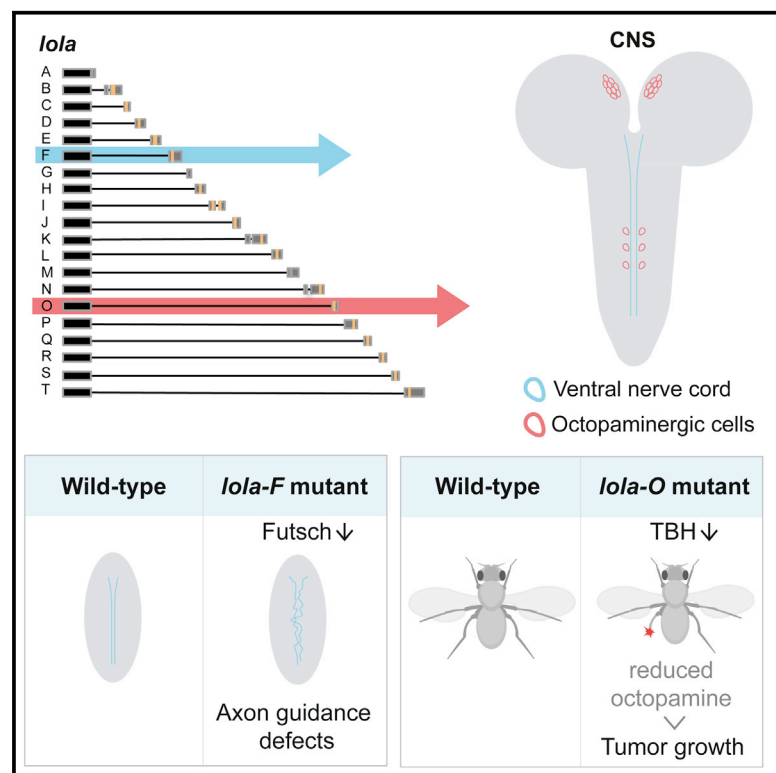


Cell Reports

Comprehensive Characterization of the Complex *lola* Locus Reveals a Novel Role in the Octopaminergic Pathway via Tyramine Beta-Hydroxylase Regulation

Graphical Abstract



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In Brief

lola is among the most complex loci in *Drosophila*, playing important roles in axonal pathfinding and neural reprogramming. Using a targeted CRISPR/Cas9 mutational screening of every *lola* isoform, Dinges et al. revisit Lola functions, and they identify a critical role for one specific variant in the octopaminergic pathway.

Highlights

- A systematic mutational screen of every Lola isoform
- Lola-F is the main isoform controlling axonal pathfinding at the ventral midline
- Lola-O prevents neurodegeneration via regulation of the octopaminergic pathway
- Lola-O is required for positive regulation of *Tyramine beta-hydroxylase*

Data and Software Availability

GSE97836



Comprehensive Characterization of the Complex *lola* Locus Reveals a Novel Role in the Octopaminergic Pathway via Tyramine Beta-Hydroxylase Regulation

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SUMMARY

Longitudinals lacking (lola) is one of the most complex genes in *Drosophila melanogaster*, encoding up to 20 protein isoforms that include key transcription factors involved in axonal pathfinding and neural reprogramming. Most previous studies have employed loss-of-function alleles that disrupt *lola* common exons, making it difficult to delineate isoform-specific functions. To overcome this issue, we have generated isoform-specific mutants for all isoforms using CRISPR/Cas9. This enabled us to study specific isoforms with respect to previously characterized roles for Lola and to demonstrate a specific function for one variant in axon guidance via activation of the microtubule-associated factor Futsch. Importantly, we also reveal a role for a second variant in preventing neurodegeneration via the positive regulation of a key enzyme of the octopaminergic pathway. Thus, our comprehensive study expands the functional repertoire of Lola functions, and it adds insights into the regulatory control of neurotransmitter expression *in vivo*.

INTRODUCTION

lola is among the most complex loci in *Drosophila*, giving rise to at least 80 different mRNA isoforms through alternative *cis*- and *trans*-splicing as well as via multiple promoter activity (Figure 1A) (Goeke et al., 2003; Horiuchi et al., 2003; Ohsako et al., 2003; Zhang et al., 2003). In total, *lola* encodes 20 known protein isoforms (Lola A–Lola T) that contain a common N-terminal BTB domain, with 17 isoforms encoding a unique zinc-finger motif in their C-terminal variable exons (Figure 1B). Lola has been shown to act as a transcription factor with regulatory roles in axon growth and guidance during embryogenesis, and it is also required for maintaining neurons in a differentiated state of the developing brain (Giniger et al., 1994; Goeke et al., 2003; Southall et al., 2014). In addition, Lola has been found to control stem cell maintenance and germ cell differentiation in the *Drosophila* testis, programmed cell death during oogenesis,

and gonad formation in early embryo (Bass et al., 2007; Davies et al., 2013; Tripathy et al., 2014).

Most experiments investigating Lola function utilized loss-of-function alleles containing mutations in the N-terminal constitutive region, which affect all 20 Lola isoforms and give pleiotropic effects *in vivo* (Giniger et al., 1994; Madden et al., 1999; Crowner et al., 2002; Goeke et al., 2003; Horiuchi et al., 2003; Spletter et al., 2007; Zheng and Carthew, 2008; Gates et al., 2011; Fukui et al., 2012; Davies et al., 2013; Southall et al., 2014). Studies of specific isoform mutant alleles, which are available only for Lola-K, -J, -L, and -T, revealed functions in distinct physiological processes (Goeke et al., 2003; Bass et al., 2007; Davies et al., 2013; Tripathy et al., 2014; Peng et al., 2016). Isoforms Lola-K and Lola-L are both involved in two unrelated mechanisms, which include motor nerve development and germline stem cell maintenance in the male testis, indicating that at least some Lola isoforms control multiple functions during development (Goeke et al., 2003; Davies et al., 2013).

To functionally characterize Lola isoforms, we generated mutations using CRISPR/Cas9 for all 20 *lola* isoforms by respectively targeting the isoform-specific C terminus. Among the 20 *lola* mutant strains, five are homozygous lethal during early development while three exhibit clear defects in adult flies. We demonstrate that mutations targeting *lola-F* result in severe disruption of axonal tracts at the embryonic ventral midline. We found that Lola-F regulates the expression of several axonal guidance genes, including the microtubule-associated factor Futsch. Furthermore, flies deficient for *lola-A* and *lola-H* display severe locomotion phenotypes. Finally, *lola-O* mutant flies are viable but display a strong degeneration phenotype due to a defective octopaminergic pathway. Lola-O is specifically expressed in the subset of neurons that produce octopamine (OA) and regulates its biogenesis by controlling the expression of *Tyramine beta-hydroxylase (Tbh)*, which encodes a key enzymatic component of this pathway. Together, our data provide a comprehensive functional characterization of Lola isoforms, revealing novel roles for previously uncharacterized isoforms.

RESULTS

CRISPR/Cas9-Induced *lola* Isoform-Specific Knockout

To comprehensively characterize Lola isoforms *in vivo*, we generated knockout (KO) flies for each isoform using the



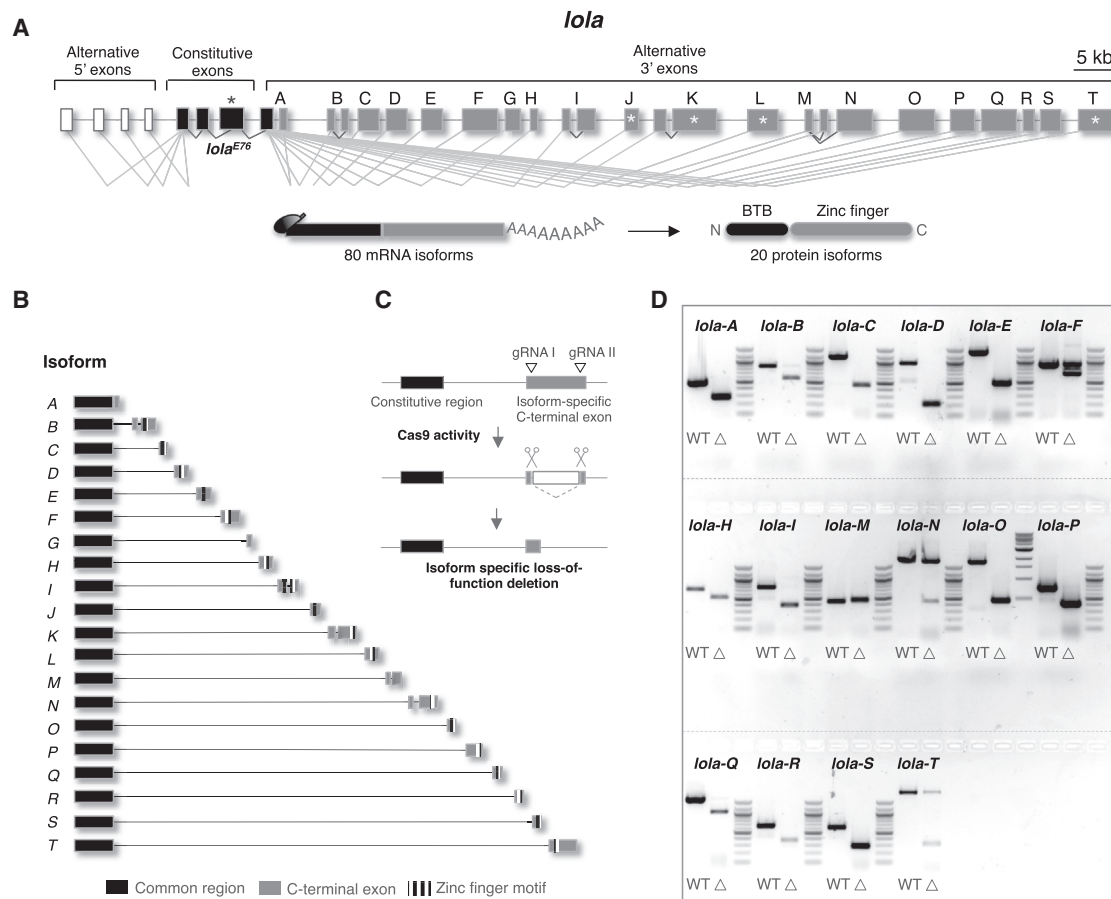


Figure 1. CRISPR/Cas9-Induced *lola* Isoform-Specific Knockout

(A) Schematic structure of the *lola* locus. *lola* comprises 32 exons including 5' UTR exons (white boxes), constitutive exons (black), and 3' alternative exons (gray). 80 putative transcripts encode for 20 protein isoforms, each sharing an N-terminal BTB domain but holding isoform-specific exons encoding for a C-terminal zinc-finger domain in 17 isoforms. Previously characterized mutations are marked by an asterisk.

