PD, we wondered whether individual stresses would also regulate expression of the fly ortholog in our model, in situations of acute exposure and within a short timeframe.

Flies lacking *GLaz* are particularly sensitive to paraquat treatment (Sanchez, Lopez-Arias et al. 2006), while flies overexpressing *GLaz* have enhanced resistance (JM unpublished data). Paraquat feeding can be viewed as a model of environmentally induced neurodegeneration. We compared, by quantitative real-time PCR (qRT-PCR), the levels of *GLaz* mRNA in flies fed paraquat plus sucrose to flies fed sucrose only, over the course of 12 hours (Fig. 5*A*). Even at the earliest time point of 2 hours, *GLaz* was dramatically upregulated, with a 7-fold induction over its normal level. This transcriptional induction decreased gradually but was still upregulated 3-fold at 12 hours.

We subsequently performed qRT-PCR on *GLaz* mRNA on young wild type flies (CS) exposed to 100% oxygen, compared with flies remaining under normoxic conditions. The transcript abundance

of *GLaz* (Fig. 5*B*) is stronglyregulated by hyperoxia exposure. Remarkably, the *GLaz* transcript signal rapidly increased 12-fold in the first 2 hours, and continued to be elevated 5-fold after 10 hours.

Given that the heat shock response lies downstream of most oxidative stress responses, we also looked at induction of *GLaz* following exposure to high temperature. Flies in standard food vials were placed in a 37°C incubator. The result in Figure 5*C* shows that *GLaz* is, once more, a strong responder, with a 4.5-fold upregulation at 2 hours, followed by a decline.

Drosophila cells are protected from Aβ42 and paraquat toxicity by _GLaz_ overexpression.

As a paradigm in which the beneficial effects of *Glaz* could be monitored more closely, at the cellular level, the effects of transient overexpression of *GLaz* in *Drosophila* S2 cell cultures were examined. We generated constructs allowing expression of HA-tagged *GLaz* under an actin promoter. Transfected cells expressed the HA-tagged protein (Fig. 6*A*), secreting it in the culture medium. As reported by a co-transfected Blue Fluorescent Protein, the

transfection efficiency was 30%. These cells were subsequently placed in serum-free medium to avoid interference by bovine lipoproteins, and exposed to 10mM paraquat for 24 hours. After this oxidative insult, only 35% of cells transfected with a sham plasmid, carrying the actin promoter alone, were still alive, but cells overexpressing *GLaz* were protected, showing no significant cell death attributable to this paraquat exposure (Fig. 6*B*).

Aβ42 overexpression is sufficient to cause AD-like pathology in animal models (Oakley, Cole et al. 2006), and it has been shown that Aβ42 can foster the production of the reactive oxygen species hydrogen peroxide and superoxide, via Fenton chemistry (Butterfield 2002). The oligomeric form of Aβ42 is toxic to neurons and glial cells in culture. It is thought to be the main cytotoxic species *in vivo*, and is found in the CSF of AD patients (Walsh and Selkoe 2007). ApoD is also upregulated in AD and found in the vicinity of plaques (Desai, Ikonomovic et al. 2005). Therefore, we wondered whether *GLaz* could protect from Aβ42 cytotoxicity. Transfected *Drosophila* S2 cells were incubated for 24 hours with 1 μM Aβ42, pre-aggregated into its cytotoxic oligomeric form. Remarkably, we found that overexpression of *GLaz* is indeed protective against Aβ42 cytotoxicity (Fig. 6*C*).

DISCUSSION

These observations are in agreement with a conserved regulation and function of ApoD and its homologs under stressful conditions. The results obtained with heat stress point to the well-recognized overlap in regulation of the oxidative stress response and the heat-shock pathway (Ahn and Thiele 2003). More studies will be needed to establish to what extent the expression of *GLaz* is under control of the heat shock or other stress-inducible pathways. Since many of the pathological situations where ApoD is upregulated in mammals are also thought to involve oxidative stress and induction of the heat-shock pathway, this is an important line of investigation. It is also intriguing to ask whether such direct stress responses can be observed in a mammalian system as well. The accompanying manuscript by Ganfornina et al. indeed shows that, for the mouse, ApoD is upregulated when the mice are exposed to paraquat, following a similar timecourse as the one we observed for *GLaz* in the fly. In a recent study of mouse ApoD in cultured cells, the authors observed induction of mouse ApoD by similar stress conditions (Do Carmo, Levros et al. 2007), along with a thought-provoking

translocation to the nucleus. The plant ortholog (*atTIL*) studied in the accompanying manuscript by Charron et al. is also induced by temperature stress. In a previous study of gene expression changes following exposure of *Drosophila* to hyperoxia (Landis, Abdueva et al. 2004) alterations in *GLaz* expression were not found. However, a critical difference between that study and ours is that we looked for changes following very short exposures to hyperoxia, whereas they examined the gene expression after 7 days under hyperoxia. Note that the levels of induction of *GLaz* observed here are similar to the 7-fold overexpression we induced in flies using the UAS/GAL4 system in a previous study (Walker, Muffat et al. 2006). That overexpression was beneficial to the organism over its entire life. The inductions we observe occur very early in a process that will eventually lead to death, whether by heat or oxidative stress.

In transgenic flies, we observe a remarkable increase in lifespan and multi-stress resistance in human ApoD overexpressors. Independently of the results obtained for *GLaz,* and the homology that rooted our interest, these results clearly indicate a beneficial function of human ApoD, and may explain its upregulation in various disorders. Such an upregulation may limit cumulative processes,

typical of age-related disorders, such as lipid peroxidation, protein carbonylation, or DNA modifications. This may take effect either by accelerating recycling, or by preventing formation of adducts in the first place. Other lipocalins have been identified as direct scavengers of potentially harmful lipophilic molecules (Lechner, Wojnar et al. 2001). The accompanying mouse study also puts lipid peroxide management in the foreground of protective functions performed by ApoD and its homologs. Our results with *Drosophila* S2 cells support the idea that ApoD homologs may also protect cells in acute stress situations, providing a possible beneficial role for chronic ApoD secretion by astrocytes in the brains of patients with AD or PD, and its acute secretion by glia in crushed peripheral nerves. As neurons strive to regenerate damaged neurites, glial cells known to express ApoD could provide local trophic support, or sequester arachidonic acid, thereby tempering inflammation. The non-cell-autonomous aspect is exemplified by our cell culture results, where a majority of cells are protected, whether they express the transgenes or not. In humans, lipoproteins produced outside of the nervous system do not pass the blood-brain barrier, therefore it is likely that glial cells would

be the most important reservoir of ApoD in stressful conditions, and could modulate the levels of this protein in the cerebro-spinal fluid.

Along with the accompanying mouse study by Ganfornina et al., we provide the first evidence that hApoD is more than a neutral bystander, let alone a deleterious actor, in pathological situations, in animals. Remarkably, the accompanying study by Charron et al. with *Arabidopsis* shows that plant orthologs of ApoD also play a role in stress resistance, and may be functionally homologous to the fly and mammalian proteins. Taken together, these three reports strongly suggest that induction of ApoD might be worthy of investigation as a possible therapeutic tool for a range of age-related diseases, including Alzheimer's disease.

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doctoral school at the University of Paris VI. D.W.W. was supported by a Development Grant from the Muscular Dystrophy Association. This research was funded by grants to S.B. from the McKnight Endowment Fund for Neuroscience, the Ellison Medical Foundation, the National Institutes of Health, and the National Science Foundation. JM and DWW are forever indebted to their mentor, SB, and will miss him dearly.

MATERIALS AND METHODS

***Drosophila* strains**: *Da-Gal4 (Da)* was obtained from the Drosophila stock center (Bloomington, IN). The *Drosophila* strain *white*^{1118} was used in all control crosses, as the background for generation of transgenic lines, and to outcross (10 times) the driver line *Da*. CS flies were used as wild type for the *GLaz* mRNA induction experiments. Male flies were used throughout the study.

Histology: Whole flies were frozen in OCT medium and horizontal serial 10μm cryosections prepared. For VFP detection, the sections were immediately mounted in DAPI-glycerol, and imaged using a

Zeiss Axioimager with Apotome (10X). For HA detection, the sections were fixed in 4% paraformaldehyde, washed in PBS, incubated with a rabbit anti-HA primary (1:100) and stained with FITC-conjugated anti-rabbit (1:250). Control sections were stained with the secondary antibody alone.

Lifespans: All crosses, including controls, were performed at 18ºC to minimize the effects of GAL4 during development. After eclosion, the adults were maintained at 25ºC. For each lifespan experiment, at least 100 2 day-old males were separated from females while anesthetized in 100% N_2. 20-30 flies were put in a single vial containing standard food(Lewis 1960), transferred every 3-4 days to a fresh vial, and the number of dead flies recorded. Survival curves were analyzed using the Graphpad Prism 4 software, yielding p-values from a logrank test.

Exposure to hyperoxia: 2-day old adult males, 20–30 flies per vial containing standard food, were maintained in a Plexiglas enclosure of 28 x 28 x 24 inches at room temperature (22–24°C). Oxygen (100%) was passed through the box at a constant rate of 300 ml/min.

Paraquat feeding: For each experiment, at least 100 2-day old flies, at 20-30 flies per vial, were transferred daily to fresh medium, and the number of dead flies recorded approximately every 2 hours. For controls, the vials contained 5 mL of 1% agar, 5% sucrose, while paraquat vials contained the same food with 20mM paraquat added.

Generation of transgenic flies: hApoD cDNA was kindly provided by Elizabeth Thomas and J. Gregor Sutcliffe (U.C. San Diego) in a pcDNA3.1 vector, and amplified by PCR. The cDNA was then subcloned into an entry vector for the Gateway system (Invitrogen, CA) using the TOPO system. Following the manufacturer's instructions, the cDNA fragments were recombined into a modified pUASt vector (Murphy Lab, Carnegie Institution), putting the hApoD cDNA (without STOP codon) 3' of UAS repeats, in frame with the HA tag motif, or with the VFP sequence. The purified vectors were sequence-verified, injected into w^{1118} embryos, and w^{+} transformants selected and mapped.

qRT-PCR and RT-PCR: Total RNA was extracted from 40 wild-type flies (CS) using Trizol reagent (Invitrogen, CA). RNA concentration was measured with a Nanodrop spectrophotometer, and sample concentrations normalized. The Retroscript kit (Ambion, TX) was used according to manufacturer's instructions, using Oligo-dT primers. For RT-PCR of hApoD and actin, primers were designed and the products were run on a 0.8% agarose gel. For qRT-PCR of *GLaz,* total cDNA from CS flies exposed to the various test conditions was amplified using a set of primers specific for *GLaz*, giving 100 bp amplicon spanning the first intron-exon boundary of the gene. The controls were rp49 and TBP, used to normalize the cDNA amounts. SYBR green mix (Biorad, CA) was used to monitor DNA amount during the PCR for 40 cycles, using an iQ5 thermal cycler (Biorad). A fluorescence threshold was chosen in the linear portion of the amplification reaction, and the cycle number (Ct) needed to cross that threshold was recorded for each sample. Analysis of *GLaz* and control gene values stressed and in normal conditions yielded the $\Delta\Delta$Ct values. Using the Pfaffl method (Pfaffl 2001), with pre-calculated primer efficiencies, the $\Delta\Delta$Ct values were converted to mRNA fold changes. Melting curves were established for all

conditions to check that no abnormal secondary structures were forming during the PCR reactions.

Cell culture: S2 cells were maintained as adherent cultures at room temperature in Schneider's medium supplemented with 10% FBS. For viability experiments, cells were transfected with either a sham pAHW plasmid (Murphy lab) containing the actin promoter and gateway cassette alone, or pAHW in which the *Glaz* cDNA had been subcloned using gateway recombination, fusing the protein with a C-terminal 3xHA tag. Transfection was performed with the Fugene HD (Roche, PA) reagent in a 9:2 ratio, per the manufacturer's instructions. All plasmids were co-transfected with pAc-BFP (blue fluorescent protein) to assess transfection efficiency. Cells were allowed to recover and express for 48 hours, before paraquat or Aβ42 were added to serum free medium at respective final concentrations of 10mM and 1uM. Viability was assessed using the live/dead assay (Invitrogen, CA), per the manufacturer's instruction. Live cells appeared green under a fluorescence microscope (Zeiss axioimager Z1), while dead cells appeared red. Experiments were done in 96 well plates, in experimental triplicates. Counting was done on aliquots of

each experimental well by an experimenter blind to the assay conditions. Results were expressed as percentage of cells alive after transfection and treatment.

Lipid peroxidation assay: we used the LPO-586 assay (Oxis research, Portland, OR) per the manufacturer's instruction. Each sample consisted of 50 flies of each genotype and age, in triplicates. Flies were homogenized in PBS with added butylated hydroxy-toluene (preventing additional lipid peroxidation during sample preparation). After centrifuging insoluble components (10 min, 13000g), the clear supernatant was incubated with the assay reagents, and a blank was prepared incubating the same sample with acetonitrile instead. Again the incubated samples were spun down, and triplicate 250uL-aliquots of each sample were loaded onto a 96-well plate. Absorbance was read at 586nm. Absorbance values of the blanks were subtracted from each sample value. A standard provided by the manufacturer was used to generate a standard curve and give a nanomolar concentration of malonyldialedhyde (MDA) and 4-hydroxynonenal (4-HNE) corresponding to the absorbances. All amounts were subsequently normalized to protein concentrations of

each sample established using a Bradford assay (Biorad, CA), yielding values in nanomoles per milligram of total protein.

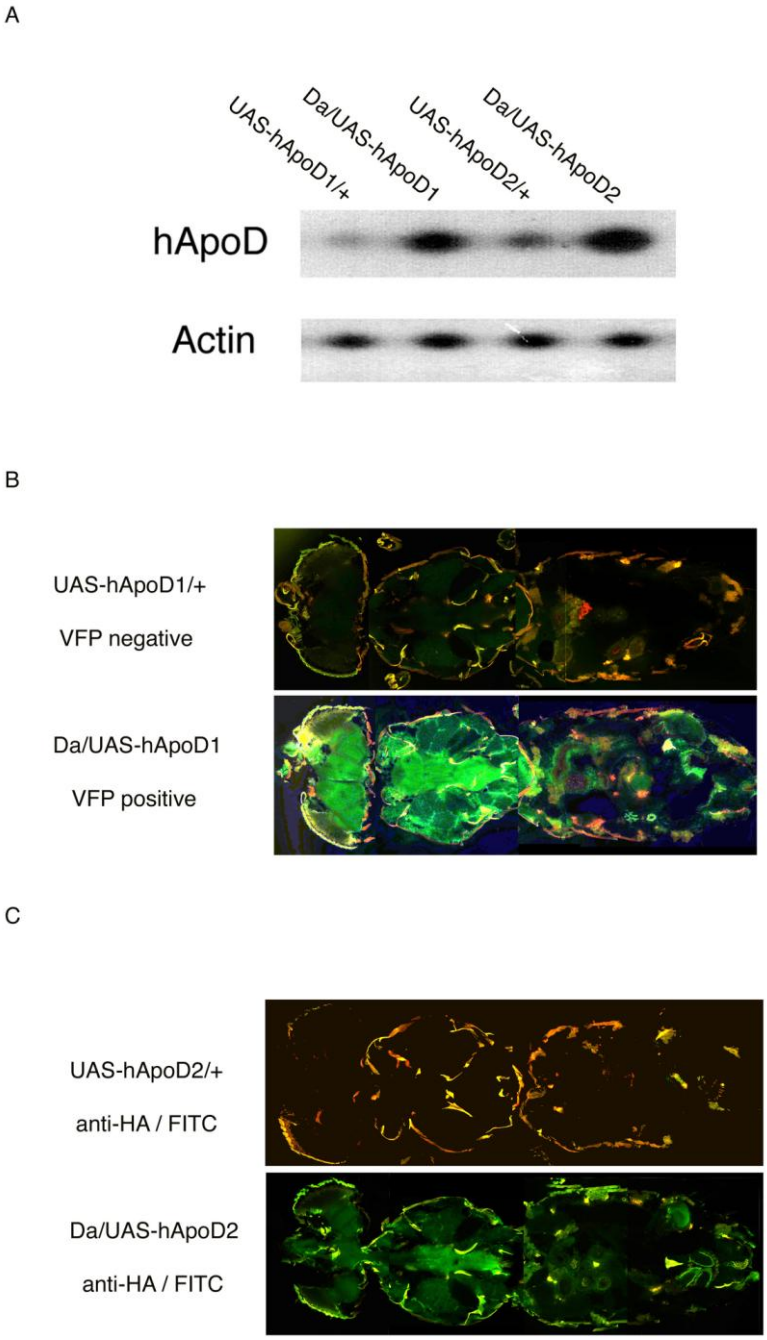

Figure 1: Generation of two human ApoD (hApoD) transgenic lines, using the UAS/GAL4 system.

