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TESIS DOCTORAL:

Lazarillo and related Lipocalins: ligands and functions

Presentada por MARIO RUIZ GARCIA para optar al grado de
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AUTORIZACIÓN DEL DIRECTOR DE TESIS

(Art. 2.1. c de la Normativa para la presentación y defensa de la Tesis Doctoral en la UVa)

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Autorizan la presentación de la misma, considerando que el candidato ha superado el nivel de formación necesario para aspirar al Título de Doctor con Mención Internacional por la Universidad de Valladolid, mediante la realización de un proyecto de investigación original en el que su contribución abarca desde el diseño de las preguntas científicas y el diseño y ejecución de los experimentos, hasta la presentación en diversos formatos (escritura de trabajos para publicación y presentaciones en congresos) de los resultados para su difusión a la comunidad científica.

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La Directora de la Tesis,

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"Sex-dependent modulation of longevity by two Drosophila homologues of human

Apolipoprotein D, GLaz and NLaz".

Abbreviations

Abbreviations:

- **4-HNE:** 4-Hydroxynonenal
- **7-T:** 7(Z)-Tricosene
- **20-HE**: 20-hydroxyecdysone
- **AA:** Arachidonic Acid
- **AEA:** Anandamide
- **AGP:** α-acid glycoprotein
- **α-1m:** α-1microglobulin
- **ApoD:** Apolipoprotein D
- **ApoM:** Apolipoprotein M
- **ATF3:** Activating Transcription Factor 3
- **BBP:** Bilin-Binding Protein
- **CALβ:** Chondrogenesis-Associated Lipocalin β
- **CG:** Computed Gene
- **CNS:** Central Nervous System
- **CRC:** Crustacyanin
- **DHA:** Docosahexaenoic Acid
- **DNA:** Deoxyribonucleic acid
- **Ex-FABP:** Extracellular Fatty Acid Binding Protein
- **FA:** Fatty Acid
- **FABP:** Fatty Acid Binding Protein
- **FOXO:** Forkhead Box Protein O
- **GLaz:** Glial Lazarillo
- **Gp93:** Glycoprotein-93
- **GPI:** Glycosylphosphatidylinositol
- **HDL:** High Density Lipoparticles
- **Hr39:** Hormone Receptor-39
- **HSD:** High Sugar Diet
- **IIS:** Insulin/IGF1 Signaling
- **Ins:** Insecticyanin
- **InR:** Insulin Receptor
- **JNK:** Jun-N-terminal Kinase
- **KO:** Knock-Out
- **Kr:** Krüppel
- **LA:** Linoleic Acid
- **Laz:** Lazarillo
- **Lcn:** Lipocalin
- **Lip-4:** Lipase-4
- **Lola:** Longitudinals Lacking Protein
- **L-PGDS:** Prostaglandin D Synthase Lipocalin
- **MPI:** Metalloproteinase Inhibitors

- **mRNA:** Messenger Ribonucleic Acid
- **MUP:** Major Urinary Protein
- **NAE:** N-acylethanolamines
- **NGAL:** Neutrophil Gelatinase-Associated Lipocalin
- **n-HETE:** Hydroxyeicosatetraenoic Acid
- **n-HpETE:** hydroperoxyeicosatetraenoic acid
- **NLaz:** Neural Lazarillo
- **OEA:** Oleoylethanolamine
- **OBP:** Odorant Binding Protein
- **PDB:** Protein Data Bank
- **PEA:** Palmitoylethanolamine
- **pI:** Isoelectric Point
- **PI3K:** Phosphatidyl-Inositol 3-Kinase
- **PQ:** Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride)
- **PUFA:** Poly-Unsaturated Fatty Acid
- **RBP:** Retinol Binding Protein
- **ROS:** Reactive Oxygen Species
- **Scn:** Siderocalin
- **SCR:** Structurally Conserved Region
- **SM:** Sphingomyelin
- **SMase:** Sphingomyelinase
- **TAG:** Triacylglycerides
- **TL:** Tear Lipocalinç
- **UTR:** Untranslated Region

Introductory words

This thesis will present a mechanistical experimental approach to understand a molecular function common to the Lipocalin protein family. As an introduction, I will first summarize their evolution, ligand-binding properties and functional implications. Following I will focus on the current knowledge about the Lipocalin Lazarillo and its homologs, my playmates for the past few years.

Introduction

The Lipocalin protein family: A brief introduction and general characteristics.

The definition of "protein family" varies according to different authors. In a simple way, a protein family can be defined as a collection of related proteins [Pfam database: 1]. This relationship was typically based on protein function and/or primary structure. However, with the increasing number of protein tertiary structures resolved, protein families are also described according to its folding. Precisely, the Lipocalins belong to a set of families mainly defined by its structural homology. The Lipocalin fold is shaped by a β-barrel, forming a cup or calyx, with a central cavity that serves as a ligand-binding site [2]. Together with fatty acid binding proteins (FABPs), avidins and metalloproteinase inhibitors (MPI), the Lipocalins assemble the Calycin superfamily [2].

Historically, the Lipocalin name was suggested by Pervaiz and Brew in 1987 [3] studying the structure-function correlations among several proteins. However, the Lipocalin protein family concept was previously proposed by Unterman et al. in 1981 [4] by comparing the amino acid sequence homology between some Lipocalin family members.

Lipocalins are typically small (160-230 amino acid residues) extracellular proteins. Family members are found in all five life kingdoms [5], and their numbers are growing quickly due to the new sequenced genomes: over six hundred Lipocalins are currently identified.

Although Lipocalins are sometimes classified as mere transport proteins, it is now clear that they are carrying out a wide variety of really important functions. Their roles include regulation of the immune response, metabolism, oxidative stress defense, coloration and control of behavior among many others. A reflection of this physiological variety is that Lipocalins are being used as clinical markers in cancer, kidney injury and lipid disorders [6].

Lipocalin Family phylogeny: Clade division.

By using several conserved features of Lipocalins, a phylogeny of the family can be inferred. Phylogenetic trees, based on both protein sequences and gene architecture, have been built (fig.1A and 1B respectively) and show a similar topology [5,7-9]. The number of tertiary structures of Lipocalins has grown lately, and it should be now possible and desirable to update the Lipocalin tree considering protein folding as a phylogenetic signal as well.

Focusing in the phylogenetic tree derived from protein sequence, the animal Lipocalins are divided into twelve monophyletic clades (defined and named in fig. 1A). Clade I is especially interesting because it encompasses the majority of arthropodan Lipocalins and ApoD (Apolipoprotein D), the most ancestral chordate Lipocalin.

Subsequently, the family can be divided in *ancestral* vertebrate Lipocalins, those more related to ApoD (clades III, IV, V and VI) and *modern* vertebrate Lipocalins (clades VII-XII) (see fig 1A) [5].

Combining phylogenetic and structural information Gutierrez et al [10] conclude that more recently evolved Lipocalins have a more flexible protein structure (based on number of disulfide bonds), and bind smaller ligands with more efficiency than the ancestral Lipocalins. These points are in agreement with the fact that modern Lipocalins show a tendency to present more gene duplications and could be more specialized.

Lipocalin structure: From genes to proteins.

Tertiary and secondary structure

As mentioned above the Lipocalin fold comprises a right hand β-barrel (composed of eight antiparallel β-strands, labeled as A-H in fig.2A) that encloses an internal ligand-binding cavity. This cavity, or pocket, is usually outlined by apolar amino acids and is ideal for binding small hydrophobic compounds. The β-strands are linked by loops (L1, L3, L5 and L7) that are located close to the opening of the binding pocket and can influence the ligand-binding properties [11,12]. At the other side of the molecule you will find loops L2, L4, L6 and L8. Loops L2-L8 are all typical short β-hairpin, whereas L1, which is a large Ω loop, forms a lid that can partially or completely covers the
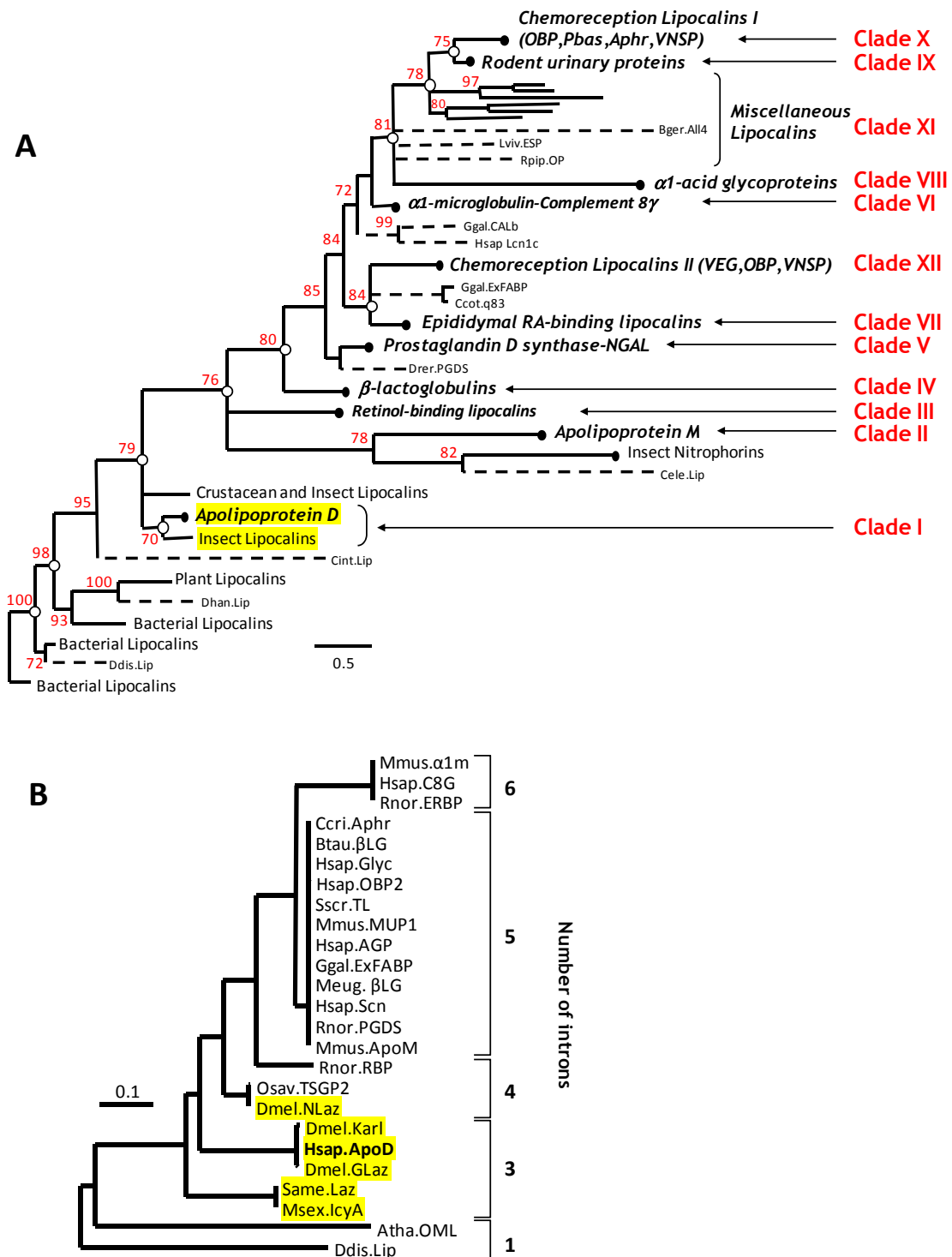
Figure 1. Two sets of independent characters have produced very similar phylogenetic relationships between Lipocalins.

(A) Phylogenetic tree of the Lipocalin family derived from a multiple alignment of protein sequences, rooted with a group of bacterial Lipocalins. **(B)** Lipocalin phylogeny based on exon-intron arrangement, rooted with a protoctist Lipocalin. Scale bars represent branch length (number of substitutions/site) [Adapted from 7].

ligand-binding site. There is a 3_{10} helix at the protein N-terminus, whereas at the C-terminus there is a canonical α-helix (fig.2) [2]. Most Lipocalins contain one or more intramolecular disulphide bonds that help to stabilize the β-barrel and are required to get the proper conformation and ligand-binding properties [13,14]. Several cysteine (Cys) residues present a pattern that is conserved in the family [7].

Within this general scheme, some Lipocalins show specific divergences in their primary and secondary structure. L-PGDS (Lipocalin-type Prostaglandin D Synthase), α-1m (α-1microglobulin) and the human version of ApoD show an unpaired Cys. The loops L5 and L7 appear elongated in RBP (Retinol Binding Protein). An unusually long N-terminus is present in the insect Lipocalin Karl.

Many Lipocalins present N- and/or O-linked glycosylation, and glycosylation residues range from none to six. Glycosylation is known to confer different properties and function, as happens for instance in Glycodelin [15], TL (Tear Lipocalin or Lcn1) [16], AGP (α-acid glycoprotein) [17], and the glycosylation-dependent secretion of α-1m [18].

<u>Primary structure</u>

Lipocalins show an extreme divergence at their amino acid sequence level, and a pairwise identity of 20-30% is common inside the family. This fact poses difficulties when assigning newly discovered sequences to the family. However, three conserved motifs, called structurally conserved regions (SCR), are revealed by multiple alignments [19].

- SCR1 is defined by the residues G-x_1-W-x_2 (being x_2 usually Y/F/W), and it is located in the first β-strand (strand A).

- SCR2 consensus sequence is T-D/N-Y-x-x-Y. Physically the SCR2 is found in the loop L6, connecting strands F and G.

- SCR3 is the shortest SCR and consists of a charged residue (R/K) in the β-strand H.

SCR1 and SCR3 appear conserved in over 90% of Lipocalins, whereas SCR2 is present in approximately 60% of them. Therefore, it is not unusual that entire groups of Lipocalins lack an individual motif. This is the case of ApoM (Apolipoprotein M) and AGP. Given that the Lipocalin motifs are so short, many proteins have been bioinformatically misclassified as Lipocalins by some protein databases. Curiously, all

three motifs are at the base of the calyx and seem to be important to achieve a proper folding. Especially essential is the SCR1 tryptophan residue [20,21].

Following the suggestion of Flower [19], Lipocalins were conceptually divided in two groups: *kernel* Lipocalins, where all SCR are present, and *outlier* Lipocalins that miss at least one SCR.

Gene Structure

Some characteristic features of Lipocalins are also extended to gene structure, exhibiting a similar arrangement of exons and introns in the coding sequence of their genes [9].

Vertebrate Lipocalins show seven exons (1-7) and six introns (A-F), usually including six coding exons (e1-e6). Exons 2-5 code for the entire set of β-strands. In some cases, e6 appears fused with e7, as happens in human ApoM or chicken Calβ genes. Additionally, the intron phase pattern is well conserved (0,2,1,1,1; each number indicates the position within the codons where the intron is inserted) [5,9].

Looking at the phylum arthropoda, there is a higher variability in gene structure, with a lower number of exons compared to vertebrate Lipocalins. However, exon sizes and the pattern of intron phases are quite similar between vertebrates and arthropods, a further genomic support for sharing a common ancestor. The Lipocalin gene architecture in other organisms, including plants, has not been studied in detail yet.
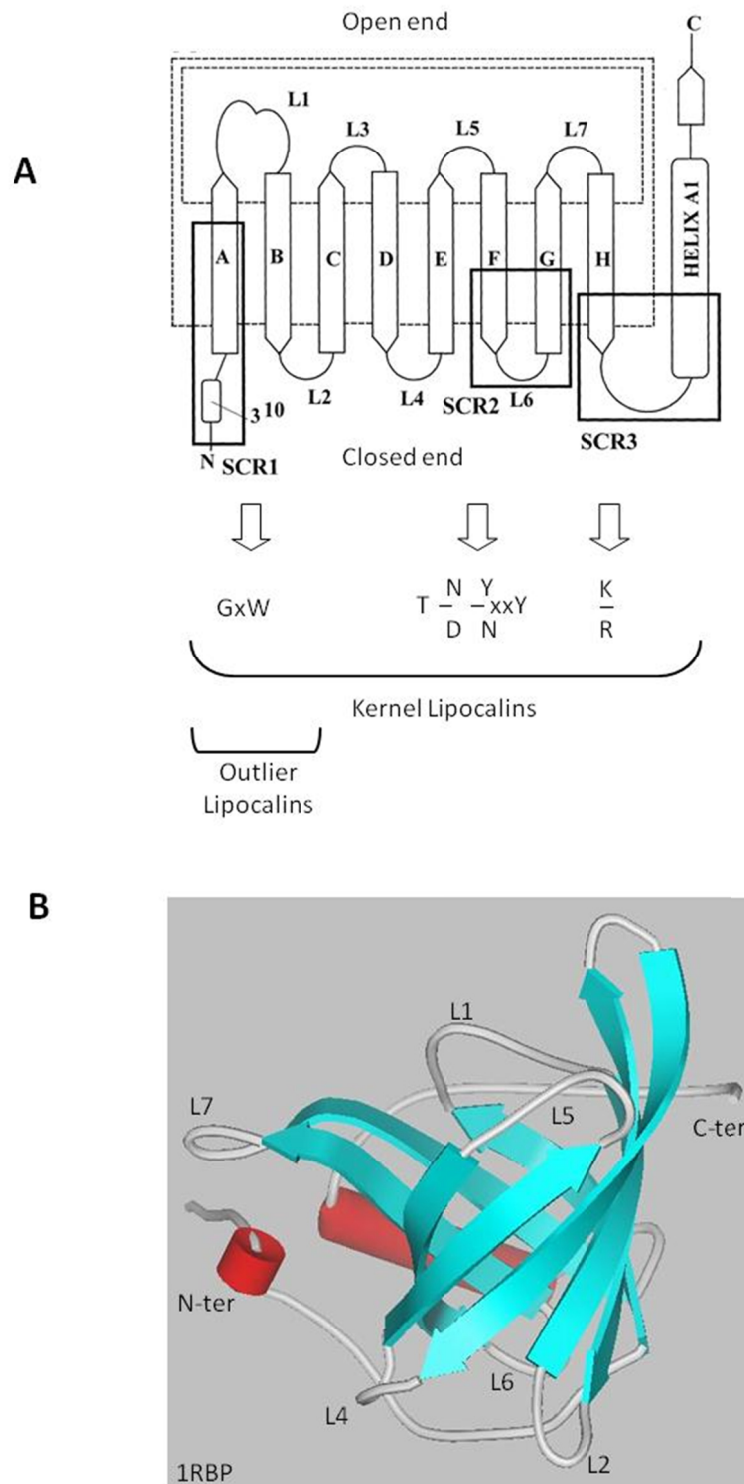
Figure 2. Lipocalin structure.
 (A) Schematic structure of the Lipocalin fold. The β-sheets are represented by arrows and labeled A-H. α-helices are also indicated. SCRs are marked and their characteristic sequences displayed. [Adapted from 2]. **(B)** Side view of the prototypical Lipocalin RBP (PDB code: 1RBP). Secondary structure is represented as red cylinders (α-helices) and blue arrows (β-sheets). Loops are also indicated as L1-L8.

Ligand binding and Lipocalin Function: A family overview.

Several properties of the binding pocket (depth, width, polarity and loop positions) determine what ligands are able to bind to each Lipocalin. Biological processes often depend on protein-ligand binding events, and this is indeed the case for Lipocalins. Their functions cannot be completely understood without taking in account their ligands. Some examples of the close relationship between ligand and function in different Lipocalin clades include:

- **MrLC** is a crustacyanin (clade I) from the prawn *Macrobrachium rosenbergii* that shows dual binding capacities. MrLC binds astaxanthin, which is responsible for the bluish prawn coloration, but MrLC also binds 20-hydroxyecdysone (20-HE) and regulates prawn molting [22].

Another example of coloration function involves Insecticyanin (**Ins,** also from clade I) and its ligand biliverdin IXγ, which confers a green color to *Manduca sexta* larvae [23-25].

- **RBP** belongs to clade III and has been identified from fish to mammals. RBP was the first crystallized Lipocalin [26] and is considered a prototypal Lipocalin. Actually, the RBP ligand retinol has been demonstrated to be a common ligand *in vitro* for most Lipocalins [27-29]. RBP transports retinol from the liver to peripheral target tissues, and plays a key role in vision and several development processes [30,31].

- Siderocalin (**Scn**), also known as Lipocalin-2 (Lcn2), neutrophil gelatinase-associated Lipocalin (NGAL) or 24p3, belongs to clade V and is a Lipocalin involved in the immune response. Scn binds siderophores, small secreted bacterial compounds that are able to chelate iron, an essential and scarce nutrient. Therefore, Scn acts as an anti-bacterial component of the innate immune response by sequestering iron away from invading pathogens [32]. This mechanism of action is shared with the avian Lipocalins Q83 [33] and Ex-FABP [34].

- Odorant binding proteins (**OBP**) and Major urinary proteins (**MUP**), clades X and IX respectively, are chemosensory proteins that act as peri-receptors transporting odorant or pheromones to the neuroepithelium, and are involved in behavior regulation [35-37]. In addition, bovine OBP has a protective role by binding 1-octen-3-ol, a potent biting insect attractant which comes from mammal breath and sweat [38].

A set of Lipocalins shows anti-oxidant binding properties. **ApoM** binds oxidized phospholipids selectively, thus serving as a scavenger of lipoprotein oxidation products [39]. **TL** and **OBP** bind to 4-hydroxy-2-nonenal (4-HNE), an end-product of ω-6 PUFA (poly-unsaturated fatty acid) peroxidation, and prevent its cytotoxicity [40,41]. Finally, **α-1m** binds heme and participates in its degradation [42]. Additionally, α-1m shows reductase/dehydrogenase properties and may act as and oxidation repair factor for important structural proteins as collagen [43].

Clade I: Arthropodan and vertebrate Lipocalin meeting point.

As discussed above, the Lipocalin clade I includes most arthropodan Lipocalins and the most ancestral chordate Lipocalin: ApoD. Clade I can be subdivided into three groups: Lazarillo-related Lipocalins, Crustacyanins (CRC) and Bilin binding-related Lipocalins. Another group of arthropodan Lipocalins, the Nitrophorins of blood-feeding insects, represents a large independent expansion of very divergent Lipocalin genes, and the phylogeny reconstruction tools usually place them among more recent Lipocalin clades (fig.1A.).

Below I will go over what is known about the 'founding member' of the Clade I group: Lazarillo. I will follow by describing the Clade I Lipocalins from the model organism *Drosophila melanogaster*, and the chordate homolog ApoD.

Lazarillo

Lazarillo (**Laz**) is a *kernel* Lipocalin from the grasshopper *Schistocerca americana*. The mature form of Laz has 170 residues, with a theoretical isoelectric point (pI) of 4.83 [ProtParam tool. 44]. Laz presents four Cys residues forming two internal disulfide bonds [45]. The Laz gene contains three introns, being A and B extremely long [9].

Laz shows a unique feature in the family. It is the only Lipocalin for what a glycosylphosphatidylinositol (GPI) anchor to the plasma membrane has been demonstrated [45]. However, a potential GPI modification was also suggested in its plant homolog AtTIL (*Arabidopsis thaliana* temperature-inducible Lipocalin, [46]). Nevertheless, other Lipocalin - plasma membrane associations have been reported [47-49].

Laz is highly glycosylated. Five N-linked oligosaccharides are predicted, and curiously all five are polarized to one side of the globular protein [50].

The natural Laz ligand or ligands are still unknown, but a set of potential candidates have been assayed *in vitro.* Recombinant Laz is able to bind retinoic acid and long chain fatty acids (FA), whereas biliverdin, farnesol or juvenile hormone do not bind [51].

Looking at the spatio-temporal expression pattern, Laz shows a restricted expression in the developing nervous system, and it is involved in axon growth and guidance. Adding a monoclonal antibody against Laz to grasshopper embryo cultures perturbs axon extension and causes growth cone misrouting. Moreover, outside the nervous system Laz is associated mainly with the excretory system: Malpighian tubules and subesophageal body [45,52].

Due to its selective expression pattern, Laz has been used as a tool for characterizing the nervous system in economically important species as the desert locust *Schistocerca gregaria* or *Locusta migratoria* [53-56].

Drosophila Lipocalins

Eight Lipocalin genes in the Drosophila genome: Glial Lazarillo (**GLaz**), Neural Lazarillo (**NLaz**), and **Karl** and five additional Lipocalin genes which are still identified only by their CG (computed gene) code number.

The chromosomal distribution of Drosophila Lipocalins is shown schematically in fig.3A. It is remarkable that the Drosophila third chromosome hosts four Lipocalins in the right arm: CG31446, CG5399, CG44013 and CG44014, found in pairs (CG31446 - CG5399 and CG44013 - CG44014), and in adjacent chromosomal regions (88 and 89). This agglomeration of Lipocalin genes is similar to that of human chromosome 9 and chicken chromosome 17 [5]. Three of these fly Lipocalins have only one intron, while CG31446 has two introns. The data above and their situation in the *Drosophila*

melanogaster Lipocalin phylogenetic tree (fig.3B) suggest that the current four Lipocalins may come from a double event of gene duplication..

Following I briefly review the data available on each Drosophila Lipocalin.
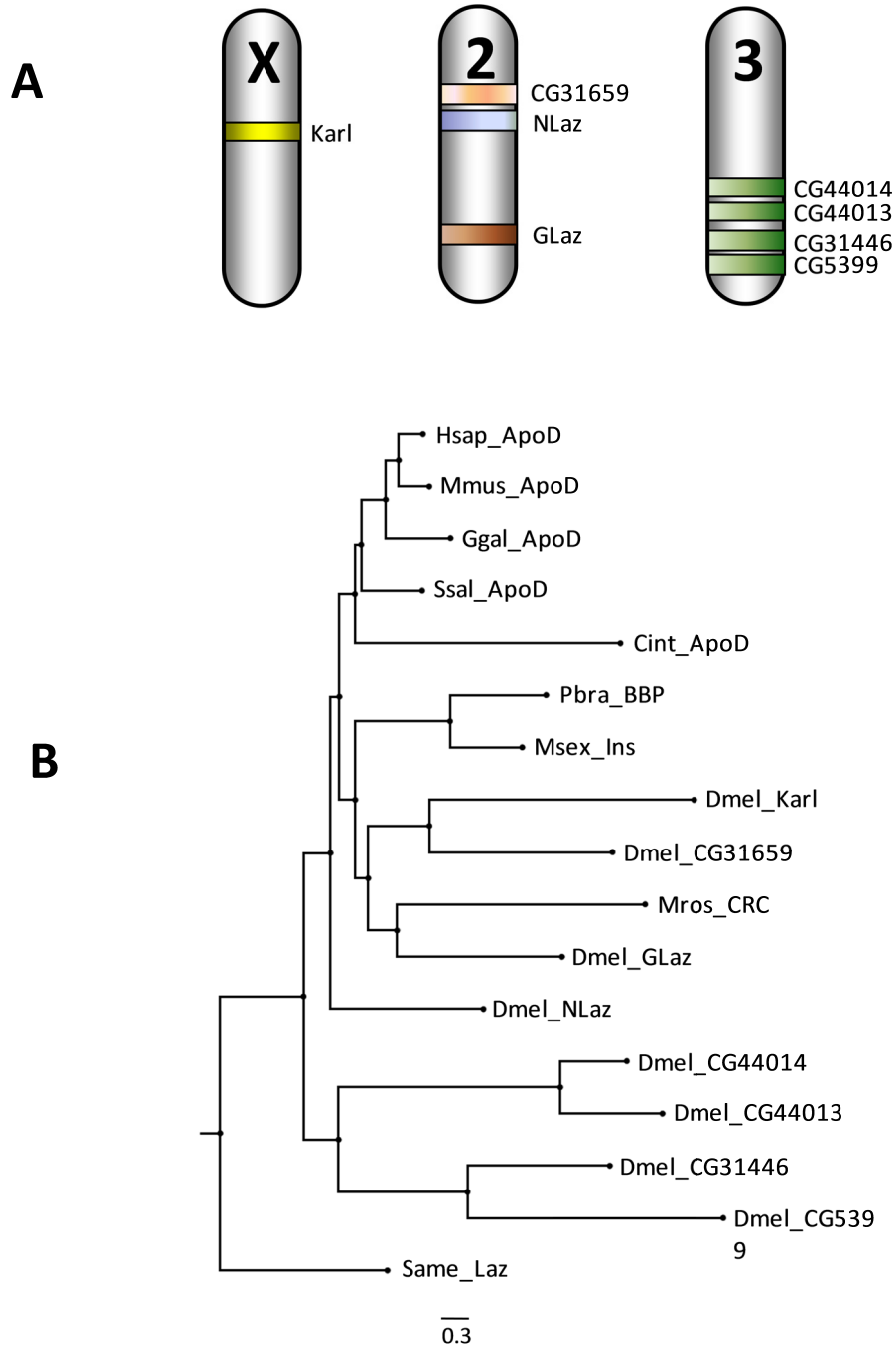

Figure 3. Drosophila Lipocalins.
(A) Schematic representation of the chromosomal location of Drosophila Lipocalin genes. **(B)** Phylogenetic tree of Lipocalin Clade I derived from a multiple alignment of protein sequences rooted with grasshopper Laz. Scale bar represents branch length (number of substitutions/site).

- **CG31446** and **CG5399** (89A2 region) show 33% identity in a pairwise alignment. At the sequence level, the C-terminus of CG5399 shows a high degree of similarity with that of Laz, and a potential GPI modification site is predicted [big-PI Predictor: The GPI Prediction Server. 57,58]. If confirmed experimentally, this would be the only GPI-linked Drosophila Lipocalin.

CG5399 and CG31446 functions are unknown. However, a couple of pieces of information are available. CG5399 expression in S2 cells appears enhanced more than ten times when cells are treated with the insecticide methoxyfenozide (RH-2485) [59]. On the other hand, CG31446 is over-expressed in a soluble guanlyl-cyclase mutant [60], and it is down-regulated in flies under long-term high magnetic field exposure [61].

- **CG44013** and **CG44014** (88F7 region) genes are separated by only sixteen base pairs. Both genes are predicted to code for *kernel* Lipocalins, showing 45% identity and 64% similarity between them. CG44013 and CG44014 were considered a single ORF called CG14872 in a previous Drosophila genome annotation.

The current functional information about these two Lipocalins comes from expression data microarrays that considered the combined CG14872 annotation. Larvae fed with wheat germ agglutinin (an anti-insect lectin) increase CG14872 transcription as part of their defensive response [62].

- **CG31659** is located in the left arm of the second chromosome (22A1 region), next to the NLaz gene. CG31659 is one of the most abundantly expressed genes in testis and male accessory gland [63]. Indeed, the CG31659 protein has been identified as a seminal fluid protein and is transferred from males to females during mating in *Drosophila melanogaster* and *D. yakuba,* but not in *D.simulans* [64].

- **Karl** (CG4139) is located in the X chromosome (10E3 region) and codes for an atypical Lipocalin. Karl N-terminal is extremely long, SCR1 is not easily identifiable and loop-2 appears expanded.

The functional information about Karl is scarce and fragmentary, coming mainly from gene expression microarrays. Karl is strongly expressed in lymph glands and circulating hemocytes [65,66]. Hemocytes are an important component of the insect immune system because they act as macrophages and produce antimicrobial peptides.

The immune role of Karl was confirmed by Hull-Thompson [67], as Karl over-expressing flies are more resistant to *Enterococcus faecalis* infection than the control fly line.

Furthermore, the insertion of a p-element in the Karl gene causes an extension of fly developing time around one hundred hours [68] and affects wing shape [69].

Finally, Karl is expressed in 25 Drosophila cell lines, including the popular Schneider's cell lines [70], at higher level than in whole flies at any developmental stage [71].

GLaz: Glial Lazarillo.

The right arm of the second chromosome (49F4 region) contains the GLaz gene. GLaz intron-exon organization is defined by 3 introns and 4 exons, as the grasshopper Laz gene. However, it differs from the typical insect gene arrangement: 4 introns and 5 exons, as the equivalent GLaz exons 4 and 5 are fused.

The GLaz mature protein has 192 residues with a theoretical pI of 8.62 [ProtParam tool. 44], and only one N-glycosylation is predicted at position 16th [72]. GLaz pairwise identities reach 26% with NLaz, 29% with Laz and 30% with ApoD. GLaz structure presents two of unique features: the loops 2 and 3 are elongated.

At the moment, GLaz can be considered an 'orphan' protein. GLaz binding properties have been reported neither *in vitro* nor *in vivo*.

In terms of expression, GLaz is found in the longitudinal glia of the developing ventral nerve cord of the central nervous system (CNS), and in specific glial precursors during late stages of embryogenesis. Subsequently, GLaz is absent in larvae, and again present in pupae and the adult nervous system [50,73]. Outside the nervous system GLaz is mainly expressed in the developing gut and salivary glands [50], and in adult hemocytes [73].

GLaz expression level has been experimentally altered in Drosophila using genetic tools. Male flies lacking GLaz exhibit multiple phenotypic defects. GLaz null-mutant (KO) flies show a shortened lifespan and a reduced resistance to starvation, H_2O_2 or paraquat (PQ) (1,1'-dimethyl-4,4'-bipyridinium dichloride). Paraquat is a generator of reactive oxygen species (ROS) that was used as herbicide, and is now broadly used in laboratories as a model to simulate Parkinson's disease. The higher sensibility to PQ is

also reflected in a lower performance in behavioral tests (phototaxis, geotropism and flight tests), an increase of apoptotic cells and lipid peroxidation levels. Furthermore, GLaz-KO male flies show a smaller body mass and a lower amount of triacylglycerides (TAG) [73].

In contrast, GLaz over-expressing flies resulted in increased lifespan and enhanced resistance to starvation and hyperoxia. Alterations in O_2 concentration produce a decline in locomotion of wild-type flies, and this deficiency is rescued by GLaz over-expression [74]. The protective properties of GLaz were corroborated in a cellular model, as Drosophila S2 cells over-expressing GLaz are protected against PQ and Aβ42 peptide toxicity [75].

Flies respond to multiple stress situations (hyperoxia, high temperatures, PQ exposure [75] and high sugar diet [76]) by increasing GLaz levels. This unspecific regulation of expression suggests that GLaz forms part of the organism first defensive line.

Due to GLaz ability to deal with oxidative stress, the effect of GLaz over-expression has been studied in the context of flies that model human neurodegenerative diseases. Friedreich's ataxia is caused by a decreased expression of the mitochondrial protein frataxin. This deficiency provokes a dysfunction of the mithocondrial respiratory chain with a subsequent increase of oxidative stress, cell damage and degeneration [77]. GLaz was able to correct the lipid peroxidation levels and mitigate the dyslipidemia and shortened lifespan caused by frataxin reduction in a Drosophila model of Friederich's ataxia [78].

Finally, it has been reported that the insertion of a p-element in the GLaz promoter does not alter ethanol sensitivity, place memory or olfaction memory [79].

NLaz: Neural Lazarillo.

The NLaz gene is located in the 22A1region of the second chromosome. Its intron-exon arrangement is slightly particular, because exon 1 is divided in two by a small intron.

The mature NLaz protein has 199 amino acids with a theoretical pI of 4.50 [ProtParam tool. 44], and shows four potential N-glycosylations sites (Asn46, 52, 71 and 172) [72,NetNGlyc 1.0 Server. 80]. Indeed, two N-glycosylations (Asn52 and Asn71) have been confirmed in head extracts [81].

Phylogenetically, NLaz is the closest Drosophila gene homolog to the grasshopper Laz and ApoD (fig. 3B). NLaz shows 39% identity with ApoD and 37% with Laz. The NLaz C-terminal sequence is extended in comparison to most Lipocalins. This 'tail' is not cleaved to GPI-anchor the protein to the plasma membrane, as it happens with grasshopper Laz. However, this peculiarity also appears in the neural Lipocalin Gallerin from the moth *Galleria mellonella* and in CG44014 and CG31446 fly Lipocalins.

In terms of expression, NLaz temporal pattern is similar to that of GLaz, but the tissue profile is quite different. While GLaz is expressed by glial cells in the CNS, NLaz is expressed by a subset of neuroblasts and neurons. Outside the nervous system NLaz is expressed in the fat body and the developing gut [72].

A NLaz-KO fly line was generated by introducing a stop codon in the NLaz coding sequence [82]. This fly line has been used to study the influence of NLaz in metabolism, signaling and stress response.

Energy storage (TAG, glucose and glycogen levels) are decreased in NLaz-KO male flies. In agreement, flies lacking NLaz show a decreased resistance to starvation, whereas over-expression of NLaz in the fat body results in an increment of starvation resistance. The same pattern, lower resistance in NLaz-KO and high in the over-expressor, was achieved in longevity and PQ toxicity assays [67].

It is known that the interaction between Jun-N-terminal kinase (JNK) and insulin/IGF1 signaling (IIS) has to be tightly regulated to ensure proper metabolic adaptation to environmental challenges [83]. NLaz is transcriptionally regulated by JNK signaling and is up-regulated under stress conditions. NLaz inhibits the IIS and it seems to act at the level of the plasma membrane modulating PI3K (phosphatidyl-inositol 3-kinase) activity. As a consequence of this IIS inhibition, FOXO (Forkhead box protein O) targets insulin receptor (InR), Lipase-4 (Lip4) and the heat shock protein 22 (hsp22) are up-regulated and contribute to promote stress tolerance and longevity. In contrast, when flies lacking NLaz are under stress conditions they cannot inhibit the IIS activity and cannot attain the appropriate protective response [67].

Flies fed with a high sugar diet (HSD) accumulate high levels of circulating glucose and show a small body size. This phenotype is due to resistance to insulin-like peptides in peripheral tissues. NLaz is up-regulated (fold change: 4.0) under HSD conditions. However, lowering NLaz gene dose (flies heterocygous for NLaz null mutation) protects against insulin resistance and returns glucose levels to normal values [76].

Since NLaz is expressed in neurons and neuroblast, as grasshopper Laz does, and it is secreted by developing axons [72], a role in axon growth and pathfinding is expected for NLaz. However, no gross defects have been observed during nervous system development in NLaz-KO flies. Several interesting relationships have been found, though, between NLaz and developmental processes in different tissues:

- The protein Lola is a critical transcription factor in nervous system development, orchestrating midline crossing by CNS axons [84]. NLaz is down-regulated (fold change: -2.40) in Lola defective embryos [85], suggesting that NLaz is a down-stream target of Lola. In addition, Lola is also involved in construction or function of neural circuits throughout the lifespan of the fly. This includes dendritic and axonal development of adult olfactory projection neurons [86]. These results may indicate a role of NLaz in axon outgrowth.

- Krüppel (Kr) is a transcription factor that plays a critical role in segmentation and organ formation during the last stages of embryogenesis. For example, a role for Kr in fat body formation is well documented [87]. A strong association between Kr and NLaz has been reported by chromatin immunoprecipitation and *in vitro* binding to DNA [88].

- Gp93 is an essential protein in Drosophila since homozygous mutant animals die at the third larval stage as a consequence of pronounced defects in midgut epithelium. Gp93 mutant larvae exhibit a starvation-like metabolic phenotype, including suppression of insulin signaling and extensive mobilization of amino acids and TAG. NLaz is almost absent in fly larvae, but it is up-regulated (fold change: 6.0) in GP93 animals [89].

- A null mutant of the activating transcription factor 3 (ATF3-KO) causes a complex larval lethal phenotype characterized by fat body lipid overload and starvation signaling from the gut. NLaz is found up-regulated (fold change: 2.24) due to the hyper-activation of stress cascades, including the JNK pathway [90].

- NLaz is also related with the development of sexual structures. The hormone receptor 39 (Hr39) is required for the normal development and function of the spermatheca in females. When Hr39 is missing, spermatheca presents obvious defects, causing sterility. NLaz expression is highly repressed in Hr39-KO animals [91]. Additionally, NLaz has been identified as a seminal fluid protein in *D. melanogaster* and *D. simulans*, being transferred from males to females during mating [64]. Thus, these findings suggest a role for NLaz in sperm maturation.

NLaz expression is also related to the circadian clock. NLaz expression cycles only in the body when head and body gene expressions were analyzed separately [92].

Finally, as it happens with GLaz, no ligands have been described for NLaz so far. Therefore, it would be critical to provide more details about the molecular mechanism of action of this Lipocalin.

ApoD: Apolipoprotein D.

ApoD orthologs are found in species from the phylum chordata. Most of the available information comes from studies with human or mouse ApoD, and this section will focus on them.

The human ApoD gene (hApoD) is located in the third chromosome (3q26.2), and is composed of 3 exons and 4 exons. Chordate Lipocalins typically contain 7 exons, but exons 4 to 7 are fused in hApoD [5]. However, 6 exons are found in the mouse ApoD (mApoD) because of a singular 5'-UTR intron [93].

ApoD is broadly expressed and present in many fluids [94]. It is remarkable the high levels of ApoD expression during development. Especially interesting is the evolutionary pattern of ApoD expression in chordates. Amphioxus ApoD is expressed in tissues derived from mesoderm and endoderm, whereas in mammals and birds there is a shift to ectodermal derivatives [95]. Besides, cell type expression changes as well. ApoD is expressed by subsets of neurons and glial cells in chicken [96], while adult mouse, rat and humans express ApoD only in glial cells [97-100]. On the other hand, ApoD is the most consistently up-regulated gene during brain aging in mammals [101,102].

The hApoD mature protein, after secretory signal peptide removal, is composed by 169 amino acids. ApoD is a *kernel* Lipocalin with two disulfide bridges formed by Cys 8-114 and 41-165 [103]. Additionally, hApoD has a fifth Cys (residue 116) which allows for the formation of ApoD homodimers, or heterodimers with Apo-AII or Apo-B [104,105].

hApoD has been recently crystallized and its structure solved. The hApoD apoprotein structure was deposited in the Protein Data Bank (PDB: 2HZR), as well as the holoprotein form in complex with progesterone (2HZQ) [106]. The crystal structure
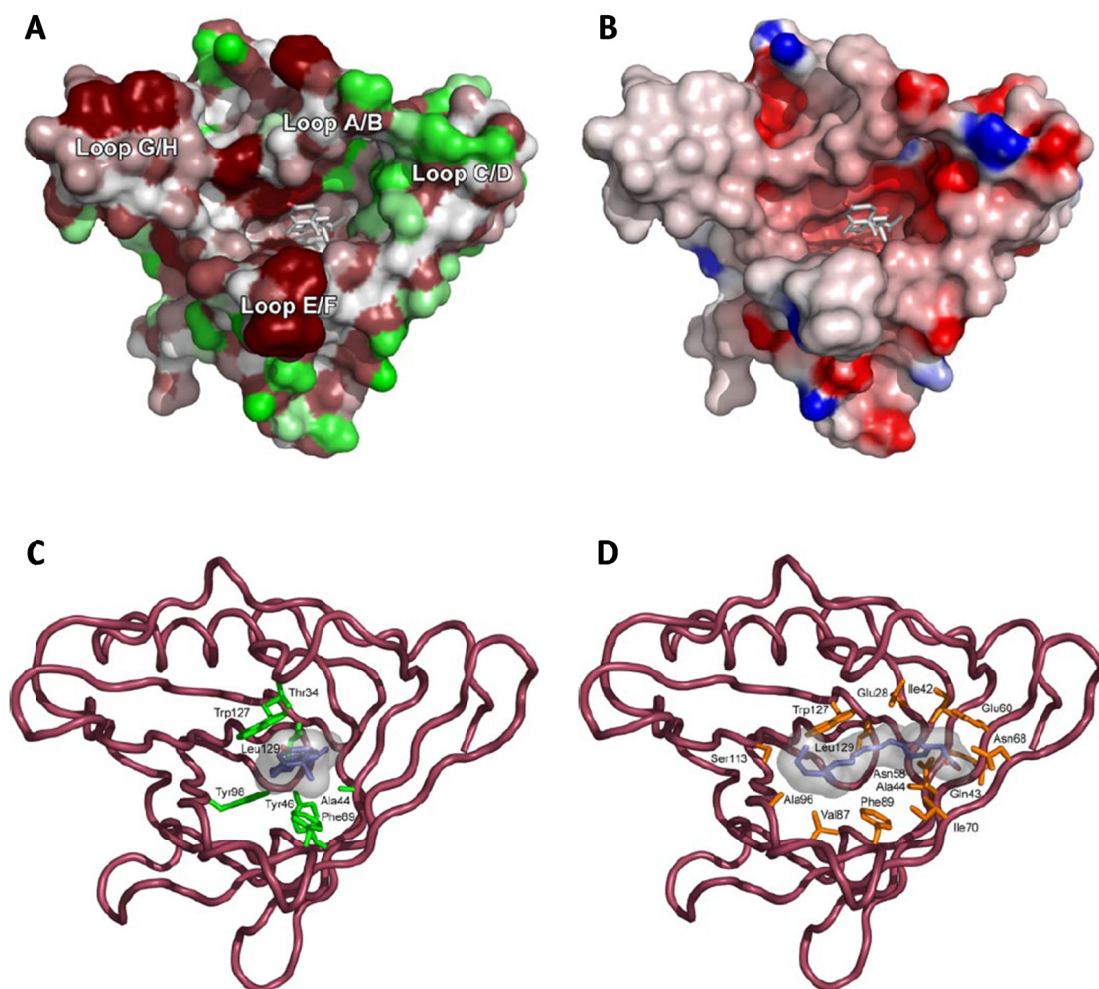
Figure 4. Representations of hApoD structure and its binding complexes.
 (A) Hydrophobic surface representation. Residues are colored according their hydrophobicity, from green (hydrophilic) over white (neutral) to brown (hydrophobic). Loops L1 (A/B), L5 (E/F) and L7 (G/H) expose hydrophobic residues. **(B)** Electrostatic surface representation. Negatively charged areas are colored in red and positively charged areas are in blue. **(C, D)** Top view of ApoD with progesterone bound (C) and a hypothetical model for AA (D). Ligand contacting amino acid side chains are represented (green in progesterone complex and brown in AA model). Ala44, Phe89, Trp127 and Leu129 are common contact points [106].

revealed a typical Lipocalin fold. The calyx has an outer diameter of around 45 Å at its broadest site and a length of around 40 Å. The cavity, mainly hydrophobic, is 15 Å deep and 10 by 15 Å wide at its entry. Loops 3 and 7 are widely open and do not limit the accessibility to the binding pocket. However, Glu-60 is close to the pocket entrance and gives a slightly negative charge. Loops 1, 5 and 7 expose bulky hydrophobic side chains [106] (fig.4A). This unusual feature in the Lipocalin family is in agreement with

the speculation of ApoD interacting directly with (high dense Lipocarticles) HDL and membranes [107].

The structures of free ApoD and its complex with progesterone are almost identical: Only small conformational changes in side-chains are detected. Progesterone, deeply introduced in the pocket, is tightly packed between the aromatic side chains of Phe89 and Trp127. Additionally, Tyr46 serves as an anchor forming a hydrogen bond with the ketone group of the progesterone [106].

Arachidonic acid (AA) is another well characterized ApoD ligand [108,109]. The ApoD crystal structure is compatible with AA binding, and a model of its binding shows ligand-protein contact points in common with the progesterone-ApoD crystal [106] (fig.4C and 4D).

The wide calyx of ApoD allows the binding of hydrophobic molecules of different nature. Besides progesterone [108-112] and AA [108,109], other molecules that ApoD can bind are retinol and retinoic acid [28]. The ability of ApoD to bind other molecules such as cholesterol [108,113] or bilirubin [109,114] is less clear.

hApoD has two glycosylation sites: Asn45 and Asn78. The hApoD crystal was obtained from recombinant protein produced in *Escherichia coli*, and thus glycosylation is absent in the resolved structure. In any case, two different glycosylation patterns have been reported: in plasma and in apocrine secretion. Interestingly, both, Asn45 and Asn78 oligosaccharide trees are terminated in sialic acid in plasma, whereas in apocrine secrection, only the Asn45 tree terminates in sialic acid [115,116].

Many studies in the mouse as a model organism have pointed out the protective properties of ApoD. Endogenously, mApoD is up-regulated under oxidative stress caused by PQ exposure [117], and this up-regulation is mediated by the JNK pathway [118], sharing activation networks with NLaz [67]. The ectopic expression of hApoD in flies and mice confers higher resistance to PQ [75,117] and OC43 coronavirus [119]. In contrast, mice lacking ApoD show a decreased resistance to PQ, higher levels of lipid peroxidation in the brain, and an altered transcriptional response to oxidative stress [117,118,120]. Moreover, ApoD presence is required to get a proper response after sciatic nerve injury [121].

A defensive ApoD capacity against oxidative insults has been corroborated in cellular and *in vitro* models. ApoD-KO astrocytes are more sensitive to PQ, and an exogenous addition of ApoD partially recovers this phenotype [118]. *In vitro* studies

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have provided a really interesting mechanistic model. n-HETE (hydroxyeicosatetraenoic acid) and n-HpETE (hydroperoxyeicosatetraenoic acid) are products of AA oxidation. ApoD will bind HpETEs and, with the involvement of Met93, will catalyze their reduction to the corresponding HETEs [122,123]. Met93 is conserved in mammalian ApoD and chicken ApoD but absent in more ancestral ApoD orthologs, as for example in salmon and amphioxus. Surprisingly, ApoD protein from amphioxus also shows antioxidant activity *in vitro* [124].

Interestingly, ApoD-KO mice show behavioral differences in locomotor and memory tests [117,118]. A putative explanation could be found in the fact that ApoD-KO mice suffer alterations in brain receptor and neurotransmiters composition [118,125-127].

In addition to the control of lipid peroxidation, many connections between ApoD and lipids are often established. Indeed, ApoD received its name because it was the fourth apolipoprotein discovered [128]. ApoD was identified in human plasma as a component of HDL, and less abundant in other lipoparticles [114,129]. Inside HDL particles, ApoD is predominant in HDL3 [130,131], which are small, dense and protein enriched lipoparticles. Typically, minor bioactive lipid components are preferentially associated with dense HDL3 particles [132].

Looking at fatty acids composition, ApoD-KO mice show an increase in a set fatty acid in brain extracts. Highlights linoleic acid (LA, 18:2), eicosadienoic acid (20:2), both are ω-6 FA, and docosahexaenoic acid (DHA, 22:6) [133]. An influence of ApoD over FA management was also observed in a cellular model. Cells tranfected with ApoD are able to incorporate higher amounts of AA. This fact suggested that ApoD could help to stabilize the plasma membrane and avoid AA oxidation [134].

In humans, ApoD is up-regulated in many pathological situations, including schizophrenia [135], Alzheimer's [136-138], Parkinson's disease [139] and multiple sclerosis [140]. Additionally, ApoD levels are altered in several types of cancers [94,141-143].

Several disease-causing mutations in ApoD have been described. Intron 1 and intron 2 polymorphisms are associated with a higher risk of Alzheimer's disease in African and Finnish populations respectively [144,145]. These polymorphisms could be

affecting mRNA processing. Three distinct missense mutations have been identified in an African-black population associated with alterations in lipid metabolism [146]. None of these mutations (F36V, Y108C, T158K) are located within the ligand-binding cavity, but the ApoD tertiary structure could still be compromised. For instance, Y108C is located really close to C114 and C116, and could likely affect the formation of disulfide bridges and result in a loss of function [106].

The study of Laz, GLaz, NLaz and ApoD experimental data discloses a number of points in common, which will synergically lead to a better understanding of their biological function.

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Objectives

Objectives

The aim of this thesis is to understand the biological processes where Lazarillo and related Lipocalins are involved, and how their function is affected by modifications in their ligand-binding ability or subcellular localization.

To accomplish this general plan, four specific objectives were carried out:

1. Analysis of GLaz and NLaz influence on the organism homeostasis, including gender and age as experimental variables. I will try to distinguish shared and specialized functions of GLaz and NLaz.

2. Comparative study of ligand-binding properties of Lazarillo and its homologs NLaz and hApoD. I will discern common binding patterns as well as specie-specific ligands that could underlie newly acquired functions.

3. *In vivo* identification of NLaz physiological roles that depend on ligand binding.

4. Evaluation of Laz protective properties. I will test whether this protection can be achieved from the plasma membrane.

Results

- # **Chapter 1**

"Sex-dependent modulation of longevity by two Drosophila homologues of human Apolipoprotein D, GLaz and NLaz".

- # **Chapter 2**

"Lipid binding properties of human ApoD and Lazarillo-related Lipocalins: functional implications for cell differentiation".

- # **Chapter 3**

"Ligand-binding dependent functions of the Lipocalin NLaz: an in vivo study in Drosophila".

- # **Chapter 4**

"Grasshopper Lazarillo, a GPI-anchored Lipocalin, increases Drosophila longevity and stress resistance, and functionally replaces its secreted homologue NLaz".

Chapter 1

"Sex-dependent modulation of longevity by two Drosophila homologues of human Apolipoprotein D, GLaz and NLaz".

Ruiz M, Sanchez D, Canal I, Acebes A, Ganfornina MD.

"Sex-dependent modulation of longevity by two Drosophila homologues of human Apolipoprotein D, GLaz and NLaz"

Sex-dependent modulation of longevity by two Drosophila homologues of human Apolipoprotein D, GLaz and NLaz.

Summary

Apolipoprotein D (ApoD), a member of the Lipocalin family, is the gene most up-regulated with age in the mammalian brain. Its expression strongly correlates with aging-associated neurodegenerative and metabolic diseases. Two homologues of ApoD expressed in the Drosophila brain, Glial Lazarillo (GLaz) and Neural Lazarillo (NLaz), are known to alter longevity in male flies. However, sex differences in the aging process have not been explored so far for these genes. Here we demonstrate that NLaz alters lifespan in both sexes, but unexpectedly the lack of GLaz influences longevity in a sex-specific way, reducing longevity in males but not in females. While NLaz has metabolic functions similar to ApoD, the regulation of GLaz expression upon aging is the closest to ApoD in the aging brain. A multivariate analysis of physiological parameters relevant to lifespan modulation uncovers both common and specialized functions for the two Lipocalins, and reveals that changes in protein homeostasis account for the observed sex-specific patterns of longevity. The response to oxidative stress and accumulation of lipid peroxides are among their common functions, while the transcriptional and behavioral response to starvation, the pattern of daily locomotor activity, storage of fat along aging, fertility, and courtship behavior differentiate NLaz from GLaz mutants. We also demonstrate that food composition is an important environmental parameter influencing stress resistance and reproductive phenotypes of both Lipocalin mutants. Since ApoD shares many properties with the common ancestor of invertebrate Lipocalins, we must benefit from this global comparison with both GLaz and NLaz to understand the complex functions of ApoD in mammalian aging and neurodegeneration.