(B) Scheme of the 20 *lola* isoforms (Lola-A–Lola-T). Stripes highlight zinc-finger motifs.

(C) CRISPR/Cas9 approach to systematically mutate each *lola* isoform.

(D) Gel analyses of established *lola* mutants. Agarose gel shows amplicons of respective genomic regions in wild-type (WT, left lane) and CRISPR/Cas9-induced *lola* KO flies (Δ, right lane). Mutation in *lola*-M resulted in partial duplications that disrupt the frame. Mutants for *lola*-G, -J, -K, and -L bear a frameshift and are not displayed. Lethal alleles are heterozygous and show both the mutant and the wild-type allele.

See also Figures S1 and S2.

CRISPR/Cas9 system. In brief, two distinct guide RNAs (gRNAs) were designed to target the isoform-specific C-terminal exon of each *lola* isoform (Figure 1C). In most cases, Cas9 activity resulted in the production of two double-strand breaks, leading to deletion of the sequence between the two Cas9 target sites and resulting in a loss-of-function mutation for the respective *lola* isoform (Figures 1D and S1).

Our targeted screen resulted in KO flies for all 20 known *Lola* isoforms, and mutations in 8 of these isoforms led to a clear phenotype (Table S1). Five isoform-specific mutations, including in the already described *lola*-K, -L, and -T as well as in *lola*-F and -N, resulted in lethality during embryonic and early larval stages. Mutations in *lola*-A and -H produced viable flies with impaired locomotion (Figure S2A). Interestingly, *lola*-H mutants displayed reduced locomotion, while *lola*-A mutant flies were hyperactive. Finally, *lola*-O mutants survived until adulthood, but they rapidly

degenerated and died around 2 weeks after hatching. With the exception of *Lola*-H and -A, we found that only isoforms conserved between *D. melanogaster* and *Anopheles gambiae* play important roles during development and in adults (Table S1), raising the question of the relevance of the non-conserved *Lola* isoforms.

Among them, *Lola*-B was previously shown to play a critical role in neural stem cell (NSC) differentiation using RNAi (Neu-müller et al., 2011). Upon depletion of *Lola*-B, NSC proliferation was shown to be drastically reduced. However, our *lola*-B KO flies did not recapitulate this phenotype, suggesting that this effect was due to off-target activity (Figure S2B). In contrast, mutations in the conserved *lola*-L and -K reproduced the reported defects in the innervation of ISNb motoneurons (Figure S2C), as well as the loss of *lola*-T in germ cell migration (Figure S2D). It has been reported that neurons deficient for all *Lola* isoforms

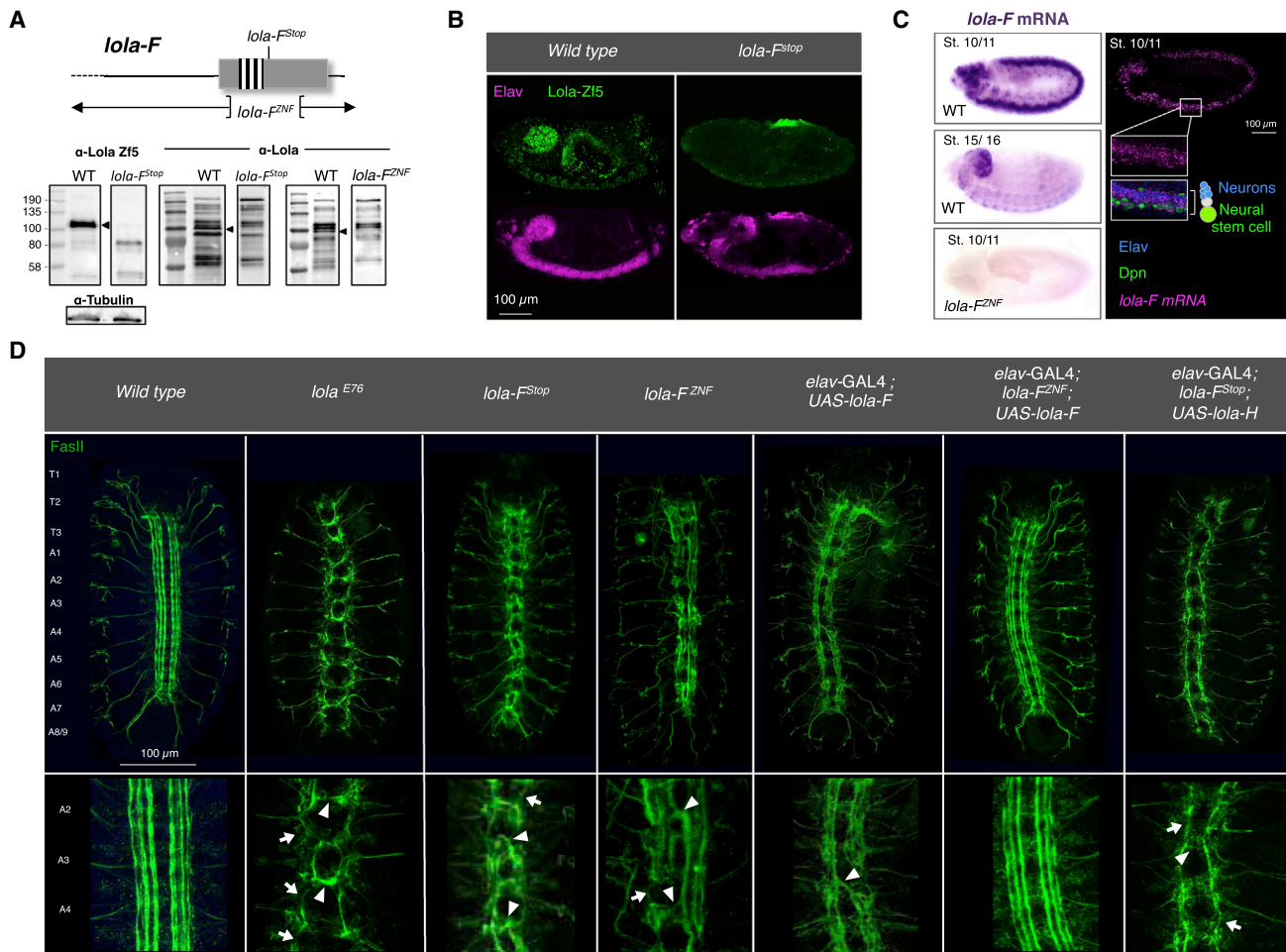


Figure 2. *lola-F* KO Embryos Display Disrupted Axonal Tracts

(A) (Top) Schematic of *lola-F* genomic region. *lola-F^{Stop}* bears a 2-bp deletion resulting in a premature stop codon downstream of the zinc-finger domain. *lola-F^{ZNF}* lacks the entire zinc-finger motif. (Bottom) Immunoblotting using the anti-Lola Zf5 and anti-Lola antibody on control and *lola-F* KO lysates of stage 15 embryos is shown. Arrowheads depict a band specific for Lola-F.

(B) Anti-Lola Zf5 immunostaining (green) on stage 13 embryos. Lola-F protein is undetectable in *lola-F^{Stop}* embryos.

(C) *In situ* hybridization shows *lola-F* mRNA (magenta) enrichment in the nervous system, which is absent in *lola-F^{ZNF}* embryos. *lola-F* mRNA co-localizes with both the neuronal marker Elav (blue) and NSC marker Dpn (green, lateral view, stage 10/11).

(D) Immunostaining of the VNC using an anti-Fasciclin II antibody (green). *lola-F* mutants display strong midline crossing of axons (arrowheads), while *lola^{E76}* embryos show more severe axon growth defects (arrows). Neuronal *lola-F* expression in wild-type disrupts axonal tracts, but it is sufficient to rescue the phenotype in a *lola-F* mutant background (stage 15/16 embryos, ventral view). Ectopic *lola-H* expression is unable to rescue the axonal defects in *lola-F^{Stop}* mutants.

See also Figures S2 and S3.

dedifferentiate into NSCs in the developing nervous system (Southall et al., 2014) and that Lola-N is able to rescue this phenotype. We found that depletion of *lola-N* in the larval or adult brain by mosaic analysis with a repressible cell marker (MARCM) did not alter the differentiation state of postmitotic neurons (Figure S2E; data not shown), hinting to possible functional redundancy between isoforms might also prevent us from uncovering additional roles for Lola. Consistent with this, reducing the level of both Lola-L and Lola-F was recently shown to alter NSC number, while reduction of either one alone had no effect (Wissel et al., 2016).

