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A Developmentally Regulated Splice Variant from the Complexlola Locus Encoding Multiple Different Zinc Finger Domain Proteins Interacts with the Chromosomal Kinase JIL-1

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Abstract

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Using a yeast two-hybrid screen we have identified a novel isoform of the *lola* locus, Lola zf5, that interacts with the chromosomal kinase JIL-1. We characterized the *lola* locus and provide evidence that it is a complex locus from which at least 17 different splice variants are likely to be generated. Fifteen of these each have a different zinc finger domain, whereas two are without. This potential for expression of multiple gene products suggests that they serve diverse functional roles in different developmental contexts. By Northern and Western blot analyses we demonstrate that the expression of Lola zf5 is developmentally regulated and that it is restricted to early embryogenesis. Immunocytochemical labeling with a Lola zf5-specific antibody of *Drosophila* embryos indicates that Lola zf5 is localized to nuclei. Furthermore, by creating double-mutant flies we show that a reduction of Lola protein levels resulting from mutations in the *lola* locus acts as a dominant modifier of a hypomorphic *JIL-1* allele leading to an increase in embryonic viability. Thus, genetic interaction assays provide direct evidence that gene products from the *lola* locus function within the same pathway as the chromosomal kinase JIL-1.

Chromatin structure as well as the differential expression of transcription factors plays an important role in the regulation of gene expression (1–3). We have recently identified a chromosomal tandem kinase, JIL-1, that modulates chromatin structure in *Drosophila* (4–6). JIL-1 is an essential kinase, and in JIL-1 nulls and hypomorphs euchromatic regions of chromosomes are severely reduced and the chromosome arms are condensed (6). These changes are correlated with decreased levels of histone H3 Ser-10 phosphorylation (6). JIL-1 has been implicated in transcriptional regulation, as it localizes to the gene-active interband regions of interphase larval polytene chromosomes (4) and has been found to associate with at least one chromatin-remodeling complex, the male specific lethal (MSL)[1] dosage compensation complex (5). The MSL complex is required for the necessary hypertranscription of genes on the male X chromosome for dosage compensation in flies (reviewed in Ref. 7). This enhanced transcription is thought to arise from MSL complex-induced histone H4 acetylation generating a more open chromatin structure (8). The increased histone H3 Ser-10 phosphorylation levels that JIL-1 promotes on the male X may also play a role in maintaining a more open and active chromatin structure (5, 6). However, it is not known whether physiological substrates of JIL-1 may include other proteins such as transcription factors or whether there are proteins directly regulating the function of JIL-1. To identify proteins that interact with JIL-1 we carried out yeast two-hybrid screens using different JIL-1 regions as baits. Here we report that a novel splice form from the *lola* locus, which we have named Lola zf5, was identified in such a screen to interact with the first kinase domain (KDI) of JIL-1.

The *lola* locus was first characterized as a mutation affecting longitudinal axon growth within the central nervous system (9). Two isoforms from the *lola* locus have been previously described, Lola long and Lola short (10). Lola long contains two zinc finger motifs and is a transcription factor with DNA binding activity (10, 11). The *lola* locus mediates decreased *copia* retrotransposon mRNA expression in the central nervous system while upregulating its expression in gonads (11). In addition, *lola* is required for proper expression of the axonal guidance proteins Robo and slit in the central nervous system (12). Although both Lola isoforms share a BTB/POZ domain (13–15), Lola short contains no zinc finger domains (10). BTB domains are known to mediate dimerization (16, 17), which includes the ability to promote heterophilic interactions of different BTB domain-containing isoforms (15, 18, 19). BTB domain-containing zinc finger proteins have been strongly implicated in regulation of chromatin structure and gene expression (20). For example, human B cell lymphoma (BCL-6) and promyelocytic leukemia zinc finger oncoproteins have been shown to act as transcriptional repressors (21–23). Specific recruitment of repressor complexes to target promoters occurs through binding of corepressors to the hydrophobic BTB dimer pocket (24). Corepressor binding recruits a complex containing a histone deacetylase (25, 26) that represses transcription by inducing condensed chromatin architecture (reviewed in Ref. 27).

In this study we characterized the genomic organization of the *lola* locus and show that it is a complex locus from which at least 17 different splice variants are likely to be transcribed. All isoforms from the *lola* locus share a common BTB domain, whereas 15 of the splice variants each contain a different zinc finger domain. We show that one of these variants, Lola zf5, physically interacts with the JIL-1 kinase and that its expres-

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[1] The abbreviations used are: MSL, male-specific lethal; mAb, monoclonal antibody; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; KDI, kinase domain I; EST, expressed sequence tag; ORF, open reading frame; UTR, untranslated region; TRITC, tetramethylrhodamine isothiocyanate.

sion is developmentally restricted to early embryogenesis. Furthermore, we show that a P element insertion in the *lola* locus enhances the viability of a hypomorphic JIL-1 allele, indicating opposing functions of the *JIL-1* and *lola* loci. Although only Lola long and Lola short have so far been studied in detail, it has long been known that P insertion mutations in *lola* that prevent the expression of some if not all the isoforms have very complex phenotypes (10–12, 28). Our findings suggest that this complexity may derive from the expression pattern of multiple gene products from the *lola* locus that are likely to serve diverse functional roles in different cellular and developmental contexts.

MATERIALS AND METHODS

Drosophila Stocks—Fly stocks were maintained according to standard protocols (29). Oregon-R was used for wild type preparations. *JIL-1$^{EP(3)3657}$* and *JIL-1^{z2}* alleles have been previously described (6). Balancer chromosomes and mutant alleles are described in (30). The *lola^{00642}* mutant stock *cn^{1} P{ry$^{+t7.2}$ = PZ}lola^{00642}/CyO*; *ry^{506}* was obtained from the Bloomington *Drosophila* Stock Center. Hatch rates were determined by counting the number of eggs laid on standard apple juice/agar plates and then counting the number of unhatched eggs at 22 h and again at 48 h after egg laying. All genetic crosses and interaction assays were conducted at 23 °C.

Identification and Molecular Characterization of Lola zf5—JIL-1 cDNA sequence encoding a 304-amino acid fragment (Tyr^{251}-Glu^{554}) comprising the first kinase domain (JIL-1 KDI) of JIL-1 was subcloned in-frame into the yeast two-hybrid bait vector pGBKT7 (Clontech) using standard methods (31) and verified by sequencing (Iowa State University Sequencing Facility). The JIL-1 KDI bait was used to screen the Clontech Matchmaker™ 0–21 h embryonic Canton-S yeast two-hybrid cDNA library according to the manufacturer's instructions. A positive cDNA clone KDIJ1 was isolated, retransformed into yeast cells containing the JIL-1 KDI bait to verify the interaction, and sequenced. Homology searches identified SW59 (GenBank™ Z97377) as well as the *lola* locus. We obtained the SW59 cDNA from Dr. D. Zhao (University of Edinburgh) and *lola* ESTs (LD28033, LD33478, and LD17361) from ResGen Invitrogen and assembled the full-length Lola zf5 coding sequence. We note a few differences between Z97377 and the Lola zf5 full-length cDNA assembled in this study: 1) 62 nucleotides at the most 5′-end of Z97377 may be a library construction artifact, because they are not present in other Lola zf5 EST clones or the reported *lola* genomic sequence (32); 2) six gaps are present between Z97377 and our KDIJ1 fragment. At all of the gaps, KDIJ1 cDNA sequence is 100% identical to the available ESTs and genomic sequence (33, 32). Sequencing of EST clones LD28033 and LD33478 support the presence of two Lola zf5 splice isoforms using alternative 5′-UTR sequences (5′-a and 5′-c, respectively) but we have not confirmed use of 5′-b and 5′-d UTR alternative exons for Lola zf5, as predicted in the November 30th, 2002 genome project update.