1. Introduction

Aging and the regulation of an organism lifespan are complex traits that are the result of multiple factors, many of which are clearly inheritable. This fact has guided the quest for genes that regulate longevity. Many genetic interventions that alter the length of life have been documented in model organisms ranging from nematodes to rodents [for recent reviews see 1,2]. They reveal the involvement of a basic set of conserved pathways, being the nutrient sensing pathways (the insulin and insulin-like growth factor signalling (IIS) and the target of rapamycin (TOR) pathway) the best known examples [3-6]. These studies are leading the search for genes in humans that perform similar functions and can become candidates for pharmacological interventions to achieve a longer and healthier life. Either genetic association studies or transcriptome analysis are the main approaches to aging research in humans [1,2,7]. It is striking that in a meta-genome study where the changes in brain transcriptome have been compared between mice, monkeys and humans, Apolipoprotein D (ApoD) was revealed as the most robust age-dependent up-regulated gene in the brain conserved across species [8]. The same is true for a meta-array analysis using mouse, rat and human expression data in different tissues, where ApoD appears as the gene most consistently over-expressed with age [9]. Not surprisingly, the expression of ApoD is highly boosted by a collection of traumatic, pathological and degenerative nervous system conditions in humans as well as by cancer and age-related metabolic diseases [reviewed by 10].

It is widely accepted that many of the genes that regulate longevity participate in defence or repair mechanisms that counteract the accumulation of damage to cell components with age. Thus, many of these genes are also thought to be important for the onset of a wide array of diseases where age is a major risk-factor. Neurodegenerative diseases are no exception. The study of ApoD and its homologous genes in model organisms should therefore prove relevant to the understanding of aging in general, and of the aging nervous system in particular.

ApoD is a member of the Lipocalin family, small secreted lipid-binding proteins. At least three other Lipocalin genes are also expressed in the nervous system of mammals: retinol binding protein, RBP [11], L type prostaglandin D synthase, L-PGDS [12], and Lipocalin 2 (also called NGAL) [13]. In Drosophila, two genes are the closest homologues to ApoD: Glial Lazarillo (GLaz) and Neural Lazarillo (NLaz). They are expressed in the nervous system in a cell-type specific manner [14]. A third Drosophila Lipocalin, Karl, is not expressed in the nervous system [15]. Both GLaz and NLaz are known to regulate stress resistance and lifespan in male flies [15-17]: loss-of-function mutants have a reduced longevity while gain-of-function manipulations result in extended lifespan. GLaz also influences fat storage [16] while NLaz regulates the metabolic response to stress by systemic inhibition of the IIS pathway [15]. Protection against oxidative stress in the nervous system has also been demonstrated for mammalian ApoD [18], revealing a sufficient degree of functional conservation between ApoD and the Lazarillo genes expressed in the nervous system of Drosophila.

Single gene analyses of aging mutants often overlook pleiotropy and effects of other gene family members expressed in the same tissues. Also, in a few cases, the original interpretations derived from studies of classical longevity mutants [19,20] have been challenged when other physiological, environmental [21], or genetic [22] factors have been taken into account. This fact highlights the need for a comprehensive understanding of the many effects of single gene mutations, as well as of the gene-environment interactions that are relevant to aging and lifespan determination.

Particularly, sex differences in the aging process are a very relevant issue that has not been explored so far for these genes. Almost all the work published on this group of Lipocalins (ApoD/Lazarillo) has been performed in males only. This work was triggered by the consistent

observation that one of the Drosophila Lipocalins (GLaz) alters longevity in a sex-dependent manner in any genetic background tested: only null mutant males reduce their longevity, while null mutant females live as long as wild type flies. The lack of NLaz, on the other hand, shortens lifespan in both sexes. This work is at the same time an attempt to understand the origin of such a sex-specific phenotype, and an exhaustive search for the physiological processes in which these Lipocalins intervene, with the final goal of uncovering the interesting labor division structure of the two Drosophila Lipocalins homologous to ApoD.

2. Material and Methods

2.1. Fly strains and husbandry.

Flies were grown in a temperature-controlled environmental incubator at 25ºC, 60% relative humidity, under a 12h light-dark cycle.

Food recipes: (A) *"Valladolid-standard" food:* wet yeast 84 g/l, NaCl 3.3 g/l, agar 10 g/l, wheat flour 42 g/l, apple juice 167 ml/l, and propionic acid 5ml/l. (B) *"Carolina" Instant Food* (Carolina Biological Supplies). (C) *"Caltech" food:* dry yeast 15 g/l, agar 4.5 g/l, dextrose 50 g/l, sucrose 25g/l, corn meal 83 g/l, phosphoric acid 0.6 ml/l, and propionic acid 4ml/l..

GLaz and *NLaz* null mutants were generated in a w^{1118} background as previously described [16,23]. Mutations were outcrossed into a *Canton-S* wild type strain for five generations to obtain a homogeneous background with less than 5% of the original genomic background. Isogenic sister lines containing the wild type allele of *GLaz* and *NLaz* (line *G10*: *GLaz* $^{+/+}$, *NLaz* $^{+/+}$), or the mutant allele (lines *G2*: *GLaz* $^{-/-}$, *NLaz* $^{+/+}$ and *N5*: *GLaz* $^{+/+}$, *NLaz* $^{-/-}$) were selected by PCR screening for GLaz, and PCR followed by SceI restriction digest for NLaz. In a last round of outcrosses, a set of fly strains with white eyes were generated in the same way outcrossing with w^{1118}-*CS₁₀.* The line w^{1118}-*CS₁₀,* a gift from Seymour Benzer (Caltech), is a 10 generation outcross of w^{1118} into the *Canton-S* background. A set of lines with white eyes were thus generated: lines *CGW* and *CNW14* as wild

type isogenic controls (*GLaz* $^{+/+}$, *NLaz* $^{+/+}$), *GW* line (*GLaz* $^{-/-}$, *NLaz* $^{+/+}$) and *NW5* line (*GLaz* $^{+/+}$, *NLaz* $^{-/-}$). Most of the experiments were carried out with *G10* as wild type control and *G2* and *N5* as experimental lines unless otherwise noted.

2.2. Wolbachia test.

The absence of infection by *Wolbachia pipiens* in all fly strains was tested by PCR of genomic DNA extracts. The following primers against the rRNA-16S gene of Wolbachia were used:
5'-GAAGATAATGACGGTACTCAC-3',
5'-GTCAGATTTGAACCAGATAGA-3' and
5'-GTCACTGATCCCACTTTA-3'.
Program: 2 min 94ºC; (30 sec 94ºC, 30 sec 60ºC, 45 sec 72ºC) x15; (30 sec 94ºC. 30 sec 52ºC, 75 sec 72Cº) x15; 7 min 72ºC. DNA from Wolbachia-infected *Dilofilaria immitis* worms was used as positive control. Primers and a positive control DNA were kindly provided by Dr. F. Simon (Univ. Salamanca).

2.3. Lifespan analysis.

At least 100 flies of each genotype were collected within 24h of eclosion, separated by sex under brief CO_2 anaesthesia, housed in groups of 25, and maintained at 25ºC. Dead flies were counted and surviving flies were transferred to new vials with fresh food twice a week.

2.4. Oxidative stress, starvation, and desiccation resistance.

Flies collected as described for the longevity analysis were separated by sex in groups of 25 when they were 3 days old. Application of different stressors was performed as follows.

Paraquat treatment: After a period of dry starvation (3 hours) flies were transferred to vials with filter papers soaked with 1 ml of 10% sucrose-20mM paraquat (Sigma). Incubation proceeded in the absence of light and deaths were scored every 4-8 h.

Wet starvation treatment: Starting at 3 days of age, flies were transferred to vials with 1% agar in water. Dead flies were scored every 4-8 h.

Dry starvation treatment (desiccation): Starting at 3 days of age, flies were transferred to empty vials. Dead flies were scored every 4-8 h.

2.5. Body weight, fat content, and protein content.

Total wet weight, dry weight after evaporation of water, and fat-free dry weight after extraction of neutral lipids with diethyl ether, was performed as previously described [16]. The protein content per fly was determined with the microBCA kit (Pierce). Three independent experiments with measurements in triplicate were performed at 3 and 30 days of age.

2.6. Lipid peroxidation levels.

A spectrophotometric assay was used to determine the concentration of free malondialdehyde (MDA-586, Bioxytech) as previously described [16]. Three independent experiments with measurements in triplicate were performed at 3 and 30 days of age.

2.7. Population reproductive output.

Three populations of 10 males x 10 virgin females per genotype were housed in 50 ml food bottles (250ml plastic bottle). Eggs laid were counted every day after transferring parents into a new bottle, and the number of adults emerging from each bottle was counted till the culture was exhausted. Egg and adult progeny production was scored during 7 days (age of parents: 5-11 days). An estimate of fecundity and fertility per female in the population per day was calculated.

2.8. Courtship behaviour and reproduction.

Courtship and mating tests were performed during 60 min using male-female pairs of 4-5 day-old flies under a watch glass used as an observation chamber, as described previously [24]. The courtship index (C.I.) represents the proportion of time a male spends actively courting the female for 10 min. Copulation features as mating time, mating latency and mating performance (%) were scored for 60 min. Offspring numbers and sex ratio (male/female) from each mated female that yielded progeny were scored during two weeks.

2.9. Hunger driven short-term food intake behaviour.

Two groups of 20 flies per sex, previously food-deprived for 19h on 1% agar in water, were fed for 5 minutes on 4ml of 10% sucrose, 1% agar, supplemented with 0.5% food dye FD&C Blue No.1 (E-133 European Union, provided by Proquimac). After feeding, the flies were anesthetized with CO_2 and homogenized in PBS. After a short spin to pellet tissue debris, absorbance of the homogenate was measured at 625nm. Background absorbance was corrected with fly samples of the same genotype subjected to the same procedure without the dye. The experiment was repeated twice and performed with the white-eyed fly lines (see above) to avoid disturbances of the absorbance spectrum due to the eye pigment.

2.10. Circadian spontaneous locomotor activity.

Individual flies at 3 days of age were transferred to monitor tubes containing fresh food. Their locomotor activity was monitored using the Drosophila Activity Monitoring System (Trikinetics, Waltham, MA) with a cumulative sampling rate of 15 min, in a temperature-controlled environmental incubator at 25ºC, 60% relative humidity, under a 12h light-dark cycle. Five monitors were used, each housing 5 flies/sex/genotype and a sixth monitor recorded light, temperature and humidity. Monitoring was performed for 6 days, but analysis included data starting on day 3 (6 days of age) to avoid variations due to habituation to the housing conditions.

2.11. Climbing ability.

Flies were collected and separated by sex in groups of 10 as described for the longevity analysis. Tests were performed as previously described [16], after a minimum of 24 hours after anaesthesia, at 3 and 30 days of age.

2.12. qRT-PCR: GLaz and NLaz gene expression.

Flies of each genotype were collected within 24 hours of eclosion, separated by sex under brief CO_2 anaesthesia, and housed in groups of 25. They were transferred to fresh food vials twice a week until the day of processing (3, 30 and 60 days). When preceded by wet starvation, gene expression measurements were performed at 15 hours of treatment (see above). We separated heads and

bodies. RNA extracted from heads should represent a good approximation to the expression in the brain, which makes most of the head tissue. RNA extracted from the body, which includes the relatively large thoracic ganglia, represents an approximation to the global expression level in whole flies.

Heads from 50 flies were cut and total RNA was extracted using TRIzol (Invitrogen). RNA concentration was measured with a Nanodrop spectrophotometer. Reverse transcription was done with the PrimeScript™ RT reagent Kit (Takara) according to the manufacturer instructions by using Oligo-dT primers and random hexamers. Primers of equal amplification efficiency were designed for GLaz (5'-GCGAACAATCGAAGTTTTCC-3' and 5'-ACAAGATGGCGAAGTTCTCG-3'), NLaz (5'-CGAGTACGCAGCCTATCCAT-3' and 5'-CCAGGTAGTTGGCCTTCGT-3') and the ribosomal protein L18 (RPL18, endogenous control) (5'-AGAACCGACCCCAAATCC-3' and 5'-CGACCACGATGGTAGACTCC-3').

Quintuplicate PCR reactions were performed for each RNA sample using the SYBR® *Premix Ex Taq™* (Takara) according to the manufacturer instructions in a Rotor-Gene RG-3000 thermal cycler (Corbett Research). Cycling conditions were 30 sec 95ºC, (5 sec 95ºC, 15 sec 55ºC, 15 sec 72ºC) x 35.

Melting curves were established for all conditions to check for the absence of unspecific amplifications.

RNA transcription levels were determined by the method of direct comparison of C_T values and relative quantities calculated by the $\Delta\Delta C_T$ method [25]. Transcripts were normalized to RPL18 for each condition. We transform the data as Log $2^{-\Delta\Delta Ct}$ and then represent them as fold changes. In this way, up- and down-regulations are symmetrically scaled around one. Statistically significant differences of pairwise gene transcriptional changes were evaluated with a Mann-Whitney U-test, using ΔC_T of each replica (calculated by subtracting the average CT of the reference gene). The level of significance was set at $p < 0.05$.

2.13. Statistics and principal component analysis.

Statistical analyses were performed with Statgraphics plus (v 5.0) software. Student's t-Test was used to assess two-sample comparisons (with the exception of qRT-PCR data, see above) with $p < 0.05$ as threshold for significant changes.

To find patterns of covariation among all the physiological parameters measured, we performed a principal component analysis (PCA). The average of each parameter was arranged in two matrices (Tables S1-S2), one with 25 variables measured at 3 days of age, and another with 7 variables measured at 3 and 30 days of age. Neither longevity data nor Lipocalin expression data were included, in order to render the analysis "blind" to the genotype (the dependent variable) and lifespan (the variable we want to explain). The analysis was also blind to sex and age, since it is performed for 6 independent entries (2 sexes per genotype) or 12 independent entries (6 entries per age).

3. Results and Discussion

3.1. The nervous system Lipocalins GLaz and NLaz influence longevity in a sex-specific manner.

The loss of either GLaz [16] or NLaz [15] is known to reduce the lifespan of male flies. However, here we report that females reduce their lifespan when NLaz is absent, but their lifespan is similar to control female flies in the absence of GLaz (Fig. 1A, B). The same results are obtained before and after changing the genetic background for both mutations (from w^{1118} to *CantonS*, not shown; median survival effects stated in Fig. 1 legend). The absence of Wolbachia infection, as a potential lifespan-reducing cause, was confirmed by PCR tests (see Fig. S1 and section 2.2. in Methods).

These observations led us to search for causal links that might explain how GLaz regulates longevity differently in males and females, and to find the fine structure of functional relationships between the two nervous system Drosophila Lipocalins.
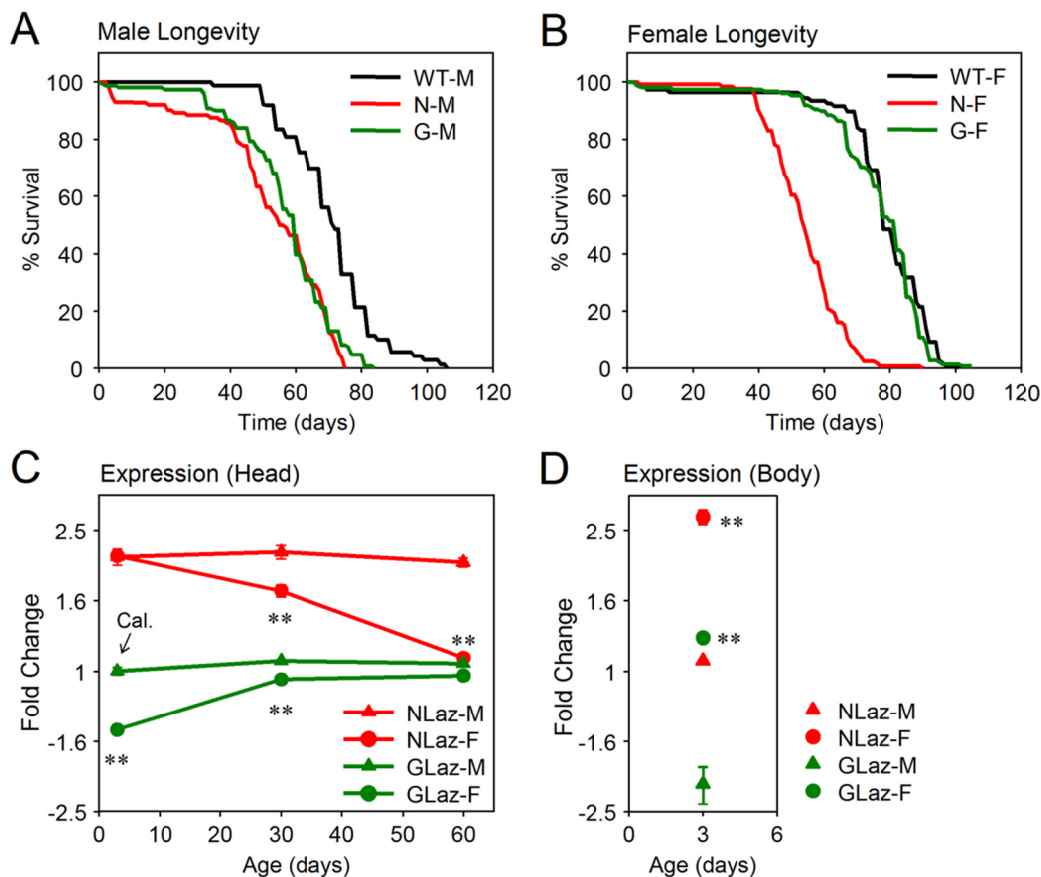
Figure 1. Lipocalin loss-of-function mutants show sex-specific patterns in longevity that can be related to their different spatiotemporal patterns of expression.

(A) Male lifespan determination of *GLaz*^{-/-} (G), *NLaz*^{-/-} (N) compared to isogenic wild type flies (G10 line). Median survival time is reduced by 15.5% in the *GLaz* mutant ($p<10^{-5}$), and by 22.5% in the *NLaz* mutant ($p<10^{-5}$). *(B)* Lifespan is also reduced in *NLaz* females (30.8% reduction, $p<10^{-5}$), but not in GLaz mutant females ($p=0.034$). Reductions in median survival in the *w1118* genetic background (not shown) were 27.8 and 34.3% for *GLaz* and *NLaz* mutant males, and 37.2% for *NLaz* mutant females. Log-rank test was used for statistical analysis. N=114-213/genotype. *(C)* Age effect in NLaz and GLaz transcription in wild type fly heads assayed by qRT-PCR. *(D)* Transcription levels of NLaz and GLaz in wild type body RNA extracts. The RPL18 gene is used as endogenous control. The expression level of GLaz in 3 day-old male heads is used as calibrator (Cal.) for both graphs. Symmetrical Fold Change was chosen for data representation for equal display of down-regulations and up-regulations. Statistical differences assayed by Mann-Whitney U-test. * $p<0.05$ ** $p<0.01$.

3.2. Male and female flies differ in their temporal and spatial regulation of Lipocalin genes expression.

Since both Lipocalins are expressed within and outside the nervous system [14-16], we investigated how much of their expression is contributed by the brain in young flies. We focused our study on the expression changes in the brain, either through aging or upon stress. These data can then guide the comparisons between the fly Lipocalins and the mammalian homologue, ApoD,

known to be the most up-regulated gene in the aged brain.

Brains of male flies have stable expression levels of both Lipocalins throughout life; NLaz expression being higher than that of GLaz at all ages explored (Fig. 1C, triangles). In contrast, the brains of females show interesting and opposite expression changes with age: GLaz increases and NLaz decreases with aging (Fig. 1C, circles). Curiously, it is the sum of the two Lipocalins what is kept roughly constant throughout aging in

females. The increase of GLaz expression with age in female brains echoes the pattern followed by ApoD in mice, macaques, and humans [8].

The fact that NLaz expression in females shows an opposite pattern, suggests that different regulatory elements are responsible for the temporal control of each Lipocalin, and that GLaz gene regulation must have commonalities with the age-dependent networks controlling ApoD expression in the mammalian CNS. Also, these gene-regulation networks differ with sex for both Drosophila Lipocalin genes.

Interestingly, the level of Lipocalins in the brain of young flies correlate with their longevity reduction when each Lipocalin is missing. Note that GLaz expression is the lowest in young wild type females, and its absence does not alter longevity. These data support the idea that early adulthood represents a critical period for Lipocalin function, affecting parameters that will result in lifespan alterations later on.

The relative contribution of head and body in Lipocalin expression also varies with sex (Fig. 1D). In young females, GLaz has the highest ratio body/head. In males, the expression of both Lipocalins is higher in the brain than in the body, with NLaz again showing higher expression levels than GLaz.

3.3. Starvation triggers a differential expression of Lipocalins in the brain.

The males of both mutants had been already described as starvation sensitive [15,16], and NLaz expression is known to be induced by starvation [15]. However, our previous works used different regimes of starvation (wet or dry starvation) and were performed only in males. Here we analyze in detail how brain expression of the two Lipocalins is altered upon food deprivation (without water deprivation, to avoid a more complex stress).

Expression of NLaz and GLaz in the fly head is differentially induced upon food deprivation in wild type flies (Fig. 2A,B). Interestingly, no induction of NLaz is observed in the head tissue where its expression is constitutively high. These data are compatible with NLaz working in the

control of systemic metabolism and with the fat body tissue being a source of NLaz under stress conditions (as we had previously shown using a dry starvation paradigm) [15]. In contrast, GLaz is highly induced in the head upon starvation, suggesting that GLaz might be involved in a nervous system regulatory loop triggered by nutrient deprivation. These results show the existence of differential domains of action of the two Lipocalins.

3.4. A multivariate analysis of the physiological parameters that contribute to lifespan modulation by Lipocalins.

Given that longevity is the result of an intricate puzzle of interactions between genetic components and physiological and environmental variables, we need to uncover the full complexity of effects of a single mutation in order to learn how each gene alters the network of causal links underlying lifespan regulation.

With that in mind, we have undertaken an analysis of multiple parameters and their dependence on NLaz and GLaz expression. We have screened variables including metabolic (levels of various metabolites, stress-nutrition relationships), behavioral (reproduction, food intake, locomotor activity), as well as stress resistance, and analyzed them by principal component analysis (PCA). PCA has been successfully used in a multitrait study by Baldal and others [26] pertaining starvation resistance and aging evolution, and by Andersen and others [27], in a study uncovering how heat stress and age induced maternal effects influence physiological parameters in the offspring. This type of analysis extracts features of the parameters variation as a set of new variables (the principal components, PCs) that are uncorrelated with one another and successively account for maximal amounts of variation. Our analysis was performed with 25 variables at 3d of age (Fig. 3A-B; Fig. S2A-B), and a subset of 7 variables mainly related to metabolic parameters at 30d of age (Fig. 3C, Fig. S2C-D). Values obtained for all variables are listed in supplementary tables (Tables S1-S2). The

PCA has uncovered sex, genotype, and age-specific patterns.

Some life history parameters clearly separate genotypes. The two principal components explaining 69% of the variation in the sample studied at 3d of age separate wild type flies from either mutant, and in turn segregate each mutant into opposite corners of the multivariate space (Fig. 3A). This pattern reflects the existence of specialized functions separating Lipocalin mutants from each other (component 1), as well as common functions separating both mutants from the wild type (component 2). For example, lipid peroxidation and relative fat content contribute to the separation of wild type from either mutant (black labels in Fig. 3B). Variables in the domain of reproduction, locomotor activity, food intake and metabolism (relative water content, starvation resistance) separate GLaz from NLaz mutants (red and green labels in Fig. 3B).

Other life history parameters contribute to sex or age differences regardless of genotype (Fig. S2). Metabolic parameters (wet weight, protein content or lipid peroxidation), response to stress (starvation, desiccation or paraquat resistance) and diurnal *vs.* nocturnal locomotor activity are important variables in the separation of sexes in all genotypes (Fig. S2A-B). In the analysis of variables at two ages (Subset 2) it is interesting that only females are segregated by age in the component 1 by 2 plot (explaining 63% of de variance). Metabolic parameters (relative water content in opposition to relative fat) and climbing ability have the most weight for the separation of young female flies from old female flies in all genotypes (Fig. S2C-D).

3.5. Sex-specific modulation of longevity by GLaz is dependent on metabolic parameters related to protein homeostasis.

Studying the multivariate space, we searched for components that would cause GLaz mutant females to cluster with WT females, and would therefore explain the observed longevity curves. Interestingly, the stronger association of *GLaz*^{-/-} females with wild type flies arises in the PCA

analysis performed for the subset of 7 variables measured at both 3 and 30d of age (Fig. 3C). The association only occurs for young GLaz mutant females, further supporting the idea that the function of Lipocalins in early adulthood is crucial for longevity determination. Young wild type females, with their low levels of GLaz expression, show a combination of parameters similar to that of *GLaz*^{-/-} young females. Old GLaz mutant females, however, are displaced to the opposite corner of the component 2 by 3 plane of the multivariate space (arrows in Fig. 3C). The variables with more weight in the "mutant GLaz females – wild type" association are the protein content and relative fat free content (with a negative weight, red labels) and the wet weight and relative water content (with a positive weight, green labels). Variables related to lipids (relative fat content or lipid peroxidation levels) do not contribute to this association.

Our analysis indicates that the patterns of metabolism, especially during early adulthood, contribute importantly to the final outcome in longevity, resulting in a shorter lifespan in NLaz mutants and in GLaz mutant males, but a similar lifespan in GLaz mutant females and wild type flies. Some of the parameters included in our PCA analysis are further described and discussed hereafter.

3.6. Energy intake and homeostasis are altered through aging in the absence of Lipocalins.

The loss-of-function mutants of both Lipocalins show pleiotropic effects at different levels of energy management (intake-balance-expenditure, Fig. 4A) that contribute to their segregation in the multivariate space (Fig. 3A-B). Particularly, the PCA analysis shows that energy management differences between genotypes are part of the functional specialization between both Lipocalins, and also contribute to the unaltered lifespan in females lacking GLaz (Fig. 3C).

In addition, the behavioral response to starvation is increased in young *NLaz*^{-/-} flies, but not in *GLaz*^{-/-} flies (Fig. 4B). We have previously
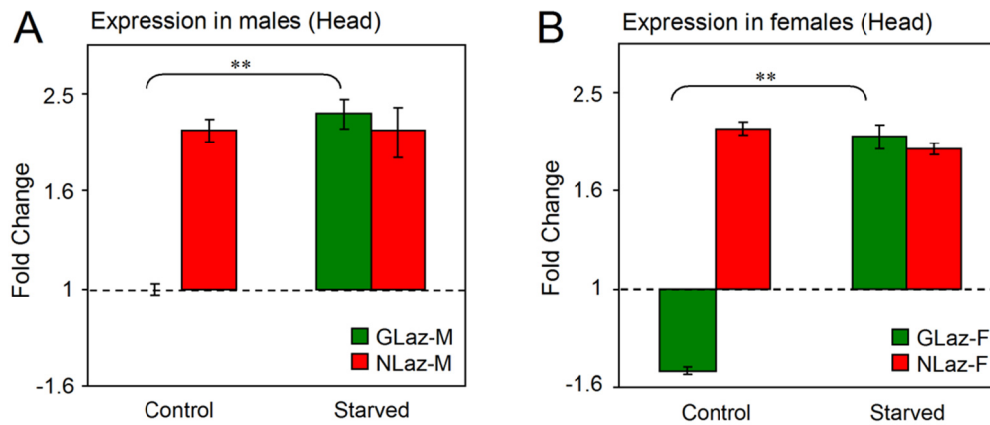
Figure 2. Differential expression of Lipocalins in the brain upon starvation.

(A) Changes in expression of GLaz and NLaz in the head of wild type male flies at 3 days of age in control conditions and after 15 hours of wet starvation treatment. *(B)* Changes in expression of both genes in the same conditions as in A, but in females. qRT-PCR using RPL18 as endogenous control and the value of GLaz in 3d wild type male heads as calibrator (Cal.) for both graphs. Symmetrical Fold Change was chosen for data representation for equal display of down-regulations and up-regulations. Statistical differences assayed by Mann-Whitney U-test. ** $p < 0.01$.
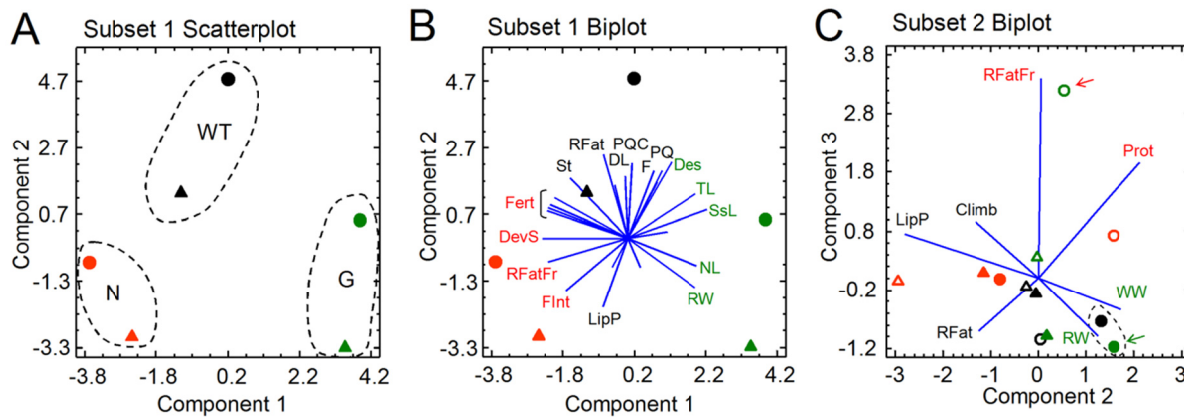
Figure 3. Principal Component Analysis of variables excluding Lipocalin gene expression and lifespan parameters.

(A-B) Scatterplot and biplot of the two principal components explaining 69% of the variation in the sample of 25 variables measured in 3 day-old flies (Subset 1). The two-dimensional space defined by these components separate wild type flies from either mutant, and sends each mutant to a separate corner of the multivariate space reflecting functional specialization. Acronyms for variables with small weight in the components are omitted in the biplot. *(C)* Biplot of components 2 by 3 extracted from subset 2 (7 variables measured in 3 day- and 30 day-old flies). Young $GLaz^{-/-}$ females are closely related to wild type females (green arrow), while the old $GLaz^{-/-}$ females are projected in the opposite corner of the two dimensional space (red arrow). Symbols: black = wild type, red = $NLaz^{-/-}$, green = $GLaz^{-/-}$, circles = females, triangles = males, filled symbols = 3 day-old flies, open symbols = 30 day-old flies. Acronyms: Climb: climbing ability; Des: dessication resistance; DevS: developmental success; DL: diurnal locomotor activity; F: fecundity; Fert: fertility; FInt: food intake; LipP: lipid peroxidation; NL: nocturnal locomotor activity; PQ: paraquat resistance; Prot: protein content; RFat: relative fat content; RFatFr: relative fat free weight; RW: relative water content; SSL: sunset locomotor activity; St: starvation resistance; TL: total locomotor activity; WW: wet weight.

described that overall weight in males is increased in the absence of NLaz [15], and decreased in the absence of GLaz [16]. Here we show that *NLaz*$^{-/-}$ flies start life with a different body size and increase their fat content with age, while *GLaz*$^{-/-}$ mutants maintain a low fat content throughout life in both sexes (Fig. 4C). These results are in line with the food intake increase observed in NLaz mutant flies (Fig. 4B).

Energy storage (in the form of fat storage) is clearly altered in opposite directions by the two Lipocalins. However, only male and not female GLaz mutants shorten their lifespan. On the other hand, NLaz mutants show both increased food intake (this work) and increased IIS activity [15]. In order to understand the relevance of food storage and food intake in lifespan determination we need to know how Lipocalins influence energy expenditure. We have analyzed locomotor activity patterns and reproduction as the major forms of energy output.

3.7. Lipocalins influence the patterns of daily energy expenditure in locomotor activity.

The patterns of daily spontaneous locomotor activity also differ with genotype and sex (Fig. 5), showing complementary patterns. Overall activity is higher in *GLaz*$^{-/-}$ males (Fig. 5A,C), which are more active than wild type flies especially at night (Fig. 5D). *NLaz*$^{-/-}$ females are overall less active (Fig. 5B,C), but the difference is mostly due to diurnal activity (see Table 1).

Shortened resting periods at night have been reported to correlate with aging [reviewed by 28]. Our data show that the highest sex difference within a given genotype is the large increase in nocturnal activity of *GLaz*$^{-/-}$ males (Fig. 5D) compared to mutant females of the same genotype. Interestingly, *GLaz*$^{-/-}$ female activity is more similar to the wild type pattern (Fig. 5 and Table 1). One could argue that this effect will contribute to an accelerated senescence of *GLaz*$^{-/-}$ males and therefore a shortening of lifespan occurring only in males of this genotype. However, the lower nocturnal activity observed in males *NLaz*$^{-/-}$ is not accompanied by an increase in lifespan. Our data support the idea that the amount of activity *per se* does not correlate with longevity, in agreement with Koh and others [29], who report sleep fragmentation rather than total sleep time as the relevant parameter influencing longevity.

Figure 4. Energy intake and homeostasis are altered in Lipocalin mutants.
(A) Variables exploring the flow of energy in Lipocalin mutants. *(B)* Behavioral response to starvation is increased in *NLaz*$^{-/-}$ but not in *GLaz*$^{-/-}$ mutants. *(C)* Age and genotype effect in relative fat content. *GLaz*$^{-/-}$ mutants have a constitutive reduction in their neutral fat stores while fat increases with age in *NLaz*$^{-/-}$ mutants. Values normalized with respect to wild type young males. Statistical differences assayed by Student's T-test. * $p<0.05$; ** $p<0.01$.
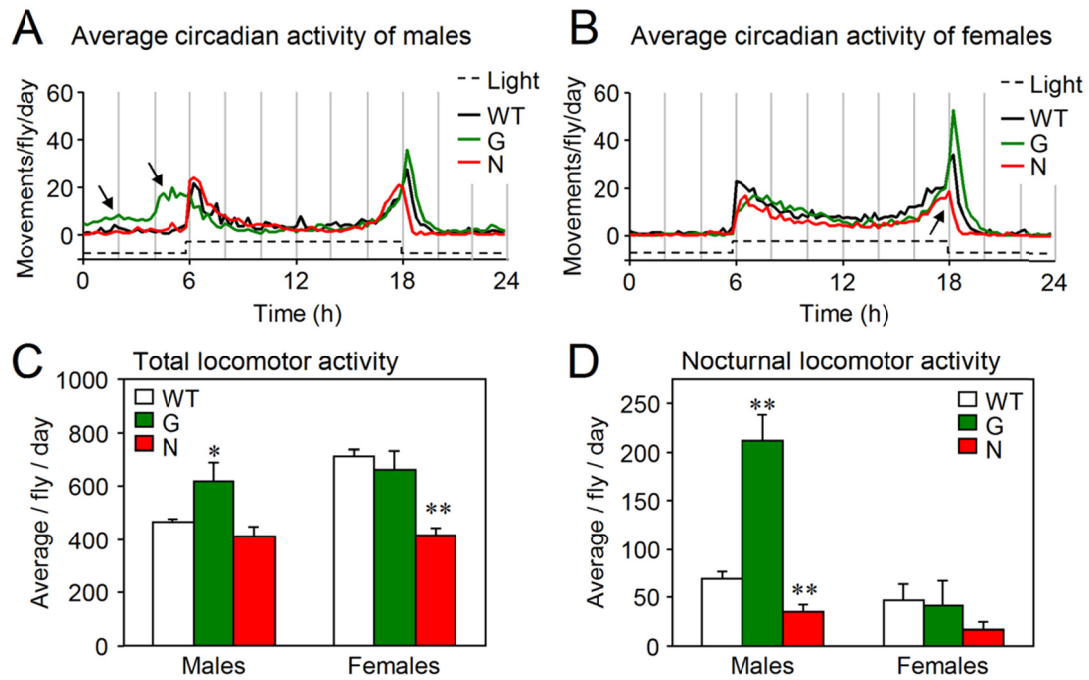
Figure 5. Lipocalin mutants show sex-specific alterations in their circadian locomotor activity.
(A-B) Average of three 24h periods of locomotor activity in wild type, *GLaz*^-/- and *NLaz*^-/- males (A) and females (B).
(C) Average total locomotor activity is higher in *GLaz*^-/- males and lower in *NLaz*^-/- females . *(D)* Locomotor activity during the dark phase. *GLaz*^-/- males are much more active at night than the wild type, while *NLaz*^-/- males reduce their activity. N=25/sex/genotype. Statistical differences assayed by Student's T-test. * p<0.05; ** p<0.01.

Genotype/Sex	Total Activity	Diurnal Activity	Nocturnal Activity	Sunrise Activity	Sunset Activity
WT males	463.1 ± 14.2	203.69 ± 9.9	69.23 ± 7.4	76.21 ± 11.6	113.97 ± 18.6
GLaz^-/- males	$\mathbf{615.22 \pm 52.3^a}$	$\mathbf{137.19 \pm 31.8^a}$	$\mathbf{212.07 \pm 36.1^b}$	$\mathbf{116.64 \pm 18.4^a}$	149.33 ± 17.8
NLaz^-/- males	412.03 ± 35.1	188.89 ± 35.7	$\mathbf{37.74 \pm 7.7^b}$	$\mathbf{99.03 \pm 3.0^a}$	89.37 ± 4.2
WT females	710.05 ± 27.5	416.43 ± 17.0	46.16 ± 17.3	93.85 ± 5.4	153.61 ± 23.7
GLaz^-/- females	660.17 ± 74.7	$\mathbf{361.89 \pm 29.4^a}$	41.79 ± 25.6	$\mathbf{53.12 \pm 1.7^b}$	203.37 ± 21.9
NLaz^-/- females	$\mathbf{415.19 \pm 28.7^b}$	$\mathbf{264.43 \pm 15.4^b}$	17.19 ± 8.0	$\mathbf{53.98 \pm 3.7^b}$	$\mathbf{59.59 \pm 2.9^b}$

Table 1. Patterns of daily locomotor activity in Lipocalin mutants.
Average of three 24h periods of locomotor activity in wild type, *GLaz*^-/- and *NLaz*^-/- flies. Sunrise and sunset are defined as the period of the light switch ±1h. N=25/sex/genotype. Bold lettering indicates statistical differences assayed by Student's T-test. [a] Different from WT (p<0.05). [b] Different from WT (p<0.01).

3.8. Reproductive output and courtship behavior is differentially altered in each Lipocalin mutant.

Reproductive parameters separate GLaz from NLaz mutants in the multivariate space analyzed at 3d of age (Fig. 3B). A lower fecundity is observed in both mutants (Fig. 6A), but fertility of $NLaz^{-/-}$ mutants does not differ from wild type (Fig. 6B). An estimation of developmental success, or "egg quality" (% of eggs reaching adulthood) is higher in $NLaz^{-/-}$ mutants and lower in $GLaz^{-/-}$ mutants compared to wild type flies (Fig. 6C). We can therefore discard the existence of major fertilization or developmental problems at least in NLaz mutants. However, given that GLaz mRNA is detected very early in embryogenesis [0-2 hours embryos, 14], the $GLaz^{-/-}$ eggs that do not reach adulthood might have developmental problems. Alternatively, they can be unfertilized eggs. We therefore tested whether mating is hampered in the absence of Lipocalins.

Mating behavior is indeed altered in both $GLaz^{-/-}$ and $NLaz^{-/-}$ flies. When paired with wild type females, males of both mutant genotypes are less involved in courting (Fig. 6D), and a lower percent of pairs actually mate (Table 2). Wild type males paired with mutant females (Fig. 6E) also display a deficient courtship, and therefore the alteration of behavior is dependent on both sexes. When $NLaz^{-/-}$ males and females pair, the courtship index is even lower (Fig. 6E), and mating success is strikingly low (Table 2), which would account for the low number of eggs per female observed in population experiments (Fig. 6A). On the contrary, pairs composed of two GLaz mutant flies are more effective in triggering the courtship behavior than pairs of $GLaz^{-/-}$ with wild type flies. This result would be compatible with pheromone signaling
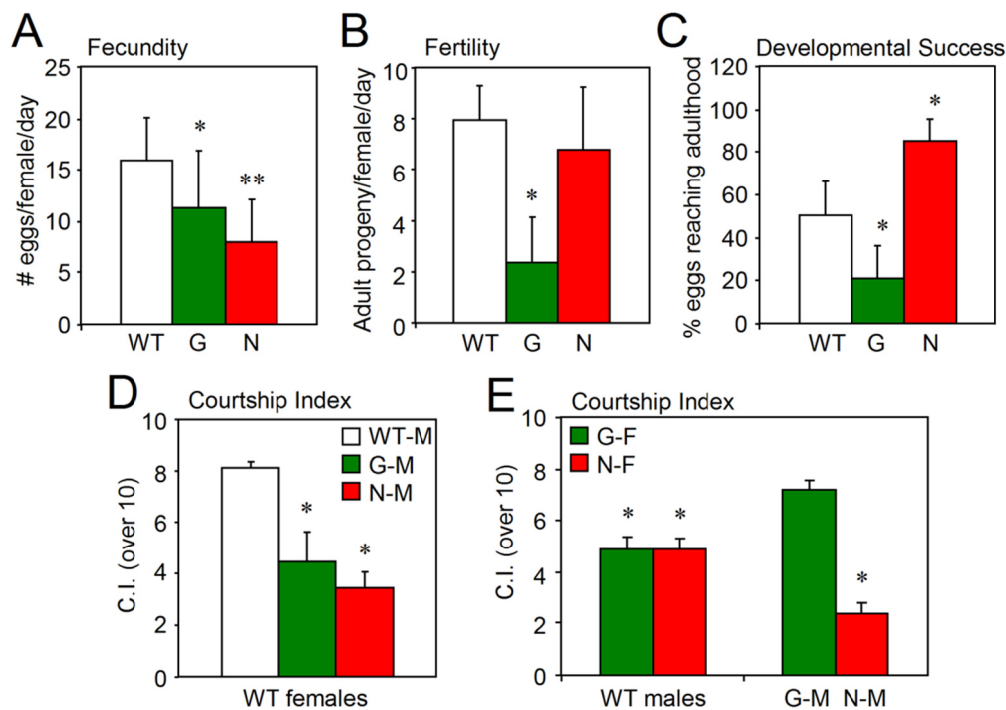
Figure 6. Reproductive output is modulated differently by GLaz and NLaz.
(A-B) The absence of both Lipocalins reduces fecundity (A) but only $GLaz^{-/-}$ mutant has a lower fertility (B). Three populations of 10 males x 10 females where scored for egg and adult progeny production during 7 days (from 5-11 days of age). The average per day is represented. *(C)* The percent of eggs reaching adulthood was calculated from the experiments shown in A-B. *(D-E)* Courtship behavior is altered in $GLaz^{-/-}$ and $NLaz^{-/-}$ mutants. Courtship index is shown for pairs of each genotype with wild type females (D) and pairs of Lipocalin mutant females with wild type or mutant males (E). Other parameters of the courtship behavior and mating success are shown in Table 2. N= 34-52 pairs per class. Statistical differences assayed by Student's T-test. * p<0.05; ** p<0.01.

Genotype/Sex	Total Activity	Diurnal Activity	Nocturnal Activity	Sunrise Activity	Sunset Activity
WT males	463.1 ± 14.2	203.69 ± 9.9	69.23 ± 7.4	76.21 ± 11.6	113.97 ± 18.6
GLaz$^{-/-}$ males	**615.22 ± 52.3^a**	**137.19 ± 31.8^a**	**212.07 ± 36.1^b**	**116.64 ± 18.4^a**	149.33 ± 17.8
NLaz$^{-/-}$ males	412.03 ± 35.1	188.89 ± 35.7	**37.74 ± 7.7^b**	**99.03 ± 3.0^a**	89.37 ± 4.2
WT females	710.05 ± 27.5	416.43 ± 17.0	46.16 ± 17.3	93.85 ± 5.4	153.61 ± 23.7
GLaz$^{-/-}$ females	660.17 ± 74.7	**361.89 ± 29.4^a**	41.79 ± 25.6	**53.12 ± 1.7^b**	203.37 ± 21.9
NLaz$^{-/-}$ females	**415.19 ± 28.7^b**	**264.43 ± 15.4^b**	17.19 ± 8.0	**53.98 ± 3.7^b**	**59.59 ± 2.9^b**

Table 2. Courtship behavior is altered in Lipocalin mutants.
Courtship behavior parameters obtained from experiments shown in Fig. 6D-E. Average ± Standard Error is represented. Bold lettering indicates statistical differences assayed by Student's T-test (p<0.05): ^{a} Different from WT-M x WT-F pairs, ^{b} Different from mutant-M x mutant-F pairs.

being off-set in GLaz mutants, both at the emission and reception levels. Interestingly, the GLaz or NLaz mutants who successfully mate show longer mating latencies (Table 2), but this effect is cancelled when mutant males are paired with wild type females.

The altered mating behaviors could modify the final reproductive output of the population. However, the fertility of successfully mated mutants does not differ from wild type values (Fig. S3A). Thus, we can conclude that in experiments performed in fly populations (Fig. 6B) the GLaz-/- eggs that do not reach adulthood are mostly unfertilized eggs, while the fertilized ones reach adulthood at a normal rate, discarding major developmental problems also for GLaz-/- mutants. In addition, we analyzed the lifespan of virgin females, which results in a pattern identical to the one observed for mated females: similar in wild type and GLaz-/- and shortened in NLaz-/- mutants (Fig. S3B). These virgin females do lay eggs, although all of them are unfertilized. The putative differential constraint to lifespan is not therefore dependent on the energy spent by the female in producing eggs proper.

Even though reproduction has been shown to correlate negatively with lifespan in many cases [21,30,31], instances of genetic interventions that alter longevity and reproductive output independently have also being documented [32,33] [34,35], challenging the existence of a direct causal relationship between reproduction and lifespan. Lipocalin mutants support the latest view, since the sex differences observed in longevity cannot be fully explained by a net reduction in the energy spent in the production of eggs. The control of courtship behaviors are part of the pleiotropic functions of the two nervous system Lipocalins clearly deserving future investigation.

3.9. Environment-genotype interactions: Food composition influence on Lipocalins phenotypes.

Food composition is an important environmental parameter that influences stress resistance and reproductive output. We have performed a nutritional analysis of three common fly food recipes including the standard one used in our laboratory, and the one used by collaborators that have also worked on NLaz or GLaz function (Fig. 7A, see Methods). Each recipe differs in the amount of yeast and the source of sugar. However, a clear pattern emerges. For wild type flies, the higher the proportion Net Fat / Total Carbohydrates (Fig. 7B), the higher the fertility (Fig. 7C) and the lower the oxidative stress resistance (Fig. 7D-F).

Characterizing this interaction in the fly strains used in our analysis has been important, since the

GLaz mutation effects on reproduction are significantly higher in diets with high or medium Fat/Carbohydrate ratio ("Valladolid" and "Carolina" foods, Fig. 7C), while sensitivity to stress observed in both mutants is only observed in diets with medium to low Fat/Carbohydrate ratio ("Carolina" and "Caltech" foods, Fig. 7D-F shows the results for NLaz mutants).

Previous studies analyzing diet effects on lifespan [36,37] have been performed with different wild type strains. Our set of experiments emphasizes the importance of controlling diet when characterizing the effects of single genes influencing lifespan [see also 21]. The effects of Lipocalin genotype in both reproductive output and stress resistance are best observed when flies are fed on our standard "Valladolid food", which contains a medium Fat/Carbohydrate proportion.

It is interesting to note that the largest differences in the stress response upon changes in diet are observed in the wild type flies. Without NLaz, the differences between feeding regimes are reduced. These results should not be surprising giving the role of NLaz in the control of metabolism in response to environmental stress [15]. In the absence of NLaz, the IIS pathway is activated and flies become less sensitive to diet modifications. On the contrary, when IIS activity is genetically reduced [34], lifespan extension becomes insensitive to nutrient supplemented diets.
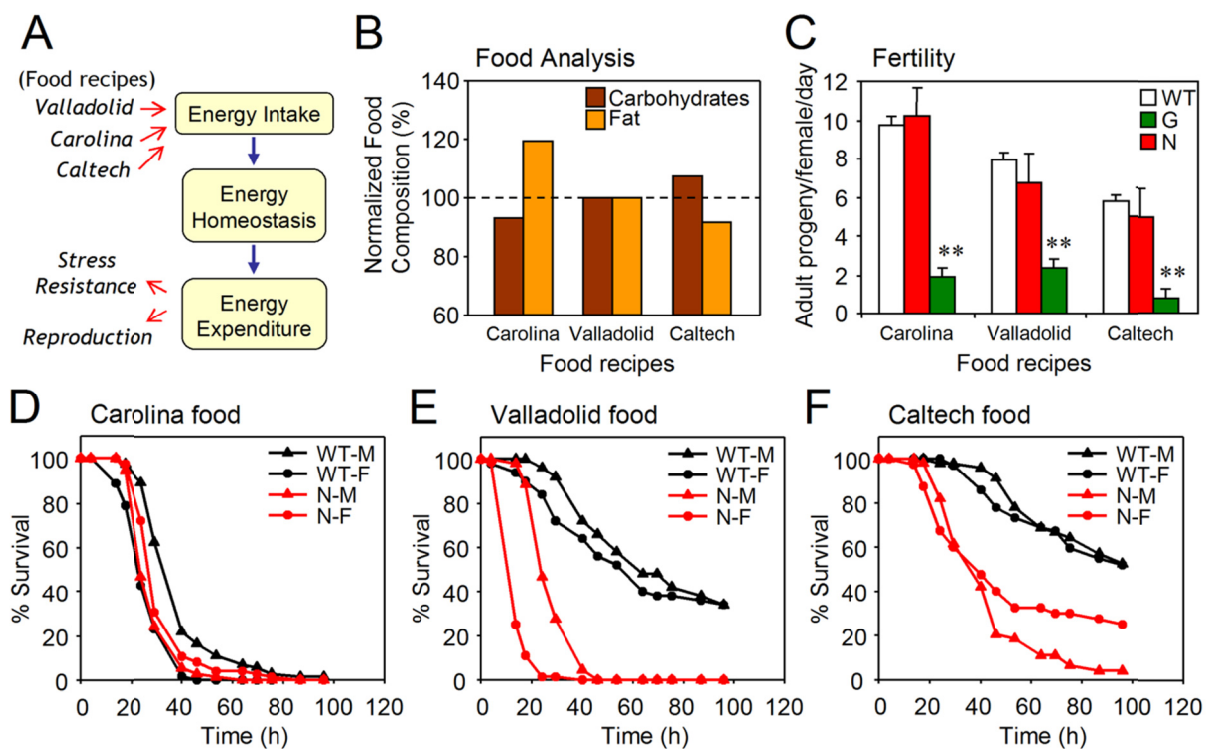
Figure 7. Food composition influences reproductive output and stress resistance.

(A) Variables exploring the flow of energy upon different nutritional regimes in Lipocalin mutants. *(B)* Nutrient analysis (performed by Aquimisa, Salamanca, Spain) of the three food recipes used in this work yielded the proportion of Net Fat vs. Total Carbohydrates as the most salient difference (represented normalized with respect to values in Valladolid food recipe). *(C)* Reproductive output (fertility) of each genotype when flies are fed in the three types of food. Three populations of 10 males x 10 females per diet condition where scored for progeny production during 2 days (from 5-6 days of age). The average per day is represented. *(D-F)* Survival curves under paraquat treatment of wild type and *NLaz*$^{-/-}$ adults born and raised in each type of food. A similar pattern is observed for *GLaz*$^{-/-}$ mutants (not shown). Log-rank test was used for statistical analysis. N=45-75/genotype/sex. Significant reduction in survival was observed only in the Valladolid and Caltech foods (p<10^{-4}).

3.10. ¿How do brain Drosophila Lipocalins help us to understand the aging process?

Several general ideas relevant to aging and lifespan determination are supported by our study.

3.10.1. Early adulthood is a crucial period for lifespan determination.

Experiments where the IIS pathway was manipulated at different time points in life, both in *C. elegans* [32] and Drosophila [33], have shown that in order for this pathway to influence lifespan, it needs to operate exclusively during adulthood, with such an influence decreasing at older ages. Two lines of evidence suggest that a brain Lipocalin critical period also exists early in adulthood for their influence on lifespan: (1) their temporal expression pattern in wild type flies, and (2) the clustering of GLaz mutant females with wild type flies only at young ages in the multivariate space of metabolic parameters.

This time period, critical for both IIS and Lipocalin function, is coincident with the reproductive period of the organism (both in worms and flies), and could be interpreted in the light of the disposable soma theory of aging [38], where natural selection forces are predicted to decline sharply once the organism is no longer engaged in passing its genes to the next generation [39]. Lipocalin function would be under selection in early adulthood only, and subsequent consequences of Lipocalin expression on the aging brain or body would be exaltations of this early adulthood selected functions [40].

3.10.2. The sex-specific lifespan modulation by Lipocalins is better explained by metabolic than by reproduction phenotypes.

In accordance to the disposable soma theory, our first working hypothesis was that GLaz mutant females maintain a lifespan similar to the wild type because they would be able to allocate more resources to maintenance and less to reproduction. We then confirmed that GLaz mutant females reproduce less and survive more. However, since NLaz mutant females reduce both fecundity and lifespan, we must conclude that survival and reproduction are in this particular case uncoupled.

Curiously, the PCA analysis shows that it is the alteration of metabolic parameters related to the protein content of the fly what mainly accounts for the observed sex differences in longevity. The final protein content and the fat free dry weight of the organism depends of protein synthesis, which has a strong evolutionary conservation among the genetic modifiers of aging [41].

NLaz is also related to the control of metabolism. When NLaz is expressed in the fat body, it is able to repress IIS activity in systemic target tissues. Also, the loss of NLaz increases signaling through IIS [15]. The experiments in this work predict that, in addition, NLaz must be a negative regulator of food intake. NLaz is thus expected to fulfill a role similar to Leptin in the nervous system, inhibiting food intake and promoting energy expenditure. Loss of NLaz mimics therefore the Leptin deficiency state in mouse models of obesity [42,43]. Curiously, mice where the neural/brain specific insulin receptor is knocked out, exhibit increased food intake and mild adiposity [44], just as the NLaz mutant flies. A direct interaction of ApoD with the cytoplasmic portion of the Leptin receptor has been suggested [45], although it is still questionable how a secreted Lipocalin can access the cytoplasmic side of the plasma membrane for such an interaction to occur.