A: RT-PCR shows hApoD driven by *Da* in each strain compared to control strains. **B:** Horizontal cryosection through *Da/UAS-hApoD1*, shows strong, ubiquitous green VFP signal (the red color is due to cuticle autofluorescence). Normal signal is seen in *UAS-hApoD1/+* control flies. **C:** Horizontal cryosection through *Da/UAS-hApoD2* which carries the HA-tag. Green fluorescence (FITC) reveals the presence of hApoD, stained by HA antibody, in most tissues (less intense than the VFP marker).

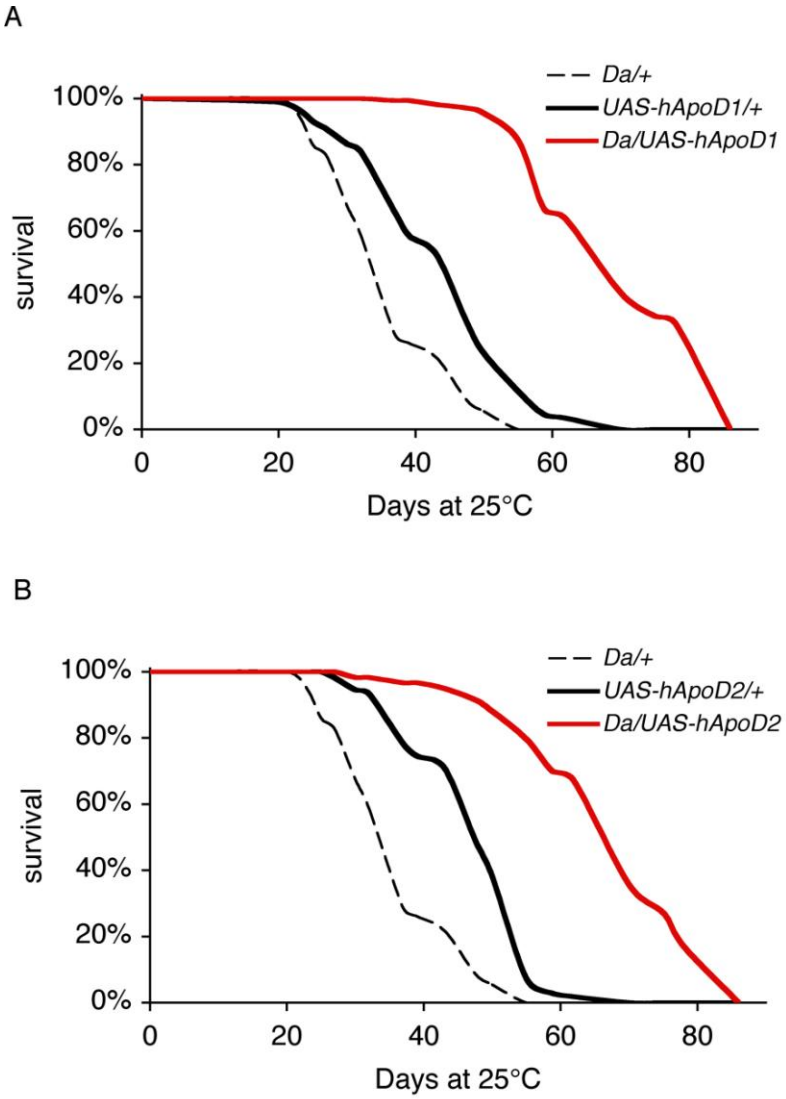

Figure 2: Overexpression of human ApoD extends normal lifespan of *Drosophila*.

Survival was recorded on standard food, in normoxia, at 25°C. **A:** *Da/UAS-hApoD1* flies had a 40% longer median lifespan than *UAS-*

hApoD1/+ controls ($p<0.001$). Maximum lifespan was also improved by 20%. **B:** *Da/UAS-hApoD2* flies had a 41% longer median lifespan than *UAS-hApoD2/+* controls ($p<0.001$). Maximum lifespan was also improved by 19%.

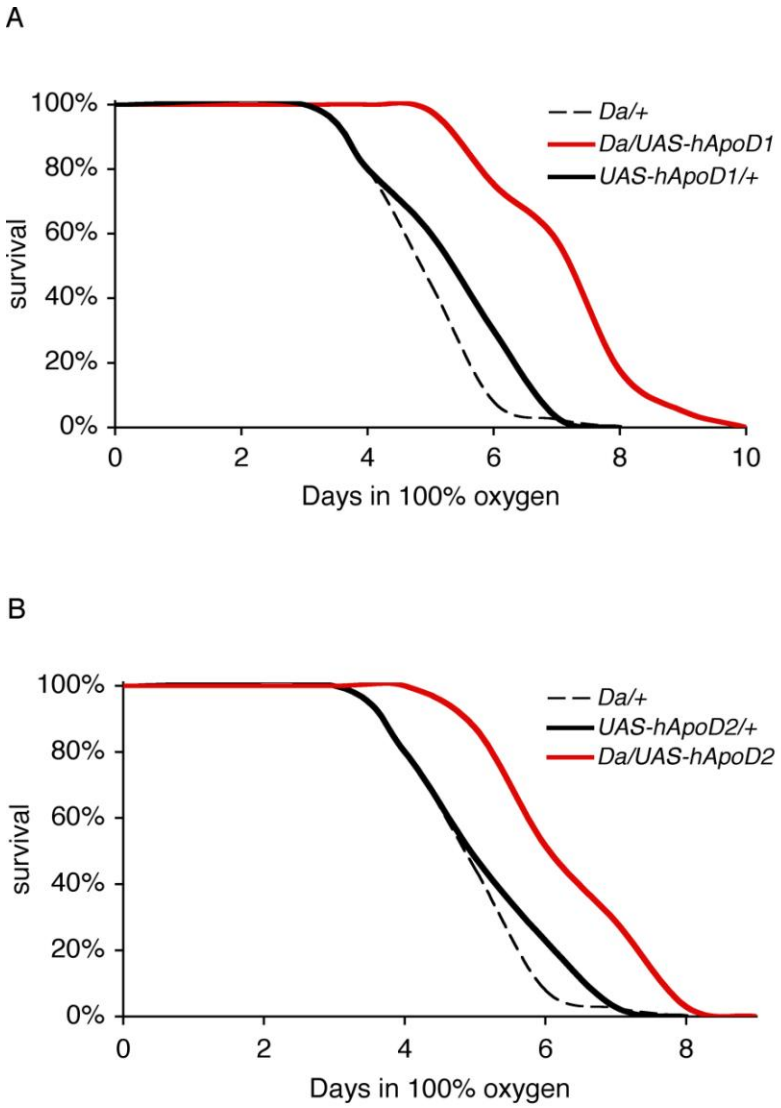

Figure 3: Overexpression of hApoD protects against hyperoxia, in *Drosophila*.

Survival was recorded on standard food, in 100% oxygen, at 25°C. **A:** *Da/UAS-hApoD1* flies had a 25% longer median lifespan than *UAS-*

hApoD1/+ controls ($p<0.001$). Maximum survival was also improved by 20%. **B:** *Da/UAS-hApoD2* flies had a 20% longer median survival than *UAS-hApoD2/+* controls ($p<0.001$). Maximum survival was also improved by 15%.

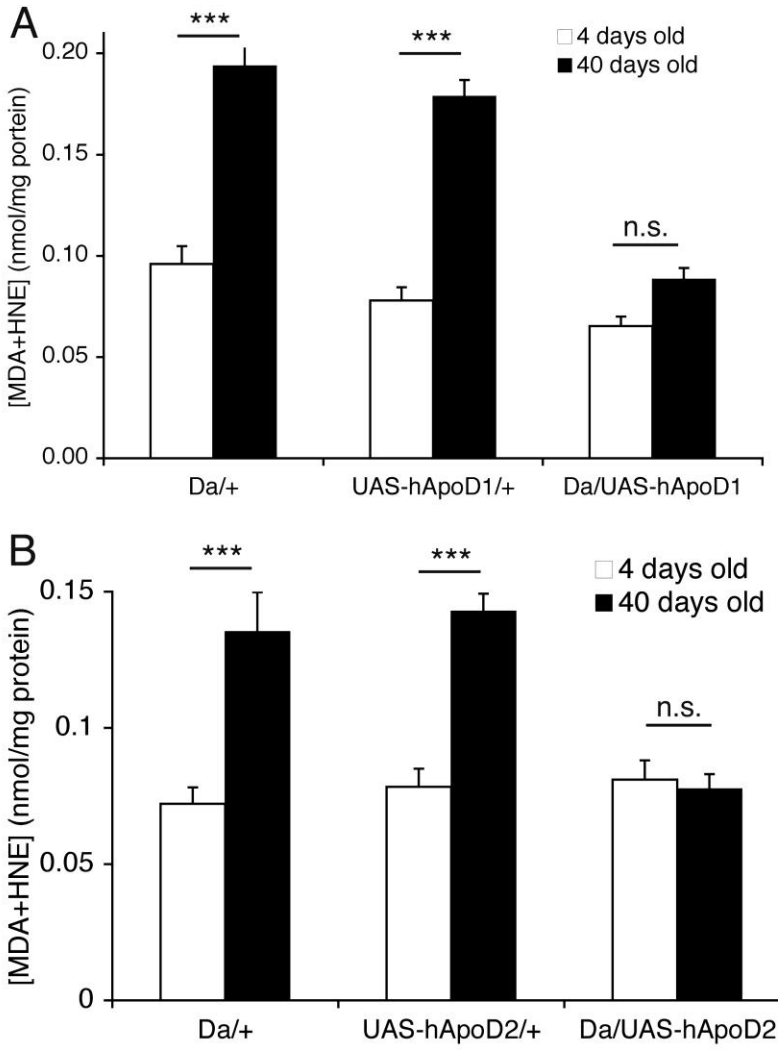

Figure 4: hApoD overexpression reduces lipid peroxide accumulation in old *Drosophila*.

Adult flies were maintained on standard food at 25°C. **A:** *Da/UAS-hApoD1* flies did not display any significant increase in lipid peroxide burden at 40 days of age, while control flies saw this burden increase

2-fold during the same period (*** p<0.005, t-test). **B:** similar results were found with the *Da/UAS-hApoD2* flies, compared to controls (*** p<0.005, t-test).

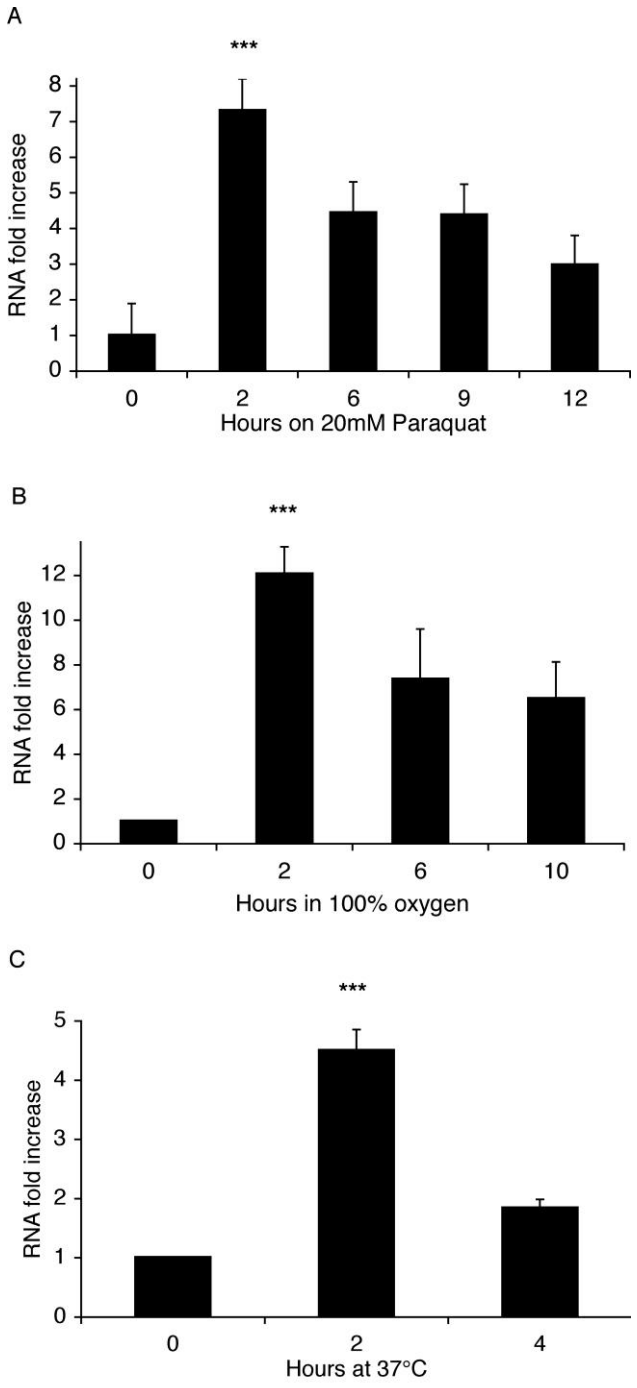

Figure 5: Induction of *GLaz* mRNA in wild-type flies (CS), by extrinsic stress.

Data represent fold increase in *GLaz* mRNA levels, compared to flies in control conditions, assessed by qRT-PCR (t=0, normoxia, standard food). **A**: On 5% sucrose/20mM paraquat, demonstrating a dramatic upregulation of *GLaz* mRNA. Induction peaked at 7-fold after 2 hours and decreased back to 3-fold after 12 hours. (control: t=0, no paraquat). **B**: In 100% oxygen, on standard fly food. *GLaz* mRNA induction peaked at 12-fold after 2 hours and stabilized at 6-fold after 10 hours. **C:** At 37°C, on standard fly food. *GLaz* mRNA was induced 4.5-fold at 2 hours, and remained upregulated 2-fold after 4 hours. During the time frame of each experiment, no death occurred. All values were normalized using housekeeping genes *rp49* and *TBP* as references (see methods). Values are averages of 3 independent experiments +/- SEM. A Student t-test performed on the Ct values yielded p-values (*** $p<0.005$).