Antibody Generation and Antibody Affinity Purification—Rabbit anti-Lola common region polyclonal antibody was a generous gift of Dr. Edward Giniger and has been previously characterized (10). Hope and Odin rabbit anti-JIL-1 polyclonal antibodies were described in Jin *et al.* (4). Affinity purification of anti-Lola and anti-JIL-1 polyclonal antibodies was as described in Giniger *et al.* (10) using lacZ-Lola and GST-JIL-1 fusion proteins, respectively. To generate Lola zf5-specific monoclonal antibody (mAb) 7F1, the KDIJ1 cDNA fragment (encoding Lola zf5 amino acid residues 427–748) was cloned in the correct reading frame into pGEX4T-1 (Amersham Biosciences), verified by sequencing, and GST-zf5 fusion protein was induced in *Escherichia coli* according to standard protocols (Amersham Biosciences). Injection of GST-zf5 fusion protein into BALB/C mice, and generation of monoclonal hybridoma lines was performed by the Iowa State University Hybridoma Facility according to standard protocols (34).

Immunohistochemistry—Embryos were dechorionated in 50% Chlorox solution, washed with 0.7 M NaCl/0.2% Triton X-100 and fixed in a 1:1 heptane:fixative mixture for 20 min with vigorous shaking at room temperature. The fixative was either 4% paraformaldehyde in phosphate-buffered saline (PBS) or Bouin's Fluid (0.66% picric acid, 9.5% formalin, 4.7% acetic acid). Vitelline membranes were then removed by shaking embryos in heptane-methanol (35) at room temperature for 30 s. Embryos were blocked in PBS with 1% normal goat serum (Cappel) and 0.4% Triton X-100 and incubated overnight in mAb 7F1 primary antibody diluted in blocking buffer. Embryos were washed in PBS with 0.4% Triton X-100, incubated for 2.5 h with TRITC-conjugated goat anti-mouse secondary antibody (1:200) (Cappell), washed in PBS with 0.4% Triton X-100 followed by a PBS-only wash. For visualization of DNA the antibody-labeled embryos were incubated in 0.2 μg/ml Hoechst 33258 (Molecular Probes) in PBS for 10 min. The final preparations were mounted in glycerol with 5% *n*-propyl gallate and viewed with a 40× NeoFluor objective on a Zeiss Axioskop equipped with filter sets optimized and selective for rhodamine and UV detection. Digital images were obtained using a Spot-cooled charge-coupled device camera (Diagnostic Instruments).

Northern and Western Blot Analysis—Approximately 1 g of wild type Oregon-R animals from different stages was collected and ground under liquid nitrogen, and total mRNA was purified using the Poly(A)$^+$ mRNA purification kit (Ambion). 5 μg of poly(A)$^+$ mRNA from each stage was fractionated on 1% agarose formaldehyde gels, transferred to Duralon-UV™ nylon membrane (Stratagene), and hybridized with ^{32}P-labeled probe overnight at 65 °C according to standard high stringency protocols (31). Lola zf5 isoform-specific probes were generated by purifying 1.8 kb of unique 3′-end Lola zf5 cDNA sequence using a QiaQuick gel extraction kit (Qiagen) and synthesizing random primer ^{32}P-labeled probe using the Prime-A-Gene kit (Promega) according to the manufacturer's instructions. As a loading control, a cDNA fragment of RP49 (ribosomal protein L32) (36) was PCR-amplified from the Clontech yeast two-hybrid cDNA library described above and confirmed by sequencing. After stripping the Lola zf5 signal, labeled RP49 cDNA was used to probe the same membrane to normalize mRNA loading levels.

Protein extracts were prepared from staged dechorionated embryos, larvae, pupae, or adults that were homogenized in immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 0.1% Nonidet P-40, 2 mM Na_3VO_4, pH 8.0) with added protease inhibitors 1.5 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (Sigma). Proteins were boiled in SDS-PAGE buffer, separated on SDS-PAGE gels, transferred to nitrocellulose, blocked in 5% Blotto, and incubated with anti-JIL-1 or anti-Lola antibody overnight. Blots were then washed three times for 10 min in TBST (0.9% NaCl, 100 mM Tris, pH 7.5, 0.2% Tween 20), incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:3000) (Bio-Rad) for 1 h at room temperature, washed in TBST, and the antibody signal was detected with the ECL chemiluminescence kit according to manufacturer's instructions (Amersham Biosciences).

In Vitro Protein Interaction and Co-immunoprecipitation Assays—Approximately 5 μg of GST-KDIJ1 (Lola zf5 C terminus) fusion protein or GST protein alone was coupled to glutathione-agarose beads (Sigma) and incubated with 0.5 ml of S2 cell lysates (3×10^6 cells) overnight at 4 °C. The beads were pelleted at low speed and washed three times with 1 ml of immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 0.1% Nonidet P-40, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 1.5 μg of aprotinin, pH 8.0) for 10 min at 4 °C. The proteins retained on the beads were analyzed by Western blot analysis using affinity-purified Hope anti-JIL-1 polyclonal antibody.

For co-immunoprecipitation experiments, anti-JIL-1, anti-Lola zf5, or control normal rabbit antibodies were coupled to protein G beads as follows: 30 μl of Odin anti-JIL-1 serum, 30 μl of normal rabbit control serum, or 1 ml of 7F1 hybridoma supernatant was coupled to 25 μl of protein G-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C on a rotating wheel in 50 μl of immunoprecipitation buffer. The appropriate antibody-coupled beads were incubated overnight at 4 °C with 300 μl of 0–6 h embryonic lysate on a rotating wheel. Beads were washed four times for 10 min each with 1 ml of immunoprecipitation buffer with low speed pelleting of beads between washes. The resulting bead-bound immunocomplexes were analyzed by SDS-PAGE and Western blotting according to standard techniques as described in Jin *et al.* (4) using Hope antibody to detect JIL-1, mAb 7F1 to detect Lola zf5, or Lola polyclonal antisera against the Lola common core domain (10) to detect all Lola isoforms.

