

lola Is an Evolutionarily New Epigenetic Regulator of *dpp* Transcription during Dorsal–Ventral Axis Formation

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Abstract

Secreted ligands in the Dpp/BMP family drive dorsal–ventral (D/V) axis formation in all Bilaterian species. However, maternal factors regulating Dpp/BMP transcription in this process are largely unknown. We identified the BTB domain protein *longitudinals lacking-like* (*lola*) as a modifier of *decapentaplegic* (*dpp*) mutations. We show that *Lola* is evolutionarily related to the Trithorax group of chromatin regulators and that *lola* interacts genetically with the epigenetic factor *Trithorax-like* during Dpp D/V signaling. Maternally driven *Lola*^{HA} is found in oocytes and translocates to zygotic nuclei prior to the point at which *dpp* transcription begins. *lola* maternal and zygotic mutant embryos display significant reductions in *dpp*, pMad, and *zerknüllt* expression, but they are never absent. The data suggest that *lola* is required to maintain *dpp* transcription during D/V patterning. Phylogenetic data revealed that *lola* is an evolutionarily new gene present only in insects and crustaceans. We conclude that *Lola* is the first maternal protein identified with a role in *dpp* D/V transcriptional maintenance, that *Lola* and the epigenetic protein *Trithorax-like* are essential for Dpp D/V signaling and that the architecture of the Dpp D/V pathway evolved in the arthropod lineage after the separation from vertebrates via the incorporation of new genes such as *lola*.

Key words: BTB domain, Dpp/Mad/BMP/TGF- β , *Drosophila*, development, new gene evolution.

Introduction

Genetic screens for modifiers of *decapentaplegic* (*dpp*) mutations have identified many highly conserved signal transduction pathway components essential for *Drosophila* dorsal–ventral axis formation (e.g., the prototype Smad protein *Mad*; Sekelsky et al. 1995). Each of these factors is maternally supplied as either RNA or protein, in effect “priming” embryonic cells for a rapid response to the earliest zygotic genes. In *Drosophila melanogaster*, the maternal to zygotic transition begins with a small number of transcribed genes during nuclear cycle 8 in the syncytial embryo (Pritchard and Schubiger 1996). Zygotic transcription ramps up slowly with dorsally restricted *dpp* expression first visible during nuclear cycle 10 (Jackson and Hoffmann 1994). Two maternal proteins required for the global activation of early zygotic genes, including *dpp*, are the ubiquitous transcription factors *Zelda* and *Stat92E* (Liang et al. 2008; Tsurumi et al. 2011).

Extracellular interactions generate the highest levels of Dpp activity in dorsal-most regions. Dpp signal transduction is stimulated when the ligand binds the type I receptors *Thickveins* (Tkv) and *Saxophone* (Sax) together with the type II receptor *Punt*. These transmembrane kinases then

phosphorylate the signal transducer *Mad*. Phosphorylated *Mad* (pMad) translocates to the nucleus where it joins its sister *Smad* protein *Medea* to regulate target genes. Maximum Dpp activity stimulates genes such as *zerknüllt* (*zen*) that drive dorsal-most cells to become *amnioserosa*. Repression of *dpp* transcription in ventral-most cells by *Dorsal* permits the activation of *Twist* and propels these cells to become *mesoderm*. One mechanism of Dpp pathway termination is monoubiquitylation of *Medea* by *Nedd4*. Subsequently, the pathway is reset by *Medea* activation via deubiquitylation by *Fat Facets* (Shimmi and Newfeld 2013).

Notwithstanding the reversal of D/V polarity between insect and vertebrate embryos (insect “nerve cords” develop on the ventral side) the signaling pathway dictating D/V polarity in both phyla is extraordinarily conserved. In vertebrates, a ventralizing gradient of *Bone Morphogenetic Protein* (BMP) employs homologous extracellular and signal transducing proteins in the same manner as the insect Dpp dorsalizing gradient (Bier and De Robertis 2015). An exception to universal conservation in the pathway was recently reported with the *Medea* ubiquitylase *Nedd4* (an HECT class enzyme) replaced in the vertebrate lineage by the vertebrate-specific *Smad4*

ubiquitylase TIF1- γ /TRIM33 (a RING class enzyme; Wisotzkey et al. 2014).

Building upon a prior genetic screen, we found that *longitudinals lacking-like* (*lola*), which encodes a BR-C, Ttk and Bab (BTB) domain protein related to the Trithorax group of epigenetic markers, has a role in Dpp D/V signaling. In the absence of maternal and zygotic *lola*, transcription of *dpp* is significantly reduced. This leads to abnormal pMad, reduced *zen* expression, and ventralization of the embryo. Phylogenetic studies revealed that *lola* is an evolutionarily new gene present only in insects and crustaceans. Overall the data reveals three new insights: that *Lolal* is the first maternal protein identified with a role in *dpp* D/V transcriptional maintenance, that *Lolal* and the epigenetic protein *Trl* are essential for Dpp D/V signaling and that the architecture of the Dpp D/V pathway evolved in the arthropod lineage after the separation from vertebrates via the incorporation of new genes such as *lola*.