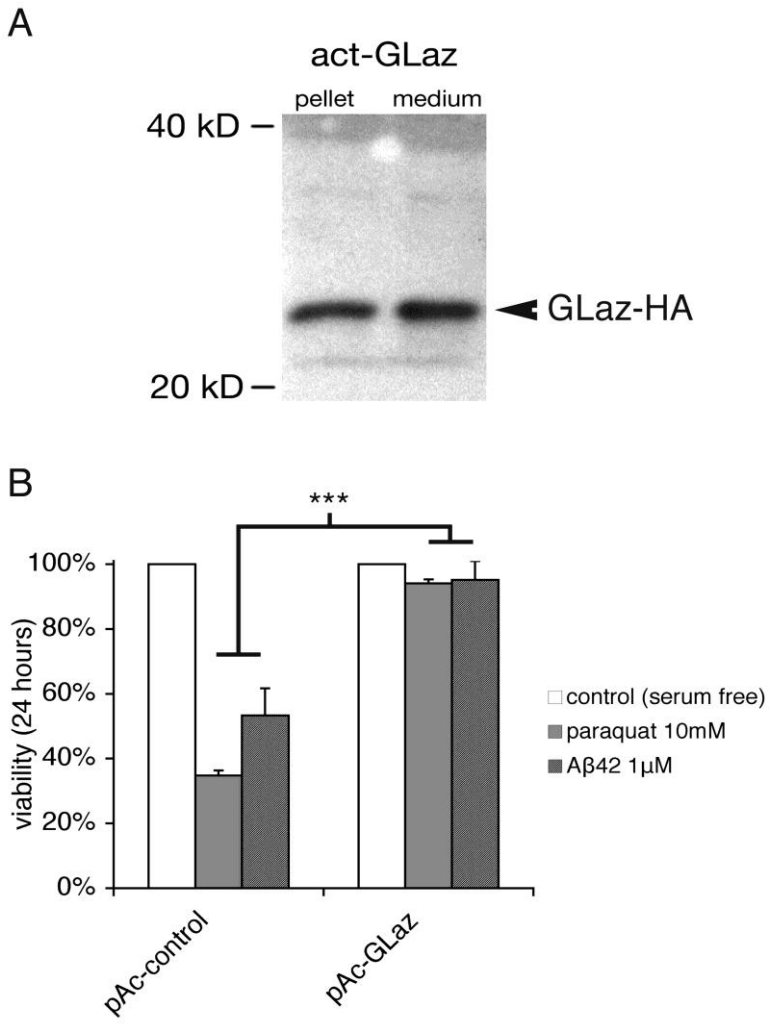

Figure 6: *GLaz* protects *Drosophila* S2 cells against death induced by paraquat and Aβ42.

A: anti-HA western blot showing that GLaz-HA is expressed under the actin promoter in *Drosophila* S2 cells and secreted into the medium. **B:** S2 cells were transfected for 48 hours, then exposed to 10mM paraquat or 1uM Aβ42 for 24 hours, and assayed for viability.

Cells transfected with a control plamid (actin promoter alone, pAc) showed low survival when exposed to paraquat or Aβ42 (respectively 35% and 53%). *GLaz* overexpression by pAc-*GLaz* was sufficient to protect the cells against the stress of both paraquat and Aβ42 (respectively 94% and 95% viability after 24 hours, *** $p<0.005$, t-test).

SUPPLEMENTARY FIGURES:

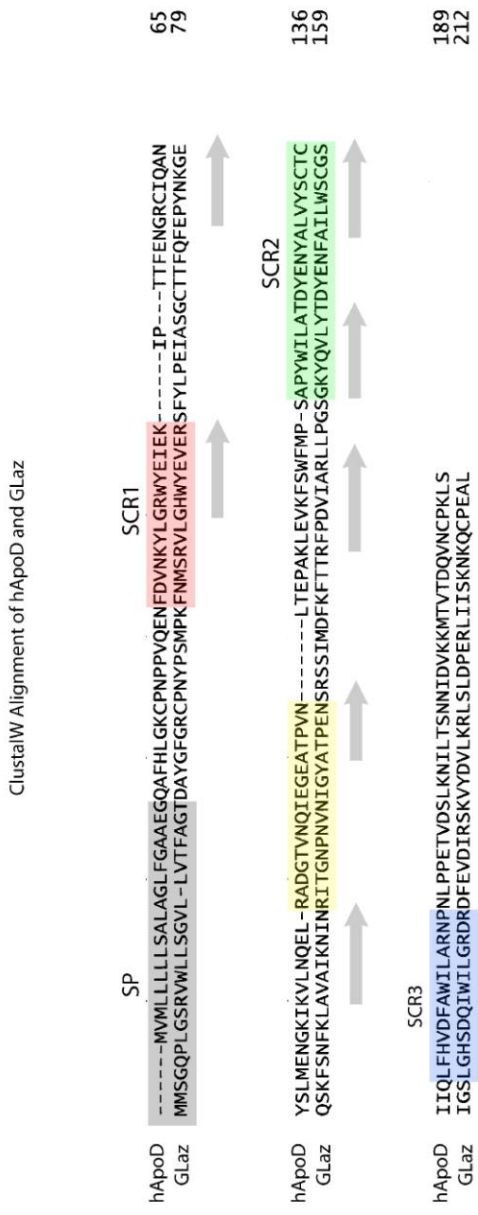

Figure S1: Alignment of human ApoD and GLaz.

ClustalW alignment of human Apolipoprotein D and the *Drosophila* ortholog GLaz. Both proteins are lipocalins, lipid carriers characterized by a chalice structure formed by 8 β-sheets (shown here as grey arrows). These proteins show significant homology, especially in 3 Structurally Conserved Regions (SCR1, 2 and 3), considered to be the backbone of the structure. In this case, the overall identity reaches 35%, while the SCRs (red, green and blue boxes) all show more than 50% identity. When amino-acid properties are taken into account, the SCRs display more than 70% similarity. The grey box represents the signal peptides (SP), while the yellow box denotes another region of significant homology not found in other lipocalins.

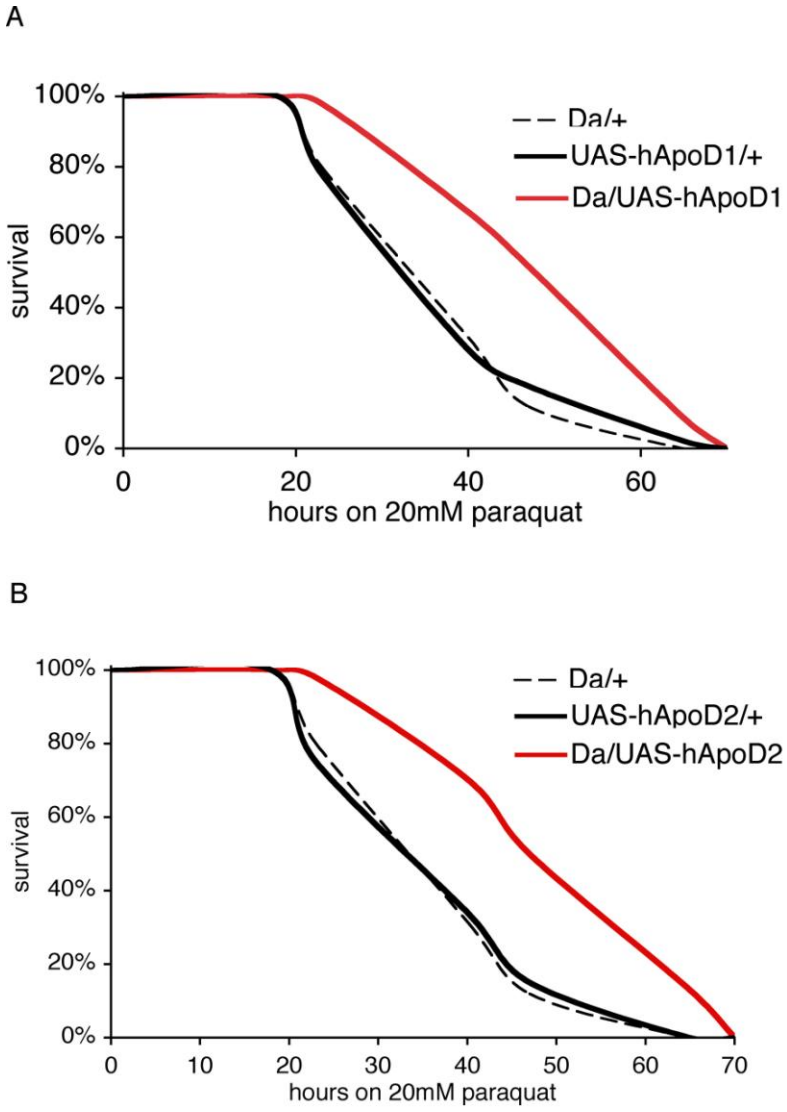

Figure S2: overexpression of human ApoD enhances resistance to paraquat, in *Drosophila*.

A: Effect of overexpressing *UAS-hApoD1,* using *Da* as a ubiquitous driver. Survival was recorded on 5% sucrose, 1% agar, at 25°C, with 20mM paraquat added. *Da/UAS-hApoD1* flies had a 40% longer

median survival than *UAS-hApoD1/+* controls ($p<0.001$). **B:** *Da/UAS-hApoD2* flies had a 38% longer median survival than *UAS-hApoD2/+* controls ($p<0.001$).

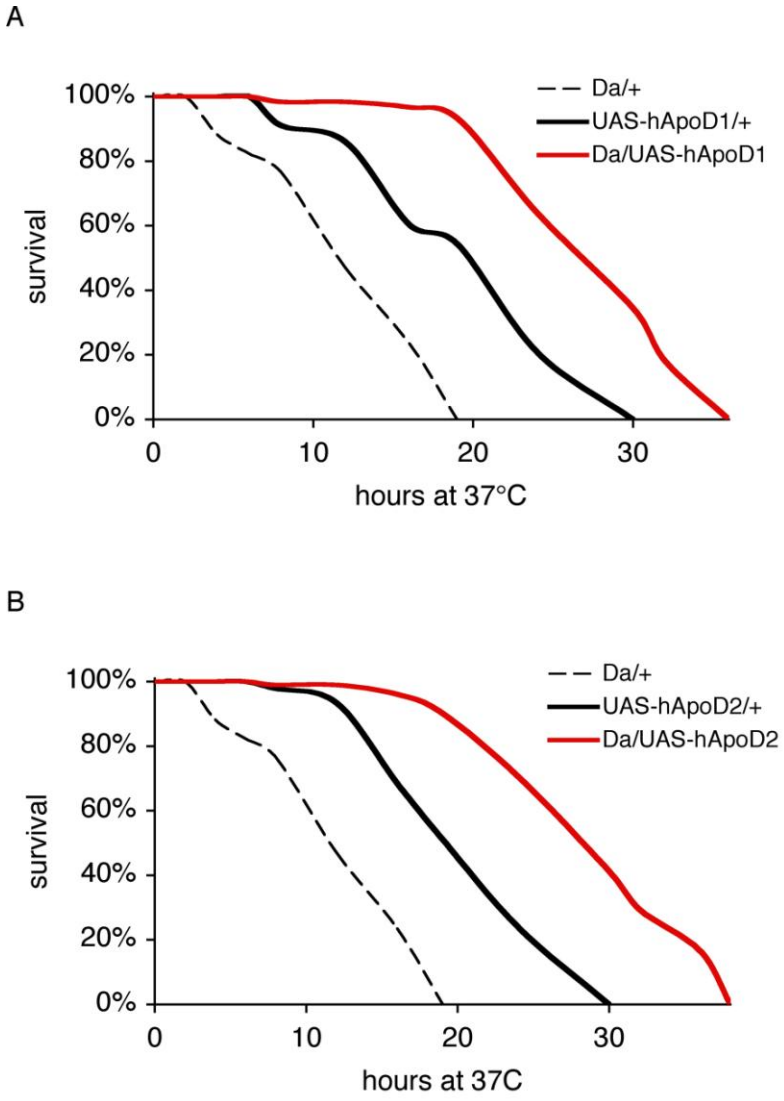

Figure S3: Overexpression of hApoD increases resistance to heat, in *Drosophila*

Survival was recorded on normal food, at 37°C. **A:** *Da/UAS-hApoD1* flies had a 35% longer median survival than *UAS-hApoD1/+* controls ($p<0.001$). Maximum survival was also improved by 25% ($p<0.001$).

B: *Da/UAS-hApoD2* flies had a 40% longer median survival than *UAS-hApoD2/+* controls ($p<0.001$). Maximum survival was also improved by 26%.

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Chapter 4

Control of metabolic homeostasis by stress signaling is mediated by the Lipocalin NLaz

Metabolic homeostasis in metazoans is regulated by endocrine interactions between different insulin target tissues. Adipose-secreted factors, such as adipokines and lipocalins, are emerging as central regulators of peripheral insulin sensitivity and have been implicated in metabolic diseases, most prominently type II diabetes. Insulin resistance, a hallmark of type II diabetes, is caused in part by adipose-specific activation of stress and inflammatory signaling pathways, such as Jun-N-terminal Kinase (JNK) signaling. Chronic activation of JNK in adipose tissue of obese animals induces systemic insulin resistance, but the physiological role of this interaction between JNK and Insulin signaling remains unclear. JNK also causes systemic insulin resistance in invertebrates, promoting stress tolerance and extending lifespan, and suggesting that the interaction between JNK and insulin signaling is an evolutionarily conserved adaptive response of the organism that allows managing energy resources under stress conditions. Here we show that JNK signaling is required for metabolic homeostasis in flies, influencing nutrient storage, glucose

homeostasis and starvation tolerance. We find that this function is mediated by the *Drosophila* Lipocalin family member Neural Lazarillo (NLaz), a homologue of vertebrate Apolipoprotein D (ApoD) and Retinol Binding Protein 4 (RBP4), which is transcriptionally regulated by JNK signaling. Nlaz is required for JNK-mediated stress and starvation tolerance, and its over-expression represses growth, promotes stress tolerance and extends lifespan, phenotypes that are consistent with reduced insulin signaling activity. Accordingly, we find elevated levels of Insulin signaling activity in NLaz mutant flies. Our results indicate that the regulation of NLaz by JNK signaling is central to the metabolic adaptation of the organism to stress.

Introduction

System-wide coordination of cellular energy consumption and storage is crucial to maintain metabolic homeostasis in multicellular organisms. It is becoming increasingly apparent that endocrine mechanisms that are required for this coordination impact the long-

term health of adult animals and significantly influence lifespan and environmental stress tolerance (Kloting and Bluher 2005; Kahn, Hull et al. 2006; Russell and Kahn 2007). Insulin/IGF signaling (IIS) is central to this regulation, as loss of Insulin signaling activity impairs metabolic homeostasis, but induces stress tolerance and increases lifespan in a variety of model organisms (Kloting and Bluher 2005; Kahn, Hull et al. 2006; Russell and Kahn 2007). Interestingly, environmental stress and cellular damage can systemically repress IIS activity, suggesting the existence of adaptive response mechanisms by which metazoans manage energy resources in times of need (Tatar and Yin 2001; Wang, Bohmann et al. 2005; Baumeister, Schaffitzel et al. 2006; Niedernhofer, Garinis et al. 2006; van der Pluijm, Garinis et al. 2007). The mechanism(s) and mediators of this endocrine regulatory system are only beginning to be understood (Kloting and Bluher 2005; Hotamisligil 2006; Kahn, Hull et al. 2006; Russell and Kahn 2007).

