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Knockdown of the neuronal gene Lim3 at the early stages of development affects mitochondrial function and lifespan in *Drosophila*

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ABSTRACT

Understanding the molecular mechanisms underlying variation in lifespan is central to ensure long life. *Lim3* encoding a homolog of the vertebrate Lhx3/4 transcription factors plays a key role in *Drosophila* neuron development. Here, we demonstrated that *Lim3* knockdown early in life decreased survival of adult flies. To study the mechanisms underlying this effect, we identified embryonic Lim3 targets using combined RNA-seq and RT-qPCR analyses complemented by *in silico* analysis of Lim3 binding sites. Though genes with neuronal functions were revealed as Lim3 targets, the characteristics of neurons were not affected by *Lim3* depletion. Many of the direct and indirect Lim3 target genes were associated with mitochondrial function, ATP-related activity, redox processes and antioxidant defense. Consistent with the observed changes in the embryonic transcription of these genes, ROS levels were increased in embryos, which could cause changes in the transcription of indirect Lim3 targets known to affect lifespan. We hypothesize that altered mitochondrial activity is crucial for the decrease of adult lifespan caused by *Lim3* knockdown early in life. In adults that encountered *Lim3* depletion early in life, the transcription of several genes remained altered, and mitochondrial membrane potential, ATP level and locomotion were increased, confirming the existence of carry-over effects.

1. Introduction

The elucidation of the molecular mechanisms that control lifespan is the most challenging biological problem. There is growing evidence that changes in the expression of key lifespan regulators during early development are crucially important for lifespan determination. The embryonic/early larval/early adulthood misexpression of genes encoding mitochondrial electron transport chain enzymes, microRNAs, the transcription factors HSF-1 and FOXO were found to have a significant effect on lifespan, suggesting the existence of developmental carry-over effects on adult survival (Alcedo et al., 2013; Vaiserman et al., 2018).

Previously, several genes that encode RNA polymerase II transcription factors involved in the development of the nervous system were shown to affect *Drosophila melanogaster* lifespan (De Luca et al., 2003; Pasyukova et al., 2004; Magwire et al., 2010); further analysis of their effects suggested that it is the decrease in their transcription during development, specifically the embryonic stage, that impacts longevity (Roshina et al., 2014; Symonenko et al., 2018). Among other genes, *Lim3* was found to be a candidate gene for lifespan control (Roshina and Pasyukova, 2007). Later, by using substitution lines containing second chromosomes from the *Drosophila* Raleigh natural

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population, we demonstrated the effect of *Lim3* on *Drosophila* lifespan. We revealed that the naturally occurring polymorphisms in the *Lim3* 5′-regulatory region were significantly associated with variation in lifespan (25% change) and in gene transcription (six-fold change) and were located exclusively in the Polycomb response element (PRE) that provides functional interactions with PC and ASH1 proteins (Rybina and Pasyukova, 2010; Rybina et al., 2018). We also demonstrated that *Lim3* misexpression in the nervous system and in muscles affected *Drosophila* lifespan. Specifically, we revealed that *Lim3* overexpression in the muscles was associated both with decreased synaptic activity in the neuromuscular junctions (NMJs) and with decreased lifespan (Rybina et al., 2017).

Lim3 encodes a Lim/homeobox protein, which is known to be a key regulator of neuron development (Thor et al., 1999). This gene participates in the specification of motor neuron subclass identity, axon pathfinding, and eventually provides proper muscle innervations (Certel and Thor, 2004). The Lim3 protein contains two LIM domains that mediate protein–protein interactions, a carboxyterminal homeodomain that is involved in protein-DNA interactions and a highly conserved 22-amino acid region called the Lim3-specific domain with an unknown function. Lim3 is highly homologous to the vertebrate Lhx3/4 transcription factors, which are similarly required for motor neuron specification (Mullen et al., 2007). The Lim3 expression pattern has been described in the embryonic nervous system (Thor et al., 1999; De Velasco et al., 2004; Fisher et al., 2012), where the level of *Lim3* transcription is high. Moreover, in flies from the Raleigh natural population, the molecular variation in the *Lim3* regulatory region most strongly affected *Lim3* expression in embryos (Rybina and Pasyukova, 2010). Additionally, deletions of the putative protein binding sites located within the PRE and containing polymorphisms associated with lifespan had significant effects on *Lim3* expression in embryos (Rybina et al., 2018). Based on these findings, we hypothesized that *Lim3* embryonic expression may be crucial for the lifespan of adult organisms.

To study the impact of *Lim3* expression during early development on *Drosophila* lifespan, we induced *Lim3* overexpression and knockdown at early developmental stages. *Lim3* overexpression was lethal, and *Lim3* knockdown caused a significant decrease in the lifespan of adult flies. To identify putative target genes of the Lim3 transcription factor and thus to define the mechanisms underlying its effect on fly lifespan, we used several approaches: whole genome *in silico* screening for conserved *Lim3* binding sites in the regulatory regions of genes that encode proteins supplemented by the analysis of DNA adenine methyltransferase identification (DamID) of Lim3 binding sites published by Wolfram et al. (2014); and genome-wide transcriptome analysis followed by real-time reverse transcription quantitative PCR (RT-qPCR) analyses of the transcription of putative target genes and functional analyses of the energy status in individuals with *Lim3* depletion early in life compared to controls. We revealed that most of the Lim3 targets belonged to GO term groups associated with redox and ATP-related activity, neuronal development, serine endopeptidase activity, cellular transport and immune response. These data allowed us to hypothesize that altered mitochondrial activity may play a crucial role in lifespan determination of *Drosophila* with depletion of *Lim3* expression early in life. To verify this allegation, we assessed the amounts of reactive oxygen spices (ROS), the ATP levels and mitochondrial membrane potential in embryos and adult flies. In embryos, the ROS production was increased, while the ATP level and mitochondrial membrane potential were not affected. In contrast, in *Drosophila* adults, ROS production was not affected, while the ATP level and mitochondrial membrane potential were significantly elevated, indicating the increase in mitochondrial activity. Consistent with the later observation, the locomotor activity was also significantly higher in the adult flies whose *Lim3* expression was depleted early in life. We concluded that the increased embryonic ROS production, mitochondrial function and related high ATP level in adult flies caused by *Lim3* depletion early in life were associated with *Drosophila* adult lifespan and locomotor behavior. Although several genes with functions related to the nervous system were revealed as Lim3 targets, no changes in the structure and function of NMJs were detected in individuals with *Lim3* deficits early in life.

2. Materials and methods

2.1. Drosophila lines and genetic crosses

To obtain flies with *Lim3* overexpression (Rybina et al., 2017), cDNA corresponding to the *Lim3A* transcript was cloned into the pBID-UASC vector (Addgene plasmid # 35200, https://www.addgene.org/35200/), which contains an attB site for the *phi31* site-specific transformation of *Drosophila* embryos and multiple UAS enhancer sequences (Wang et al., 2012). A clone with the unimpaired *Lim3* sequence and the line y^1*M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP'}ZH-68E*, with the third chromosome attP *phi31* integration site (Bischof et al., 2007), were used for the transformation performed by BestGene Inc. (https://www.thebestgene.com/HomePage.do). One of the five homozygous transgenic lines obtained was used in further experiments (Lim3+). The line y^1 *M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP'}ZH-68E* (Control +) that was initially used for the *Lim3* transformation was used as the control line with the same genetic background.

Several lines were obtained from the Bloomington *Drosophila* Stock Center (USA) (http://flystocks.bio.indiana.edu/).

$y^1 w^*$*;P{en2.4-GAL4}e22c;P{tGPH}4/TM3,Ser*^{1} (en-Gal4) line was used to induce the expression of transgenic constructs during early *Drosophila* development.

$y^1 v^1$*;P{TRiP.JF02125}attP2* (Lim3–) line was used to provide *Lim3* RNAi knockdown;

y^1 v^1; $P\{y^{+t7.7} = CaryP\}attP2$ (Control–) line without a transgene providing RNAi was used as a control line for *Lim3* RNAi knockdown, as suggested by the manufacturer (http://flystocks.bio.indiana.edu/Browse/TRiPtb.htm).

To induce the expression of the transgenic constructs, females of the en-Gal4 driver line were crossed to males of Lim3+, Control+, Lim3– and Control– lines. Prior to these crosses, all lines were tested for the presence of *Wolbachia*, a *Drosophila* symbiont known to affect life history traits (McGraw and O'Neill, 2004). Negative results were obtained for all lines. In all experiments, flies were kept at 25 °C on a medium of semolina, sugar, raisins, yeast and agar with nipagin, propionic acid and streptomycin.

2.2. Tests for Wolbachia

Wolbachia was detected via RT-qPCR with primers targeted to the 16S rRNA gene, 5′-CATACCTATTCGAAGGGATAG-3′ and 5′-AGCTTCG AGTGAAACCAATTC-3′ (Werren and Windsor, 2000).