In contrast with NLaz, GLaz is not regulated by JNK in the peripheral tissues [15]. Here we demonstrate that it is instead induced by food deprivation in the brain, where no NLaz transcriptional response is detected. This pattern of differential transcriptional response in the brain further supports the existence of specialized functional domains for these two Lipocalins.

3.11. Two brain Lipocalins in Drosophila: a labor division strategy in longevity regulation.

Our work highlights that the two Drosophila Lipocalins expressed in the nervous system have both functional redundancies and specializations. The response to oxidative stress and accumulation of lipid peroxides are among their common functions, while the transcriptional and behavioral

response to starvation, the pattern of daily locomotor activity, storage of fat along aging, fertility, and courtship behavior differentiate NLaz from GLaz mutants. This framework is guiding our current research, as more details need to be elucidated. However, we can already start questioning: how many of these shared or unique functions are conserved in the mammalian homologues also expressed in the brain?

The basal position of ApoD in the phylogenetic tree of vertebrate Lipocalins [46,47] suggests that ApoD shares many properties with the common ancestor of invertebrate Lipocalins. However, we have to take into account that neither GLaz nor NLaz is a true orthologue of ApoD. Our molecular phylogenetic analyses strongly suggest that the Drosophila Lipocalins originated from an independent duplication event, taking place within the invertebrate lineage. Subsequently, the resulting genes have diverged both in their protein coding sequence and their regulatory sequences.

Since ApoD in the adult mammalian brain is expressed in glial cells, one might be tempted to directly conclude that GLaz is the closest Drosophila homologue. Furthermore, the expression data we report in this work strongly suggest that GLaz regulation through aging is most similar to the robust increase of mammalian ApoD in the aged brain [8,9]. Interestingly, ApoD is most similar to GLaz in protein sequence, but to NLaz in the intron-exon structure of the gene [48]. We have also data showing that ApoD is up-regulated by oxidative stress in astrocytes, and that this induction is mediated through the JNK pathway (Bajo-Grañeras, Ganfornina and Sanchez, unpublished observations), which is comparable to the NLaz JNK-mediated induction by stress in Drosophila [15]. Thus, if we want to extrapolate the Drosophila data to learn about the functions of ApoD in mammalian aging and neurodegeneration we must benefit from a global comparison with both GLaz and NLaz, as the one reported here.

To understand the multigenic control of aging, we need to take into account that a layer of complexity is added due to the fact that each gene has pleiotropic effects, and each one has differing degrees of specialization or redundancy with members of the same gene family. This fact represents a daunting complication for the task of predicting the actions of putative anti-aging or anti-neurodegeneration drugs. However, complexity should not keep us from investigating till we get a comprehensive understanding of the aging process.

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Supplementary Information
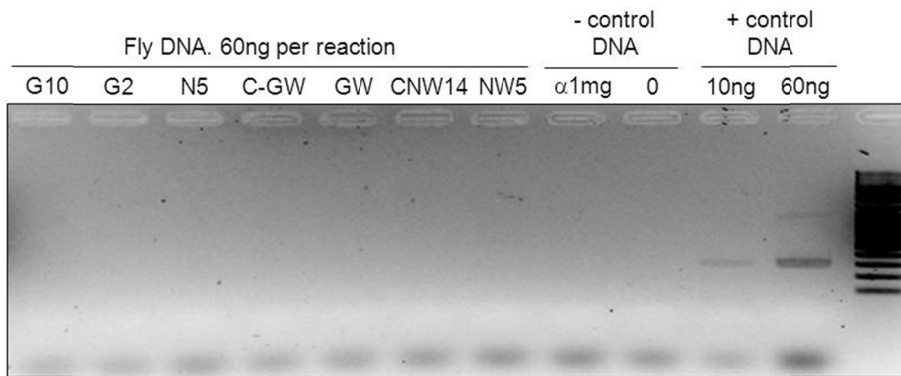
Figure S1. PCR test for Wolbachia infection.

PCR of genomic DNA extracts from all wild type and mutant strains used in this work. Primers against the rRNA-16S gene of Wolbachia endosymbiont are used. DNA from *Dilofilaria immitis* worms infected with Wolbachia is used as positive control. A plasmid containing the cDNA of α-1-microglobulin (a1m, also a Lipocalin) or no DNA are used as negative controls.
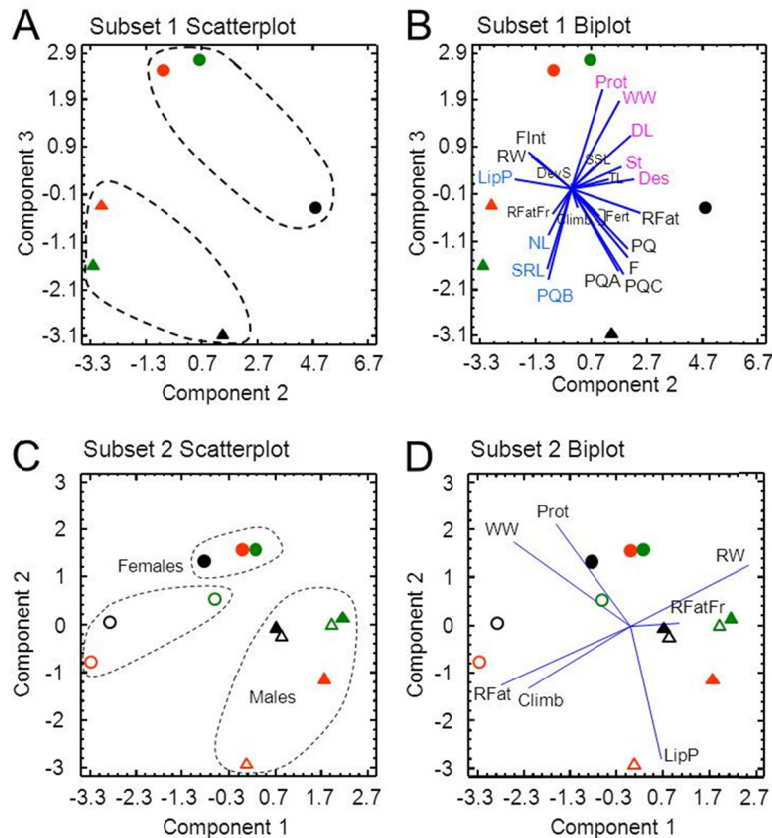
Figure S2. Principalcomponents separating sex and age.

(A-B) Scatterplot and biplot of components 2 by 3 extracted from the sample of 25 variables measured in 3 day-old flies (Subset 1). The two-dimensional space defined by these components separate males from females of the three genotypes. *(C-D)* Scatterplot and biplot of the two principal components explaining 63% of the variation in the sample of 7 variables measured in 3 day- and 30 day-old flies (Subset 2). These components are also able to separate samples according to sex. In addition, they separate females according to age. Symbols: black = wild type, red = $NLaz^{-/-}$, green = $GLaz^{-/-}$, circles = females, triangles = males, filled symbols = 3 day-old flies, open symbols = 30 day-old flies. Acronyms for the variables are listed in Table S1 and S2.

Pair Genotypes	Descendence per couple	Sex ratio (males/females)
WT-M × WT-F	40.33 ± 4.43	1.11 ± 0.09
N-M × N-F	51.5 ± 1.5	1.10 ± 0.02
N-M × WT-F	29.91 ± 3.55	**0.81 ± 0.08 [a]**
WT-M × N-F	46.88 ± 2.12	1.23 ± 0.15
G-M × G-F	36.0 ± 4.69	1.07 ± 0.049
G-M × WT-F	32.67 ± 3.45	1.11 ± 0.13
WT-M × G-F	55.08 ± 7.32	1.11 ± 0.09

[a] different from WT-M x WT-F (p<0.05)
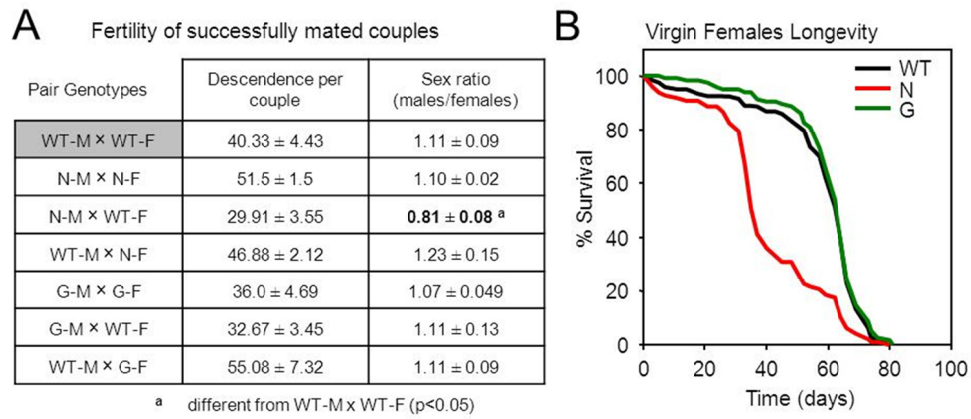
Figure S3. Reproduction –Lifespan relationship.
(A) Fertility of successfully mated mutant pairs does not differ from wild type when scoring the offspring of mated couples from courtship behavior analysis (Fig. 6D-E). N= 34-52 pairs. Statistical differences assayed by Student's T-test (p<0.05): [a] Different from WT-M x WT-F pairs. *(B)* Lifespan of virgin females is reduced in *NLaz*^{-/-} mutants (p<10[-5]), but is similar to wild type in *GLaz*^{-/-} mutants. Log-rank test was used for statistical analysis. N=97-120/genotype.

Genotype_Sex_Age	STRESS RESISTANCE			METABOLISM					
	Paraquat Resistance MedianSurv	Starvation Resistance MedianSurv	Desication Resistance MedianSurv	Lipid Peroxidation	Wet Weight	Relative Water Content	Relative Fat Content	Relative FatFree Weight	Protein content
WT_M_3	110,0	54,3	22,8	57,3	100,0	100,0	100,0	100,0	33,62
WT_F_3	108,0	79,0	30,5	45,3	141,4	101,0	105,5	95,4	53,32
G_M_3	64,8	25,0	18,0	132,9	94,5	106,2	68,9	95,9	33,33
G_F_3	76,2	45,0	28,0	100,0	136,3	105,8	81,9	92,5	56,11
N_M_3	29,9	58,5	10,0	275,9	95,7	103,1	75,6	99,8	33,12
N_F_3	29,9	66,0	16,5	119,3	138,7	101,2	85,4	101,7	64,42
Variable acronism:	PQ	Starv	Desic	LipidPerox	Wet W	RelWater	RelFat	RelFatFree	Prot

Genotype_Sex_Age	REPRODUCTION		DEVELOPMENT	BEHAVIOURAL RESPONSES						
	Fecundity	Fertility	Developmental succes	Climbing ability	Total Locomotor Activity	Diurnal Locomotor Activity	Nocturnal Locomotor Activiy	Sunrise Locomotor Activity	Sunset Locomotor Activiy	Food Intake Response
WT_M_3	15,9	7,19	66,21	7,2	771,84	546,64	225,20	127,02	189,96	10,10
WT_F_3	15,9	7,19	66,21	8,2	1183,42	986,96	196,47	156,42	256,02	7,17
G_M_3	11,3	3,75	41,66	7,5	1025,38	407,56	617,82	194,40	248,89	18,24
G_F_3	11,3	3,75	41,66	8,4	1100,29	825,13	275,16	88,53	338,96	7,05
N_M_3	7,9	7,19	84,45	8,7	706,33	600,71	105,62	169,76	153,21	34,63
N_F_3	7,9	7,19	84,45	5,6	709,13	643,53	65,60	96,16	132,93	43,20
Variable acronism:	Fecund	Fertility	DevSucces	Climbing	TotalLocom	DiurLocom	NoctLocom	SunriseLocom	SunsetLocom	FoodIntake

Genotype_Sex_Age	AUTOPHAGY		STRESS RESISTANCE AND REPRODUCTION VS. NUTRITION					
	Autophagy Induction Head	Autophagy Induction Head Starvation	Fertility FoodA (Valladolid)	Fertility FoodB (Carolina)	Fertility FoodC (Caltech)	PQ Resistance FoodA (Valladolid)	PQ Resistance FoodB (Carolina)	PQ Resistance FoodC (Caltech)
WT_M_3	-1,763	-2,059	18,5	18,2	7,2	63,0	33,0	100,0
WT_F_3	-1,184	-1,369	18,5	18,2	7,2	56,0	23,0	100,0
G_M_3	-2,075	-2,103	4,7	3,7	1,5	36,7	35,4	56,7
G_F_3	-1,180	-1,932	4,7	3,7	1,5	36,7	13,8	33,3
N_M_3	-2,059	-2,063	17,7	19,0	6,2	24,0	24,0	35,5
N_F_3	-0,923	-2,008	17,7	19,0	6,2	10,0	27,0	38,0
Variable acronism:	Autoph	AutophStarv	FertilityA	FertilityB	FertilityC	PQA	PQB	PQC

Table S1. Matrix for Principal Component Analysis. Subset 1.
Averages of the 25 variables measured in 3 day-old flies. Transcription of GLaz or NLaz and longevity parameters are not included.

Genotype_Sex_Age	METABOLISM						BEHAVIOR
	Lipid Peroxidation	Wet Weight	Relative Water Content	Relative Fat Content	Relative FatFree Weight	Protein content	Climbing ability
WT_M_3	57.32	100	100	100	100	33.62	7.16
WT_F_3	45.32	141.39	101.02	105.45	95.41	53.31	8.15
G_M_3	132.89	94.45	106.21	68.90	95.89	33.32	7.46
G_F_3	99.96	136.27	105.83	81.87	92.45	56.11	8.42
N_M_3	275.92	95.66	103.05	75.56	99.79	33.11	8.65
N_F_3	119.33	138.73	101.15	85.42	101.68	64.42	5.61
WT_M_30	112.26	100	100	100	100	36.48	6.31
WT_F_30	81.71	140.74	97.85	146.81	90.36	56.88	14.45
G_M_30	122.10	91.50	104.90	59.30	101.18	37.50	11.24
G_F_30	160.12	114.25	99.05	80.63	108.78	78.66	14.24
N_M_30	404.30	101.78	98.82	125.06	96.77	32.68	12.07
N_F_30	109.95	143.30	95.91	156.52	96.40	49.68	17.64
Variable acronism:	LipP	WW	RW	RFat	RFatFr	Prot	Climb

Table S2. Matrix for Principal Component Analysis. Subset 2.
Averages of the 7 variables measured in 3 day-old and 30 day-old flies. Transcription of GLaz or NLaz and longevity parameters are not included.

Chapter 2

"Lipid binding properties of human ApoD and Lazarillo-related Lipocalins:

functional implications for cell differentiation"

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"Lipid binding properties of human ApoD and Lazarillo-related Lipocalins: functional implications for cell differentiation"

Submitted

Lipid binding properties of human ApoD and Lazarillo-related Lipocalins: Functional implications for cell differentiation.

Summary

Apolipoprotein D (ApoD) and its homologues, Lazarillo (Laz) and Neural Lazarillo (NLaz), are involved in longevity regulation, stress resistance and lipid metabolism. They belong to the Lipocalin family, characterized by a conserved tertiary structure: an eight-stranded β-barrel wrapping around a ligand binding pocket.

Using tryptophan fluorescence titration we have uncovered several novel ligands for NLaz, ApoD and Laz, all with apparent dissociation constants in the low micromolar range.

Retinoic acid (RA), retinol, fatty acids and sphingomyelin are shared ligands. Though frequently associated with ApoD, cholesterol did not show strong binding to human ApoD, while NLaz and Laz did bind the related sterol, ergosterol. Searching for species-specific ligands, ApoD shows a selective affinity for the endocannabinoid anandamide, whereas 2-acylglycerol does not bind. NLaz binds the pheromone 7-tricosene, but not 7,11-heptacosadiene or 11-cis-vaccenyl acetate.

To clarify the functional relevance of ligand-binding at the cellular level, we analyzed the effect of ApoD, Laz and NLaz preloaded with RA on neuronal differentiation. Our results show that ApoD is necessary and sufficient to allow for RA differentiating activity. Both ApoD and NLaz successfully deliver RA to immature neurons, driving neurite outgrowth.

We conclude that ApoD, NLaz and Laz bind selectively to a different but overlapping set of lipid ligands. This multispecificity can explain the varied functions they carry physiologically.

1. Introduction

L ipocalins are an ancient and functionally diverse family of proteins characterized by their ability to bind and transport a variety of ligands, including in some cases bioactive lipids. Noted examples of functional Lipocalin-lipid associations include (i) Retinol Binding Protein (RBP) and retinol [1], (ii) Apolipoprotein M (ApoM) and sphingosine-1-phosphate (S1P) [2] and (iii) the enzyme Prostaglandin D Synthase (L-PGDS) and its substrate prostaglandin-H_2 (PGDH_2) [3]. Despite limited sequence identity, the Lipocalin three-dimensional structure: an eight-stranded antiparallel β-barrel with accessory α- and 3$_{10}$ helices, is well conserved through evolution. The Lipocalin fold forms a cup, or calyx, with a central cavity, which serves as a ligand-binding site [4].

Apolipoprotein D (ApoD) is an extracellular glycosylated Lipocalin expressed with a wide temporal and tissue distribution. Although detected in liver, spleen, skin and placenta, ApoD is mostly present in serum and nervous system [reviewed by 5]. Plasma ApoD is mainly associated with high-density lipoproteins (HDL), but can also be found in very low- and low-density lipoproteins (VLDL, LDL) [6,7]. ApoD nervous system expression has been studied during development [8-10], and it is known to increase during prefrontal cortex maturation, a period marked by active lipid metabolism [11]. On the other hand, ApoD is the most consistently up-regulated gene during brain aging in mammals [12,13]. ApoD levels are also elevated in many neurodegenerative diseases, including Alzheimer's [14], Parkinson's [15], schizophrenia [16] and multiple sclerosis [17]. In the mouse model of Niemann-Pick type C (NPC) disease [18], caused by deficiencies in cholesterol metabolism and management, ApoD expression is also up-regulated.

ApoD is considered the most ancient vertebrate Lipocalin and has homologues in many other phyla [19,20]. Neural Lazarillo (NLaz) from the fruitfly *Drosophila melanogaster* and Lazarillo (Laz) from the grasshopper *Schistocerca americana* are two of the most studied ApoD homologues. Many ApoD, NLaz and Laz functions are conserved during evolution, and include protection against oxidative stress, and longevity and metabolism regulation [21-27]. Oxidative stress protection has been established for ApoD homologues *in vivo*, and the ApoD molecule itself exhibits anti-oxidant properties *in vitro* [28,29]. The regulation of ApoD and NLaz expression are also conserved, as both are controlled by the JNK signaling pathway under stress conditions [22,26]. Furthermore, ApoD and NLaz have been proposed as functional links between the nervous system and adipose tissue both in mammals and insects [22,30].

The biochemical mechanism by which ApoD and related Lipocalins perform their function is not understood, though their hydrophobic pockets and potential ligands are obvious candidates to be explored. The wide range of tissue expression and physiological roles of ApoD, NLaz and Laz suggest that particular functions are dependent on specific ligands. Thus, determining the ligand specificity of these Lipocalins is essential to define their molecular mechanism.

Several ligands have been described for ApoD, but not without controversy. Progesterone-ApoD binding has been documented [31-35] and the complex co-crystallized [36]. In addition, arachidonic acid (AA) [34,35], retinol, and retinoic acid (RA) [37] are *in vitro* ApoD ligands. However, a consensus has not been reached on whether bilirubin [7,35], odorants like E-3-methyl-2-hexenoic acid [35,38] or other steroids, such as cholesterol [34,39] and pregnenolone [33,35], are *bona fide* functional ApoD ligands.

Laz shows positive binding for RA, AA, linoleic acid (LA) and palmitic acid (PA) [40]. However, the Drosophila NLaz ligand preferences have not been characterized.

The aim of this work is to identify new putative lipophilic ligands for the ApoD-related Lipocalins, which should contribute to understand how they perform their physiological functions in different tissues and cellular contexts. Placing these results in an evolutionary context will help us distinguish derived versus ancestral protein-lipid functional associations. Finally, we assay at the cellular level the effect of ApoD-RA binding pair

on a particular biological function: the differentiation of neurons in culture.

2. Materials and Methods

2.1. Cell cultures.

Drosophila S2 cells were cultured as previously described [27]. Human neuroblastoma cell lines SH-SY5Y and M17 were grown at 37ºC in a saturated humidity atmosphere containing 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium, Lonza Iberica) supplemented with glucose (4.5 g/l), heat-inactivated 10% FBS (fetal bovine serum, Lonza Iberica), 1% L-Glutamine, antibiotics (penicillin, 10 U/μl; streptomycin, 10 μg/μl; amphotericin B, 25 μg/ml; Lonza Iberica), and 1% non-essential amino acids (NEAA, Lonza Iberica). The culture medium was replaced twice a week, and cells were subcultured at 90% confluence.

2.2. Cloning and protein purification of NLaz, Laz and ApoD.

The NLaz full-length cDNA, translating into residues 1-224 (CG33126, Uniprot reference Q9NAZ4, FlyBase entry FBgn0053126), and a fragment of Lazarillo cDNA encoding a 192 residue fragment missing the GPI signal peptide (Uniprot reference P49291), were subcloned into the pRmHa3 vector using EcoRI and NotI sites. This system expresses the cloned sequences under the control of an inducible *Drosophila* metallothionein promoter and incorporates a C-terminal poly-histidine sequence allowing for protein purification from conditioned medium.

NLaz-pRmHa3 or Lazarillo-pRmHa3 plasmids were co-transfected with a pCoBlast selection vector (conferring blasticidin resistance) into *Drosophila* S2 cells. Protein expression induction and purification were performed as previously described [27], adding two rounds of size exclusion chromatography purification after the initial metal affinity chromatography (Fig. S1B-D).

Bacterial recombinant Laz was purified from the periplasmic space of *E. coli* as previously described [40]. Human ApoD (hApoD) was purified from breast cystic fluid (Fig. S1A and S1D) by ion-exchange chromatography (UNO-Q, BioRad) in 25 mM Tris pH 8.0 followed by size exclusion chromatography (P60, BioRad) in 50 mM Tris pH 8.0, 75 mM NaCl. Protein purity was determined by SDS-PAGE and integrated optical density of the protein bands stained with Coomassie was measured, resulting in 91% purity for ApoD and >98% purity for NLaz and Laz (Fig. S1).

2.3. Ligand binding assays by tryptophan fluorescence titration.

Fluorescence measurements were conducted with a Shimadzu RF-5301PC spectrofluorometer in a quartz cuvette (Hellma 105.251-QS, 3 mm-path length). Temperature was held at 22 ± 0.1ºC. The excitation wavelength was 295 nm (selective for tryptophan residues). Emission was recorded from 327 to 400 nm with slit width set at 5 nm. The peak observed at 340 nm was measured and used to calculate the apparent equilibrium dissociation constant (K_D). Data analysis was performed with the RFPC software package (Shimadzu). NLaz and the control protein α1-microglobulin were diluted to a concentration of 1 μM in 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA at pH 7.0. Laz and hApoD were diluted to a concentration of 0.5 μM in the same buffer, so that the starting intrinsic fluorescence of all proteins lies within the same dynamic range of measurement. The ligands tested were: retinoic acid, retinol, non-esterified cholesterol, ergosterol, β-estradiol, palmitic acid (Sigma); palmitoyl-sphingomyelin, arachidic acid, 7(z)-tricosene, 7(z),11(z)-heptacosadiene, 11-cis-vaccenyl acetate, 2-arachidonylglycerol, anandamide, palmitoyl-linoleyl-phosphoethanolamine (Cayman); and 20-hydroxyecdysone (Santa Cruz Biotechnology). All ligands were dissolved in N,N-dimethylformamide (DMF; Sigma) to make concentrated stock solutions within their solubility range. A four step titration was performed, adding each time 1 μl aliquots of the ligand to a 100 μl volume of protein solution. The mixtures were equilibrated for 3 min in the dark before the fluorescence was recorded.

The fluorescence spectrum in the presence of a ligand was subtracted from a DMF baseline obtained by titration of the protein with the same

amounts of carrier without ligand. The corrected fluorescence at 340 nm versus ligand concentration was fitted as previously described [37,40]. K_D was calculated under the assumption of a single binding site, consistent with known Lipocalin structural properties, regardless of whether ligand binding enhances or quenches tryptophan fluorescence. A tryptophan residue conserved along the entire Lipocalin family is located at the binding pocket surface, and is predicted to be the residue responsible for most of the intrinsic fluorescence changes observed. The specificity of protein fluorescence variation was tested by performing the assay with the protein denatured with 5 M guanidine hydrochloride in the same buffer for 21 h before measurement (Fig. S2).

2.4. Neuronal cell line differentiation assay.

SH-SY5Y or M17 cells were seeded at 20,000/cm^2 in 24 well plates (Nunc) with standard medium. After 12 h, the medium was replaced. New medium was added with or without serum or additives (protein, ligand or equimolar amounts of both protein and ligand). Retinoic acid and retinol stock solutions were prepared in ethanol and diluted in medium to a final concentration of 1 or 10 μM. The carrier concentration was kept at 0.1 % (v/v). The concentrations of hApoD, NLaz and Lazarillo were measured by MicroBCA-assay (Pierce) and the proteins were diluted in sterile PBS to prepare the working protein stocks. Equimolar protein-ligand mixtures were incubated in the dark at room temperature for five minutes to allow for complexation before addition to cells. SH-SY5Y cultures were analyzed after 4-6 days of growth in the presence of additives, and M17 cultures were analyzed after 3-4 days of growth. Photomicrographs were analyzed with NISelements software (Nikon) with morphological differentiation determined by the number of neurites (processes longer than 50 μm) present per field [41].

2.5. Statistical analysis.

Statistical analyses were performed with Sigma Plot software (v 11.0). Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's multiple comparison post-hoc tests were used in the analysis of neurite promoting activity of Lipocalins. A *p-value* < 0.05, marked with an asterisk, was used as a threshold for significant changes. Two asterisks were used for *p* <0.001.

3. Results and Discussion

3.1. Binding properties of ApoD and its related insect homologues.

Intrinsic fluorescence titration was used to characterize the ligand binding capacities of ApoD, NLaz and Laz, an approach that has been successfully employed in many other Lipocalin binding studies [e.g.: 37,40]. A panel of 15 small hydrophobic molecules was tested, including lipids with different size, shape, chemical properties and biological functions. Ligands identified in other studies were also included.

Previous studies were carried out using proteins recombinantly expressed in *E.coli* [35,40]. Since the three Lipocalins are glycoproteins, we performed our study with their glycosylated forms, avoiding undesired effects on the calyx structure or binding properties due to the lack of glycosylation. To perform the binding assays we purified the proteins either from native sources (ApoD) or from a eukaryotic expression system (NLaz and Laz).

3.1.1. Retinol and retinoic acid: classical Lipocalin ligands.

Retinol and retinoic acid (RA) are common ligands for most Lipocalins, including ApoD and Laz [37,40]. The Lipocalin retinol binding protein (RBP) delivers retinol to several target tissues [42], and the physiological relevance of RBP-retinol interaction has been reported, for RBP mutations preventing retinol binding cause night blindness [43].

RA and especially retinol bind to ApoD with high affinity [37]. Our binding studies confirm that ApoD tightly binds retinol and RA (Fig. 1A and 1D) with K_D values of 0.2 μM for the ApoD-retinol and 4 μM for ApoD-RA interactions (Table 1). The higher affinity of ApoD for retinol over RA reported here is in agreement with previous results [37].

A study of bacterially-expressed recombinant grasshopper Laz binding to RA reported a K_D in the low micromolar range [40]. Here we have confirmed the Laz-RA interaction with bacterially-expressed Laz (Fig. S3A), as well as with the glycosylated eukaryotic Laz produced by S2 cells (Fig. 1C). Binding to both forms results in similar K_D (Table 1). Additionally, retinol was evaluated as a Laz ligand with positive results (Fig. 1F). RA and retinol have well known critical roles during vertebrate development [44]. The recent quantification of RA in locust embryos [45], previously thought to be absent in insects, suggests an involvement of Laz-retinol or -RA interactions in the Laz known functions during insect development [46].

Binding specificities for retinol and RA were also conserved in NLaz (Fig. 1B and 1E), with K_D values similar to those for ApoD or Laz (Table 1). Taken together, the binding of retinol or RA to both NLaz and Laz suggests a putative conserved physiological role for ApoD-related Lipocalins in insects, which lack a recognizable RBP ortholog [19].

To determine whether ApoD, NLaz or Laz binding to RA was dependent on the protein tertiary structure, denatured forms of all three Lipocalins were used in parallel binding assays, with the result that GuHCl denaturation hinders RA binding to ApoD, NLaz and Laz (Fig. S2). We conclude that an intact Lipocalin calyx is required for specific binding.
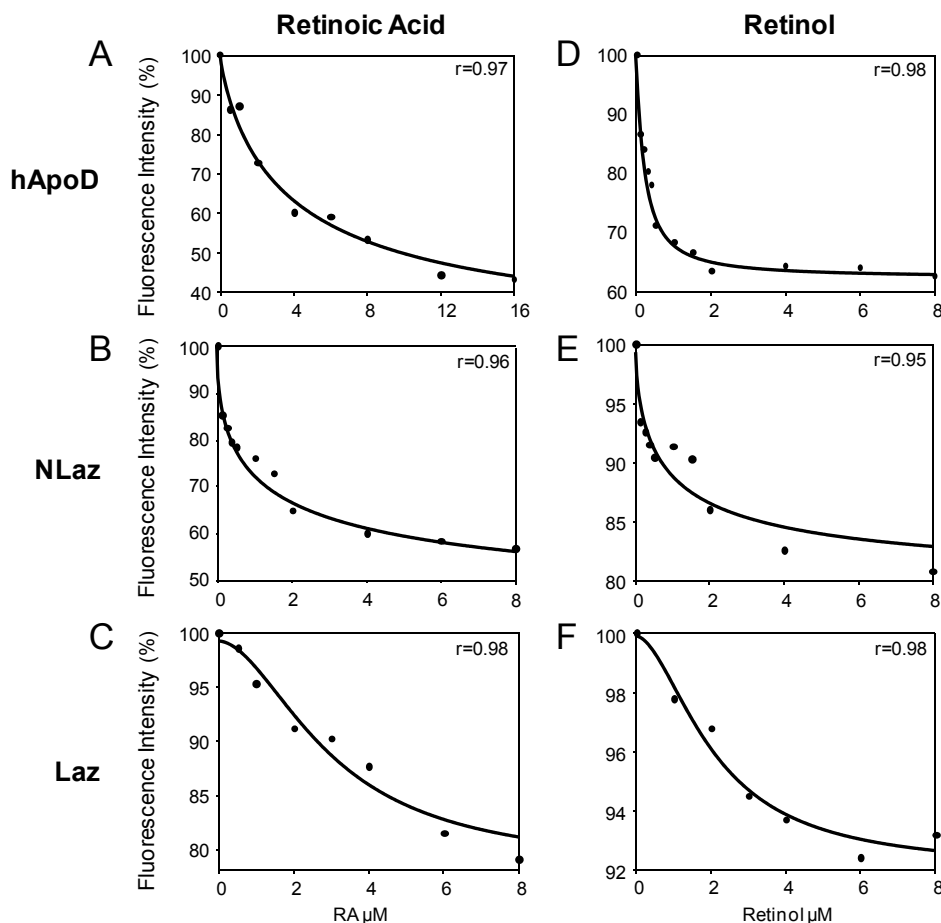
Figure 1. Relative fluorescence intensity of hApoD and its insect homologues NLaz and Laz exposed to increasing concentrations of retinoic acid or retinol.
hApoD and Laz protein concentration was 0.5 μM in 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA at pH 7.0. NLaz concentration was 1 μM in the same buffer. (A) hApoD-RA; (B) NLaz-RA; (C) Laz-RA; (D) hApoD-retinol; (E) NLaz-retinol; (F) Laz-retinol. Dots represent the mean of at least 5 independent titrations.

3.1.2. Lipids present in HDL particles and plasma membrane are putative ligands for ApoD-related Lipocalins.

ApoD was initially discovered as a component of HDL [47], where it is disulfide-linked to ApoA-II [48]. This discovery suggested that cholesterol is a potential ApoD ligand. Here we find no ApoD-cholesterol interaction in the micromolar range (Fig. 2A) in agreement with previous results where no interaction [34] or interaction in the millimolar range [39] were found, far from the low micromolar to nanomolar K_D values typical of physiologically relevant Lipocalin-ligand interactions [49]. Although ApoD is not able to bind non-esterified cholesterol with high affinity, many studies have shown a relationship between ApoD expression and cholesterol levels in tissues or organisms, or have proposed cholesterol binding as a physiological mechanism [e.g.: 50,51]. For example, ApoD is up-regulated in a mouse model of NPC disease [18] where cholesterol metabolism is impaired. Also, the addition of ApoD to organotypic hippocampal cultures reduced the amount of 7-ketocholesterol (an oxidized from of cholesterol) after kainate-induced excitotoxicity [52]. Although these results are suggestive of cholesterol binding, the observed correlations between ApoD and cholesterol levels cannot be ascribed to a direct cholesterol-ApoD interaction.

Drosophila cannot synthesize sterols and requires a dietary source. Ergosterol is the major sterol in the yeast-based *Drosophila* food used in laboratories [53]. Interestingly, NLaz and Laz can bind ergosterol with K_D values of 2 and 2.1 µM respectively (Fig. 2B-C). This finding differs from that obtained for hApoD-cholesterol interaction, and invites to test *in vivo* the physiological relevance of NLaz/Laz-ergosterol binding.

Sphingomyelin (SM) is the major sphingolipid in HDL particles and plasma membranes. SM has high affinity for cholesterol, and both lipids pack tightly forming lipid rafts and caveolae [54]. SM also regulates cholesterol efflux from cellular membranes to HDL via the ATP-binding cassette [55]. A connection of ApoD with SM is supported by the enrichment in SM of ApoD-containing blood lipoparticles [6], and by the role played by ApoD in maintaining peripheral nerve myelin sheath [56], a cell compartment that contains a high concentration of SM. Here we find that ApoD and the related insect Lipocalins NLaz and Laz bind palmitoyl-SM (Fig. 2D-F) with K_D values of 1.3 µM (ApoD), 4.6 µM (NLaz) and 2.7 µM (Laz). The Laz-SM interaction has a similar K_D value when using the bacterially-expressed form of Laz (Fig. S3B). Thus, SM is a common ligand for this group of Lipocalins, with ApoD exhibiting a higher affinity for SM than NLaz or Laz (Table 1). The number of carbons in the sphingoid base differ across species [54] and could potentially explain the differential affinities observed. Here, we used SM with a C18:1 sphingoid base, one of the typical forms found in humans, whereas C14:0 and C14:1 are the major sphingoid base isoforms in insects [54].

Since this is the first report of binding of SM to any Lipocalin, and SM binding is conserved for ApoD, NLaz and Laz, SM could be a ligand for other Lipocalins. We tested SM binding to α1-microglobulin (α1-mg), a Lipocalin more distantly related to the ApoD group [19]. No binding interaction was detected for α1-mg and SM (Fig. S3D), demonstrating the specificity of this assay and suggesting that SM could be a specific ligand restricted to the ancient metazoan Lipocalins, including ApoD and its insect related Lipocalins (NLaz and Laz). Retinol binding to α1-mg was used as a positive control (Fig. S3C). With these findings, the question of whether ApoD-cholesterol relationships found *in vivo* [18,52] are indirectly mediated by ApoD control of SM content of membranes or lipoprotein particles is also open for further research.

Membrane phospholipids have been also suggested as ApoD ligands, though previous studies failed to show binding to phosphocholine [34]. We tested the binding of ApoD, NLaz and Laz to phosphoethanolamine, with PA as substituent in position 1 and LA in position 2 (PLPE). Neither ApoD nor its insect homologues (NLaz and Laz) showed positive binding (Fig. S4). Thus, with the available data, we can discard phospholipids as probable ligands for the ApoD Lipocalin group. An interesting antecedent is found in the Lipocalin literature: ApoM is only able to bind phospholipids with high affinity when

they have been previously oxidized [57]. This property is still open for ApoD and other Lipocalins and might be related to the protective functions of many family members.

3.1.3. Binding to steroid hormones.

ApoD was characterized as a progesterone-binding protein from the cystic fluid of women with breast gross cystic disease [31]. This was confirmed by co-crystallizing ApoD and progesterone [36], and many groups have studied the role of ApoD in breast cancer [reviewed by 58]. Steroid hormone regulatory elements have been identified in the ApoD promoter, also suggesting such a link [59]. However, we show here that ApoD does not bind 17β-estradiol (Fig. 3A), the other main steroid hormone involved in reproduction, as previously reported [32].

The steroid hormone 20-Hydroxyecdysone (20-E) plays crucial roles in larval molting and metamorphosis [reviewed by 60]. No binding was detected for NLaz with 20-E in the micromolar range of concentrations (Fig. 3B), as we reported for bacterially-expressed recombinant Laz [40].

Therefore, ApoD, NLaz and Laz show a varied specificity for steroids. ApoD binds progesterone with high affinity, but it does not bind cholesterol or 17β-estradiol. A possible explanation could be the presence of a hydroxyl substituent in position 3 of the cholesterol and 17β-estradiol molecules, which is replaced with a carboxyl group in progesterone. On the other hand, NLaz and Laz bind ergosterol but not 20-HE, which has two hydroxyl groups in positions 2 and 3.

3.1.4. Fatty acids and related lipids.

Fatty acids have been frequently identified as Lipocalin ligands [reviewed by 49]. Both ApoD and Laz bind AA (20:4) [34,35,40], which is released from membrane phospholipids by phospholipase-A2, is a substrate for eicosanoids synthesis, and plays a critical role in inflammation and tissue homeostasis. Protection of AA oxidation by binding to ApoD, preventing its conversion to isoprostanes, has been suggested as a putative ApoD mechanism of action [52].

The relationship between ApoD and AA is also extended to neurological disorders. ApoD

levels are increased in certain brain areas of patients with Alzheimer's disease [14], Parkinson's disease [15] or schizophrenia [16]. A common characteristic in these three pathologies is a reduction in the levels of AA in the brain [61-63]. Increased ApoD levels could be interpreted as an attempt to correct for low AA levels, to maintain the fluidity properties of plasma membranes and the function of AA signaling pathways.

As ApoD-AA binding has already been well established, we tested if NLaz can bind other fatty acids such as arachidic acid (ArA) or PA, which are abundant in Drosophila sphingolipids and glycerophospholipids respectively [53]. Figure 4B-C shows a positive binding with ArA and PA. The estimated K_D were 4.5 µM and 4.7 µM for NLaz-ArA and NLaz-PA respectively.

NLaz-PA binding and the previously known Laz-PA binding [40], led us to test whether PA binding was also conserved in ApoD. Figure 4A shows the titration of PA with ApoD, resulting in a calculated K_D of 3.3 µM. These results contrast with a previous report where ApoD does not measurably bind to PA [34].

3.1.5. Endocannabinoids and ApoD.

Endogenous cannabinoids, or endocannabinoids (EC), are lipid modulators of synaptic activity and are involved in memory, anxiety, movement and pain. Predominant ECs include anandamide (AEA) and 2-arachidonoylglycerol (2-AG). AEA and 2-AG are both synthesized from membrane AA glycerophospholipid precursors [reviewed by 64].

How ECs are released from cells and how they reach their targets is a major issue in the field. Recent studies have identified intracellular AEA transport proteins, but the question is still open on whether extracellular EC-transporter proteins exist [see 65 for a review]. It has been suggested that Lipocalins, due to their lipid binding pocket, could be responsible for EC mobilization inside the brain [66]. Three Lipocalins are normally expressed in the human brain: L-PGDS, RBP and ApoD. RBP and PGDS ligands are well known and their functions have been established. ApoD appears therefore as a likely candidate for EC binding. NLaz is also a brain Lipocalin, but the classical

cannabinoid receptors are absent in the *Drosophila* brain [67].

These results, along with ApoD binding to the EC precursor AA [34,35], point to this Lipocalin as an endocannabinoid carrier. We tested whether ApoD binds to 2-AG or AEA, and found measurable binding with AEA (K_D 1.6 µM) (Fig. 4D, E). This is the first experimental evidence showing an interaction between ApoD and the endocannabinoid system. This selective binding profile of ApoD is particularly noteworthy, and future *in vivo* experiments with ApoD-KO and ApoD-overexpressor mice might confirm a physiological role for ApoD in the endocannabinoid system.
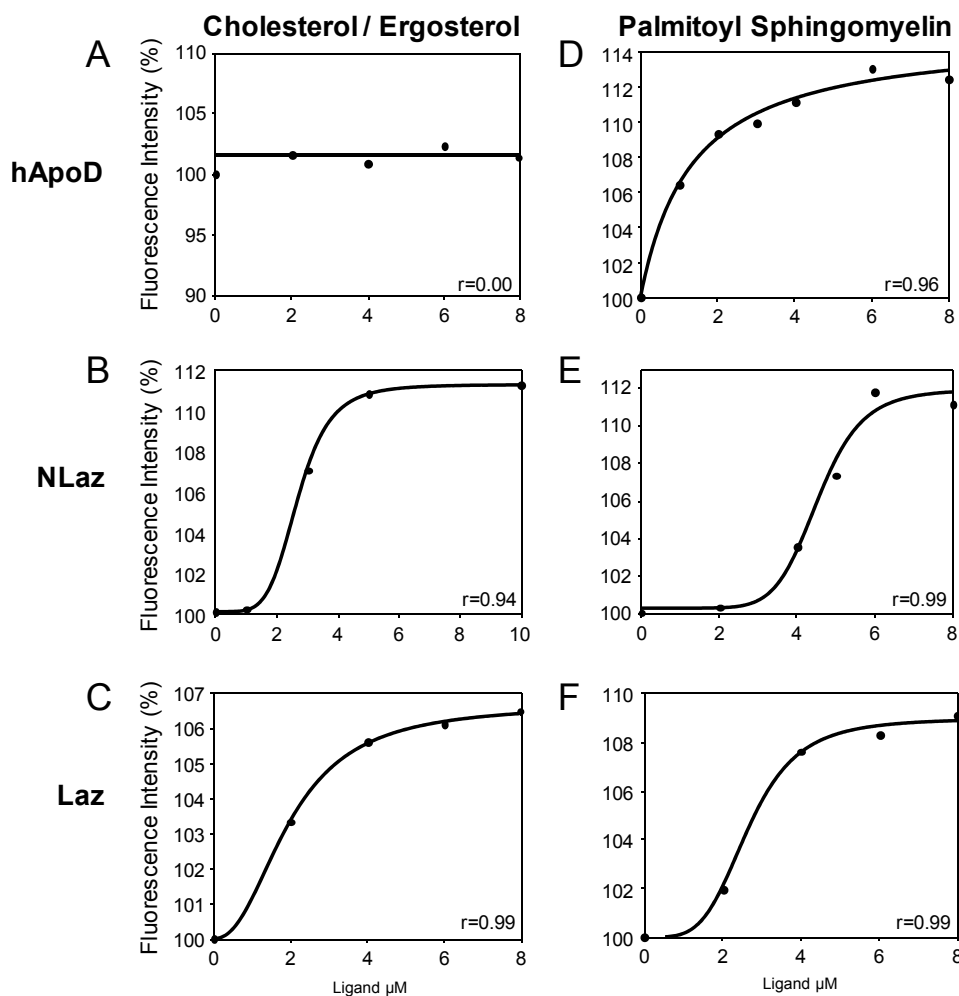
Figure 2. Intrinsic fluorescence changes of hApoD, NLaz and Laz upon incubation with different lipids present in biological membranes.
(A) Stable fluorescence of hApoD upon exposure to increasing concentrations of cholesterol. (B) NLaz fluorescence enhancement by ergosterol. (C) Laz fluorescence enhancement by ergosterol. (D-F). Palmitoyl-sphingomyelin enhances the fluorescence of hApoD, NLaz and Laz. Dots represent the mean of at least 3 independent titrations.

3.1.6. NLaz binding to *Drosophila* pheromones.

Pheromones are bioactive lipids involved in the regulation of courtship behavior and mating. NLaz null mutant (NLaz-KO) flies exhibit a dramatically low courtship index compared to their isogenic controls [23,27]. As a first step to understand the mechanistic origin of this phenotype, we tested whether NLaz is able to bind *Drosophila* pheromones.

11-cis-vaccenyl acetate (cVA) is the most studied *Drosophila* pheromone, and acts selectively through the Or67b odorant receptor in both sexes [68]. LUSH is the extracellular carrier for cVA and undergoes a conformational change that triggers cVA release and binding to the Or67b receptor [69]. We tested NLaz as an alternative pheromone-binding protein for cVA, but did not detect significant binding (Fig. 3E).

Other *Drosophila* cuticular sex pheromones are unsaturated long-chain hydrocarbons synthesized from fatty acid precursors. PA, a ligand of NLaz, is also the substrate of Desat1, a PA desaturase that triggers an enzymatic pheromone synthesis pathway [70].

Drosophila hydrocarbon pheromones are dimorphic: monoene hydrocarbons are predominant in males, and dienes in females [71]. We tested whether NLaz is able to bind two of the major *Drosophila* pheromones: the 7-tricosene (7-T, 23:1) and 7,11-heptacosadiene (7,11-HD, 27:2). Figure 3C shows positive binding of NLaz to monoene 7-T, whereas no binding was detected with the diene 7,11-HD (Fig. 3D). The estimated K_D of 7-T binding to NLaz is 4.5 μM. 7-T increases *Drosophila* female sexual receptivity, making flies to mate faster and more often with males that have higher levels of 7-T, while low levels of 7-T reduces courtship success [72]. We propose that NLaz could be acting as a pheromone carrier protein for 7-T, in a way similar to the LUSH-cVA pair [69].

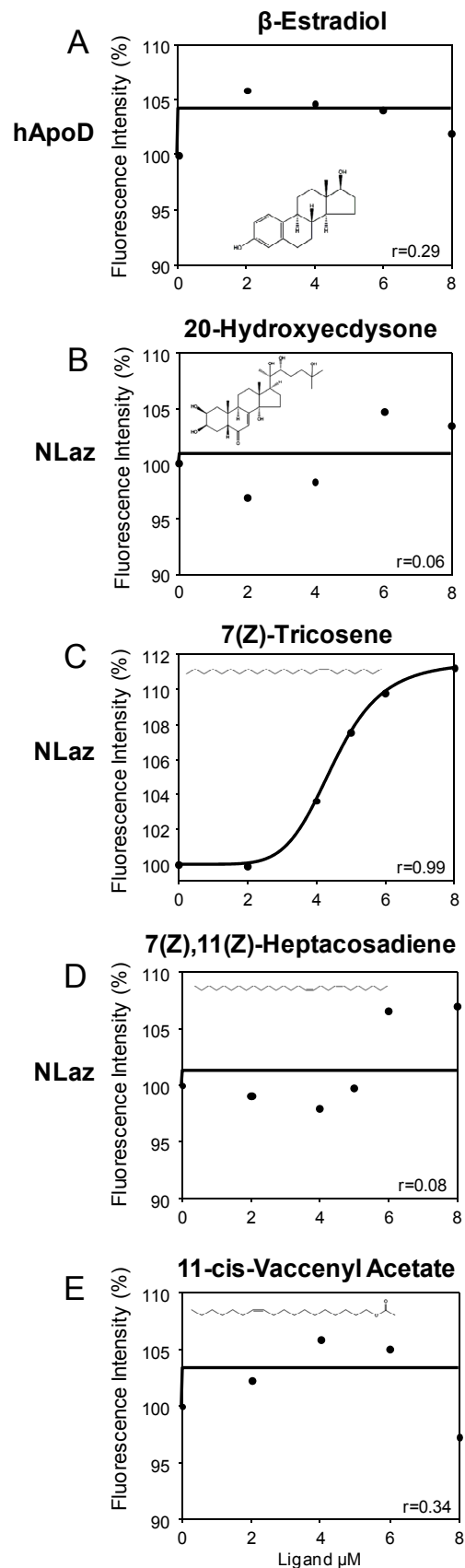**Figure 3. Binding assays of hApoD and NLaz with hormones and pheromones.**
(A) β-estradiol does not alter the intrinsic fluorescence of hApoD. (B) NLaz does not bind 20-hydroxyecdysone. (C) NLaz selectively binds the pheromone 7(z)-tricosene, whereas no interaction was detected for 7(z),11(z)-heptacosadiene (D), or 11-cis-vaccenyl acetate (E). Dots represent the mean of at least independent 3 titrations.
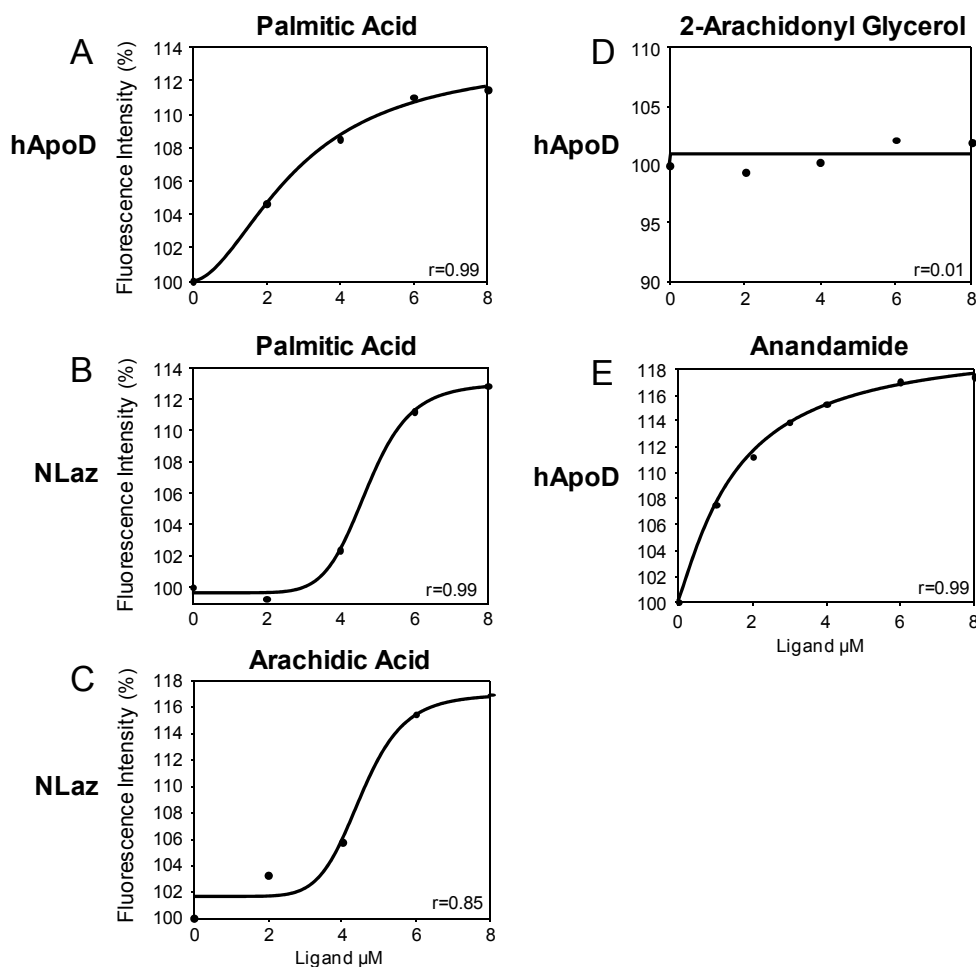
Figure 4. Interaction study of fatty acids and arachidonic acid-related molecules with hApoD and/or NLaz.
(A, B) hApoD and NLaz bind palmitic acid. (C) NLaz is also able to bind arachidic acid. (D-E) Binding of ApoD to endocannabinoids. hApoD does not bind 2-arachidonyl glycerol (D), but anandamide successfully interacts with hApoD enhancing its fluorescence (E). Dots represent the mean of at least independent 3 titrations.

3.2. ApoD-RA interaction is required for neuronal differentiation in culture.

Finally, we assayed whether any of the binding properties of the ApoD-related Lipocalins have direct *in vivo* consequences. For that purpose, we chose a neuronal cell model (SH-SY5Y and M17 cell lines), and RA as a well-defined common ligand for these proteins.

RA has a crucial role during brain development, being required to induce early progenitors to adopt a neural fate. During embryogenesis there is a requirement for a precise timing of exposure to RA and a coordinated pattern of expression of retinoic acid receptors (RARs) [see 44 for a general review].

SH-SY5Y is a human neuroblastoma cell line that has been established as a model of neuronal differentiation [41,73]. Under RA treatment SH-SY5Y cells develop long processes, and cells with processes longer than 50 μm have been considered as morphologically differentiated [41]. The presence of at least 1% serum [74] or exogenous albumin is required to obtain differentiation of SH-SY5Y cells by RA [75]. On the contrary, SH-SY5Y cells quickly enter the apoptosis pathway under serum deprivation [76].