Results

Lolal Is a Dominant Maternal Enhancer of *dpp* in D/V Patterning

The deletion *Df(2R)Pcl-11B* was identified as a dominant maternal enhancer of the recessive allele *dpp^{hr4}* but the responsible gene was not shown (Nicholls and Gelbart 1998). We examined ten lethal P-element insertions within the deleted region for maternal enhancement of *dpp^{hr4}*. *P{lacW}^{k02512}* an insertion in the 5' UTR of *lola* (CG5738; also known as *batman*) displayed strong dominant maternal enhancement (fig. 1; supplementary table S1, Supplementary Material online). We generated two new *lola* mutations via P element excision (*lola¹¹²²* and *lola¹⁷²²*) and confirmed that these did not affect the adjacent gene *adipose* via obesity tests (Wisotzkey et al. 2003). The two excision alleles and the *lola* insertion allele *P{EP}^{G9603}* failed to complement each other or *Df(2R)Pcl-11B* (supplementary table S2, Supplementary Material online). Repeating the dominant maternal enhancement assay revealed that *Df(2R)Pcl-11B* and the *lola* alleles phenocopied the effect of mutations in *Mad* and *Medea*. The reverse cross with maternal *dpp^{hr4}* had no effect (supplementary table S1, Supplementary Material online).

To extend these results, we performed dominant maternal enhancement rescue experiments with UASP transgenes of *Lolal*, activated *Tkv*, activated *Sax*, activated *Medea*, and *Trithorax-like* (*Trl*) under the control of the maternal driver *nos.Gal4*. In every case, a substantive rescue was observed. *Lolal* and *Lolal^{HA}* rescue of *lola¹¹²²* enhancement generated roughly 75% of expected progeny versus 6% without *Lolal*. Rescue of *lola¹¹²²* enhancement by activated *Tkv*, activated *Medea* and *Trl* was 100% of expected. Significant rescue in all cases was also achieved in maternal enhancement assays with the more severe allele *dpp^{hr27}* (fig. 2, supplementary fig. S1, and supplementary tables S2 and S3, Supplementary Material online). It appears that the presence of activated receptors or signal transducers can supplement the attenuated Dpp signal in enhanced embryos to the point at which feedback mechanisms (Wang and Ferguson 2005) are able to restore the

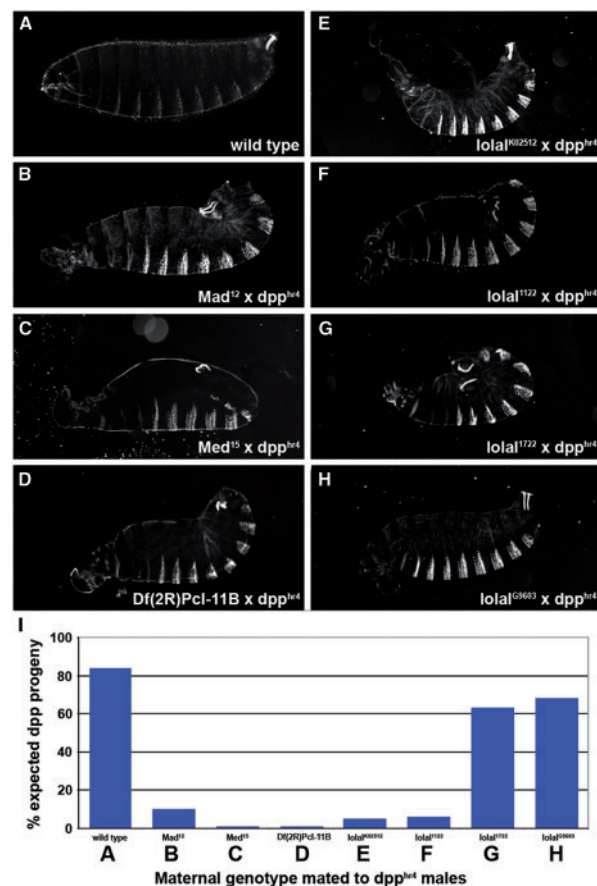


FIG. 1. *lola* dominant maternal enhancement of *dpp*. Cuticles from the progeny of crosses between the indicated heterozygous females (except *Med¹⁵* females were homozygous) and males heterozygous for *dpp^{hr4}* are shown. Anterior is to the left and dorsal up with the maternal genotype listed first. (A) Wild-type with the head skeleton (left), ventral denticle belts (bottom), and Filzkörper (top right) visible. (B) *Mad¹²* progeny with a ventralized phenotype containing a curved body, dorsally extended denticles, herniated head, and defective Filzkörper. (C) *Med¹⁵* progeny shows a more pronounced ventralized phenotype. (D) *Df(2R)Pcl-11B* progeny are similar to those of *Mad¹²*. (E) *lola^{k02512}*, (F) *lola¹¹²²*, (G) *lola¹⁷²²*, and (H) *lola^{G9603}* progeny are also ventralized. (I) Graph of progeny adult viability from the crosses shown above. Bars are connected to panels by letters and numerical data is shown in supplementary table S1, Supplementary Material online.

pathway. From this perspective, the rescue data suggest that *lola* enhancement of *dpp* occurs upstream of Dpp receptors and signal transducers.