Bioinformatics—Lola genomic DNA sequence corresponding to nucleotides 118,125–182,622 of *Drosophila melanogaster* genomic scaffold AE003829.3 (GI: 21627529, updated on September 20, 2002) (32) was used to search for zinc finger motifs. Consensus zinc finger motifs include C*XX*C, H*XXXX*C, and H*XXXX*H (*X* represents any amino acid) using the "Find" function under "Edit" menu of Microsoft Word 98 software. In most cases, when an open reading frame (ORF) contained two of the three consensus sequences, it was considered a potential zinc finger motif and further analyzed. PCR primers were designed to amplify Lola sequences containing the putative cDNA fragments specific to the predicted zinc finger motifs using standard PCR protocols (31). The forward PCR primer ZnF5P (5′-GGATGAACTTGGACTAATGGC-3′) consists of Lola common core sense sequence derived from exon IV,

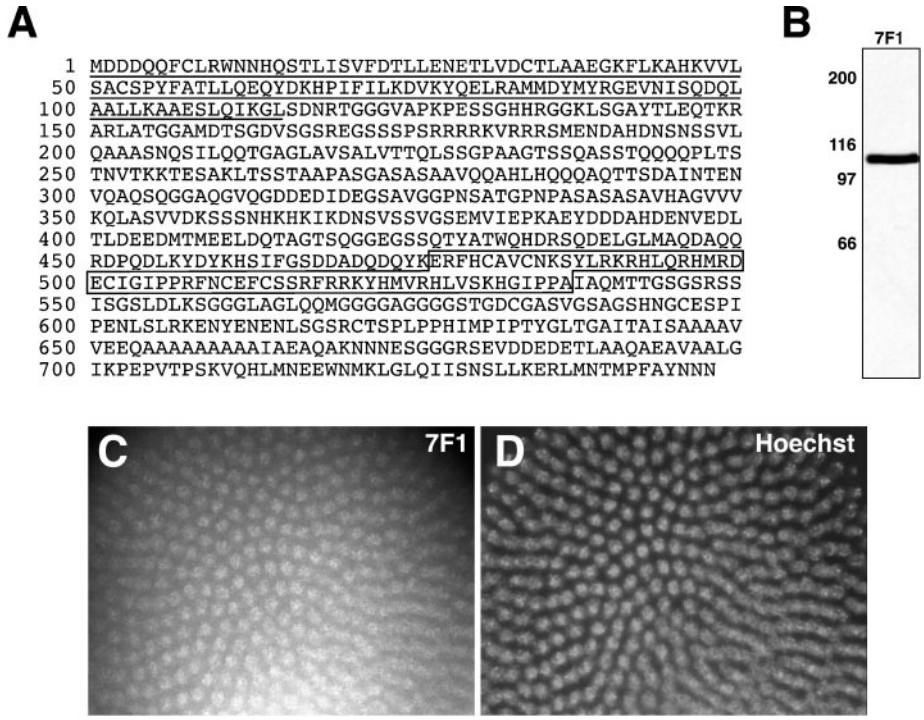

FIG. 1. **The predicted sequence of the Lola zf5 isoform and Lola zf5 antibody labeling.** *A*, the complete predicted amino acid sequence of Lola zf5. Lola zf5 is a 748-residue protein with a calculated molecular mass of 79.4 kDa. The BTB domain is *underlined*, and the zinc finger domains are *boxed*. *B*, immunoblot of SDS-PAGE fractionated 0- to 6-h embryo extracts labeled with the Lola zf5-specific mAb, 7F1. The antibody recognizes a single band migrating at 105 kDa. The migration of molecular mass markers in kilodaltons is indicated to the *left*. *C* and *D*, double labeling of a syncytial embryo with mAb 7F1 and Hoechst. The labeling of mAb 7F1 (*C*) shows that Lola zf5 is localized in a pattern overlapping with that of the Hoechst labeled nuclei (*D*).

whereas reverse PCR primers were designed to be isoform-specific, with individual reverse primers comprised of antisense sequence based on the 3′-end of each separate predicted zinc finger motif. Oligonucleotides were designed using the Oligo 5.0 program. cDNA templates for PCR reactions were from the 0- to 21-h cDNA Matchmaker™ yeast two-hybrid library (Clontech) or a 3- to 12-h cDNA library (Stratagene). PCR conditions were optimized for each primer pair, respectively, and PCR products were directly sequenced.

The *lola* genomic sequence was also used to search for homologs in the *Drosophila* EST databases with FLYBLAST (available at www.fruit fly.org/blast/) (33) and with the Geneseqer program (available at bioinformatics.iastate.edu/cgi-bin/gs.cgi) (37). Returned ESTs were compared with *lola* genomic DNA sequence using BLAST2 (available at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) at NCBI (National Center for Biotechnology Information) (38). Putative zinc finger motifs were aligned using Clustal W available at www2.ebi.ac.uk/CLUSTALW (39).

RESULTS

The JIL-1 Kinase Interacts with a Novel Isoform of the lola Locus—To identify proteins that have direct interactions with the JIL-1 kinase, we performed a yeast two-hybrid screen of a Canton-S 21-h embryonic library using the first kinase domain of JIL-1 as bait. True positive clones detected in the primary screen were confirmed by β-galactosidase two-hybrid interaction assays on filter paper following retransformation of the candidate clones and JIL-1 KDI bait plasmid into the yeast strain AH109 (data not shown). One of the positive clones identified in this way was sequenced, and searches of the *Drosophila* genome database revealed it to be the COOH-terminal domain of a novel splice variant from the *lola* locus, which we have named Lola zf5. Subsequently, the full-length cDNA sequence for Lola zf5 was assembled from overlapping ESTs obtained from the *Drosophila* genome project and is currently available as AY058586 as well as the SW59 clone (Z97377). Fig. 1*A* shows the amino acid sequence of the predicted open reading frame of a protein of 748 residues with a calculated molecular mass of 79.4 kDa. The BTB domain and zinc finger domains are *underlined* and *boxed*, respectively. To further characterize the protein, a Lola zf5-specific monoclonal antibody, 7F1, was generated against a GST fusion protein containing the COOH-terminal region unique to Lola zf5. On immunoblots of embryo protein extracts (0–6 h) mAb 7F1 detects Lola zf5 as a single band migrating at 105 kDa (Fig. 1*B*). The Lola zf5 protein is highly acidic with a pI of 5.54 accounting for its anomalous gel migration. Immunocytochemical labeling of early *Drosophila* embryos with mAb 7F1 revealed the Lola zf5 protein to be localized to nuclei in a pattern similar to that obtained by Hoechst labeling (Fig. 1, *C* and *D*).

To further explore the interaction between Lola zf5 and JIL-1 that we observed in the yeast two-hybrid assays we performed pull-down assays with the Lola zf5 COOH-terminal GST fusion protein using protein extracts from the S2 cell line. The Lola zf5-GST fusion protein or a GST-only control were coupled with glutathione-agarose beads, incubated with S2 cell lysate, washed, fractionated by SDS-PAGE, and analyzed by immunoblot analysis using JIL-specific antibody (Fig. 2*A*). Whereas the GST-only control showed no pull-down activity, Lola zf5-GST was able to pull down JIL-1 as detected by the JIL-1 antibody. In addition, we performed co-immunoprecipitation experiments using embryonic lysates. For these immunoprecipitation experiments, proteins were extracted from 0–6 h embryos, immunoprecipitated using either JIL-1- or Lola zf5-specific antibodies, fractionated on SDS-PAGE after the immunoprecipitation, immunoblotted, and probed with antibodies to Lola zf5 and JIL-1, respectively. Fig. 2*B* shows an immunoprecipitation experiment using Lola zf5 antibody where the immunoprecipitate is detected by JIL-1 antibody as a 160-kDa band that is also present in the embryo lysate. This band was not present in lanes where immunobeads only were used for the immunoprecipitation. Fig. 2*C* shows the converse experiment: JIL-1 antibody immunoprecipitated a 105-kDa band detected by Lola zf5 antibody that was also present in embryo lysate but not in control immunoprecipitations with immunobeads only. These results strongly indicate that Lola zf5 and the JIL-1 kinase are present in the same protein complex.