ApoD plays an active role during nervous system development, where it is mainly expressed by glial cells, but is also present in neurons [8-10]. ApoD has been previously related with cell differentiation. Lopez-Boado el al. [77] first

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Figure 5. Physiological role of RA and hApoD in neuronal differentiation.
(A) SH-SY5Y cells growing in complete medium (10% FBS) with ethanol carrier. (B-C) Cells cultured in complete medium after 4-6 days of incubation with 10 μM RA (B) or 1 μM RA (C) in ethanol. (D) Cells cultured in serum-free medium plus carrier. (E-G) Serum-free medium was supplemented with 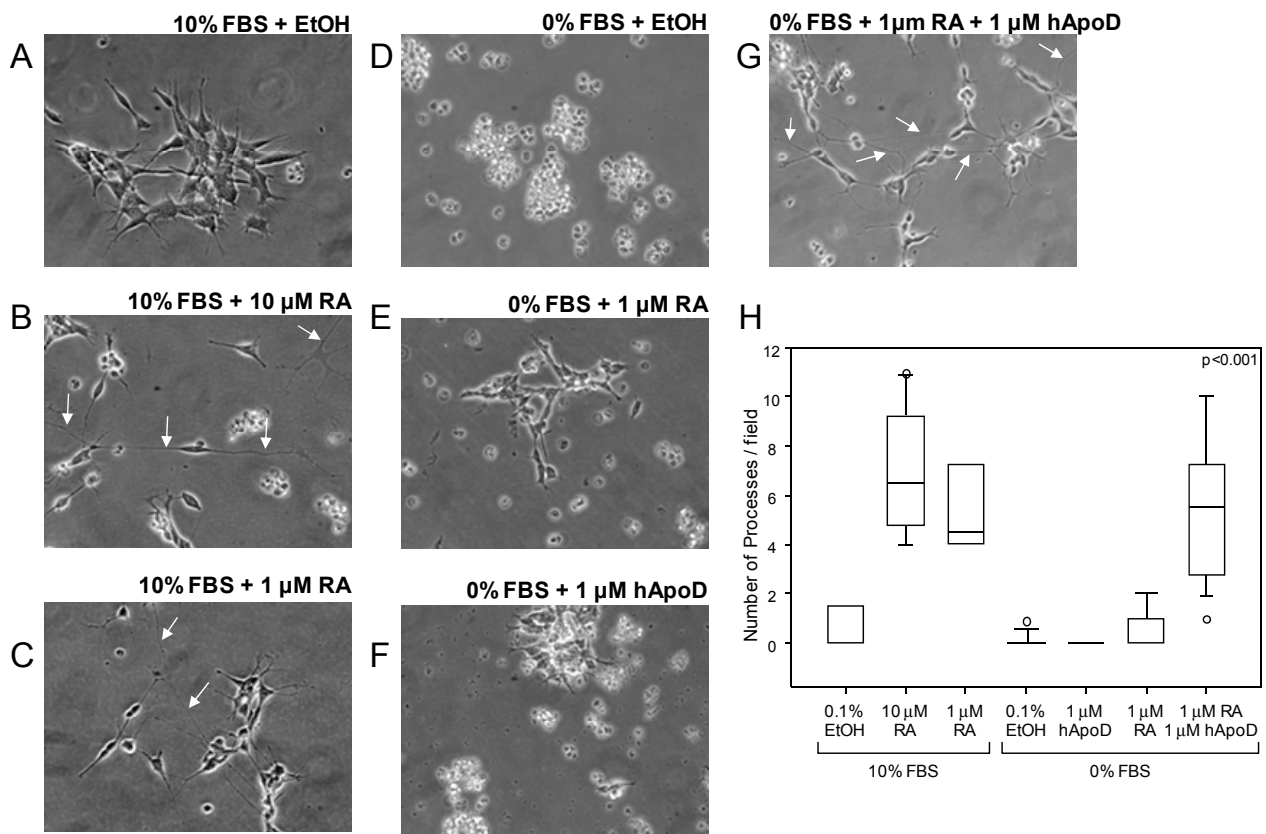1 μM RA (E), 1 μM hApoD (F) or a mixture of 1 μM RA and 1 μM hApoD (G). Cell growth was monitored after 4-6 days in culture. (H) Quantification of the number of neurites observed per field. Bars represent the quantification of 8-18 fields per condition from 5-6 independent experiments. Kruskal-Wallis one-way ANOVA on Ranks ($p < 0.001$) followed by Dunn's multiple comparison post-hoc test. In SH-SY5Y cells hApoD is able to substitute for FBS to allow maturation and neurite growth.

described that human breast cancer T-47D cells differentiate under RA treatment. In these conditions T-47D cells accumulate lipid droplets and increase ApoD levels, both at mRNA and protein levels. This process is RARα-dependent [78]. Moreover, Kosacka et al. [30,51] have shown that ApoD might act as a neurotrophic adipokine and exerts differenciating and synaptogenic effects in dorsal root ganglion neurons. On the other hand, when SH-SY5Y cells are transfected with p73, a transcription factor of the p53 family involved in neuronal differentiation, cells differentiate to mature neurons in an ApoD-dependent manner [79].

Initially, we confirmed that SH-SY5Y extend neurites under standard differentiation conditions (Fig. 5B and 5C). When serum was removed from the medium, cell survival decreased dramatically

and no differentiation was achieved even in the presence of RA, ApoD, NLaz or Laz (Fig. 5E-F and 6A-B). However, cells do differentiate and develop numerous long processes if we add ApoD preloaded with RA (Fig. 5G) in the absence of serum. These results show that ApoD is necessary and sufficient to allow for RA differentiating activity.

To validate as general the effects of ApoD-RA on neuronal differentiation, we used another human neuroblastoma cell line, M17, and obtained identical results (Fig. S5 and S6B).

Thus, ApoD loaded with RA produces neuronal differentiation and promotes neuritogenesis even under serum deprivation conditions. Since RA solubility in water is limited, we propose that ApoD could be acting as a carrier protein for RA. Astrocytes act as a source of ApoD

[26] and also provide RA and contribute to RA homeostasis [80]. ApoD could therefore deliver, or make available, RA from astrocytes to neuronal precursors *in vivo*.

Finally, we tested whether NLaz or Laz can also mediate the differentiation effect of RA on SH-SY5Y cells. RA-preloaded NLaz also induces neuronal differentiation, but in a less efficient way than ApoD-RA or serum-RA (Fig. 6C and E). However, the lengths of processes produced by NLaz-RA are similar to those under ApoD-RA or serum-RA treatment (Fig. S6A). On the other hand, SH-SY5Y cells treated with Laz-RA do not show a different number of processes than control cells (Fig. 6D and E).

In summary, SH-SY5Y cells treated with ApoD or NLaz, but not Laz, preloaded with RA are able to survive and differentiate in serum free medium, developing long processes. These results indicate that not every RA binding protein is able to trigger the differentiation program. Since NLaz-RA is not as successful as ApoD-RA in triggering SH-SY5Y differentiation, our results also suggests that a specific interaction, perhaps with an ApoD receptor, is further required to obtain the full effect. NLaz interaction with this putative receptor in mammalian cells might not be as favorable.
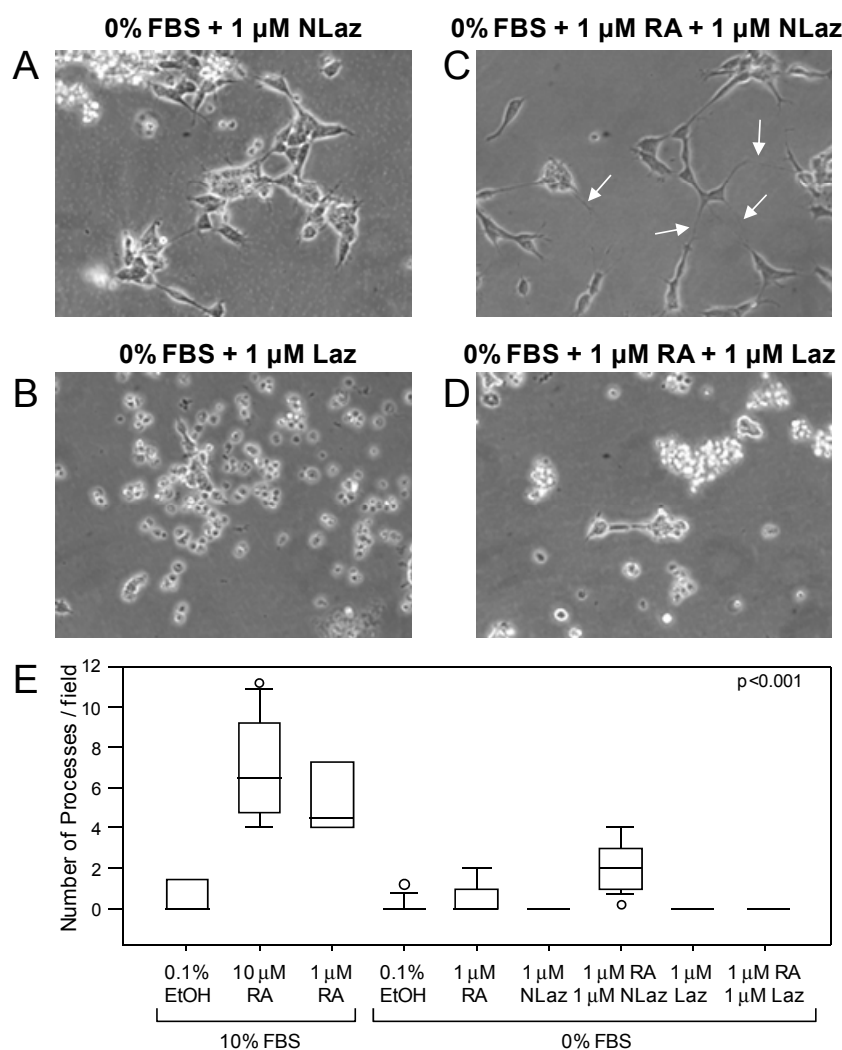
Figure 6. Effects of long-term RA and/or NLaz-Laz treatments on SH-SY5Y cell differentiation. Cells grown in serum-free medium containing.
(A) 1 μM NLaz, (B) 1 μM Laz, (C) 1 μM RA plus 1 μM NLaz or (D) 1 μM RA plus 1 μM Laz. (E) Quantification of neurites observed per field normalized by the number of cells per field in each condition. Bars represent the quantification of 8-16 photographs from 4-5 independent experiments. Kruskal-Wallis one-way ANOVA on Ranks (p<0.001) followed by Dunn's multiple comparison post-hoc test. NLaz and RA are able to induce some degree of differentiation on SH.SY5Y cells, whereas Laz does not produce any effect.

3.3. ApoD and its insect related Lipocalins: conservation and divergence of ligand binding properties.

Many studies have shown that human ApoD, *Drosophila* NLaz and *Schistocerca* Laz share functional properties in the organism: regulation of lifespan, lipid and carbohydrate metabolism control and protection against oxidative stress or starvation [21,22,26,27,56]. This work extends the knowledge of ligand binding for ApoD and Laz, and presents an extensive ligand screening for NLaz.

Our ligand-binding studies show that ApoD-NLaz-Laz similarities also extend to their biochemical and lipid binding properties. Homology modeling of the structures of Laz and NLaz (Fig. 7) also show the presence of predicted ligand binding sites, broadly consistent with the conserved structures of Lipocalins and the ligand specificities reported here. The modeled calyces of the insect Laz and NLaz are somewhat smaller in total volume and with moderately greater polar character than the ApoD calyx. As most Lipocalins, NLaz binds retinol and RA. Besides, fatty acids and SM are ligands common to ApoD and its insect related Lipocalins. These ligands can be therefore predicted to be related with *in vivo* common functions of ApoD, NLaz and Laz. In addition, species-specific ligands are first described here. AEA binding to ApoD and 7-T to NLaz might represent new functions acquired by these Lipocalins during their divergent evolution.

ApoD, NLaz and Laz can be considered as moonlighting proteins, and that pleiotropy is reflected on the high number of putative hydrophobic ligands. Their ligands belong to different groups of lipids, exhibiting different shapes and biochemical properties. In spite of this high variability and apparently promiscuous binding, ApoD, NLaz and Laz show a quite exquisite selectivity between very similar lipidic species. For example, ApoD binds the endocannabinoid AEA, but not 2-AG, and NLaz binds the pheromone 7-T but not 7,11-H.

Taken together, our ligand screening and the neuronal cell based assay should help us understand how ApoD, NLaz and Laz perform

their known biological functions, and how much of each function can be dependent on the particular lipidic ligand involved. Furthermore, the discovery of new putative ligands for these Lipocalins opens the door to explore new functions of ApoD and Lazarillo *in vivo*.
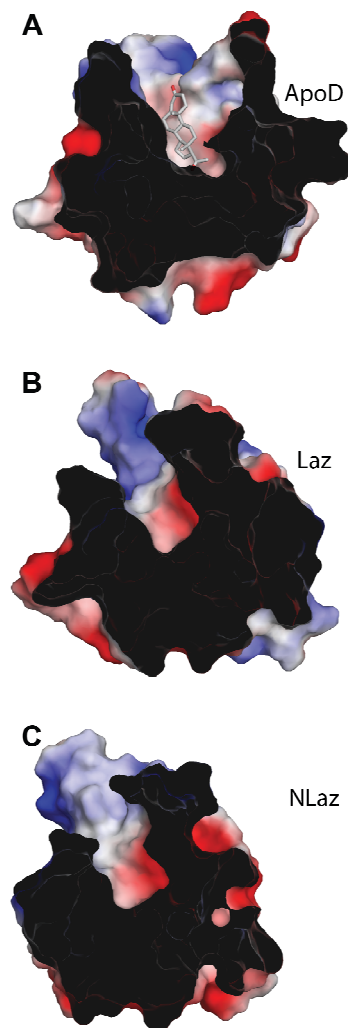
Figure 7. Homology models of Laz and NLaz.
Cutaway views of the molecular surfaces of the crystal structure (PDB accession code 2HZQ, [36]) of ApoD (A) and the homology models of Laz (B) and NLaz (C) are shown, colored by electrostatic potential, highlighting the calyces. Images were prepared with the PyMOL Molecular Graphics System, Version 1.3 (Schrödinger, LLC.). The progesterone ligand of ApoD is shown in a licorice stick representation, colored by atom type. Homology models were generated with SWISS-MODEL using the crystal structure of insecticyanin from the tobacco hornworm *Manduca sexta* (PDB accession code 1Z24, [81]) as a template.

Table 1.
Comparative of apparent K_D values for the binding of various ligands to Lipocalins
(average value ± S.D.).

Protein	Ligand	K_D	n
hApoD	All-*trans*-Retinoic Acid	4.0±2.6	8
	Retinol	0.2±0.1	7
	Cholesterol	n/d	3
	Palmitoyl Sphingomyelin	1.3±0.5	6
	Palmitic Acid	3.3±0.6	8
	PLPE	n/d	3
	β-Estradiol	n/d	7
	2-Arachidonyl-Glycerol	n/d	3
	Anandamide	1.6±1.3	10
NLaz	All-*trans*-Retinoic Acid	1.40±1.6	9
	Retinol	0.9±1.2	10
	Ergosterol	2.7±0.7	6
	Palmitoyl Sphingomyelin	4.6±0.2	8
	Palmitic Acid	4.7±0.2	6
	Arachidic Acid	4.5±0.7	3
	PLPE	n/d	8
	7(Z)-Tricosene	4.5±0.6	6
	7(Z),(11)-Heptacosadiene	n/d	6
	11-cis-Vaccenil-Acetate	n/d	5
	20-Hydroxyecdysone	n/d	3
Lazarillo (S2)	All-*trans*-Retinoic Acid	3.0±1.1	8
	Retinol	2.1±0.6	5
	Ergosterol	2.0±0.1	7
	Palmitoyl Sphingomyelin	2.7±0.2	7
	PLPE	n/d	5
Lazarillo (*E.coli*)	All-*trans*-Retinoic Acid	2.6±1.8	3
	Palmitoyl Sphingomyelin	3.0±2.6	3
α1-microglobulin	Retinol	1.4±0.1	4
	Palmitoyl Sphingomyelin	n/d	3

5. References

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Supplementary Figures
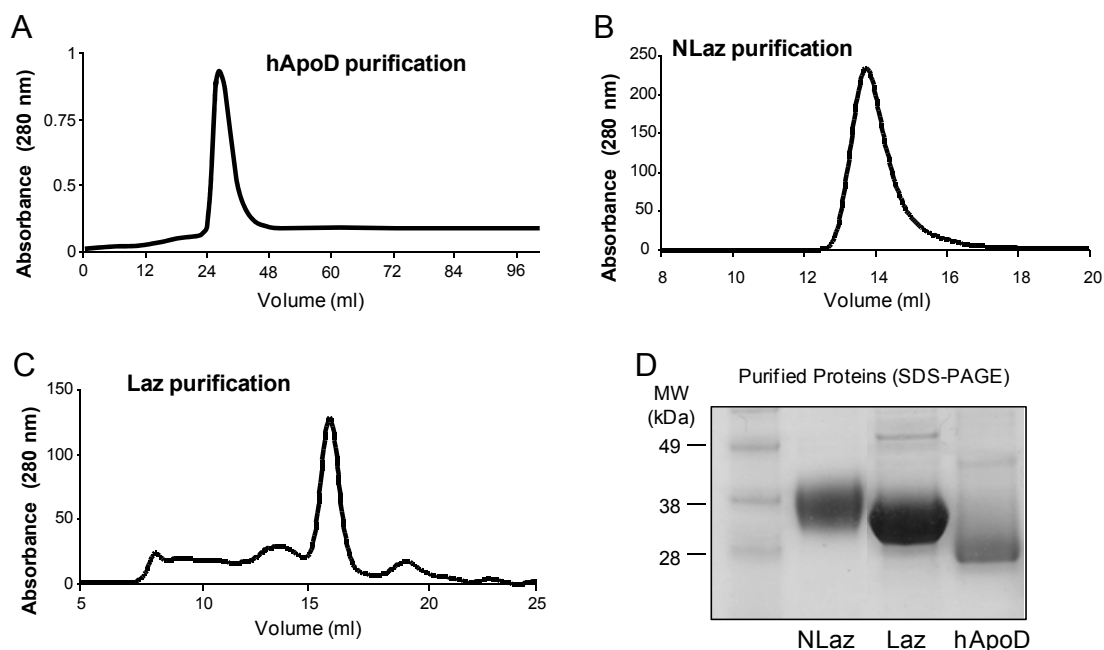
Figure S1.
Expression and purification of hApoD from human breast cystic fluid, and NLaz and Laz from S2 cells.
Size-exclusion chromatography elution profile of hApoD (P60) (A), NLaz (analytical grade-200 column) (B), and Laz (analytical grade-200 column) (C). (D) SDS-PAGE analysis of the purified Lipocalins.
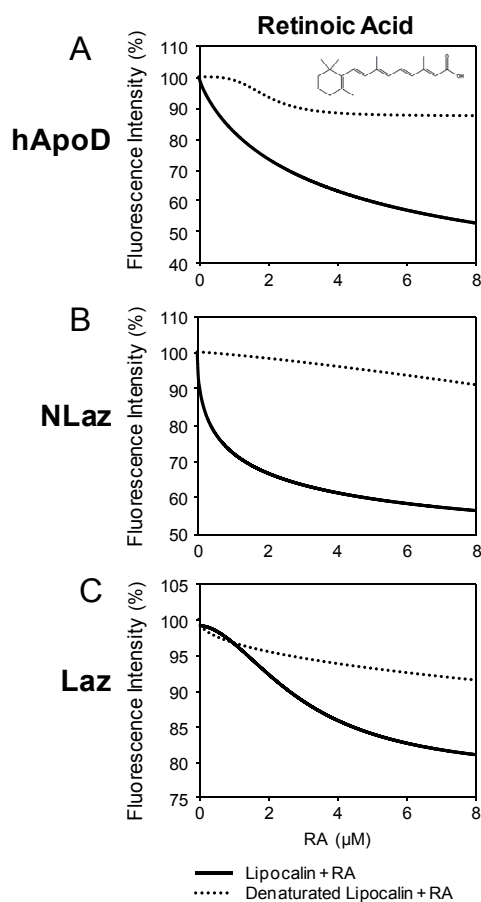
Figure S2.
Lack of retinoic acid binding to hApoD (A), NLaz (B) and Laz (C) under denaturing conditions. Binding assays were performed either in the standard buffer or after 21 h incubation with 5 M guanidine hydrochloride. Lipocalins-RA interaction depends on the protein tertiary structure.
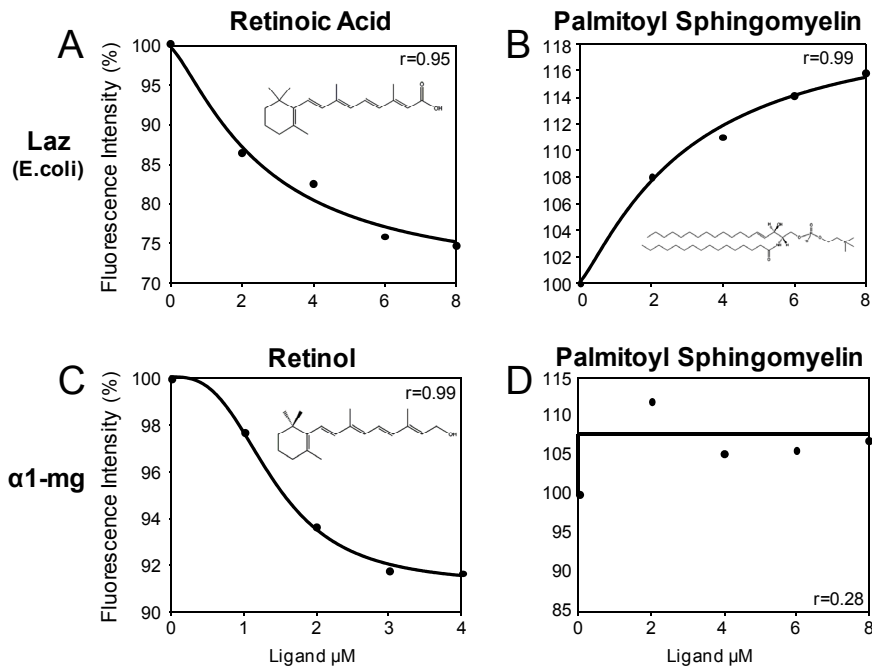
Figure S3. Additional intrinsic fluorescence titration studies.
Recombinant Laz purified from *E. coli* reproduces the binding pattern of Laz purified from S2 cells. (A) Laz binds retinoic acid and (B) palmitoyl-sphingomyelin. A different Lipocalin (α1-mg) also binds retinol (C), but does not bind palmitoyl-sphingomyelin (D).
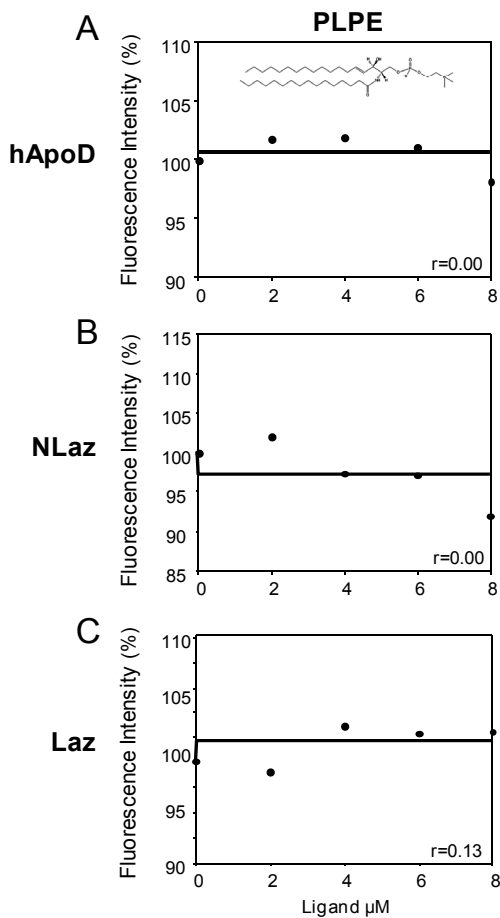
Figure. S4. **Additional intrinsic fluorescence titration studies.** (A) hApoD, (B) Nlaz and (C) Laz do not bind the phospholipid PLPE.
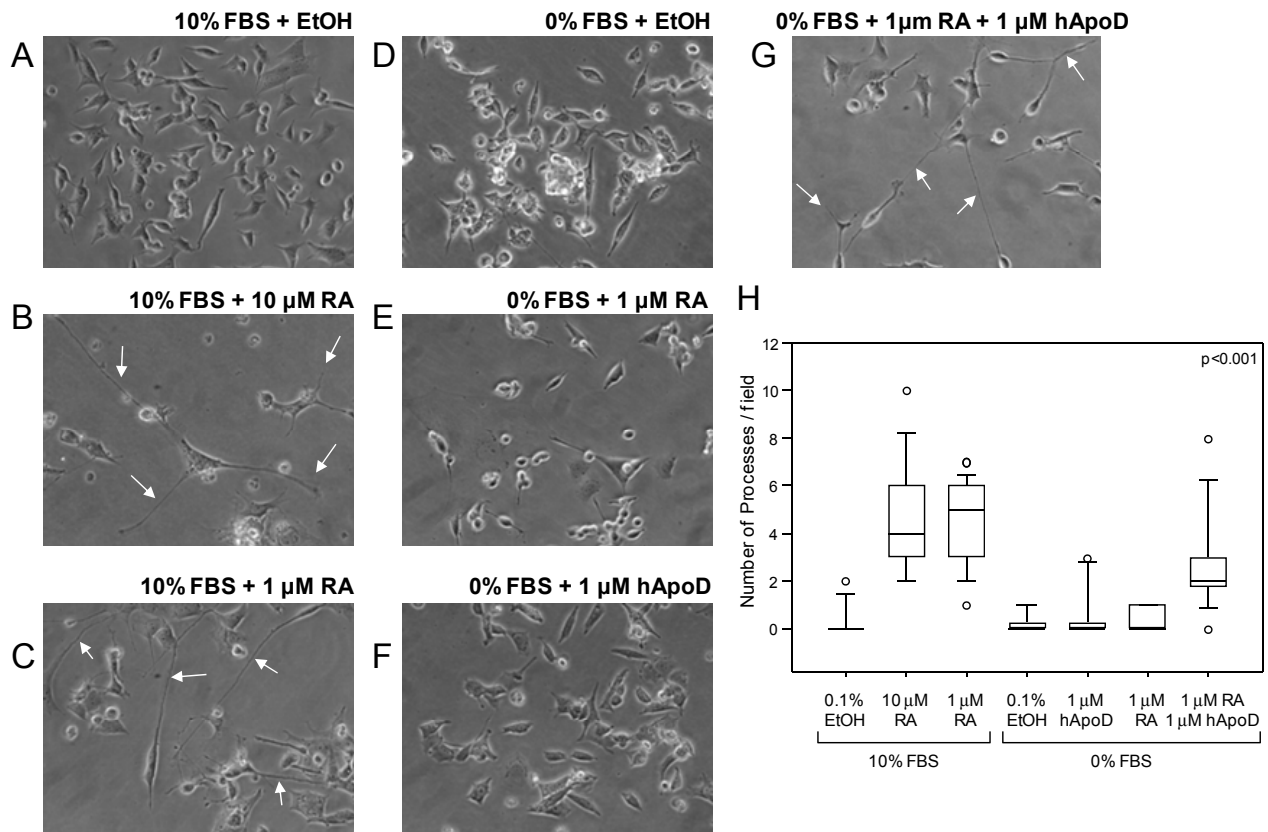
Figure S5.
Confirmation of the physiological role of RA and hApoD in M17 cells, a different human neuronal cell line.
Morphology changes of M17 cells growing in complete medium with (A) ethanol carrier, and after treatment with (B) 10 µM RA or (C) 1 µM RA in complete medium. (D) Cells in serum-free medium with ethanol carrier. (E-G) Cell differentiation after 4-6 days of treatment with (E) 1 µM RA, (F) 1 µM hApoD, or (G) 1 µM RA plus 1 µM hApoD. (H) Quantification of the number of neurite processes /field normalized by the number of cells/field in each condition. Bars represent the quantification of 10-25 fields per condition from 3 independent experiments. Kruskal-Wallis one-way ANOVA on Ranks ($p<0.001$) followed by Dunn's multiple comparison post-hoc test. In M17 cells, hApoD is also able to substitute for serum and to allow maturation and neurite growth.
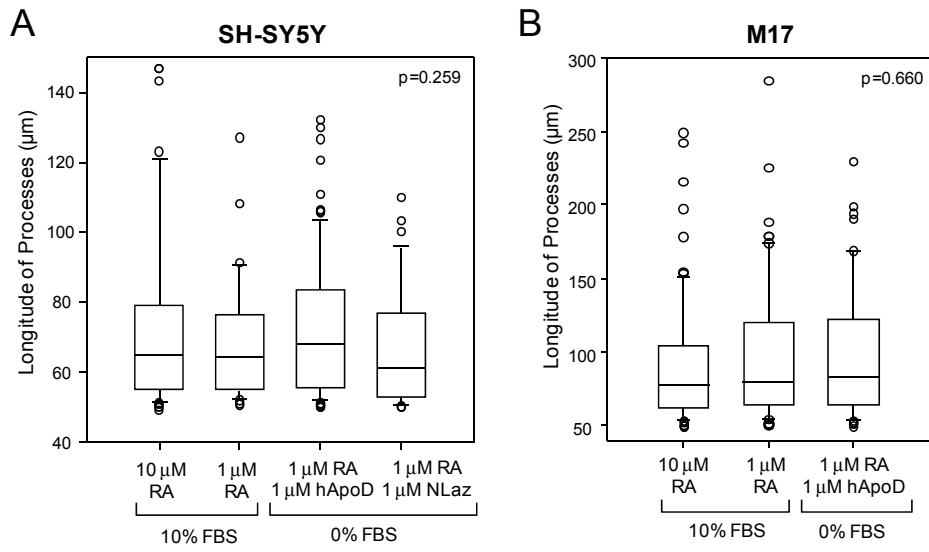
Figure S6.
Quantification of neurite length upon RA-induced differentiation of (A) SH-SY5Y or (B) M17 neuronal cell lines. No changes are observed in the length of neurites between treatments. Kruskal-Wallis one-way ANOVA on Ranks ($p>0.05$).

Chapter 3

"Ligand-binding dependent functions of the Lipocalin NLaz:

An in vivo study in Drosophila".

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"Ligand Binding-Dependent Functions of the Lipocalin NLaz: an in vivo Study in Drosophila."

Submitted

Ligand binding-dependent functions
of the Lipocalin NLaz:
An *in vivo* study in Drosophila.

Summary

NLaz (Neural Lazarillo) is a Drosophila Lipocalin induced upon stress, and able to regulate longevity, stress resistance, and locomotor and mating behaviors. NLaz modulates the IIS pathway; however its biochemical mechanism of action is still unknown. Here we test whether ligand binding to the Lipocalin hydrophobic pocket is required to perform NLaz functions. We use point-mutant versions altering the binding pocket (NLaz^{L130R}, NLaz^{Y47A}) and other control mutant versions of NLaz. We express the proteins in Drosophila S2 cells and assay their survival-promoting potential. Ligand binding, tested by tryptophan fluorescence titration, revealed NLaz^{WT} binds retinoic acid, ergosterol and the pheromone 7(z)-tricosene, while NLaz^{L130R} binds only retinoic acid. Using site-directed transgenesis in Drosophila we analyze which physiological functions depend on NLaz ligand binding ability, constituting the first organism test of a ligand binding-deficient Lipocalin. NLaz-dependent lifespan reduction, paraquat and starvation sensitivity, aging markers accumulation, and deficient courtship are rescued by ubiquitous expression of NLaz^{WT}, but not of NLaz^{L130R}. The transcriptional responses to aging and paraquat show a larger set of aging-responsive genes dependent on the integrity of NLaz ligand binding properties. Inhibition of IIS activity by NLaz, and modulation of oxidative-stress and infection responsive genes appear as ligand binding-dependent processes, all of them impacting the process of aging.

1. INTRODUCTION

Lipocalins are a large family of small extracellular proteins characterized by their well conserved β-barrel structure embracing an internal cavity able to bind small ligands, most of them hydrophobic or amphipathic [1]. For some Lipocalins we have a good approximation to the role played by their native ligand. For instance, Retinol Binding Protein (RBP) transports vitamin A within the retina [2]; Siderocalin (Lcn2 or NGAL) sequesters iron-binding molecules and provides a bacteriostatic mechanism against infections [3]; and Crustacyanins carry the pigment giving color to the carapace of crustaceans [4]. However, the panoply of different physiological functions in which Lipocalins are involved keeps increasing, and in the vast majority of cases a lipid transport function is presupposed but not tested.

A set of Lipocalins, including the most ancient within the metazoan lineage [5-7], has been functionally linked to the response of organisms or cells to different forms of stress and to the modulation of metabolism, key parameters regulating senescence and longevity [reviewed by 8]. Among these are the Lazarillo related Lipocalins, a group including the Drosophila Lipocalins Neural Lazarillo (NLaz) and Glial Lazarillo (GLaz) [9] and the vertebrate Apolipoprotein D (ApoD). The expression of these Lipocalins, both within and outside the nervous system, is boosted by oxidative or metabolic stress and they provide an endogenous mechanism of protection particularly important in situations of aging and neurodegeneration [10-17].

NLaz is induced under the control of the stress responsive JNK pathway, and is able to negatively regulate the IIS pathway [13,18]. NLaz expression increases stress resistance and longevity, but also serves other apparently unrelated functions by regulating fecundity, food-intake and locomotor and courtship behaviors [15,19]. Whether all NLaz functions are carried out using a common biochemical mechanism or this Lipocalin can exert different functions depending on the ligand available for binding in different physiological situations is still unknown.

Studies testing Lipocalins ligand binding abilities *in vitro* are abundant [20-22], including ApoD and Lazarillo-related Lipocalins ([23-27], and unpublished work by the authors). In addition, many Lipocalins have been extensively studied at the molecular level, revealing particular residues and regions of the protein scaffold that are important for their ligand binding properties [20,22,28-31]. The biochemical and biophysical properties of Lipocalin-ligand interactions have been reproted for some family members, either by solving their crystal structure with the ligand bound to the pocket [32,33] or by site-directed mutagenesis of residues contributing to the pocket interactive surfaces [21,22,30,31,33-37]. Amino acid substitutions that naturally occur in the binding pocket of RBP result in vitamin A deficiency causing night blindness [38]. Also, thanks to their very robust folding, the Lipocalins have been used as starting point for protein engineering. Using site-directed random mutagenesis followed by selection techniques, an increasing set of so-called "Anticalins" has been generated with various specific target-binding activities, many of them with potential therapeutic benefits [for a review see 39].

In spite of the many known Lipocalin mutant forms that result in deficient ligand binding *in vitro*, the studies analyzing their effects outside the test tube are rare. Mutant forms of Prostaglandin D synthase (L-PGDS) have recently been tested for their ability to promote cell survival in H_2O_2 treated neuronal cultures [40]. A mechanism involving the titration of a free thiol in the protein without alterations of ligand-binding capacity has been found. Modifications of the binding-pocket of the Lipocalin C8γ have been tested for hemolytic activity [30]. Also, Correnti et al. [35] have found that Siderocalin binding pocket mutants that cannot bind siderophores *in vitro* lose their bacteriostatic activity when added to bacterial cultures.

The purpose of this study is to test whether NLaz functions are dependent on ligand binding. We designed different mutant forms of NLaz and tested them at three levels of analysis. We assayed their ligand binding capacity *in vitro*, their cell survival promoting activity in a cell culture system, and their effect on longevity, stress resistance,

behavior and downstream transcriptional responses *in vivo*. We were able to compare the physiological effects of wild type and mutant NLaz expression without the confounding effect of transgene insertion site in the genome by the use of site-directed transgenesis in Drosophila. This is, to our knowledge, the first *in vivo* test of the physiological outcome derived from a ligand binding-deficient Lipocalin at the organism level.

2. MATERIAL AND METHODS

2.1. Homology modeling of NLaz 3D structure.

The NLaz model was obtained from the Swiss Model Repository platform [41,42], built using the atomic coordinates of hApoD [PDB entry: 2HZQ] as the best template [26].

2.2. S2 cells culture.

Drosophila S2 cells were maintained as semi-adherent cultures at 27ºC in Express-Five medium (Gibco) supplemented with 10% L-Glutamine, 50 U/ml Penicillin, and 50 μg/ml Streptomycin. The culture medium was replaced twice a week.

2.3. Cloning and cell lines generation.

NLaz cDNA, translating into residues 1-224 (CG33126, Uniprot reference Q9NAZ4, FlyBase entry FBgn0053126), was subcloned using the EcoRI and NotI sites into the pRmHa3 vector (Bunch et al. 1988; Sanchez et al. 2008). This system expresses the cloned sequence under the control of the inducible Drosophila metallothionein promoter, and the presence of a C-terminal His-tag sequence allows for protein purification from the culture medium.

NLazL130R-pRmHa3 was generated by direct mutagenesis of NLazWT-pRmHa3 following the Quick Change Site Direct Mutagenesis method (QIAgen). Table S1 lists the oligonucleotides used for mutagenesis, cloning, and sequencing.

The NLazWT-pRmHa3 or NLazL130R-pRmHa3 plasmids were co-transfected with the selection vector pCoBlast (conferring blasticidin resistance) into Drosophila S2 cells using FuGENE6 (Roche) at 3:1 (v:w) ratio according to

the manufacturer instructions. Transfected cells were selected with 25 μg/ml blasticidin-S for 3 weeks (Invitrogen).

2.4. Purification of NLaz^{WT} and NLaz^{L130R} proteins.

S2 cells expressing the recombinant protein NLaz^{WT} or NLaz^{L130R} were grown in spinner flasks with blasticidin-free medium, upon induction with 1mM $CuSO_4$ for 5-7 days. The secreted NLaz proteins were purified from the cell medium by immobilized metal affinity chromatography using Ni-NTA resin (5-Prime). The NLaz proteins were further purified by two rounds of size exclusion chromatography (Superdex preparative grade 75 and analytical grade 200, Amersham Biosciences) in PNEA buffer (25 mM PIPES, 150 Mm NaCl, 1mM EDTA, 0.02% NaN_3) (Fig. S1-A). The purified proteins were deglycosylated by treatment with peptide-N-glycosidase F (PNGase-F) from *Flavobacterium meningosepticum* (New England Biolabs) after denaturation following the protocol supplied by the manufacturer. The purified proteins were analyzed by 12% SDS-PAGE (Fig. S1-B).

2.5. Ligand binding assays by tryptophan fluorescence modification

Fluorescence measurement were conducted in a Shimadzu RF-5301PC spectrofluorometer with a quartz cuvette (Hellma; type 105.251-QS, light path length of 3 mm). Temperature was held at 22 ± 0.1ºC by a Peltier thermostat. Excitation wavelength was 295 nm (mainly selective for tryptophan). Fluorescence emission was recorded from 327 to 400 nm with slits width set at 5 nm. The peak at 340 nm was measured and used to calculate K_d. Each titration was performed in 100 μl.

The NLaz protein was diluted at 1 μM in 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA at pH 7.0. The ligands tested were Retinoic Acid (Sigma), Ergosterol (Sigma) and 7(z)-Tricosene (Cayman), and were dissolved in N,N-dimethylformamide (DMF) (Sigma).

The fluorescence spectrum in the presence of the ligand was subtracted from a DMF baseline obtained by titrating the protein with the same

amounts of carrier without ligand. The data of corrected fluorescence at 340 nm vs. the total concentration were fitted as previously described [23,24], and a K_d was estimated with the assumption of a single binding site.

2.6. Generation of transgenic flies by site-specific transgenesis.

NLaz loss-of-function mutant (NLaz-KO) was generated in a w^{1118} background [43] and crossed with a w^{1118}-CS wild type line to generate the NW5 line used in this study [13].

The full wild type NLaz cDNA (coding for a 224 residue polypeptide) was subcloned into the pUASt-attB vector [44] (Gen Bank entry EF362409 and FlyBase entry FBmc0003002) using the EcoRI site and sequenced to confirm the right orientation. $NLaz^{L130R}$ and $NLaz^{Y47A}$ point mutations were generated by site-directed mutagenesis of the wild type NLaz. A C-terminal truncated version of NLaz was generated by amplifying a cDNA fragment translating into residues 1-176 of NLaz. $NLaz^{N165S}$ was identified by sequencing as a polymorphism in some of our wild type fly stocks. All these NLaz versions were subsequently subcloned into the pUASt-attB vector. The constructs were sequenced to confirm the absence of undesirable mutations. See table S1 for oligonucleotides used for mutagenesis and cloning.

The PhiC31 system-mediated [44] transformation of *Drosophila* strain "y^1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb" (BL4749) was used to generate fly lines with the UAS:NLaz^{WT} or UAS:NLaz mutated versions, yielding transgene integration into chromosomal position 86Fb.

2.7. Fly Handling.

Flies were grown in a temperature-controlled environmental incubator at 25 ºC under a 12h light-dark cycle. The stocks were routinely maintained in tubes containing our standard medium [16].

To drive the expression of the NLaz transgenes, the driver da:Gal4 (ubiquitous expression) line was crossed with pUASt-NLaz fly lines or w^{1118} flies (to generate driver-only control flies). Only males

were employed (except for courtship experiments where both sexes are involved).

The absence of infection by *Wolbachia pipiens* in our fly strains was tested by PCR of genomic DNA extracts (Fig. S1-C) as previously described [15]. Primers against the rRNA-16S gene of *Wolbachia* were used (Table S1). The PCR conditions were: 2 min 94°C; (30s 94°C; 30 s 60°C; 45 s 72 °C)×30; 7 min 72°C. DNA from *Wolbachia*-infected *Dirofilaria immitis* worms was used as a positive control. Primer sequences and a positive control DNA were kindly provided by Dr. F. Simon (Univ. Salamanca).

2.8. Drosophila Lifespan Analysis.

Flies were collected within 24 h of hatching and allowed to mate for 2-3 days. They were then separated by sex under brief CO_2 anesthesia and housed in groups of 20-25. Every three days, flies were transferred to fresh media without anesthesia and scored for viability. From 76 to 147 individuals were analyzed per genotype. The viability tests were carried out at 25ºC.

2.9. Oxidative Stress, Toxicity and Desiccation Stress Assays.

2.9.1. *Assays in flies.* Flies were collected and separated by sex as described for the longevity analysis. All tests were carried out at 25ºC. From 67 to 156 individuals were analyzed per genotype and condition. The following treatments were used:

2.9.1.1. Starvation-desiccation Stress Assay. Starting at 3 days of age, flies were transferred to empty plastic vials. Dead flies were scored every 2–7 hours.

2.9.1.2. Paraquat Toxicity Assay. Flies were kept on regular food until the experiment started (3 or 30 days-old), starved 3h at 25ºC and transferred to vials with filter papers soaked in 10% sucrose-20 mM paraquat (Sigma) solution in water. Dead flies were scored every 2–7 hours.

2.9.2. *Assays in cell cultures.* S2 cells were seeded at a density of 2×10^6/ml in 24-well plates, and 20 mM hydrogen peroxide (H_2O_2) was used as an oxidant. Fifty nM of pure NLaz^{WT} or NLaz^{L130R} protein were added to the culture medium 30 min before H_2O_2 treatment. Trypsin Inhibitor (TI) from soybean (Sigma) was assayed at the same concentration as a control protein. Protein concentrations were determined with the Micro-BCA^{TM} protein assay (Pierce). Two hours after treatment, cells were stained with Trypan Blue (Invitrogen) and counted in a haemocytometer. Cell viability was determined as the percentage of live cells. Each condition was assayed in quadruplicate, and at least two measurements were made in each culture well.

2.10. Behavioral Assays.

2.10.1. Climbing Assay. Flies (50 days-old) were placed in empty vials, and after a resting period of 10 min the animals were tapped to the bottom of the tube and the number of individuals able to climb 5 cm in 6 sec was recorded. The assay was repeated three times at 1 min intervals.

2.10.2. Courtship behavior. Courtship and mating tests were performed during 60 min using virgin male–female pairs of 3-4 day-old flies under a glass clock cover (40 mm diameter) used as an observation chamber. The courtship index (C.I.) represents the proportion of time a male spends actively courting the female for the first 10 min. Copulation features such as mating time, mating latency and mating performance (%) were scored for 60 min. The test was performed in the first 1-3 h of the fly daylight period. 25-30 couples/genotype were included in this study.

2.11. Metabolic Measurements.

Flies (4-5 days-old) were analyzed in basal situation and after 8 hours of starvation. Two independent groups of 15 flies per genotype and condition were analyzed. A set of kits were employed for triglycerides (BioSystems, Spain), glucose (Sigma, USA) and glycogen (Sigma, USA) measurement, following the manufacturer's

instructions, and absorbance was registered by a Versamax microplate reader (Molecular Devices).

2.12. AGE assay.

Fluorescent AGEs (advanced glycation end products) were measured according to Oudes et al. [45] with minor modifications. Flies were homogenized in 1 ml of PBS containing 10 mM EDTA. Cuticle and other debris were removed by centrifugation for 5 min at 11000 g. Supernatants were digested by addition of 10 mg/ml trypsin (Sigma). Following incubation for 24 h at 37ºC the digested homogenates were centrifuged and filtered through a 0.22 μm sterile filter. Filtrates were diluted up to an absorbance range of 0.02-0.05 units at 365 nm. Fluorescence (excitation and emission wavelengths of 365 and 440 nm, respectively) was measured in three aliquots of each filtrate with a Shimadzu RF-5301PC spectrofluorophotometer at 25ºC.

2.13 Quantitative RT-PCR gene expression array.

RNA from 25 homogenized flies per genotype and condition was extracted with QIAzol and RNeasy Mini Kit (Qiagen) according to the manufacturer instructions. We used SYBR Green and designed a custom qRT-PCR array (SABiosciences) with 4 replicas per sample to study gene expression changes generated by the expression of NLaz^{WT} or its mutant versions. RNA transcription profiles were determined by the method of direct comparison of Ct values and relative quantities calculated by the $\Delta\Delta$Ct method [46]. Table S3 shows the list of genes explored and their relationship to the physiological output that motivated its selection. A fold regulation ≥ 2 and p < 0.05 for at least one genotype was established for the gene to be considered as a PQ or aging-responsive gene.

2.14. Immunoblots

Fly proteins were extracted from the phenol phase after QIAzol RNA isolation. Protein concentrations were determined with the Micro-BCA^{TM} protein assay (Pierce). 25 μg of total protein/lane where transferred to a nitrocellulose membrane (BioRad). Membranes where blocked

with 2.5% BSA, 2.5% non fat milk and 0.4% gelatin. Mouse anti-ubiquitin (P4D1) (Cell Signaling) was used as primary antibody, and HRP-conjugated goat anti-mouse IgG (Dako) as secondary antibody. Non-transferred proteins that remained in the SDS-PAGE where stained with Coomassie R-250 (BioRad) for normalization purposes. Membranes were developed with ECL (Millipore) and the integrated optical density of the inmunoreactive protein bands was measured in images taken within the linear range of the camera (VersaDoc, BioRad) avoiding signal saturation. Blots were run as duplicates, and one representative experiment is shown.

2.15. Statistical Analysis.

Statistical analyses were performed with SigmaPlot (v 11.0) software. A $p < 0.05$ was defined as a threshold for significant changes. Particular tests used are stated in figure legends.

3. RESULTS

In order to design mutations that could result in altered ligand-binding properties we used NLaz tertiary structure model generated in the Swiss Model platform (Fig. 1). Using the well-known ligand-protein interactions of the closely related ApoD [26] we designed two types of binding-pocket mutants: 1) L130R (Fig. 1B) is predicted to obstruct binding by introducing a large side change and charged residue facing the pocket. 2) Y47A (Fig. S2 A) is predicted to enlarge the binding-pocket size by introducing a smaller side change residue of similar biochemical properties and that might consequently alter ligand binding interaction in more subtle ways. As a control, another point mutation was selected. A NLaz allele with a serine in position 165 was found in our wild-type fly stocks, while an asparagine in that position appear in the databases by more recent genome sequencing. In this work the N165 version was selected as the reference wild-type protein, matching the current entries in genomic databases. The NLaz^{N165S} version might represent a polymorphism present among laboratory flies. Since N165 lies outside the binding pocket (Fig.

S3 A), it serves to analyze the physiological outcomes of expressing an NLaz point mutant unrelated to ligand binding.

In addition to binding pocket mutants, we explored the effect of deleting a C-terminal extension (residues 177-192, Fig. S4 A) that is unique to NLaz within the Lipocalin family [47]. This C-terminal tail is similar in size to the GPI-anchoring signal peptide present in the ancient Lazarillo of grasshoppers and other insects [19,48], but shows a quite different amino acid composition. Aside of serving as a different control for the binding pocket mutants, the function of this putative evolutionary vestige is also explored in this work.

3.1. Monitoring NLaz-ligand interactions *in vitro* by intrinsic fluorescence analysis

The wild type and mutant versions of the NLaz pocket were expressed using an inducible expression system in Drosophila S2 cells. The proteins were purified from the culture medium by affinity chromatography against a C-terminal His-tag followed by two rounds of size exclusion chromatography to reach a purity > 99% (Fig. S1 A). The proteins produced by S2 cells were successfully secreted into the culture medium, are glycosylated and show signs of intramolecular disulfide bonds, as expected for a correctly folded native NLaz protein (Fig. S1 B). We centered our biochemical analysis in the ligand-binding properties of NLaz^{WT} and NLaz^{L130R} versions.

We used tryptophan fluorescence titration to assay the interaction of NLaz with different ligands. One of the two tryptophan residues in NLaz, W128, is predicted to be located within the binding pocket. Its modification by interaction with ligands in the pocket could either quench or enhance its fluorescence emission. All-trans-retinoic acid (RA) produced quenching of the emitted fluorescence (Fig. 2 A-B) in both NLaz^{WT} and NLaz^{L130R}. Since RA is known to bind most Lipocalins tested so far [24,49-51], it is a good probe of the correct folding of the S2 expressed proteins. We then tested ergosterol, the functional equivalent to mammalian cholesterol in insects, and proved that it produces an enhanced fluorescence over the carrier baseline in NLaz^{WT}

(Fig. 2C), but not in NLaz^{L130R}(Fig. 2D). The same pattern was observed with the pheromone 7(z)-tricosene [52] (Fig. 2E-F) demonstrating that the alteration of the pocket introduced by the L130R mutation was compromising the binding to ligands of different sizes, molecular properties and biological functions. Table 1 shows the estimated apparent Kd for the ligand–protein interaction in each case (assuming binding of one ligand per protein molecule). Similar Kd values in the μM range have been obtained for RA-Lipocalin interaction by us and others [23,24]. Ergosterol and 7(z)tricosene have been tested for the wild type version of NLaz (unpublished observation) showing a Kd of 2.7 μM and 4.5 μM respectively. Here, no binding was detected in the micromolar range for NLaz^{L130R}.

A

```
Signal Peptide:
MNHHSSSHLLLLISVVFGAVWVAHA

QVPFPGKCPDVKLLDTFDAEAYMGVWYEYAAYPFAFEIGKKCIYANYSLI

DNSTVSVVNAAINRFTGQPSNVTGQAKVLGPGQLAVAFYPTQPLTKANYL

VLGTDYESYAVVYSCTSVTPLANFKIVWILTRQREPSAEAVDAARKILED

NDVSQAFLIDTVQKNCPRLDGNGTGLTGEDGLDVDDFVSTTVPNAIEKA
```

B

NLaz homology-based models
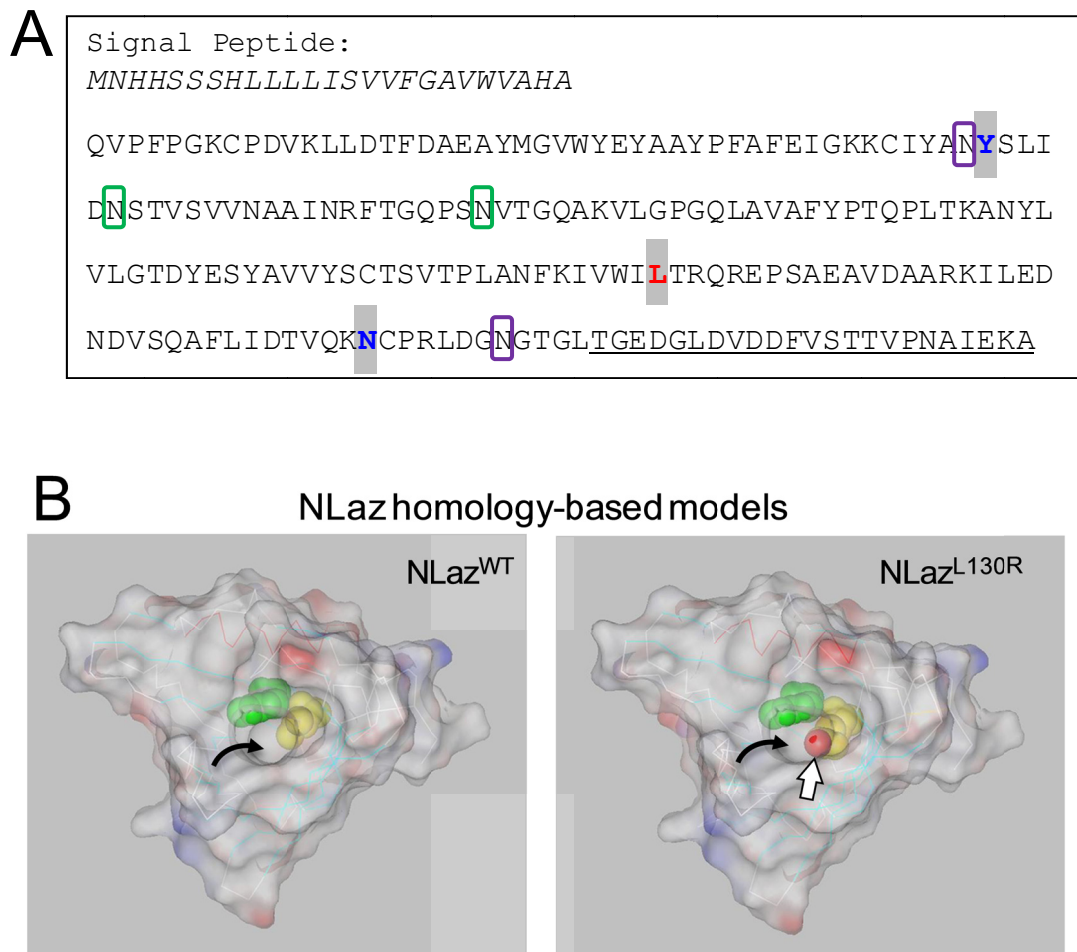
Figure 1. **NLaz primary and tertiary structures.**

(A) NLaz^{WT} amino acid sequence structure. Residues selected to generate point mutant versions of NLaz are boxed in gray and highlighted (L130 in red, Y47 and N165 in blue). The C-terminal fragment deleted in NLaz$^{\Delta177-192}$ mutant is underlined. Some Asn residues are highlighted by rectangles; green for known, and purple for predicted glycosylation.