2.3. Lifespan assay

Lifespan was measured as described by Roshina et al. (2014). Five virgin flies of the same genotype and sex, all collected on the same day from cultures with moderate density, were placed in replicate vials. Flies were relocated weekly to vials with fresh food containing approximately 5 mL of medium without live yeast on the surface. The number of dead flies was recorded daily. Experiments comparing fly lifespans were conducted simultaneously. Sample sizes were 100 flies/sex/genotype. All experiments were repeated twice. Lifespan was estimated for each fly as the number of days alive from the day of eclosion to the day of death. Mean and median lifespan and survival curves were primarily used to characterize the lifespan.

2.4. RNA-seq analysis

Total RNA was extracted from 0- to 16-h embryos (50–100 μg) using ExtractRNA (Evrogen). The poly-A-containing mRNA fraction was

isolated from the total RNA samples using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB) according to the manufacturer's protocol. The sequencing libraries were constructed from the poly-A containing mRNA fraction using the Next Ultra Directional RNA Library Prep Kit for Illumina (NEB) following the manufacturer's protocol with some modifications, namely, fragmentation was performed at 94 °C for 15 min; Agencourt Ampure XP beads (Beckman Coulter, Inc.) were used to purify double-stranded cDNA of more than 200 bp after adapter ligation. The obtained sequencing libraries with fragment sizes between 250 and 400 bp were purified by gel extraction using QIAquick Gel Extraction Kit (Qiagen). cDNA libraries were normalized and pooled together in equal volumes and sequenced with 100 bp single-end reads on the HiSeq 2000 platform (Illumina, USA) using the TruSeq SBS sequencing kit, version 3. The Illumina HiSeq Analysis Software was used to obtain raw sequencing reads. The sequencing data were stored in the FASTQ format. At least 43 million reads were obtained for each pool of embryos. The sequencing reads were aligned to the annotated *Drosophila* transcripts (FlyBase r5.57) using TopHat with zero mismatches. All sample preparations and sequencing were performed by Evrogen (http://evrogen.com). The differentially expressed genes were identified by the tool Cuffdiff. The total expression level for each transcript, measured in FPKM (fragments per kilobase of exon model per million reads mapped), was calculated based on the mapped reads. Genes with values under 0.6 FPKM and expression alterations under 1.7-fold were discarded, and the remaining genes were further analyzed. Gene set enrichment analysis (GSEA v3.0, http://software.broadinstitute.org/gsea/index.jsp) was used to identify differentially expressed gene sets, including pathways (Mootha et al., 2003; Subramanian et al., 2005). Raw and processed RNA-seq data were deposited in the NCBI GEO under the accession number GSE116910.

2.5. Phylogenetic footprinting analysis

To reveal genes that have conserved Lim3 binding sites in 5′ regulatory regions and, thus, are suggested to be regulated by Lim3, we scanned the genomes of 12 fly species: *D. melanogaster* (FlyBase r5.57), *D. simulans* (FlyBase r1.4), *D. sechelia* (FlyBase r1.3), *D. yakuba* (FlyBase r1.3), *D. erecta* (FlyBase r1.3), *D. ananassae* (FlyBase r1.3), *D. pseudoobscura* (FlyBase r3.2), *D.* persimilis (FlyBase r1.3), D. willistoni (FlyBase r1.3), D. mojavensis (FlyBase r1.3), D. virilis (FlyBase r1.2), D. grimshawi (FlyBase r1.3). The core Lim3 position weight matrix (PWM) from JASPAR (Portales-Casamar et al., 2010) with the highest score (TAATTA) that corresponded to the core Lhx3 (Lim3 ortholog) consensus site (Certel and Thor, 2004) was used to find the Lim3 binding sites located from -3000 bp to +1000 bp from the transcription start sites of gene transcripts. The Lim3 binding sequence was searched in reverse complementary and forward orientation. A gene transcript was considered to have *Lim3* binding sites if it contained at least two or more Lim3 binding sites in *D. melanogaster* and in *D. simulans*, *D. sechelia*, *D. yakuba*, *D. erecta*, *D. ananassae*, i.e., in species that separated from D. *melanogaster* not more than 45 million years ago and at least one or more Lim3 binding sites in more evolutionarily distant species (*D. pseudoobscura*, *D.* persimilis, D. willistoni, D. mojavensis, D. virilis, D. grimshawi). Gene transcripts for which orthologs were not found more than in three *Drosophila* species were not included in the analysis (Supplementary Table 1).

2.6. Real-time RT-qPCR analysis

RT-qPCR was performed using 30–50 μg of the initial material (0- to 16 -h embryos; carcasses of adult males) as previously described (Symonenko et al., 2018).The *Gdh* housekeeping gene was used as a reference gene to normalize the differences in total cDNA between samples. The primers utilized in the RT-qPCR analysis are listed in Supplementary Table 2. At least three biological replicates were performed for each measurement.

2.7. Larva dissection, neuromuscular junction morphology, immunostaining and microscopy

The procedure of third-stage larvae dissection followed by fixation, immunostaining and microscopy was performed as previously described (Rybina et al., 2017). The following primary antibodies were used: Alexa Fluor 647-conjugated goat anti-HRP (1:400, Jackson ImmunoResearch) against horseradish peroxidase (HRP), which was used as a marker of presynaptic membranes (Franco et al., 2004); mouse anti-Brp (mAb NC82, 1:200; Developmental Studies Hybridoma Bank (DSHB)) against Bruchpilot (BRP), which was used as a marker of active synaptic zones (Wagh et al., 2006); mouse anti-Dlg (mAb 4F3, 1:200; DSHB) against Disc Large 1 (DLG), which was used as a marker of postsynaptic zones (Budnik et al., 1996); mouse anti-α-acetylated tubulin (1:200; Santa Cruz Biotechnology) against a tubulin isoform; and mouse anti-Futsch (mAb 22C10, 1:200; DSHB) against the microtubule-associated protein Futsch, which was used as a marker of microtubule networks (Sherwood et al., 2004; Roos et al., 2000). The secondary antibodies used were goat anti-mouse Cy3 conjugated (1:400, Jackson ImmunoResearch). Antibodies obtained from the DSHB were developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Sample sizes were 5–7 larvae/genotype/experiment. Each experiment was performed in triplicate.

2.8. ATP measurement

ATP levels were evaluated according to (Vernace et al., 2007), with modifications. Embryos from 0 to 16 h of age (30–50 μg) were washed with phosphate-*buffered saline* solution (PBS) (1×, pH 7.4) and the carcasses of 15-day-old males and females (4 per trial) were harvested with 100 μl of 2.5% trichloroacetic acid followed by homogenization on ice and centrifugation (11,500 *g*, 10 min at 4 °C). ATP levels were determined in cleared supernatants (20 μl) upon neutralization of the samples with 1 M Tris − HCl, pH 9.5 (6 μl). Samples were then diluted 1:500 in ATP-free water, and 20 μl of each sample was mixed with 80 μl of the ATP assay reagents from the luciferin–luciferase assay kit (Enliten®ATP Assay System, Promega). Luminescence was measured with a Modulus Microplate Luminometer (Turner BioSystems, Inc.). To normalize ATP levels to protein concentration, 80 μl of the remaining total fly lysates were neutralized by the addition of 24 μl of 1 M Tris − HCl, pH 9.5, and 1 μl was used to perform a Bradford Assay (BioRad). At least six biological replicates were performed for each measurement.

2.9. Reactive oxygen species assay

The total amount of cellular ROS was measured using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Molecular Probes™) according to the manufacturer's instructions. Embryos from 0 to 16 h of age (30–50 μg) and carcasses of 15-day-old males and females (4 per trial) were harvested with 100 μl of PBS (1×, pH 7.4) followed by homogenization and centrifugation (2500 *g*, 5 min at room temperature). ROS production was determined in cleared supernatants (50 μl) that were diluted in 1950 μl PBS (1:40) and mixed with 2 μl of CM-H2DCFDA. The fluorescence of the samples was measured at an excitation/emission of 495/525 nm every 5 min using an MPF-4 fluorescence spectrophotometer (Hitachi Ltd.). To normalize ROS levels to the protein concentration, 1 μl of each sample was used to perform a Bradford Assay (BioRad). At least three biological replicates were performed for each measurement.