Lola-F Is the Main Isoform Required for Axon Guidance in the Developing Embryo

We generated two different *lola-F* alleles that were both lethal during late embryogenesis (Figures 1D, 2A, and S1). One allele specifically lacks the zinc-finger motif (*lola-F^{ZNF}*), whereas the second one carries a 2-bp deletion downstream of the zinc-finger domain (*lola-F^{Stop}*), leading to a frameshift and premature stop codon. We confirmed the depletion of Lola-F protein in both homozygous mutant embryos, while the overall levels of other isoforms were virtually unchanged or only slightly reduced (Figure 2A). Immunostaining using a Lola-F-specific antibody further confirmed the absence of Lola-F protein in *lola-F^{Stop}* mutant

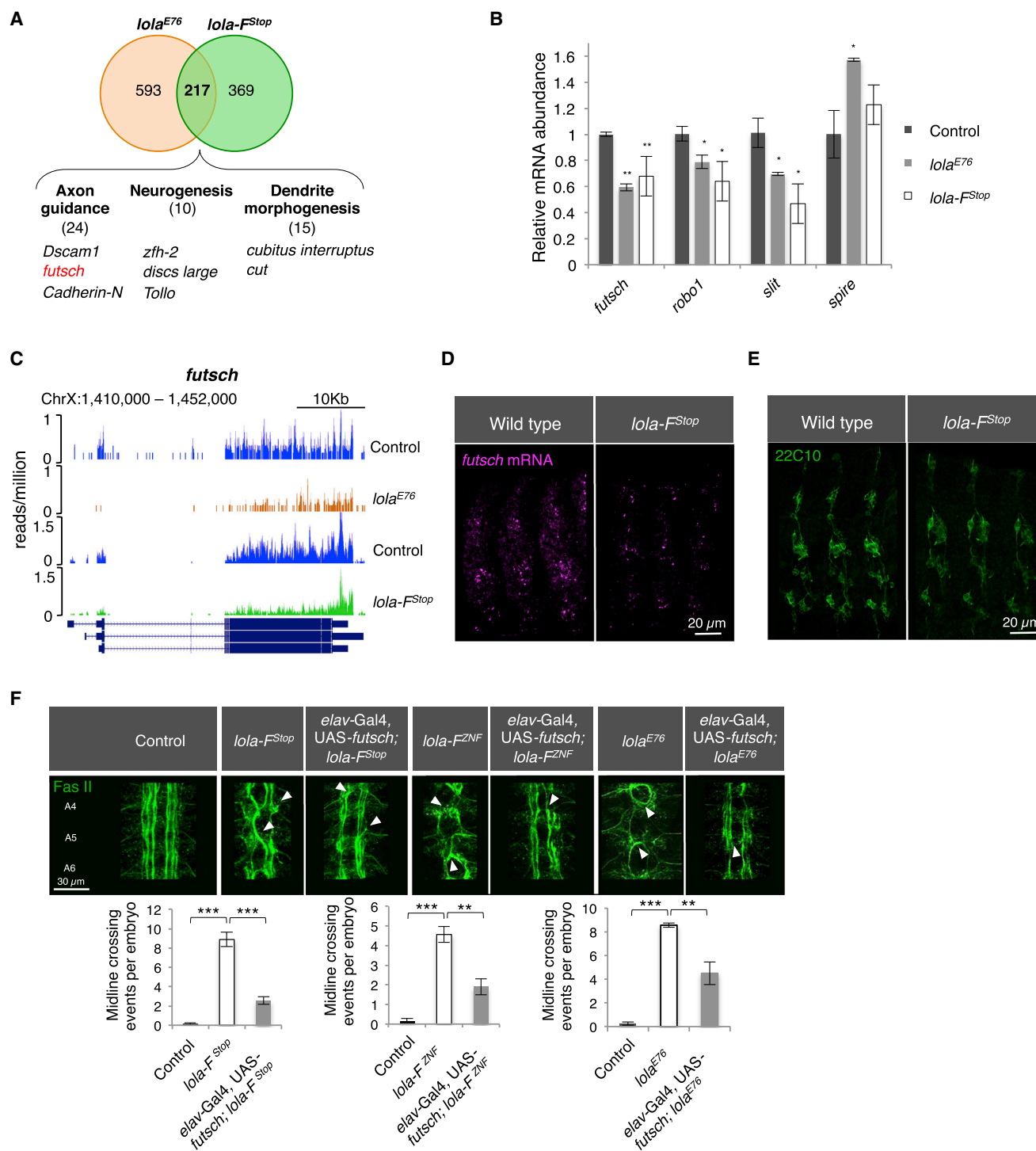


Figure 3. Lola-F Regulates Neuronal Projection by Activation of Axon Guidance Genes

(A) Transcriptome analysis of *lola-F^{Stop}* and *lola^{E76}* embryos reveals 217 commonly downregulated genes. Highlighted are examples of shared target genes involved in neuronal development.

(B) qRT-PCR for selected genes on *lola^{E76}* and *lola-F^{Stop}* mutant RNA extracted from stage 15 embryos. ANOVA t test was performed (**p < 0.01 and *p < 0.05). Data are represented as median ± SD from three technical replicates.

(C) Track example of poly-A-selected RNA-seq at the *futsch* locus.

(D) *In situ* hybridization reveals reduced *futsch* mRNA (magenta) in *lola-F^{Stop}* embryos (stage 15, lateral view).

(E) Anti-22C10 immunostaining (green) detects reduced Futsch levels in *lola-F^{Stop}* mutants (stage 15 embryo, lateral view).

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embryos (Figure 2B). Moreover, expression of a *lola* genomic construct rescued the lethal effect of both mutants, confirming the specificity of our alleles (data not shown). Expression analysis by *in situ* hybridization revealed a strong enrichment of *lola-F* mRNA in the developing CNS, which was lost in *lola-F^{ZNF}* embryos (Figure 2C). More precisely, *lola-F* localized in both NSCs and differentiated neurons, suggesting possible functions during neurogenesis. At late embryogenesis and in larvae, *lola-F* RNA expression became mainly restricted to NSCs (Figures 2C and S3A).

To address the role of Lola-F, we stained control and *lola-F* KO embryos with anti-Fasciclin II to label axon tracts of stage 15 embryos (Figure 2D). The loss of all Lola isoforms was previously shown to impair axonal growth and pathfinding at the ventral midline, a phenotype we could recapitulate using the *lola^{E76}*-null mutant allele. Remarkably, depletion of Lola-F also resulted in severe disruptions of the ventral nerve cord (VNC). Compared to the null mutant, axons extended slightly further but several crossing defects were observed. The number of neurons, NSCs or glial cells, was, however, unaffected (Figures S3B and S3C). Both alleles and transheterozygous combinations gave indistinguishable phenotypes (Figure 2D; data not shown), and expression of a *lola*-BAC was sufficient to rescue the axonal guidance defects (data not shown), confirming the specific role of this isoform in this process. We further generated a transgenic line expressing Lola-F under the control of UAS promoter. Strikingly, neuronal Lola-F expression in wild-type embryos disrupted the VNC, mimicking the *lola-F* mutant phenotype (Figure 2D). However, neuronal Lola-F cDNA expression in *lola-F* KO embryos completely restored the axonal guidance defects, which was not the case upon neuronal expression of Lola-H or Lola-O cDNA (Figure 2D; data not shown). Altogether, these data indicate that Lola-F plays a major role in establishing axonal guidance at the ventral midline, and they suggest that its physiological levels must be tightly controlled to ensure its correct function.

We next wondered whether other isoforms that are essential during embryonic development were also involved in controlling axonal pathfinding. As previously shown by *in situ* hybridization (Goeke et al., 2003), we confirmed by RNA sequencing (RNA-seq) of neuronal sorted cells from stage 15/16 embryos that most isoforms were expressed in neurons. Lola-A, -F, -H, -K, and -N were most strongly expressed, while Lola-J, -M, -O, and -B were barely detectable (Figure S4A). Lola-K and -L were already shown to control muscle innervation by ISNb motoneurons, a phenotype we could recapitulate; we also could demonstrate that Lola-F plays a similar function (Figure S2C). However, neither the depletion of Lola-K and -L nor the absence of Lola-N and -T altered axon guidance at the ventral midline (Figure S2F). Only *lola-K* mutants occasionally displayed crossing defects between the A5 and A6 hemisegments. Hence,

our data indicate that Lola-F is the major isoform required in axonal pathfinding at the ventral midline for which *lola* is named.

Lola-F Activates Expression of Several Genes Involved in Axon Guidance

Previous studies reported the involvement of Lola in axon guidance, in part by upregulating the levels of the repulsive ligand Slit and its receptor Robo at the ventral midline (Crown et al., 2002), while its effect on axon growth could partly be explained by downregulation of the actin nucleation factor Spire (Gates et al., 2011). To get further insights into the mechanisms by which Lola controls axon growth and guidance, we took advantage of our specific *lola-F^{stop}* allele to perform transcriptome analysis at stage 15, when the majority of axon guidance events are taking place. We also included samples from *lola^{E76}*-null mutant embryos to compare the affected genes with the absence of *lola-F*. We found that 465 and 586 genes were significantly upregulated and downregulated in *lola-F* KO embryos, respectively (adjusted p value < 0.01). Intriguingly, gene ontology (GO) term analysis revealed enrichment for genes involved in cell adhesion and axon extension specifically for downregulated genes, underlining the role of Lola-F in regulating axonogenesis (Figures 3A and S5A). 217 (37%) downregulated genes were mutually found in the *lola*-null mutant, including the previously described targets *slit* and *robo1* (Figures 3A, 3B, S5B, and S5C). However, in contrast to the null allele, *lola-F* mutants showed no significant change in *spire* expression (Figures 3B and S5D), indicating that other isoforms must control its level. The absence of *spire* regulation by Lola-F might account for the milder effect observed on axon growth compared to the complete loss of *lola* (Figure 2D). Furthermore, potential antagonistic isoform-specific Lola functions might nullify each other's effect, thus explaining that a subset of genes affected in the *lola-F* mutant is not present in the null allele.