Consistent with the rescue data, in two *Drosophila* S2 cell assays *ds-lolal* did not prevent pMad activation or Dpp-dependent reporter gene activation. These cell-based assays indicate no role for *lola* in Dpp signal transduction (supplementary fig. S2, Supplementary Material online). Analysis of *lola* mutant embryos did not reveal any Dpp-dependent phenotypes and stage of lethality assays showed that *lola* mutants die as pupae rather than as larva, like mutants for *Mad* and *Medea* (supplementary figs. S3 and S4 and supplementary tables S5 and S6, Supplementary Material online). These mutant studies suggest there is no role for *lola* in

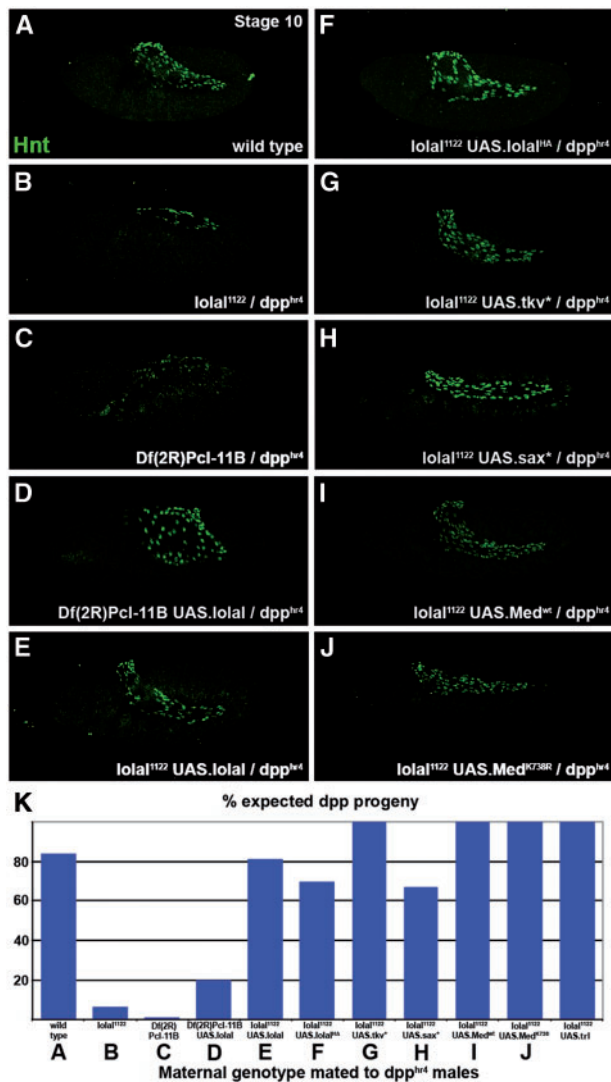


FIG. 2. Maternal expression of Lolal, Dpp pathway components or Trl rescues *lolal* dominant maternal enhancement of *dpp*. Stage 10 embryos with anterior to the left and dorsal up displaying Dpp-dependent Hindsight (green) in amnioserosa cells. The maternal genotype is listed first as a heterozygous female or a heterozygous female carrying a UASP transgene driven maternally by nos.Gal4. The paternal genotype is *dpp^{hr4}*. (A) Wild type. (B) Maternal *lolal¹¹²²* yields little Hindsight. (C) Maternal *Df(2R)Pcl-11B* yields no Hindsight. (D) Maternal *Df(2R)Pcl-11B* with Lolal partially rescues Hindsight. (E) Maternal *lolal¹¹²²* with Lolal fully rescues Hindsight. (F) *Lolal^{HA}*, (G) *Tkv^{*}*, (H) *Sax^{*}*, (I) *Med^{wt}*, and (J) *Med^{K738R}* also fully rescue Hindsight. (K) Graph of progeny adult viability from the crosses above. Bars are connected to panels by letters and numerical data is shown in [supplementary table S3, Supplementary Material](#) online. The bar at the far right shows complete rescue of *lolal¹¹²²* dominant maternal enhancement of *dpp^{hr4}* by maternal expression of Trl.

embryonic development beyond D/V patterning. Analysis of *lolal* somatic clones in larval and pupal wing disks indicated that loss of *lolal* had no effect on *dpp* transcription, pMad expression, or Dpp target gene activation (e.g., *brinker-lacZ*; [supplementary fig. S5, Supplementary Material](#) online). Collectively the S2 cell assays, zygotic mutant data, and

wing disk clone results imply that *lolal* only plays a role in Dpp signaling during D/V patterning.

Maternal *Lolal^{HA}* Translocates to the Nucleus Prior to *dpp* Transcription

lolal dominant maternal enhancement of *dpp* suggests that *lolal* RNA and protein are generated during oogenesis and deposited in unfertilized eggs as are *Mad* and *Medea*. We found *lolal* RNA in ovarioles plus *lolal* maternal RNA and protein ubiquitously distributed in unfertilized eggs (detected as translated *Lolal^{HA}* expressed via nos.Gal4; [fig. 3](#) and [supplementary fig. S6, Supplementary Material](#) online). This is consistent with a study reporting that *lolal* RNA is present in the embryo prior to the maternal to zygotic transition (Fisher et al. 2012). At stage 5, *lolal* zygotic RNA is ubiquitously present, a spatial distribution indicating that *lolal* is not a target of Dpp signaling.

We then examined *Lolal^{HA}* expression during the maternal to zygotic transition ([fig. 3](#) and [supplementary fig. S6, Supplementary Material](#) online). *Lolal^{HA}* rescues *lolal* enhancement of *dpp^{hr4}* providing confidence that *Lolal^{HA}* mimics the activity of endogenous Lolal. In these assays, we employed Bonus as a marker for developmental timing. Bonus migrates into nuclei at the maternal to zygotic transition (Wisotzkey et al. 2014). If *Lolal* migrates into nuclei coincidentally with Bonus, then it will be in place to modulate *dpp* transcription. At nuclear cycle 8 prior to the initiation of zygotic transcription (Pritchard and Schubiger 1996), *Lolal^{HA}*, and Bonus are ubiquitous in the cytoplasm and absent from nuclei. At cycle 9, the initiation of the transition, *Lolal^{HA}*, and Bonus are present in both the cytoplasm and nuclei indicating that at least a fraction of each protein has translocated into nuclei. This is prior to the initiation of *dpp* transcription in cycle 10 (Jackson and Hoffmann 1994). By cycle 12, *Lolal^{HA}* and Bonus are further concentrated in nuclei and nuclear localization is complete by cycle 14. The RNA and protein expression data suggested the hypothesis that *lolal* dominant maternal enhancement of *dpp* mutations is due to Lolal regulation of *dpp* transcription, an unprecedented result for a dominant maternal enhancement screen.