2.10. Mitochondrial membrane potential assay

The mitochondrial membrane potential (Δψm) was measured using 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzamidazol-carboncyanine (JC-1, Invitrogen) which is a cationic fluorescent dye that aggregates

with intact and negatively charged mitochondria emitting red fluorescence, whereas in cells with depolarized, non-functional mitochondria JC-1 is present in a form of cytoplasmic monomer showing green fluorescence (Smiley et al., 1991; Reers et al., 1995). Embryos from 0 to 16 h of age (30–50 μg) and carcasses of 15-day-old males and females (4 per trial) were harvested with 200 μl of PBS (1 ×, pH 7.4) followed by homogenization and centrifugation (1000 *g*, 5 min at room temperature). Δψm was quantified in cleared supernatants (40 μl) that were diluted in 1960 μl PBS (1:50) and incubated with 3 μl of JC-1 (5 mg/ml in DMSO) for 10 min. JC-1 was analyzed at an excitation/emission 488/525 nm (green) and 590 nm (red) using Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies). Relative fluorescence of each sample was quantified as red/green ratio and normalized to citrate synthase activity for mitochondrial content. At least three biological replicates were performed for each measurement.

The protonophore Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma) that disrupts mitochondrial membrane potential was used at a final concentration of 2 μM as the negative control. Disodium succinate (10 mM, Sigma) was used as the positive control that provides a compensatory upregulation of Δψm.

To evaluate the mitochondrial content, embryos from 0 to 16 h of age (30–50 μg) and carcasses of 15-day-old males and females (4 per trial) were used to measure citrate synthase activity according to the protocol described previously (Kuznetsov et al., 2010). Citrate synthase activity was normalized to total protein amount using Bradford Assay (BioRad). At least three biological replicates were performed for each measurement.

2.11. Locomotion assay

Flies were collected and maintained as for the lifespan assays. Locomotion was analyzed in unmated females and males at day 15, 30, 45, and 60 at the same time each day. Experiments comparing locomotion were conducted simultaneously. One day before the measurements, five flies of the same age, sex and genotype were placed in special vials. To measure locomotor activity, vials were placed horizontally in a *Drosophila* Population Monitor (TriKinetics). Fly movements that interrupted infrared beam rings along the length of the vial were detected and counted electronically, and totals were reported every five minutes to the host computer. Two measurements were made for each vial. The sample size was 50 flies/sex/genotype/age. Locomotion was characterized as the mean number of beam interruptions per vial per five minutes.

2.12. Statistical analyses

To compare the control and misexpressed genotypes, the nonparametric, distribution-free Kruskal-Wallis test was used for the analyses of the transcript amounts measured by RT-qPCR, locomotion, satellite bouton and active zone numbers in NMJs, and ATP, ROS levels and Δψm. Survival curves were estimated using the Kaplan–Meier procedure. The nonparametric, distribution-free Mann-Whitney test and Kolmogorov-Smirnov test were used to evaluate the statistical significance of the difference between the survival curves (Supplementary Table 3).

3. Results

3.1. Lim3 misexpression during early Drosophila development

To assess the early developmental impact of *Lim3* on *Drosophila* lifespan, we used the en-Gal4 driver, which provided GAL4 expression according to the *engrailed* expression pattern, i.e., predominantly during embryonic and larval development (http://www.modencode.org, Graveley et al., 2011).

To obtain individuals with *Lim3* overexpression, we crossed en-Gal4 with Lim+, which was the line with the transgenic DNA copy of the functional *Lim3A* transcript (Thor et al., 1999; Rybina and Pasyukova, 2010) under UAS regulation. No larvae were detected in this progeny. The progeny of the cross between en-Gal4 and Control+, which was the corresponding control line, was fully viable. This result indicated that *Lim3* overexpression during development was lethal.

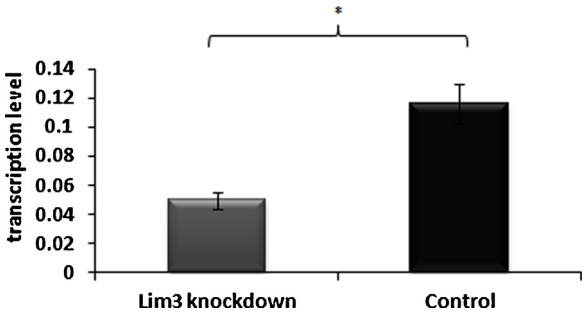

Fig. 1. RT-qPCR results confirm the significantly reduced transcription level of *Lim3* due to its knockdown in *Drosophila* embryos. Data represent the mean ± s.e. $^{*}P < 0.05$ was determined by Kruskal-Wallis test. Three biological replicates were performed for each measurement.

To obtain individuals with *Lim3* RNAi knockdown, we crossed en-Gal4 with Lim3–, which was the line with the transgenic construct containing the *Lim3* hairpin sequence ($Lim3^{JF102125}$) under UAS regulation. en-Gal4 was also crossed with Control–, which was the corresponding control line. Progenies of both crosses were viable.

To evaluate the effectiveness of *Lim3* knockdown, the amounts of the *Lim3A* transcript were measured in *Lim3* knockdown embryos and in control embryos. The expression level of *Lim3A* as evaluated by RT-qPCR was significantly decreased (2.3-fold) by the knockdown compared to the control levels (Fig. 1).

Analysis of the RNA-seq data revealed that the normalized read count on *Lim3* gene body confirmed the high expression level of the $Lim3^{JF102125}$ transgene, when compared to the control fly line (Fig. 2).

These results demonstrated that the Gal4-UAS system effectively decreased *Lim3* expression *in vivo*. Accordingly, individuals with *Lim3* knockdown during early development were used to study the relationship between *Lim3* depletion early in life and lifespan.

3.2. Lifespan in flies with Lim3 knockdown during early Drosophila development

We assessed the effect of *Lim3* knockdown during early development on the lifespan of unmated males and females. Standard descriptive statistical analyses of lifespan (Wilmoth and Horiuchi, 1999) for two experimental replicates with males were consistent (Supplementary Table 3), and the replicates were combined for further analysis. A significantly decreased lifespan was detected in males with *Lim3* knockdown at the early stages of *Drosophila* development compared to that of control flies (Fig. 3, Supplementary Table 3).

The shapes of the survival curves indicated that the decrease in *Lim3* expression during early development did not substantially accelerate aging in males; indeed, the maximum lifespans were similar in the knockdown and control males (Fig. 3, Supplementary Table 3). Conversely, *Lim3* depletion deteriorated male survival, predominantly at the middle adult stage, as evidenced by comparing the survival curves (Fig. 3, Supplementary Table 3).

An effect of *Lim3* knockdown in females was found only in one of the two experimental replicates, and in that replicate, the effect was very low (3%) but significant (Supplementary Table 3). On the one hand, it is possible that this result objectively demonstrates the presence of a small effect of *Lim3* misexpression on female lifespan. It is well known that small effects are extremely difficult to reproduce. For example, previously, we demonstrated that out of 18 identical

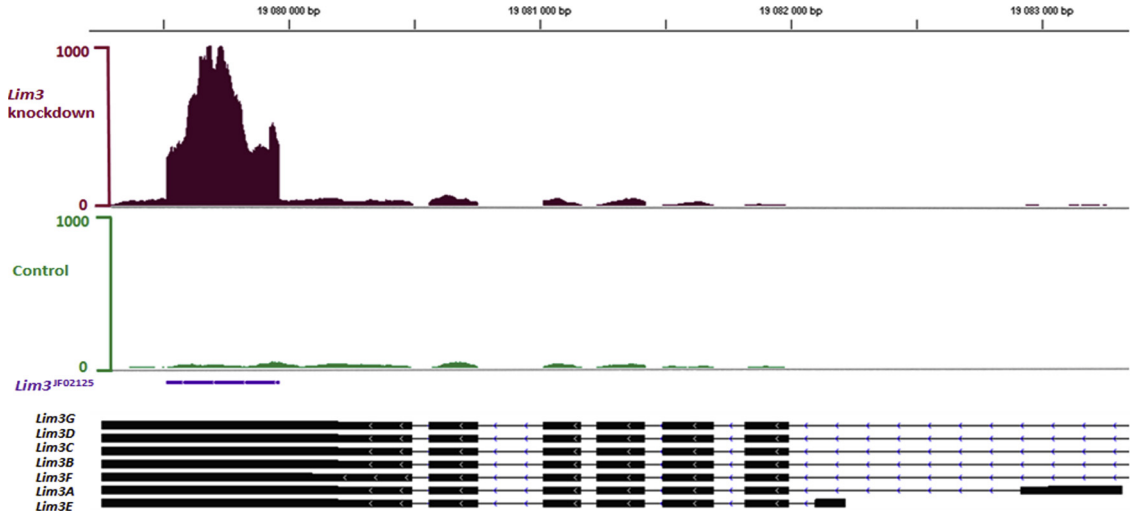

Fig. 2. The screenshot of RNA-seq read density shows the high expression level of the *Lim3*^{JF102125} transgene in embryos with *Lim3* knockdown compared to the control embryos.