Among the top 15 downregulated genes in both *lola^{E76}* and *lola-F^{stop}* mutants is *futsch*, a well-characterized and conserved axon guidance gene (Figure 3A). The axonal defects observed for *lola-F* KO embryos mimicked the previously characterized *futsch^{K68}* mutant phenotype, as embryos showed disrupted VNC motoraxons and stalling of the peripheral ISNb motoneurone (Hummel et al., 2000; Figure S2C). The reduction in *futsch* levels was confirmed by qRT-PCR and *in situ* hybridization (Figures 3B–3D). Immunostaining using a Futsch-specific antibody on *lola-F^{stop}* embryos showed an overall reduced fluorescent intensity, indicating that the protein level was also decreased (Figure 3E). To examine whether the effect on *futsch* expression in the absence of Lola-F activity contributes to the observed phenotypes, we wondered whether restoring its levels could rescue some of the *lola* defects. To this purpose, an EP element insertion line containing UAS-regulatory elements located in the promoter region of *futsch* was crossed with the neuronal *elav*-GAL4

(F) (Top) Immunostaining of VNC using anti-Fasciclin II antibody (green) to analyze midline-crossing events. Ectopic neuronal Futsch expression partially rescues axonal midline crossing of *lola-F^{stop}*, *lola-F^{ZNF}*, and *lola^{E76}* embryos. Arrowheads indicate axon guidance defects. (Bottom) Quantification of midline-crossing events is shown (n = 9 for *lola-F^{stop}* and *lola^{E76}*; n = 8 for *lola-F^{ZNF}*). Significance was tested using ANOVA one-way t test (***p < 0.001 and **p < 0.01). Data are represented as average ± SEM.

See also Figure S5.

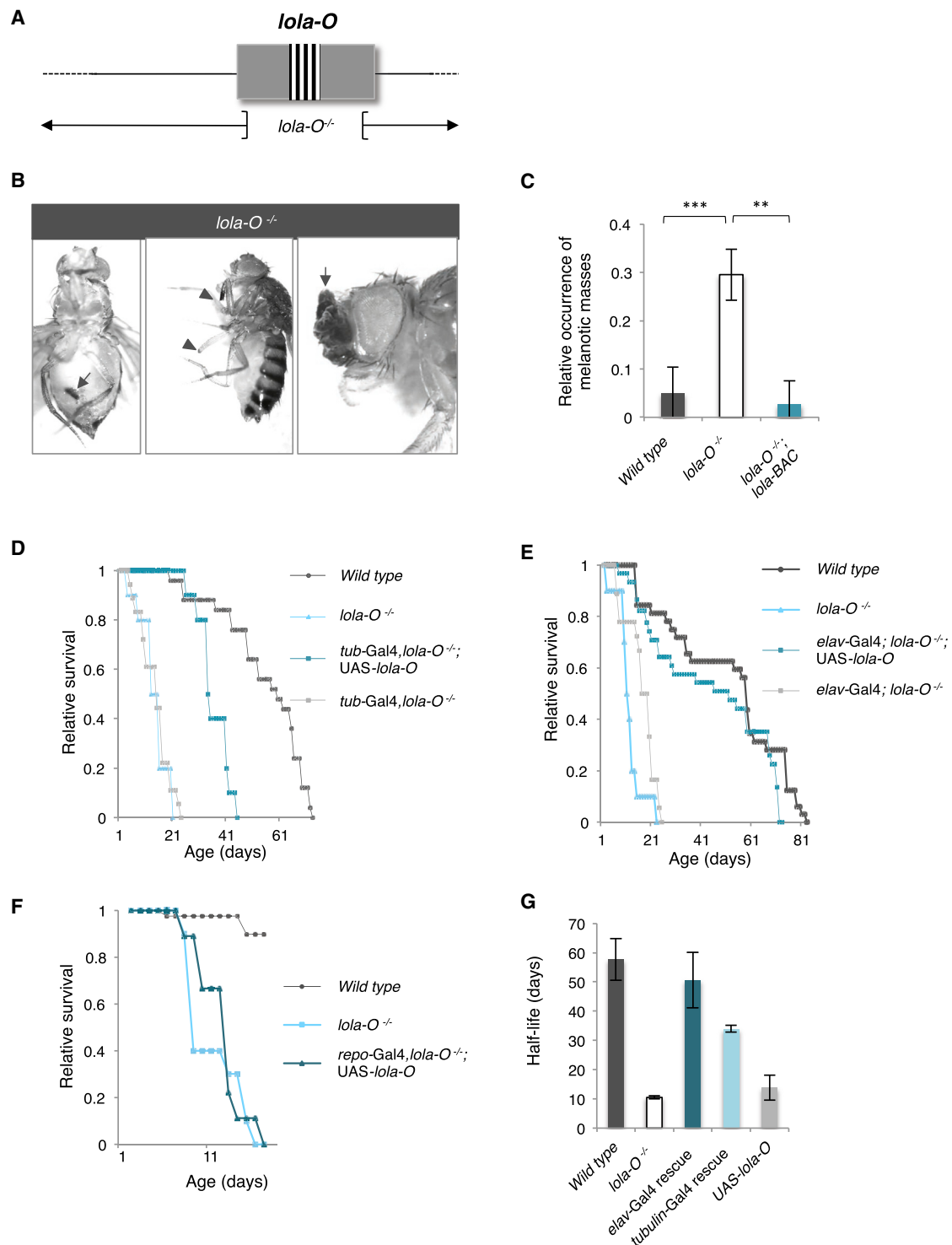


Figure 4. *lola-O* KO Flies Display a Severe Neuronal Degeneration Phenotype

(A) Scheme of the *lola-O* genomic region and expected deletion.

(B) *lola-O* KO adult flies display melanotic masses (arrows), eventually leading to the loss of affected limbs (arrowhead).

(C) Phenotypic penetrance of melanotic masses is reduced to control levels by recombination with a *lola* genomic construct. 10 flies were examined in four replicates at 11 days of age. ANOVA one-way t test was performed (** $p < 0.001$). Data are represented as average $\pm$ SEM.

(D–F) Survival curves of adult *Drosophila*. The average of three biological replicates is shown, and *lola-O* mutants recombined with the driver line serve as a control. (D) Ubiquitous ectopic expression (*Tub-GAL4*) of *lola-O* cDNA elongates the lifespan of *lola-O* mutants by 2-fold. (E) Neuronal *lola-O* expression (*elav-*

(legend continued on next page)

driver line. We found that embryonic progeny derived from this cross displayed ectopic Futsch levels, yet axonal guidance was normal (Figures S5E and S5G). Similar elevated Futsch levels were observed in embryos deficient for Lola-F. Remarkably, this ectopic Futsch expression was sufficient to partially restore both VNC and ISNb axonal defects for both *lola-F* alleles and the *lola*-null mutant (Figures 3F and S5F). It is noteworthy that neuronal overexpression of *lola-F* did not result in elevated *futsch* levels, indicating that ectopic *lola-F* expression alone was not sufficient to transcriptionally activate *futsch* (Figures S5H and S5I). Furthermore, previous findings have shown that Futsch expression is negatively regulated by a splice variant of Tramtrack (Ttk) (Giesen et al., 1997). To analyze whether the observed axon guidance phenotype in *lola-F* mutants was mediated by a change in Ttk levels, we analyzed *ttk* expression in *lola-F^{Stop}* embryos (Figure S5J). We found that both *ttk* isoforms displayed a slight downregulation upon Lola-F depletion, indicating that the observed axon guidance phenotype was not a consequence of increased Ttk levels. Collectively, our findings demonstrate that Lola-F regulates axonal pathfinding by activating numerous axon guidance genes, including the microtubule-associated encoded gene *futsch*.

Lola-O Mutant Flies Display a Severe Degeneration Phenotype

We next investigated the function of Lola-O *in vivo*. We generated one mutant allele that was expected to disrupt the entire zinc-finger domain (Figure 4A). The lack of mRNA expression was confirmed by qRT-PCR and RNA-seq using RNA extracts from control and mutant strains (Figures S6A and S6B). Depletion of *lola-O* results in homozygous viable animals, yet adult flies displayed several abnormalities, including reduced lifespan and severe locomotion defects (Figures S6C and S6D). In addition, females exhibited cuticle malformation and suffered from partial sterility (Figures S6E and S6F). Both males and females also frequently formed melanotic masses (also called pseudotumors), which occurred predominantly on abdomen and limbs (Figures 4B and 4C). Melanotic masses were also occasionally observed in third instar larvae (Figure S6G). Importantly, all these phenotypes could be rescued by a genomic construct restoring *lola-O*, ruling out off-target effects. Likewise, ubiquitous or ectopic neuronal *lola-O* expression was sufficient to restore the lifespan and reduce phenotypic penetrance of *lola-O* KO flies (Figures 4D, 4E, 4G, and S6H). In contrast, glial expression had no effect (Figure 4F). Taken together, these findings indicate a neuronal role for Lola-O in regulating multiple physiological functions.