Studies in flies and worms have recently identified the stress-responsive Jun-N-terminal Kinase (JNK) signaling pathway as an important component of such an adaptive metabolic response to stress. JNK activation, which can be induced by a variety of

environmental stressors, including oxidative stress, represses IIS activity, extending lifespan but limiting growth (Oh, Mukhopadhyay et al. 2005; Wang, Bohmann et al. 2005). Interestingly, similar effects of JNK signaling are also observed in mammals, in which it represses Insulin signal transduction by various mechanisms, including an inhibitory phosphorylation of Ser-307 of the insulin receptor substrate, as well as activation of the transcription factor FoxO (Aguirre, Uchida et al. 2000; Essers, Weijzen et al. 2004). This inhibition contributes to Insulin resistance and the metabolic syndrome in obese mice (Hirosumi, Tuncman et al. 2002), suggesting that chronic inflammatory processes (which result in activation of JNK signaling) are central to the etiology of metabolic diseases in obese individuals (Hotamisligil 2006).

Endocrine interactions between Insulin-producing and various Insulin-responsive tissues are likely to coordinate the adaptive metabolic response described above (Kloting and Bluher 2005; Kahn, Hull et al. 2006; Russell and Kahn 2007). JNK-mediated activation of Foxo in Insulin Producing Cells (IPCs) of flies, for example, represses the expression of insulin-like peptide 2 (dilp2), regulating growth and longevity (Wang et al., 2005). At the same time, Foxo activation in the

fatbody results in lifespan extension, presumably by an endocrine mechanism that feeds back to IPCs (Giannakou, Goss et al. 2004; Hwangbo, Gersham et al. 2004).

Adipose tissue is increasingly being recognized as an important regulator of metabolic homeostasis. It secretes a variety of so-called adipokines, including the inflammatory cytokine TNF-alpha (Hotamisligil 2006). TNF-alpha activates JNK signaling, contributing to JNK-mediated insulin resistance in mouse models for obesity (Hirosumi, Tuncman et al. 2002; Hotamisligil 2006). While the chronic inhibition of insulin signaling by adipose-derived TNFalpha thus has deleterious effects in obese individuals, it is likely that such endocrine interactions have evolved to govern metabolic homeostasis systemically in an adaptive manner (Hotamisligil 2006). Supporting this view, adipose tissue is an important regulator of lifespan in worms, flies, and mice, and it is emerging that systemic inhibition of Insulin signaling by adipose-derived factors is involved in this effect (Kloting and Bluher 2005; Kahn, Hull et al. 2006; Russell and Kahn 2007).

An endocrine role for adipose tissue in metabolic regulation has further been demonstrated in mice with adipose-specific deletion of

the glucose transporter GLUT4, in which secretion of the Lipocalin family member RBP4 from fat cells induces insulin resistance throughout the organism (Abel, Peroni et al. 2001; Yang, Graham et al. 2005). Such an endocrine system is expected to be adaptive, since it preserves glucose for only the most essential functions during starvation or environmental stress. At the same time, mis-regulation of this system is likely to contribute to metabolic diseases like type II diabetes. Accordingly, increased serum levels of RBP4 are found in obese and diabetic individuals (Graham, Yang et al. 2006), and polymorphisms in the *rbp4* locus are associated with type II diabetes (Munkhtulga, Nakayama et al. 2006).

The Lipocalins are a large family of secreted proteins that bind small hydrophobic ligands (Flower 1996)(Åkerström et al., 2006). Lipocalin family members are characterized by a low sequence similarity (reflecting diversification of biological functions), but a highly conserved tertiary protein structure and similar arrangement of exons and introns in the coding sequence of their genes (Sanchez et al., 2006b; Sanchez, Ganfornina et al. 2003). Recent studies implicate various Lipocalins as regulators of systemic insulin action and of stress responses (Yang, Graham et al. 2005; van Dam and Hu 2007;

Yan, Yang et al. 2007; Ganfornina, Do Carmo et al. 2008; Muffat, Walker et al. 2008). The *Drosophila* genome contains three Lipocalin genes: *NLaz*, *GLaz*, and *karl*. Recent studies have identified an important role for GLaz in metabolic regulation, stress tolerance and lifespan control (Sanchez, Lopez-Arias et al. 2006; Walker, Muffat et al. 2006). While the function of NLaz remains unclear, *in situ* hybridization in *Drosophila* embryos suggest that it is expressed in a subset of neuronal cells, and, interestingly, in the developing fat body (Sanchez, Ganfornina et al. 2000).

Here, we show that *NLaz* transcription is induced by JNK signaling and in response to oxidative stress, influencing metabolic homeostasis in the fly. Importantly, NLaz induces stress and starvation tolerance downstream of JNK signaling, and negatively regulates Insulin signaling, disrupting glucose homeostasis, repressing growth, and extending lifespan. Our results thus indicate that NLaz is part of a stress response mechanism in flies that adjusts metabolism and growth in response to environmental insults.

Results

JNK signaling is required for metabolic homeostasis in flies.

Based on the ability of JNK signaling to antagonize IIS activity in flies and worms (Oh, Mukhopadhyay et al. 2005, Wang, 2005 #957; Wang, Bohmann et al. 2005), and on the starvation tolerance of flies with increased JNK signaling activity (Wang et al., 2003), we hypothesized that this pathway plays a role in regulating metabolic homeostasis under physiological conditions. To start characterizing such a role, we analyzed the maintenance of nutrient stores under starvation conditions in wild-type flies and in flies mutant for the JNK activating Kinase Hemipterous (JNKK/Hep). Interestingly, males hemizygous for the *hep* loss-of-function allele hep^{1} exhibited significantly reduced energy stores (lipids and carbohydrates) in *ad libitum* conditions compared to wild-type control flies, suggesting impaired metabolic homeostasis in these animals (Figure 1 A-C, Figure S1). Accordingly, we found that in hep^{1} mutants, nutrient stores were rapidly depleted upon starvation. Interestingly, hep^{1} mutants also exhibited an accelerated and increased gluconeogenic response to starvation (Figure 1 D), measured by phosphoenolpyruvat carboxykinase (PEPCK) expression (Zinke, Kirchner et al. 1999), supporting the idea that JNK signaling mutants suffer a rapid decline in available free sugars upon starvation.

Consistent with this view, *hep*^{1} hemizygotes are significantly more sensitive to starvation than wild-type controls (Figure 1 E). Similarly, flies in which JNK signaling was repressed by ubiquitous over-expression of a dsRNA against the *Drosophila* JNK Basket (Bsk) were sensitive to starvation, confirming a loss of metabolic homeostasis in JNK loss-of-function conditions (Figure 1 F).

Interestingly, these findings recapitulate similar observations in IIS gain-of-function conditions, which lead to a decrease in steady-state metabolic stores and starvation sensitivity, as well as IIS loss-of-function conditions, which show the opposite results (Bohni, Riesgo-Escovar et al. 1999; Clancy, Gems et al. 2001; Oldham, Stocker et al. 2002; Rulifson, Kim et al. 2002; Broughton, Piper et al. 2005). The effects of JNK signaling on metabolism thus support the perceived antagonism between JNK signaling and IIS in the regulation of energy homeostasis (Oh, Mukhopadhyay et al. 2005, Wang, 2005 #957; Wang, Bohmann et al. 2005). Since we had previously found that JNK represses *dilp2* transcription in IPCs in response to oxidative stress (Wang et al., 2005 and Hull-Thompson et al., in preparation), we tested whether the starvation sensitivity in *hep*^{1} mutants correlates with elevated *dilp2* transcription. Surprisingly, we

found no difference in *dilp2* (nor *dilp3* or *dilp5*) transcript levels in *hep*^{1} mutants compared to wild-type controls under *ad libitum* conditions, and no changes in *dilp2* transcription in response to starvation (Figure S1B and data not shown; *dilp2* transcript levels are insensitive to nutritional conditions, see (Gershman, Puig et al. 2007)). These results suggest that, while JNK regulates *dilp2* transcription to regulate systemic responses to stress, other targets of JNK might be mediating the control of metabolic homeostasis. Since mammalian JNK acts in adipose tissue to induce Insulin resistance, we focused on the fatbody as a potential site of action for JNK in flies.

To identify potential mediators of JNK-induced metabolic changes, we tested the transcriptional response of a number of candidate genes to JNK activation. We focused on the putative adipokines and secreted regulators of Insulin signaling, dALS, IMP-L2, GLaz, Karl, and NLaz, since these molecules and their mammalian homologues have been implicated in systemically governing metabolic homeostasis. Activation of JNK was achieved by over-expression of a constitutively active Hep (Hep^{act}) in larvae using the TARGET system, which allows heat-inducible expression of UAS-linked transgenes (McGuire, Le et al. 2003). We expressed Hep^{act} ubiquitously (using

the ubiquitous driver T80-Gal4), or specifically in the fatbody (using the fatbody driver ppl-Gal4, (Zinke, Kirchner et al. 1999)), for a short period of time, increasing the likelihood of observing direct transcriptional effects of increased JNK activity. Transcript levels of potential JNK target genes were then assessed by real-time RT-PCR (Figure 2 and Figure S1C).

Among the tested molecules, we found that transcription of the Lipocalin NLaz was potently induced in the fatbody in response to JNK activation, within a timeframe that resembles the induction of *puc*, a *bona fide* JNK signaling target gene (Figure 2). Another Lipocalin, *Karl*, was also induced by JNK signaling, albeit to a lesser extent (Figure S1C).

NLaz acts downtream of JNK to maintain metabolic homeostasis.

These results, and the known effects of the NLaz homologue Rbp4 in mice, suggested that NLaz or Karl might act downstream of JNK signaling to regulate metabolic homeostasis. To test this hypothesis, we measured carbohydrate and lipid levels in homozygous NLaz mutant flies (using the knock-out allele $NLaz^{NW5}$, derived from the $NLaz^{SceI}$ allele (Rong, Titen et al. 2002)) and isogenic controls. Similar to hep^{1} mutants, *NLaz* mutants exhibited reduced stores and

rapid starvation-induced decline of glucose, trehalose, glycogen, and triglyceride levels (Figure 3A–C and Figure S1D). Likewise, NLaz mutants showed an accelerated gluconeogenic response (induction of PEPCK, (Zinke, Kirchner et al. 1999)) and were sensitive to starvation (Figure 3D, E).

Since NLaz is expressed in the fatbody (Sanchez, Ganfornina et al. 2000), we tested whether NLaz or Karl over-expression in the fatbody would be sufficient to protect the organism from starvation sensitivity. Indeed, we found that expression of NLaz using ppl-Gal4 promotes starvation tolerance (Figure 3 F). Expression of Karl, on the other hand, did not protect against starvation (Figure S2A).

These results suggested that JNK-mediated induction of NLaz in the fatbody regulates metabolic homeostasis. Supporting this view, we found that fatbody expression of NLaz was sufficient to restore starvation tolerance and Glucose and Triglyceride levels in *hep*^{1} mutants (Figure 4). Since NLaz is secreted (see Sanchez et al., 2000, and Figure S6), it is impossible to rule out that NLaz induction in other tissues also contributes to metabolic homeostasis. Indeed, when expressed in pericardial cells and hemocytes of flies (using the

dorothy-Gal4 driver, dot-Gal4 (Kimbrell, Hice et al. 2002)), NLaz also protects against starvation (Figure S2B).

NLaz promotes stress tolerance.

JNK signaling promotes oxidative stress tolerance of flies and worms. Similarly, reduced IIS activity also leads to stress tolerance, and the known crosstalk between these two pathways indicates that metabolic and stress responses are tightly linked, allowing organisms to balance protective and growth responses in accordance with available resources (Kloting and Bluher 2005; Kahn, Hull et al. 2006; Russell and Kahn 2007). Based on these studies and on our findings described above, we reasoned that NLaz-mediated metabolic changes can influence stress tolerance of flies. To test this hypothesis, we first assessed whether NLaz transcription would be induced in response to oxidative stress, and found that NLaz expression is indeed elevated in flies exposed to the reactive-oxygen inducing compound Paraquat (resembling the induction of *puc;* Figure 5 A). We further tested stress sensitivity of NLaz mutants, and found that these flies exhibit increased sensitivity to Paraquat compared to wild-type control flies (Figure 5 B). Over-expression of NLaz both ubiquitously and in the fatbody, on the other hand, confers resistance

to Paraquat as well as hyperoxic conditions, supporting a protective role for NLaz in the fly (Figure 5C and Figure S3). To test whether this function of NLaz was specific for oxidative stress, we assessed the sensitivity of NLaz over-expressing flies to infection with Enterococcus faecalis. E. faecalis infection is lethal to flies, and JNK promotes resistance against it (Libert, Chao et al. 2008). Interestingly, NLaz over-expression in hemocytes (which mediate immune responses in flies; (Agaisse, Petersen et al. 2003; Lemaitre and Hoffmann 2007)) was not sufficient to induce infection tolerance, suggesting that NLaz acts specifically to protect against oxidative stress (Figure S3C). Karl expression in hemocytes, on the other hand, is both sufficient and required for infection tolerance, suggesting that these two Lipocalins mediate specific stress responses downstream of JNK signaling (Figure S3D). Further studies will be needed to confirm a role for Karl downstream of JNK in this context.

JNK activity in the fatbody confers NLaz-dependent stress tolerance.

Stress protection by JNK signaling has been observed in *puc*^{E69} mutants, which exhibit elevated JNK signaling throughout the organism (Wang et al., 2003), but also in flies over-expressing

JNKK/Hep in neuronal tissue exclusively. This suggests that secreted molecules promote stress tolerance downstream of JNK signaling (Oh, Mukhopadhyay et al. 2005, Wang, 2005 #957; Wang, Bohmann et al. 2005). One endocrine mechanism by which JNK activity promotes stress tolerance is downregulation of *dilp2* expression in IPCs (Wang et al., 2005, Hull-Thompson et al., in preparation). Interestingly, however, increased stress tolerance can also be observed when JNKK/Hep is specifically over-expressed in the fatbody (Figure 5 D). To test whether NLaz induction might mediate the endocrine effects of fatbody-specific JNK activation, we assessed stress tolerance of NLaz mutant flies in which JNKK/Hep was over-expressed in the fatbody. Remarkably, we found that lack of NLaz completely abolished the ability of JNKK/Hep expression to promote stress tolerance (Figure 5 D). These results indicate that Nlaz induction is an integral component of the JNK-mediated adaptation to environmental stress in *Drosophila*.

NLaz promotes longevity.

Our results thus support a model in which Nlaz is required downstream of JNK signaling to promote metabolic homeostasis and stress tolerance. Since elevated JNK signaling is associated with

increased longevity in the fly, we tested the effect of NLaz on lifespan. Consistent with our other findings, flies lacking NLaz function are short-lived relative to isogenic controls (Figure 5E), while over-expression of NLaz with a ubiquitous driver increases lifespan (Figure 5F and Figure S5). These results further support the view that the effects of NLaz on metabolic homeostasis and stress tolerance are an important adaptive mechanism to preserve energy resources and optimize the fitness of the organism.

NLaz represses growth and affects hemolymph glucose levels.