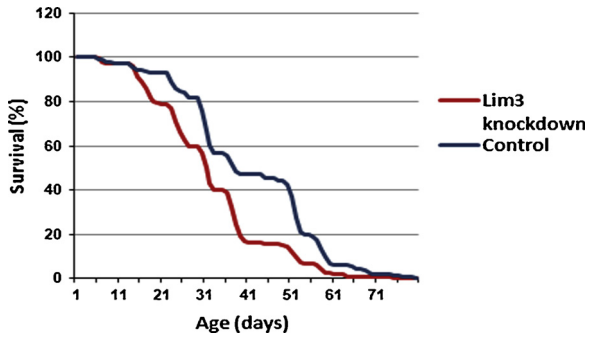

Fig. 3. Lifespan of unmated males with *Lim3* knockdown during early stages of *Drosophila* development compared to that of control fly lines. Lifespan measurements were repeated twice. Survival curves were drawn based on the combined data of two replicates. Sample sizes were 100 flies/genotype/replicate.

independent experiments, only 12 allowed us to observe significant effects of a chemical on the mean lifespan (Krementsova et al., 2012). The size of the effect was about 10–15%, which is 3–5 times larger than the effect of *Lim3* knockdown on female lifespan. On the other hand, the complete absence of the effect of *Lim3* depletion on female lifespan cannot be excluded. The quantitative differences between males and females in the effects of *Lim3* knockdown on the lifespan can be, at least partially, explained by the sex-specificity of the expression of the en-Gal4 driver, which was previously observed in Ni et al., 2007. Generally, sex-specific differences in lifespan are quite common (for example, Tricoire et al., 2009; Ruiz et al., 2011; Roshina et al., 2014; Schriner et al., 2014).

3.3. Genome-wide screening for Lim3 target genes

To elucidate the mechanisms of lifespan control underlying the effect of *Lim3* knockdown during early *Drosophila* development, we performed genome-wide transcriptome analysis of *Drosophila* embryos with depleted and normal expression of *Lim3*. In total, 217 genes changed their expression by 1.7-fold or more and had an FPKM of 0.6 or more after *Lim3* knockdown (Supplementary Table 4).

The differentially transcribed genes included either primary (direct) or secondary (indirect) Lim3 targets. The transcription of the primary target genes is directly controlled by Lim3 through protein-DNA or protein-protein interactions; the transcription of the indirect target genes is controlled by proteins encoded by direct Lim3 target genes. To distinguish between the direct and indirect Lim3 target genes, we compared the 217 misexpressed genes that were revealed by transcriptome analysis with 1) direct Lim3 target genes identified with FDR of < 0.01 by DamID analysis, which was performed in embryos with depleted *Lim3* by Wolfram et al., 2014 (Supplementary Table 4) and 2) genes whose regulatory regions possessed putative Lim3 binding sites that were conserved among the 12 *Drosophila* species, according to a phylogenetic footprinting analysis (Supplementary Table 1, Supplementary Table 4). Each of the methods, and especially the combination of these methods, can indicate the most likely Lim3 target genes. Among the 217 genes with altered transcription following *Lim3* knockdown early in life, we found 15 genes that were identified by both DamID and phylogenetic footprinting analyses as direct Lim3 targets, and 73% of these were upregulated (Fig. 4A, Supplementary Table 4). Another 34 genes were identified as direct Lim3 targets solely by DamID analysis, 72% of which were upregulated. Overall, 49 genes were identified as direct Lim3 target genes. The 22 genes that had Lim3 binding sites revealed by footprinting analysis but were not confirmed to be direct Lim3 targets by DamID analysis were excluded from further consideration because of their uncertain status. The remaining 146 genes were considered indirect Lim3 targets, and 65% of these genes were upregulated (Fig. 4A, Supplementary Table 4). Altogether, the number of upregulated genes (67%) was higher than the number of downregulated genes.

Using Gene Set Enrichment Analysis (GSEA), the 195 misexpressed genes that were found to be direct and indirect Lim3 targets were clustered into 12 groups according to their GO terms (Fig. 4B, Supplementary Table 5). In each group, the ratio of the direct Lim3 target genes did not exceed 50% of the total number of genes in the GO term group.

Thirty percent or more direct Lim3 target genes were revealed in groups involved in cuticle development, cellular transport, ATP-related activity and behavior, while in groups involved in redox metabolism, serine type endopeptidase activity, defense response, determination of adult lifespan and DNA repair, 13% or less direct Lim3 target genes were found (Fig. 4B). Interestingly, a group composed of genes involved in the determination of adult life span was revealed (Fig. 4B, Supplementary Table 4), including the following: the heat shock protein gene *hsp68* (Wang et al., 2003), which is involved in protein folding and heat and chemical stress resistance; the glutamate transporter gene *Eaat1* (Rival et al., 2004), which controls neuronal functionality and oxidative stress resistance; and the gene encoding an inhibitor of apoptotic protein antagonists *grim,* (Zheng et al., 2005; Bedoukian et al., 2009), which regulates apoptosis of cells in the central nervous system. This

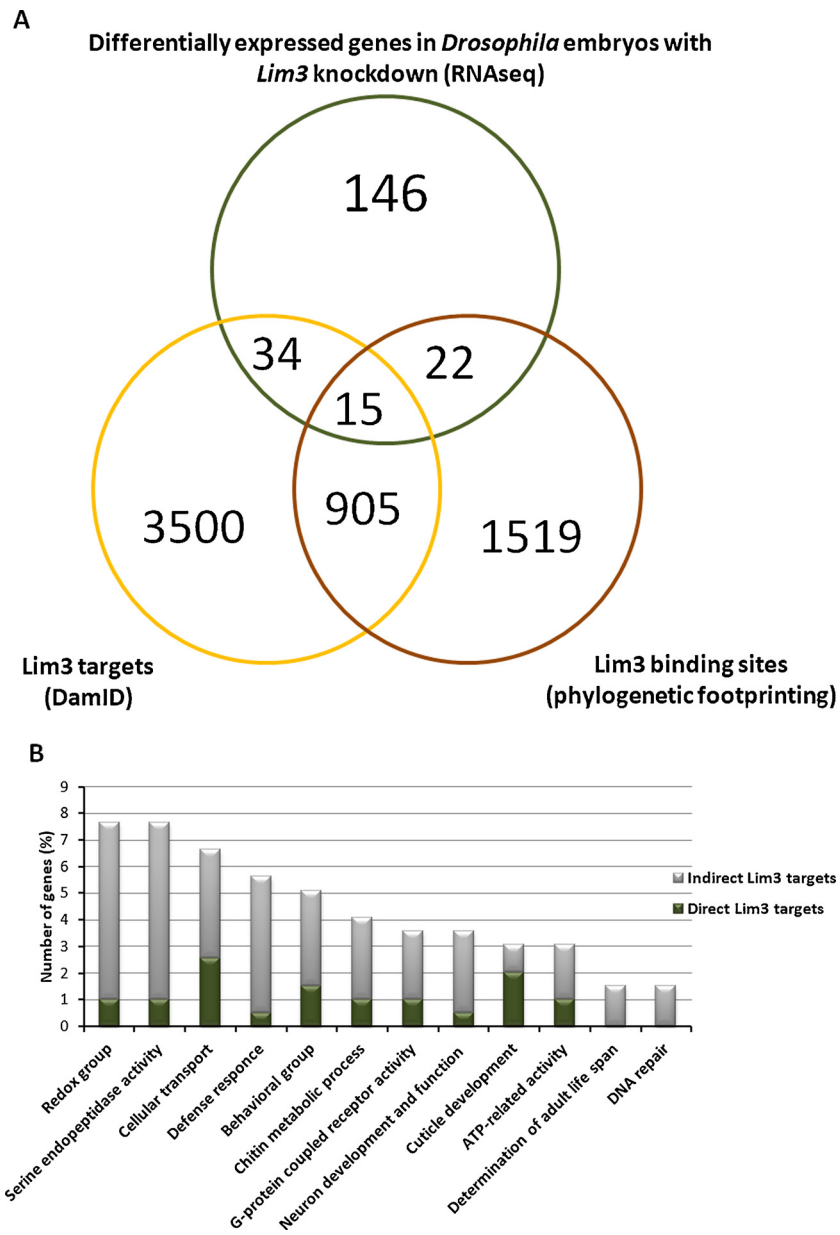

Fig. 4. (A) An overlap between Lim3 targets determined by different methods. Putative direct Lim3 targets were determined by 1) the overlap between the gene sets revealed by phylogenetic footprinting, DamID and RNAseq analyses; 2) the overlap between the gene sets revealed by RNAseq and DamID analyses. Putative indirect Lim3 targets were determined by RNAseq. (B) Functional classification of differentially expressed genes in *Drosophila* embryos with *Lim3* knockdown relative to control. The list of the GO terms that are clustered into groups including redox, serine endopeptidase activity, cellular transport, defense response, behavioral, chitin metabolic process, G-protein coupled receptor activity, neuron development and function, cuticle development, ATP-related activity, determination of adult lifespan and DNA repair is provided in Supplementary Table 5.

result indicated that the Lim3 effect on lifespan might be, at least partially, mediated by pathways involved in the control of neuronal functions and stress resistance.