The Complex lola Locus Encodes Multiple BTB Domain-containing Proteins Each with Different Zinc finger Motifs—Two alternatively spliced isoforms of *lola*, Lola long and Lola short, have been previously characterized (10). Lola long is a sequence-specific DNA-binding protein with C_2HC and C_2H_2

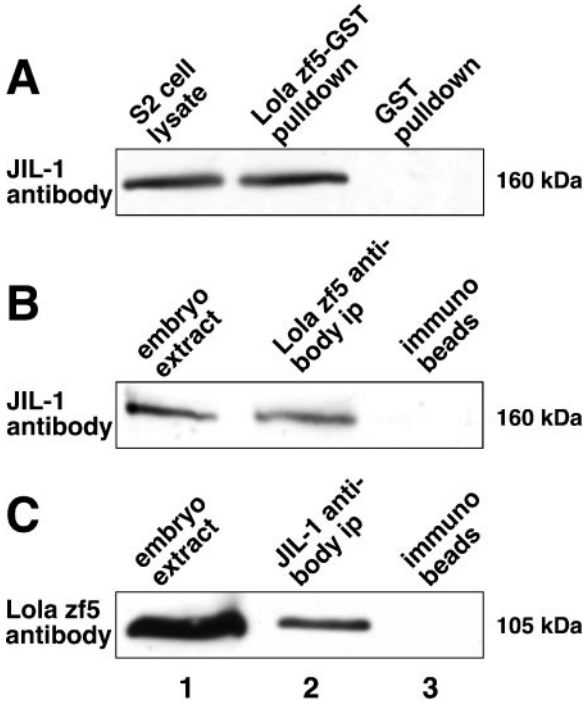

FIG. 2. **Lola zf5 and JIL-1 pull-down and immunoprecipitation experiments.** *A*, S2 cell lysate incubated with a Lola zf5-GST fusion construct or a GST only control was pelleted with glutathione-agarose beads and the interacting protein(s) fractionated by SDS-PAGE, Western blotted, and probed with JIL-1 antibody. Non-incubated S2 cell lysate was included as a control (*lane 1*). The Lola zf5-GST construct was able to pull down the 160-kDa JIL-1 protein as indicated by detection with JIL-1 antibody (*lane 2*), whereas no interaction was observed with the GST only control (*lane 3*). *B*, immunoprecipitation of lysates from 0- to 6-h embryos was performed using Lola zf5 antibody (mAb 7F1) coupled to immunobeads (*lane 2*) or with immunobeads only as a control (*lane 3*) and analyzed by SDS-PAGE and Western blotting using JIL-1 antibody for detection. JIL-1 is detected as a 160-kDa band in embryo extracts (*lane 1*) as well as in the Lola zf5 antibody immunoprecipitation sample (*lane 2*) but not in the control sample (*lane 3*). *C*, immunoprecipitation of lysates from 0- to 6-h embryos were performed using JIL-1 antibody coupled to immunobeads (*lane 2*) or with immunobeads only as a control (*lane 3*) and analyzed by SDS-PAGE and Western blotting using Lola zf5 antibody for detection. Lola zf5 is detected as a 105-kDa band in embryo extracts (*lane 1*) as well as in the JIL-1 antibody immunoprecipitation sample (*lane 2*) but not in the control sample (*lane 3*).

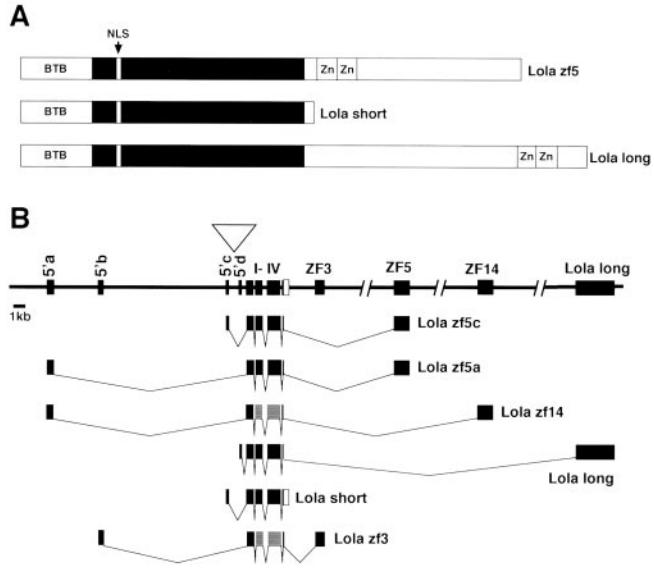

FIG. 3. **Comparison of Lola isoforms.** *A*, schematic diagrams of the domain organization of Lola zf5 and the two Lola isoforms, Lola long and Lola short, drawn to scale. Each Lola isoform shares a common region consisting of an NH_2-terminal BTB domain and a core domain (in *black*) with a nuclear localization signal (*NLS*). In addition, Lola zf5 and Lola long have tandem zinc finger domains (*Zn*). *B*, diagram of the different 5′-UTR usage by Lola splice forms. The *lola* locus has four exons (*I–IV*) that code for the common region. The 5′-UTR utilization for six Lola splice forms are diagrammed. Exons in *black* have been confirmed by sequencing, whereas exons in *gray* are inferred. In addition, the COOH-terminal sequence of Lola short (in *white*) is generated by alternative read through of exon IV of the common region (10). The insertion site of the P-element of the $lola^{00642}$ mutation is indicated by the triangle.

zinc finger motifs, whereas Lola short only has a very short COOH-terminal tail segment without zinc finger motifs (10, 11). Fig. 3*A* shows the domain structure of these two isoforms as compared with Lola zf5. Lola zf5 has an NH_2-terminal common region shared by all cloned Lola isoforms that is followed by an isoform-specific COOH-terminal region (Fig. 3*A*). The Lola common region is encoded by four exons and contains a 120-amino acid NH_2-terminal BTB domain as well as a nuclear localization signal (Fig. 3, *A* and *B*). The COOH-terminal domain of Lola zf5 contains tandem C_2HC and C_2H_2 zinc finger motifs (Fig. 3*A*). The position of the Lola zf5 zinc fingers are very close to the common region in contrast to the Lola long isoform in which the zinc fingers are positioned at the COOH-terminal end.