Lola-O Is Specifically Expressed in OA Neurons

To obtain insights into Lola-O function, we sought to address its localization *in vivo*. For this purpose, we took advantage of a fly line carrying a *lola*-BAC encoding a Lola-O-GFP fusion

protein (Spokony and White, 2012). We found that Lola-O was generally expressed at a very low level in the larval brain, just at the limit of its detection. However, the low Lola-O-GFP expression was refined to only a subset of cells, which included the midline and lateral midline of the ventral ganglion and a few groups of cells in the central brain (Figure 5A). To reveal the identity of these cells, we crossed flies carrying a UAS-GFP transgene reporter with flies expressing GAL4 under the control of various neuronal promoters, and the resulting GFP expression was subsequently compared to the expression pattern of Lola-O. Interestingly, GFP expression driven by the *Tdc2*-GAL4 driver was reminiscent of the expression of Lola-O-GFP (Figure 5B).

Tdc2 stands for Tyrosine-decarboxylase and encodes for an enzyme required for the synthesis of OA (Figure 6A). OA acts as a neurotransmitter, neuromodulator, and neurohormone in insects, and it is involved in diverse physiological functions. Notably, perturbation of its level results in phenotypes reminiscent of the loss of Lola-O, including locomotion defects, cuticle deformity, the appearance of pseudotumors, female sterility, and the shortening of lifespan (Figure 6B; Monastirioti et al., 1996; Stathakis et al., 1999; Saraswati et al., 2004; Li et al., 2016). Its synthesis requires the amino acid tyrosine, which is modified to tyramine by the Tyrosine-decarboxylase 2 (*Tdc2*). Subsequently, Tyramine β -hydroxylase (TBH) hydrolyzes tyramine to produce OA (Figure 6A; Barron et al., 2010; Li et al., 2016).

To confirm the expression of Lola-O in OA neurons, larval brains were stained for both Lola-O-GFP and an antibody that recognizes TBH, which was shown to be a faithful marker of OA cells (Selcho et al., 2012). We found that TBH and Lola-O-GFP co-localized in a cluster of dorsomedial cells in the larval brain and along the midline of the VNC. Single cells lateral of the midline were only positive for Lola-O-GFP (Figure 5C). Therefore, our data suggest that Lola-O function may be linked to the OA pathway. We also performed transcriptome analysis on OA cells sorted via fluorescence-activated cell sorting (FACS) of stage 17 embryos. We found that *lola-A*, *lola-K*, and *lola-L* were strongly expressed, while the read coverage was not sufficient to detect *lola-O* mRNA, confirming that this isoform is expressed at low levels in OA cells (Figure S4B).

Expression of *lola-O* cDNA in OA Neurons Is Sufficient to Rescue Most *lola-O* Mutant Defects

To address a potential role of Lola-O in the OA pathway, we performed a drug-feeding assay in which *lola-O* KO flies were supplied with OA-enriched food. Interestingly, ectopic feeding of OA to *lola-O* mutants was sufficient to elongate the half-life by 2-fold (12 days $\pm$ 2.6 versus 21 days $\pm$ 3.6, *p* value < 0.05), while feeding the same flies with tyrosine had no effect (Figures 6C and 6D). We verified that feeding *Tbh^{nm18}* mutant flies with OA rescues the half-life to a similar extent (Figure 6B). Furthermore,

GAL4) restores longevity of *lola-O* mutant flies to wild-type levels. (F) Glial cell-specific (*repo*-GAL4) *lola-O* expression is unable to rescue premature lethality of *lola-O* KO flies.

(G) Half-life quantification. Half-life is defined as the day with 50% survival. Four biological replicates were analyzed. Data are represented as average $\pm$ SEM. See also Figure S6.

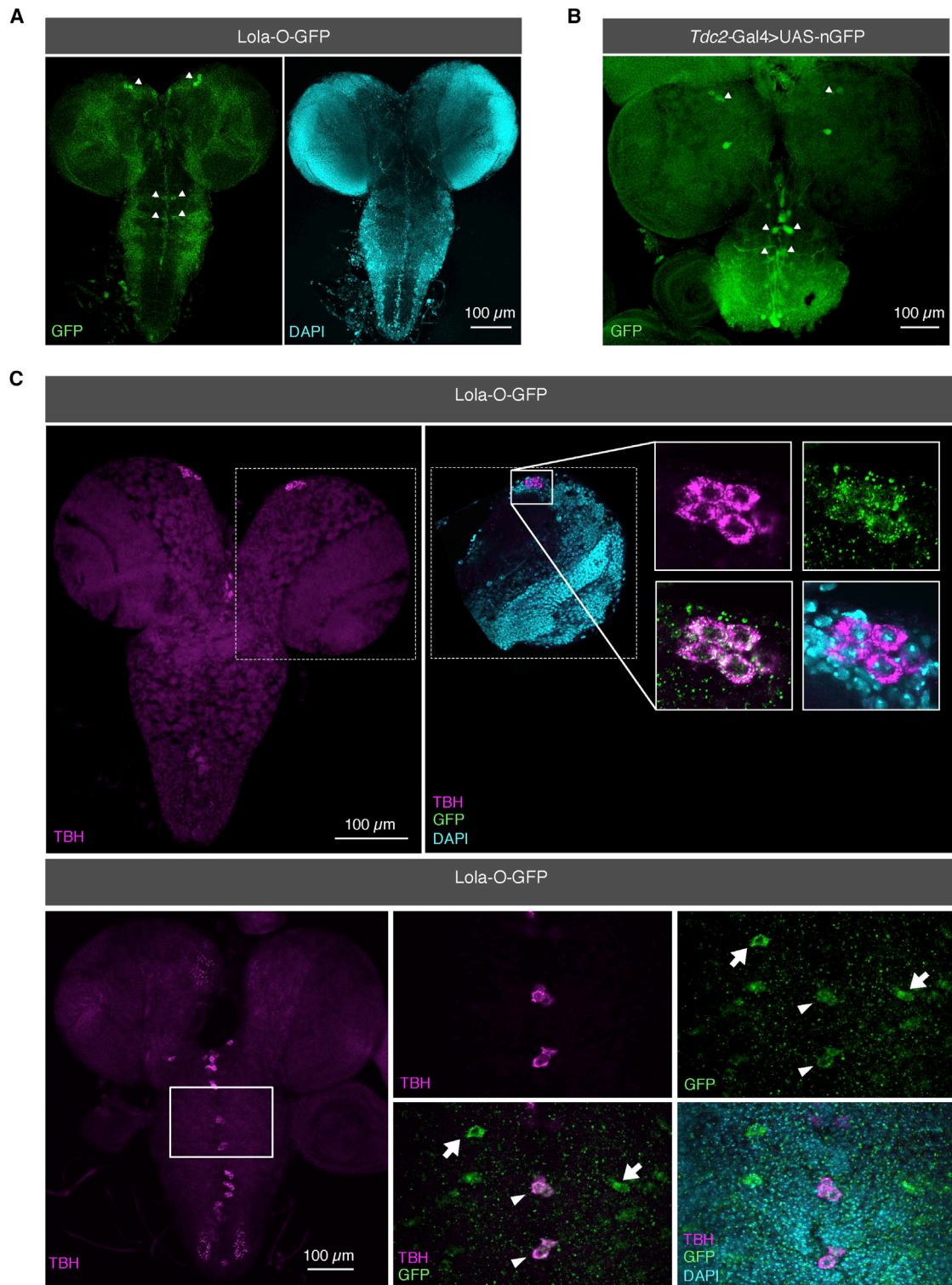


Figure 5. Lola-O Co-localizes with OA Neurons in the Larval Brain

(A) Immunostaining of Lola-O-GFP using an anti-GFP antibody (green; DAPI, cyan).

(B) *Tdc2-GAL4* expression pattern in the larval brain revealed with an anti-GFP antibody (green). OA cells are present in the VNC along the midline and in a subset of cells in the central brain (arrowheads), similar to the Lola-O-GFP expression.

(C) Lola-O (anti-GFP, green) and TBH (magenta) co-localize in the larval central brain and in cells along the VNC (arrowheads). Individual Lola-O-positive cells at the lateral midline show no TBH immunoreactivity (arrows).