To validate changes in the expression of genes from many different GO term groups, we selected genes that simultaneously belonged to several GO term groups and evaluated the transcription level of these genes in individuals with *Lim3* depletion early in life compared to controls by RT-qPCR (Fig. 5).

The genes that were previously shown to be involved in lifespan regulation, *Eaat1* and *grim*, were upregulated. Among the genes involved in neuronal development and function, *nrv3*, which encodes one of three beta subunits of the sodium-potassium pump, was also upregulated, while *nAChRalpha1*, which encodes the nicotinic acetylcholine receptor, was downregulated. Two genes encoding enzymes belonging to the cytochrome P450 family, *Cyp4p2* and *Cyp6A20* (redox group), and *Pkg21D*, which encodes a Mg^{+} dependent kinase that is activated by cGMP (ATP-related activity group), were upregulated, while *GstD8* and *GstE4,* which are essential for the deactivation of reactive oxygen species (redox group), were downregulated. The following genes were upregulated: *Jon65Aiv* and *Jon25Bi*, which are involved in proteolysis

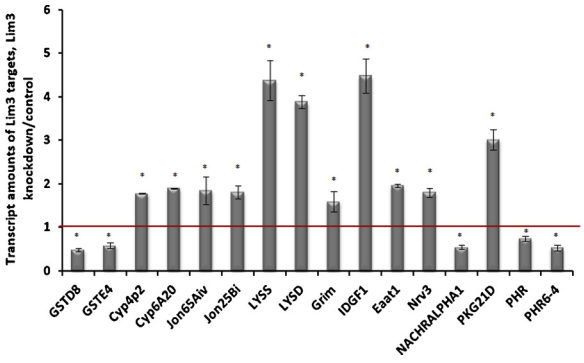

Fig. 5. Validation of RNA-seq by RT-qPCR. Expression levels of differentially expressed genes in *Drosophila* embryos with *Lim3* knockdown relative to controls. Data represent the mean ± s.e. $^{*}P < 0.05$ was determined by Kruskal-Wallis test. The red line designates the nominal control level. Three biological replicates were performed for each measurement (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

essential for immune defense (serine endopeptidase activity group); *LysS* and *LysD,* which are involved in antimicrobial humoral response (defense response group); *Idgf1* from the chitin metabolic process group. The genes *phr*, *phr6-4*, which are involved in the repair of pyrimidine dimers, excision repair and UV resistance (DNA repair group), were downregulated.

Overall, the results of the RT-qPCR analysis confirmed the data obtained by the transcriptome analysis. In some cases, the observed changes in gene expression (for example, downregulation of *GstD8, GstE4, phr*, *phr6-4*) were in line with the phenotypic effect of *Lim3* knockdown during early *Drosophila* development, i.e., with decreased lifespan. However, the observed changes in the transcription of *Eaat1* were expected to be associated with increased lifespan. Previously, the inactivation of *Eaat1* was shown to decrease lifespan (Rival et al., 2004). In Rival et al. (2004), the inactivation was carried out using a native *Eaat1* promoter which provides GAL4 expression predominantly in the glial cells of adult flies. In our study, the increase in the *Eaat1* was detected in embryos due to *Lim3* knockdown performed using an *engrailed* promoter which activates GAL4 expression during the embryonic and larval development. In addition, the expression of mutant Eaat1 transgene in different types of neurons was shown to have no effect on *Drosophila* lifespan (https://escholarship.org/uc/item/89t3v131). Thus, the observed discrepancy may be due to the complexity of the functions of the gene, which is reflected in the difference of the results obtained using different experimental systems (Dworkin et al., 2009; Chandler et al., 2014). In addition, it was unclear whether the changes in gene transcription observed in the embryos persist into adulthood, which was when lifespan was measured.

3.4. Transcript amounts of genes affected by Lim3 knockdown during early Drosophila development in the carcasses of adult males

The driver en-Gal4, which was used in crosses with Lim3– and Control– lines, is expressed during the early stages of *Drosophila* development. Accordingly, changes in gene transcription were not anticipated in adult flies. To verify whether gene transcription was perhaps still changed in flies with altered lifespan, we measured the transcript amounts of Lim3 targets whose expression was evaluated by qRT-PCR in the embryos.

Indeed, in adult flies that experienced *Lim3* depletion during early development, the expression of many of the genes that were affected in embryos was not changed in adulthood and the expression of only a few genes were affected (Fig. 6 vs. Fig. 5).

The transcript levels of *Cyp4p2* (redox group), *Pkg21D* (ATP-related activity group) and *nrv3* (neuronal development and function group) remained increased in adult males that experienced *Lim3* knockdown during early development. *Jon65Aiv*, *Jon25Bi* (serine endopeptidase activity group), *LysS*, and *LysD* (defense response group) appeared to be downregulated in adult males, and *nAChRalpha1* (neuronal development and function group) appeared to be upregulated, as opposed to the observations in embryos.

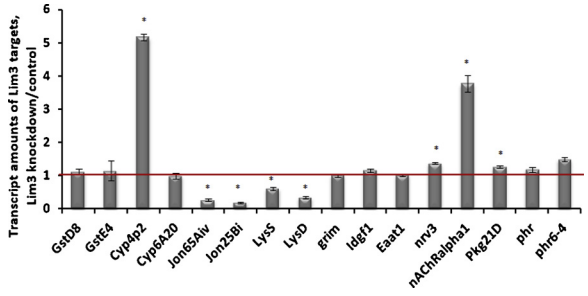

Fig. 6. Transcription levels of the genes affected by *Lim3* knockdown during early stages of *Drosophila* development, measured by RT-qPCR in carcasses of adult males. Data represent the mean ± s.e. $^{*}P < 0.05$ was determined by Kruskal-Wallis test. The red line designates the nominal control level. Three biological replicates were performed for each measurement (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

In recent years, accumulating data suggested that several key regulators affected lifespan predominantly during early larval development and early adulthood (Alcedo et al., 2013; Vaiserman et al., 2018). On the one hand, our results could imply that epigenetic memory might maintain the elevated level of *Cyp4p2*, *Pkg21D* and *nrv3* expression after the removal of the inducing agent, as suggested in Turner (2009), thus explaining the long-term effects originating during the development. On the other hand, it is well known that gene transcription is extremely flexible due to many positive and negative feedbacks, and changes appearing in response to internal and external cues become apparent across a broad range of time scales (Sneppen et al., 2010; Yosef and Regev, 2011). Genes that, in adults, changed their expression in the opposite direction compared to embryos and are associated with mitochondrial activity (*nAchalpha1*), immune defense (*Jon65Aiv*, *Jon25Bi*), and antimicrobial humoral activity (*LysS*, *LysD*) are generally involved in the fast response to different stimuli to stabilize and maintain homeostasis (Schuliga et al., 2002: Li et al., 2013; Lopez et al., 2018), and rapid adaptation of their expression to the needs of the body arising in the process of aging might be highly demanded. Thus, sophisticated hypotheses based on both epigenetic inheritance and complex spatial and temporal interactions between affected genes and their products are required to explain the observed facts.

3.5. Neuronal structure and function in individuals with Lim3 knockdown during early Drosophila development

According to the RNA-seq analysis, GO terms associated with neuronal development and function were identified (Fig. 4B, Supplementary Table 4), and changes in the transcript levels of several genes involved in the control of neuronal function (*Eaat1, grim, nrv3, nAChRalpha1*) were confirmed by RT-qPCR. To approach the issue under study in a different way, we investigated whether certain structural and functional properties of the nervous system changed as a result of the *Lim3* knockdown during early *Drosophila* development. For this experiment, we analyzed the structural and functional properties of NMJs, based on the fact that *Eaat1* (Stacey et al., 2010), *nrv3* (Doi and Iwasaki, 2008) and *nAChRalpha1* (Heiny et al., 2010) are involved in the regulation of effective synaptic transmission and neural network activity, and considering that NMJs are often used as a model system to study synaptic development and function (Ruiz-Canada and Budnik, 2006).

The abdominal body wall muscles are innervated by motor neurons that form synaptic boutons containing glutamate (type-1b, type-1 s and satellite boutons) (Menon et al., 2013). We determined the number of type 1b and satellite boutons and the number of active synaptic zones in stage III larvae that were exposed to *Lim3* knockdown during development (Supplementary Table 6). No deviations from the corresponding control values were observed. Synaptic function depends on membranous structures called the subsynaptic reticulum (SSR), which surround NMJs (Menon et al., 2013). Among other proteins and molecular complexes, DLG, a homolog of mammalian postsynaptic density protein 95 that encodes a membrane-associated guanylate kinase scaffolding protein, is present in SSRs and participates in the regulation of the synaptic reticulum (Budnik et al., 1996). DLG staining, which is commonly used to characterize the postsynaptic density, was observed as a halo-like structure around the HRP-staining of type 1b boutons in individuals of all genotypes and had the same intensity (data not shown). The functional integrity of NMJs depends on the correct status of their cytoskeletons. Antibody staining of components of the microtubule network, α-acetylated tubulin and Futsch, which is a neuronal microtubule-associated protein (Roos et al., 2000), failed to reveal visual differences in the morphology of NMJs between individuals who

encountered *Lim3* depletion early in life and control individuals (data not shown).