Lolal Is Required to Maintain Normal Levels of *dpp* Transcription

To test the hypothesis that *lolal* is necessary for *dpp* transcription, we generated *lolal¹¹²²* maternally mutant eggs via germline clone bearing females (*lolal* GLC). These females were mated to heterozygous *lolal¹¹²²* males. The enhancement and expression data suggests that expression of *dpp* will be reduced during D/V patterning in the half of progeny that are *lolal* GLC homozygous mutant embryos. These have neither a maternal nor a paternal source of functional *lolal* and will display D/V defects. The other half, the *lolal* GLC heterozygous mutant embryos, will have a paternal copy of *lolal*, and the zygotic mutant data suggests that they will appear wild type. Analysis of GLC cuticles ([supplementary table S7,](#)

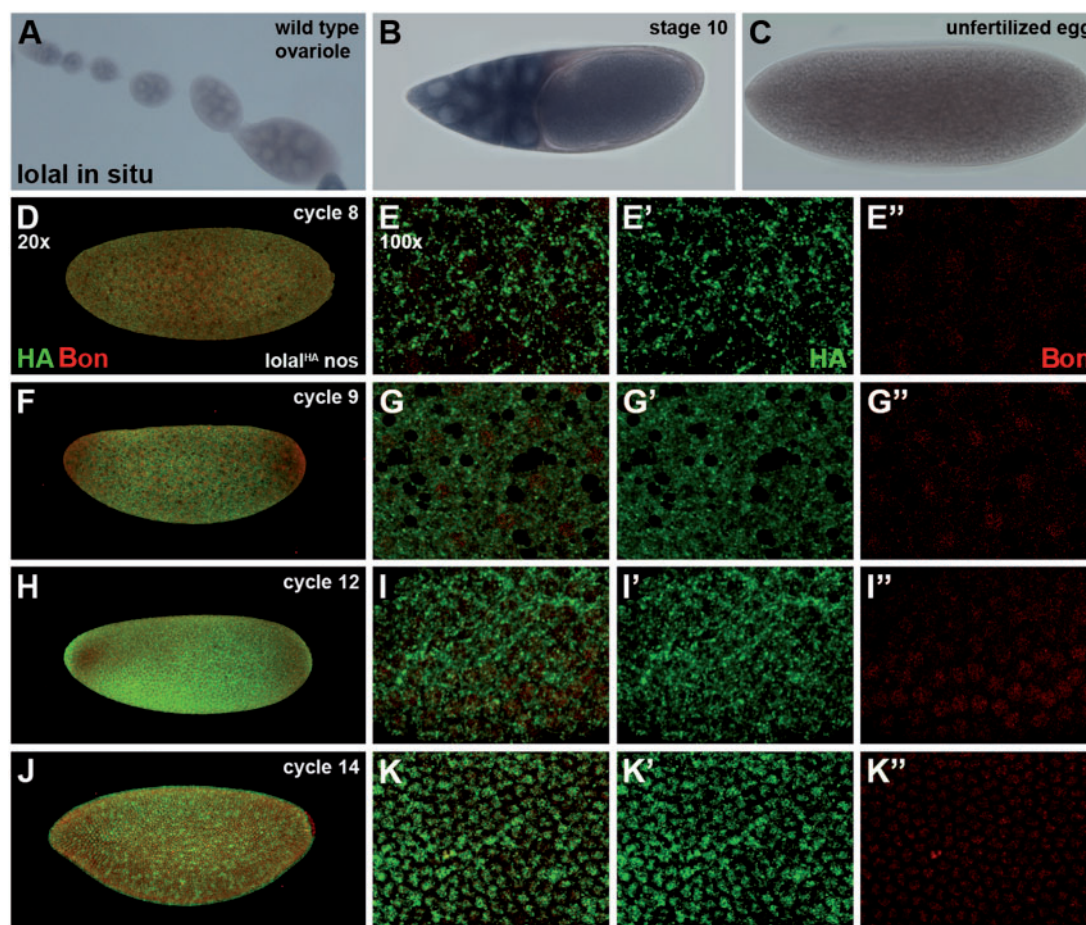


Fig. 3. *lolal* is expressed during oogenesis, is maternally supplied as RNA and protein, and enters nuclei prior to *dpp* transcription. *lolal* maternal RNA expression in wild type revealed via alkaline phosphatase (blue). (A) Ovariole, (B) Stage 10 egg chamber, and (C) Unfertilized egg contain ubiquitous *lolal* RNA. Opposite strand controls are in [supplementary fig. S6, Supplementary Material](#) online. (D–K) *lolal*^{HA} maternal protein expression in embryos shown anterior to the left and dorsal up displaying HA (green) and Bonus (Bon; red) at 20 × (stack) or 100 × (single slices shown as two colors and individual channels). Bonus migrates into nuclei at the maternal to zygotic transition (Wisotzkey et al. 2014) and is employed here as a marker for developmental timing. Unfertilized egg, wild type, and DAPI controls are in [supplementary fig. S6, Supplementary Material](#) online. Each row is from the same embryo. (D, E) Nuclear cycle 8 (Stage 2) embryo has ubiquitously cytoplasmic HA and Bonus. (F, G) Nuclear cycle 9 (Stage 3) embryo revealing Bonus has begun to concentrate in nuclei whereas HA displays roughly equal concentrations in the cytoplasm and nuclei. (H, I) Nuclear cycle 12 (latter part of Stage 4) embryo with Bonus completely nuclear whereas HA is still equally present in the cytoplasm and nuclei. (J, K) Nuclear cycle 14 (Stage 5) embryo with Bonus and HA both completely nuclear.

[Supplementary Material](#) online) revealed that half had D/V defects and assays of GLC adult viability did not identify any *lolal* homozygous mutants suggesting the two classes of progeny are as predicted.