Our identification of Lola zf5 as a novel zinc finger-containing protein within the locus prompted us to survey the region for additional exons with zinc finger motifs. Consensus zinc finger motifs include the sequences C*XX*C, H*XXXX*C, and H*XXXX*H. We searched the *lola* genomic region for long open reading frames (ORFs) containing any of these zinc finger motif consensus sequences. A total of fifteen putative exons containing zinc finger motifs were identified following this strategy as summarized in Fig. 4*A*. From this analysis we predict that at least 17 splice variants are generated from the *lola* locus. Fifteen of these each have a different zinc finger domain (zf1–zf15), whereas two use exons without zinc finger motifs. Fig. 4*B* shows an alignment of the 15 different zinc finger domains within the locus. Two isoforms, Lola zf4 and zf14, have only a single C_2H_2 zinc finger domain, whereas the remaining splice variants have tandem C_2HC/C_2H_2 or C_2HC/C_2HC zinc finger domains (Fig. 4*B*). We propose to name the various novel zinc finger domain-containing Lola isoforms according to the order of the zinc finger domain they contain, hence the name Lola zf5. To verify that these putative isoforms, the majority of which were not predicted by the genome project, were indeed expressed, we used PCR to amplify isoform-specific cDNA fragments from the 0- to 21-h yeast two-hybrid cDNA library based on the assumption that all of the Lola isoforms share the same common regions as the known isoforms. In this way the expression of thirteen out of the fifteen predicted zinc finger-containing exons was confirmed by direct sequencing of such PCR products (Fig. 4*A*, indicated in *black*). In addition, we searched the *Drosophila* EST database with the genomic DNA sequence of each isoform and found further EST support for expression of six of the 13 exons containing zinc finger motifs. We also identified an EST clone (GM27815) representing the second Lola isoform without a zinc finger domain and have named it Lola short-like (Fig. 4*A*).

The coding regions of most of the Lola isoforms are generated by splicing the four common exons together with a single exon containing the different zinc finger domains. However, by sequencing the ESTs and the PCR amplification products we identified a number of smaller exons without zinc finger motifs that are utilized by Lola zf1 and zf8 (Fig. 4*A*). In addition, the locus has four different 5′-UTRs (5′a through 5′d) that may

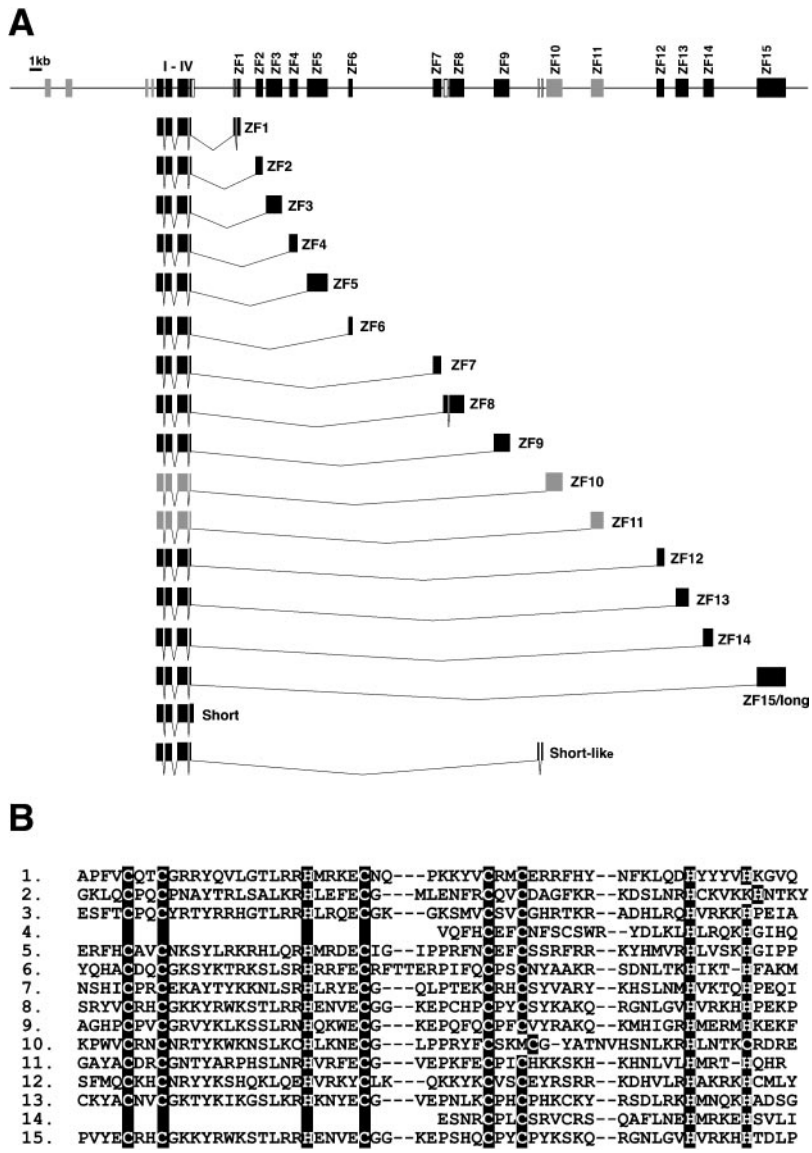

FIG. 4. **Diagram of the *lola* genomic locus and alignment of zinc finger motifs.** *A*, the *lola* locus has at least 27 potential exons: four coding for 5′-UTRs, four coding for the Lola common region (*I–IV*), and 19 alternatively spliced exons coding for the variable COOH-terminal region of the Lola isoforms. Fifteen of these exons contain zinc finger domains (*ZF1–ZF15*). The coding sequence for 17 potential ORFs of Lola isoforms generated from the locus are diagrammed below. The existence of the isoforms depicted in *black* was supported by *Drosophila* ESTs from the genome project or by PCR-amplified isoform-specific cDNA fragments. Lola long and Lola short have both been previously characterized (10). In addition, a homolog of Lola zf8 has been identified in *D. hydei* (11). *B*, alignment of the 15 different zinc finger domains from the zinc finger containing exons. The conserved coordinating cysteines and histidines are in *white typeface outlined in black*.

further amplify the diversity of transcripts generated from this locus (Fig. 3*B*). By sequencing ESTs of the various Lola splice variants, we have obtained evidence that each of the four 5′-UTRs has been utilized into at least one transcript (Fig. 3*B*). Interestingly, we identified two ESTs for Lola zf5 that used different 5′-UTRs (5′a and 5′c, respectively). Transcripts with different 5′-UTRs may allow for the fine regulation of their spatial and temporal expression patterns. Although Lola zf5 is the only example where this alternative utilization has been confirmed, it is likely that other isoforms are regulated by the same splicing mechanisms.

Developmental Expression of Lola zf5—To determine the expression pattern of Lola zf5, we carried out Northern blot analysis using mRNA samples from representative developmental stages (Fig. 5). As a probe we used a Lola zf5 isoform-specific cDNA fragment from the COOH-terminal coding region and 3′-UTR. Lola zf5 mRNA migrates as a single band with an approximate molecular size of 3.9 kb on 1% agarose-denaturing gels. Potential differences in size between transcripts using alternative 5′-UTRs would not be resolved on these gels. Lola zf5 mRNA was abundant in 0- to 2-h embryos, and this level of transcript was maintained in embryos 2–6 h after egg laying (Fig. 5). However, the Lola zf5 mRNA level began to decrease in 6- to 12-h embryos and could not be