Thus, the analyses of different NMJ characteristics did not allow us to determine whether synaptic properties were affected by *Lim3* depletion early in life and could be related to alterations in the lifespan of adult flies.

3.6. ROS production, ATP level and mitochondrial membrane potential in individuals with Lim3 knockdown during early Drosophila development

According to the transcription analysis data, the most over-represented GO term group was associated with redox processes (Fig. 4B); in addition, the GO term group associated with ATP metabolism was overrepresented. Changes in the transcript level of several genes from these functional groups were confirmed by RT-qPCR. ROS production and ATP synthesis, which are associated with redox processes, are tightly connected with mitochondrial function, and it is well known that mitochondria play a crucial role in lifespan control (Cui et al., 2012; Orr et al., 2013). Following the logic of research, we decided to determine whether ROS generation, ATP production and mitochondrial membrane potential (Δψm), a key indicator of mitochondrial function and energy metabolism, were affected by *Lim3* knockdown during early stages of *Drosophila* development. ATP levels and mitochondrial membrane potential were not affected in embryos with depleted *Lim3* compared to that of the control, while ROS production was significantly increased (Figs. 7A, 8 A, 9 A).

ROS production has been shown to affect lifespan in a complicated way. At present, it is generally accepted that the moderate reduction of mitochondrial functions and, consequently, moderate decrease in ROS production by mitochondria increases lifespan, whereas a strong reduction shortens lifespan (Orr et al., 2013). The increased level of ROS observed here is in line with a negative effect on lifespan. The mechanism of how the ROS production in embryos can affect the adult lifespan remains an open question.

It was of interest to investigate whether the difference in ROS production holds in adult flies whose lifespan was changed as a result of *Lim3* knockdown during early stages of development. We hypothesized that the alteration of energy metabolism would be particularly apparent in the carcasses of adult flies, which are mainly composed of muscles and the peripheral nervous system. ATP levels, mitochondrial membrane potential and ROS production were quantified in the carcasses of 15-day-old males and females.

In the carcasses of adult males who had *Lim3* knockdown during early *Drosophila* development, the ATP levels and mitochondrial membrane potential were increased about five-fold compared to the control, while ROS amounts were not changed (Figs. 7B, 8 B, 9 B). In the carcasses of adult females with *Lim3* knockdown during early development, alterations in ATP levels, mitochondrial membrane potential and ROS production were small and not significant (Figs. 7C, 8 C, 9 C).

Overall, changes in the level of ATP and Δψm were well correlated with each other across developmental stages and sexes. It is also worth noting that, similar to the effect on the level of ATP and mitochondrial membrane potential, *Lim3* knockdown early in life caused a pronounced effect on male lifespan, whereas female lifespan was either marginally affected or not affected at all. The fact that both ATP levels and Δψm were not affected in the carcasses of adult females with depleted Lim3 early in life pointed to possible sex-specific effects of Lim3 on mitochondrial function and strengthened the possibility of sex-specific effects of Lim3 on lifespan.

It remains to be formally proved that a decrease in male lifespan can be causally related to increased ATP levels and elevated mitochondrial membrane potential.

Thus, data describing changes in gene expression, ATP levels, Δψm and ROS production indicated that redox processes, ATP metabolism and mitochondrial function were affected by the *Lim3* knockdown during early *Drosophila* development. We suggest that these changes could be causally related to alterations in the lifespan of adult flies.

3.7. Locomotion in flies with Lim3 knockdown during early Drosophila development

We hypothesized that *Lim3* knockdown during early *Drosophila* development, which increased ATP levels and Δψm in carcasses of adult flies, could affect *Drosophila* muscle activity. Additionally, although we did not find effects of *Lim3* depletion during early *Drosophila* development on the functional properties of synapses, we presumed that NMJs might be changed in some way that was not yet characterized. Changes in both muscle and NMJ activity should affect the locomotion of flies. To verify this, we assessed the impact of *Lim3* knockdown during early *Drosophila* development on locomotor activity in unmated males and females.

Locomotion was first measured in young 15-day-old flies; measurements were repeated every 15th day until flies reached an age close to the maximum lifespan (Supplementary Table 7). Both in males and females with altered *Lim3* transcription during early *Drosophila* development and in control flies, locomotion decreased with age (Fig. 10).

In flies with *Lim3* depletion, locomotion was elevated, mainly at younger ages, compared to controls (Fig. 10, Supplementary Table 7). On average, the effect was less pronounced in females than in males, similar to the effect on the amount of ATP and lifespan.

Overall, data on locomotor activity were in line with other results and testified that redox processes, ATP metabolism and mitochondrial function were affected by the *Lim3* knockdown during early *Drosophila* development. Importantly, these data, together with the results on ATP levels and Δψm in adults, confirmed the existence of carry-over effects caused by *Lim3* depletion early in life.

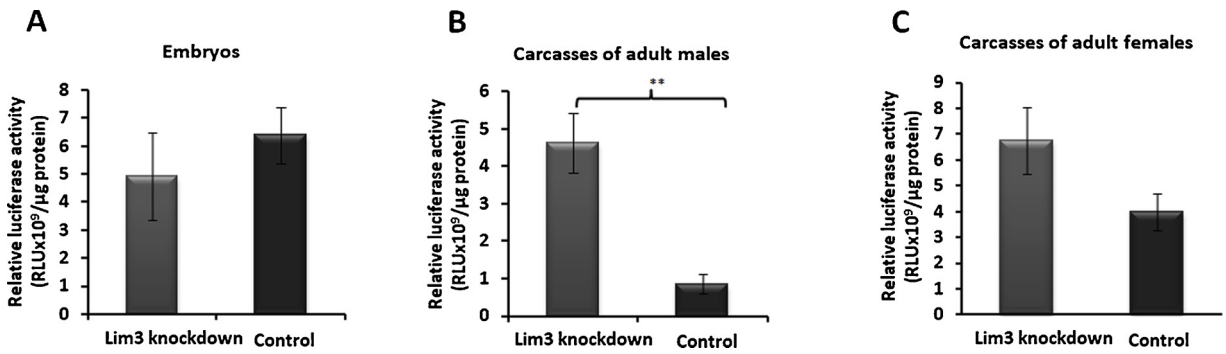

Fig. 7. ATP levels in *Drosophila* embryos (A), carcasses of adult males (B) and females (C) with *Lim3* knockdown during early stages of *Drosophila* development relative to control. Data represent the mean ± s.e. ** $P < 0.01$ was determined by the Kruskal-Wallis test. Three biological replicates were performed for each measurement.

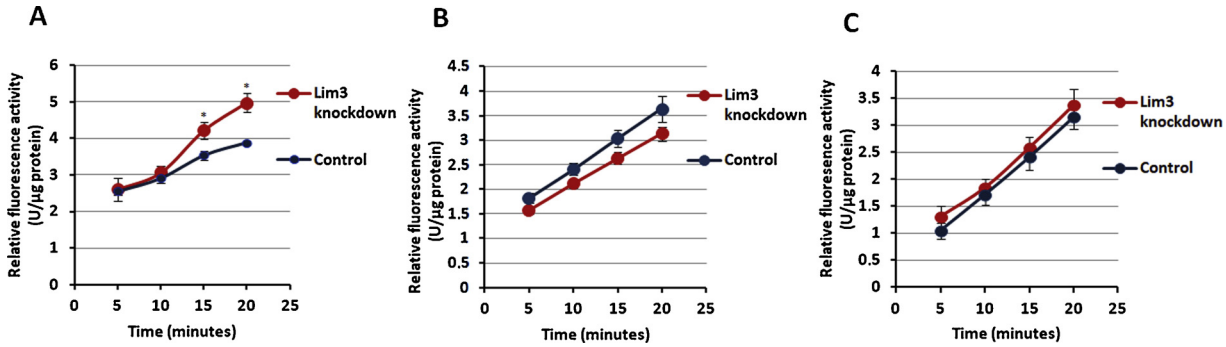

Fig. 8. ROS production in *Drosophila* embryos (A) and carcasses of adult males (B) and females (C) with *Lim3* knockdown during early stages of *Drosophila* development relative to control. Data represent the mean ± s.e. $^{*}P < 0.05$ was determined by the Kruskal-Wallis test. Three biological replicates were performed for each measurement.