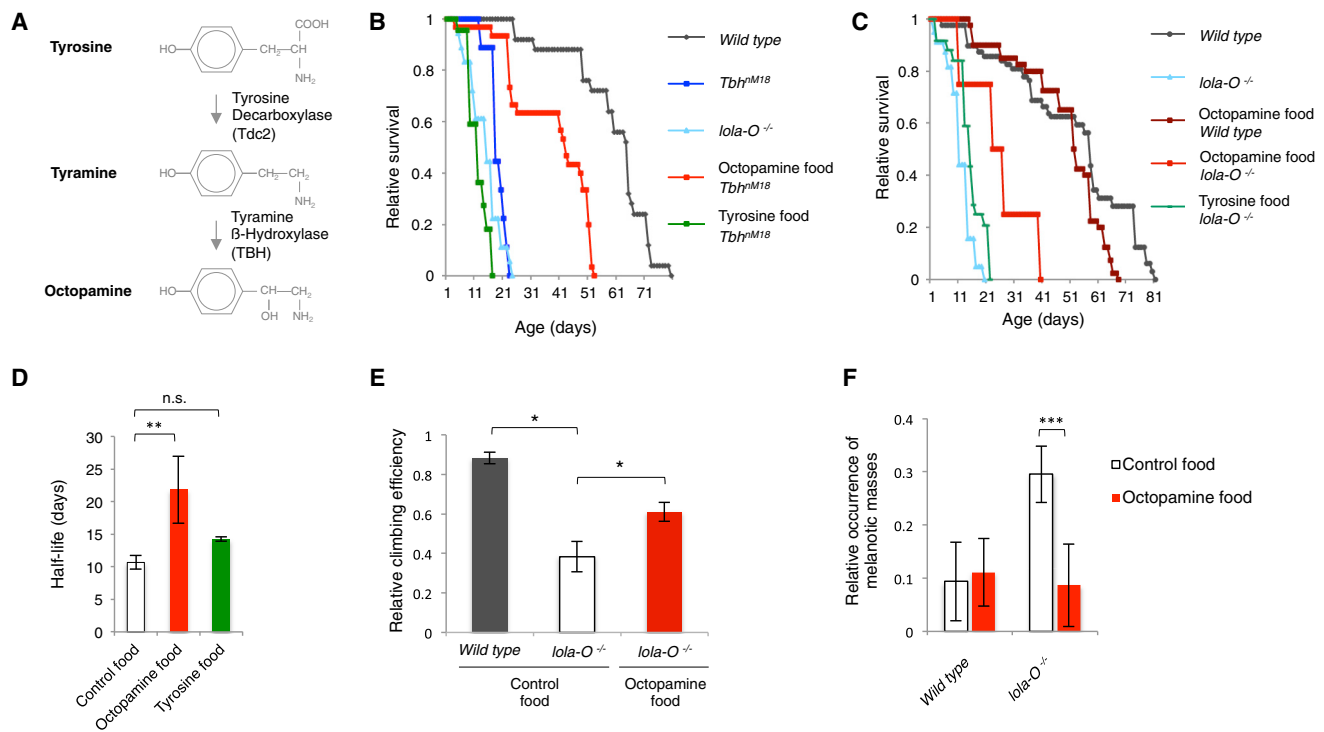


Figure 6. *lola-O* KO Flies Can Be Rescued by Feeding OA

(A) Scheme of the OA pathway.
(B) Longevity is drastically reduced in *Tbh^{M18}* flies. Feeding OA extends the lifespan by more than 2-fold, while supplementing tyrosine has no effect. The average of two biological replicates is shown.
(C) Rearing *lola-O* KO flies on OA-enriched food elongates longevity by 2-fold, while tyrosine feeding has no effect on survival. The average of three biological replicates is shown.
(D) Quantification of the half-life deduced from (C). Feeding OA to *lola-O* mutants increases the half-life from 10.6 to 21.8 days. ANOVA t test was applied (** $p < 0.001$). Data are represented as the average of three biological replicates $\pm$ SD.
(E) Feeding OA to *lola-O* mutants enhances climbing efficiency. Three-day-old male flies were used for locomotion quantification in three biological replicates. ANOVA one-way t test was performed to test statistical significance (* $p < 0.05$). Data are represented as average $\pm$ SD.
(F) 10 freshly hatched males and females were analyzed for melanotic masses at 11 days. ANOVA one-way t test was applied (*** $p < 0.0001$). Data are represented as the average of four biological replicates $\pm$ SEM.
See also Figure S6.

OA-enriched food improved locomotion of *lola-O* mutants, and it reduced the occurrence of pseudotumors close to wild-type levels (9.86% on OA-enriched food compared to 35.71% on control food) (Figures 6E and 6F). Thus, these results strongly suggest that the defects observed in *lola-O* KO flies result from reduced levels of OA.

We reasoned that if Lola-O function was solely required in OA cells, expressing Lola-O specifically in these cells should be sufficient to rescue all defects associated with its loss. To test this hypothesis, UAS-Lola-O flies were crossed with the *Tdc2*-GAL4 driver, which completely restored survival of *lola-O* mutants as well as their climbing ability and the appearance of pseudotumors (Figures 7A, S7A, and S7B). Furthermore, the fertility of mutant flies was also partially rescued, demonstrated by the improved egg-laying rate (Figure S7C). In contrast, expression of either UAS-Lola-F or UAS-Lola-H in OA cells was unable to restore the survival of *lola-O* KO flies (Figure S7D). Altogether, these experiments demonstrate that the primary activity of Lola-O is restricted to OA neurons.

Lola-O Regulates the OA Pathway via the Control of *Tbh* Expression

OA is synthesized via the activity of two enzymes, Tdc2 and TBH. To test whether Lola-O controls their expression *in vivo*, we analyzed transcript levels from RNA isolated from adult heads of control and *lola-O* KO flies by qRT-PCR. Intriguingly, while *Tdc2* expression was unaffected in *lola-O* mutants, the level of *Tbh* mRNA was significantly reduced (Figure 7B). A similar result was observed in a transheterozygous combination in which *lola-O* mutants were crossed with a *lola* deficiency line (data not shown). We also found that TBH protein levels were decreased, as shown by reduced TBH immunoreactivity in brains of *lola-O* KO larvae (Figure S7E). The specificity of the TBH staining was confirmed, as the signal was essentially absent in the brains of *Tbh^{M18}* mutant larvae (Figure S7F). Collectively, these results indicate that Lola-O is required to maintain proper *Tbh* levels.

Given the reduced abundance of *Tbh* in *lola-O* mutants, we wondered whether ectopic expression of *Tbh* cDNA would restore longevity and phenotypic penetrance of these flies. We

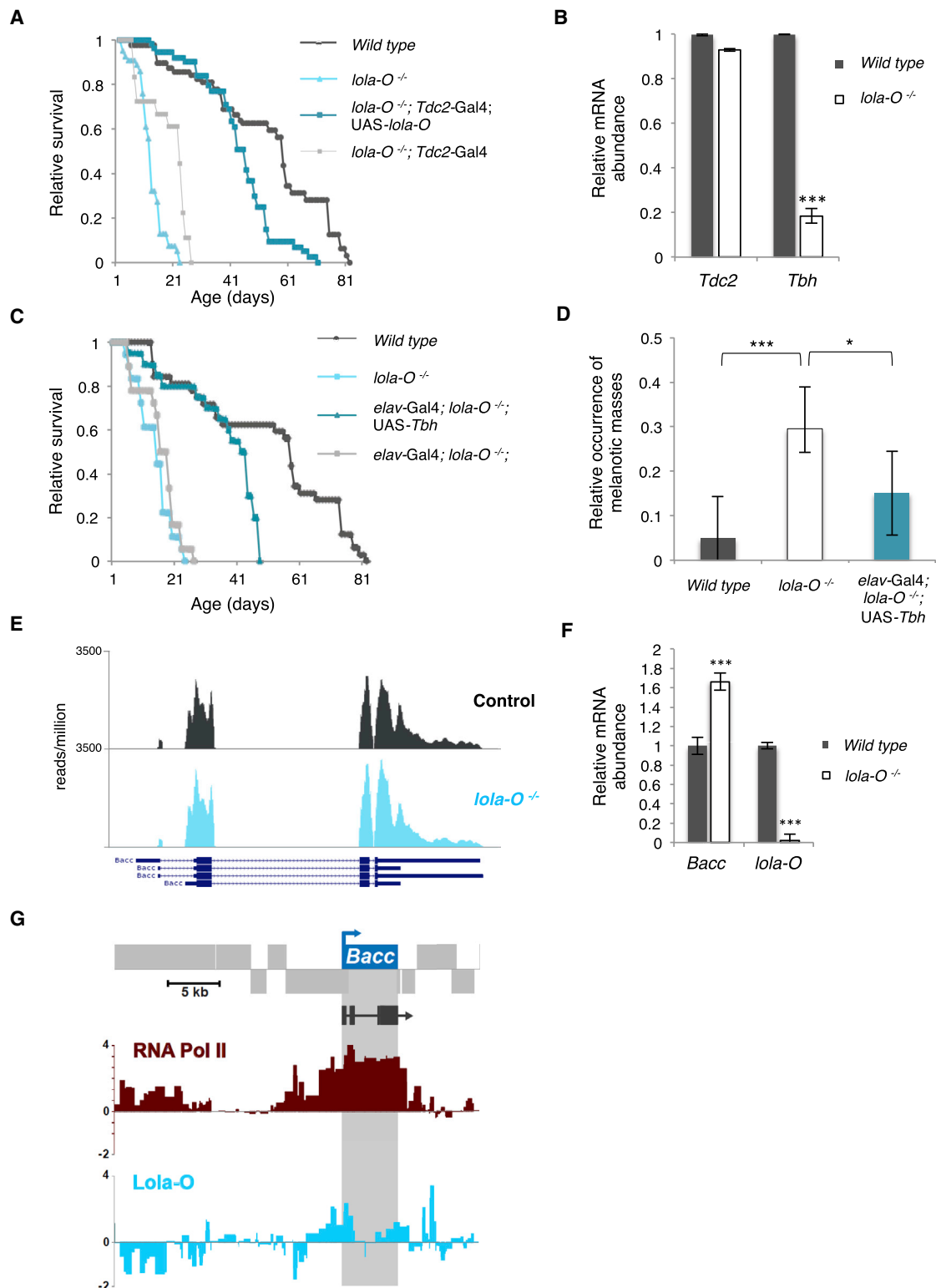


Figure 7. Lola-O Regulates the OA Pathway via the Activation of *Tbh* Expression

(A) Expression of *lola-O* in OA neurons restores wild-type longevity. The average of three biological replicates is shown.