Interestingly, the phenotypes we observed in flies over-expressing Nlaz (starvation tolerance, stress resistance and extended lifespan) are also phenotypes associated with reduced IIS activity in the fly (Clancy, Gems et al. 2001; Rulifson, Kim et al. 2002; Broughton, Piper et al. 2005), suggesting that the effects of NLaz might be mediated by inhibition of IIS.

To test this notion, we asked whether NLaz would affect other IIS-regulated processes in the fly. Indeed, we found elevated membrane localization of the reporter for PI3K activity, GFP-PH, in fatbody cells of NLaz mutant flies, suggesting that Insulin signaling is increased in these cells in the absence of NLaz (Figure 6 A-C; PI3K activity is

indicative of Insulin signaling activity in flies and GFP-PH is widely used as a reliable reporter for this activity (Britton, Lockwood et al. 2002)). Further supporting a role for NLaz in repressing Insulin signaling, increased expression of NLaz in the fatbody also leads to elevated hemolymph glucose levels in third-instar larvae (Figure 6 D), a phenotype that is associated with reduced IIS activity and that is reminiscent of defects in Glucose homeostasis observed in Insulin resistant vertebrates (Rulifson, Kim et al. 2002). In addition, we found that over-expression of NLaz could not further promote starvation tolerance of heterozygotes for the *chico* loss-of-function allele $chico^{1}$ (Figure 6E; Chico is the *Drosophila* homologue of Insulin receptor substrates (Bohni, Riesgo-Escovar et al. 1999; Clancy, Gems et al. 2001)). Furthermore, over-expression of NLaz in the fatbody reduces growth of the animal (measured as fresh weight as well as wing size) in a heterozygous NLaz mutant background (Figure 6 F-H), and NLaz over-expression induces the expression of the Foxo target gene *l(2)efl* in larvae (Figure S4A). Supporting the view that NLaz acts upstream of IIS in metabolic regulation, we did not observe changes in *NLaz* expression in $chico^{1}$ heterozygous animals (Figure S4B).

Taken together, these results support the notion that NLaz represses Insulin signaling systemically. Interestingly, however, transcript levels of the three major insulin-like peptides dilp2, dilp3, and dilp5, were unaffected by NLaz loss-or gain-of-function conditions, and by over-expression of Hep in the fatbody (Figure S4C, D), indicating that the repression of IIS by NLaz is not mediated by regulation of *dilp* transcription, but by downstream events that promote insulin resistance.

Discussion

Our findings support a role for JNK – mediated NLaz induction in the fatbody as a central part of an adaptive endocrine system that coordinates metabolism in response to environmental stress (Figure 6). Recent studies have highlighted the role of adipose-derived endocrine factors in such adaptive responses (Hotamisligil 2006; Russell and Kahn 2007). For example, over-expression of the transcription factor dFoxo in the *Drosophila* fatbody leads to lifespan extension and stress tolerance, presumably mediated by systemic

repression of IIS (Giannakou, Goss et al. 2004; Hwangbo, Gersham et al. 2004). Similar effects of adipose-specific activation of Foxo have been observed in *C.elegans* and mice (Bluher, Kahn et al. 2003; Libina, Berman et al. 2003). Additionally, amino acid deprivation of the *Drosophila* fat body leads to marked decreases in PI3K activity in wing imaginal discs and in the epidermis (Colombani, Raisin et al. 2003). While it is unclear what factors mediate this systemic repression of IIS by adipose-specific Foxo activity, our studies implicate NLaz as a potential candidate.

Interestingly, our studies indicate that JNK-mediated repression of IIS in flies is not only mediated by its function in IPCs (where it represses *dilp2* transcription; Wang et al., 2005; Hull-Thompson et al., in preparation), but that JNK activity in adipose tissue separately regulates Insulin resistance through NLaz induction. This dual function of JNK is intriguing, as it indicates that adaptive regulation of metabolism requires coordinated control of both Insulin-like peptide production and Insulin sensitivity of Insulin target tissues. How the relative contribution of these effects regulates the organism's metabolic homeostasis, stress resistance and lifespan, is an intriguing question that will require further investigation.

Recent reports, and our results presented here further indicate that Lipocalins have a conserved role in the control of stress tolerance and metabolic homeostasis. Vertebrate Lipocalins are important modulators of insulin action in mammals, and recent studies suggest a protective role of these molecules (Yang, Graham et al. 2005; Yan, Yang et al. 2007; Ganfornina, Do Carmo et al. 2008; Muffat, Walker et al. 2008). Our finding that NLaz promotes stress and starvation tolerance downstream of JNK signaling by repressing IIS activity, predicts that similar functions are conserved in mammalian homologues and has important implications for potential therapeutic targeting of these molecules. Interestingly, our results suggest functional specificity between different Lipocalins. While NLaz and GLaz both regulate stress sensitivity, only NLaz was found to be regulated by JNK signaling. Regulation of Karl, on the other hand, does not influence oxidative stress resistance or starvation tolerance, but promotes resistance against infection. Further investigation of this intriguing diversification of Lipocalin function promises to provide important insight into the systemic regulation of adaptation to environmental challenges.

In humans, dysregulation of Lipocalins has been correlated with obesity, insulin resistance, and type II diabetes (Yang, Graham et al. 2005; van Dam and Hu 2007; Wang, Lam et al. 2007). The cause for this mis-regulation remains unclear, however. Our results implicate the evolutionarily conserved JNK signaling pathway, which is activated chronically in obese conditions, as a possible cause. The finding that mammalian lipocalin-2, which impairs insulin action, is induced by the JNK activator TNFalpha, is especially intriquing (Yan et al., 2007). Additional studies in vertebrates, as well as in the *Drosophila* model, are needed to provide further insight into the physiological role of Lipocalins, their regulation by stress signaling, as well as their interaction with Insulin signaling. Such insight promises to provide a deeper understanding of the coordination of metabolic adaptation in metazoans as well as of the etiology of diabetes and other metabolic diseases.

Materials and Methods

Fly lines and handling

Fly lines were as follows: OreR and Da-Gal4 from Bloomington stock center; hep1/FM6, gift from S. Noselli; ppl-Gal4, (Zinke, Kirchner et al. 1999), gift from Michael Pankratz; dot-Gal4, gift from W.X. Li; UAS-Hep, gift from M. Mlodzik; UAS-bsk^{RNAi}, from Vienna *Drosophila* RNAi Center (Transformant ID 34138).

Generation of the NLaz deletion strain (NLaz^{SceI}) is described in (Rong, Titen et al. 2002). Isogenic knock-out and control lines were generated by outcrossing NLaz^{SceI} knock-out flies into a w^{1118}-CS_{10} wild type strain. Sister lines containing either the wild type allele of NLaz (line NLaz^{CNW14}) or the mutant allele (line NLaz^{NW5}) were selected by PCR and subsequent SceI restriction digest. The line w^{1118}-CS_{10} is a 10 generations outcross of w^{1118} into the CS background.

P-element mediated transformation of w^{1118} mutant flies was used to generate pUASt-NLaz. The full-length NLaz cDNA was amplified using PCR (as annotated in flybase), and it was inserted into pUASt

via ligation into EcoRI and XbaI sites. Three different independent insertion lines of pUASt-NLaz were used in our experiments, producing identical results. Flies were fed a cornmeal and molasses based diet, and were reared at 25°C. For each experiment, care was taken to ensure flies developed at an equal larval density.

Starvation tolerance and nutrient measurement

Starvation experiments were performed by placing flies in empty vials or vials with water-soaked filters, as indicated in figure legends. For glucose, trehalose, glycogen, and triglyceride measurements, cohorts of 10 male flies were weighed prior to homogenization in 100 µL homogenization buffer (0.01M KH_2PO_4, 1 mM EDTA, pH 7.4). Homogenates were spun for 2 min. at 3,000 rpm, and the supernatant was collected. 10µL of homogenate was used in each of the following assays: ***Glucose***: Homogenate was pipetted into 500 µL glucose reagent (Glucose (HK) Assay Kit, Sigma). Samples were incubated at room temperature for 15 min. and absorbance was measured at 340 nm versus deionized water. ***Trehalose***: 0.5 µL Trehalase from porcine kidney (Sigma) was added to homogenate.

After 1 hour incubation at 37°C, glucose was measured as above, and the concentration of glucose prior to trehalase digestion was subtracted. ***Glycogen***: 10 µL of starch assay reagent (Starch assay kit, Sigma) was mixed with homogenate. Sam ples were shaken at 60°C for 15 minutes. 10 µL of sample was used in glucose assay already described. Absorbance of glucose prior to digestion with starch assay reagent was subtracted from final absorbance.

Triglyceride assay

Homogenate was added to 500 µL of activated triglyceride reagent (Liquicolor Triglycerides, Stanbio) and reaction was incubated at room temperature for 10 minutes. The absorbance was measured at 500 nm relative to activated triglyceride reagent. All metabolic measurements were normalized to fly weight.

Paraquat and hyperoxia treatments

Flies were starved for 3-6 hours prior to re-feeding with 25 mM or 50 mM paraquat (Methyl Viologen, Sigma) in 5% sucrose. Paraquat

solution was administered on soaked filters. Flies were kept in the dark once re-fed.

For hyperoxia, 20–30 2-day old adult males, were maintained in vials containing standard food within a Plexiglas enclosure of 28 x 28 x 24 inches at room temperature (22–24°C). Oxygen (100%) was passed through the box at a constant rate of 300 ml/min. Survival was assayed daily.

Lifespan analysis

Flies were collected within 24 hr of eclosion and were separated by sex at 2-3 days of age in groups of 20. They were raised at 25ºC under a 12hr:12hr light cycle and transferred to fresh food vials every 2-3 days.

To ensure identical genetic backgrounds*, Da-Gal4* was out-crossed 10 times to w^{1118}. *Similarly,* UAS-*NLaz*^{4} and UAS-*NLaz*^{8} were generated and maintained in the w^{1118} background, minimizing effects of genetic background variations and hybrid vigor in the progeny of the crosses studied. Remaining effects of genetic background and hybrid vigor can be assessed in lifespan differences between the

progenies of the two control crosses (UAS-NLaz x w^{1118} and Da-GAL4 x w^{1118}). Any GAL4-dependent modification of lifespan observed in the test cross (Da-GAL4 x UAS-NLaz), is thus due to the over-expression of the transgene.

Real Time PCR

RNA was prepared from whole flies or larvae using Trizol reagent (Invitrogen) according to package instructions. Subsequently, Superscript reverse transcriptase (Invitrogen) and oligodT were used to generate cDNA. cDNA, diluted 1:100, served as template for real time PCR using SYBR green based detection. All reactions were performed in triplicate, and melting curves were examined to ensure single products. Primer pairs utilized were as follows:

rp49: 5'-CGGCACTCGCACATCATT,

5'-AGCTGTCGCACAAATGGC;

actin5C: 5'-CTCGCCACTTGCGTTTACAGT,

5'-TCCATATCGTCCCAGTTGGTC;

puc: 5'-CGAGGATGGGTTTGATTACGA,

5'-TCAGTCCCTCGTCAAATTGCT;

NLaz: 5'-GCCAGAAGTAGAACGGATACCA,

5'-ACTGGTGCAGCTGTAGACGAC;

pepck: 5'-CCAGGACAATTGCGGTCTGT,

5'-CTGCAGCATCCATGTCGCT;

l(2)efl: 5'-AGGGACGATGTCACCGTCTCT,

5'-CGAAGCAGACGCGTTTATCC;

Quantification of tGPH membrane intensity

Nuclear fluorescence was used to normalize membrane fluorescence. Fat bodies of 5 individual wandering third instar larvae were imaged using confocal microscopy and average fluorescence of membrane and nuclei was measured using the histogram function of NIH ImageJ. Intensity ratios were calculated for n=6-10 individual

cells from different fatbodies, and overall averages and standard deviations were calculated.

Weight and wing measurements

Sibling flies were used for comparisons. Flies were weighed as cohorts of 10 flies in pre-weighed eppendorf tubes. Wings were photographed, and size was determined by quantifying the number of pixels within each wing with adobe Photoshop.

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Figures and Legends

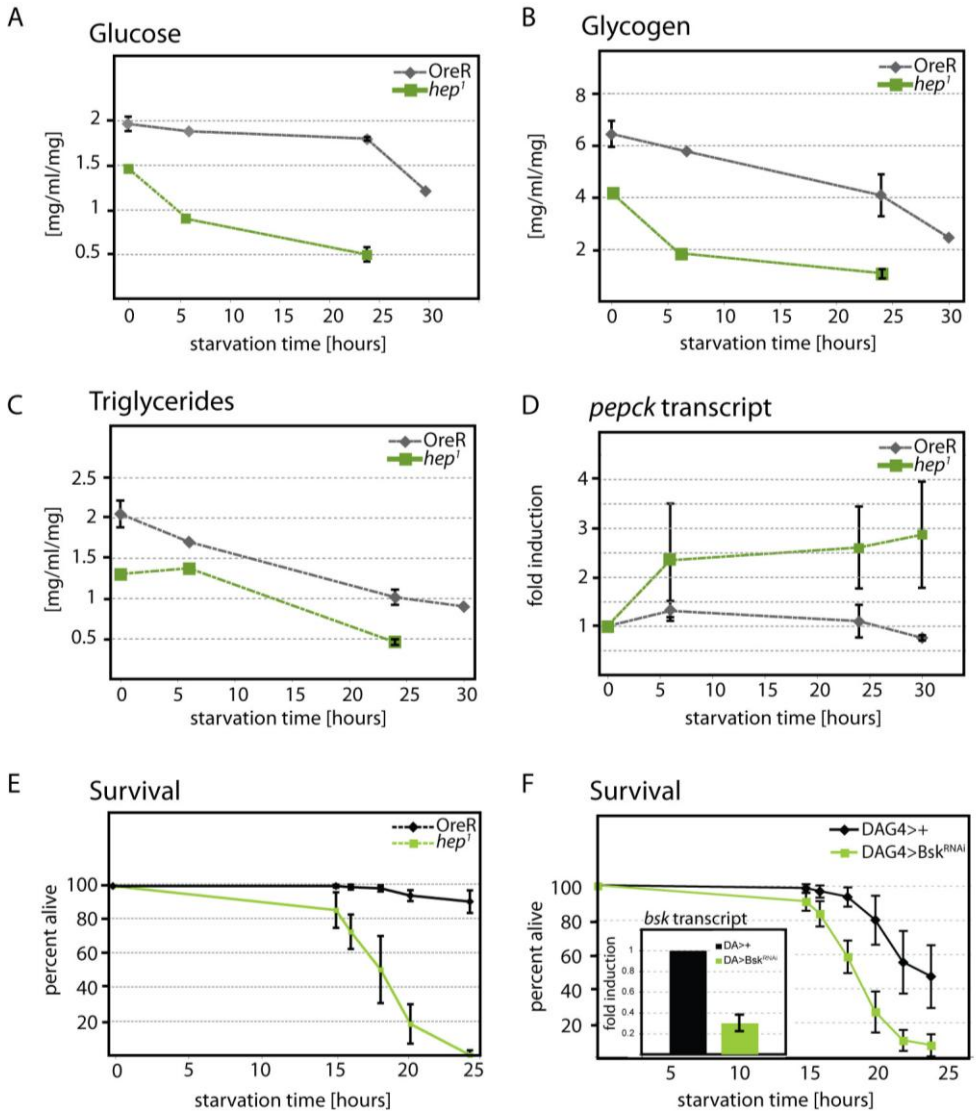

Figure 1: JNK is required for maintenance of metabolic homeostasis.