4. Discussion

Transcription factors are global regulators that affect the expression of a large number of genes through different regulatory networks and therefore have a great effect on various biological processes and functions of the organism. Lim3, one of the transcription factors participating in the development and function of the *Drosophila* nervous system, affects fly lifespan when it is misexpressed in nervous and muscle tissues (Rybina et al., 2017). In this study, we demonstrated that the alteration of *Lim3* expression resulting in a deviation from the optimal level of *Lim3* transcript amounts during the early stages of the *Drosophila* life cycle has a deleterious effect on the survival rate of adult flies, emphasizing the special role of *Lim3* in the early development of *Drosophila* and the particular importance of the early stages of the fly life cycle for adult life. Indeed, *Lim3* overexpression early in life led to lethality, and *Lim3* knockdown significantly decreased male survival and lifespan.

To determine the mechanisms underlying the *Lim3* effect on *Drosophila* lifespan, we attempted to identify direct and indirect Lim3 target genes. Most of the 195 targets revealed were upregulated upon *Lim3* knockdown, suggesting that Lim3 acts mainly as a repressor. The Lim3 targets were annotated by function, and most of them appeared to belong to redox and serine protease activity groups followed by cellular transport and defense response groups. Similar results were also obtained in genome-wide studies of gene expression patterns during aging (Pletcher et al., 2002; Doroszuk et al., 2012; Zhan et al., 2007; Lai et al., 2007), oxidative stress (Landis et al., 2004; Girardot et al., 2004; Gruenewald et al., 2009) and neurodegenerative processes caused by mutations in the gene *sall* (Ferreiro et al., 2012), confirming the existence of molecular mechanisms that are common for aging, stress response and neurodegeneration. The predominance of the genes involved in redox processes together with the genes with ATP-related activity among the Lim3 targets affected by Lim3 deficit early in life led us to believe that an alteration of mitochondrial function could underlie the effect of *Lim3* knockdown on *Drosophila* lifespan. Importantly, *Lim3* depletion also affected the transcription of genes that were formally attributed to other groups that were functionally related to mitochondria (for example, *nrv3*).

Mitochondria produce energy in the form of ATP molecules through oxidative phosphorylation and in this way regulate cellular metabolism. A natural byproduct of this process is the formation of ROS, which play an important role in cell signaling but, in greater amounts, induce oxidative stress, which promotes aging and shortens lifespan (Cui et al., 2012; Orr et al., 2013). In our study, we used the Δψm, ROS and ATP levels in embryos and carcasses of adult flies and locomotion of adult flies as markers of mitochondrial function. Importantly, these four parameters are not independent. It was shown that the increased levels of ROS was associated with decreased mitochondrial electron transport chain activity, reducing its capacity for ATP production (Stefanatos et al., 2012), while high levels of ATP correlated with low amounts of ROS and increased of Δψm (Korshunov et al., 1997). It is also clear that the Δψm and related ATP concentration are closely allied to locomotor activity since any action requires energy provided by ATP. We found that in embryos, the ROS amounts were increased, while the Δψm and ATP levels were not affected. In adult flies, on the contrary, the amount of ROS was not changed, while the Δψm, ATP levels and locomotion were increased. The main thing for us was to interpret these data in the context of lifespan changes found in flies that encountered *Lim3* deficits early in life. We suggested that the increased ROS production was induced in embryos with *Lim3* knockdown as a result of changes in the transcription of genes responsible for redox processes, ATP production and mitochondrial function, which might also be responsible for the decreased adult lifespan due to carry-over mechanisms providing long-lasting effects of oxidative damage. At the same time, the elevated Δψm and ATP levels in adults also should be a consequence of changes early in life induced by *Lim3* depletion. The molecular mechanisms of these

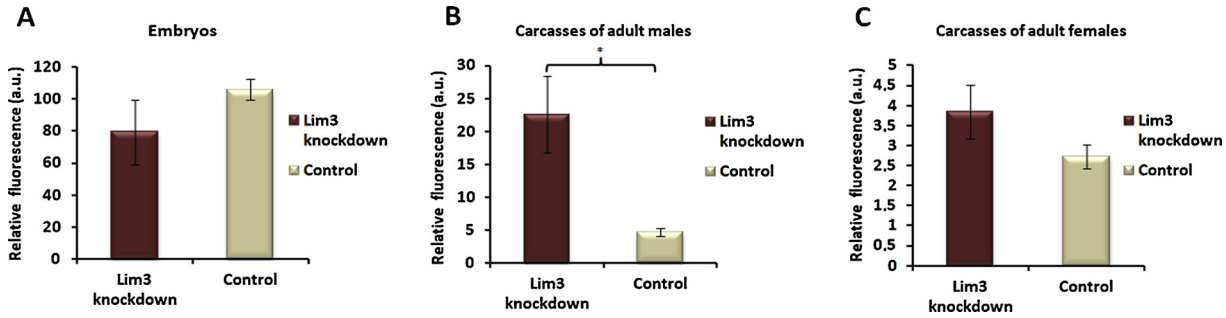

Fig. 9. Mitochondrial membrane potential in *Drosophila* embryos (A), carcasses of adult males (B) and females (C) with *Lim3* knockdown during early stages of *Drosophila* development. Data represent the mean ± s.e. $^{*}P < 0.05$ was determined by the Kruskal-Wallis test. Three biological replicates were performed for each measurement.

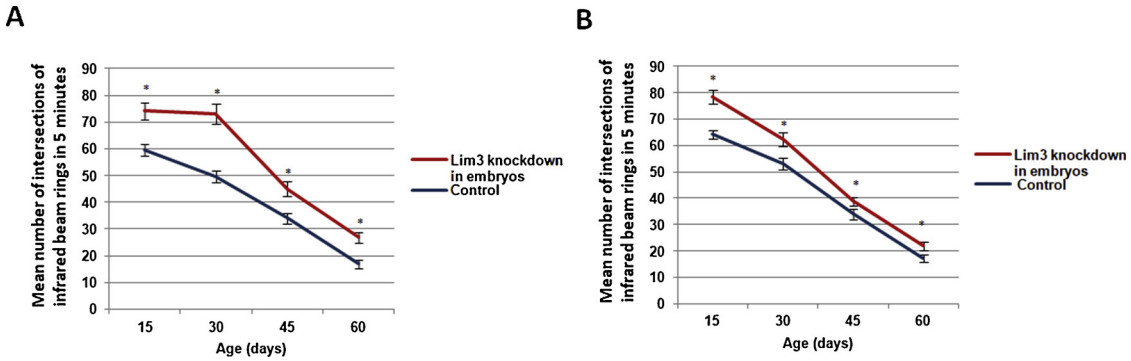

Fig. 10. Locomotion of males (A) and females (B) with *Lim3* knockdown during early stages of *Drosophila* development. Data represent the mean ± s.e. $^{*}P < 0.05$ was determined by the Kruskal-Wallis test. Measurements of locomotion were repeated twice. Graphs were drawn based on the combined data of two replicates. The sample size was 50 flies/sex/genotype/age/replicate.

changes remain unknown.

Interestingly, in flies with *Lim3* knockdown in the nervous system and muscles, which were also characterized by positive effects on locomotion, ATP levels were not affected (Rybina et al., 2017). The nature of such tissue-specific differences in the effects of the *Lim3* depletion remains largely unclear. The obvious suggestion would be that Lim3 targets in the nervous system and muscles were different from the Lim3 targets in embryos and did not include genes involved in the energy metabolism, at least as much as in embryos, and changes in locomotion were determined by other molecular mechanisms. For example, increase in locomotion might be caused by altered expression of genes encoding proteins of ion channels (Hodge, 2009) or transporters (Kume et al., 2005; Parinejad et al., 2016). However, we believe that in flies that experienced *Lim3* deficit early in life elevated Δψm and related ATP levels in muscles but not functional alterations of the nervous system were responsible for the increase in locomotion.

ROS levels could become elevated in embryos with *Lim3* knockdown as a result of changes in the transcription of several genes analyzed in our study.

P450 enzymes are known to participate in different developmental processes and in the detoxification of xenobiotics. They catalyze numerous hydroxylation reactions of a diverse range of substrates, such as fatty acids, fatty acid epoxides and steroid hormones, which determines their influence on the expression of various genes (Chung et al., 2009), and they participate in the control of diverse processes. For example, *Cyp6a20* is associated with aggressive behavior (Dierick and Greenspan, 2006; Wang et al., 2008), and *Cyp4p2* is considered to play an important role in *Drosophila* reproduction (Allen and Spradling, 2008). The cytochrome P450 gene 4 family (CYP4) in parallel with the cytochrome P450 gene 6 family (CYP6) is involved in the ω-oxidation of fatty acids (Hardwick, 2008; Helvig et al., 2004). The members of both of these families (*Cyp4p2, Cyp6a20*) were activated in embryos with *Lim3* knockdown and one of them, *Cyp4p2*, in adults that encountered *Lim3* depletion early in life. On the one hand, the increase in ROS levels could be promoted by cytochrome P450 s (Lewis, 2002). On the other hand, the stimulation of fatty acid oxidation is known to increase ATP levels (Landree et al., 2004; Zhao et al., 2014) and locomotor activity, which is what we observed in adult males with *Cyp4p2* activation.