(B) 3D-surface model of NLaz using hApoD crystal coordinates as a template. The left panel shows wild-type NLaz with the pocket shown with a curved arrow, and residues Y47 and L130 highlighted in green and yellow respectively. The right panel shows a similar view of the model for NLaz^{L130R} protein, with the Arg basic group marked in red and pointed by a white arrow.
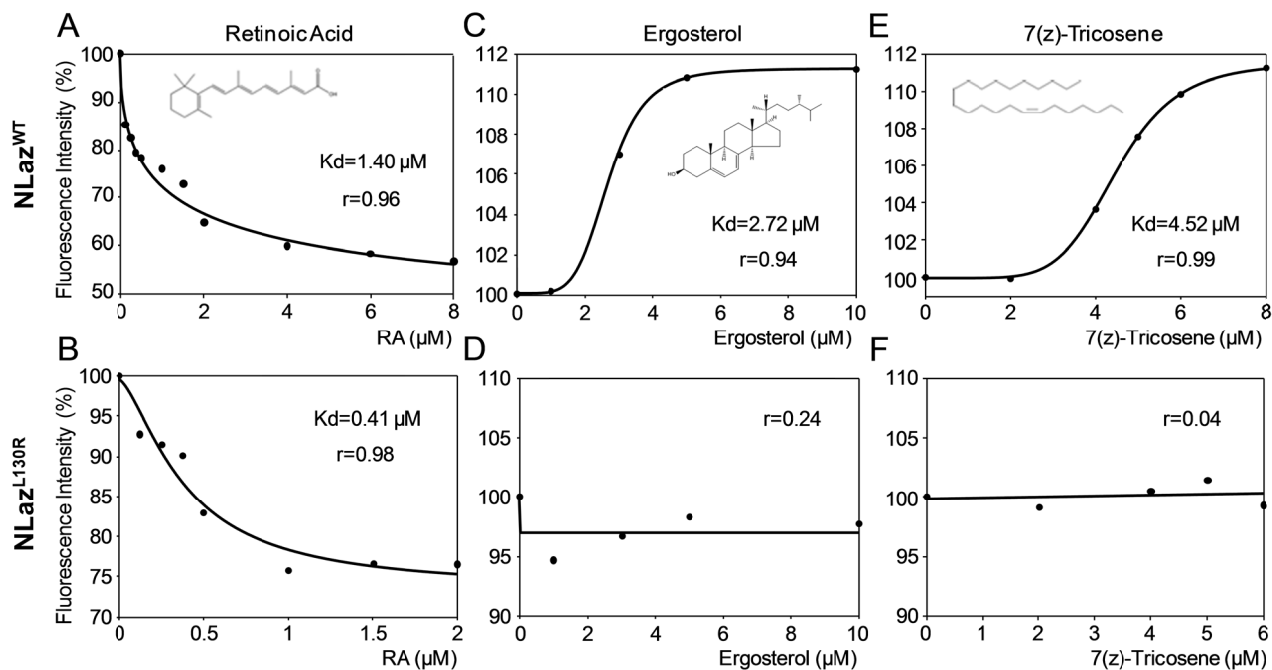
Figure 2. Intrinsic fluorescence analysis of NLaz proteins.
Changes in intrinsic fluorescence of NLaz^{WT} and NLaz^{L130R} with different hydrophobic compounds are shown. NLaz^{WT} fluorescence is quenched by retinoic acid (A), and enhanced by ergosterol (B) and the Drosophila pheromone 7(z)-tricosene (C). NLaz^{L130R} fluorescence is quenched by retinoic acid (D), but no evidence of interaction was found for NLaz^{L130R} with ergosterol (E) or 7(z)-tricosene (F).

Protein	Ligand	K_d	SD	n
		μM		
NLaz-WT	All-*trans*-retinoic acid	1.40	1.62	9
	Ergosterol	2.72	0.72	6
	7-(z)-tricosene	4.52	0.60	6
NLaz-L130R	All-*trans*-retinoic acid	0.41	0.14	10
	Ergosterol	n/d	-	6
	7-(z)-tricosene	n/d	-	6

Table 1. Kd values for the binding of various ligands to NLaz.
Estimated Kd (apparent dissociation constant) for the ligand–protein interaction for NLaz^{WT} and NLaz^{L130R} assuming a single binding site per protein molecule.

3.2. NLaz^{WT} has stronger cell-survival promoting activity than NLaz^{L130R} under oxidative stress in a cell-culture based assay.

In order to test the biological activity of the wild type and ligand binding-deficient NLaz, we treated non-transfected S2 cell cultures with H_2O_2 and measured cell viability by trypan blue exclusion analysis (Fig. 3). Neither version of NLaz protein nor an unrelated control protein (Trypsin Inhibitor, TI) had effects on the viability of untreated cells. Upon a pro-oxidant treatment the viability of control cells was reduced to 45%. No rescue was observed when the culture was supplemented with the control protein TI, but a significant increase in viability was obtained with both NLaz^{WT} and NLaz^{L130R}. The increase in viability produced by NLaz^{WT} was nevertheless significantly higher than that obtained with the pocket mutant NLaz^{L130R}.
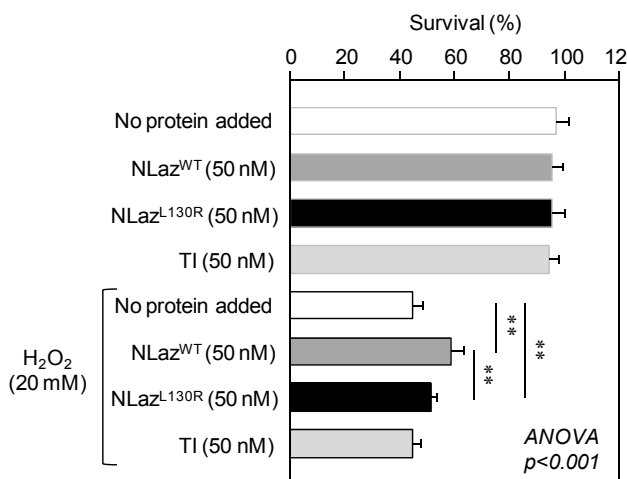
Figure 3. NLaz^{WT} has stronger cell survival promoting activity than NLaz^{L130R} over S2 cells under oxidative stress.
Drosophila S2 cell viability measured by trypan blue dye exclusion. S2 cells improve their survival upon exposure to hydrogen peroxide (20 mM H_2O_2) when 50 nM of exogenous pure NLaz^{WT} or NLaz^{L130R} proteins are added to the culture medium. Trypsin Inhibitor (TI, 50 nM), added as a control unrelated protein, does not influence cell survival. One-way ANOVA (p<0.001) followed by Holm-Sidak multiple comparison post-hoc test indicates that the recovery of viability obtained with NLaz^{WT} is significatively larger than that obtained with NLaz^{L130R}.

3.3. Monitoring survival-promoting activity of NLaz^{WT} and mutant forms *in vivo*.

The wild type and the four mutant versions of NLaz were placed downstream of an UAS (upstream activated sequence) containing a promoter to make its expression controlled by the Gal4/UAS system in Drosophila. Using PhiC31-mediated transformation all transgenes were integrated into the fly genome in position 86Fb of chromosome 3, an otherwise neutral site with no phenotypic effects known due to insertion mutations. With this strategy, genetic background differences and positional effect differences in expression levels are avoided, allowing for a proper comparison of the effects of different versions of NLaz on the physiological parameters of the fly. In this work we analyze the effects of ubiquitous expression of the transgenes using the daughterless:Gal4 (da:Gal4) driver.

3.3.1. Drosophila resistance to paraquat throughout aging is differentially modulated by NLaz mutant versions.

We have previously demonstrated that NLaz expression, and that of its mammalian homologue ApoD, is induced via JNK signaling activation upon exposure to the oxidative stress generator paraquat [10,13]. This expression of NLaz/ApoD is required to promote survival in oxidative stress conditions [12,13,53].

When flies are exposed to paraquat and NLaz^{WT} is overexpressed with a ubiquitous pattern (da:Gal4 driver), young 3 day-old flies increase only slightly their survival (Fig. 4A), but aged flies (30 day-old) experience a 13.6 and 40.2% increase in median and maximal survival respectively (Fig. 4B and Table 2). In contrast, over-expression of the pocket mutant NLaz^{L130R} even decreases survival of flies (13.5 and 8.9% decrease in median and maximal survival of aged flies, Fig. 4A-B and Table 2). When the expression of NLaz^{WT} or NLaz^{L130R} is performed in a NLaz null background (flies homozygous for the allele NLaz^{NW5}) only a partial rescue is obtained with the ligand-binding impaired version while the wild type NLaz completely restores sensitivity to PQ to the same level attained with a single copy of wild type NLaz in its native locus (heterozygous +/NLaz^{NW5} flies) (Fig. 4C).

On the contrary, over-expression of the mutant version $NLaz^{Y47A}$, that alters the binding pocket in a different way (Fig. S2A), does not differ from $NLaz^{WT}$ expression, being able to increase median and maximal survival (52.6 and 24.9% respectively, Fig. S2B-C and Table S2) in aged flies treated with PQ. In the absence of native NLaz, however, the rescue obtained with this version of NLaz is also partial (Fig. S2D). Taken together, these results are in agreement with a need for proper ligand binding activity in order for NLaz to exert its protective effect upon oxidative stress.

$NLaz^{N165S}$, bearing a mutated residue located outside the binding pocket (Fig. S3 A), results notwithstanding in extended survival upon PQ treatment in both young and aged flies and in flies lacking native NLaz as well (Fig. S3 B-D, and Table S2). Whether this NLaz allele represents a natural beneficial polymorphism existing in naturally occurring fly populations deserves further studies.

The mutant $NLaz^{\Delta177-192}$ is also predicted not to alter the ligand binding properties of NLaz. However, it has a survival-promoting activity similar to $NLaz^{WT}$ in young flies and larger than $NLaz^{WT}$ in aged flies (Fig. S3 A-B). In the NLaz null mutant background this truncated version of NLaz produces a partial rescue of median survival but a complete rescue of maximal survival (Fig. S3 C and Table S2). These data again reinforce the idea that the interference of ligand binding activity generated by the L130R mutation, but not other NLaz protein modifications, decreases significantly the NLaz protective effects upon PQ exposure. The milder modification of the ligand binding pocket caused by the Y47A mutation produces a protein that *in vivo* has functional properties more similar to the wild-type version of NLaz.

3.3.2. An intact binding pocket is required to rescue the Drosophila lifespan-reduction phenotype of NLaz null mutation.

Since resistance to oxidative stress is often correlated with extended longevity, we tested the ability of each mutant form to rescue the decrease in lifespan caused by the absence of NLaz [13].

The mutants that do not alter the ligand-binding pocket show either similar ($NLaz^{N165S}$) or enhanced ($NLaz^{\Delta177-192}$) lifespan-expanding activity compared with flies expressing $NLaz^{WT}$ from the same locus (Fig. S3-S4; Table S2). The pocket mutant $NLaz^{Y47A}$ is able to rescue median survival but not maximal survival, in contrast with the ubiquitous expression of $NLaz^{WT}$, which rescues maximal but not median survival (Fig. S2, Table S2). Interestingly, expressing the ligand binding-deficient $NLaz^{L130R}$ in NLaz null background results in a lifespan shorter than that of the null mutant (Fig. 4D, Table 2). This result is compatible with a requirement for adequate ligand binding activity in NLaz in order to obtain a wild type-like longevity pattern. However, it also suggests a potential dominant-negative effect of the $NLaz^{L130R}$ protein. Interactions of $NLaz^{L130R}$ with NLaz functional partners without carrying the ligand might be more deleterious than the absence of NLaz.

3.3.3. Drosophila resistance to metabolic stress is partially dependent on the ligand-binding pocket structure and requires the C-terminal tail of NLaz.

Resistance to metabolic stress is also correlated with an extended longevity, and we have described how NLaz expression can be triggered upon starvation-desiccation conditions and modulate metabolism by acting on the IIS pathway [13]. Consequently, a loss of NLaz decreases resistance of flies to the lack of nutrients and water.

As expected, the over-expression of $NLaz^{WT}$ significantly increases the resistance of NLaz null flies to starvation-desiccation stress (Fig. 4E, Table 2) producing a survival curve similar to that of the heterozygous $+/NLaz^{NW5}$ control. However, all point mutant versions of NLaz produce only a partial rescue of the null mutant phenotype (Fig. 4E, S2-F and S3-F, Table 2).

Interestingly, the truncation of the C-terminal tail of NLaz in $NLaz^{\Delta177-192}$ does not rescue the starvation-desiccation sensitivity phenotype (Fig. S4, Table 2), suggesting that this region might be important for an NLaz molecular interaction required to attain its survival-promoting activity upon metabolic stress
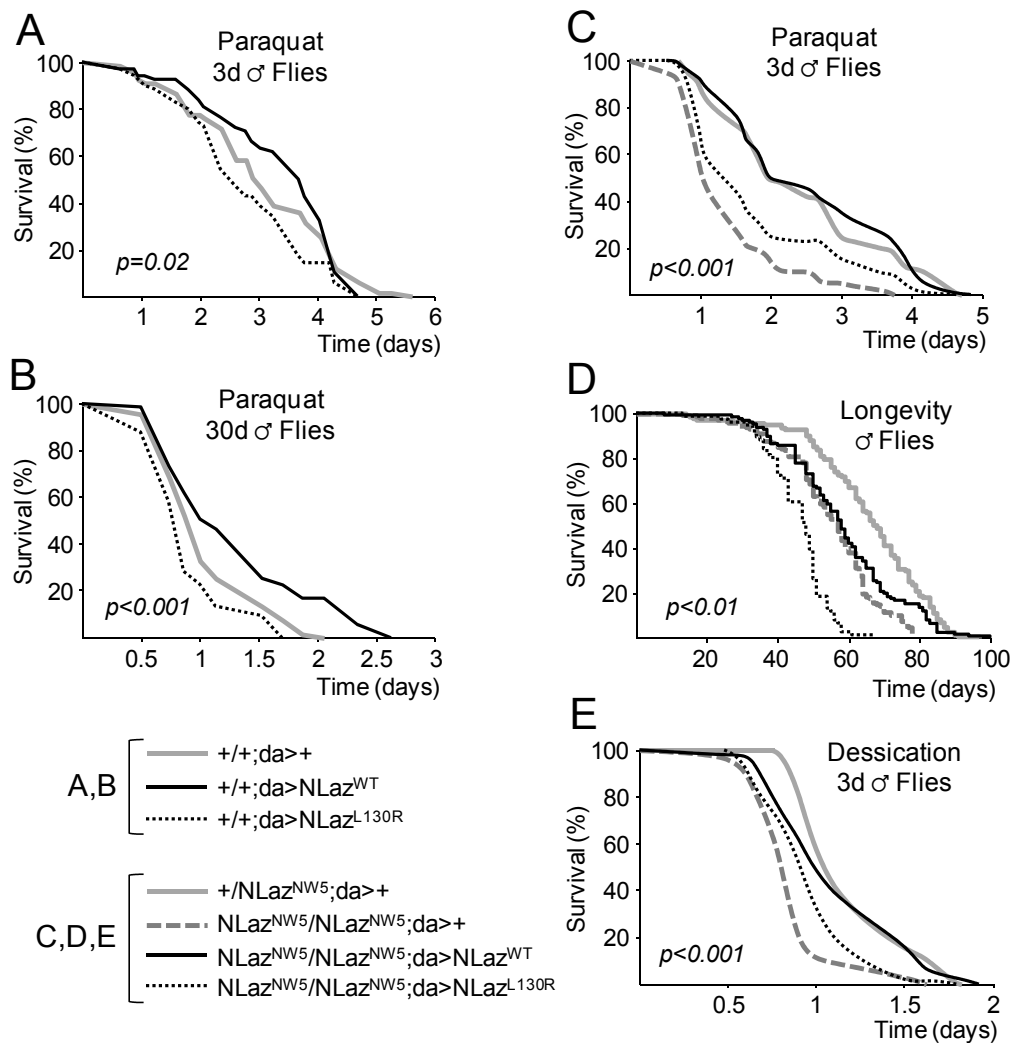
Figure 4. NLaz^{L130R} shows lower survival promoting activity than NLaz^{WT} upon aging or exposure to oxidative and metabolic stress.

(A) Resistance to paraquat of 3 day old flies with ubiquitous over-expression of NLaz^{WT} or NLaz^{L130R} in wild type background is not significantly different from that of control flies. However, over-expression of NLaz^{WT} confers more resistance to paraquat than over-expression of NLaz^{L130R}.

(B) Upon aging (30 day-old flies) resistance to paraquat is increased by ubiquitous over-expression of NLaz^{WT}. However, old flies over-expressing NLaz^{L130R} are still more sensitive to paraquat than control flies. (

C) Three day-old NLaz^{NW5} homozygous flies are more sensitive to paraquat exposure than control flies. NLaz^{WT} ubiquitous over-expression rescues this phenotype completely. However, NLaz^{L130R} expression is only able to exert a partial rescue.

(D) In the absence of added stress, null NLaz fly mutants show a reduced lifespan compared with control flies. This longevity reduction is rescued by ubiquitous over-expression of NLaz^{WT} (evident in terms of maximal survival; see Table 2). However, over-expression of NLaz^{L130R} fails to rescue NLaz loss-of-function shortened longevity, showing a lifespan even shorter than that of NLaz^{NW5}homozygous flies.

(E) Three day-old NLaz^{NW5} flies are more sensitive to starvation-desiccation stress than control flies. NLaz^{WT} ubiquitous over-expression also rescues this phenotype while NLaz^{L130R} expression is only able to exert a partial rescue. To compare survival distributions, log-rank tests were performed (*p*-values shown in each panel) followed by Holm-Sidak multiple comparison post-hoc tests.

Background	♂ da> UAS transgene	N	Experiment	ANOVA Sig	Median Survival or Behavior Success		Maximal Survival	
					Sig	%Change	Sig	%Change
WT (w^{1118})	NLaz^{WT} [2M] NLaz^{L130R} [3M]	67-68 67-70	Paraquat (3 d)	*	n/s n/s	23.14 -13.92	n/s *	-6.50 -6.50
WT (w^{1118})	NLaz^{WT} [2M] NLaz^{L130R} [3M]	67-71 67-74	Paraquat (30 d)	**	** *	13.56 -13.46	** *	40.16 -8.90
NLaz^{NW5}	NLaz^{WT} [2M] NLaz^{L130R} [3M]	147-126 147-76	Longevity	**	** **	1.75 -15.79	** **	17.65 -19.72
NLaz^{NW5}	NLaz^{WT} [2M] NLaz^{L130R} [3M]	153-128 153-93	Paraquat (3 d)	**	** **	90.4 36.09	** **	46.04 23.68
NLaz^{NW5}	NLaz^{WT} [2M] NLaz^{L130R} [3M]	119-150 119-76	Desiccation	**	** *	22.26 22.26	** n/s	21.74 0.00
NLaz^{NW5}	NLaz^{WT} [2M] NLaz^{L130R} [3M]	29-29 29-25	Courtship	**	* n/s	600 200		
NLaz^{NW5}	NLaz^{WT} [2M] NLaz^{L130R} [3M]	95-88 95-29	Climbing (50 d)	*	n/s n/s	46.17 -23.23		

Table 2. Effects of NLaz^{WT} and NLaz^{L130R} ubiquitous expression in flies survival and behavior.
Summary of the survival parameters and behavioral scores obtained with different experimental paradigms in flies. In over-expression experiments (WT background) the statistics are presented for the comparisons of each NLaz version with the driver alone control (w^{1118};da:Gal4/+). In rescue experiments, the comparisons presented are with the driver alone in heterozygous null NLaz mutant background (w^{1118};+/NLaz^{NW5};da:Gal4/+). All UAS:NLaz transgenes are located in the same locus of the genome (86Fb in chromosome 3). ** = p<0.001, * = p<0.05.

3.4. Fly locomotor and reproductive behaviors also depend on NLaz binding pocket structure.

Locomotor behavior can be used to evaluate the extent of neurodegeneration or functional decline of the nervous system with age. We have shown that a loss of NLaz leads to an accelerated aging [19] and that young NLaz mutants have defective daily locomotor activity patterns [15]. Here we use climbing ability to assay locomotor performance. Upon aging, flies bearing the null mutation of NLaz lose significantly their ability to climb (Fig. 5A). Flies expressing NLaz^{L130R} perform as poor as the null mutant in the climbing test, while ubiquitous overexpression of NLaz^{WT} is able to

produce a partial rescue when replacing the absence of native NLaz. Therefore, ligand binding disruption does interfere with the contribution of NLaz to the regulation of locomotor abilities.

Male courtship performance, also significantly reduced in the null NLaz flies ([15] and Fig. 5B), is rescued by NLaz^{WT}, while the courtship index of NLaz^{L130R} expressing males is more similar to the null mutant flies than to the wild type control (Fig. 5B). This result, together with the inability of NLaz^{L130R} to bind a key male pheromone (Fig. 2F), indicates that NLaz binding to specific ligands is required as part of the mechanisms controlling courtship behavior.
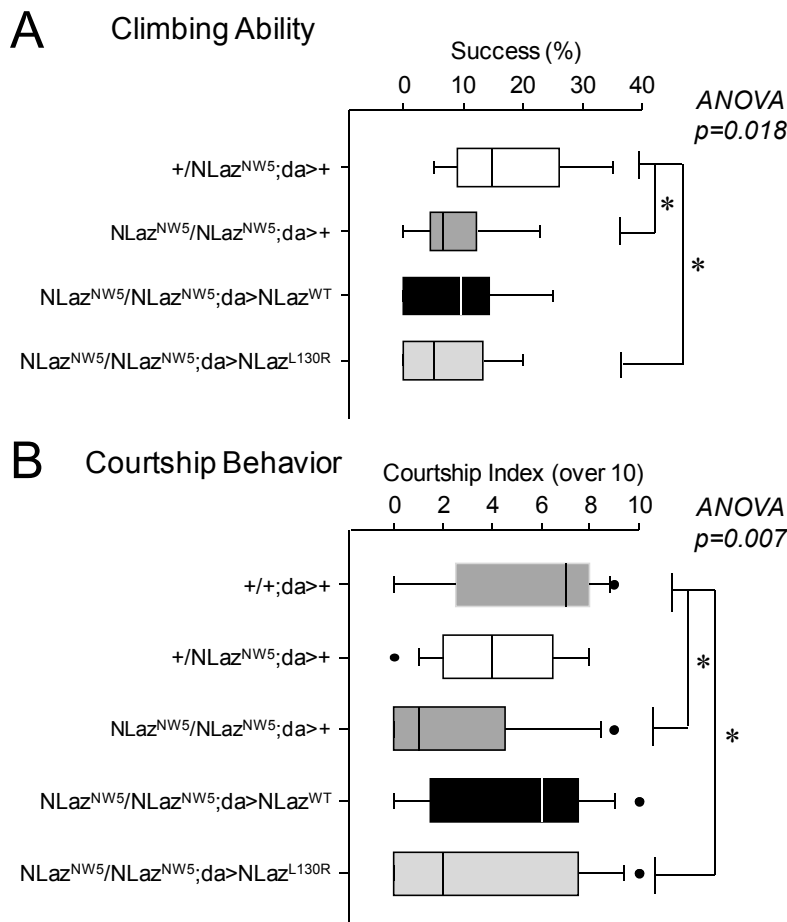
Figure 5. Fly locomotor and reproductive behaviors depend of NLaz pocket structure.
(A) Fly climbing ability was tested in 50 day old individuals. NLaz^{NW5} flies show a reduced success in the climbing test than that of control flies. This phenotype is partially rescued by a ubiquitous over-expression of NLaz^{WT}, but not of NLaz^{L130R}. Kruskal-Wallis one-way ANOVA on ranks (p=0.018) followed by Dunn's multiple comparison post-hoc test.
(B) Courtship behavior index is reduced in NLaz^{NW5} flies. NLaz^{WT} ubiquitous over-expression rescues this phenotype completely. However, NLaz^{L130R} expression results in a courtship index more similar to the null mutant than to the wild type control.

3.5. Measurements of metabolic state: NLaz binding ability conditions energy storage and the metabolic response to starvation.

Energy management and metabolism is tightly linked to longevity regulation and the rate of aging [reviewed by 8]. We have previously shown that NLaz null mutants have alterations of triglycerides, glucose and glycogen levels both in basal conditions and upon starvation [13]. In response to stress [13] or to a high sugar diet [18] NLaz is induced and exerts a negative regulation on the IIS pathway.

Here, we explore the effects of expressing $NLaz^{WT}$ and $NLaz^{L130R}$ in the null mutant background. A decrease in basal levels of triglycerides is produced by the absence of NLaz. This phenotype is rescued by $NLaz^{WT}$, but not by $NLaz^{L130R}$ (Fig. 6A). However, the alteration of the binding pocket of NLaz does not influence glucose or glycogen basal stores (Fig. 6B and 6C), since $NLaz^{L130R}$ expression results in basal levels similar

to those obtained with $NLaz^{WT}$. In contrast, the expression of $NLaz^{Y47A}$, $NLaz^{N165S}$ or $NLaz^{\Delta177-192}$ rescues the loss of triglycerides phenotype as effectively as the $NLaz^{WT}$ transgene (Fig. S2-G, S3-G and S4-G). Thus, only the regulation of triglyceride stores is dependent on NLaz ligand-binding abilities, and particularly on the ligand binding impairment caused by the $NLaz^{L130R}$ mutation.

The metabolic response to eight hours of starvation-desiccation stress is also altered by the absence of NLaz ([13,18] and Fig. 6). However, both $NLaz^{WT}$ and $NLaz^{L130R}$ produce similar rescue effects on metabolite concentrations in starvation conditions, with a complete rescue of triglyceride and glycogen and a partial rescue of glucose levels. Therefore, energy storage management in response to starvation appears as independent of NLaz ligand binding pocket in these flies, where the transgenes lack the regulatory regions of the native gene and the native gene expression is absent.
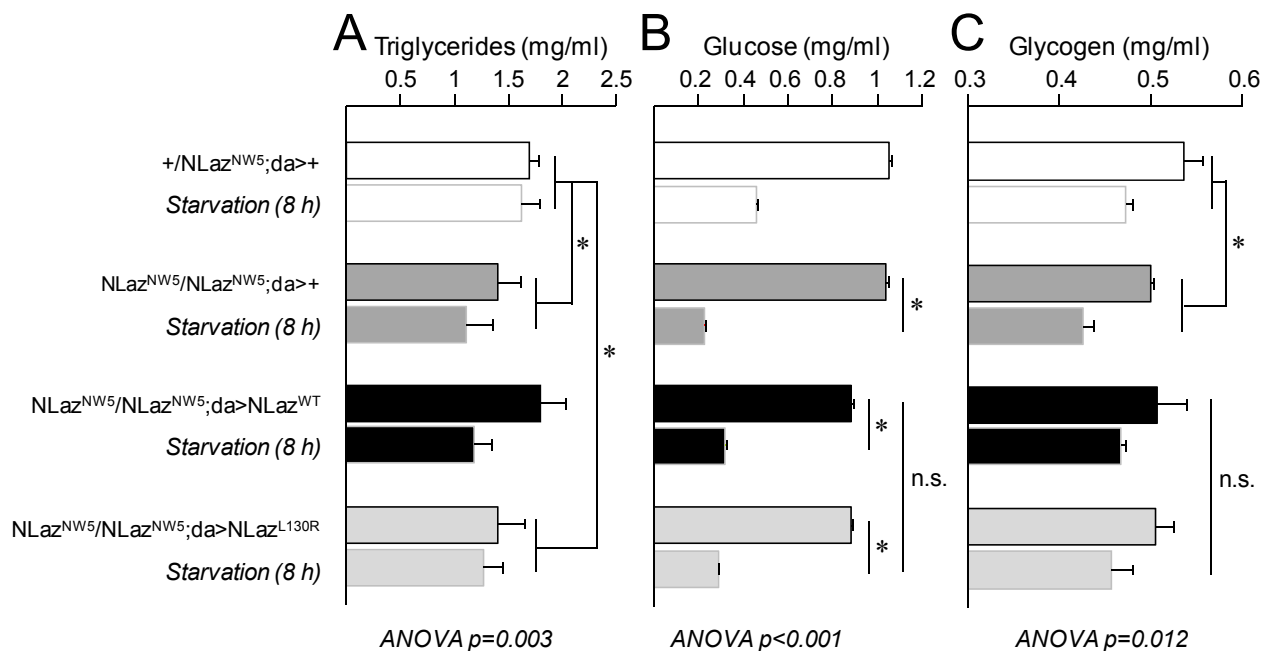
Figure 6. Effects of $NLaz^{WT}$ and $NLaz^{L130R}$ in the metabolic homeostasis of the fly.
(A) Glycogen storage levels only show differences between wild type and $NLaz^{NW5}$ flies. Two-way ANOVA (p=0.012) followed by Holm-Sidak multiple comparison post-hoc test. (B) TAG amounts are dependent on NLaz genotype and NLaz binding pocket structure. TAG levels are reduced in $NLaz^{NW5}$ flies compared to control flies. When $NLaz^{WT}$ and $NLaz^{L130R}$ are ubiquitously over-expressed in the $NLaz^{NW5}$ genetic background, differences in TAG with respect to control are only found for $NLaz^{L130R}$ flies. Two-way ANOVA (p=0.003) followed by Holm-Sidak multiple comparison post-hoc test. (C) Glucose levels depend on NLaz genotype, but not on NLaz binding pocket structure. $NLaz^{NW5}$ flies present lower levels of glucose compared with control flies. No differences were found between $NLaz^{WT}$ and $NLaz^{L130R}$ over-expressing flies. Two-way ANOVA (p<0.001) followed by Holm-Sidak multiple comparison post-hoc test.

3.6. Aging related markers accumulate early in NLaz null mutant and their rescue is dependent on NLaz pocket integrity.

We have previously shown that the accumulation of oxidized forms of sugars (AGEs), which are reliable biomarkers of normal aging-related damage, is accelerated in the NLaz null mutants [19]. The increase in fluorescent AGEs caused by the lack of NLaz is fully rescued by the ubiquitous expression of NLaz^{WT}, but not by NLaz^{L130R} (Fig. 7A), indicating that the beneficial action of NLaz in the delay of age-related damage is dependent of its ligand-binding ability.

Likewise, the accumulation of polyubiquitinated proteins, which also occur upon normal aging mostly due to a decreased efficiency of the protein quality control systems (autophagy and proteasome), is accelerated in the NLaz^{NW5} null mutant and only NLaz^{WT} is able to partially rescue this phenotype (Fig. 7B).

3.7. Transcriptional responses to oxidative stress or aging are modulated by NLaz and are differentially rescued by NLaz^{WT} and NLaz^{L130R} transgenes.

The expression of a set of 40 genes was explored in flies of four genotypes (Fig. 8) using a custom qRT-PCR array (see Methods). Genes were selected to monitor the state of metabolism-regulating pathways, aging and stress biomarkers, lipid and carbohydrate metabolism, and pheromonal and neuropeptide signaling. The other two known Drosophila Lipocalin genes (GLaz and Karl) were also included together with genes related to the innate immune response, given the infection-protective effect known for Karl [13]. In addition to technical controls, four potential housekeeping genes were included. Among those, in agreement with the criteria of Ponton et al. [54], RpL32 is the one with least variation among genotypes and conditions, and was used as reference for the analysis. Each genotype was explored under basal conditions (3 day-old flies), upon PQ exposure (17 h treatment, 3 day-old flies), and with aging (30 day-old flies).

In untreated young flies, the differences between genotypes are small, and only three genes fulfill our criteria of altered expression (see Methods) in the NLaz null mutant with respect to heterozygous control flies (Table S4). Among these, the expression pattern of the Lipocalin Karl is of particular interest: its expression decreases in the NLaz null mutant and is recovered by both NLaz^{WT} and NLaz^{L130R} ubiquitous expression.

PQ exposure provoked a transcriptional response in 23 of the 40 genes studied, while aging modified the expression of 24 genes. In Figure 8 we analyze the subsets of responding genes where NLaz^{WT} or NLaz^{L130R} revert the transcriptional change observed in the NLaz null mutant. NLaz ubiquitous expression rescues the effects of PQ in 12 genes (Fig. 8A) representing approximately half the sample of responding genes (Fig. 8B). Interestingly, the NLaz effect on aging-responding genes is wider, with a rescue observed in 17 genes (Fig. 8C) representing three quarters of the sample (Fig. 8B). Furthermore, a differential rescue by the NLaz^{WT} transgene is particularly enriched in the aging-responding gene sample with approximately half the rescuing effects being dependent on a functional ligand binding pocket of NLaz.

In the transcriptional response to PQ we found three genes whose expression is differentially rescued by the NLaz^{WT} transgene (boxed in Fig. 8A). They are functionally related to lifespan modulation (Lsp2, [55]), detoxification, oxidative and heat stress response (GstE1, [56]), and regulation of neurogenesis by metabolism (Ilp4, [57]). In the response to aging, more genes show binding pocket-dependent rescue by NLaz (boxed in Fig. 8C). They include genes directly involved in the IIS pathway activity (InR, [8]) and oxidative stress responsive genes that are in turn regulated by the IIS pathway (Gadd45, [58]), genes downstream of the stress-responsive JNK pathway (puc, Karl and Hsp68, [13,59]), genes related to the immune response (the antibacterial peptide Drs and Karl, [13,60]), aging-modulated neuropeptides and synaptic proteins (Nplp3 and synaptogyrin [55]), as well as neuropeptides controlling food-intake (sNPF, also downstream of IIS pathway [61-63]).

Among the tested genes, NLaz binding pocket-dependent transcriptional changes were not observed for genes that we had selected for their direct relationship to pheromone synthesis, lipid homeostasis or lipid metabolism.
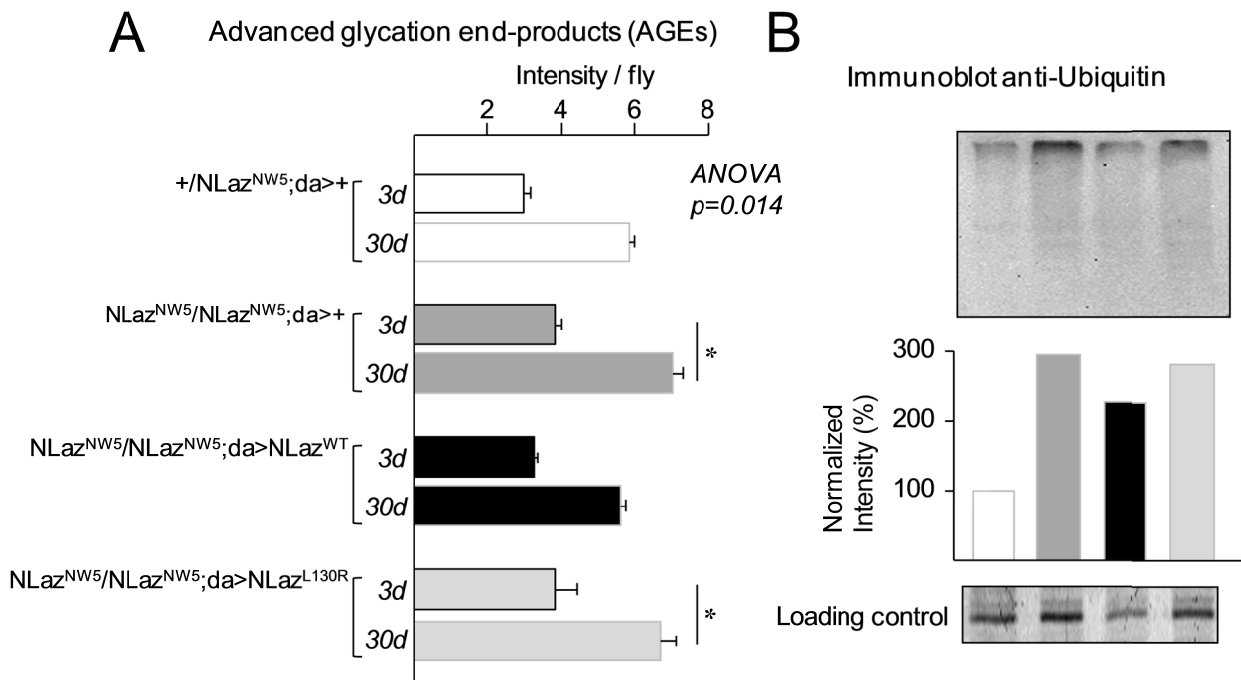
Figure 7. Aging-related markers accumulation is dependent on NLaz ligand-binding ability.
(A) Advanced glycation end products (AGEs), which normally accumulate upon aging, are increased in 3 and at 30 days old NLaz null mutants, reflecting an accelerated aging process. NLaz^{WT} over-expression rescues this phenotype, whereas NLaz^{L130R} over-expression is not able to reduce AGEs accumulation. Two-way ANOVA (p=0.014) followed by Holm-Sidak multiple comparison post-hoc test. (B) Ubiquitin-conjugated proteins, which also accumulate upon aging, are increased in 30 day-old NLaz^{NW5} flies compared to wild type flies. NLaz^{WT} ubiquitous expression partially rescues this phenotype, whereas NLaz^{L130R} is not able to reduce the accumulation of ubiquitinated proteins. A representative anti-ubiquitin immunoblot is shown.

4. DISCUSSION

In this study we tested *in vivo* the requirement of a proper ligand-binding activity for the Lipocalin NLaz to fulfill many of its physiological functions. Interestingly, it also highlights that a subset of functions are not dependent on the particular binding pocket alterations we have tested, and that other protein surfaces are important for the biological interactions of NLaz. Of particular interest are the potential novel functions dependent on the C-terminal tail of NLaz, a probable vestigial derivative of the ancient GPI-linkage signal peptide present in grasshopper Lazarillo and other insect Lipocalins. We have found that this C-terminal region of NLaz is required to promote survival upon metabolic stress in young flies, but its absence is beneficial for aged flies to resist oxidative stress. Further analysis of the binding interactions of this NLaz protein motif should grant interesting age-dependent survival mechanisms. Also intriguing is the finding that NLaz^{N165S}, a putative natural polymorphism, confers extended protection against oxidative stress. The residue involved is one of the asparagines whose protein sequence context does not predict to be a N-linked glycosylation site, and its location facing one external side of the ß-barrel is suggestive of potential molecular interactions also worth to study.

The mutation L130R has been especially informative in our study. We have shown that it renders NLaz unable to bind different types of lipidic ligands (ergosterol and 7(z)-tricosene). This modification hinders the survival promoting activity of NLaz, both in a cell-based culture system and *in vivo*, where it is unable to rescue the effects of the NLaz null mutation upon oxidative or metabolic stress. NLaz^{L130R} does not rescue the
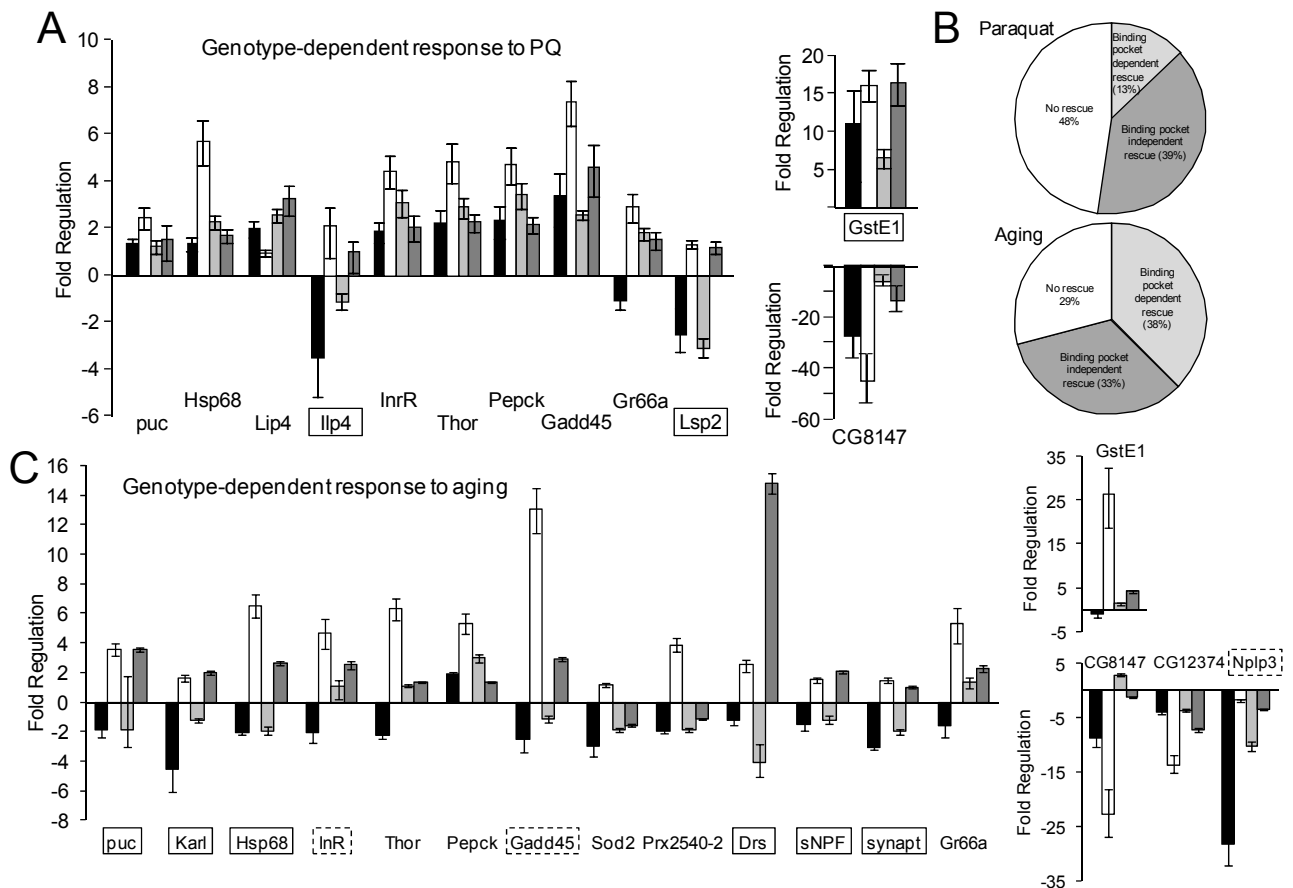
Figure 8. Transcriptional profile analysis shows ligand-dependent and independent NLaz modulation of stress and age-responsive pathways.

(A) Subset of genes where the response to PQ shows dependence on NLaz genotype. Only genes with expression changes≥2, $p < 0.05$, and showing a rescuing effect by either of the NLaz transgenes are shown. Boxes point to genes with a rescue dependent on the binding pocket mutation (differential rescue). Genes with large expression changes are shown in separate graphs for scaling purposes.

(B) Percent distribution of genes with transcriptional changes upon PQ or aging classified according to the ability of NLaz transgenes to revert the changes observed in the NLaz^{NW5} null mutant genotype.

(C) Subset of genes whose response to aging is NLaz-dependent. Inclusion criteria as in A. Boxes point at genes with binding pocket-dependent rescue, with dashed boxed showing cases with partial dependence (NLaz^{L130R} causing a partial rescue of the transcriptional change provoked by the null mutation). Genes with large expression changes are shown in separate graphs for scaling purposes.

Student's t-test was performed on the replicate $2^{-\Delta Ct}$ values for each gene in each genotype. Fold regulation by either PQ or aging is shown as $2^{-\Delta\Delta Ct}$ when the experimental condition produced an increase in expression, and as $-1/2^{-\Delta\Delta Ct}$ when the condition produced down-regulation of the gene.

accumulation of age-dependent markers, and it appears to show a dominant-negative effect in lifespan modulation. Therefore we demonstrate *in vivo* that the residue L130 is a key element in the ligand-binding abilities of NLaz, as it has been shown *in vitro* for its human homologue. The crystal structure of hApoD bound to progesterone, a ligand of the same structural class as ergosterol, shows the residue L129 (equivalent to L130 in NLaz) in direct contact with the ligand [26]. When modeling the binding of a very different lipid, arachidonic acid, the same residue shows up as an interacting partner in hApoD [26]. L130 lies in close proximity to a tryptophan residue conserved in the entire Lipocalin family (W127 in hApoD, W128 in NLaz), which is also known to be involved in the protein-ligand interaction and is probably responsible for most of the intrinsic fluorescence changes observed upon ligand-binding in our assays. The residue Y47, equivalent to Y46 in hApoD, is known to interact with progesterone, but modeling predicts no interaction with arachidonic acid. This fact, plus its substitution by a non-obstructing residue, supports the milder physiological effects of this NLaz version when expressed in flies.

Our analysis also shows that the ligand binding-deficient version of NLaz (NLaz^{L130R}) cannot rescue particular behavioral phenotypes. Our data strongly support a specific function of NLaz as a mediator of pheromone signals, either at modulating their availability in the extracellular milieu or its reception by the sensory neurons in charge.

Finally, the transcriptional changes monitored in our study clearly show that NLaz acts upstream of key pathways modulating the management of resources upon stress or aging. Many of the genes with NLaz-dependent responses to aging or PQ are in fact genes regulated by the IIS pathway, either as direct FOXO targets (Lip4, Gadd45, Sod2, Pepck, and sNPF) or downstream of the TOR pathway (Thor). This further supports our previous findings of a negative control of IIS activity by NLaz [13], and suggests additional consequences derived from NLaz function, some of which are dependent on the ligand-binding modification of NLaz^{L130R}.

In light of the new findings we can refine our previous conclusions about a modulation of lifespan through the control of metabolism under stress conditions by NLaz. We previously proposed that the lifespan-expanding activity of NLaz was due to the systemic action of fat body-derived NLaz on peripheral target tissues, inhibiting IIS and therefore repressing growth [13]. Also, we showed that a constitutive lack of NLaz function significantly increases the hunger-driven food intake behavior of the flies, and that they gain weight (particularly fat) with aging [15]. Now we find that NLaz mutants have increased expression of sNPF upon aging. This fact provides an explanation for the food intake and "age-related obesity" phenotypes, and highlights new actions of NLaz within the CNS. NLaz might exert a negative regulation of the expression of sNPF downstream of FOXO in the neuropeptidergic neurons, thus contributing, in addition to its systemic control, to the final metabolic-nutritional status and lifespan of the flies.

We also find that under oxidative stress NLaz modulates the expression of Ilp4 in a binding-pocket dependent manner. Ilp4 is one of the insulin-like peptides described to be part of the CNS specific pool, and it is known to contribute to neurogenesis reactivation during larval development [57], but not to organismal growth. Curiously, NLaz does not alter the expression of Ilp2, Ilp3 or Ilp5 [13], the systemic pool of insulin-like peptides in Drosophila that control organismal growth.

Our data therefore demonstrate that NLaz modulatory actions on various signaling processes, including IIS and pheromone signaling, have cell type-dependent responses within and outside the nervous system. Some, but not all functional outcomes are dependent on NLaz proper ligand-binding capacity, conflicting with a simple hypothesis of NLaz as a lipid-carrier. The present work should place the foundations for a detailed dissection of the molecular mechanisms of NLaz and the contribution of its lipid ligands in each physiological system, with Drosophila as a powerful tool for *in vivo* analysis.

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SUPPLEMENTARY FIGURES AND TABLES
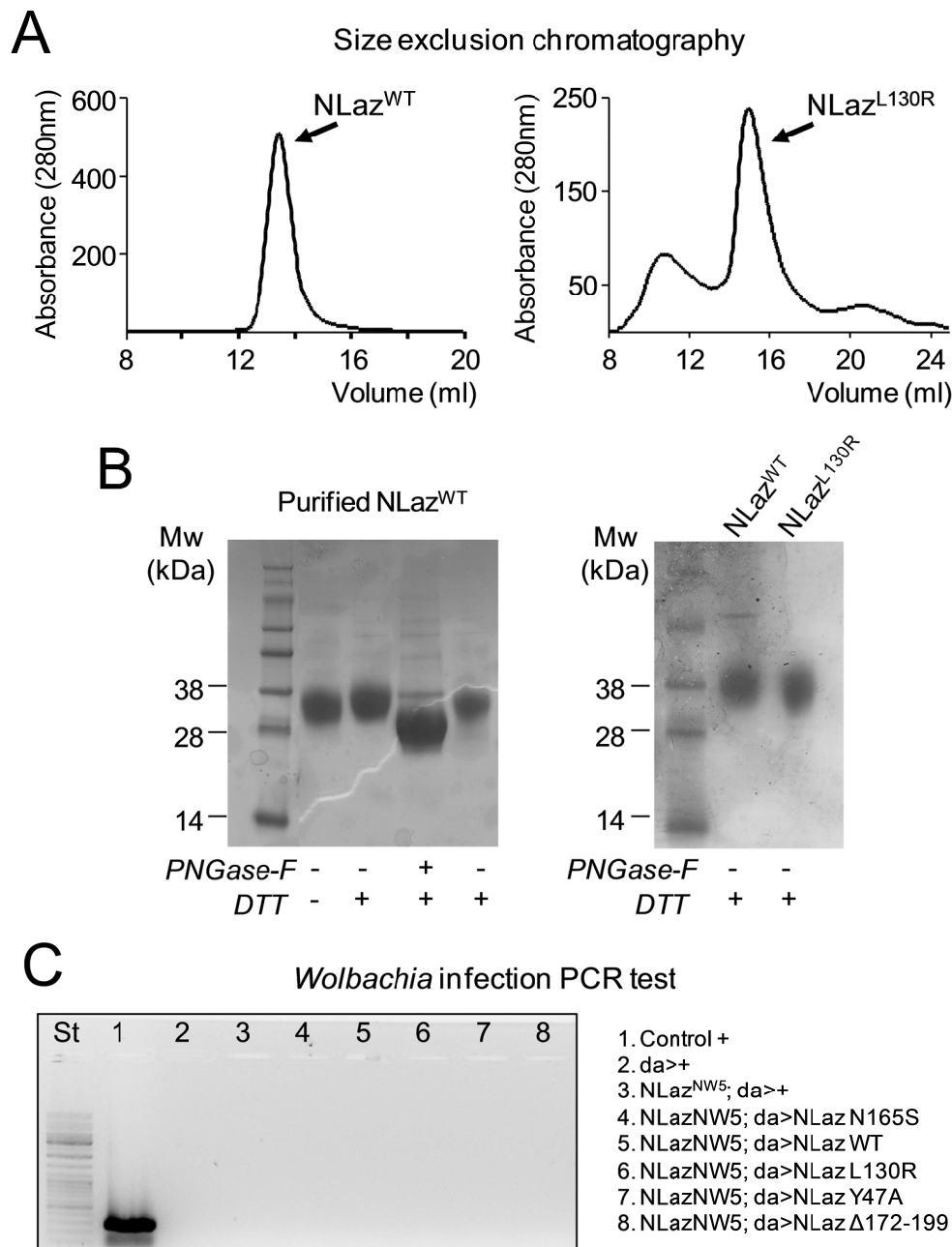
Figure. S1. Protein purifications and fly lines characterization tests.
(A) Purification of NLaz^{WT} and NLaz^{L130R} from S2 cells. Size-exclusion chromatography elution profiles of enriched extracts obtained after metal affinity chromatography of culture media from stably transfected S2 cells.
(B) SDS-PAGE analysis of NLaz^{WT} purified from S2 cells (right panel). The redox-state of the protein affects slightly its electrophoretic mobility, while a large shift is observed after deglycosylation. No differences in electrophoretic mobility are detected between NLaz^{WT} and NLaz^{L130R} purified from S2 cells (left panel).
(C) Fly lines used in this study are free of *Wolbachia* infection. PCR of genomic DNA extracts from all lines employed. Primers against the rRNA-16S gene of *Wolbachia* endosymbiont were used (Table S1). DNA from *Dirofilaria immitis* worms infected with *Wolbachia* was used as a positive control.
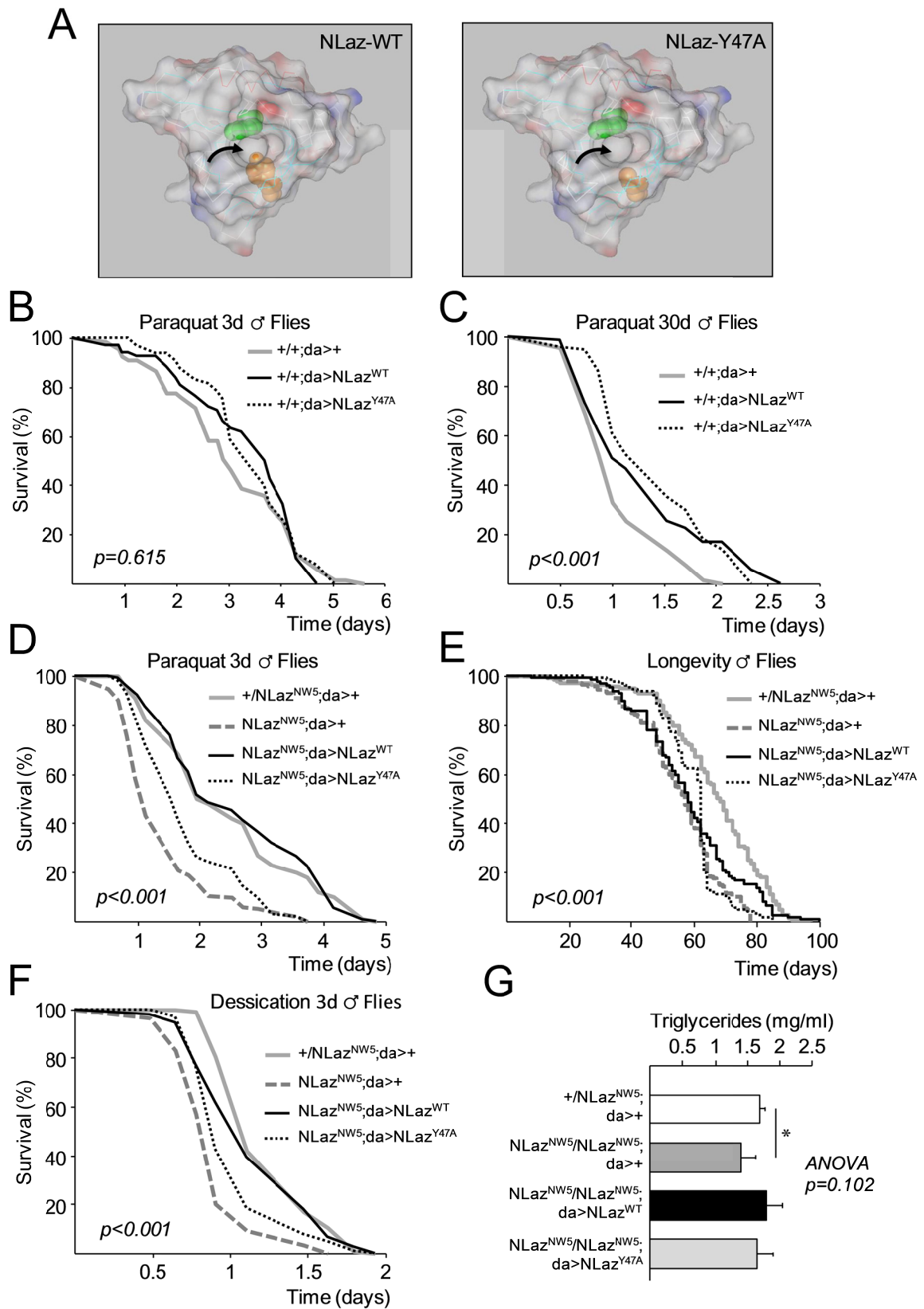
Figure S2: Legend at page 130

A

NLaz-WT

NLaz-N165S

B Paraquat 3d ♂ Flies

Survival (%)

+/+;da>+
+/+;da>NLaz^{WT}
+/+;da>NLaz^{N165S}

p=0.005

Time (days)

C Paraquat 30d ♂ Flies

Survival (%)

+/+;da>+
+/+;da>NLaz^{WT}
+/+;da>NLaz^{N165S}

p<0.001

Time (days)

D Paraquat 3d ♂ Flies

Survival (%)

+/NLaz^{NW5};da>+
NLaz^{NW5};da>+
NLaz^{NW5};da>NLaz^{WT}
NLaz^{NW5};da>NLaz^{N165S}

p<0.001

Time (days)

E Longevity ♂ Flies

Survival (%)

+/NLaz^{NW5};da>+
NLaz^{NW5};da>+
NLaz^{NW5};da>NLaz^{WT}
NLaz^{NW5};da>NLaz^{N165S}

p<0.001

Time (days)

F Dessication 3d ♂ Flies

Survival (%)

+/NLaz^{NW5};da>+
NLaz^{NW5};da>+
NLaz^{NW5};da>NLaz^{WT}
NLaz^{NW5};da>NLaz^{N165S}

p<0.001

Time (days)

G

Triglycerides (mg/ml)

+/NLaz^{NW5};da>+

NLaz^{NW5}/NLaz^{NW5};da>+

NLaz^{NW5}/NLaz^{NW5};da>NLaz^{WT}

NLaz^{NW5}/NLaz^{NW5};da>NLaz^{N165S}

ANOVA p=0.038

*

Figure S3: Legend at page 130

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Figure S2. Mutation Y47A in NLaz binding pocket alters fly survival but does not influence TAG basal levels.
(A) Model of NLaz^{Y47A} protein structure compared to NLaz^{WT}. Backbone and surface models are superimposed. A view facing the entrance of the binding pocket is shown. W128 (green). Y47 or A47 (orange). (B-C) Ubiquitous over-expression of either NLaz^{WT} or NLaz^{Y47A} in wild-type background increases resistance to paraquat, particularly in aged flies (C, 30 day old). (D) Three day-old NLaz^{NW5} flies are more sensitive to PQ exposure than control flies (heterozygous for NLaz native locus). NLaz^{WT} ubiquitous over-expression rescues this phenotype completely while NLaz^{Y47A} is only able to exert a partial rescue. (E) Longevity analysis of NLaz null-mutant flies in the presence or absence of NLaz^{Y47A} and NLaz^{WT} transgenes. NLaz^{Y47A} is able to rescue median survival but not maximal survival. NLaz^{WT} rescues maximal but not median survival. (F) NLaz^{Y47A} partially rescues the starvation-desiccation stress sensitivity of NLaz^{NW5} flies. (G) The Y47A mutation of NLaz does not show any effect in triglycerides levels. Log-rank tests followed by Holm-Sidak multiple comparison post-hoc tests were used in B-F. One-way ANOVA followed by Holm-Sidak multiple comparison post-hoc test was used in G.