dpp transcription in the progeny was then analyzed by fluorescent RNA in situ hybridization assays that included DAPI to identify nuclei for staging. Given reports of stochastic variation in *dpp* expression at this stage (Karim et al. 2012), we employed an unbiased empirical standard of *dpp* pixel intensity to distinguish between the “bottom” and “top” *dpp* expressing groups of embryos corresponding to the two classes of predicted progeny—*lolal* GLC homozygous mutant and *lolal* GLC heterozygous embryos (i.e., computational application of Mendelian ratios to progeny to determine an embryo’s genotype). Employing wild-type embryos that were analyzed in parallel and with common reagents as controls, statistical tests were applied to identify differences between the bottom and top classes within *lolal* GLC progeny

and within wild-type progeny, as well as differences between the bottom and top *lolal* GLC class and pooled wild-type embryos.

The pixel intensity method easily distinguished two classes of *dpp* expressing embryos with nonoverlapping distributions among *lolal* GLC progeny at early stage 6 ([fig. 4](#), quantitation in [supplementary fig. S7, Supplementary Material](#) online). *dpp* was always present in the bottom group, but expression was significantly below the top group ($P = 0.003$). There was no significant difference between the top and bottom groups of wild-type progeny ($P = 0.151$). The *lolal* GLC bottom group was also significantly below wild type ($P = 0.001$), whereas *dpp* expression in the top group of *lolal* GLC progeny was indistinguishable from wild type ($P = 0.785$). Our interpretation is that *lolal* GLC homozygous mutant embryos are the bottom group with reduced expression and heterozygous mutants are the top group since the top group’s expression matches wild type. The data show a statistically significant

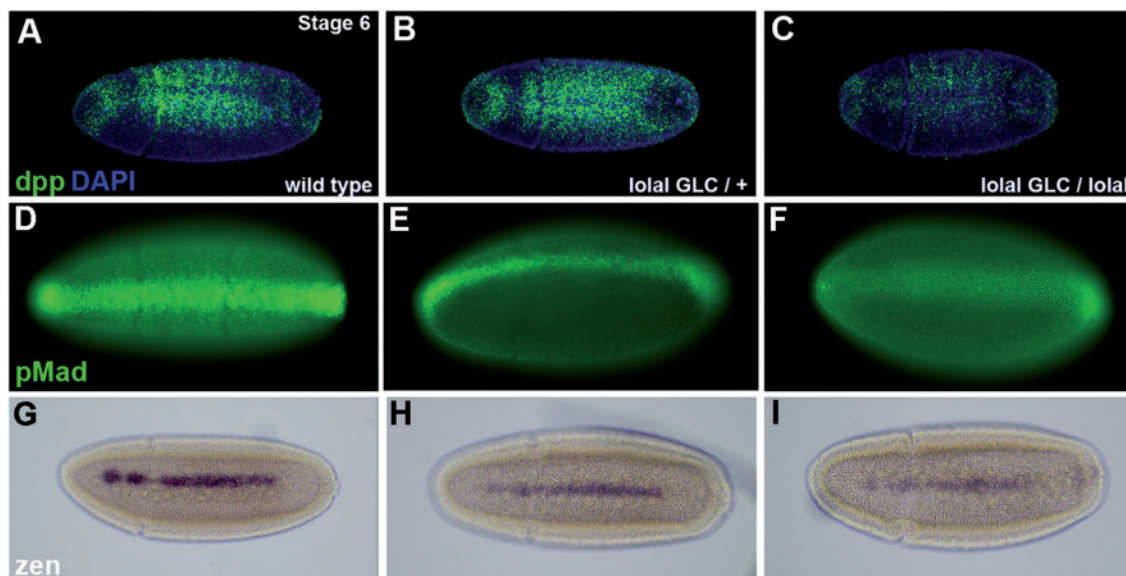


FIG. 4. Loss of maternal and zygotic *lola* significantly reduces *dpp* and *zen* transcription as well as a pMad expression at stage 6. Stage 6 embryos with anterior to the left and dorsal up. Left column shows wild type embryos stained side-by-side with *lola* embryos. Middle column shows heterozygous *lola*¹⁷²² germline clone (GLC) embryos with paternal rescue via wild-type *lola* on the balancer chromosome (*lola* GLC/+). Right column shows homozygous *lola* mutant GLC embryos with *lola*¹⁷²² on the paternal chromosome (*lola* GLC/*lola*). Detailed methods and quantitative data in [supplementary fig. S7, Supplementary Material](#) online. (A–C) Embryos in dorsal view analyzed by confocal microscopy for *dpp* RNA (green) and DAPI (nuclei; blue). The homozygous GLC embryo contains significantly reduced *dpp* transcription. (D–F) Embryos in dorsal view analyzed by confocal microscopy for pMad expression (green). The homozygous GLC embryo contains significantly reduced pMad expression. (G–I) Embryos in dorsal view displaying the narrow *dpp*-dependent stripe of *zen* RNA analyzed by light microscopy and alkaline phosphatase staining (blue). The homozygous GLC embryo contains significantly reduced *zen* transcription.