(B) qRT-PCR using mutant RNA extract isolated from freshly eclosed adult male heads. ANOVA one-way t test was performed (**p < 0.001 and *p < 0.05). Data are represented as the median of three technical replicates ± SD.

(legend continued on next page)

generated flies with integrated *Tbh* under the control of a UAS-promoter, and we induced expression using different GAL4 lines. Strikingly, expression of *Tbh* using a neuronal driver line rescued fly survival and significantly decreased the occurrence of pseudotumors (Figures 7C and 7D). These findings therefore demonstrate that Lola-O regulates the OA pathway via the control of *Tbh* expression.

To uncover the transcriptional network regulated by Lola-O, we aimed to identify the Lola-O genome-wide binding sites, and we compared our findings with genes differentially expressed upon Lola-O depletion. For this purpose, we took advantage of the Targeted DamID (TaDa) approach to identify direct target genes in OA neurons (Southall et al., 2013). Expression of N-terminally tagged Dam-Lola-O was induced by the driver line *Tdc2*-GAL4, and embryos were processed at stage 17. The same experiment was repeated with Dam-RNA polymerase II (Pol II) to identify transcriptionally active genes in OA neurons. We found 1,449 genes bound by Pol II and 7,327 by Lola-O, while 905 were shared by Lola-O and Pol II (with a false discovery rate [FDR] of < 0.01). Furthermore, we found an overlap of 32.7% with differentially expressed genes (296) in *lola-O* mutants of the same developmental stage (Figure S7G). While *Tbh* was not among the Lola-O targets (Figure S7H), we identified the gene *Bacc*, which encodes a repressor of *Tbh* (Chen et al., 2013), as directly bound and repressed by Lola-O (Figures 7E–7G). Therefore, our results suggest a model whereby Lola-O regulates TBH levels indirectly, possibly via the repression of *Bacc* expression.

DISCUSSION

Here we describe a comprehensive functional characterization of the different Lola isoforms *in vivo*. Using the CRISPR/Cas9 approach, we generated mutants for every *lola* isoform, and we demonstrated that *lola-F* mutants mimic the characteristic *lola*-null mutant phenotype observed during embryogenesis. We further uncovered a function for Lola in the OA pathway mediated by Lola-O activity via the regulation of *Tbh*. In addition to revealing novel Lola functions, this study demonstrates that the recently developed CRISPR/Cas9 system can be used to systematically address isoform-specific functions *in vivo*.

Assigning Lola Function to Specific Isoforms

Lola is among the most complex loci in *Drosophila*, encoding for 20 different protein isoforms via the usage of 3' alternative exons. Its complete loss of function gives rise to pleiotropic defects *in vivo*, which have been difficult to analyze at the molecular level due to the paucity of specific mutant isoforms. First described in 1993 by Giniger and colleagues, Lola was shown to control axon growth and guidance in both the CNS and peripheral nervous system (PNS) of the *Drosophila* embryo (Giniger et al., 1993). However, it remained unclear how it exerts these functions and

whether these effects depend on a specific Lola isoform or on the activities of multiple ones. Our results clearly establish Lola-F as being the main isoform required in these early developmental processes.

A previous transcriptomic analysis from *lola*-null mutant extracts suggested that Lola controls axonal guidance by fine-tuning the expression of many genes involved in this process, and it is the sum of small changes on many genes that give rise to the severe *lola*-null mutant phenotype (Gates et al., 2011). Nevertheless, several key targets could be identified. For instance, in the CNS, Lola was suggested to repel longitudinal axons away from the midline by increasing the expression of the midline repellent Slit and its axonal receptor Robo (Crowner et al., 2002). In the PNS, Lola controls ISNb axonal growth partially via reducing the expression of the actin nucleation factor Spire (Gates et al., 2011). In spite of Lola-F being involved in both processes, we found that only *slit* and *robo* expression was altered, while *spire* was unchanged. Furthermore, our study revealed *futsch* as being a key target of Lola-F in axon growth. Futsch is a microtubule-associated protein whose expression levels need to be tightly controlled. For instance, heterozygous animals display mutant phenotypes, including slower growth rates and motor system abnormalities, indicating that its dosage is critical for its function (Edelmann et al., 1996). Moreover, its expression in the somatic and visceral musculature is repressed by Tramtrack (Giesen et al., 1997). It is interesting to note that Tramtrack, like Lola, encodes for a zinc-finger and BTB domain-containing protein, suggesting that this class of proteins plays a prominent role in either repressing or activating Futsch expression. Previous studies on several BTB-containing proteins demonstrated that the BTB domain mediates homomeric and heteromeric dimerization and transcriptional repression through the recruitment of diverse co-repressor proteins (Bardwell and Treisman, 1994; Dhordain et al., 1997). Our data, however, indicate that Lola-F is a general activator of neuronal genes. Further interactome studies will, therefore, be necessary to understand how different BTB proteins exert antagonistic molecular functions on gene expression.

The expression of Lola-F drops as *Drosophila* development progresses and becomes primarily restricted to NSCs. This finding suggests that neuronal gene expression outside the NSCs must be maintained by different means, such as via the activity of other transcription factors or via an epigenetic mechanism. Intriguingly, Lola-F was shown to interact early in development with the histone H3S10 kinase JIL-1 (Zhang et al., 2003). Via this molecular activity, JIL-1 maintains euchromatic regions by antagonizing Su(var)3-9-mediated heterochromatin formation. Therefore, it is possible that, during early development, Lola-F establishes an active epigenetic state via its association with JIL-1, and, once established, its function may become dispensable. The remaining Lola-F expression in NSCs appears

(C and D) Neuronal *Tbh* expression (*elav*-GAL4) restores longevity (C) and the occurrence of melanotic masses (D) of *lola-O* KO flies. The average of two biological replicates is shown in (C).

(E and F) Track example of poly-A-selected RNA-seq at the *Bacc* locus. (E) *lola-O* KO embryos show upregulated *Bacc* levels, which were quantified by qRT-PCR in (F). RNA was isolated from stage 17 embryos (20–22 hr AEL). ANOVA one-way t test was performed and data are represented as average $\pm$ SD (* p < 0.05).

(G) Lola-O and Pol II binding to the *Bacc* locus in OA cells. Vertical bars show log2 ratio between the Dam-only and Dam-fusion signal.

See also Figure S7.

important to maintain their differentiation capacity, as the double knockdown (KD) of Lola-F and Lola-L leads to dramatic overproliferation of NSCs in the central brain (Wissel et al., 2016). It will be important to further identify the direct targets of Lola-F and Lola-L in this process to understand how these two isoforms cooperate to allow NSC differentiation.

Connection between Lola and the OA Pathway

Our findings demonstrate a regulatory role for Lola-O in the OA pathway by regulating *Tbh* expression, which encodes an enzyme required for the synthesis of OA, a monoamine that acts as a neurohormone, neuromodulator, and neurotransmitter (Nathanson, 1979). Monoamine neurotransmitter levels are usually tightly regulated, as their misregulation can lead to a wide range of disorders in humans (Ng et al., 2015). Accordingly, several lines of evidence indicate that the absolute levels of OA must also be tightly controlled. For instance, reducing its levels leads to diverse defects, including female sterility, locomotion, aggressiveness, and pseudotumor formation (Monastirioti et al., 1996; Saraswati et al., 2004; Zhou et al., 2008), while increasing its level also leads to similar abnormalities (Stathakis et al., 1999). Despite this dosage-function dependency, very little is known about the mechanisms controlling OA synthesis throughout the *Drosophila* life cycle. Studies on honeybees have shown that OA synthesis increases with age (Harris and Woodring, 1992), which might be also the case in *Drosophila*. Additionally, it has been shown that stress can induce OA synthesis in the hemolymph of both locusts and cockroaches (Davenport and Evans, 1984), implying that differential concentrations of OA are required in response to altered environmental circumstances. Recent reports demonstrated a FOXO-mediated increase in OA metabolism upon elevated temperatures in *Drosophila* (Gruntenko et al., 2016). It would, therefore, be interesting to test whether TBH expression is also stress induced and whether Lola-O contributes to this effect.

We show that Lola-O binds and negatively regulates *Bacc*, a previously characterized repressor of *Tbh*. Heterozygous *Bacc* mutants display only mild upregulation of *Tbh* levels, which is, however, sufficient to induce elevated OA synthesis, leading to acute ethanol sensitivity (Chen et al., 2013). These findings suggest that *Bacc* expression requires a precise regulation to control OA synthesis.

Future Directions

Our study illuminates a novel role for Lola in controlling neurotransmitter signaling. Interestingly, Lola-O expression appears to be completely restricted to TBH-positive cells in the larval CNS, albeit at very low levels. The mechanisms that restrict Lola-O expression to this subset of cells are currently unknown. It is widely known that promoter activity can influence and determine tissue-dependent gene expression, suggesting that expression of Lola-O in OA neurons might be a consequence of alternative promoter activity. *lola* is encoded by four promoters, with expression of all isoforms from all four promoters (Ohsako et al., 2003); yet, cell-type-specific promoter activity has not been addressed. Alternatively, a splicing regulator might be specifically expressed in OA-responsive cells, promoting the expression of Lola-O exclusively in these cells. Additional exper-

iments are needed to further reveal the mechanism of the restricted expression of Lola-O in the brain and to address whether analogous mechanisms apply in vertebrates to control the level of norepinephrine upon normal and disease conditions.