Increasing ROS levels should be detoxicated by the antioxidant defense system. Several different antioxidant enzymes provide the first line (phase I) of defense against ROS. The most important among them are superoxide dismutase (Sod) and catalase (Cat). We did not observe any significant decrease in *Sod1* or *Cat* expression, which could cause an increase in ROS amounts in embryos with *Lim3* knockdown. However, we found a significant decrease in the expression of genes encoding enzymes providing the second line (phase II) of defense against ROS, such as glutathione-S-transferases (*GstD8, GstE4*), which indicated a substantial deterioration of the antioxidant defense system in embryos with *Lim3* knockdown.

ROS appear to be essential for a wide range of innate immune functions, including antiviral, antibacterial, and antiparasitic responses (Sena and Chandel, 2012; Ferreiro et al., 2012). We revealed changes in the expression of several genes belonging to the defense response group (Fig. 4B), including genes involved in antibacterial humoral responses and genes with serine endopeptidase activity (Supplementary Table 5). Most of these genes are indirect *Lim3* targets, which confirms that *Lim3* does not affect the expression of defense response genes directly but, probably, due to its impact on ROS production and maintenance. The upregulated Jonah gene family (*Jon25Bi, Jon65Aiv*), which consists of serine proteases and belongs to the serine endopeptidase activity group, was shown to be involved in proteolysis, which is essential for immune defense (Lopez et al., 2018). Interestingly, one of these genes (*Jon25Bi*) has been identified as a factor associated with mitochondrial function (Fernández-Ayala et al., 2010). *Jon25Bi* has been suggested to contribute to aging by facilitating mitochondrial activities that reduce survival (Cho et al., 2011; Zhan et al., 2007). This suggestion is in good agreement with our data. The other upregulated genes associated with antimicrobial humoral activity were *LysS*, *LysD* (Daffre et al., 1994) and *Idgf1,* which is known to be upregulated in response to septic injury (De Gregorio et al., 2001). *Idgf1* has been identified as a candidate marker of aging (Doroszuk et al., 2012; Lai et al., 2007; Landis et al., 2004; Pletcher et al., 2005). Increasing oxidative stress can also induce defense responses through the activation of genes participating in chitin metabolic processes (Fig. 4B). These genes are associated with the function of the peritrophic matrix, a semipermeable layer composed of chitin and glycoproteins that lines the insect intestinal lumen. It functions as an immune barrier against pathogens (Kuraishi et al., 2011). It has been recently proposed that the upregulation of immune genes in *Drosophila* might be related to inflammation and other immunological reactions (Chung et al., 2006), which could eventually lead to shortening of *Drosophila* lifespan, as we also observed in our study.

ROS are highly cytotoxic. They can cause impairment of protein function, peroxidation of lipids and damage to DNA. We found that genes involved in DNA repair were downregulated in embryos with *Lim3* knockdown, suggesting that the repair of DNA damage may be not effective. Furthermore, the inability of the antioxidant and DNA repair systems to defend cells from ROS damage leads to the induction of apoptosis through the activation of mitochondrial apoptotic signals (Circu and Aw, 2010; Redza-Dutordoir and Averill-Bates, 2016). One of the main regulators of apoptosis is Grim, which induces apoptosis through the activation of the caspase pathway (Chen et al., 1996; Clavería et al., 2002). *Grim* was upregulated in adult *Drosophila* during aging (Zheng et al., 2005). Overexpression of *grim* led to shortening of lifespan (Bedoukian et al., 2009). In our study, *grim* was one of the *Lim3* targets that was upregulated in embryos with *Lim3* depletion (Fig. 5, Supplementary Table 4). Our results are in line with other data on the

role of *grim* in lifespan control. We suggested that *Lim3* can affect lifespan due to the induction of high ROS levels, which results in proapoptotic activity causing a decrease in lifespan.

Redox and ATP-related groups of genes were mainly composed of indirect Lim3 targets, suggesting that Lim3 mostly regulates Δψm and related ATP, ROS production indirectly through other genes. The direct Lim3 target from these groups, *Pkg21D*, which encodes a protein kinase, cGMP-dependent (PKG), was upregulated in embryos with *Lim3* depletion and stayed upregulated in adults that encountered *Lim3* depletion during early development. PKG utilizes ATP to phosphorylate its target proteins, which are involved in the signaling pathways associated with cell migration, axon guidance, cytoskeletal remodeling and muscle development (Patel et al., 2012). PKG has a high homology to another *Drosophila* PKG, DG2 (Kalderon and Rubin, 1989). Consistent with our results, *dg2* activation was shown to be associated with the increased locomotion of adult flies (Osborne et al., 1997).

PKG also activates Na^+/K^+-ATPases (McKee et al., 1994). In our study, we demonstrated that *nrv3*, a gene encoding the Na^+, K^+-ATPase β-subunit, was upregulated both in embryos with *Lim3* knockdown and in adults that encountered *Lim3* depletion early in life. This ATPase is known to be a plasma membrane enzyme of the nervous system. The gene *nrv3* couples ATP hydrolysis with Na + and K + exchange against their respective chemical gradients, which provides energy for different cellular functions, e.g., for the cation-coupled uptake of various molecules (glucose, glutamate and other neurotransmitters), to ensure mitochondrial motility in neurons (Baumann et al., 2010; Zhang et al., 2010). Na^+/K^+-ATPase also interacts with EAAT1 (Hayashi and Yasui, 2015), encoded by *Eaat1,* which is upregulated in embryos with *Lim3* knockdown. EAAT1 is an excitatory amino acid transporter that regulates the extracellular glutamate level. Inactivation of EAAT1 resulted in a shortened lifespan (Rival et al., 2004). *Eaat1* expression in the *Drosophila* nervous system was also shown to be essential for larval locomotion (Stacey et al., 2010). Na^+/K^+-ATPase was also shown to specifically regulate the expression and localization of the nicotinic acetylcholine receptor (nAchR) (Doi and Iwasaki, 2008). Na^+/K^+-ATPase and nAChR functionally interact to provide effective synaptic transmission and to control neural network activity (Heiny et al., 2010). *nAChRalpha1,* encoding one of the nAchR subunits, is downregulated in embryos with *Lim3* knockdown and upregulated in adults that experienced *Lim3* depletion during early development.

It is well known that *Lim3* is a key regulator of neuron development (Thor et al., 1999). However, although *Lim3* misexpression in the nervous system, muscles (Rybina et al., 2017) and at the early stages of development (this paper) affected lifespan, we failed to find any accompanying changes in the nervous system in all cases. Moreover, we found that naturally occurring polymorphisms located in the *Lim3* 5′-regulatory region and significantly associated with variation in lifespan affected *Lim3* expression in embryos, larvae, carcasses and gonads but not in the brains of both male and female larvae and adults (Rybina et al., 2018). Our data on Lim3 targets also did not allow us to specifically and reliably determine the main neuronal pathways by which *Lim3* might control survival and aging. In this paper, we used a restricted number of tests to assess the properties of the nervous system. We believe that a separate, systematic, carefully thought out analysis is needed to further study the neuronal role of *Lim3* in the lifespan control. With this regard, our data combined with published information on the interactions between Na^+/K^+-ATPase and other genes in the nervous system can provide us with preliminary insights into the molecular mechanisms underlying the neuronal role of *Lim3* in lifespan control and help in sorting out this issue.

Identifying genetic mechanisms that control lifespan is important for the ability to manipulate the rate of aging. Our data demonstrated that *Lim3* expression early in *Drosophila* development can affect the lifespan of adult flies. This effect was associated with alterations in the transcription of genes involved in mitochondrial function, ATP-related activity and redox processes. Due to changes in the transcription of genes involved in ROS induction and antioxidant defense, ROS was affected. The increase in ROS levels could most likely be a cause of changes in the transcription of genes involved in the defense response (proteolysis, which is essential for immune defense; antimicrobial humoral activity; immune barrier against pathogens), which are known to be involved in lifespan control. Our data showed that *Lim3* knockdown during early *Drosophila* development affected mitochondria in adult males, demonstrating the causal relationship between the *Lim3* expression and mitochondrial function. In addition, the transcription of genes participating in DNA repair, apoptosis and neuron development and function was affected by *Lim3* depletion early in life. Our results underscore the complexity and interdependence of mechanisms controlling lifespan.

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The authors declare the absence of any conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mad.2019.111121.

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