(A-E) Comparison of hemizygous *hep*^{1} males (*hep*^{1}*/y*) to wild type OreR males.

(A-C) Carbohydrate and lipid content in homogenates prepared from populations of 10 flies prior to and after 6, 24, and 30 hours of wet starvation. All measurements were normalized to the average weight of a single fly in its population. (A) Glucose. (B) Glycogen. (C) Triglycerides.

(D) Real time PCR results from cDNA prepared from starved adult males collected 6, 24, and 30 hours after wet starvation. Levels of *PEPCK* are compared in starved conditions to fed controls (0 hours). All transcripts are normalized to *actin5C*.

(E,F) Percent of male flies surviving after prolonged dry starvation. Population sizes were (E) OreR: n=151; *hep*^{1}: n=103. (F) DaG4/+: n=179; BskRNAi/+;DaG4/+: n= 138.

(F, inset) Real time PCR on cDNA prepared from Bsk^{RNAi}/+;DaG4/+ larvae. Levels of *Bsk* are compared to DAG4/+ controls. All transcripts are normalized to *rp49*.

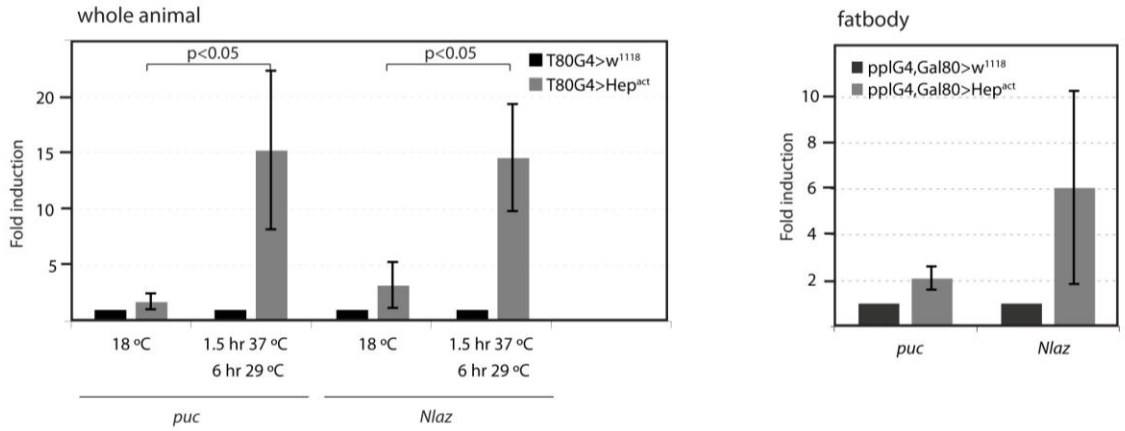

Figure 2: JNK regulates transcription of NLaz.

(A, B) Real time PCR measuring transcript levels of *puc* and *NLaz* in whole larvae (A) and dissected fatbody (B). Larvae express Hep^{act} under the control of the ubiquitous T80-Gal4 driver (A) or the fatbody pplG4 driver (B) combined with tubGal80^{ts}. Genotypes: (A) *w^{1118}*; T80Gal4, tubGal80^{ts}/UAS-Hep^{act}; control genotype: *w^{1118}*; T80Gal4, tubGal80^{ts}/+; (B) *w^{1118}*; pplGal4, tubGal80^{ts}/UAS-Hep^{act}; control genotype: *w^{1118}*; pplGal4, tubGal80^{ts}/+. Larvae were reared at 18ºC until 96 hours after egg laying, heat shocked for 1.5 hours at 37ºC and left at 29ºC for 6 hours (A) or 3 hours (B) to activate the driver. Transcript levels are normalized to *rp49*. Averages and Standard

Deviations of three independent experiments are shown. p values were calculated using Student’s T test.

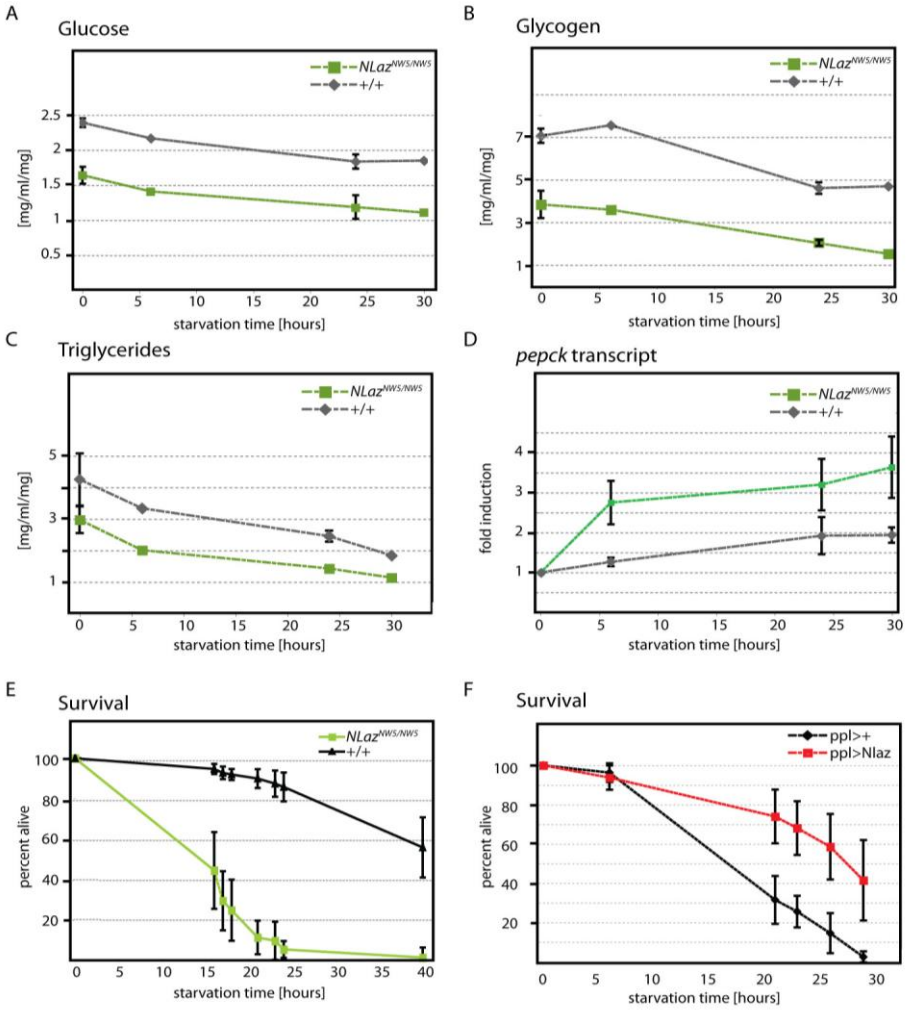

Figure 3: NLaz is required for maintenance of metabolic homeostasis.

(A-E) Comparison of homozygous NLaz loss of function mutants (NLaz$^{NW5/NW5}$) to wild type isogenic controls (*NLaz*$^{C\text{-}NW\text{-}14/C\text{-}NW\text{-}14}$).

(A-C) Carbohydrate and lipid content in homogenates prepared from populations of 10 male flies prior to and after 6, 24, and 30 hours of wet starvation. All measurements were normalized to the average weight of a single fly in its population. (A) Glucose. (B) Glycogen (C) Triglycerides.

(D). Real time RT-PCR results from cDNA prepared from starved adult males collected 6, 24, and 30 hours after wet starvation. Levels of *PEPCK* are compared in starved conditions to fed controls (0 hours). All transcripts are normalized to *actin5C*.

(E,F) Percent of male flies surviving after being exposed to prolonged dry starvation. Genotypes: (E) +/+: n=227; $NLaz^{NW5/NW5}$: n=254. (F) *pplG4/+;+/+*: n=102; *pplG4/+;*UASNLaz/+: n=115. To equalize culture conditions and genetic backgrounds, sibling F1 progeny derived from out-crossed w^{1118}; pplGal4/pplGal4 with out-crossed w^{1118}; UAS-NLaz/+ are compared.

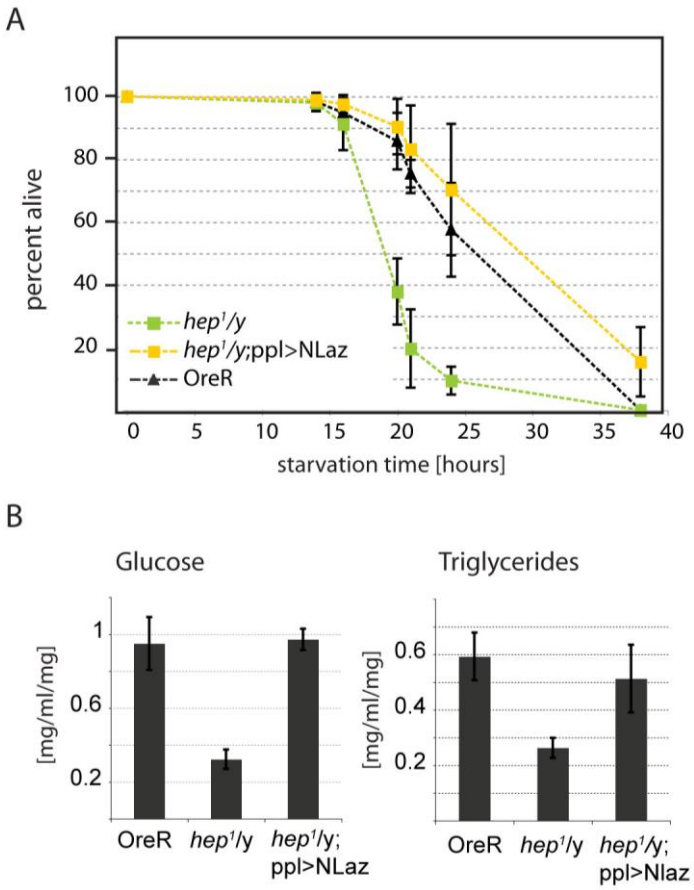

Figure 4: Nlaz acts downstream of JNK to maintain metabolic homeostasis.

(A) Percent of male flies surviving after being exposed to prolonged dry starvation.

(B) Glucose and lipid content in homogenates prepared from populations of 10 male flies after 24 hours of wet starvation.

(A, B) Genotypes: *hep^{1}/y: n=105*; *hep^{1}/y;pplG4/+;UASNLaz/+: n=74*; *OreR: n=114*.

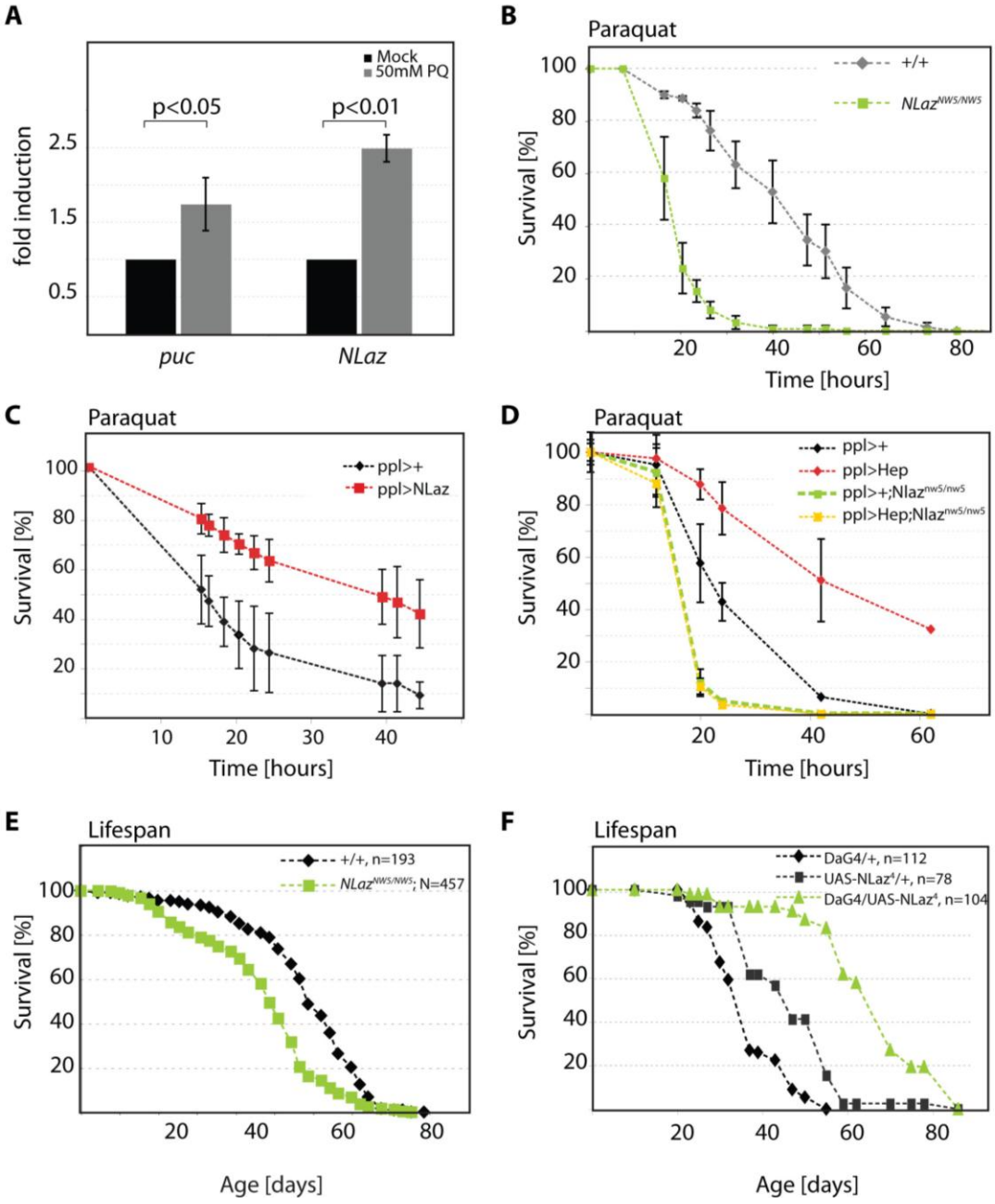

Figure 5: NLaz acts downstream of JNK to promote oxidative stress tolerance.

(A) NLaz is induced in flies exposed to oxidative stress. Levels of *NLaz* are compared using cDNA prepared from wild type OreR flies fed 50 mM paraquat in 5% sucrose to those fed 5% sucrose alone for 24 hours. Levels of *puc* are shown for comparison. All transcript levels are normalized to *actin5C*. p values are calculated using Student's T test.

(B) Oxidative stress sensitivity in NLaz mutants. Survival after exposure to paraquat. $NLaz^{CNW14}$ N=70, $NLaz^{NW5}$ N=117. Log rank test, $p<0.001$.

(C) Over-expressing UAS-NLaz using the ppl-Gal4 driver promotes tolerance to paraquat. Comparison of siblings of the following genotypes: *pplG4/+,* n=103; pplG4/+;UASNLaz/+, n=134.

(D) Stress sensitivity of NLaz mutants cannot be improved by JNK activation. Survival after exposure to paraquat. Populations of the following genotypes and numbers of individuals were used: *pplG4/+* n= 61; *pplG4/UASHep n=97*; *pplG4,NW5/NW5 n=135*; *pplG4,NW5/UASHep,NW5 n=59*. Log rank test for pplG4/UASHep: p<0.001.