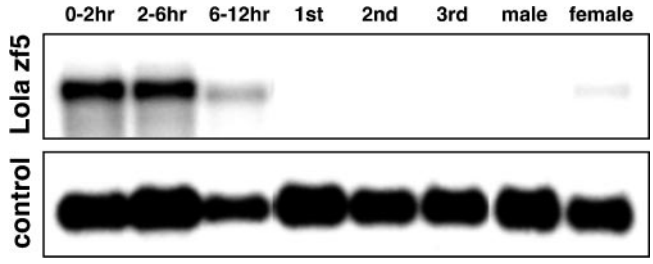

FIG. 5. **Developmental Northern blot analysis of Lola zf5 mRNA.** Poly(A)$^+$ mRNA from various stages of *Drosophila* development was fractionated on a 1% agarose denaturing gel, transferred to nylon filter paper, and probed with random primer-labeled Lola zf5-specific sequences spanning the COOH-terminal region (*upper lanes*) or with ribosomal protein RP49 control probe (*lower lanes*). A single band of ~3.9 kb was detected in 0- to 12-h embryos with Lola zf5 probe. Lola zf5 transcripts were not detected in postembryonic stages except at low levels in female adults.

detected in postembryonic stages except at a low level in female adults, which may reflect the maternal deposition of mRNA into eggs. These findings correlated well with the results from developmental immunoblots using the Lola zf5-specific mAb 7F1 (Fig. 6*A*). We detected high levels of Lola zf5 protein in early embryos (0–12 h); however, the protein level decreased

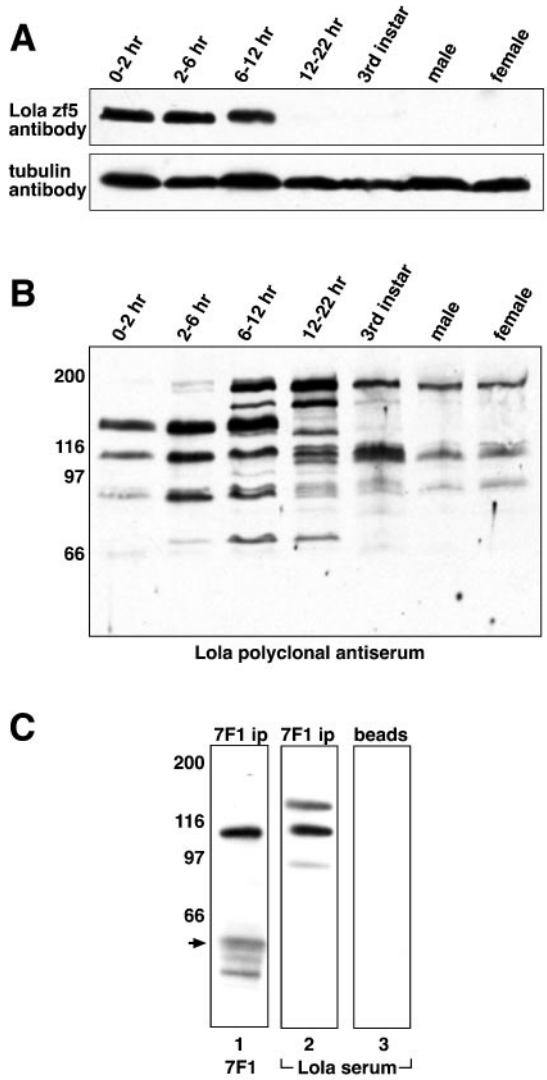

FIG. 6. **Western blot analysis of Lola proteins.** *A*, protein extracts from selected stages of *Drosophila* development were fractionated by SDS-PAGE, immunoblotted, and labeled with the Lola zf5-specific mAb 7F1 (*upper lanes*) or with tubulin antibody as a control (*lower lanes*). The Lola zf5 protein was only detectable during early embryogenesis and was absent at postembryonic stages. *B*, developmental Western blot as in *A* but probed with a Lola polyclonal antiserum likely to recognize all the different Lola isoforms. Multiple Lola isoforms were labeled by the antiserum at the various developmental stages. *C*, immunoprecipitation (*ip*) of lysates from 0- to 6-h embryos were performed using Lola zf5 antibody (mAb 7F1) coupled to immunobeads (*lane 1* and *2*) or with immunobeads only as a control (*lane 3*) and analyzed by SDS-PAGE and Western blotting using mAb 7F1 (*lane 1*) and Lola antiserum (*lane 2* and *3*) for detection. Lola zf5 was detected as a 105-kDa protein by mAb 7F1 in *lane 1*. The *lower bands* (*arrow*) are comprised of 7F1 IgG antibody, which was pulled down in the immunoprecipitation. Rabbit polyclonal Lola antiserum also recognized the 105-kDa Lola zf5 band but additionally labeled two protein bands co-immunoprecipitating with Lola zf5. None of the three bands recognized by the Lola antiserum were detected in the beads only control lane.

~12 h after egg laying and could not be detected at postembryonic stages, including adult females (Fig. 6*A*). The lack of Lola zf5 protein in female ovaries suggests that Lola zf5 is not translated from maternally stored transcripts until after fertilization. These results indicate that the functional expression of Lola zf5 is restricted to early embryogenesis.

In previous studies a polyclonal Lola antibody was made to the Lola common region that would be expected to recognize all the Lola isoforms (10). In developmental immunoblots using this Lola polyclonal antibody we detected multiple bands throughout all developmental stages (Fig. 6*B*). In early embryos (0–6 h) three major bands, including one migrating at 105 kDa, can be detected on the immunoblots. In 12- to 22-h embryos at least 15 bands can be recognized by the polyclonal Lola antibody suggesting that most of the Lola isoforms are expressed at this stage. Some isoforms also appear to be present in later developmental stages such as third instar larvae as well as adults (Fig. 6*B*). To test whether the 105-kDa protein detected by the Lola antiserum corresponded to Lola zf5 as we would predict, we performed immunoprecipitation experiments with the mAb 7F1 of protein extracts from 0- to 6-h embryos. The immunoprecipitations were fractionated by SDS-PAGE, immunoblotted, and detected with mAb 7F1 and Lola antiserum, respectively (Fig. 6*C*). As shown in Fig. 6*C* Lola antiserum recognizes three major bands, including one of 105 kDa that is also labeled by mAb 7F1 and thus is likely to represent the Lola zf5 protein. Interestingly, the presence of the two additional bands labeled by the Lola antiserum and not present in the control lane suggests that Lola zf5 may be involved in heterodimer formation with other Lola isoforms (Fig. 6*C*, *lane 2*).