Figure S3. NLaz^{N165S} variant expression alters stress resistance, but does not affect triglyceride basal levels.
(A) Model of NLaz^{N165S} protein structure compared to NLaz^{WT}. Backbone and surface models are superimposed. A lateral view is shown. The binding pocket is pointed by an arrow and is outlined by a dashed line. N165 or S165 residues are labeled in green. (B-C) Over-expression of NLaz^{N165S} in a wild-type genetic background is able to increase fly resistance against PQ when compared to control flies both at 3 days of age (B) and at 30 days of age (C). Log-rank tests followed by Holm-Sidak multiple comparison post-hoc tests. (D) Resistance to paraquat of young flies expressing NLaz^{N165S} in NLaz^{NW5} null background is higher than that of heterozygous controls or flies rescued by NLaz^{WT}. (E) Null NLaz fly mutants show a reduced longevity compared with wild-type flies. The reduction in maximal lifespan is rescued by the ubiquitous over-expression of NLaz^{WT} and NLaz^{N165S}. (F) NLaz^{N165S} expression is only able to exert a partial rescue in starvation-desiccation test. (G) The N165S polymorphism of NLaz does not show any effect in triglyceride concentration in basal conditions. Log-rank tests followed by Holm-Sidak multiple comparison post-hoc tests were used in B-F. One-way ANOVA followed by Holm-Sidak multiple comparison post-hoc test was used in G.
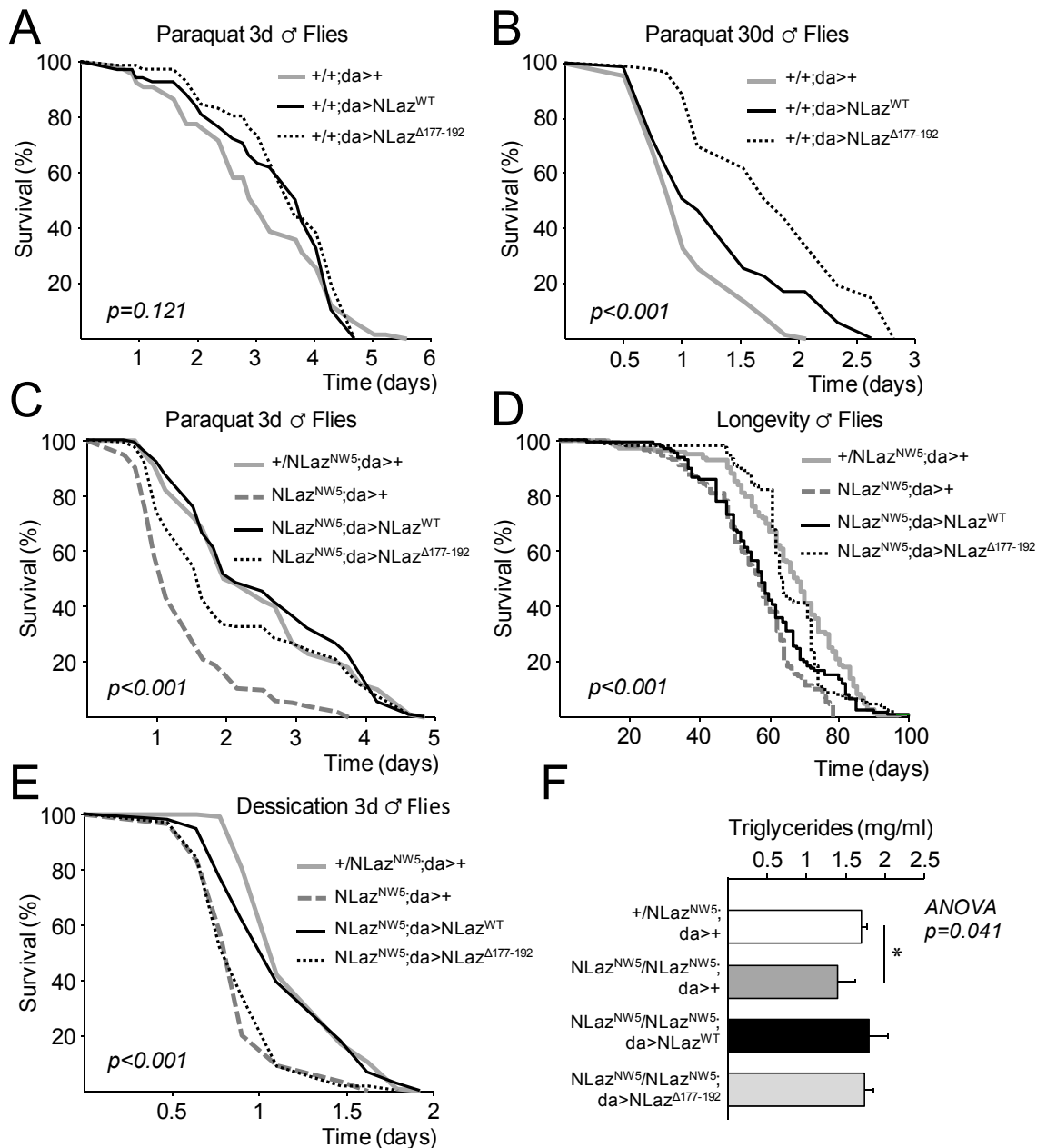
Figure S4. A deletion of the C-terminal portion (177-192) of NLaz affects stress resistance, but it does not modify triglycerides storage.

(A-B) Ubiquitous over-expression of both NLaz^{WT} and NLaz$^{\Delta177-192}$ in wild-type background increases resistance to PQ in young flies (A). NLaz$^{\Delta177-192}$ is more effective than NLaz^{WT} in reducing sensitivity to PQ in 30 day-old flies (B). (C) NLaz$^{\Delta177-192}$ expression is only able to partially rescue against PQ exposure when expressed in NLaz^{NW5} null mutant background. (D) The effect of NLaz null mutation in both median and maximal survival are rescued by NLaz$^{\Delta177-192}$.

(E) No rescue is achieved by ubiquitous NLaz$^{\Delta177-192}$ expression in a starvation-desiccation test. (F) Deletion of NLaz C-terminal portion does not have any effect in triglyceride basal amounts. Log-rank tests followed by Holm-Sidak multiple comparison post-hoc tests were used in A-E. One-way ANOVA followed by Holm-Sidak multiple comparison post-hoc test was used in F.

Table.S1. Primer sequences employed in this work.

Application	Primer Sequence
Mutagenesis L130R	5'-gccaatttcaaaattgtttggatc<u>cgc</u>actcgtcagcgtgaaccttcagc-3'
	5'-gctgaaggttcacgctgacgagt<u>gcg</u>gatccaaacaattttgaaattggc-3'
Mutagenesis Y47A	5'-gaagtgcatctacgccaac<u>gcc</u>agtctcatagacaacagc-3'
	5'-gctgttgtctatgagact<u>ggc</u>gttggcgtagatgcacttc-3'
From pBS-II to pRmHa-3	5'-caagaattctcagtggatagcgac-3'
	5'-caagcggccgcggagccgcgcggcaccagtgccttttcaatggcatttgc-3'
NLaz-Δ177-199 from pBSII-KS to pUASt-attB	5'-cgaattctcagtggatagcgac-3'
	5'-cgaattctcaaccatccaaccg-3'
Sequence pBSII-KS	Universal Primer Set (CIC)
Sequence pUASt-attB	5'-gcagtaaagtgcaagttaaagtga-3'
	5'tgtccaattatgtcacaccac-3'
Sequence pRmHa-3	5'ccagagcatctggccaatgtgc-3'
Wolbachia rRNA-16S	5′-gaagataatgacggtactcac-3′
	5′-gtcagatttgaaccagataga-3′

Background	♂ da > UAS transgene	N	Experiment	ANOVA Sig	Median Survival		Maximal Survival	
					Sig	%Change	Sig	%Change
WT (w^1118)	NLaz^N165S	67-72	Paraquat (3 d)	*	*	38.74	n/s	-6.50
	NLaz^WT	67-68			n/s	23.14	n/s	-6.50
	NLaz^L130R	67-70			n/s	-13.92	*	-6.50
	NLaz^Y47A	67-65			n/s	21.49	n/s	0.00
	NLaz^Δ177-192	67-71			n/s	21.49	n/s	-6.50
WT (w^1118)	NLaz^N165S	67-81	Paraquat (30 d)	**	**	52.64	**	65.00
	NLaz^WT	67-71			**	13.56	**	40.16
	NLaz^L130R	67-74			*	-13.46	*	-8.90
	NLaz^Y47A	67-94			**	52.64	**	24.89
	NLaz^Δ177-192	67-89			**	86.94	**	50.55
NLaz^NW5	NLaz^N165S	147-126	Longevity	**	*	3.50	**	17.65
	NLaz^WT				*	1.75	**	17.65
	NLaz^L130R	147-76			**	-15.79	**	-19.72
	NLaz^Y47A				n/s	8.77	n/s	2.42
	NLaz^Δ177-192				**	10.53	**	17.65
NLaz^NW5	NLaz^N165S	153-106	Paraquat (3 d)	**	**	181.51	**	46.04
	NLaz^WT	153-128			**	90.4	**	46.04
	NLaz^L130R	153-93			**	36.09	**	23.68
	NLaz^Y47A	153-103			**	47.20	n/s	0.00
	NLaz^Δ177-192	153-120			**	47.20	**	46.04
NLaz^NW5	NLaz^N165S	119-122	Desiccation	**	**	0.00	*	10.39
	NLaz^WT	119-150			**	22.26	**	21.74
	NLaz^L130R	119-76			*	22.26	n/s	0.00
	NLaz^Y47A	119-103			**	0.00	*	21.74
	NLaz^Δ177-192	119-122			n/s	-6.97	n/s	0.00

Table S2. Survival parameters. Comparison between NLaz^WT and NLaz mutant transgenic flies.
Summary of the survival parameters obtained with different experimental paradigms in flies. Wild-type flies (expressing the driver alone, w^1118 ; da:Gal4/+) were used as control in over-expression experiments, whereas NLaz-KO flies (w^1118 ; NLaz^NW5/NLaz^NW5 ; da:Gal4/+) are the control in rescue experiments. All UAS:NLaz transgenes are located in the same locus of the genome (86Fb in chromosome 3).** = $p < 0.001$, * = $p < 0.05$.

Table S3. List of genes explored for NLaz-dependent transcriptional regulation.

Gene Symbol	Refseq #	Official Name / Mamalian orthologue	RT2 Cat.No.	Functional category
Act5C	NM_001014725	Actin 5C	PPD00467	Housekeeping
Akt1	NM_169705	serine/threonine-protein kinase	PPD09200	Metabolism control (IIS pathway)
bsk	NM_164900	Basket / JNK	PPD11004	Stress response pathway (JNK pathway)
Cat	NM_080483	Catalase	PPD07567	Oxidative stress response (Foxo target)
CG12374	NM_136975	Carboxypeptidase-like (Zn) CG12374	PPD04510	Age/Oxiative stress specific response (vs. starvation/infection)
CG8147	NM_001038951	Alkaline phosphatase-like CG8147	PPD08516	Age/Oxiative stress specific response (vs. starvation/infection)
Ddc	NM_078876	Dopa decarboxylase	PPD03531	Neurotransmitter/Hormonal systems
desat1	NM_144474	Desaturase 1	PPD11642	Lipid / Hormonal systems
Dpt	NM_057460	Diptericin	PPD05159	Immune response
Drs	NM_079177	Drosomycin	PPD06186	Immune response
foxo	NM_142073	Forkhead box, sub-group O	PPD08986	Metabolism control (IIS and Sir2 pathways)
Gadd45	NM_136420	Growth arrest and DNA damage-inducible	PPD03876	Oxidative stress response-DNA repair (Foxo target)
Gapdh1	NM_001038847	Glyceraldehyde 3 phosphate dehydrogenase 1	PPD03944	Housekeeping
GLaz	NM_079003	Glial Lazarillo	PPD04541	Lipocalin family / Oxidative stress response
Gr66a	NM_079247	Gustatory receptor 66a	PPD06616	Chemoreception in pheromone sensing neurons
GstE1	NM_137479	Glutathione S transferase E1	PPD05094	Oxidative stress response
GstS1	NM_079043	Glutathione S transferase S1	PPD66725	Oxidative stress response
Hr96	NM_079769	Hormone receptor-like in 96	PPD10079	Lipid homeostasis
Hsp68	NM_001031945	Heat shock gene 68	PPD13069	Oxidative stress response (JNK pathway)
Ilp4	NM_140104	Insulin-like peptide 4	PPD06800	Metabolism control / Neurogenesis control (IIS pathway)
InR	NM_079712	Insulin-like receptor	PPD09691	Metabolism control (IIS pathway)
Jafrac1	NM_058162	Thioredoxin peroxidase 1	PPD11559	Oxidative stress response (Foxo target)
Karl	NM_132520	Karl	PPD00966	Lipocalin family / Immune response
Lip4	NM_135574	Lipase 4	PPD02897	Metabolism control (Foxo target)
Lsd-1	NM_142926	Lipid storage droplet-1	PPD09917	Lipid metabolism
Lsp2	NM_080077	Larval serum protein 2	PPD11062	Lifespan-Aging modulation
Mgstl	NM_079957	Microsomal glutathione S-transferase-like	PPD10883	Oxidative stress response

Gene Symbol	Refseq #	Official Name / Mamalian orthologue	RT2 Cat.No.	Functional category
mio	NM_134812	Missing oocyte	PPD02026	Lipid metabolism
Nplp3	NM_144453	Neuropeptide-like precursor 3	PPD11605	Neural activity/ Lifespan-Aging modulation
Prx2540-2	NM_078959	Peroxiredoxin 2540-2	PPD04249	Oxidative stress response
puc	NM_079549	Puckered (JNK-specific phosphatase)	PPD08362	Stress response pathway (JNK pathway)
RpL18	NM_139834	Ribosomal protein L18	PPD06501	Housekeeping
RpL32	NM_079843	Ribosomal protein L32	PPD10569	Housekeeping
S6k	NM_079217	RPS6-p70-protein kinase	PPD06382	Metabolism control pathway
Sir2	NM_058003	NAD-dependent histone deacetylase Sir2	PPD03110	Lifespan-Aging modulation / Metabolism control
sNPF	NM_078881	Short neuropeptide F precursor	PPD03613	Neuropeptide hormones / Food intake regulation / Stress response
Sod2	NM_057577	Superoxide dismutase 2 (Mn-SOD)	PPD04905	Oxidative stress response
Spt-I	NM_136998	Serine palmitoyltransferase subunit I	PPD04542	Lipid metabolism
synaptogyrin	NM_137064	Synaptogyrin	PPD04615	Neural activity/ Lifespan-Aging modulation
Thor	NM_057947	Thor / 4E-BP (eIF4E binding protein)	PPD02171	Metabolism control (IIS - TOR pathways)
Tk	NM_141884	Tachykinin	PPD08785	Neuropeptide hormones / Stress response
to	NM_079773	Takeout	PPD10117	Lipid metabolism / Lifespan-Aging modulation
whd	NM_078961	Withered	PPD04258	Lipid metabolism
Controls				
DGDC	SA_00146	Fly Genomic DNA Contamination	PPD66893	Technical control
RTC	SA_00104	Reverse Transcription Control	PPX63340	Technical control
PPC	SA_00103	Positive PCR Control	PPX63339	Technical control

Table S3. List of genes explored for NLaz-dependent transcriptional regulation.

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Gene Symbol	-/- ; da>+		-/- ; da>Nlaz-WT		-/- ; da>Nlaz-L130R	
	Fold Regulation	*p-value*	Fold Regulation	*p-value*	Fold Regulation	*p-value*
Act5C	1,001	*0,96843*	1,289	*0,08226*	1,281	*0,06374*
Akt1	-1,172	*0,03538*	-1,298	*0,00568*	-1,447	*0,00460*
bsk	-1,011	*0,92925*	-1,069	*0,71340*	-1,032	*0,69246*
Cat	-1,132	*0,37087*	-1,057	*0,56660*	1,145	*0,45657*
CG1237	-1,038	*0,45605*	-1,534	*0,00009*	-2,228	*0,00002*
CG8147	-1,483	*0,00259*	-10,367	*0,00000*	-3,809	*0,00001*
Ddc	1,008	*0,93340*	1,047	*0,68979*	1,168	*0,14218*
desat1	-1,051	*0,46832*	-1,243	*0,04395*	-1,078	*0,27147*
Dpt	4,410	*0,00002*	63,570	*0,00000*	13,513	*0,00000*
Drs	-1,113	*0,65387*	4,599	*0,00004*	1,456	*0,00750*
foxo	-1,345	*0,00976*	-1,313	*0,00438*	-1,450	*0,00158*
Gadd45	-1,307	*0,12868*	-1,220	*0,13537*	-1,060	*0,72584*
Gapdh1	-1,352	*0,19108*	-1,425	*0,05864*	-1,303	*0,07286*
GLaz	1,009	*0,87518*	-1,082	*0,10049*	-1,263	*0,00782*
Gr66a	-1,340	*0,33279*	-1,177	*0,49413*	1,247	*0,47097*
GstE1	-1,191	*0,68953*	1,382	*0,20157*	1,241	*0,29282*
GstS1	-1,301	*0,00370*	-1,962	*0,00010*	-1,838	*0,00038*
Hr96	-1,076	*0,48599*	-1,295	*0,02979*	-1,015	*0,83008*
Hsp68	-1,268	*0,00516*	-1,203	*0,02425*	-1,371	*0,00223*
InR	-1,586	*0,00060*	-1,929	*0,00016*	-1,383	*0,01687*
Jafrac	1,101	*0,19687*	1,079	*0,14682*	1,022	*0,76704*
Karl	-2,434	*0,00022*	2,674	*0,00000*	1,026	*0,77165*
Lip4	1,311	*0,02100*	-1,253	*0,02485*	-1,203	*0,06966*
llp4	-4,167	*0,02306*	-4,076	*0,03266*	-3,060	*0,04019*
Lsd-1	-1,271	*0,00975*	-1,992	*0,00001*	-1,618	*0,00012*
Lsp2	1,088	*0,56817*	2,084	*0,00010*	-1,009	*0,88738*
Mgstl	-1,023	*0,89408*	1,203	*0,17277*	1,254	*0,09054*
mio	-1,895	*0,03709*	-2,085	*0,06450*	-2,276	*0,04994*
Nplp3	-1,825	*0,00030*	-2,665	*0,00002*	-2,892	*0,00003*
Pepck	-1,624	*0,00040*	1,115	*0,08810*	1,276	*0,00589*
Prx254	1,049	*0,54468*	-1,223	*0,03380*	1,150	*0,09191*
puc	-1,142	*0,03390*	-1,366	*0,02986*	-1,132	*0,37226*
RpL18	1,160	*0,55173*	1,392	*0,14750*	1,757	*0,00801*
RpL32	1,000	*0,00000*	1,000	*0,00000*	1,000	*0,00000*
S6k	-1,372	*0,04942*	-1,623	*0,00530*	-1,499	*0,02345*
Sir2	1,050	*0,60638*	-1,093	*0,17760*	-1,019	*0,72289*
sNPF	-1,154	*0,37812*	-1,115	*0,33652*	-1,154	*0,22055*
Sod2	-1,129	*0,37138*	-1,099	*0,33513*	-1,122	*0,35336*
Spt-l	1,262	*0,02655*	1,009	*0,90201*	-1,075	*0,41873*
synapt	-1,226	*0,00053*	-1,169	*0,00209*	-1,163	*0,20411*
Thor	-1,277	*0,00136*	-1,354	*0,00163*	1,154	*0,02945*
Tk	-1,003	*0,87159*	-1,880	*0,01431*	-1,525	*0,07503*
to	-1,221	*0,04269*	-1,030	*0,54535*	-1,349	*0,00015*
whd	-1,473	*0,00002*	-1,579	*0,00004*	-1,353	*0,03662*

Table S4. Gene expression comparisons in basal conditions.
Expression in flies of each genotype was compared with the w[1118] ; NLaz[NW5]/+ ; da:Gal4/+ control genotype at 3 days of age. *p* values are calculated based on a Student's t-test of the replicate $2^{-\Delta Ct}$ values for each gene in the control group and treatment groups. *p* values less than 0.05 are indicated in red. Up-regulations are shown in red and down-regulations in blue.

Gene Symbol	+/- ; da>+ (PQ vs. Control)		-/- ; da>+ (PQ vs. Control)		-/- ; da>Nlaz-WT (PQ vs. Control)		-/- ; da>Nlaz-L130R (PQ vs. Control)	
	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	*p-value*
Act5C	1,080	*0,64586*	1,084	*0,67628*	-1,137	*0,14188*	1,567	*0,00000*
Akt1	1,017	*0,82186*	1,450	*0,00007*	1,380	*0,00123*	1,571	*0,00027*
bsk	-1,349	*0,22559*	1,046	*0,86969*	1,143	*0,44734*	1,279	*0,03281*
Cat	-1,039	*0,76239*	1,556	*0,00022*	1,183	*0,00295*	1,524	*0,01534*
CG1237	-9,970	*0,00000*	-13,674	*0,00000*	-7,387	*0,00000*	-5,051	*0,00000*
CG8147	-27,606	*0,00000*	-44,839	*0,00000*	-5,805	*0,00000*	-13,511	*0,00000*
Ddc	-1,516	*0,01410*	-1,337	*0,02769*	-1,354	*0,00493*	1,056	*0,32784*
desat1	-2,420	*0,00010*	-2,027	*0,00001*	-1,600	*0,00248*	-1,396	*0,00021*
Dpt	2,255	*0,00004*	1,991	*0,00004*	-20,740	*0,00000*	-2,257	*0,00008*
Drs	1,812	*0,02333*	2,340	*0,00036*	-2,473	*0,00026*	1,512	*0,00036*
foxo	-1,774	*0,00019*	-1,251	*0,01420*	-1,442	*0,00010*	-1,072	*0,16082*
Gadd45	3,419	*0,00037*	7,415	*0,00000*	2,620	*0,00000*	4,614	*0,00001*
Gapdh1	-3,602	*0,00342*	-1,567	*0,07232*	-2,174	*0,00263*	-1,045	*0,44976*
GLaz	-2,141	*0,00002*	-2,221	*0,00000*	-1,667	*0,00000*	-1,181	*0,04135*
Gr66a	-1,025	*0,88273*	2,954	*0,00068*	1,821	*0,00593*	1,535	*0,01218*
GstE1	10,947	*0,00440*	16,078	*0,00007*	6,604	*0,00080*	16,503	*0,00000*
GstS1	-1,759	*0,00054*	-1,230	*0,00161*	1,028	*0,74450*	1,217	*0,10148*
Hr96	-1,127	*0,16779*	-3,406	*0,37900*	1,063	*0,54653*	1,172	*0,01844*
Hsp68	1,399	*0,00282*	5,755	*0,00000*	2,288	*0,00001*	1,732	*0,00000*
InR	1,897	*0,00025*	4,467	*0,00000*	3,141	*0,00091*	2,096	*0,00084*
Jafrac	1,425	*0,00138*	1,278	*0,00169*	1,183	*0,00266*	2,353	*0,00001*
Karl	-1,894	*0,00325*	1,347	*0,02570*	-1,951	*0,00005*	1,719	*0,00298*
Lip4	2,035	*0,00002*	1,002	*0,96271*	2,575	*0,00000*	3,274	*0,00000*
llp4	-3,488	*0,03697*	2,117	*0,18010*	-1,140	*0,58492*	1,010	*0,93531*
Lsd-1	-1,962	*0,00002*	-2,408	*0,00005*	-1,036	*0,27274*	1,496	*0,00007*
Lsp2	-2,490	*0,00216*	1,346	*0,00192*	-3,066	*0,00000*	1,225	*0,06080*
Mgstl	-1,434	*0,05709*	-1,209	*0,15701*	-1,551	*0,00013*	1,107	*0,01213*
mio	1,025	*0,96199*	2,531	*0,00023*	-1,079	*0,79606*	2,002	*0,16243*
Nplp3	-3,814	*0,00001*	-4,428	*0,00002*	-6,671	*0,00000*	-1,409	*0,02443*
Pepck	2,379	*0,00021*	4,731	*0,00000*	3,471	*0,00002*	2,212	*0,00000*
Prx254	2,950	*0,00000*	2,509	*0,00001*	1,732	*0,00012*	1,245	*0,00331*
puc	1,371	*0,00356*	2,508	*0,00001*	1,271	*0,23545*	1,576	*0,09594*
RpL18	1,146	*0,58380*	1,026	*0,91643*	1,106	*0,51049*	1,432	*0,00059*
RpL32	1,000	*0,00000*	1,000	*0,00000*	1,000	*0,00000*	1,000	*0,00000*
S6k	-1,581	*0,01124*	-1,090	*0,48048*	-1,149	*0,32823*	1,051	*0,74253*
Sir2	-1,113	*0,27683*	1,311	*0,01832*	1,203	*0,01185*	1,151	*0,00934*
sNPF	-1,148	*0,22731*	1,291	*0,09971*	1,122	*0,18456*	1,235	*0,00856*
Sod2	-1,663	*0,00942*	-1,314	*0,02937*	-1,523	*0,00000*	1,064	*0,46572*
Spt-l	-1,404	*0,00161*	-1,628	*0,00102*	-1,245	*0,00952*	-1,080	*0,36353*
synapt	-1,571	*0,00005*	-1,012	*0,81790*	-1,014	*0,86483*	1,034	*0,85412*
Thor	2,277	*0,00007*	4,880	*0,00000*	2,923	*0,00001*	2,299	*0,00000*
Tk	-1,561	*0,04728*	-1,402	*0,00588*	1,484	*0,01579*	1,383	*0,06986*
to	-1,183	*0,08093*	1,092	*0,36494*	1,318	*0,00477*	1,476	*0,00005*
whd	-1,184	*0,00271*	1,270	*0,00164*	1,200	*0,00603*	1,115	*0,53095*

Table S5. Gene expression comparisons of flies treated with PQ with respect to control flies of each genotype.
Flies were treated with PQ for 17 h at 3 days of age. *p* values are calculated based on a Student's t-test of the replicate $2^{-\Delta Ct}$ values for each gene in the control group and treatment groups. *p* values less than 0.05 are indicated in red. Up-regulations are shown in red and down-regulations in blu.

Gene Symbol	+/- ; da>+ (30 d vs. 3 d)		-/- ; da>+ (30 d vs. 3 d)		-/- ; da>Nlaz-WT (30 d vs. 3 d)		-/- ; da>Nlaz-L130R (30 d vs. 3 d)	
	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value
Act5C	-1,542	0,06321	1,197	0,29363	-1,401	0,02413	2,156	0,00001
Akt1	-1,672	0,00040	1,625	0,00192	-1,183	0,02144	1,806	0,00007
bsk	-1,837	0,02556	1,376	0,07002	-1,777	0,06654	1,875	0,00001
Cat	-1,140	0,59566	2,002	0,00058	1,043	0,64200	1,371	0,04269
CG1237	-4,017	0,00000	-13,712	0,00000	-3,657	0,00000	-7,343	0,00000
CG8147	-8,803	0,00000	-22,907	0,00000	2,798	0,00001	-1,316	0,00001
Ddc	-1,995	0,00307	1,068	0,58010	-1,713	0,00064	1,256	0,02046
desat1	-1,288	0,02715	-1,771	0,00094	1,444	0,00859	-1,115	0,05137
Dpt	1,171	0,23333	-1,130	0,22003	-3,122	0,00000	42,877	0,00000
Drs	-1,228	0,38725	2,511	0,00032	-4,091	0,00006	14,817	0,00000
foxo	-1,542	0,00102	-1,159	0,33855	-1,211	0,02149	1,387	0,00010
Gadd45	-2,492	0,00519	13,009	0,00000	-1,167	0,08599	2,936	0,00001
Gapdh1	-9,964	0,00018	1,433	0,08215	-5,559	0,00020	-3,726	0,00000
GLaz	-1,743	0,00095	-1,909	0,00002	-1,544	0,00000	1,054	0,37975
Gr66a	-1,385	0,36476	5,332	0,00015	1,324	0,20643	2,294	0,00013
GstE1	-1,217	0,80575	26,472	0,00006	1,313	0,14101	4,203	0,00000
GstS1	-4,133	0,00002	-1,053	0,49674	-2,662	0,00364	-1,360	0,01648
Hr96	-1,678	0,00351	1,214	0,15648	-1,093	0,21807	1,335	0,00199
Hsp68	-2,046	0,00005	6,524	0,00002	-1,970	0,00004	2,660	0,00000
InR	-2,084	0,00230	4,690	0,00011	1,042	0,59538	2,500	0,00004
Jafrac	-1,330	0,01034	1,632	0,00333	-1,189	0,00028	1,491	0,00054
Karl	-4,532	0,00007	1,622	0,01491	-1,255	0,00156	1,960	0,00002
Lip4	-1,181	0,12714	1,067	0,51384	1,045	0,10813	1,686	0,00004
llp4	-4,712	0,02160	3,039	0,02089	-3,311	0,29345	1,306	0,46516
Lsd-1	-1,848	0,00003	-1,924	0,00016	1,934	0,00087	1,004	0,94163
Lsp2	1,167	0,32893	1,717	0,00036	3,499	0,00000	2,975	0,00001
Mgstl	-1,816	0,01465	-1,060	0,55326	-1,362	0,01045	-1,145	0,01262
mio	-2,228	0,02669	2,815	0,00309	-2,186	0,52800	2,559	0,00214
Nplp3	-28,416	0,00000	-1,792	0,00253	-10,340	0,00000	-3,613	0,00027
Pepck	1,936	0,00003	5,351	0,00002	2,970	0,00011	1,335	0,00043
Prx254	-2,002	0,00019	3,876	0,00011	-1,912	0,00001	-1,184	0,00754
puc	-1,840	0,00880	3,589	0,00002	-1,854	0,65336	3,579	0,00000
RpL18	1,142	0,55893	1,465	0,12492	1,546	0,01499	1,307	0,01551
RpL32	1,000	0,00000	1,000	0,00000	1,000	0,00000	1,000	0,00000
S6k	-1,825	0,00455	1,061	0,68089	-1,090	0,17141	1,473	0,00468
Sir2	-1,373	0,00288	1,927	0,00048	-1,024	0,70739	1,790	0,00002
sNPF	-1,473	0,08927	1,492	0,01589	-1,206	0,10719	2,058	0,00002
Sod2	-3,013	0,00072	1,194	0,13122	-1,899	0,00000	-1,563	0,00231
Spt-l	-1,284	0,03626	-1,303	0,05097	-1,448	0,00049	1,355	0,00208
synapt	-3,063	0,00000	1,473	0,00262	-2,000	0,00001	1,018	0,96504
Thor	-2,229	0,00001	6,340	0,00002	1,088	0,14582	1,326	0,00044
Tk	-2,567	0,00561	-1,181	0,06543	-1,490	0,04258	1,233	0,24832
to	-1,403	0,00485	1,492	0,00536	1,641	0,00029	2,100	0,00000
whd	-1,503	0,00081	1,548	0,00278	1,068	0,57877	1,382	0,02675

Table S6. Gene expression of 30 day-old flies with respect to 3 day-old flies of each genotype.

p values are calculated based on a Student's t-test of the replicate $2^{-\Delta Ct}$ values for each gene in the control group and treatment groups. *p* values less than 0.05 are indicated in red. Up-regulations are shown in red and down-regulations in blue.

Chapter 4

"Grasshopper Lazarillo, a GPI-anchored Lipocalin, increases Drosophila longevity and stress resistance, and functionally replaces its secreted homologue NLaz".

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Grasshopper Lazarillo, a GPI-anchored Lipocalin, increases Drosophila longevity and stress resistance, and functionally replaces its secreted homologue NLaz.

Summary

Lazarillo (Laz) is a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein first characterized in the developing nervous system of the grasshopper *Schistocerca americana*. It belongs to the Lipocalins, a functionally diverse family of mostly secreted proteins. In this work we test whether the protective capacity known for Laz homologues in flies and vertebrates (NLaz, GLaz and ApoD) is evolutionarily conserved in grasshopper Laz, and can be exerted from the plasma membrane in a cell-autonomous manner. First we demonstrate that extracellular forms of Laz have autocrine and paracrine protecting effects for oxidative stress-challenged *Drosophila* S2 cells. Then we assay the effects of overexpressing GPI-linked Laz in adult *Drosophila* and whether it rescues both known and novel phenotypes of NLaz null mutants. Local effects of GPI-linked Laz inside and outside the nervous system promote survival upon different stress forms, and extend lifespan and healthspan of the flies in a cell-type dependent manner. Outside the nervous system, expression in fat body cells but not in hemocytes results in protection. Within the nervous system, glial cell expression is more effective than neuronal expression. Laz actions are sexually dimorphic in some expression domains. Fat storage promotion and not modifications in hydrocarbon profiles or quantities explain the starvation-desiccation resistance caused by Laz overexpression. This effect is exerted when Laz is expressed ubiquitously or in dopaminergic cells, but not in hemocytes. Grasshopper Laz functionally restores the loss of NLaz, rescuing stress-sensitivity as well as premature accumulation of aging-related damage, monitored by advanced glycation end products (AGEs). However Laz does not rescue NLaz courtship behavioral defects. Finally, the presence of two new Lipocalins with predicted GPI-anchors in mosquitoes shows that the functional advantages of GPI-linkage have been commonly exploited by Lipocalins in the arthropodan lineage.

1. Introduction

Lazarillo (Laz) is a glycoprotein linked to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) tail, and first characterized in the developing nervous system of the grasshopper *Schistocerca americana* [1-3]. Laz is expressed in a specific subset of neuroblasts, neurons and ganglion mother cells of the central nervous system (CNS). In the peripheral nervous system (PNS) Laz is detected in all sensory neurons and in a group of enteric nervous system neurons [2,4]. Its expression is neither limited to the embryonic stage, since it continues throughout adulthood, nor to the nervous system. Outside the nervous system Laz is associated mainly with the excretory system: malpighian tubules and subesophageal body. During nervous system development, Laz is involved in the growth and guidance of developing pioneer axons. Grasshopper embryos treated with a monoclonal antibody against Laz show axonal aberrant growth and misdirection [2]. Lazarillo has been used as a marker of subsets of pioneer neurons also in other orthopterans, as in the economically important desert locust *Schistocerca gregaria* [5-8]. However a test for grasshopper Laz functions during adulthood has not been performed yet.

Lazarillo belongs to the Lipocalin protein family, an ancient and functionally diverse family of secreted proteins that share a similar structural fold, often in the presence of low sequence similarity [9]. Their structure is defined by an eight-stranded antiparallel β-barrel that forms a binding pocket, usually hydrophobic [10-12]. Molecular phylogenetic studies have shown that arthropodan Lipocalins have diversified in five main groups [13]: a Laz-related clade of insect Lipocalins, a clade of Lipocalins found in Crustacea, a clade of biliproteins and related insect Lipocalins, and two large family expansions occurring in blood-feeding insects and arachnids. Grasshopper Laz has a basal position in the arthropodan Lipocalins phylogenetic tree, and the Laz-related clade is the closest relative to the chordate Lipocalin Apolipoprotein D (ApoD) [9,14].

Lipocalins play a great number of different roles: bacteriostatic effect, retinoids transport, prostaglandins synthesis, control of reproductive behavior, and arthropod coloration among others [reviewed by 15]. The function of a given Lipocalin must be intimately related both with its hydrophobic ligands and with its site of expression. The nature of Laz physiological ligand(s) is still unknown, but a set of candidates has been studied *in vitro*. Lazarillo is able to bind retinoic acid and fatty acids [16].

The domain of expression of all insect Lipocalins analyzed so far commonly includes, although is not restricted to, ectodermal derivatives, with nervous system and epidermal tissues been recurrent sites of expression [reviewed by 13]. Lepidopteran Lipocalins like Bilin binding protein from *Pieris brasssicae* and *Samia cynthia* or Insecticyanin from *Manduca sexta* are expressed in epidermis, and some of them have been demonstrated to play roles in cuticle coloration [17-19] and prevention of oxidative damage [20]. Brain Lipocalins have been identified both in Diptera and Lepidoptera. The lepidopteran protein Hyphantrin from *Hyphantria cunea* is up-regulated in the brain in response to injury [21]. Also, Bombyrin and Gallerin were isolated from pupal brain extracts of *Bombyx mori* and *Galleria mellonella* [22,23], but their function remains unknown.

In *Drosophila melanogaster*, two Lipocalins expressed in the nervous system are the Lazarillo homologs Neural-Lazarillo (NLaz) and Glial-Lazarillo (GLaz) [9,14,24]. NLaz and GLaz have a key role in the regulation of longevity and defense against oxidative stress [25-27], and over-expressing NLaz or GLaz increases lifespan and survival against different forms of oxidative stress [26,28].

The function of ApoD, the closest chordate Laz homolog, has also been studied extensively in diverse model organisms as well as in humans. Its expression in the brain is the most consistently up-regulated upon aging in different mammalian species [29,30], and it is also up-regulated in many human neurodegenerative diseases [reviewed by 31]. Moreover, ApoD have demonstrated

protective effects in the mouse brain upon oxidative stress [32-34]. That this protective function of ApoD is a conserved trait is supported by the results with transgenic flies expressing human ApoD. Like NLaz and GLaz, over-expressing human ApoD increases fly longevity and resistance against different stresses [35].

So far the Lazarillo GPI-anchor is a unique feature within metazoan Lipocalins, which are mainly secreted proteins. Lipocalins linked to membranes through other mechanisms have been reported in bacteria and plants [36,37], and indirect interactions of Lipocalins with membranes have been described for α1-acid-glicoprotein [38], β-Lactoglobulin [39] and Tear Lipocalin [40]. These data point to the existence of membrane receptors for Lipocalins, some of which have been already characterized, mostly for vertebrate Lipocalins [41-43], but also for Insecticyanin in *Manduca sexta* [44].

In this work we test the protective potential of grasshopper Lazarillo in a cell culture system using the MT promoter system, and in adult fruit flies expressing grasshopper Laz with the UAS/GAL4 system. We test whether the GPI-linked Lazarillo, locally acting within and outside the nervous system, is able to promote survival upon different stress forms and extend the lifespan and healthspan of flies. Fat storage and hydrocarbon profiles are also explored as variables potentially controlled by the Lazarillo-related Lipocalins and contributing to their mechanism of regulating stress resistance and longevity. We also test whether the membrane-anchored Lazarillo is able to functionally restore the loss of NLaz by exploring stress resistance, biomarkers of aging-related damage, and behavioral phenotypes. Our purpose is to assay whether the protective capacity of NLaz, GLaz and ApoD is evolutionarily conserved in the grasshopper Lazarillo protein, and if this role can be exerted linked to the plasma membrane by a GPI-tail in a cell-autonomous manner. Finally, we found two new Lipocalins with predicted GPI-anchors in mosquitoes that point to the functional advantages of GPI-linkage as being commonly exploited by Lipocalins in the arthropodan lineage.

2. Material and Methods

2.1. Cell culture.

Drosophila S2 cells were maintained as semi-adherent cultures at 27ºC in Express-Five medium (Gibco) supplemented with 10% L-Glutamine, 50 U/ml Penicillin, and 50 μg/ml Streptomycin. The culture medium was replaced twice a week.

2.2. Cloning and Purification of the Lazarillo Protein.

A fragment of the Lazarillo cDNA, translating into residues 1-192 of the precursor (Uniprot reference P49291), and thus missing the GPI signal peptide, was subcloned using the EcoRI and NotI sites into the pRmHa3 vector [16,45]. This system expresses the cloned sequence under the control of the inducible Drosophila metallothionein promoter, and the presence of a C-terminal His-tag sequence allows for protein purification from the culture medium.

The Laz-pRmHa3 plasmid was co-transfected with the selection vector pCoBlast (conferring blasticidin resistance) into Drosophila S2 cells using FuGENE6 (Roche) at 3:1 ratio according to the manufacturer instructions. Transfected cells were selected with 25 μg/ml blasticidin-S for 3 weeks (Invitrogen).

The recombinant protein was expressed in spinner flasks with blasticidin free medium, upon induction with 1mM $CuSO_4$ for 5-7 days. The secreted Lazarillo protein was purified from the cell medium by immobilized metal affinity chromatography using Ni-NTA resin (5-Prime). The Laz protein was further purified by size exclusion chromatography (Superdex prep grade 75, Amersham Biosciences) in PNEA buffer (25 mM PIPES, 150 Mm NaCl, 1mM EDTA, 0.02% NaN_3). The purified protein was deglycosylated by treatment with peptide-N-glycosidase F (PNGase-F) from *Flavobacterium meningosepticum* (New England Biolabs) after denaturation following the protocol supplied by the manufacturer.

Bacterial recombinant Lazarillo was purified from the periplasmic space of *Escherichia. coli* as previously described [16].

2.3. Fly Generation and Handling.

Flies were grown in a temperature-controlled environmental incubator at 25 ℃ under a 12h light-dark cycle. The stocks were routinely maintained in tubes containing our standard medium [yeast 84 g/l, sugar 84 g/l, NaCl 3.3 g/l, agar 10 g/l, wheat flour 42 g/l, apple juice 167 ml/l, propionic acid 5 ml/l; [25]], or a yeast/cornmeal/agar medium [maize flour 83 g/l, agar 11 g/l, yeast 8.3 g/l, nipagine 5 g/l; [46]] in the case of hydrocarbon and triglyceride measurements. NLaz loss-of-function mutant (NLaz-KO) was generated in a w^{1118} background [47] and crossed with a w^{1118}-CS wild type line to generate the NW5 line used in this study [26].

2.3.1. Generation of Lazarillo transformed Drosophila fly lines

P-element mediated transformation of w^{1118} flies was used to generate a pUASt-Laz fly line using standard procedures (BestGene Inc. USA). An EcoRI/XhoI fragment containing the full length Lazarillo cDNA [1] was subcloned into the pUAST vector (FlyBase annotation FBmc0000383) to generate the UAS-Lazarillo transgene, with Upstream Activating Sequences (UAS) upstream of Lazarillo cDNA. Two different independent insertion lines of pUASt-Laz were used in our experiments, producing identical results. pUASt-Laz-8F has the insertion on X-chromosome, whereas pUASt-Laz-7F has the insertion on the third chromosome.

2.3.2. Expression of grasshopper Lazarillo under the control of GAL4 drivers

To drive the expression of the Laz transgene a set of GAL4 lines, expressing the yeast transcription factor GAL4 in different spatiotemporal patterns, were crossed with pUASt-Laz. To generate driver-only controls with minimal background differences we crossed each GAL4 line with wild-type flies (the strain w^{1118} in which the Laz transgenic lines were generated). For the hydrocarbon and triglycerides measures drivers on the third chromosome were used with the TM3 balancer. The Laz expression was achieved by crossing GAL4/TM3 flies with UAS-Laz flies. The resulting progeny bears the same genotype, excepting the third chromosome; UAS-Laz; TM3 was used as control and UAS-Laz; GAL4 was used as overexpressing Laz.

The following GAL4 drivers were used according to their main domain of expression: da-GAL4 (ubiquitous expression); GAL4$^{109(2)80}$ (expression in brain and muscle); BL7415 Repo-GAL4 and M1B-GAL4 lines (for glial expression), BL7009 Ddc-GAL4 (expression in dopaminergic and serotonergic neurons in the CNS and epidermis), BL 8699 He-GAL4 (expression in hemocytes), BL5820 31-1-GAL4 (expression in embryonic neuroblasts and neurons in the CNS and PNS), BL8816 D42-GAL4 (expression in motoneurons) and ppl-GAL4 (expression in fat body).

2.4. *Wolbachia* Test.

The absence of infection by *Wolbachia pipiens* in fly strains was tested by PCR of genomic DNA extracts. The following primers against the rRNA-16S gene of *Wolbachia* were used: 5′-GAAGATAATGACGGTACTCAC-3′, 5′-GTCAGATTTGAACCAGATAGA-3′ and . The PCR conditions were: 2 min 94°C; (30 s 94°C; 30 s 60°C; 45 s 72 °C)×30; 7 min 72°C. DNA from *Wolbachia*-infected *Dirofilaria immitis* worm was used as a positive control. Primer sequences and a positive control DNA were kindly provided by Dr. F. Simon (Univ. Salamanca).

2.5. Quantitative RT-PCR.

Procedures, primers, PCR parameters, and other reagents were previously described [27]. Briefly, total RNA was extracted from 3 days old flies (n=25/sample) using TRIzol (Invitrogen), and reverse transcription was performed with PrimeScript™ RT reagent Kit (Takara) according to the manufacturer instructions. PCR reactions were performed in quintuplicate for each RNA sample using the SYBR® Premix Ex Taq™ (Takara). Expression of RPL18 was use to normalized the experimental samples. Transcriptional levels were calculated following the ΔΔCT method [48,49].

2.6. Drosophila Lifespan Analysis.

Flies were collected within 24 h of eclosion and allowed to mate for 2-3 days. They were then separated by sex under brief CO_2 anesthesia and housed in groups of 25. Every three days, flies were transferred to fresh media without anesthesia and scored for viability. Over 140 individuals were analyzed per sex, genotype and condition. The viability tests were carried out at 25ºC.

2.7. Oxidative Stress, Toxicity and Desiccation Stress Assays.

2.7.1. *Assays in flies*

Flies were collected and separated by sex as described for the longevity analysis. All tests were carried out at 25ºC. Over 60 individuals were analyzed per sex, genotype and condition. The following treatments were used:

2.7.1.1. Starvation-desiccation Stress Assay

Starting at 3 days of age, flies were transferred to empty plastic vials. Dead flies were scored every 2–7 hours.

2.7.1.2. Hyperoxia Stress Assay

Adult flies (3 days old) were placed in plastic vials (20–25 flies per vial) containing our standard food and maintained in a plastic box enclosure at room temperature (25ºC) in a humidified 99.5% O_2 atmosphere. Dead flies were scored every 12 hours.

2.7.1.3.Iron Toxicity Assay

Flies were kept on regular food until the experiment started (3 days of age), starved 3 h at 25ºC and transferred to vials with filter papers soaked in 1% glucose-15 mM $FeSO_4$ solution in water. Flies were moved everyday to new vials with fresh iron-glucose solution. Dead flies were scored every 2–7 hours.

2.7.2. *Assays in cell cultures*

2.7.2.1. Oxidative Stress Assay

S2 cells were seeded at a density of $2x10^6$/ml in 24-well plates, and 20 mM hydrogen peroxide (H_2O_2) was used as oxidant. When Lazarillo was generated by S2 cells, its expression was induced with 1mM $CuSO_4$ 24 h. In other experiments, 50 nM of pure Lazarillo protein was added to the culture medium 30 min before H_2O_2 treatment. As a control protein, Trypsin Inhibitor (TI) from soybean (Sigma) was added at the same concentration. Laz and TI concentrations were determined with the Micro-BCA^{TM} protein assay (Pierce). Two hours after treatment, cells were stained with Trypan Blue (Invitrogen) and counted in a hemocytometer. Cell viability was determined as the percentage of live cells. Each condition was assayed in triplicate, and three measurements were made in each culture well.

2.8. Climbing Assay.

The flies climbing ability was tested after 48 (for males), 72 and 96 hours (for females) of iron treatment. Flies were placed in empty vials, and after a resting period of 10 min the animals were tapped to the bottom of the tube and the number of individuals able to climb 5 cm in 6 sec was recorded. The assay was repeated three times at 1 min intervals.

2.9. Courtship behavior.

Courtship and mating tests were performed during 60 min using virgin male–female pairs of 4–5 day-old flies under a Petri cover dish (40 mm diameter) used as an observation chamber. The courtship index (C.I.) represents the proportion of time a male spends actively courting the female for the first 10 min. Copulation features such as mating time, mating latency and mating performance (%) were scored for 60 min. The test was performed in the first 1-3 h of the fly daylight period.

2.10. Immunohistochemistry.

Flies were fixed either with 5% glacial acetic acid, 4% formaldehyde, 85% ethanol, or 4% paraformaldehyde, and embedded in paraffin following standard procedures. Sections (5 μm) were performed with a rotary microtome (Microm), mounted in series on Polysine^{TM} slides (Menzel-Gläser), and dried. Sections were dewaxed with xylene, and rehydrated through an ethanol series into phosphate buffered saline (PBS). Sections were then permeabilized and blocked with Tween20 (0.2% in PBS), 1% BSA and 1% normal goat serum.

The monoclonal antibody 10E6 against Lazarillo was used as primary antibody. Cy3 or

HRP-conjugated goat anti-mouse IgG (Abcam) were employed as secondary antibodies. HRP development was performed with DAB (0.03%) and H_2O_2 (0.002% in 50 mM Tris, pH 8.0). For fluorescence immunohistochemistry, sections were mounted with Vectashield-DAPI (Vector Labs).

HRP immunohistochemistry sections were visualized with an Eclipse 90i (Nikon) microscope equipped with a DS-Ri1 (Nikon) digital camera. Images were acquired and processed with the NISElements BR 3.0 software (Nikon). Images from fluorescence immunohistochemistry preparations were obtained in a DMI 6000B microscope with a TCS SP5 X confocal system and a WLL laser (Leica) controlled by LAS AF software (Leica).

2.11. Immunoblots.

Flies were homogenized in RIPA lysis buffer [1% NP-40, 0.1% SDS, 0.5% Na-DOC and 10% complete protease inhibitors (Roche) in PBS]. Protein extracts were separated on 12% SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were washed (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% NaN_3) and blocked for 2 hours with 0.2% gelatin, 2.5% BSA and 2.5% dry non-fat milk in the previous buffer [1]. Membranes were then incubated with primary antibody 10E6 (1:200 in blocking solution) for 2 hours, washed again, and incubated with HRP-conjugated goat anti-mouse (1:10000) (Dako) for 1h at RT. Blots were developed with ECL (ImmobilonTM Western, Millipore), and the signal visualized with a digital camera (VersaDoc, BioRad).

2.12. AGE assay.

Fluorescent AGEs (advanced glycation end products) were measured according to [50] with minor modifications. Flies were homogenized in 1 ml of PBS containing 10 mM EDTA. Cuticle and other debris were removed by centrifugation for 5 min at 11.000 g. Supernatants were digested by addition of 10 mg/ml trypsin (Sigma). Following incubation for 24 h at 37ºC the digested homogenates were centrifuged and filtered through a 0.22 μm sterile filter. Filtrates were diluted up to an absorbance range of 0.02-0.05 units at 365 nm.

Fluorescence (excitation and emission wavelengths of 365 and 440 nm, respectively) was measured in three aliquots of each filtrate with a Shimadzu RF-5301PC spectrofluorophotometer at 25ºC.