EXPERIMENTAL PROCEDURES

Fly Stocks

Drosophila melanogaster *w¹¹¹⁸* was used as the wild-type control. For rescue experiments, the following driver lines were used: *Tdc2-GAL4*, *Tub-GAL4*, and *elav^{C155}-GAL4*. *Df(2R)ED2076* served for the generation of *trans*-heterozygous *lola* mutants. *PBac(lola.J-GFP.FLAG)VK00033* and *PBac(lola.GR-GFP.FLAG)* were used for rescue experiments (Spokony and White, 2012). For transcriptome analysis, *lola^{E76}* and *lola-F^{stop}* flies were balanced with *w⁺*; *P[sqh-mCherry.M]* to enable selection of homozygous embryos based on fluorescence (Martin et al., 2009). *P[w[+mC] = EP]futsch[EP1419]* served for the rescue experiment of *lola-F^{stop}* embryos. MARCM clones were generated using the following lines: *lola-N*, *FRT42D/CyO*, *FRT42D/CyO* and *elav-GAL4*, *UAS-mCD8-GFP*, *hsFLP*; *FRT42D*, *tub-Gal80* (kind gift from C. Berger). Flies were obtained at the Bloomington *Drosophila* Stock Center. *Tbh^{nm18}* mutant flies were a kind gift from M. Monastirioti and *UAS-LT3-Dam-Pol II* and *UAS-LT3-Dam* flies were a kind gift from A. Brand.

CRISPR/Cas9 Mutant Flies

gRNA sequences were cloned into pBFv-U6.2B (Kondo and Ueda, 2013), sequenced, and injected in our lab into *y1 v1 P(nos-phiC31\int.NLS)X; attP40*. Transgenic flies were further crossed with *y² cho² v¹; attP40(nos-Cas9)/CyO*, and flies from the F1 generation were PCR-screened for the expected mutation using primer sequences flanking the gRNA sequences (Table S2). Obtained PCR amplicons were sequenced at GATC Biotech.

UAS Constructs

Coding sequences of *lola-A*, *-O*, *-F*, and *-H* and *Tbh* were amplified from cDNA and inserted into Gateway plasmids with N-terminal FLAG-Myc or FLAG-HA tag (pPFMW or pPFHW, respectively; obtained from *Drosophila* Genomics Resource Center at Indiana University). All constructs were sequenced prior to injection into *w¹¹¹⁸*. *Drosophila* germline injection for *lola-A* and *-F* and *Tbh* was performed in house. The construct for UAS-Lola-O and UAS-Lola-H was injected at BestGene.

Drug-Feeding Assay

Flies were collected within 10 hr of eclosion, gender-separated, and placed on medium containing 5 or 7.5 mg/mL OA or tyrosine for males and females, respectively. Flies were examined daily for survival rate and phenotypic penetrance. 20 flies were used for each condition.

Locomotion Assay

20 freshly hatched male and female flies were separated and staged until desired age. The locomotion was assessed using the climbing assay described previously (Bahadorani and Hilliker, 2008). Flies were taped to the bottom, and flies passing 8 cm in 10 or 5 s, respectively, were counted. Measurements were repeated five times in three biological replicates.

Lifespan Assay

20 control or experimental flies were gender-separated within 10 hr of eclosion and maintained at 25°C. Survival was analyzed every 2 days and flies were transferred to new vials twice a week.

Fertility Assay

10 female virgin flies were mated with 5 males for 3 days. Upon fertilization, females were transferred onto fresh agar plates every 12 hr, and the number of eggs laid was counted for 5 days.

Cell-Type-Specific Transcriptome Analysis

UAS-*mCherry* expression was induced in neurons by *elav-GAL4* and in OA cells by *Tdc2-GAL4*. Eggs were collected for 3 hr and aged for 13 or 20 hr,

respectively. Cell isolation was performed as described previously (Salmand et al., 2011), and cells were sorted using the Becton Dickinson Aria III SORP flow cytometer. For RNA preparation, 10 cells per replicate were collected in lysis buffer and subjected to library preparation using the Smart-Seq2 Kit for Illumina.

Transcriptome Analysis

lola-F^{Stop} and *lola-E⁷⁶* embryos were collected at 25°C for 2 hr and aged for 13 hr. Embryos were hand-sorted based on mCherry expression. *lola-O* KO embryos were collected at 25°C for 2 hr and developed for 20 hr. Mutant and control embryos were transferred into TRIzol reagent (Thermo Fisher Scientific), and they were subjected to RNA isolation using the manufacturer's protocol.

cDNA Library Preparation

RNA was isolated, DNase I- (New England Biolabs) treated, and subjected to library preparation using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina. 1 µg total RNA was used as starting material for cDNA library preparation.

Computational Analysis

Libraries for transcriptome analyses were sequenced on the NextSeq500. Cells sorted via FACS were sequenced as single-read 76 bp; the other samples were sequenced as 42-bp paired end. Demultiplexing and fastq conversion were done with bcl2fastq (version [v.2.19]). Reads were mapped using STAR (v.2.5.2b) against ensembl release 87 (BDGP6). For the cells sorted via FACS, the reads were filtered for rRNA reads before mapping. Counts per gene were calculated using featureCounts (v.1.5.1) with ensembl release 87 as a reference. Differential expression analysis was done using DESeq2 (v.1.16.1) with an FDR filter of 1%. Transcript quantification for the cells sorted via FACS was performed using stringtie (v.1.3.3b) and visualized using ballgown (v.2.8.4). Splice junction quantification and visualization was done using SGSeq (v.1.10).

GO Term Method and Plot Outline

GO terms overrepresentation was calculated using GOSTats (v.2.38.1) requiring a minimal amount of 5 genes per GO term and adjusted p value smaller than 1%. Afterwards, terms were summarized using semantic similarity (GOSemSim 1.30.3). Only GO terms with an odds ratio larger than 5 are displayed.

Targeted DamID

UAS-Dam-lola-O flies were generated by amplifying and cloning the *Lola-O* coding sequences into pUASTattB-LT3-NDam (kind gift from A. Brand). *UAS-LT3-Dam-lola-O* and *UAS-LT3-Dam-Pol II* flies and *UAS-LT3-Dam* flies (control) were crossed with *Tdc2-GAL4* to induce octopaminergic expression of the Dam fusion proteins. DNA was isolated from embryos (20–22 hr after egg laying [AEL]), and subsequent treatments were performed as described (Marshall et al., 2016). Purified and processed genomic DNA of two biological duplicates was subjected to library preparation using the NebNext DNA Ultra II library kit (New England Biolabs) and sequenced on a NextSeq500. The first read was mapped to *Drosophila melanogaster* genome (BDGP6) using bowtie (v.2.2.9), binned to GATC fragments, and normalized against the Dam-only control (Marshall and Brand, 2015). Peaks were called and mapped to genes using a custom Perl program (available upon request).

qRT-PCR

Total RNA was transcribed into cDNA using MMLV reverse transcriptase (Promega). qRT-PCR analysis was performed using a ViA7 real-time PCR system (Applied Biosystems). Measurements were done in triplicates. Relative RNA levels were normalized to *rp15* levels. Primer sequences are listed in Table S2.

In Situ Hybridization

In situ hybridization was performed as previously described (Lence et al., 2016). For fluorescent *in situ* hybridization, anti-DIG-HRP (1:1,000, Roche) was applied, and probes were visualized using the tyramide signal amplification (Alexa Fluor 568, Thermo Fisher Scientific).

Whole-Mount Embryo Immunostaining

Embryos were fixed and washed with PBS with 0.3% Triton X-100. Primary antibodies used were the following: mouse anti-Fas II (1:20; 1D4, DSHB), rat anti-Elav (1:100; 7E8A10, DSHB), mouse anti-Repo (1:100; 8D12, DSHB), mouse anti-22C10 (1:50; DSHB), mouse anti-Lola z5 (1:100; 1D5, DSHB), guinea pig anti-Dpn (1:50, kind gift from C. Berger), rat anti-TBH (1:75, kind gift from M. Monastirioti), and rabbit anti-GFP (1:500; TP401, Torrey Pines Biolabs). Appropriate combinations of secondary antibodies (Jackson ImmunoResearch Laboratories) were applied. Samples were analyzed with a Leica SP5 confocal microscope.

Larval L3 Brain Immunostaining

Larval CNS was dissected in cold PBS, fixed for 20 min in 4% paraformaldehyde in PBS, and subsequently treated as embryonic samples. A confocal stack was recorded using a Leica SP5 confocal microscope.

Western Blotting

Staged embryos were collected and homogenized in lysis buffer (140 mM NaCl, 10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8], and 0.5% Triton X-100). The protein extract was separated on 8% SDS-PAGE gel followed by western blot analysis with affinity-purified anti-Lola antibody (1:500, kindly provided by E. Giniger) or Lola-Zf5 antibody (1:100; DSHB). For visualization, ultra-sensitive enhanced chemiluminescent reagent (Thermo Fisher Scientific) was used.

Statistical Analysis

Statistical parameters and significance are reported in the figures and figure legends. For comparisons of the means of two groups, Student's t test was used. For comparisons among more than two groups, the one-way ANOVA was performed followed by multiple comparisons using t tests with Bonferroni normalization of p values.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq and DamID-seq data reported in this paper is GEO: GSE97836.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.11.015>.

AUTHOR CONTRIBUTIONS

Conceptualization, J.-Y.R. and N.D.; Methodology, J.-Y.R., N.D., and V.M.; Investigation, N.D. and V.M.; Computation Analysis of RNA-Seq, N.K.; Computational Analysis of DamID-Seq, T.D.S.; Writing – Original Draft, N.D.; Writing – Review & Editing, J.-Y.R. and N.D.; Funding Acquisition, J.-Y.R.; Supervision, J.-Y.R.

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