The Lethal lola^{00642} Mutation Is a Dominant Modifier of the Hypomorphic JIL-1$^{EP(3)3657}$ Allele—To further study whether JIL-1 and Lola zf5 interact *in vivo* we explored genetic interactions between mutant alleles of *lola* and *JIL-1* by generating double-mutant individuals containing both *lola*^{00642} and *JIL-1*$^{EP(3)3657}$. The *lola*^{00642} allele contains a recessive lethal P element insertion and fails to complement the lethality of many P element insertion alleles of *lola* (40). By PCR amplification of the flanking region of *lola*^{00642} followed by direct sequencing, we found that the insertion site is 438 bp downstream to 5′-UTRc and 54 bp upstream to 5′-UTRd (Fig. 3*B*). Because Lola zf5 mRNA contains either the 5′-UTRa or the 5′-UTRc at the 5′-end of the transcript, the insertion of a P element within the Lola zf5 transcription unit is likely to disrupt expression of both splicing alternatives of Lola zf5. The *JIL-1*$^{EP(3)3657}$ allele is a hypomorphic allele that can be maintained in a homozygous stock for only a few generations due to the low hatch rate and recessive semi-lethality (6). The hatch rate of *JIL-1*$^{EP(3)3657}$ homozygous embryos produced by homozygous parents is as low as 4–7% when compared with the hatch rate of wild type Oregon-R embryos (Ref. 6 and this study). We generated double mutants by crossing a chromosome containing the *lola*^{00642} allele into a homozygous *JIL-1* hypomorphic *EP(3)3657* background. Interestingly, individuals homozygous for *JIL-1*$^{EP(3)3657}$ that also contain a *lola*^{00642} allele can be maintained indefinitely as a stock. This suggests that heterozygous *lola*^{00642} may function as a dominant suppressor of the *JIL-1*$^{EP(3)3657}$ phenotype. To quantify the extent of rescue, we compared the numbers of *JIL-1*$^{EP(3)3657}$ homozygous progeny with or without *lola*^{00642} from a single cross (Fig. 7*A*). Although equal numbers of curly- and straight-winged phenotypic classes are expected in matings of *lola*^{00642}/*CyO*; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ males with +/+; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ females, 2.3 times more flies with straight wings were observed than curly wings (Fig. 7*A*). In this cross straight-winged flies carry a *lola*^{00642} allele, whereas curly-winged flies do not. The difference in numbers observed for the two classes was statistically significant ($p < 0.005$, χ^2 test). To exclude the possibility that the apparent rescue phenotype is due to an enhancer of *JIL-1*$^{EP(3)3657}$ phenotype on the *CyO* second chromosome balancer, we also set up a control cross in which the *lola*^{00642} chromosome is not present. In the control cross, we did not observe any statistically significant evidence ($p > 0.1$, χ^2-test) that the *CyO* balancer chromosome affects the viability of *JIL-1*$^{EP(3)3657}$ homozygotes (Fig. 7*A*). The control crosses were per-

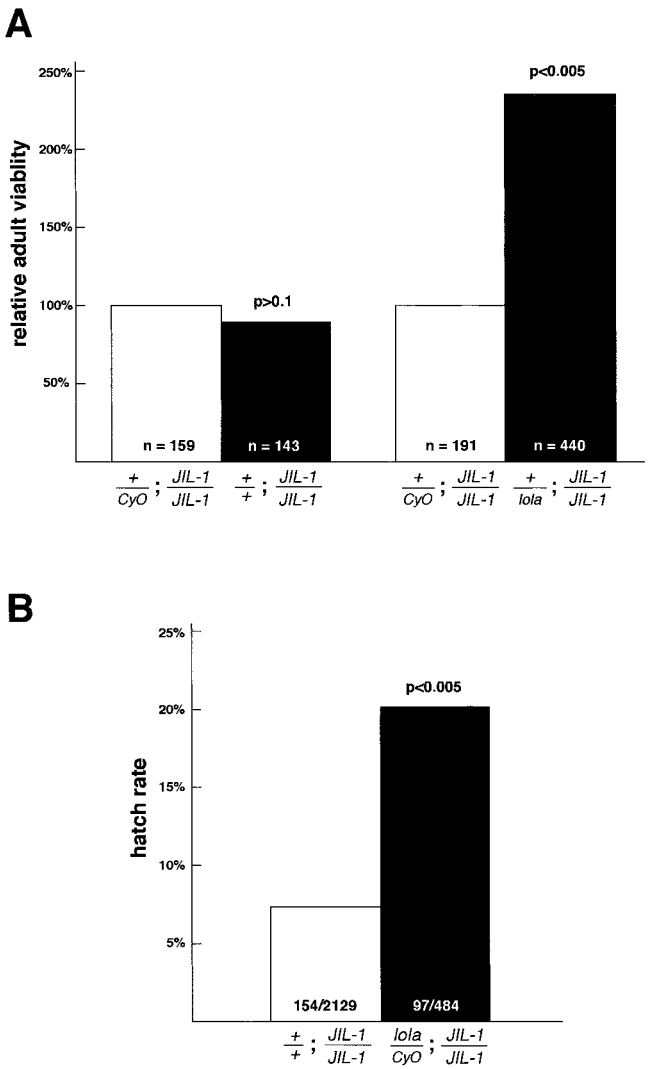

FIG. 7. **Genetic interaction between *lola*^{00642} and *JIL-1*$^{EP(3)3657}$.** *A*, presence of a heterozygous *lola*^{00642} (*lola*) allele increases the viability of *JIL-1*$^{EP(3)3657}$ (*JIL-1*) homozygous animals (histograms to the *right*). *lola*^{00642}/*CyO*; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ males were mated with +/+; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ females. *JIL-1*$^{EP(3)3657}$ homozygotes with a *lola*^{00642} allele (histogram in *black*) enclosed at a rate 2.3 times greater than *JIL-1*$^{EP(3)3657}$ homozygotes with a wild type *lola* allele (histogram in *white*; normalized to 100%). The difference in numbers observed for the two classes was statistically significant ($p < 0.005$, χ^2 test). Control crosses were performed by mating +/*CyO*;*JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ males with +/+; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ females. Presence of the *CyO* balancer alone did not affect viability of homozygous *JIL-1*$^{EP(3)3657}$ animals, as statistically equivalent ($p > 0.1$, χ^2 test) numbers of curly (normalized to 100%) and straight-winged flies were observed in the F1 progeny (*left white* and *black histograms*). *B*, rescue of *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ lethality by *lola*^{00642} occurs during embryogenesis. Only 7.2% of embryos from control matings of *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ flies hatched into larvae (*white histogram*). In contrast, when *lola*^{00642}/*CyO*; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ flies were mated, the hatching rate of those embryos not homozygous for embryonic lethal *lola*^{0062}/*lola*^{0062} or *CyO*/*CyO* chromosomes increased to 20% (*black histogram*). Thus, presence of the heterozygous *lola*^{00642} allele increased the hatch rate of *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ flies 2.8-fold. This difference was statistically significant ($p < 0.005$, χ^2 test). The numbers of animals counted in all classes are indicated at the *bottom* of each histogram. In *B* the number of embryos hatching from the total number of *lola*^{00642}/*CyO*; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ individuals are shown.

formed by mating +/*CyO*; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ males with +/+; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ females. To study the strength of rescue of the *JIL-1* mutant phenotype by *lola*^{00642}, we also crossed the *lola*^{00642} mutant chromosome into a heterozygous *JIL-1*^{z2} null background (6). However, we did not observe any eclosion of *z2* homozygotes (data not shown). These results suggest that either the interaction between *lola*^{00642} and *JIL-1* is mild or the genetic interaction depends on the presence of a minimal level of JIL-1 protein.

If the observed genetic rescue is a consequence of normalized relative levels of Lola zf5 and JIL-1, we would expect that some or all of this rescue occurs during embryogenesis, because Lola zf5 is expressed only in early embryos (Fig. 6*A*). We, therefore, quantified embryonic rescue by determining hatch rates for *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ embryos that were either heterozygous for the *lola*^{00642} allele or homozygous for the wild type allele. Homozygous *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ embryos produced by homozygous mothers typically hatch at a low (7%) rate (Fig. 7*B*). Adjusting for the fact that none of the embryos with a *lola*^{00642}/*lola*^{00642} or *CyO*/*CyO* genotype hatch, the hatch rate of *lola*^{00642}/*CyO*; *JIL-1*$^{EP(3)3657}$/*JIL-1*$^{EP(3)3657}$ embryos is 20% (Fig. 7*B*). Thus, homozygous *JIL-1*$^{EP(3)3657}$ embryos that were heterozygous for *lola*^{00642} hatched at a statistically significant 2.8-fold greater rate than embryos that did not carry the *lola* mutation ($p < 0.005$, χ^2-test). Therefore, the increase of viability observed for *lola* and *JIL-1* double mutants is at least partially due to an increase in the frequency with which such individuals survive embryonic development and hatch.