2.13. Hydrocarbon profile and triglyceride content assays.

Flies were separated by sex just after emergence and kept in groups of ten flies for 5 days at 25°C. Each single fly was extracted with 100 μl heptane and 500 ng n-C26 (internal standard) for 5 min. Samples were injected into a Perichrom Pr200 gas chromatograph, as previously described [46]. Hydrocarbon profiles and total amount of hydrocarbons (between 23 and 29 carbons) was calculated.

The total triglyceride content of 10 flies/sample was analyzed using the Triglyceride Liquicolor kit (Stanbio Laboratory) as described [51]. Two to four independent measures were performed for each genotype (with flies coming from independent crosses).

2.14. Statistical Analysis.

Statistical analyses were carried out with Statistica (v 5.5) software. Survival curves were analyzed using the Log-Rank Test. In maximal survival and quantitative PCR experiments the nonparametric Mann-Whitney U test was performed. Student's t-Test was used for the analysis of the climbing assays, cell survival, triglycerides and AGEs assays. One-way ANOVA followed by Tukey's multiple comparison post-hoc test was used for hydrocarbon profile comparisons. Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's multiple comparison post-hoc test was applied to analyze courtship experiments. Differences were considered statistically significant at $p<0.05$ (*) $p<0.005$ (**), and $p<0.0005$ (***).

2.15. Sequence alignment and prediction of GPI-anchoring signal peptides.

Multiple sequence alignments were performed with CLUSTAL-X2 [52]. Potential GPI cleavage sites were predicted by the big-PI Predictor [53]. Hydrophobicity profiles were determined by Kyte and Doolittle's method [54] using a window of 9 residues.

3. Results and Discussion

3.1. Grasshopper Lazarillo promotes survival in S2 cells upon oxidative stress.

To test whether the ability of NLaz, GLaz and ApoD to protect organisms under oxidative stress is an evolutionarily conserved function in grasshopper Laz, we used two different recombinant forms of Laz to assay their effects on S2 cell survival (Figure 1). A secreted form of Laz was generated in *E. coli* and purified from the bacterial periplasmic space. A glycosylated and soluble form of Laz (Figure S1), lacking the GPI tail in order to make it a secreted form, was expressed by Drosophila S2 cells with an inducible expression system.

We first tested the effect of expressing Laz on the survival of S2 cells exposed to oxidative stress. After 24 h of induction, S2 cells expressing soluble grasshopper Laz are more resistant to H_2O_2 (58.1% survival, Figure 1A) than control non-transfected S2 cells (35.9% survival). The exogenous addition of purified recombinant Laz protein also promotes cell survival (Figure 1B). Non-transfected control S2 cells show 51.4% and 51.6% survival upon H_2O_2 treatment when either no addition was performed or an unrelated protein was added to the culture. Survival increases to 71% and 69.7% respectively when Laz, either purified from S2 cells (glycosylated) or from bacteria (non-glycosylated), is added to the culture medium. A similar protective effect has been demonstrated for the mammalian Lazarillo homolog, ApoD, on primary astrocytes [33], where it contributes to lower the levels of lipid peroxides and to modify the transcriptional response of cells to oxidative stress.

3.2. Expression of GPI-linked grasshopper Lazarillo in Drosophila docks the protein to the cell membrane and does not alter native Drosophila Lipocalins expression.

We generated a full-length cDNA construct downstream of UAS promoter sequences in order to use the GAL4/UAS system to express the GPI-linked grasshopper Laz in Drosophila with different expression patterns according to the

GAL4 driver used. Two independent single-insertion transgenic lines carrying the pUASt-Laz transgene were chosen for the study (lines 7F and 8F, mapped to the 3rd and X chromosome respectively). These transgenic lines as well as other lines used in this study were tested for the presence of *Wolbachia*, an endosymbiont of arthropods known to alter longevity related traits [55]. No infection was detected (Figure S2 A).
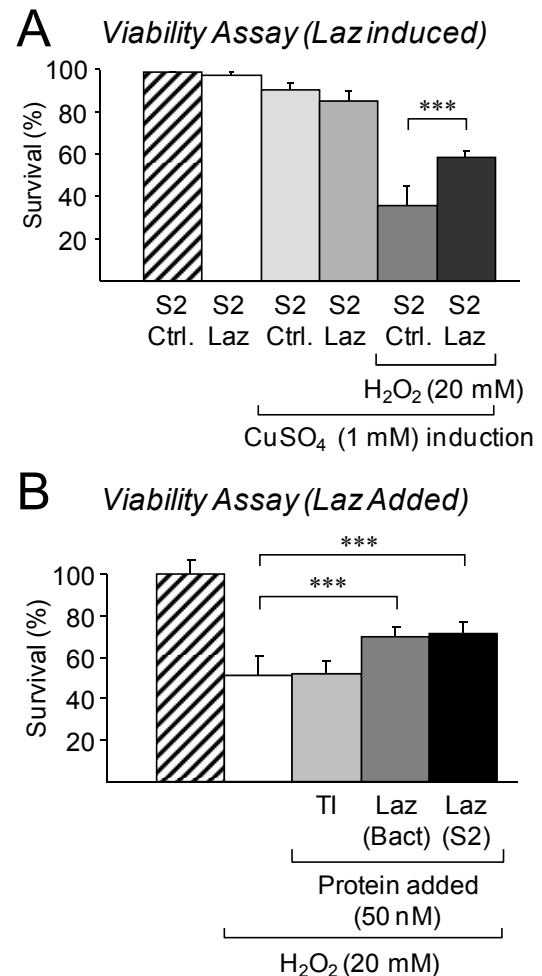

Figure 1. Grasshopper Lazarillo promotes survival in S2 cells upon exposure to hydrogen peroxide.
(A) S2 cells over-expressing Laz without its GPI tail, therefore secreted to the medium, are more resistant than wild-type cells to the oxidative stress generated by 20 mM H_2O_2. Expression is induced upon exposure to 1 mM $CuSO_4$. **(B)** Non-transfected S2 cells also improve their survival under oxidative stress upon addition of exogenous Lazarillo (50 nM) purified from either S2 cells culture medium or bacterial periplasm. Addition of Trypsin Inhibitor (TI, 50 nM) as a control unrelated protein does not influence cell survival. (*** Student's t-Test, p<0.0005).

Laz expression was tested by immunoblot (Figure 2A). The protein shows the characteristic smear due to its glycosylation and an electrophoretic mobility (40 kDa) similar to the native grasshopper protein. Both transgenic lines have similar Laz expression levels. The bacterially produced non-glycosylated Laz protein is shown for comparison. Immunohistochemistry experiments (Figure 2B-C) show the protein present in the expected cell type (glial cells in the particular case shown), and localized at the cell surface (arrows in Figure 2C). Negative controls performed to ascertain that the monoclonal antibody 10E6 against grasshopper Laz is not cross-reacting with any Drosophila epitope are shown in Figure S2 B. These experiments confirmed that all the post-translational modifications that take place in the native grasshopper Laz, including anchoring to the cell membrane, were correctly reproduced in the transgenic flies.

Since we intended to assay the effects of overexpressing Laz in wild type flies, we quantified the expression of the native homologous Drosophila genes (GLaz and NLaz) under these circumstances. NLaz gene expression was unaltered (Figure 2D), while GLaz gene was slightly (1.6-fold) up-regulated in flies overexpressing Laz ubiquitously. Increases of 7-10 fold in GLaz ubiquitous expression have been reported to produce mild longevity extensions [28]. Therefore, though we cannot discard a contribution of the mildly altered expression of GLaz upon transgenic Laz over-expression, other results presented and discussed below cast doubt about the functional significance of these minor changes.
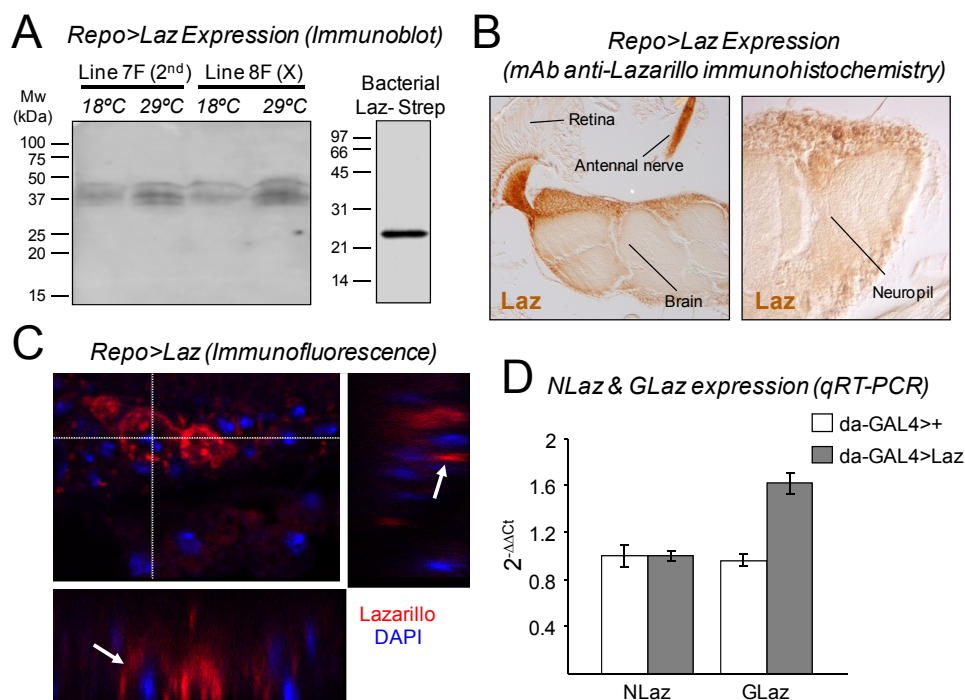

Figure 2. Expression of grasshopper Lazarillo in transgenic flies. Influence on the expression of the *Drosophila* homologs NLaz and GLaz.
(A) Immunoblot against Lazarillo with MAb 10E6 confirms its expression in flies at the protein level. Two lines representing independent insertions of the Laz gene in the *Drosophila* genome were tested (mapped to the 3rd and X chromosome). The level of expression is subject to the known temperature sensitivity of the GAL4/UAS expression system in Drosophila. Bacterially produced non-glycosylated Laz (Laz-Strep) was used as positive control and shows the difference in electrophoretic mobility with the protein produced in Drosophila. **(B-C)** MAb 10E6 also recognizes grasshopper Laz expressed under the control of a glial driver (Repo-GAL4). Membrane localization of the labeling is evident in confocal sections (C). **(D)** Quantitative PCR of NLaz and GLaz in transgenic flies over-expressing Laz compared to their driver-only controls. NLaz expression is not altered, while GLaz is slightly up-regulated when Lazarillo is present (Mann-Whitney U test, $p<0.0005$).

3.3. Membrane bound grasshopper Lazarillo increases lifespan with both ubiquitous and nervous system-enriched expression.

Laz overexpression produces a significant increase in fly longevity. Median survival increases in female flies by 28%, 43.5% and 31.4% when Laz is expressed either ubiquitously (da-GAL4 driver, Figure 3A), in the nervous and muscular system (GAL4$^{109(2)80}$ driver, Figure 3B), or in the dopaminergic-serotonergic neurons and epidermis (Ddc-GAL4 driver, Figure 3C). Effects in male flies (Table 1) are less prominent when Laz is expressed ubiquitously (8.5%), but very similar when expressed under the control of GAL4$^{109(2)80}$ or Ddc-GAL4 drivers (32.7% and 30% respectively). Similar results were obtained with both Laz transgenic lines. The survival curves shown in Figure 3 are the average of data obtained independently in cohorts from both lines. Survival curves obtained independently for each UAS:Laz line are shown in Figure S3. Table 1 summarizes the results of all tests performed with male and female flies.

The magnitude of the effects obtained with Laz overexpression contrasts lifespan extension obtained in transgenic male flies producing GLaz with similar expression patterns: 0-21% in ubiquitous patterns (actin or Hsp70 drivers), 23-29% when driven by GAL4$^{109(2)80}$, and no effect when expressed panneuronally (elav driver) or in motor neurons (D42 driver) [28]. Thus, grasshopper Laz is able to extend lifespan of flies more efficiently than the native GLaz, especially when the expression domain included neurons (GAL4$^{109(2)80}$ and Ddc-GAL4 drivers). So far, no analysis of NLaz effects on longevity have been performed with neuronally enriched patterns. On the other hand, ubiquitous overexpression of NLaz with the da-GAL4 driver produces a 42-50% increase in median longevity of male flies [26], a larger effect than that obtained with Laz.

The Ddc-GAL4 line used drives the expression of UAS-transgenes with the dopa decarboxylase promoter, the enzyme controlling the synthesis of dopamine (DA). DA is not only a neurotransmitter in insect CNS, but has also a role in the sclerotization of pigmented cuticle. The Ddc gene is expressed in late embryogenesis, when cuticle

hardening is taking place [56], and in the epidermis of third instar larvae [57]. Also, DA levels peak within the first hours after pupa eclosion [58]. DA contributes to adult cuticle hardening in this period, but also regulates cuticular sex-specific hydrocarbon biosynthesis in female flies [59,60]. Curiously, the later effect is dependent on DA production in the brain, and not on local production of DA by Ddc activity in the epidermis. In addition, Ddc expression can be induced in the larval and adult epidermis upon local infection, as part of the immune response [61]. Ddc basal expression in adult epidermis is small compared to the expression in the CNS domain [61]. In view of these data the large extension of lifespan caused by Laz expressed under the control of the Ddc driver (Figure 3C) has probably an important contribution of the neuronal domain of expression (dopaminergic and serotonergic neurons in the CNS), where Ddc is expressed throughout life. However, below we describe sex-dependent effects of Laz on stress resistance when expressed under the control of Ddc-GAL4. The putative contribution of Laz epidermal expression will be discussed.

3.4. Lazarillo protects flies against different forms of stress in a cell-type dependent manner within the nervous system.

Laz increases survival of flies upon starvation-desiccation stress when expressed with ubiquitous or wide range patterns (23.1-30% median survival increase, Figure 4A and Table 1). Protective effects are obtained when Laz is expressed by dopaminergic-serotonergic neurons and epidermis (Ddc-GAL4 driver, Figure 4B), or by a subset of embryonic neuroblasts and neurons in the CNS and PNS (31-1-GAL4 enhancer trap insertion; Figure S4). However, no protection is obtained when Laz is driven to motoneurons by D42-GAL4 (Figure 4B). These effects are, moreover, sex-dependent (see below). In contrast, glial cell expression of grasshopper Laz consistently protects both male and female flies from starvation-desiccation stress with survival extensions ranging 10.4-38.3% (Figure 4C and Table 1).
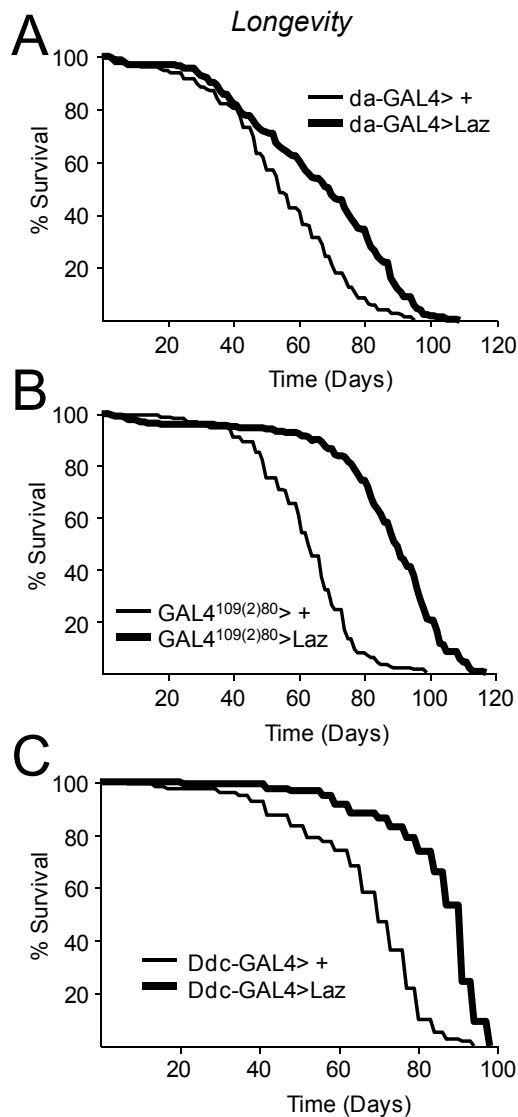
Figure 3. The overexpression of grasshopper Lazarillo extends lifespan.

(A) Drosophila lifespan increases when Laz is expressed using the ubiquitous da-GAL4 driver (da-GAL4>Laz) compared to da-GAL4> + control flies. Median survival time is increased by 28.3% in the Lazarillo expressing flies (Long-rank test: p<0.0005). **(B)** Median lifespan is also increased by 43.5% when GAL4$^{109(2)80}$ drives the expression of Laz in muscles and brain (Log-rank test: p<0.0005). **(C)** When Laz is expressed in dopaminergic and serotonergic neurons and epidermis, fly survival increases by 31.4% (Log-rank test: p<0.0005). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.
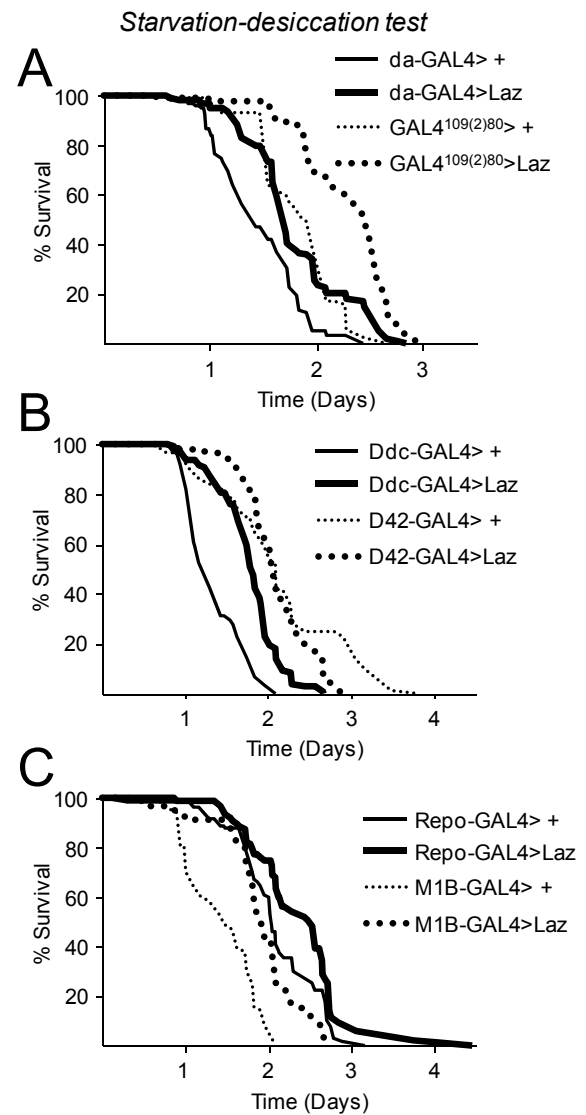
Figure 4. Lazarillo protects flies against starvation-desiccation stress.

(A) Resistance against starvation-desiccation increases when Lazarillo is expressed in flies using da-GAL4 or GAL4$^{109(2)80}$ drivers. Median survival increases by 25.8 and 30% respectively (Log-rank test: p<0.0005 in both cases). **(B)** Median survival is increased by 37.9% (Log-rank test: p<0.0005) when Lazarillo expression is driven by Ddc-GAL4. However, using a motoneuron driver (D42-GAL4) no changes in median-survival upon starvation-desiccation are observed. **(C)** Expression in glial cells (Repo-GAL4 or M1B-GAL4 drivers) also promotes survival upon starvation-desiccation (survival increases 24% and 38.3% respectively; Log-rank test: p<0.0005 in both cases). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.

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When flies are subjected to oxidative stress (exposure to 15 mM $FeSO_4$, Figure 5), Laz is able to extend their survival when expressed with the glial driver M1B (Figure 5C), the Ddc driver (Figure 5B), the nervous and muscular systems driver ($GAL4^{109(2)80}$, Figure 5A) or the ubiquitous driver (da-GAL4). These protective effects are therefore also cell type-dependent and could be sensitive to variations in timing or magnitude of overexpression.

We also assayed whether Laz expression either ubiquitously or in neuron-enriched patterns would modify the locomotor performance of Fe^{2+}-intoxicated flies. Generic overexpression of Laz (da-GAL4 driver) improves climbing performance after 72 h of iron treatment (Figure 5D and Table 1) and the protecting effect is maintained up to 93 h of treatment (Table 1). However, expression of Laz with D42 or Ddc drivers shows no significant effect (Figure 5D and Table 1). Thus, the ubiquitously-expressed grasshopper Laz is not only able to extend the flies' lifespan under normal or stress conditions, but also to improve their healthspan.

Finally, when oxidative stress is provoked by hyperoxia, neuronal or glial enriched expression of Laz is beneficial for flies (Figure 6 and Table 1) with significant effects ranging from a mild 6.8% to a substantial 23.7% increase in survival. However, no statistically significant survival extension was obtained with a wide expression of Laz (Figure 6A).

3.5. Local effects of GPI-linked Lazarillo outside the nervous system promote survival under starvation and oxidative stress.

The expression domains of Drosophila GLaz and NLaz and grasshopper Laz include cells outside the nervous system. GLaz is expressed in mesodermal cells, gut epithelium, and salivary glands primordia during embryogenesis [24], and in adult hemocytes [25]. NLaz is expressed in developing mesodermal cells and sessile cells in the gut lumen [24], and in the developing and adult fat body [24,26]. Finally grasshopper Laz is expressed in cells at the tip of the Malpighian tubules, nephrocytes of the subesophageal body, and in a group of mesodermal cells that might be the precursors of sessile hemocytes, nephrocytes, or fat body cells [2]. Thus, systemic effects of Lipocalins might be part of their longevity-regulation and stress-protection functions.

Interestingly, when expressed in hemocytes, grasshopper Laz has no effect in starvation-desiccation resistance (Figure S4 B). However, flies expressing Laz in the fat body increase their resistance to both starvation-desiccation and hyperoxia stress (Figure S5 A-B and Table 1). Iron exposure appears to be deleterious for flies overexpressing Laz in fat-body (Figure S5 C).

Expression of NLaz in the fat body is triggered by the JNK signaling pathway and promotes resistance to starvation and oxidative stress [26]. Since native NLaz is a secreted protein, we have proposed NLaz as an important component of the systemic control of metabolism necessary under stress conditions. In support of that idea, expression of NLaz in pericardial cells or hemocytes also protect against a starvation-desiccation stress [26]. On the other hand, GLaz is not expressed by fat body cells, but controls fat storage in these cells [25], probably acting at a distance once secreted to the hemolymph by hemocytes. Our results show that the GPI-linked grasshopper Laz protects against stress when expressed in fat body cells (Figure S5 and Table 1), but not in hemocytes (Figure S4 B and Table 1), suggesting that not only systemic effects, but also local effects of these Lipocalins in the fat body tissue are important for stress resistance.

3.6. Lazarillo protects flies against different forms of stress in a sex dependent manner.

Using loss-of-function mutants we have previously shown that (i) lifespan extension by GLaz is sex dependent, (ii) NLaz and GLaz expression change in opposite directions (only in females) upon aging, and (iii) the relative contribution of head and body in Lipocalin expression also varies with sex [27]. A multivariate analysis uncovered that differences in metabolic parameters, particularly protein and relative water content, account for the observed sex-specific patterns of longevity.

Upon different types of stress, grasshopper Laz also shows sex-dependent effects. When expressed by the Ddc driver, resistance to starvation-

desiccation stress is promoted only in females (Figure S6 A), resistance to hyperoxia is promoted in both sexes (Figure S6 B), and resistance to iron exposure is slightly enhanced in females and decreased in males (Figure S6 C). Some of these differences could be explained by functional differences of Laz in the Ddc cuticular domain of expression. Since epidermal DA production is triggered in stress situations [58,62] and Ddc functions downstream of the stress-responsive JNK signaling cascade upon a septic cuticle injury [61], a contribution to epidermal Laz expression might not be negligible under the stress situations used in our experiments. DA production is particularly important in females, where it controls the last biochemical steps toward the production of female-specific long-chain hydrocarbons with

pheromonal functions [60]. Although a quantitative profile of Ddc expression in the epidermis has not been reported yet, our data are compatible with a significant expression of membrane-bound Laz expression in female epidermis (driven by the Ddc promoter) as a putative cause for the sex-specific effects in starvation-desiccation resistance.

Similarly, females expressing Laz in the nervous system and muscles (109-GAL4 driver) show a larger resistance to starvation-desiccation than males, but neuronal Laz (31-1 or D42 drivers) shows larger protective effects in males than in females (Table 1).

Interestingly, overexpression of grasshopper Laz extends lifespan in both males and females (Figure 3 and Table 1).
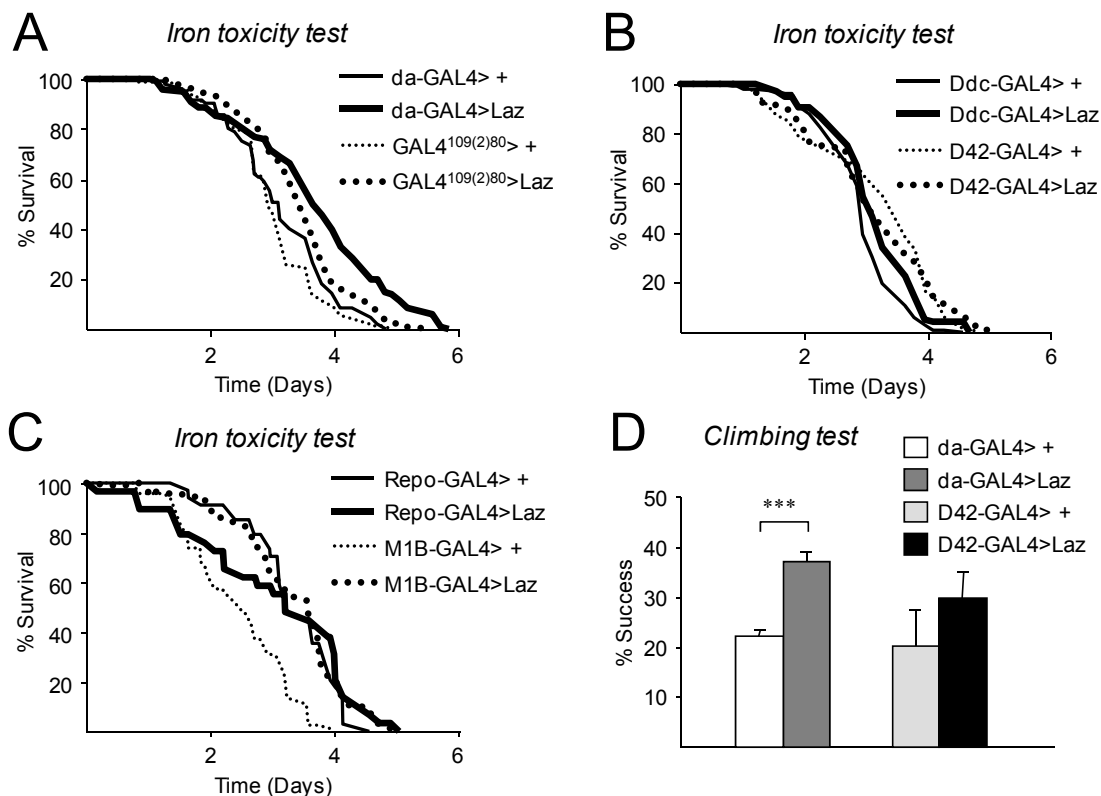

Figure 5. Expression of Lazarillo improves survival and locomotor behavior of flies exposed to iron.
(A) Wide range expression of Lazarillo (da-GAL4>Laz or GAL4$^{109(2)80}$>Laz) increase median survival upon FeSO$_4$ treatment by 17 and 24.1% (Log-rank test p<0.0005). **(B)** A small increase in median lifespan (5.3%; Log-rank test: p<0.05) is observed when Lazarillo is expressed by dopaminergic and serotonergic neurons and the epidermis of flies exposed to FeSO$_4$. In contrast, D42-GAL4>Laz flies show no change in median survival. **(C)** Expression in glial cells with the repo-GAL4 driver does not modify fly resistance to iron intoxication. However, M1B-GAL4>Laz flies significantly increase their survival 42.3% (Log-rank test: p<0.0005). **(D)** Climbing ability was assayed after 72h of iron exposure in ubiquitous and motoneuron overexpressor flies. Only ubiquitous expression of grasshopper Laz improves fly climbing ability after iron exposure (Student's T-test: p<0.0005). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.
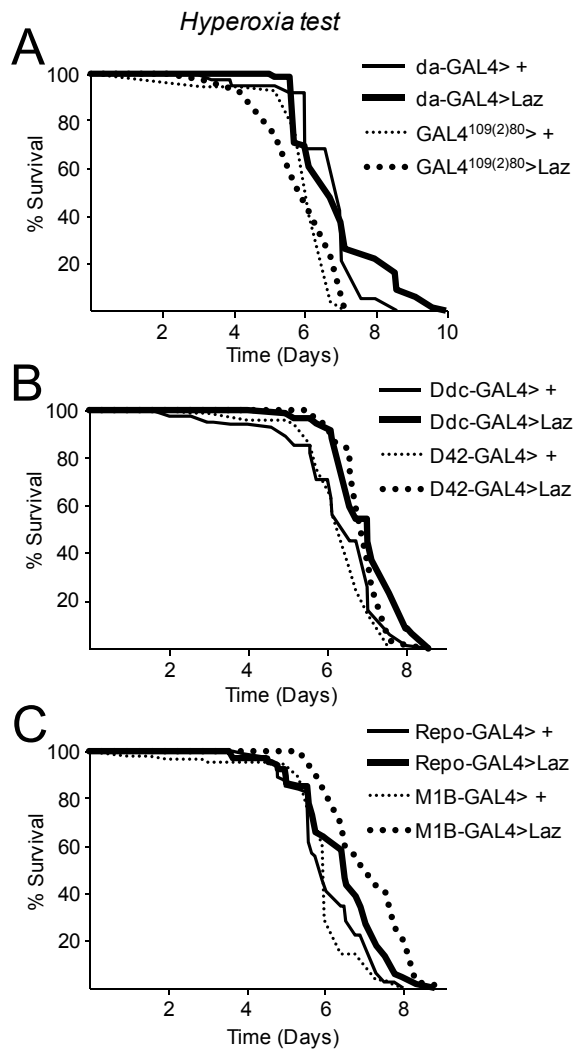
Figure 6. Grasshopper Laz protects against hyperoxia in a cell-type dependent manner.
(A) Wide range expression of Lazarillo (da-GAL4>Laz or GAL4$^{109(2)80}$>Laz) does not modify median survival upon hyperoxia. **(B)** The expression of Laz in dopaminergic and serotonergic neurons and epidermis (Ddc-GAL4>Laz) or in motoneurons (D42-GAL4>Laz) increases slightly their median survival: 7.1 and 6.8% (Log-rank test: p<0.0005 and p<0.005 respectively). **(C)** When Lazarillo is expressed in glial cells using M1B-GAL4 driver, it produces a significant survival expansion in female flies exposed to hyperoxia: 22% increase in median survival (Log-rank test: p<0.0005). Conversely, repo-GAL4>Laz flies show a milder increase (9.3%) in median-survival (Log-rank test: p<0.05). Female data is shown in all panels. The complete data for both sexes is shown in Table 1.

3.7. Grasshopper Lazarillo rescues stress-sensitivity and premature AGEs accumulation in NLaz null mutant flies.

At least two arthropodan Lipocalins are predicted to be the minimal ancestral set of Lipocalins [63]. However, awaiting full genome sequencing, no other grasshopper Lipocalins have been reported so far. Among the Drosophila Lipocalins, NLaz and grasshopper Laz show many shared properties, in addition to their neuronal expression domain. They both have higher % similarity in protein sequence [9] and intron-exon structure similarity index [14] than the Laz-GLaz pair. Also NLaz, but not GLaz, bears an extended C-terminal segment similar in size to the GPI-anchoring signal of Laz [13] that could represent a vestigial NLaz tail (Figure 9).

We therefore set to examine whether the ancient Laz is able to rescue the phenotypes caused by the loss of NLaz. The NLaz loss-of-function mutation is known to reduce longevity, and to increase the flies sensitivity to oxidative and starvation-desiccation stress [26,27]. Ubiquitous expression of the GPI-linked grasshopper Laz (da-GAL4 driver) is in fact able to restore stress resistance to or above control levels, both under starvation-desiccation stress and iron induced oxidative stress (Figure 7A-B). Median survival increases by 71.7% and 83.2% respectively with respect to control flies.

A slight increase in GLaz mRNA expression (1.6 fold increase) is observed when NLaz is absent (Figure 7C). As it has been described for the vertebrate homologue ApoD in the mouse [32-34,64], without additional stress the absence of NLaz might cause an imbalance in tissue homeostasis, generating a basal level of stress. This in turn might cause the observed slight GLaz up-regulation. Since both proteins are secreted within and outside the nervous system, though from different cell types, they might be functionally compensating for each other. However, GLaz expression is equivalent to control levels when membrane-linked grasshopper Laz is expressed with the da-GAL4 driver. Therefore, the rescue of NLaz null mutation by grasshopper Laz cannot be ascribed to functional compensation by GLaz. These GLaz expression changes, together with those observed when grasshopper Laz is

expressed in wild type flies (Figure 2D), are indicative of the existence of stochastic small variations in the expression levels for GLaz. Whether they represent biologically relevant variations would need further study.

During normal aging, reducing sugars react nonenzymatically with proteins or nucleic acids forming advanced glycation end-products (AGEs) that accumulate within cells. A 40% increase in AGEs has been reported in Drosophila between 10 and 75 days of age [50]. Fluorescent AGEs are reliable biomarkers of aging-related damage. They track the effects of factors that condition mortality rates (e.g., temperature in the case of ectothermic animals like Drosophila), but are irreversible and thus not affected by other potentially reversing factors like dietary restriction [65]. If the lack of NLaz does impose a basal stress in the tissues, we should expect an accelerated aging occurring in the null mutant flies. This hypothesis is confirmed, since young 3-5 day old NLaz mutant flies exhibit 31.6% more fluorescent AGEs than control flies (Fig. 7D). Interestingly, the ubiquitous expression of grasshopper Laz fully rescues the acceleration of aging in mutant NLaz flies (Fig. 7D). The beneficial action of grasshopper Laz therefore must prevent age-related damage to occur.
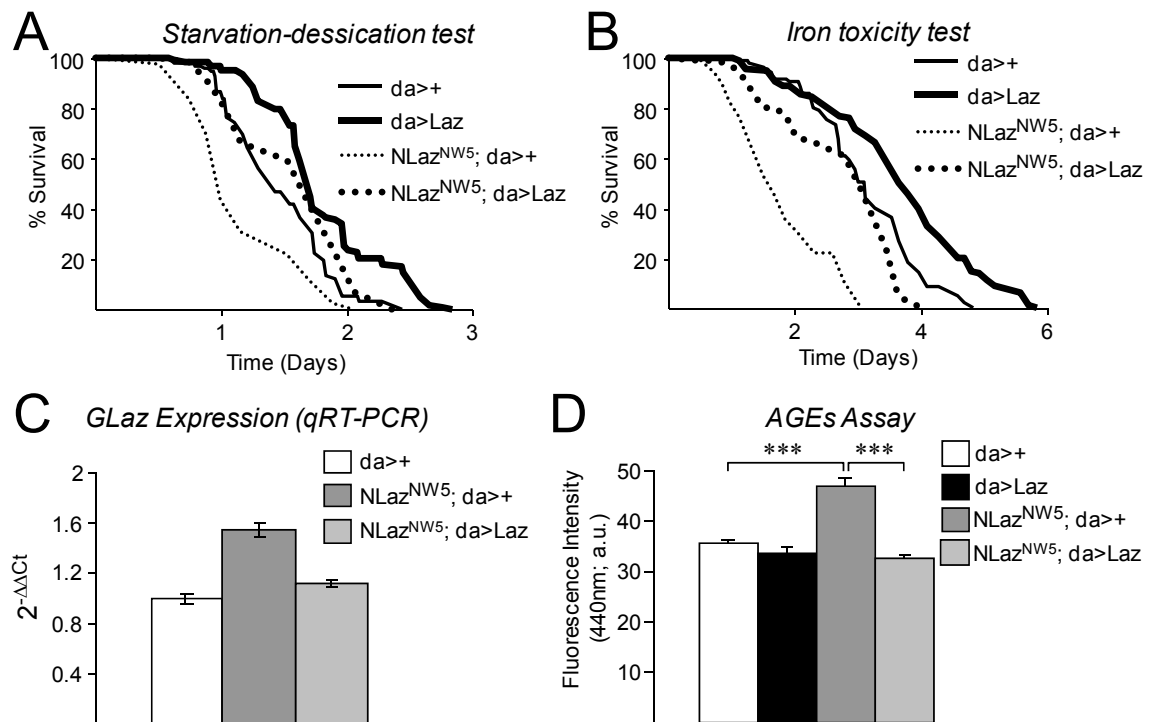

Figure 7. Grasshopper Lazarillo expression rescues NLaz null mutant flies from stress-sensitivity and accelerated-aging phenotypes.
(A, B) Homozygous NLaz[NW5] flies are more sensitive to starvation-desiccation and iron exposure than control flies. Ubiquitous expression of grasshopper Laz returns NLaz null mutant flies to or above control levels. Median survival increases by 71.7% (NLaz [NW5]; da-GAL4>+ versus NLaz [NW5]; da-GAL4>Laz) in the starvation-desiccation test and 83.2% in the iron toxicity assay (Log-rank test: $p < 0.0005$ in both cases). Female data is shown. The complete data for both sexes is shown in Table 1. **(C)** GLaz transcript levels slightly increase in homozygous NLaz[NW5] flies (da-GAL4>+ *versus* NLaz [NW5]; da-GAL4>+). Overexpression of grasshopper Laz returns GLaz expression back to control levels (NLaz [NW5];da-GAL4>+ *versus* NLaz[NW5];da-GAL4>Laz flies). **(D)** Advanced glycation end products (AGEs), which normally accumulate upon aging, are basally increased in NLaz null mutant young female flies reflecting an accelerated aging process. Grasshopper Laz expression rescues this phenotype completely (Student's t-Test, $p < 0.0005$ in both cases).

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Table 1. Summary of the survival parameters and behavioral scores obtained with different experimental paradigms in flies of different sex and genotypes

Background	Driver (*main site of expression*)	N	Sex	Experiment	Median Survival or Climbing Success		Maximal Survival	
					Sig.	% Change	Sig.	% Change
WT		266-418	♀	Longevity	***	**28.302**	***	**21.413**
WT		239-294	♂	Longevity	***	8.475	**	9.962
WT		93-123	♀	Desiccation	***	**25.806**	***	**24.184**
WT		105-144	♂	Desiccation	***	**23.112**	***	10.677
WT		38-69	♀	Hyperoxia	ns	-3.736	*	15.926
WT		45-63	♂	Hyperoxia	ns	0.000	ns	-0.925
WT	Da (*Ubiquitous*)	105-116	♀	Iron	***	17.056	***	**21.210**
WT		99-100	♂	Iron	***	**28.205**	*	5.370
WT		42-77	♀	Climbing (72h)	***	67.532		
WT		15-40	♀	Climbing (96h)	*	275.00		
WT		24-61	♂	Climbing (48h)	*	16.065		
NLaz^{NW5}		87-118	♀	Desiccation	***	**71.678**	***	17.098
NLaz^{NW5}		85-119	♂	Desiccation	***	**45.038**	***	**23.149**
NLaz^{NW5}		68-110	♀	Iron	***	**83.167**	***	**23.811**
NLaz^{NW5}		62-112	♂	Iron	***	**94.130**	***	**31.214**
WT		171-286	♀	Longevity	***	**43.548**	***	**40.937**
WT		136-294	♂	Longevity	***	**32.727**	***	**32.455**
WT		89-140	♀	Desiccation	***	**30.055**	***	**20.732**
WT		79-138	♂	Desiccation	ns	3.333	ns	4.409
WT	109 (*CNS / Muscle*)	43-55	♀	Hyperoxia	ns	0.000	ns	3.577
WT		27-42	♂	Hyperoxia	***	-12.859	ns	-9.266
WT		90-105	♀	Iron	***	**24.113**	**	12.789
WT		80-97	♂	Iron	*	0.000	**	7.230
WT		24-73	♀	Climbing (72h)	ns	-7.347		
WT		24-43	♂	Climbing (48h)	ns	-17.395		
WT	31-1 (*Neurons*)	68-95	♀	Desiccation	*	5.849	***	**35.647**
WT		61-79	♂	Desiccation	*	14.815	***	**20.192**
WT		117-119	♀	Longevity	***	**31.479**	***	13.410
WT		118-121	♂	Longevity	***	**30.000**	***	11.753
WT		233-289	♀	Desiccation	***	**37.903**	***	**21.689**
WT	Ddc (*Dopaminergic and Serotonergic Neurons*)	262-312	♂	Desiccation	ns	2.105	ns	6.748
WT		82-94	♀	Hyperoxia	***	7.154	*	6.412
WT		86-98	♂	Hyperoxia	***	**23.680**	***	**24.910**
WT		97-102	♀	Iron	*	5.282	*	7.091
WT		94-101	♂	Iron	***	-14.450	*	-3.725
WT		40-54	♀	Climbing (72h)	ns	-1.234		
WT		23-59	♂	Climbing (48h)	*	-45.735		

Background	Driver (*main site of expression*)	N	Sex	Experiment	Median Survival or Climbing Success		Maximal Survival	
					Sig.	% Change	Sig.	% Change
WT		93-100	♀	Desiccation	*	-1.990	***	-20.659
WT		80-95	♂	Desiccation	**	4.444	**	**20.815**
WT		69-86	♀	Hyperoxia	**	6.772	ns	2.785
WT	D42	87-96	♂	Hyperoxia	ns	7.155	**	-1.509
WT	(*Motoneurons*)	81-93	♀	Iron	ns	-14.571	*	5.864
WT		72-85	♂	Iron	ns	0.000	***	-23.170
WT		59-61	♀	Climbing (72h)	ns	47.766		
WT		8-31	♂	Climbing (48h)	ns	-55.288		
WT		87-109	♀	Desiccation	***	**23.980**	***	**21.809**
WT		62-74	♂	Desiccation	**	10.484	**	**41.286**
WT	Repo	67-81	♀	Hyperoxia	*	9.282	*	7.018
WT	(*Glial cells*)	57-88	♂	Hyperoxia	***	-23.077	**	-9.964
WT		29-34	♀	Iron	ns	-1.29	ns	11.745
WT		13-57	♂	Iron	ns	0.000	ns	7.831
WT		84-91	♀	Desiccation	***	**38.298**	***	**28.791**
WT		83	♂	Desiccation	**	**23.256**	**	7.483
WT	M1B	81-90	♀	Hyperoxia	***	**22.049**	***	12.899
WT	(*Glial cells*)	83-91	♂	Hyperoxia	***	7.618	**	7.028
WT		73-93	♀	Iron	***	**42.324**	***	**28.193**
WT		48-51	♂	Iron	ns	0.000	ns	4.141
WT	He	82-112	♀	Desiccation	ns	-3.468	ns	1.692
WT	(*Hemocytes*)	81-87	♂	Desiccation	ns	0.000	*	2.688
WT		77-89	♀	Desiccation	***	**21.212**	***	**22.865**
WT		81-83	♂	Desiccation	***	18.868	***	**20.139**
WT	ppl	81-99	♀	Hyperoxia	***	13.117	**	12.258
WT	(*Fat body*)	79-94	♂	Hyperoxia	***	7.877	***	**25.376**
WT		81-93	♀	Iron	***	-8.450	ns	-1.679
WT		82-94	♂	Iron	***	-25.535	*	-8.242

Values in bold indicate changes in median or maximal survival larger than 20%. Survival curves differences tested with Log-rank test. Climbing success differences tested with Student's T-test. $p < 0.05$ (*); $p < 0.005$ (**); $p < 0.0005$ (***).

3.8. Courtship behavior and cuticular sex pheromones are not altered by Lazarillo expression.

NLaz not only modulates longevity and regulates the metabolic response to stress [26,27], but also participates in fly mating behavior [27]. Loss of NLaz leads to deficiencies in courtship that are dependent on functional alterations in both sexes, since both wild type males paired with mutant females and mutant males paired with wild type females are less involved in courting. The courtship index decreases to even lower levels when both flies are NLaz null mutants [27]. Here we show that a ubiquitous overexpression of Laz is not able to rescue the courtship defects produced by the lack of NLaz (Figure 8A), indicating that Lazarillo is not able to substitute NLaz in all of its functions.

We have proposed that courtship defects in NLaz mutants might be due to its role as a transporter of pheromones either at the emission or reception level. As mentioned above, the main sex-pheromones in insects are cuticular long-chain unsaturated hydrocarbons (HC) synthesized from fatty acid precursors in epidermal cells. They, together with visual and acoustic cues, play an important role in male courtship behavior [46]. Interestingly, HC are also important factors modulating survival upon desiccation. A causal association between HC composition, their resulting global melting point and desiccation survival has been well documented [66,67].

We assayed the profile of HC in Laz overexpressing flies, comparing global expression (da-GAL4 driver) with nervous system enriched expression (Ddc-GAL4 driver). In no case the expression of Laz produced major changes in the profile of HC in males or females (Tables S1 and S2). The total amount of HC (between 23 and 29 carbons), calculated for da, and Ddc-driven expression of Laz, also showed no significant differences (Figure 8B and Table 2).

3.9. Neutral fat stores are promoted by Lazarillo.

Since Laz does not change the profiles or total amounts of HC, they cannot account for its ability to increase starvation-desiccation resistance in flies. We therefore tested whether greater metabolic stores, particularly fat stores, could be the mechanism by which flies can better resist the lack of nutrients and water in our starvation-desiccation test.

A significant increase in total triglycerides content is actually observed when grasshopper Laz is expressed ubiquitously (da-GAL4 driver) or in dopaminergic cells (Ddc-GAL4 driver) both in males and females (Figure 8C and Table 2). However, the effect is not attained when Laz is expressed in hemocytes (He-GAL4 driver).

Opposite to Laz overexpression, NLaz and GLaz null mutant adults exhibit reduced triglycerides stores and are sensitive to diverse forms of starvation [25,26]. Also, the fat body tissue is markedly reduced in GLaz mutants [25]. Our results support a model in which the control of fat storage by Lipocalins is a conserved ancestral function, promoting both starvation and desiccation resistance.

3.10. Tissue specificity and range of action of Lazarillo related Lipocalins: Contributions to the control of the aging process.

The ubiquitous expression of grasshopper Laz, anchored to cell membranes, is able to accomplish many functions. In wild type background it promotes lifespan extension, decreases starvation-desiccation sensitivity, and improves survival and locomotor activity upon iron treatment. In addition it decreases AGEs formation in the accelerated-aging NLaz mutant background, and rescues its sensitivity to starvation-desiccation and oxidative stress.

Some of these functions are also possible when grasshopper Laz is expressed by subsets of cells, within or outside the nervous system. We have reasoned above that the sex-independent longevity extension obtained with the Ddc-GAL4 driver could be mostly dependent on the neuronal expression, while the female-specific resistance to starvation-desiccation stress is better explained by the epidermal expression of Laz. On the other hand, the results obtained with restricted patterns of expression (e.g. hemocytes or fat body) clearly show that Laz can perform its protection against
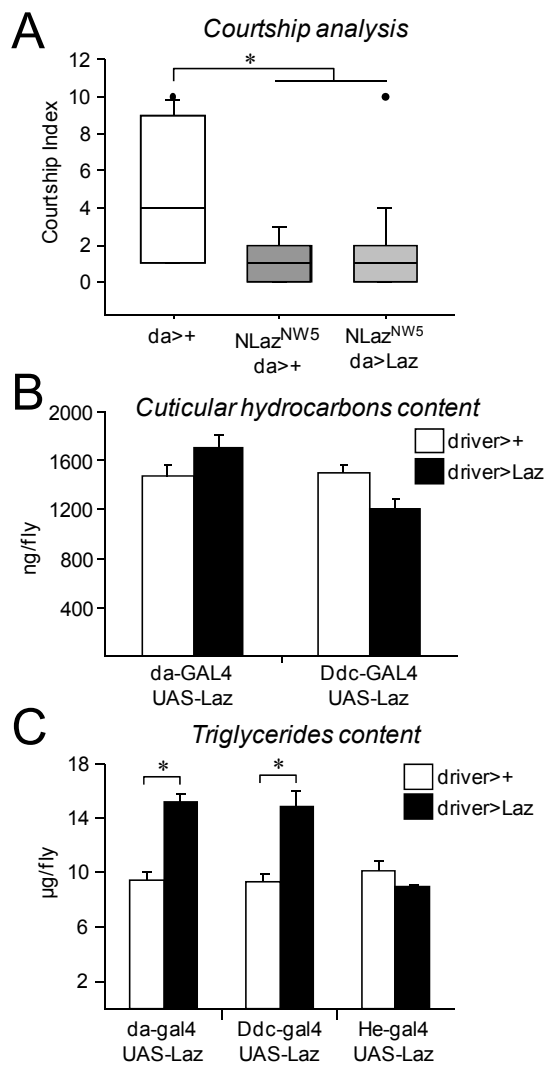
A

Courtship analysis

B

Cuticular hydrocarbons content

C

Triglycerides content

Figure 8. Ubiquitous expression of Lazarillo promotes fat storage, but does not alter phenotypes related to pheromonal signaling.
(A) Homozygous NLaz[NW5] flies show a decreased courtship index and expression of Lazarillo under the control of da-GAL4 does not rescue this phenotype. **(B)** Total amounts of cuticular hydrocarbons are not altered by Laz expression neither ubiquitously nor when expressed in a nervous system enriched pattern (Ddc-GAL4>Laz). **(C)** Triglyceride contents increase in flies overexpressing Laz with da-GAL4 or Ddc-GAL4 drivers. Laz has no effect on fat storage when expressed only in hemocytes (He-GAL4>Laz). (* Student's T-test: p<0.05). Female data is shown in all panels. The complete data for both sexes is shown in Table 2.

starvation-desiccation when locally expressed in the fat body and not in hemocytes, which contrasts with the long-range actions of the secreted GLaz or NLaz homologs [25,26]. We have shown that secreted forms of grasshopper Laz are also able to protect insect S2 cells in culture when exposed to oxidative stress, both in an autocrine and a paracrine manner. Thus, Lazarillo has the potential of acting both, from the extracellular space or linked to the cell membrane. Interactions of the secreted GLaz and NLaz with cell membranes upon which they exert their protective functions are predicted as a result.

The restricted neuronal expression of the 31-1-GAL4 driver (similar to the embryonic expression pattern of grasshopper Laz) consistently, though moderately, improves survival upon starvation-desiccation in a sex-independent manner. Glial restricted expression of Laz does cause large survival increases upon different forms of stress, reflecting the wider range of protective functions exerted by glial cells. As we have shown for mouse ApoD [33], protecting glial cells might represent a beneficial strategy to maintain the nervous system homeostasis and ultimately improve the whole organism survival. Our results with grasshopper Laz are in agreement with the protecting effects of glial-derived ApoD on oxidation-challenged dopaminergic neurons in the mouse [33], as well as global effects on survival upon systemic oxidative stress [32].

3.11. Ancestral vs. derived functions of the Lazarillo-related Lipocalins. Is the GPI-linked grasshopper Lazarillo an evolutionary rarity?

We have shown that grasshopper Laz is able to perform protective functions when expressed in a variety of patterns. Moreover, a ubiquitous expression of Laz rescues NLaz null mutants from stress sensitivity and accelerated aging phenotypes. However, functional equivalence between grasshopper Laz and Drosophila NLaz is not all-inclusive since deficient courtship phenotypes of NLaz null mutants are not rescued. A divergent evolution of hydrophobic ligand binding specificities could account for the differences between grasshopper Laz and NLaz.

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Table 2. Total hydrocarbon and triglyceride content in Laz over-expressing vs. control flies.

	Control males	*Laz males*	*Control females*	*Laz females*
Hydrocarbons (ng/fly)				
da-GAL4 x UAS-Laz	1474 ± 203	1207 ± 71	1471 ± 90	1704 ± 104
Ddc-GAL4 x UAS-Laz	1289 ± 66	1186 ± 65	1499 ± 60	1207 ± 73
Triglycerides (µg/fly)				
da-GAL4 x UAS-Laz	7.04 ± 0.25	**10.77 ± 0.3[a]**	**9.48 ± 0.8[b]**	**14.9 ± 0.3[a]**
Ddc-GAL4 x UAS-Laz	7.03 ± 0.23	**8.06 ± 0.49[a]**	**9.41 ± 0.44[b]**	**14.91 ± 1.09[a]**
He-GAL4 x UAS-Laz	7.04 ± 0.20	6.62 ± 0.25	10.12 ± 0.70	9.00 ± 0.13

Average ± SEM is represented. Values in bold and shaded in gray indicate significant differences assayed by Student's T-test.
[a] Difference between Laz overexpression and driver-only control $(p < 0.05)$.
[b] Difference between females and males of the same genotype $(p < 0.05)$.