(E and F) NLaz gene dose influences lifespan. (E) Male longevity at 25ºC. Groups of 20 flies per vial. $NLaz^{CNW14/CNW14}$ N=193, $NLaz^{NW5/NW5}$

N=457. Log-rank test, $p < 0.001$. (F) Overexpression of NLaz increases normal survival of males at 25ºC. Overexpressing *UAS-NLaz*^{4} using *DaG4* as a ubiquitous driver increases mean and maximum life spans in normal conditions. DaG4/+, n=112; UAS-NLaz^{4}/+, n=78; DaG4/UAS-NLaz^{4}, n=104; Log-rank test: $p<0.001$ (comparing UAS-NLaz and DaG4/UAS-NLaz).

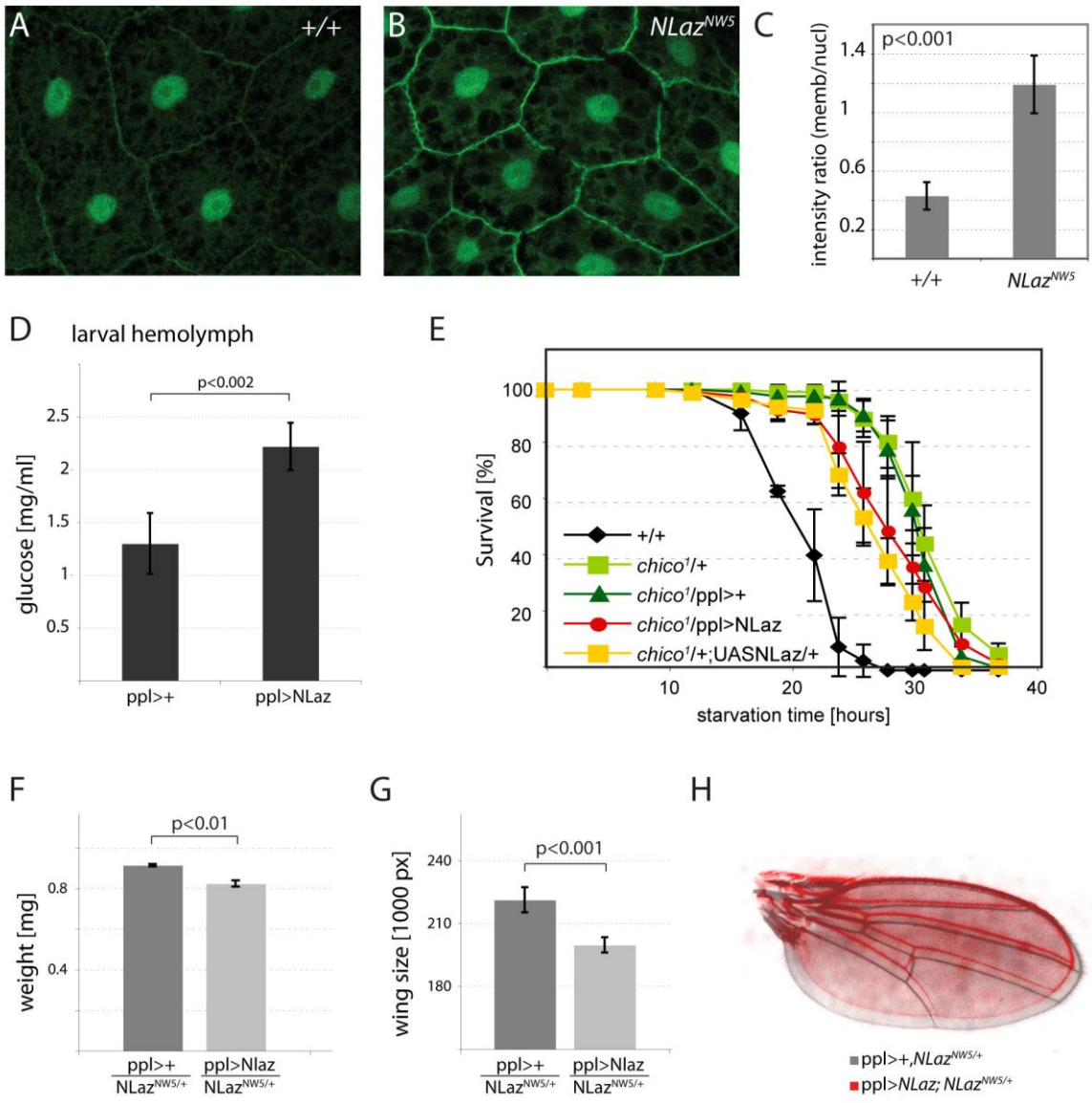

Figure 6: NLaz antagonizes IIS.

(A-C) tGPH fluorescence (Britton, Lockwood et al. 2002) in larval fatbodies. (A) +/+. (B) *NLaz*$^{NW5/NW5}$. Membrane localization of tGPH is increased in NLaz mutants compared to isogenic wild-type

controls, indicating elevated PI3K activity. (C) Ratios of average membrane vs. nuclear fluorescence as determined using NIH ImageJ on images of fatbody cells of independent individuals. n=6 for +/+, n=10 for *NLaz*^{NW5}. P value from Student's T test.

(D) Over-expression of NLaz in the fatbody results in elevated hemolymph glucose levels. Experiments were performed in larvae of the following genotypes: *pplG4/+;+/+*; *pplG4/+; UASNlaz/+*.

(E) NLaz cannot promote further starvation tolerance of *chico1* mutant flies. Percent survival of males of the following genotyps in response to prolonged dry starvation: +/+, n=112; *chico/+*, n=124, *chico/pplG4*, n=122; *chico/pplG4;UASNLaz/+,* n=124; *chico/+;UASNLaz/+*, n=78.

(F-H) Comparison of adult sibling males of the following genotypes: *ppl,NW5/+;+/+*; *ppl,NW5/+;UASNlaz/+*.

(F) Overall weight is decreased in NLaz over-expressing animals. Fresh weight of males of the indicated genotypes.

(G and H) NLaz levels affect wingsize. Comparison of wing sizes of sibling NLaz mutant flies with or without over-expression of NLaz in the fatbody. (A) Wing sizes were measured using Adobe Photoshop. (B) Overlay to visualize wing size differences.

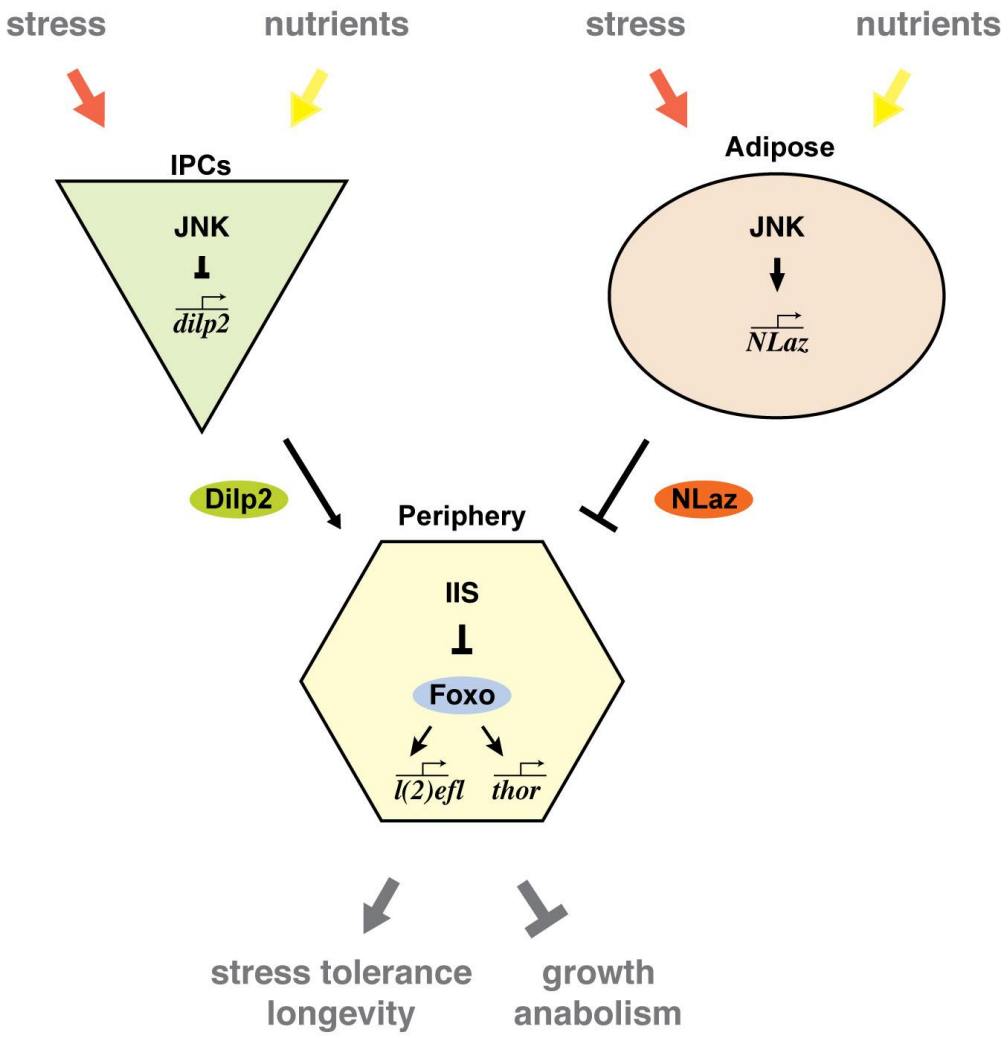

Figure 7: Proposed role of NLaz in promoting stress tolerance and metabolic homeostasis in the fly.

JNK-mediated induction of NLaz in the fatbody is required for regulation of metabolic homeostasis and stress tolerance. Accordingly, NLaz over-expression promotes stress tolerance and extends lifespan. As suggested by our data and reported for related

vertebrate Lipocalins, NLaz might interfere with Insulin signaling activity, thus coordinating metabolic changes throughout the organism. At the same time, JNK represses transcription of *dilp2* in IPCs. Together, these two mechanisms antagonize IIS in the periphery, thereby promoting stress tolerance systemically.

Supplemental Figures and Legends

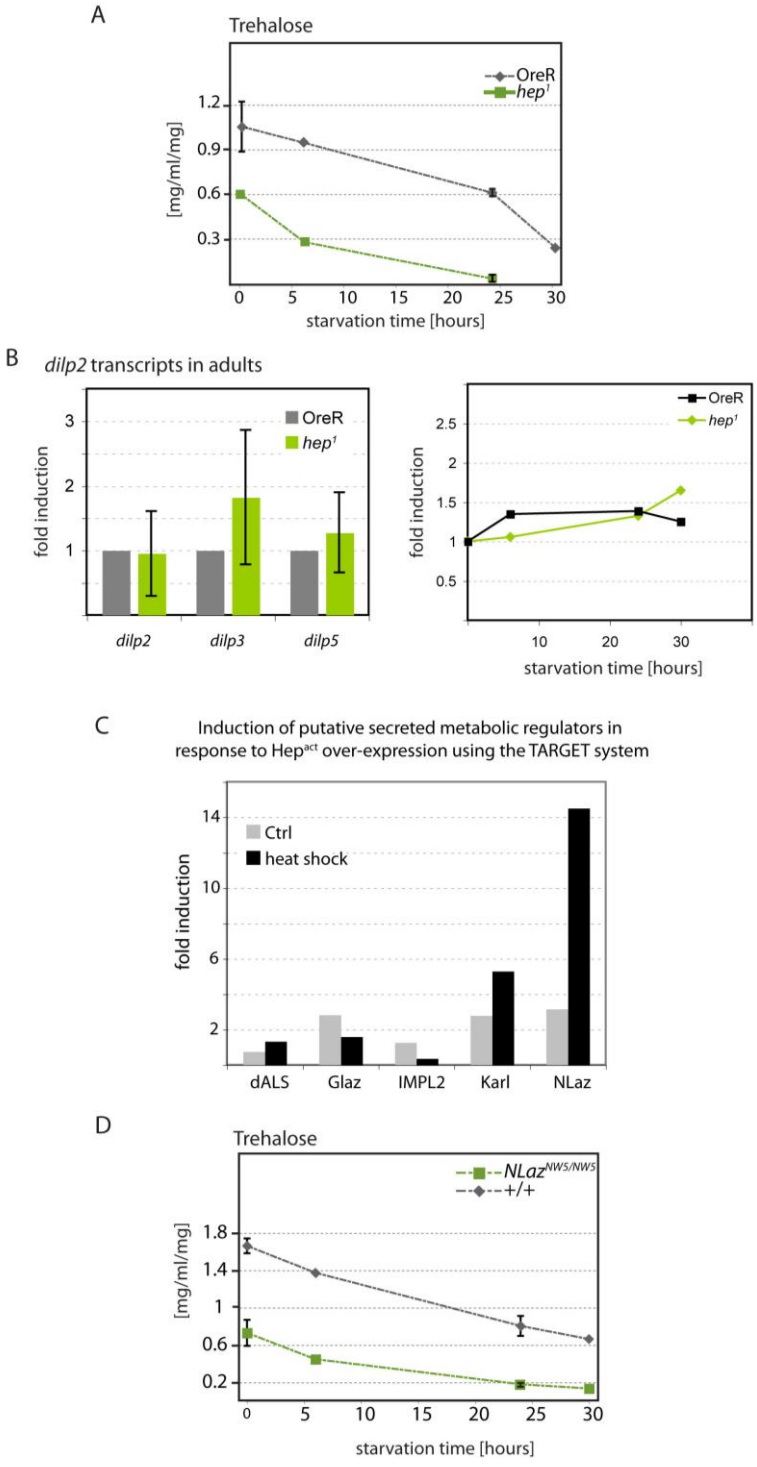

Figure S1.

(A) Trehalose content in homogenates prepared from populations of 10 flies prior to and after 6, 24, and 30 hours of wet starvation. (A) *hep1* and +/+. All measurements were normalized to the average weight of a single fly in its population.

(B) *dilp2, dilp3 and dilp5* transcript levels in hep^{1} mutants relative to +/+ controls determined by real time RT-PCR. All transcript levels were normalized to *actin5C*. No significant differences are observed in transcript levels of ILPs between hep1 and wild-type in *ad libitum* conditions (left panel). No differences are observed in *dilp2* transcript levels in starved flies (right panel).

(C) Real time RT-PCR to measure changes in transcription of selected potential secreted metabolic regulators in response to JNK activation. The heat shock-inducible TARGET system was utilized. Average fold induction (between Hep^{act} expressing and wild-type controls) of non-heat shocked (reared at 18 degrees) and heat shocked samples are shown. Actin5C expression was used for normalization.

(D) Trehalose content of *NLaz*$^{NW5/NW5}$ compared to isogenic wild-type controls.