reduction in *dpp* transcription intensity in *lola* GLC homozygous mutants. We conclude that maternal and zygotic *lola* are each necessary and sufficient for proper *dpp* transcription since the phenotype is visible only in the absence of both.

Consistent with the reduction in *dpp* transcription, the pixel intensity method easily distinguished two classes of pMad expressing embryos with nonoverlapping distributions among *lola* GLC progeny at early stage 6 ([fig. 4](#), quantitation in [supplementary fig. S7, Supplementary Material](#) online). pMad was always present in the bottom group but expression intensity was significantly below the top group ($P = 0.002$). There was no significant difference between the top and bottom groups of wild-type progeny ($P = 0.220$). The *lola* GLC bottom group was also significantly below wild type ($P = 0.004$), whereas pMad expression in the top group of *lola* GLC progeny was indistinguishable from wild type ($P = 0.992$). The data show a statistically significant reduction in pMad expression intensity in *lola* GLC homozygous mutant embryos.

Although frequently employed as an on/off measure, pMad is known to be temporally highly dynamic and moderately variable between embryos of the same age. This makes it less reliable as a quantitative readout ([Umulis et al. 2010](#)). A more sensitive indicator of Dpp D/V signaling is the narrow dorsal stripe of *zen* expression at stage 6, a direct transcriptional target of pMad ([Rushlow et al. 1987](#)). We applied the quantitative approach to examine the pixel area of *zen* transcription ([fig. 4](#), quantitation in [supplementary fig. S7, Supplementary Material](#) online). For *zen*, the pixel area is

more relevant than pixel intensity because *zen* expressing cells become the amnioserosa whereas the adjacent non-expressing cells become the dorsal ectoderm ([Rusch and Levine 1997](#)).

Consistent with the reduction in *dpp* transcription and pMad expression, the pixel intensity method easily distinguished two classes of *zen* expressing embryos with nonoverlapping distributions among *lola* GLC progeny at early stage 6 ([fig. 4](#), quantitation in [supplementary fig. S7, Supplementary Material](#) online). *zen* was always present in the bottom group but expression was significantly below the top group ($P = 0.005$). There was no significant difference between the top and bottom groups of wild-type progeny ($P = 0.083$). The *lola* GLC bottom group was also below wild type ($P = 0.001$), whereas *zen* transcription in the top group was indistinguishable from wild type ($P = 0.131$). The data show a statistically significant reduction in *zen* expression area in *lola* GLC homozygous mutants. The reduction in *dpp* transcription leading to decreased pMad and *zen* expression in *lola* homozygous GLC mutant embryos provides a mechanistic explanation for *lola* dominant maternal enhancement of *dpp*.

The fact that *dpp*, pMad, and *zen* were always present but at significantly reduced levels in *lola* GLC homozygous mutants at stage 6 suggested a defect in maintenance of *dpp* transcription rather than initiation. To determine if *lola* GLC homozygous mutants display a defect in the initiation of *dpp* transcription at stage 5, we examined *lola* GLC embryos at mid and late-stage 5. This is when the *dpp*-dependent pMad dorsal stripe first becomes visible and then strengthens. The

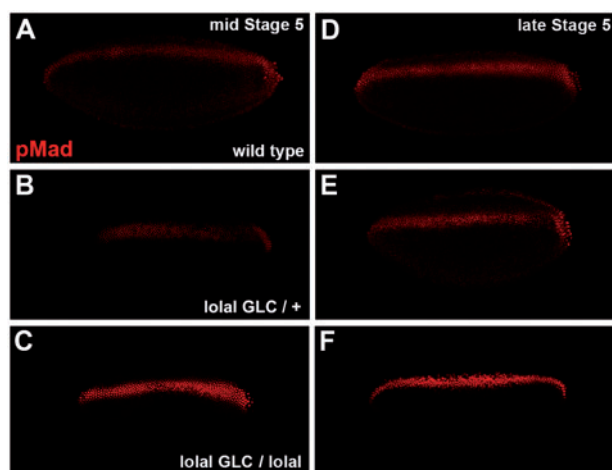


FIG. 5. Loss of maternal and zygotic *lolal* significantly increases pMad expression at stage 5. Mid and late-stage 5 embryos, in the left and right column respectively, with anterior to the left and dorsal up. Top row contains wild type embryos stained side-by-side with *lolal* embryos. Middle row shows heterozygous *lolal*¹⁷²² GLC embryos with paternal rescue via wild type *lolal* on the balancer chromosome (*lolal* GLC/+). Bottom row shows homozygous *lolal*¹⁷²² GLC embryos with *lolal*¹⁷²² on the paternal chromosome (*lolal* GLC/*lolal*). Detailed methods and quantitative data in [supplementary fig. S8, Supplementary Material](#) online. (A–C) Midstage 5 embryos in dorsal view analyzed by confocal microscopy for pMad expression (red). The homozygous GLC embryo (*lolal* GLC/*lolal*) contains significantly increased pMad expression. (D–F) Late stage 5 embryos analyzed similarly. The homozygous GLC embryo (*lolal* GLC/*lolal*) also contains significantly increased pMad expression.