DISCUSSION

In this study we provide evidence that the JIL-1 tandem kinase molecularly interacts with a novel isoform of the *lola* locus, Lola zf5. This interaction was first detected in a yeast two-hybrid screen and subsequently confirmed by pull-down and cross immunoprecipitation assays. Furthermore, immunocytochemical labeling of *Drosophila* embryos shows that Lola zf5 is localized to nuclei. This localization is compatible with a direct interaction with JIL-1, because JIL-1 has been shown to be a nuclear kinase expressed throughout embryogenesis (4). Northern and Western blot analyses show that the expression of Lola zf5 is developmentally regulated and is only expressed during early embryogenesis.

An interesting feature of the *lola* locus is its complex splicing pattern, and we demonstrate that it contains at least 27 exons. Four of these code for a BTB domain and sequences with a nuclear localization signal common to all Lola splice forms. In addition, fifteen of the exons code for sequences with different zinc finger domains. From this analysis, we predict that a minimum of 17 different protein products are generated by the *lola* locus, fifteen of which contain zinc finger domains and two that do not. By PCR amplification of cDNAs from 0- to 21-h embryos and sequencing of ESTs, we have obtained confirming evidence that at least 15 different Lola polypeptides are likely to be encoded. We were not able to verify the existence of the two remaining isoforms; however, this could be due to their being only expressed at developmental stages or in tissues that we did not examine. We further provide evidence that the number of transcripts from the locus is enhanced by alternative splicing of four different 5′-UTRs. The utilization of different 5′-UTRs and exon shuffling may provide a way to finely regulate stage- and tissue-specific expression of multiple gene products from the locus that serve different functional roles. This kind of complex gene organization has previously been observed at other loci. The most extreme example may be the *Dscam* locus that codes for cell adhesion receptors in the *Drosophila* nervous system and that potentially can generate more than 38,000 Dscam receptor isoforms (41). Another example related to control of gene expression is the locus of the trithorax group protein mod(mdg4). This locus encodes at least 21 different BTB domain-containing protein isoforms (42). At least one of these isoforms has been shown to associate with

Su(Hw) to exert gypsy insulator function preventing enhancer-promoter communication (43). Thus, differential splicing may be a general mechanism for BTB domain proteins to generate functional diversity.

The BTB domain has been shown to promote dimerization and the residues necessary for this function have been identified for the Bab protein (16). Comparison between the Lola and Bab BTB domains show that all these residues are conserved in the Lola BTB domain indicating that it has the capacity for homodimer formation. Furthermore, our immunoprecipitation experiments strongly suggest that Lola zf5 forms heterodimers with other Lola isoforms. Formation of homo- or heterodimers between Lola isoforms, including Lola zf5 is likely to lead to different developmental consequences by modifying DNA binding specificities and/or affinities of the Lola zinc finger isoforms. The majority of BTB-containing zinc finger proteins described thus far have been observed to function as transcriptional repressors, and several of these have been shown to directly bind co-repressor components of histone deacetylase complexes to their dimerized BTB domains (reviewed in Ref. 44). Consistent with such a repressive activity it has been suggested that Lola long may reduce expression of *copia* transposable elements in the *Drosophila* nervous system (11). However, Cavarec *et al.* (11) also observed Lola-mediated positive regulation of *copia* transcription in the gonads indicating that Lola isoforms also can act as transcriptional activators. In support of this notion, they identified a *D. hydei* isoform of Lola that binds directly to the *copia* enhancer and positively regulates transcription in transfected S2 cells. However, this effect was abrogated in a dose-dependent manner when the *D. melanogaster* Lola long coding sequence was co-transfected. Our analysis shows that the zinc finger domains of the *D. hydei* Lola isoform are nearly identical to those of *D. melanogaster* Lola zf8, with only a single conservative substitution of serine to threonine in the second zinc finger domain. Thus, the expression of alternative Lola isoforms may determine whether Lola acts as an activator or a repressor within different tissues and at specific stages during development.

C_2H_2 zinc fingers are one of the most common DNA binding motifs found in eukaryotic transcription factors and are characterized by a small number of conserved residues that generate the folded $\beta\beta\alpha$ domain structure necessary to align the residues that coordinate binding activity (reviewed in Ref. 45). The identification of alternatively spliced isoforms of the *lola* locus containing different zinc finger arrangements in conjunction with the ability of Lola isoforms to generate heterodimers suggests that Lola complexes functioning as transcription factors may recognize a wide range of potential DNA regulatory sequences. However, it should be noted that many of the Lola isoforms have a very unusual arrangement of zinc finger domains that suggest they have the potential to be involved in protein-protein interactions as well. Although most zinc fingers are of the C_2H_2 class, in each of the Lola isoforms where the zinc finger is present as a tandem array, the first finger is of the unusual C_2HC class. This atypical zinc finger domain is also found in MYST family histone acetyltransferases such as MOF, where it has been shown to be essential for nucleosome interactions (46). Additionally, this domain has been implicated in binding non-histone proteins (47), RNA (48–50), and DNA (51, 52).

Our genetic experiments suggest that a reduction of protein levels resulting from mutation in the *lola* locus can act as a dominant modifier of a hypomorphic *JIL-1* allele leading to an increase in embryonic viability. However, in these experiments the P element insertion into the *lola* locus is likely to perturb transcription of many of the Lola isoforms. Thus, we do not know which of the Lola isoforms are responsible for the genetic interaction or whether JIL-1 acts upstream or downstream. Thus, several scenarios for a functional interaction between Lola proteins and JIL-1 that enhances viability can be envisioned. On one hand, JIL-1 may act as a derepressor to counteract a potential repressive function of Lola zf5 or other Lola isoforms on gene expression. Derepression of the gene products from these loci may lead to enhanced viability. On the other hand, Lola zf5 or other Lola isoforms may normally act to down-regulate JIL-1 kinase activity by physically interacting with JIL-1. Consequently, the decrease in JIL-1 kinase activity observed in a hypomorphic mutant background may be alleviated by the reduction of its negative regulator in the *lola* mutant. In a third scenario, the interaction of JIL-1 with Lola zf5 may indeed enhance transcription at some genes, but the loss in the *lola* mutant of other Lola isoforms that normally function to down-regulate gene expression in other contexts may counterbalance the reduced JIL-1 activity in the JIL-1 hypomorph. Thus, the interaction between JIL-1 and the *lola* locus may be highly complex and promises to provide new experimental avenues into exploring the mechanisms of modification of chromatin and/or the regulation of gene expression during early embryogenesis.

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A Developmentally Regulated Splice Variant from the Complex*lola* Locus Encoding Multiple Different Zinc Finger Domain Proteins Interacts with the Chromosomal Kinase JIL-1

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