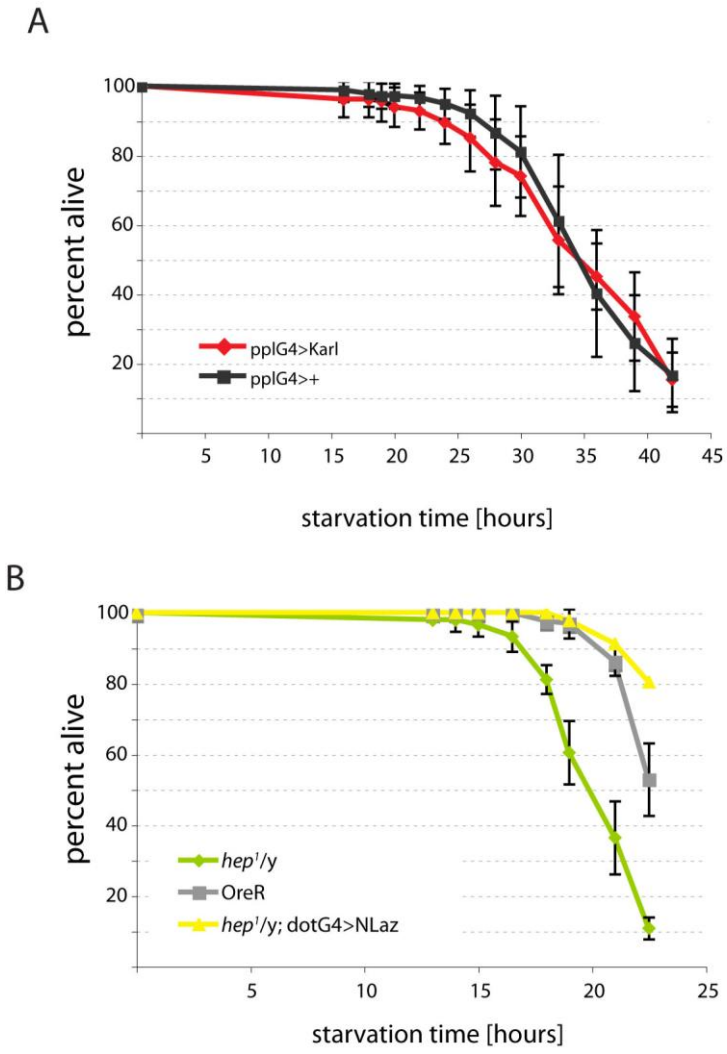

Figure S2.

(A, B) Percent survival in response to prolonged dry starvation. Genotypes: (A) pplG4/+, n= 181, pplG4/+;UASKarl/+, n= 181. (B) *hep1/y*, n=134, +/+, n=130, hep1/y;DorothyG4/+;UASNLaz/+, n=46.

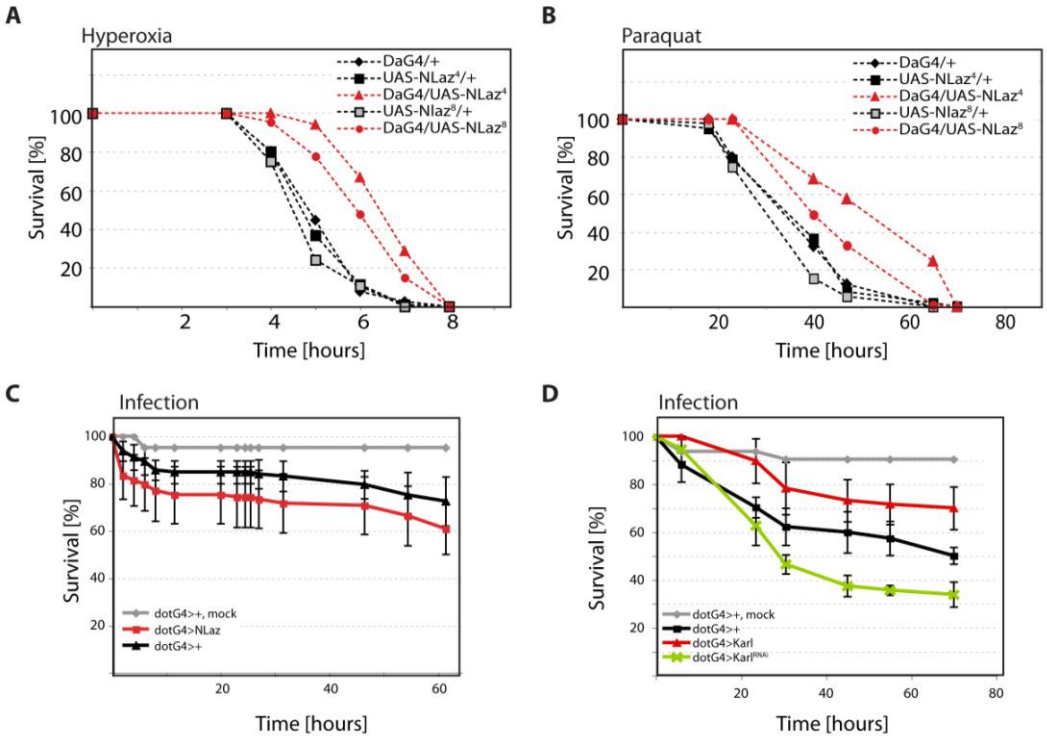

Figure S3.

(A) Over-expression of NLaz enhances resistance to hyperoxia. Overexpressing *UAS-NLaz*^{4} *and UAS-NLaz*^{8}, using *DaG4* as a ubiquitous driver protects from 100% oxygen-induced mortality. DaG4/+, n=157; UAS-NLaz^{4}/+, n=106; DaG4/UAS-NLaz^{4}, n=84; UAS-NLaz^{8}/+, n=104; DaG4/UAS-NLaz^{8}, n=107. Log rank test for UAS-NLaz4: p<0.001. Log rank test for UAS-NLaz^{8}: p<0.001.

(B) Overexpressing *UAS-NLaz* using *DaG4* protects from paraquat-induced mortality. UAS-NLaz^{4} and UAS-NLaz^{8} are independent insertion lines of the same construct. DaG4/+, n=118; UAS-NLaz^{4}/+,

n=102; DaG4/UAS-NLaz^{4}, n=132; UAS-NLaz^{8}/+, n=94; DaG4/UAS-NLaz^{8}, n=116. Log rank test comparing UAS-NLaz4 and DaG4/UAS-NLaz^{4}: p<0.001. Log rank test for UAS-NLaz^{8}: p<0.001

(C, D) Percent surival in response to infection with *E. faecalis*. Genotypes: (C*).* *DorothyG4/*+ mock, n=21*, DorothyG4/*+, n=111; *DorothyG4/+;UASNLaz/+,* n=108. (D) *DorothyG4/+,* mock, n= 31; *DorothyG4/*+, n=84; *DorothyG4/+; UASKarl/*+, n=60, *DorothyG4/+;pWizKarl/*+, n=56.

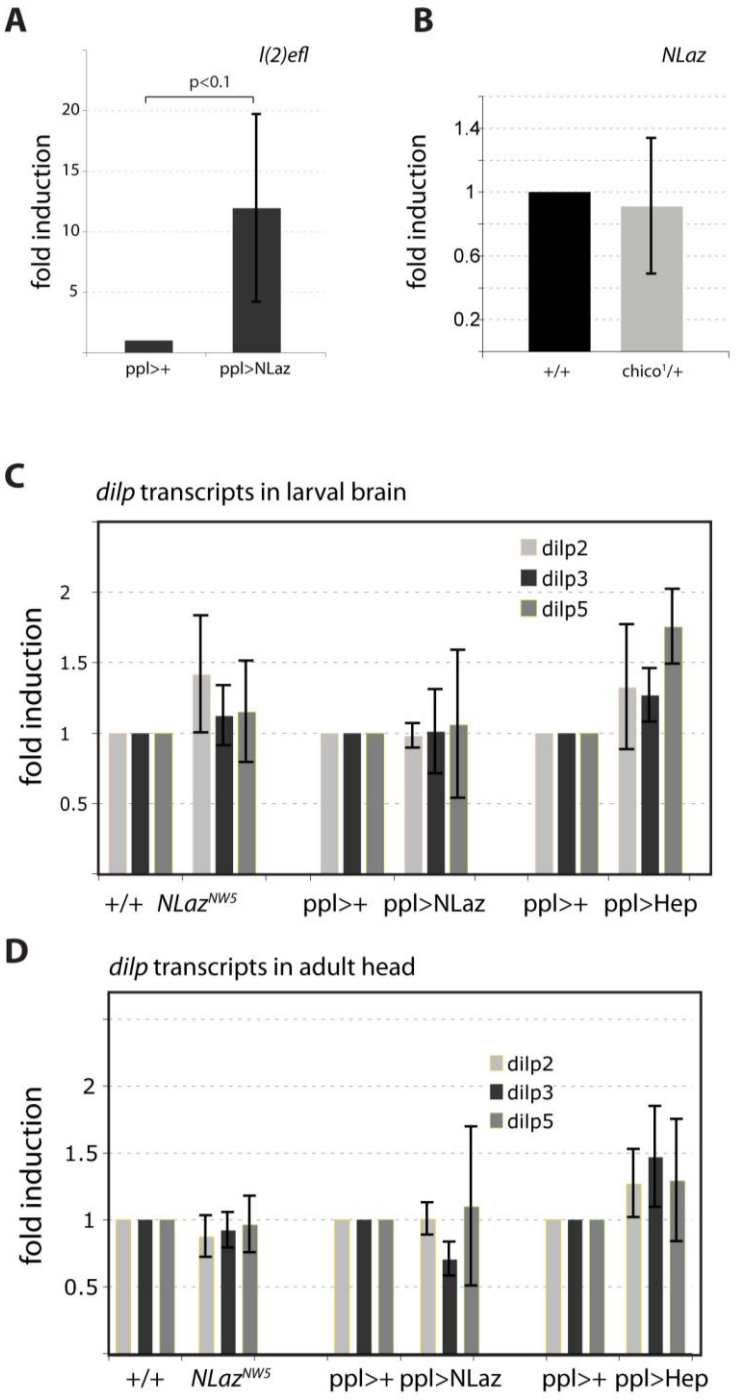
A
l(2)efl
p<0.1
fold induction
20
15
10
5
ppl>+
ppl>NLaz
B
NLaz
fold induction
1.4
1
0.6
0.2
+/+
chico¹/+
C
dilp transcripts in larval brain
dilp2
dilp3
dilp5
fold induction
2
1.5
1
0.5
+/+
NLaz^NW5
ppl>+
ppl>NLaz
ppl>+
ppl>Hep
D
dilp transcripts in adult head
dilp2
dilp3
dilp5
fold induction
2
1.5
1
0.5
+/+
NLaz^NW5
ppl>+
ppl>NLaz
ppl>+
ppl>Hep

Figure S4.

(A) Over-expression of NLaz induces expression of the FoxO target gene l(2)efl. Real time RT-PCR in whole larvae of the following genotypes: pplG4/+;UASNLaz/+; pplG4/+.

(B) Real time RT-PCR demonstrates that *NLaz* transcript levels are unchanged in *chico1* heterozygous mutants.

(C) Real time RT-PCR measuring levels of *dilp2*, *dilp3*, and *dilp5* in cDNA prepared from dissected larval brains. Larval genotypes were as follows: +/+; $NLaz^{NW5/NW}$; *pplG4/+*; *pplG4/+;UASNLaz/+*; *pplG4/UASHep* . Transcript levels were normalized to *Actin5C*.

(D) Real time RT-PCR measuring levels of *dilp2*, *dilp3*, and *dilp5* in adult heads from flies of the following genotypes: *+/+, $NLaz^{NW5/NW5}$ pplG4/+, pplG4/+;UASNLaz/+, pplG4/UASHep*. All transcript levels are normalized to *Actin5C*.

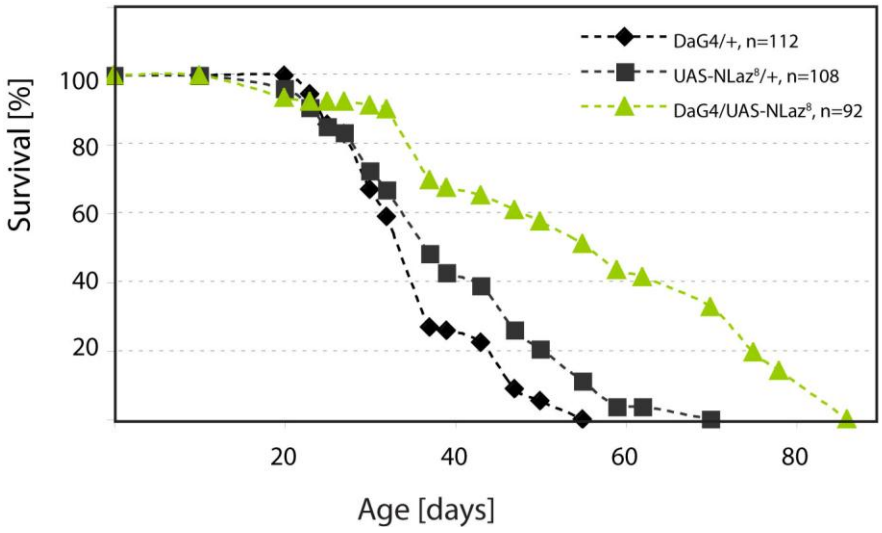

Figure S5. NLaz over-expression from an alternative transgenic line promotes longevity.

Overexpressing *UAS-NLaz*^{8}, using *DaG4* as a ubiquitous driver, increases mean and maximum lifespans in normal conditions.

UAS-NLaz^{8}/+, n=108; DaG4/UAS-NLaz^{8}, n=92. Log-rank test: $p<0.001$.

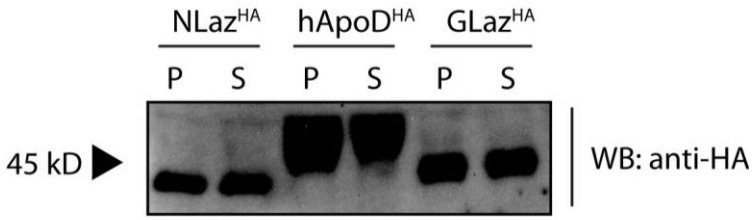

Figure S6. NLaz is secreted

HA-tagged NLaz (lanes 1 and 2) can be detected in the medium of S2 cells after 6 hrs of conditioning. Cell pellet (P) and supernatant (S) are shown. Related lipocalins are also secreted: human ApoD (lanes 3 and 4) and *Drosophila* GLaz (lanes 5 and 6).

Supplemental Material and Methods

Transfection of S2 cells and detection of secreted proteins

S2 cells were maintained as adherent cultures at room temperature in Schneider's medium supplemented with 10% FBS. Cells were transfected with the pAHW plasmid (Murphy Laboratory), into which the Lipocalin cDNA had been subcloned using gateway recombination, fusing the protein with a C-terminal 3xHA tag. Regulation of trafficking of the fusion protein by the N-terminal signal sequence was thus maintained. Transfection was performed with the Fugene HD (Roche) reagent in a 9:2 ratio per the manufacturer's instructions.

S2 cells were transfected with this construct and allowed to express the protein for 48 hours. The transfection medium was removed, and new medium was conditioned for 6 hours. Cells were separated from the conditioned medium by centrifugation (10', 5000rpm), and lysed in standard lysis buffer with protease inhibitors. An aliquot of the cell lysate and one from the conditioned medium were assayed for protein concentration (BCA assay, Pierce), and each adjusted to

1ug/uL. 100uL of these protein samples were mixed with SDS sample buffer, and denatured by heating to 95°C for 5 minutes. 10 ug of total protein from each sample were run on a denaturing SDS-page gel (Nupage, Invitrogen), and transferred to PVDF membrane following standard protocols. The membrane was then probed for HA reactivity using an HRP-conjugated anti-HA antibody (Roche), and detected using ECL West Dura substrate (Pierce).

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