A

```
Same.Laz    1   -QETMG-CADRTAINDENATLYMGKWYEYAKMGSMPYEEGGVCVTAEYSMSSN--NITVV  56
Aaeg.Lip    1   -INVQGPCRDLPVENQFNITQYMGTWYEIKRYENE-YQPNGDCVTAQYTLNSTTMEVTVE  58
Cqui.Lip    1   -LDVAGPCRTLPVVQSEDVTQYMGMWYEIQRYPHR-NQPNGDCVVNYTLNQTTGEVAIL  58
Dmel.NLaz   1   QVPFPGKCPDVKLLDTEDAEAYMGVWYEYAAYPFA-FEIGKKCIYANYSLIDN-STVSVV  58

Same.Laz   57   NSMKDNTTHEVNTTTGWAEFASELHTDGKLSVHFPNSPSVG---NYWILSTDYDNYSIVW  113
Aaeg.Lip   59   NTMKKLPDQKPSVARGRAVLAHPTSGEAKLLVRFESTPEAAPSSIYWVLKTDYQKYAVVW  118
Cqui.Lip   59   NRMRVIADDATVEARGTAVTDPDYPGQGVLRVRFEETPPEVPASYYRILGVVYDRYAVVW  118
Dmel.NLaz  59   NAAINRFTGQPSNVTG----QAKVLGPGQLAVAFYPTQPLT-KANYLVLGTDYESYAVVY  113

Same.Laz  114   SCVKRPDSAASTEISWILLRSRNSSNMTLERVEDELKNLQLDLNKYTKTEQS--AKYCAG  171
Aaeg.Lip  119   SCHA--VGENSTESAWILSRSPVMEQASEVVVENLIK-QHLKPESFRNTKQG--DEFCSG  173
Cqui.Lip  119   SCRQ--NGANSVESAWVLSRAPTLEGVSVSNVKAIID-QHLDQESFTITKQG--EQYCGG  173
Dmel.NLaz 114   SCTS-VTPLANFKIVWILTRQREPSAEAVDAARKILEDNDVSQAFLIDTVQKNCPRLDGN  172

Same.Laz  172   AEHVVGA----MLSVAIASLFALLH--  192
Aaeg.Lip  174   APAAKIS----CVLLILFVLLICKKS-  195
Cqui.Lip  174   AAALTTS----IVVFILSVFVMLQSNN  196
Dmel.NLaz 173   GTGLAGEDGLDVDDFVSTTVPNAIEKA  199
```
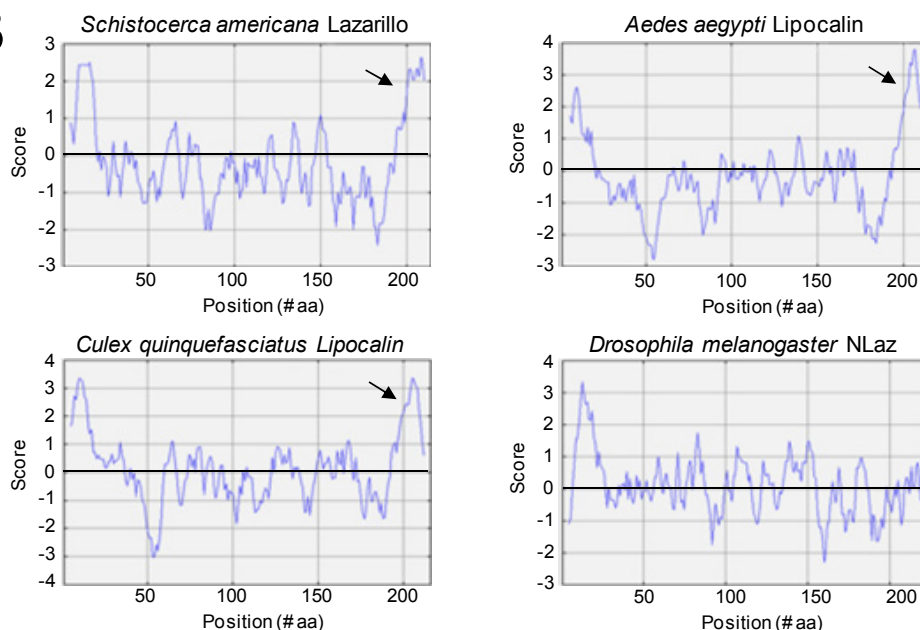
B

Schistocerca americana Lazarillo

Aedes aegypti Lipocalin

Culex quinquefasciatus Lipocalin

Drosophila melanogaster NLaz

Figure 9. **GPI-linkage is a shared character in Orthoptera and Diptera Lipocalins.**
(A) Multiple sequence alignment of Lazarillo (Same.Laz), NLaz (Dmel.NLaz) and the two predicted GPI-linked Lipocalins from the mosquitoes *Aedes aegypsi* (Aaeg.Lip) and *Culex quinquefasciatus* (Cqui.Lip). Sequences were aligned with CLUSTAL-X2. Black and gray boxes show residue identities and similarities respectively. Potential GPI cleavage sites for Aaeg.Lip and Cqui.Lip were predicted by the big-PI Predictor software and are highlighted with an asterisk. They align with the experimentally demonstrated GPI-signal peptide cleavage site of Same.Laz. (B) Hydrophobicity plot of the Lipocalin protein sequences determined by Kyte and Doolittle's method using a window of 9 residues. The hydrophobic domains of the GPI-anchoring signal are indicated by arrows.

These results strongly support the hypothesis that some functions (e.g., lipid storage control, protection against oxidative stress, and oxidative age-related damage), all of them impacting on lifespan regulation, represent the ancestral suite of functions for this group of Lipocalins. The Lazarillo-related Lipocalins include ApoD, which also extends lifespan and protects against oxidative stress in the fly [35]. Other functions, such as the specific signaling required for proper courtship, might be part of the autapomorphic characters evolved independently in different lineages, like the NLaz-dependent courtship behavior in Drosophila, or the axillary odorant binding by human ApoD [68].

The features currently present in the extant Drosophila Lipocalins might be the result of a gene duplication occurring before the divergence of Orthoptera and Diptera. As mentioned above this would predict the existence of a GLaz-like gene in grasshoppers, but it also predicts that more Lipocalins derived from a GPI-linked ancestor might exist in insects. Thanks to the genome sequencing of diverse insect species, we were able to identify two Laz-like mosquito Lipocalins with an extended C-terminal segment that is not only similar in size to the GPI-anchoring signal of Laz, but also has the characteristic hydrophobicity profile and predicted cleavage site (Figure 9). This finding strongly supports the hypothesis of an ancestral GPI-signal peptide as the origin of the vestigial NLaz C-terminal tail, and that insects have exploited the functional possibilities provided by GPI-linked Lipocalins in more lineages than previously thought.

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Supplementary Information
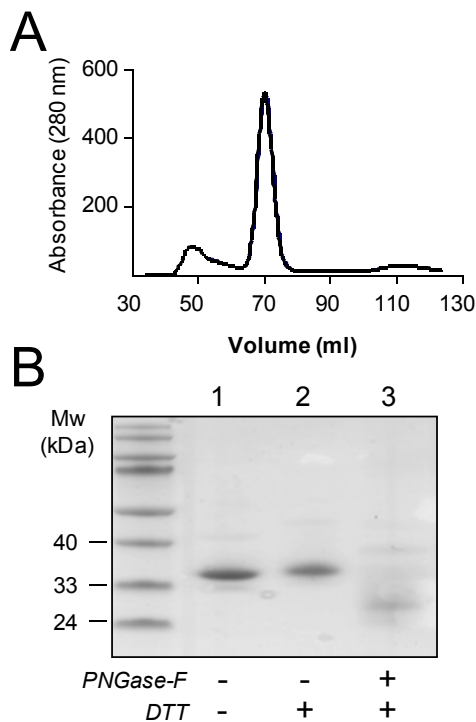
Figure S1. Expression and purification of grasshopper Lazarillo from S2 cells.

(A) Size-exclusion chromatography elution profile of Laz enriched extracts obtained after metal affinity chromatography of culture medium harvested from stably transfected S2 cells. The main peak eluting at 70 ml correspond to Laz protein (98% pure) secreted by the cells. **(B)** SDS-PAGE analysis of purified Laz protein. The redox state of the protein affects only slightly its electrophoretic mobility while a large shift in relative mobility is observed in Lazarillo after deglycosylation. Lane 1: No reducing agent added. Lane 2: The electrophoresis sample buffer contains DTT. Lane 3: Proteins treated with PNGase-F.
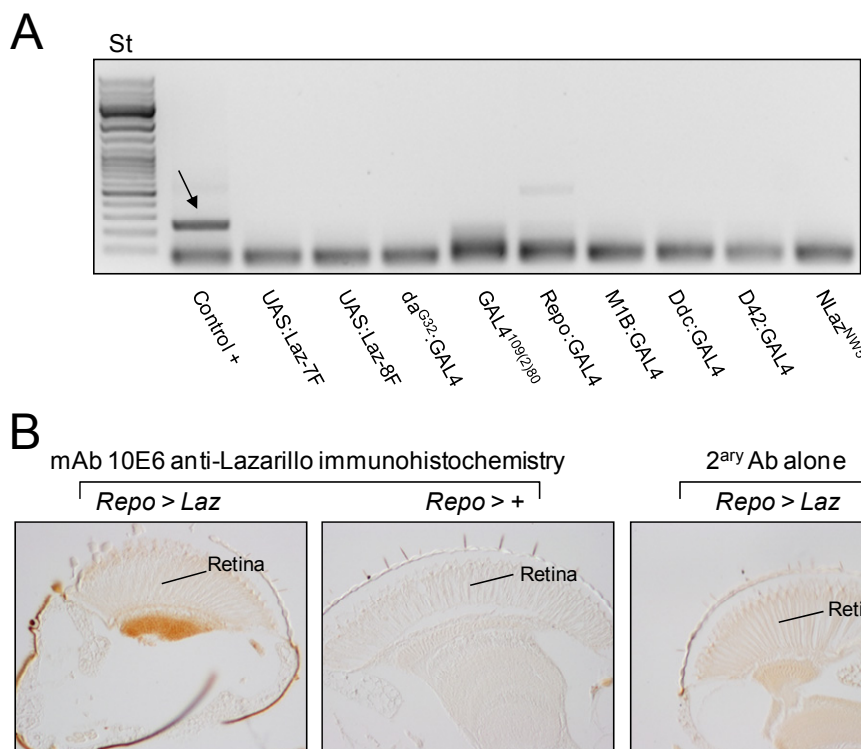
Figure S2.

(**A**) Fly lines used in this study are free of *Wolbachia* infection. PCR of genomic DNA extracts from drivers, responders and mutant fly lines used in this work. Primers against the rRNA-16S gene of *Wolbachia* endosymbiont were used. DNA from *Dirofilaria immitis* worms infected with *Wolbachia* is used as positive control. (**B**) Controls of MAb 10E6 specificity against grasshopper Laz. No labeling is detected in control flies not expressing Laz and the secondary antibody used does not yield any detectable signal either.
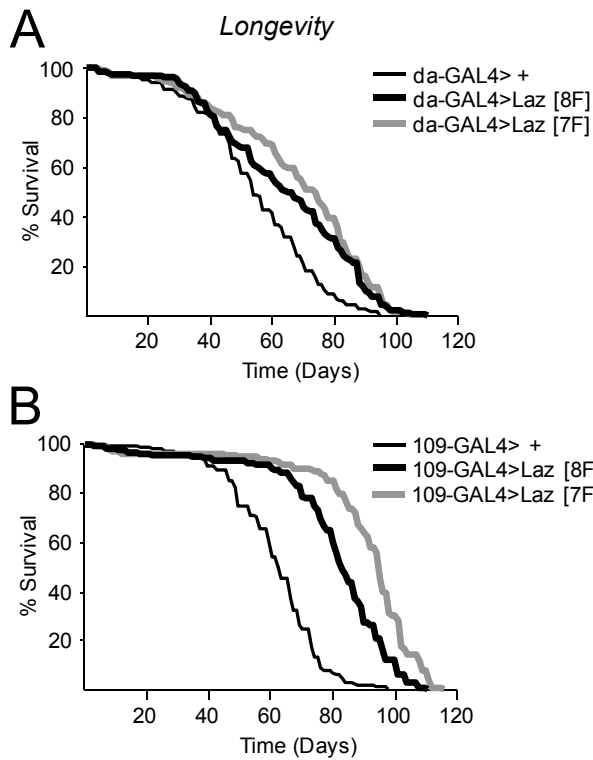
A *Longevity*

B

Figure S3. Overexpressing grasshopper Lazarillo using different transgenic lines extends lifespan.
(A-B) Two lines with independent insertions of the Laz gene in the *Drosophila* genome were tested (mapped to the 2nd and X chromosome). Drosophila lifespan increases when Laz is expressed from both transgenes using the ubiquitous da-GAL4 driver or the muscles and brain driver $GAL4^{109(2)80}$. Median survival time is increased by 25.65% and 17.17% in da-GAL4>Laz (7F) and da-GAL4>Laz (8F) respectively, compared to da-GAL4> + control flies, and 49.56% and 32.30% in $GAL4^{109(2)80}$>Laz (7F) and $GAL4^{109(2)80}$>Laz (8F) compared with the $GAL4^{109(2)80}$>+ control flies (Log-rank test: $p < 0.0005$ for all comparisons).

Starvation-desiccation test

A Males

B Females

C Males

D Females
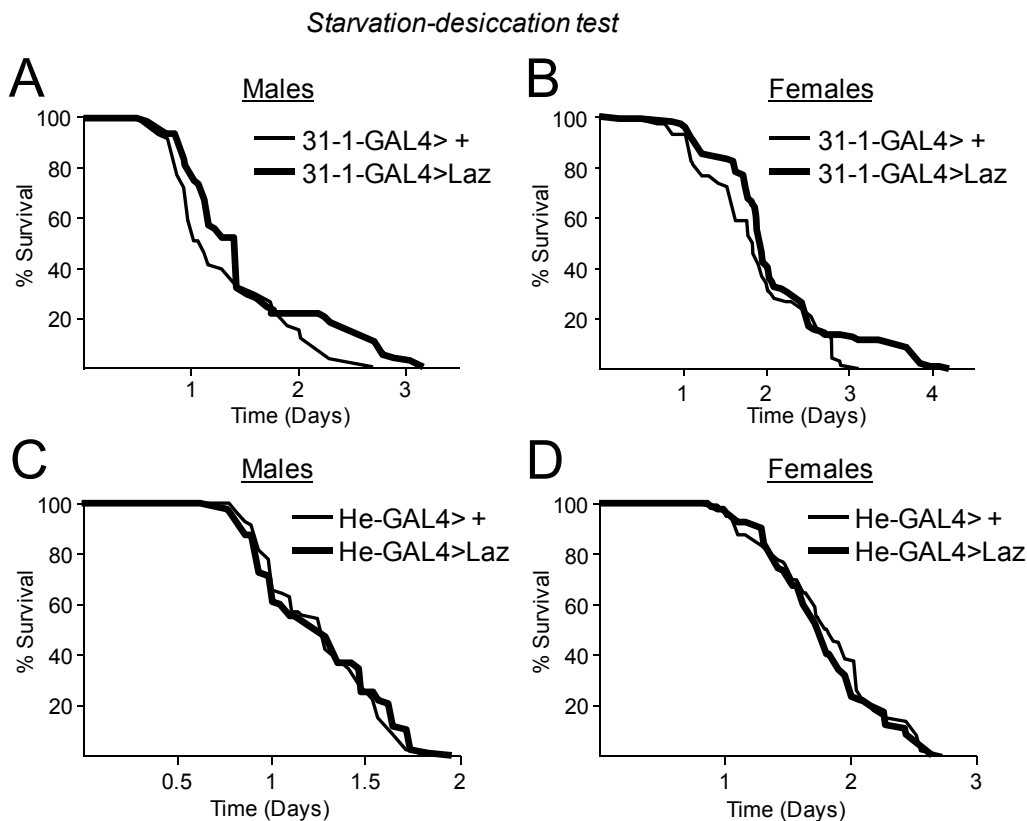
Figure S4. Expression of Lazarillo in restricted patterns within or outside the nervous system has different consequences on starvation-desiccation resistance.
(A,B) Pan-neuronal expression of grasshopper Laz under the control of 31-1-GAL4 produces a 5.8% extension of median survival in females and 14.8% in males (Log-rank test $p < 0.0005$ in both cases). **(C,D)** In contrast, when Lazarillo expression is targeted only outside the nervous system with He-GAL4 driver (expression by hemocytes) no significant changes were found in median or maximal survival in either sex.
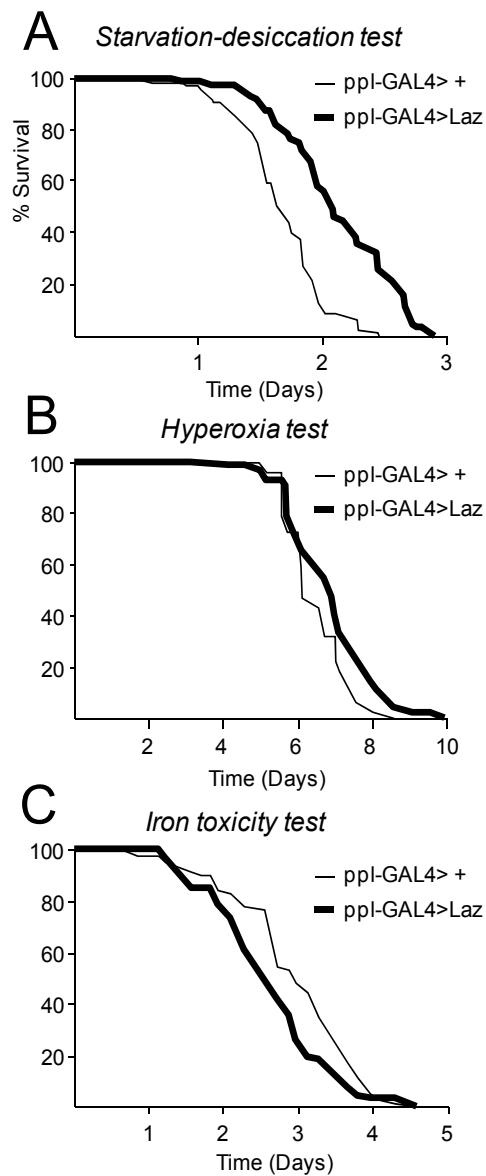
Figure S5. Local expression of the membrane-linked grasshopper Lazarillo in the fat body increases survival upon starvation-desiccation and hyperoxia stress but not upon iron treatment.

(A) Median survival upon starvation-desiccation stress increases by 21.2% in flies expressing Laz in the fat body (ppl-GAL4>Laz; log-rank test: $p<0.0005$). **(B)** Fat body expressed Lazarillo increases resistance against oxidative stress provoked by hyperoxia (13.1% median survival increase; log-rank test: $p<0.0005$). **(C)** Survival under iron treatment does not change significantly by expressing Laz in the fat body. Female data is shown in all panels. The complete data for both sexes is shown in Table-1.
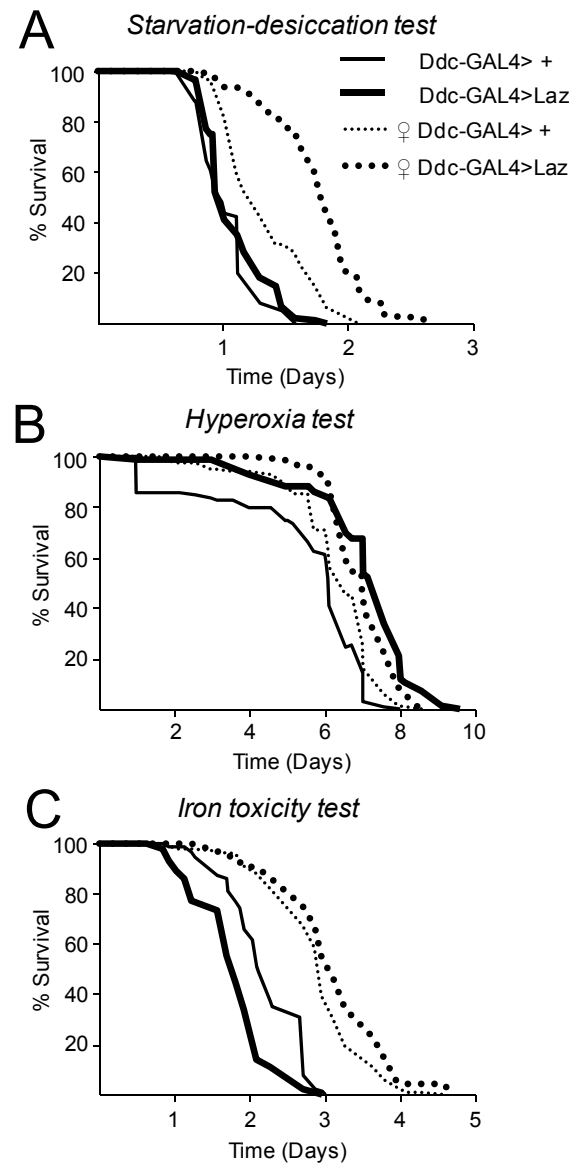
Figure S6. **Beneficial effects of Grasshopper Lazarillo are stress and sex-specific.**

(A) Expression of Lazarillo in dopaminergic and serotonergic neurons and in the epidermis increases resistance to starvation-desiccation in female but not in male flies (Log-rank test:$p<0.0005$ in females). **(B)** In contrast, both Ddc-GAL4>Laz females and males flies resist more under hyperoxia than control flies (Ddc-GAL4> +): 7.2% and 23.7% median increase respectively (Log-rank test: $p<0.0005$). **(C)** Ddc-GAL4>Laz female flies increase their resistance to iron exposure (Log-rank test: $p<0.05$), but males expressing Laz in the same pattern show a decrease of 14.5% in their median-survival (Log-rank test: $p<0.0005$).

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Discussion

General Discussion and Future Perspectives

In this thesis I have explored the physiological roles of Laz and Laz-related Lipocalins and their molecular mechanisms.

This work has confirmed and even broadened the functions in which Laz and Laz-related Lipocalins are involved. Among these functions, I would like to highlight their role in the process of aging.

Aging is defined as a gradual impairment of biological functions. Thus, it is a universal, deleterious and progressive process [1]. Aging is a complex and multi-factorial process that can be influenced by both, external and genetic factors. The expression levels of Laz and Laz-related Lipocalins is one of the critical genetic factors that determine not only lifespan, but also the quality of life upon aging, measured as behavioral outputs.

The contribution of GLaz, NLaz and ApoD to the aging process and to the protection against external stress has been extensively studied [2-8]. However, the results obtained in Chapter 4 have added grasshopper Laz to the aging-related group of Lipocalins. Especially interesting is the fact that Laz is able to increase fly lifespan and stress resistance as a protein attached to the membrane, reinforcing the idea that the role of ApoD, NLaz or GLaz could be associated with the plasma membrane. A tridimensional model of ApoD associated with the cell membrane is available at the OPM database [9]. Another interesting connection is that Laz expression in dopaminergic cells is enough to extent Drosophila lifespan, and dopaminergic cells are one of the most affected systems in ApoD-KO mice under oxidative stress [2]. These data suggest what can be considered a more general principle: that protecting dopaminergic cells is critical to slow down aging.

Surprisingly, in the absence of external stress, lifespan is not reduced in GLaz-KO female flies (Chapter1), but in the presence of metabolic or oxidative stress, their survival decreases dramatically. Thus, the lifespan of GLaz-KO flies might be also reduced in the absence of ideal living conditions, that is, in a real setting.

Moreover, the results presented in Chapter 3 make it possible to go one step further: A functional ligand-binding protein is required to achieve a proper response to oxidative or metabolic stress. The newly developed fly and cell lines expressing NLaz point-

mutants are useful tools for future research on NLaz biological functions and its molecular mechanisms. Likewise, solving the structures of NLaz (as apoprotein and as holoprotein in complex with different ligands) and of the NLaz[L130R] mutant would be critical to fully understand the ligand-binding interactions.

ApoD is the gene most up-regulated among those with conserved expression responses upon brain aging in mammals [10,11]. Additionally, ApoD transcription and protein levels are increased in the stroma of prostate during aging in mice [12]. It would be really interesting to analyze which tissues might require ApoD to slow down the aging process and to study what they have in common.

In the works presented in this thesis, control of the aging process and defense against oxidative stress have emerged as shared functions of Laz and Laz-related Lipocalins and these functions are probably part of the ancestral abilities of this protein family.

The protective properties of Laz and Laz-related Lipocalins have also been tested in cell-culture models upon oxidative stress (Chapters 3 and 4, and [2]). A protective mechanism has been reported for hApoD that involves binding of the oxidized lipid molecule and a detoxification reaction through Met93 [13,14]. This mechanism could be combined with the conventional hypothesis of binding and protecting easily oxidable compounds. Alternatively, binding to products of lipid peroxidation would stop peroxidative chain reactions. A mechanism relying on the binding of toxic products has been already described for other Lipocalins: OBP and TL bind 4-HNE [15,16] and ApoM binds oxidized phospholipids [17].

The ApoD residue Met93 is absent in the insect proteins Laz, NLaz and GLaz. Therefore, a catalytic mechanism is not expected. A non-enzymatic alternative mechanism may explain the anti-oxidative and pro-survival properties of the Laz-related insect Lipocalins.

Therefore, the group of mammalian Lipocalins with enzymatic activity (L-PGDS, α-1m and TL [18-20]) now includes ApoD. Enzymatic activity could be influenced by the presence of a second ligand. Indeed, in the chicken Lipocalin ExFABP the binding cavity is subdivided, with lysophosphatidic acid bound to the lower part, and siderophore-iron complexes bound to the upper part [21]. Given that the Met93 residue is located close to the rim of the binding pocket in ApoD, the situation described for L-PGDS can potentially have resemblances with that of ApoD. The lower part of L-PGDS

cavity is highly hydrophobic and can fit several different ligands, whereas the residue Cys65 catalyzes the isomerization of PD-H$_2$ in PD-D$_2$ in the upper part of the cavity [19].

The analysis of the binding properties of Laz, NLaz and hApoD (Chapter2) has opened a door to discover novel functions, and has also helped to explain phenotypes characterized in the null-mutants of mice and fly.

For instance, the selective binding of the pheromone 7-T by NLaz can explain the deficit in courtship behavior that NLaz-KO flies exhibit. 7-T increases Drosophila female sexual receptivity, making female flies to mate faster and more often with males with higher levels of 7-T, while low levels of 7-T in males reduce their courtship success [22]. NLaz can be acting as a soluble receptor transporting 7-T to sensory organs (Chapter 1 and 3). This proposal can also explain why grasshopper Laz, anchored to the plasma membrane, cannot rescue the courtship behavior deficits in NLaz null-mutants (Chapter 4).

The discovery of AEA binding to ApoD may implicate Lipocalins in the endocannabinoid system, suggesting new functions in the central nervous system. AEA binding to ApoD invites to think of other *N*-acylethanolamines (NAE) as potential ApoD ligands. OEA and PEA (oleoylethanolamine and palmitoylethanolamine) are known to regulate food intake and lipid metabolism, either by acting in the brain or in signaling from the gastrointestinal tract or the pancreas [23]. Moreover, low levels of NAE occur under dietary restriction, a condition that leads to lifespan extension [24]. The inhibitory action over the IIS pathway, known for NLaz and proposed for ApoD [5,25-27], could be acting synergically with a putative sequestration of NAE. This situation would create a 'state of dietary restriction' and, consequently, an extension of lifespan.

NLaz inhibits the IIS pathway at the PI3K level [5], but no one knows how. PI3K carries out the phosphorylation of PIP2 to PIP3 at the plasma membrane. Thus, modifications in the plasma membrane composition might lead to a different IIS/PI3K activity [28,29]. The ability of Laz and Laz-related Lipocalins to bind FA and SM (Chapter 2 and [30-32]), together with the reported capacity of hApoD to influence AA signaling [33], strongly suggest that NLaz inhibition of PI3K activity could be achieved through modifications of plasma membrane lipid composition. Additionally, it has been described that there is a progressive enhancement of neutral sphingomyelinase (SMase)

in the hippocampus of aged mice. SMase hydrolyses SM in phosphocholine and ceramide, being ceramide a pro-apoptotic molecule. This mechanism underlies neuronal death in the *substantia nigra* in Parkinson's disease [34], a tissue where Laz/ApoD protection is fundamental (Chapter 4 and [2]). Another connection between ApoD and SM is that, hApoD lipoparticles are enriched in SM [35]. Thus, Laz/ApoD may be binding SM and keeping it at the right level to control cell-death in age-sensitive neural circuits.

Therefore, Laz and Laz-related Lipocalins can control the IIS pathway and the process of aging, and can deal with oxidative stress through the management of a set of different hydrophobic ligands.

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Conclusions

Conclusions

This thesis demonstrates that:

1. GLaz and NLaz have both common and specialized functions that influence lifespan in a sex-dependent way.

2. ApoD, NLaz and Laz bind selectively to a different but overlapping set of lipid ligands. This multispecificity can explain the multiple functions they possess.

3. NLaz requires an intact ligand-binding cavity to carry out most of its biological functions. Further molecular interactions prove to be also essential for NLaz actions *in vivo*.

4. Grasshopper Laz, GPI-linked to the plasma membrane, increases lifespan and functionally replaces NLaz of its protective functions.

5. Ligands, and their management, are critical to understand Laz and Laz-related Lipocalins functions.

Resumen

(Summary in Spanish)

Resumen

Lazarillo (Laz) de saltamontes, Lazarillo Glial (GLaz) y Lazarillo Neural (NLaz) de Drosophila, y Apolipoproteina D (ApoD) de vertebrados, son proteínas homólogas pertenecientes a la familia de las Lipocalinas. Funcionalmente se han asociado con un papel protector frente al estrés oxidativo y al envejecimiento [1-6]. Además tienen un papel activo en la regulación del metabolismo [4,5,7-9].

La familia de las Lipocalinas está presente en todos los reinos. Sus miembros tienen entre 160 y 230 aminoácidos y son secretados al espacio extracelular. Los integrantes de la familia típicamente presentan una baja identidad de secuencia, pero sin embargo una alta conservación en su estructura terciaria. El plegamiento de las Lipocalinas se caracteriza por un barril-β que forma una cavidad o bolsillo donde se van a unir pequeños compuestos o ligandos, que serán habitualmente de carácter hidrofóbico. El ligando (o ligandos) es un elemento clave en las Lipocalinas y habitualmente define sus funciones biológicas. Es frecuente encontrar que muchos miembros de la familia presentan un amplio espectro de ligandos y por tanto están implicados en múltiples procesos biológicos [10].

El estudio de la filogenia de las Lipocalinas integra a Laz y a las Lipocalinas relacionadas (GLaz, NLaz y ApoD) en el clado I del árbol filogenético de proteínas. El clado I por tanto se convierte en punto de unión de las Lipocalinas de invertebrados y vertebrados [11-13].

En esta tesis he estudiado a la Lipocalina Lazarillo y a sus homólogos como grupo. La identificación de sus ligandos y su influencia en las funciones que llevan a cabo es el objetivo global de esta tesis. Para alcanzar este objetivo global se desarrollaron cuatro objetivos concretos que han sido resumidos anteriormente junto a los resultados, estos últimos organizados según los capítulos en que se organiza esta tesis.

Para la consecución del objetivo global he empleado distintos niveles de aproximación experimental: 1) Ensayos *in vitro*: expresión génica, medidas de peroxidación lipídica, medidas de triglicéridos y glucosa, ensayos de unión ligando-proteína, etc. 2) Cultivos celulares: neuroblastomas humanos y líneas celulares de Drosophila. 3) Ensayos *in vivo* utilizando *Drosophila melanogaster* como organismo

modelo. Entre los ensayos *in vivo* se incluyen estudios de longevidad, resistencia a estrés, análisis del comportamiento, etc.

El primer objetivo específico (**objetivo 1**) consistió en la caracterización de las funciones biológicas en que están implicadas las Lipocalinas de *Drosophila* GLaz y NLaz. Para ello se utilizaron mutantes nulos de GLaz o NLaz y se estudió qué fenotipos están alterados, prestando especial atención a su relación con el envejecimiento y a los dimorfismos sexuales. La ausencia de NLaz reduce la supervivencia en machos y en hembras. Sin embargo, la falta de GLaz reduce la longevidad en machos, pero no en hembras. El análisis de los mutantes revela que los cambios en la homeostasis de proteínas son los responsables de las diferencias observadas en longevidad. Tanto GLaz como NLaz están implicadas en la respuesta a estrés y el control de lípidos peroxidados, sin embargo, los ensayos de comportamiento y el almacenaje de grasas a los largo del tiempo diferencian a los mutantes de GLaz y NLaz. Por tanto se observa una división parcial de trabajos entre GLaz y NLaz.

Dado que el ligando es el responsable de muchas de las funciones que llevan a cabo las Lipocalinas, en el **objetivo 2** estudié las capacidad de unión de Laz, NLaz y ApoD. Para ello, cloné estas Lipocalinas en un sistema de expresión en células S2 de Drosophila, las purifiqué, y las enfrenté a una batería de posibles ligandos. La capacidad de unión fue monitorizada mediante fluorimetría. Laz, NLaz y ApoD son capaces de unir ligandos hidrofóbicos dentro un gran rango de estructuras y tamaños, pero a su vez muestran una alta especificidad. Por ejemplo, ApoD une selectivamente el cannabinoide anandamida y NLaz une específicamente la feromona 7-T. Por otra parte, ApoD, NLaz y Laz presentan ligandos en común, como es el caso del ácido retinoico o la esfingomielina.

En el **objetivo 3** estudié *in vivo* la relevancia de la unión de los ligandos. Para ello diseñé una serie de mutaciones puntuales en la secuencia de NLaz que podrían alterar la capacidad de unión a ligandos. En concreto, la mutación L130R elimina la unión *in vitro* del ergosterol y la feromona 7-T a NLaz. El estudio *in vivo* de los mutantes puntuales L130R mostró que, en muchos fenotipos, se asemejan más a individuos mutantes nulos para NLaz que a individuos silvestres. Los fenotipos en los que se observó este patrón incluyen la regulación de la longevidad, la respuesta a estrés, las habilidades comportamentales (test de escalada y de cortejo) y, por último, el

almacenaje de triglicéridos. Sin embargo, la homeostasis de la glucosa no se vio alterada por la pérdida de la capacidad de unión a ligandos de NLaz^{L130R}.

En el último objetivo específico (**objetivo 4**), me planteé si estas Lipocalinas pueden llevar a cabo sus funciones protectoras desde otra localización sub-celular. La Lipocalina escogida fue Laz, ya que Laz de saltamontes en su contexto natural presenta la exclusividad de aparecer unida a la membrana plasmática mediante una cola GPI. Mediante transgénesis obtuve moscas que expresan Laz anclado a la membrana plasmática. Estos individuos, mostraron una mayor resistencia a distintas formas de estrés y una longevidad incrementada. Por tanto, las funciones protectoras de este grupo de Lipocalinas pueden ser ejercidas desde la membrana. Sin embargo, la presencia de Laz en individuos deficientes en NLaz no fue capaz de restaurar el fenotipo silvestre en los ensayos de cortejo. En este último caso sí que es necesario la presencia de la proteína libre, probablemente debido a que es necesario el transporte del ligando.

De todos estos resultados se concluye que Laz y las Lipocalinas relacionadas están implicadas en un gran número de procesos biológicos, jugando tanto su localización sub-celular como el ligando un papel fundamental en la correcta ejecución de sus funciones.

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Annex

Bajo-Grañeras R, **Ruiz M.**
*Both authors contribute equally

"Apolipoproteina-D / Lazarillo, una pieza clave en el envejecimiento"

APOLIPOPROTEÍNA-D / LAZARILLO, UNA PIEZA CLAVE EN EL ENVEJECIMIENTO
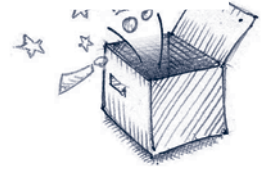
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La apolipoproteína-D (ApoD) pertenece a la familia de las lipocalinas. En general, las apolipoproteínas se producen fundamentalmente en el hígado; sin embargo, ApoD se encuentra ampliamente distribuida en el organismo y tiene un alto nivel de expresión en determinadas situaciones fisiológicas y patológicas. El aumento de la expresión de ApoD con la edad es un fenómeno conservado en los mamíferos. Sabemos que los homólogos de ApoD en Drosophila, Lazarillo Glial (GLaz) y Lazarillo Neural (NLaz), son capaces de modular la longevidad y los niveles de oxidación tisular, y que el envejecimiento esta íntimamente relacionado con un incremento de la oxidación de biomoléculas. Además, esta situación se exacerba en patologías asociadas a la edad.
En esta revisión analizamos los conocimientos actuales sobre ApoD-Lazarillo y el papel que juegan en el envejecimiento y en las enfermedades neurodegenerativas, cardiovasculares y el cáncer, donde la edad es un importante factor de riesgo.

El envejecimiento es la pérdida gradual de las funciones biológicas con el paso del tiempo. Esta pérdida aumenta el riesgo de sufrir enfermedades y de morir. Es un proceso deletéreo, progresivo, endógeno e irreversible. El envejecimiento se asocia con un aumento de las especies reactivas de oxígeno (ROS) y de nitrógeno (RNS). Estas moléculas son capaces de reaccionar con proteínas, lípidos y ácidos nucleicos provocando su deterioro funcional (Oliveira y cols., 2010). Este estrés oxidativo está íntimamente ligado a la inflamación, y ambos lo están a patologías como la aterosclerosis, a la resistencia a insulina y a la diabetes (Uranga y cols., 2010).

Pese al carácter irreversible del envejecimiento, el ser humano siempre ha buscado la manera de evitarlo o, al menos, retrasarlo lo máximo posible. Esto ha convertido al envejecimiento en uno de los temas centrales de la investigación. Se han identificado recientemente distintas maneras de incrementar la longevidad, muchas de ellas gracias a las herramientas genéticas disponibles en organismos modelo. La longevidad aumenta cuando: (1) se inhibe la actividad de la vía de la insulina (IIS) (Berryman y cols., 2008; Clancy y cols., 2001; Tatar y cols., 2001); 2) se disminuye la tasa de respiración mitocondrial (Dillin y cols., 2002; Lee y cols., 2003); y, por último, (3) cuando se realiza un patrón de ingesta de alimentos restringida (DR), sin caer en la malnutrición. Esta última manipulación permite ampliar la esperanza de vida de un modo conservado desde levaduras hasta mamíferos (Fontana y cols., 2010; Skorupa y cols., 2008).

Analizando en conjunto el perfil de expresión génica del cerebro de humanos, macacos y ratones envejecidos, se han descrito los genes en común que aumentan su expresión con el envejecimiento. Apolipoproteína D (ApoD) es el gen que más aumenta su expresión (Loerch y cols., 2008) en todas las especies analizadas. Un resultado idéntico se obtuvo de un meta-análisis de transcriptomas de distintos tejidos de ratón, rata y humanos envejecidos (de Magalhaes y cols., 2009), por lo que la elevación de ApoD con la edad es generalizable a muchos tejidos. Esto convierte a ApoD en un elemento clave en el estudio del envejecimiento y sus mecanismos.

Biología molecular de ApoD

ApoD es una glicoproteína secretada que fue inicialmente identificada en el plasma humano asociada a las lipoproteínas de alta densidad (HDL) (McConathy y Alaupovic, 1973). Se ha detectado su expresión en numerosos tejidos. Al contrario que otras apolipoproteínas, sus niveles de expresión en el hígado e intestino no son altos. Destaca el sistema nervioso como uno de los principales tejidos de expresión, siendo producida principalmente por las células de la glia (Van Dijk y cols., 2006). En su promotor se han identificado elementos reguladores de respuesta a estrés, estrógenos, progesterona y glucocorticoides de entre otros muchos (Do Carmo y cols., 2002).

ApoD se sintetiza como un polipéptido de 189 aminoácidos, correspondiendo los 20 primeros al péptido señal que será eliminado dando lugar a la proteína madura, que se secreta al medio extracelular. ApoD ha sido recientemente cristalizada y su estructura resuelta con una resolución de 1,8Å (Eichinger y cols., 2007). Como ya se había predicho por su estructura primaria, ApoD pertenece a la familia de las lipocalinas, Esta familia se caracteriza por su estructura terciaria altamente conservada: un barril-β formado por 8 láminas-β antiparalelas. El barril-β forma una cavidad o bolsillo, típicamente delimitado por aminoácidos apolares, lo que permite la unión de pequeños ligandos hidrofóbicos (Flower, 1996, 2000).

Experimentos in vitro han descrito varios ligandos potenciales de ApoD: progesterona (con la que ha sido cristalizada) (Eichinger y cols., 2007), colesterol (Patel y cols., 1997), retinol (Breustedt y cols., 2005) y ácido araquidónico -por el que muestra la mayor afinidad- (Morais Cabral y cols., 1995; Vogt y Skerra, 2001). Únicamente se conoce un ligando fisiológico: el odorante ácido E-3-metil-2-hexanoico (E3M2H) de la piel de la axila humana (Zeng y cols., 1996). Además, ApoD presenta dos puntos de glicosilación: Asn45 y Asn78 y presenta cadenas de azúcares cuya longitud puede variar considerablemente en función del tejido

(Zeng y cols., 1996). Esto hace que el peso final de la proteína varíe entre 19 y 32kDa (Rassart y cols., 2000).

Papel de ApoD y sus homólogos en el envejecimiento

ApoD es la lipocalina de mamíferos más ancestral dentro del árbol filogenético de la familia, siendo sus homólogos más cercanos las proteínas Lazarillo de artrópodos (Ganfornina y cols., 2000; Gutierrez y cols., 2000). Lazarillo es un homólogo del saltamontes Schistocerca americana que se encuentra anclado a la membrana plasmática de las neuronas (Ganfornina y cols., 1995; Sanchez y cols., 1995). Los estudios de unión a ligandos han demostrado que, al igual que ApoD, tiene una gran afinidad por ácidos grasos de cadena larga, entre ellos el ácido araquidónico (Sanchez y cols., 2008). Se han identificado dos homólogos de ApoD en la mosca del vinagre Drosophila melanogaster: Lazarillo Glial (GLaz) y Lazarillo Neural (NLaz), que reciben este nombre debido a las células del sistema nervioso que los expresan. Sin embargo, al igual que ApoD, también se expresan ampliamente fuera del sistema nervioso (Sanchez y cols., 2000).

Para un mejor estudio de ApoD y sus homólogos, y su contribución en el envejecimiento, se han generado mutantes de pérdida (KO) y ganancia de función. La caracterización de las moscas GLaz-KO ha revelado una importante disminución de la esperanza de vida, una mayor sensibilidad al ayuno y a distintas formas de estrés oxidativo: al agente generador de ROS, paraquat (PQ) y al H_2O_2. Además los niveles de apoptosis neuronal y de lípidos peroxidados aumentan en estos mutantes (Sanchez y cols., 2006). Opuestamente, la sobre-expresión de GLaz aumenta la longevidad y otorga mayor resistencia frente a un potente estrés oxidativo como es la hiperoxia (Walker y cols., 2006). También en células S2 de Drosophila, la sobre-expresión de GLaz aumenta la viabilidad cuando son expuestas a PQ o al péptido Aβ42 (Muffat y cols., 2008), ampliamente utilizado para simular la enfermedad de Alzheimer en modelos experimentales.

Recientemente se ha descrito un papel importante de NLaz en la regulación metabólica, ya que es capaz de reprimir la actividad de la vía IIS a nivel de la PI3K (fosfoinositol-3-cinasa) en larvas y moscas adultas. Al igual que sucede en los mutantes GLaz-KO, los individuos NLaz-KO tienen una menor esperanza de vida y resistencia al estrés, mientras que la sobre-expresión de NLaz incrementa su resistencia y su longevidad (Hull-Thompson y cols., 2009).

Los genes Lazarillo tienen características ancestrales, demostrando una función protectora en otros organismos como en amphioxus (Branchiostoma belcheri) (Zhang y cols., 2010) e incluso en plantas (Arabidopsis thaliana) (Charron y cols., 2008). La conservación funcional es clara, ya que la propia ApoD humana confiere un fenotipo de mayor resistencia al estrés, aumento de la longevidad y disminución de los lípidos peroxidados cuando se sobre-expresa en Drosophila (Muffat y cols., 2008).

El papel de ApoD también ha sido estudiado en mamíferos. Los ratones ApoD-KO poseen una menor resistencia al PQ, un aumento de lípidos peroxidados en el cerebro y un menor éxito en pruebas de locomoción y memoria (Ganfornina y cols., 2008). Estos animales ApoD-KO presentan alteraciones en la composición y distribución de varios receptores de neurotransmisores (Boer y cols., 2010; Rajput y cols., 2009).

Por otra parte, la ausencia de ApoD prolonga la respuesta inflamatoria retrasando la regeneración en el nervio periférico tras un daño (Ganfornina y cols., 2010). En situaciones inflamatorias se ha visto un aumento de la expresión de ApoD en células tratadas con lipopolisacárido bacteriano (LPS) (Do Carmo y cols., 2007). También se ha comprobado su capacidad de reducir los niveles de ácido araquidónico en membranas (Thomas y cols., 2003b), lo que disminuye la señalización inflamatoria. Los ratones sobreexpresores de hApoD son capaces de resistir la infección por el coronavirus OC43 (Do Carmo y cols., 2008).

La sobre-expresión de hApoD en las neuronas de ratón provoca intolerancia a la glucosa y resistencia a la insulina (Do Carmo y cols., 2009). Se ha descrito recientemente que la sobre-expresión de ApoD en el hígado de ratones silvestres u obesos provoca una disminución de los triglicéridos en plasma, ambos casos mediados por el aumento de la actividad de la enzima lipoproteína-lipasa (Perdomo y cols., 2010). El control del metabolismo es un punto clave que determina la tasa envejecimiento y la esperanza de vida. Todo apunta a que ApoD-Lazarillo es un elemento esencial en este control.

ApoD y las patologías asociadas al envejecimiento

Existe un gran número de patologías asociadas a la edad que en los últimos cien años han cobrado gran relevancia, ya que la esperanza de vida en los países industrializados se ha incrementado considerablemente. Las más relevantes pueden agruparse en tres categorías: enfermedades del sistema nervioso, cardiovasculares y cáncer.

Sistema Nervioso

Las enfermedades del sistema nervioso tienen un gran impacto social, principalmente aquellas que cursan con un déficit cognitivo.

La enfermedad de Alzheimer (AD) es una demencia progresiva que lleva a la pérdida de funciones cognitivas como son la memoria y el lenguaje. A nivel

tisular la AD se caracteriza por la degeneración de neuronas hipocampales y corticales, observándose ovillos neurofibrilares y placas de agregados del péptido β-amiloide (Parihar y Hemnani, 2004).

En pacientes con AD se detectan niveles elevados de ApoD en el líquido cerebroespinal y en diversas regiones cerebrales afectadas: el hipocampo (Glockner y Ohm, 2003; Terrisse y cols., 1998), el córtex temporal (Kalman y cols., 2000) y prefrontal (Thomas y cols., 2003a). ApoD aumenta selectivamente en el córtex entorrinal de pacientes con AD, pero la proteína no se detecta en las neuronas con ovillos (Belloir y cols., 2001). Esto sugiere que la expresión de ApoD se incrementa en las neuronas que están sufriendo un estrés pero antes de estar verdaderamente dañadas. En un modelo de AD en ratón aumenta la expresión de ApoD en mayor medida en los cerebros patológicos que en sus controles silvestres envejecidos (Thomas y cols., 2001). Navarro y cols. (2003) han detectado la presencia de ApoD en los depósitos de β-amiloide de pacientes con AD. Existen correlaciones entre pacientes con AD y determinados polimorfismos de ApoD en poblaciones de Finlandia, afroamericanas y del norte de China (Chen y cols., 2008; Desai y cols., 2003; Helisalmi y cols., 2004). Varios trabajos que estudian ApoD y AD han intentado encontrar alguna relación con apolipoproteina E (ApoE) sin encontrar una clara relación entre ellas (Glockner y Ohm, 2003; Kalman y cols., 2000; Terrisse y cols., 1999; Thomas y cols., 2003c).

Otra enfermedad neurodegenerativa asociada a la edad y de diferente origen es el Parkinson. Únicamente existe una publicación donde se describe una mayor cantidad de ApoD en la glia que rodea la sustancia negra de estos pacientes (Ordonez y cols., 2006).

Los accidentes cerebro-vasculares son frecuentes en edades avanzadas. Con la edad aumentan las moléculas de adhesión en el endotelio provocando inflamación endotelial, y en última instancia llevando a sufrir ateroesclerosis e ictus (Uranga y cols., 2010). En un modelo de ictus oclusivo en rata se observa como la glia y principalmente los oligodendrocitos sobre-expresan ApoD en el área peri-infartada. No se observa ApoD en las neuronas piramidales hasta varios días tras la reperfusión, lo que indica que las neuronas en regeneración captan ApoD de la glia de la escara (Rickhag y cols., 2008). Ya que la presencia de ApoD inicialmente se sitúa en la sustancia blanca, se postula que tiene que ver con la reparación de las sinapsis y el aporte de colesterol y lípidos para la biogénesis de las membranas, así como con la formación de la escara astrocitaria.

Como ya se ha comentado, ApoD aumenta en el cerebro ante el envejecimiento normal y patológico, pero es una reacción generalizada ante cualquier tipo de daño o patología sin ser necesaria una vinculación con la edad. Este incremento ocurre, por ejemplo, en enfermedades genéticas como Niemann-Pick (Suresh y

cols., 1998), enfermedades infecciosas como la encefalopatía 'scrapie'(Dandoy-Dron y cols., 1998) o idiopáticas como la esquizofrenia (Sutcliffe y Thomas, 2002; Thomas y Yao, 2007) y ante el daño por excitotoxicidad con ácido kaínico (Ong y cols., 1997).

Enfermedades cardiovasculares

La edad es un factor de riesgo muy importante en la aterosclerosis. Esta patología está habitualmente asociada a otras como son la hipertensión, la hipertrigliceridemia o hipercolesterolemia y la diabetes tipo 2. Esta asociación se conoce con el nombre de síndrome metabólico (Duvnjak y Duvnjak, 2009). La desregulación del metabolismo lipídico o la obesidad son uno de los principales factores de riesgo en la aterosclerosis. ApoD como componente de las HDL ha suscitado estudios sobre su relación con el colesterol y los lípidos (Perdomo y Dong, 2009). Se han detectado variantes alélicas del gen en la población afroamericana. Dos de ellas elevan el riesgo de enfermedad cardiovascular: Una de ellas reduce las cantidades de HDL y la otra eleva los triglicéridos y ApoA-I en plasma (Desai y cols., 2002).

Otros polimorfismos se asocian con los niveles de insulina basal y la obesidad. Por todo ello, se propone a ApoD como marcador de obesidad y hiperinsulinemia (Vijayaraghavan y cols., 1994). Se ha propuesto que ApoD interacciona con el receptor de leptina en el hipotálamo, donde la expresión de ApoD se estimula con dietas ricas en grasa y se correlaciona con la ganancia de peso, con la cantidad de grasa corporal y con los niveles de leptina circulantes (Liu y cols., 2001).

En el inicio de la aterogénesis los lípidos juegan un papel importante, junto con la proliferación de las células de músculo liso de la íntima de las arterias. Las señales proliferativas son citocinas y factores de crecimiento secretados por los leucocitos infiltrantes activados por las lipoproteínas (sobre todo por LDL oxidadas). Uno de los factores típicos es el factor de crecimiento de origen plaquetario (PDGF). Se ha demostrado que ApoD se encuentra en la placa de ateroma y que si se sobre-expresa en células de músculo liso, éstas no reaccionan al PDGF. El mecanismo de acción de ApoD para inhibir la acción de PDGF y la proliferación de las células se basa en bloquear la entrada al núcleo de la cinasa ERK activa (Sarjeant y cols., 2003). Sin embargo otro trabajo propone un efecto aditivo entre PDGF y ApoD (Leung y cols., 2004). Un detalle importante es el hecho de que una dieta rica en grasa aumenta la proporción de ApoD en las LDL plasmáticas (Perdomo y Dong, 2009), lo que podría concordar con una mayor presencia de ApoD en la placa de ateroma.

Cáncer

La parada del crecimiento celular induce la expresión de ApoD, lo cual la relaciona directamente con el cáncer (Do Carmo y cols., 2007). Además, su expresión está controlada por p73 y p63 (Sasaki y cols., 2009), proteínas de la familia del supresor de tumores p53, ambas implicadas en el control del desarrollo.

Muchos trabajos sobre distintos tipos de cáncer coinciden en señalar a ApoD como un marcador de buen pronóstico. Entre ellos se encuentran los de mama (Diez-Itza y cols., 1994), ováricos (Vazquez y cols., 2000), hepáticos (Utsunomiya y cols., 2005; Vizoso y cols., 2007), colon (Ogawa y cols., 2005), astrocitomas (Hunter y cols., 2002; Hunter y cols., 2005b) y neurofibromas (Hunter y cols., 2005a). En otros tipos de cáncer se ha usado como marcador diagnóstico apareciendo en los dermofibrosarcomas protuberantes (Linn y cols., 2003; West y cols., 2004) y encontrándose ausente en los fibromixomas (Lisovsky y cols., 2008). Existe controversia en cuanto a la aparición de ApoD en el cáncer de próstata (Hall y cols., 2004).

Figura 1. ApoD en el laberinto de la vida. Esquema representativo de ApoD a lo largo del complejo entramado de la vida: del desarrollo a la vejez, destacando su elevada expresión en el envejecimiento.

Conclusiones y perspectivas

Existe una clara relación entre ApoD, sus homólogos, el envejecimiento y las patologías asociadas. El envejecimiento es un proceso global del organismo en el que la interrelación entre los distintos sistemas y tejidos es fundamental (Murphy y Partridge, 2008). ApoD se incrementa con la edad (Loerch y cols., 2008) y, además, tiene un amplio patrón de distribución, permitiendo ser una proteína muy pleiotrópica y desempeñar distintos papeles en los diferentes tejidos (Rassart y cols., 2000). Todo esto la convierte en una excelente candidata para el estudio y comprensión del envejecimiento.

La gran conservación funcional de ApoD y los Lazarillos ha permitido trabajar con modelos animales. Todos los resultados indican un papel protector y de mantenimiento de la homeostasis para este grupo de proteínas. Merece destacarse (1) la capacidad para reducir los lípidos peroxidados y modular la longevidad (Ganfornina y cols., 2008; Muffat y cols., 2008; Sanchez y cols., 2006; Walker y cols., 2006) y (2) la regulación de la vía de la insulina por parte de NLaz (Hull-Thompson y

cols., 2009).

Frecuentemente se ha sugerido la posibilidad de utilizar ApoD como una diana terapéutica en patologías asociadas a la edad de muy distinta índole, ya sea modulando sus niveles o mediante las llamadas "anticalinas". Las anticalinas son lipocalinas mutagenizadas con la intención de transportar un ligando específico y ejercer un efecto terapéutico (Skerra, 2008; Vogt y Skerra, 2004).

Sin embargo, permanecen muchos aspectos desconocidos acerca de ApoD y sus homólogos. ¿Cómo modulan un proceso tan multifactorial como la longevidad? ¿Cuál es su papel exacto en las enfermedades neurodegenerativas y el cáncer?... El futuro…¡Esperamos llegar a viejos para conocerlo!

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Notes