pixel intensity method easily distinguished two classes of pMad expressing embryos with nonoverlapping distributions among *lolal* GLC progeny at both time points ([fig. 5](#), quantitation in [supplementary fig. S8, Supplementary Material](#) online). pMad was always present in the bottom group at both times but expression was significantly below the top group (midstage $P = 0.002$; late-stage $P = 0.031$). There was no significant difference at either time between the top and bottom groups of wild type progeny (midstage $P = 0.258$; late-stage $P = 0.175$). The *lolal* top group was significantly above wild type (midstage $P = 0.002$; late-stage $P = 0.007$), whereas pMad expression in the bottom group was indistinguishable from wild type (midstage $P = 0.566$; late-stage $P = 0.809$). Our interpretation is that *lolal* GLC homozygous mutant embryos are the top group with increased pMad expression and *lolal* heterozygous mutants are the bottom group since the bottom group's expression matches wild type. The mid and late-stage 5 stage data show a statistically significant increase in pMad expression intensity in *lolal* GLC homozygous mutants.

The initial increase in Dpp signaling revealed by the mid and late-stage 5 pMad data together with the subsequent decrease in *dpp* transcription, pMad, and *zen* expression at stage 6 strongly supports our initial thought that *lolal* GLC homozygous mutants have a defect in *dpp* transcription maintenance and not *dpp* transcription initiation. In a final GLC assay, we tested the possibility that *lolal* influences the transcription of other genes in D/V patterning by examining

Twist (stage 6), *short gastrulation* (late stage 5), and *zen* (early stage 5)—the wide dorsal stripe that is independent of Dpp signaling). For each gene, the two classes of *lolal* GLC progeny were not significantly different from each other nor was either class significantly different from wild type ([supplementary fig. S9, Supplementary Material](#) online). This implies that *lolal* function in D/V patterning may be specific to *dpp* transcription, though this conclusion requires additional verification. Overall, from the GLC data, we conclude that *lolal* does not affect *dpp* transcript initiation but instead is required for maintenance of proper *dpp* transcription levels.

Lolal Is a New BTB Domain Protein

Lolal is a small protein of 123 amino acids. Eighty-six residues are devoted to a BTB domain. BTB (also known as POZ) is a well-established homo- and hetero-multimerization domain ([Bonchuk et al. 2011](#)). The BTB domain is present in a very large family of proteins, with members in eukaryotes and prokaryotes characterized by rapid birth-and-death evolution ([Domman et al. 2014](#)). BTB proteins are highly diverse with N- and C-terminal extensions containing other domains ([Stogios et al. 2005](#)). In eukaryotes, BTB domains are often found in chromatin proteins ([Bonchuk et al. 2011](#)). Upstream of the BTB domain 27 residues form a Pipsqueak (Psq) domain found in Lolal's closest relatives but that has no known function ([Siegmond and Lehmann 2002](#)). To better understand Lolal origins, a phylogenetic tree containing fly, nematode, and human proteins with BTB domains similar to Lolal was constructed ([fig. 6](#)).

The Lolal family tree has two subfamilies and the Lolal subfamily has two branches. The Lolal branch contains 11 fly proteins, including the well-known chromatin/epigenetic protein Trithorax-like (Trl; a Zinc-finger protein also known as GAGA factor) as part of the Lolal group. The Lolal branch contains a single human protein, BTBD18 (hCG1730474; [Alonso et al. 2010](#)) that does not contain a Zinc-finger but has a recognizable Psq domain. BTBD18 was identified in a single patient as a fusion with the *myeloid/lymphoid leukemia* gene but has no known function. The sister to the Lolal branch contains 11 human proteins and a single fly protein. The asymmetric topology of these branches is consistent with previously noted lineage-specific expansions in the BTB family ([Aravind and Koonin 1999](#)). Of the 24 proteins in the Lolal subfamily, 19 contain a DNA-binding Zinc finger domain whereas the other primary subfamily is composed of non-DNA-binding Math/Bath and Kelch domain proteins.

Overall, the tree topology suggests that the common ancestor of the Lolal subfamily contained Zinc-finger, Psq, and BTB domains. Then, in the Lolal branch the arthropod, but not the vertebrate lineage, experienced multiple gene duplications with the Zinc-finger lost in a few instances including Lolal. The Lolal cluster, consisting of Lolal and its closest relatives Tramtrack (Ttk), Lola, and Modifier of *mdg4* (Mmd4), is the most distant from HsBTBD18 indicating that it resulted from the most recent arthropod duplications. This inference that Lolal is arthropod-specific is supported by unsuccessful searches for Lolal cluster sequences in other vertebrate

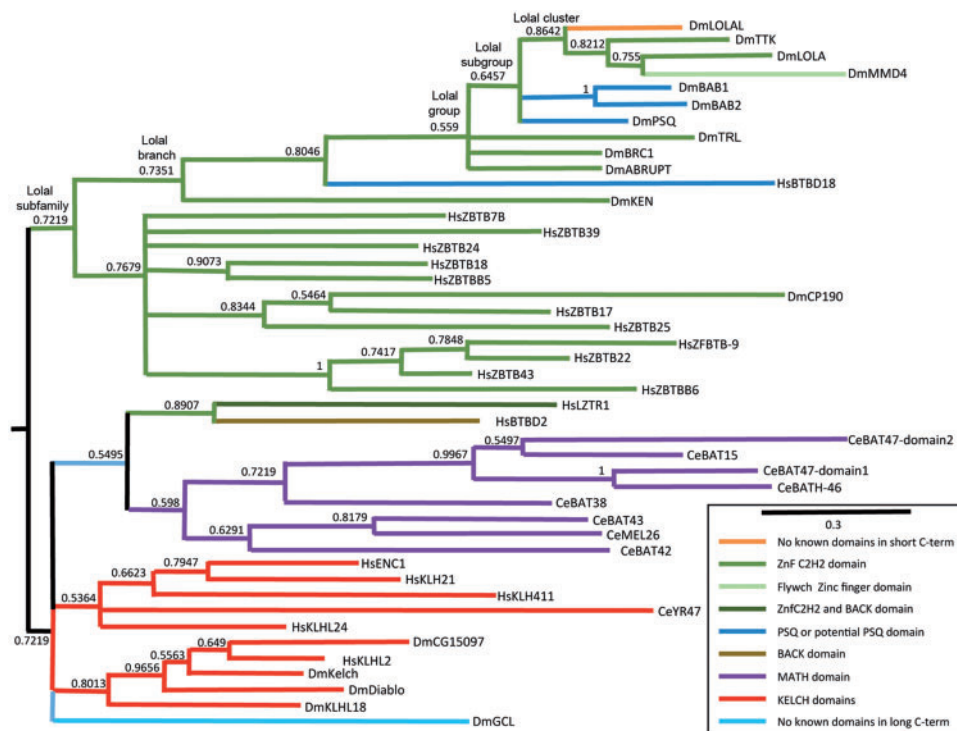


Fig. 6. Lolal is a new arthropod-specific BTB protein. Bayesian tree displaying 45 human (Hs), fly (Dm), and nematode (Ce) BTB domain proteins. Twenty-three proteins (Lolal subfamily) were selected due to the similarity of their BTB domain to Lolal. The other 21 proteins (bottom subfamily) contain Math/Bath or Kelch domains in addition to a BTB domain to provide a robust outgroup and allow the tree to be midpoint rooted. Lolal has no close vertebrate relatives indicating it is a new family member born after the arthropod-vertebrate divergence. Accession numbers are in [supplementary table S8a, Supplementary Material](#) online. Nodes defining tree features associated with Lolal are named. A scale bar showing amino acid substitutions per site is present. Colors of branches indicate the presence of additional functional domains as described. Posterior probabilities above 0.5 are shown. The alignment contained 77 informative positions. As described in the Materials and Methods, for alignments between 50 and 100 informative characters posterior probabilities ≥ 0.75 should be considered statistically significant.

genomes and the genomes of vertebrate siblings the sea urchin and tunicate.

The phyla Arthropoda consists of four subphyla—insects, crustaceans, chelicerates (mites/spiders), and myriapods (centipedes/millipedes). Phylogenetic studies indicate that the first to diverge were the chelicerates (543 million years ago [Mya]) then the myriapods (539 Mya) with insects and crustaceans separating 470 Mya (Rota-Stabelli et al. 2013). Utilizing this information as a scaffold, additional analyses such as reciprocal BLASTs, amino acid conservation frequency, and intron–exon structure comparisons suggested a serial duplication scenario that matches the topology for the Lolal subgroup: 1) Bab is oldest (present in all four subphyla), 2) Bab generated Ttk (present in insects and chelicerates but lost in crustaceans), 3) Ttk generated Lolal (present in insects and crustaceans), 4) Bab later generated Psq (present in insects and crustaceans), 5) Ttk later generated Mmd4 (only present in insects but has a longer branch than Lola), and 6) Mmd4 generated Lola (only present in insects).

The specificity of Lolal to insects and crustaceans is shown in a representative tree of Lolal homologs (four insect and five crustacean species; [fig. 7](#)). Overall the phylogenetic data shows that Lolal was generated in the arthropod lineage 230 Mya after the arthropod-vertebrate split (700 Mya; Parfrey et al. 2011). Although not the newest gene in its subgroup, it is newer

than any known *Drosophila* gene in D/V axis formation as all of the others have vertebrate homologs (e.g., Dorsal, Sog, Twist, Dpp, Mad, and Medea).

Discussion

dpp Transcription in D/V Patterning

The genetic screen that pointed us to *lola* also identified a second deletion, *Df(3L)66C-G28*, displaying dominant maternal enhancement. Employing the same candidate gene approach, we determined that *moleskin* is the dominant maternal enhancer of *dpp* in that deletion ([supplementary fig. S10, Supplementary Material](#) online). *moleskin* is a nuclear importer for Mad that was not previously implicated in D/V patterning (Xu et al. 2007). These two discoveries, 20 years after the identification of *Mad* and *Medea* utilizing the same enhancement screen, reinforce the value of genetic analyses in *Drosophila* as the premier method for gene discovery.

Genetic screens have also identified the maternal transcription factors Zelda and Stat92E as general activators of early zygotic genes including *dpp*, *sog*, *zen*, and *twist* (Liang et al. 2008; Tsurumi et al. 2011). Lolal differs from Zelda/Stat92E in three ways: 1) *lola* displays dominant maternal enhancement of *dpp* mutants whereas Stat92E does not, 2) *dpp* transcription initiates but goes awry in *lola* GLC homozygous embryos whereas it does not initiate in Zelda or

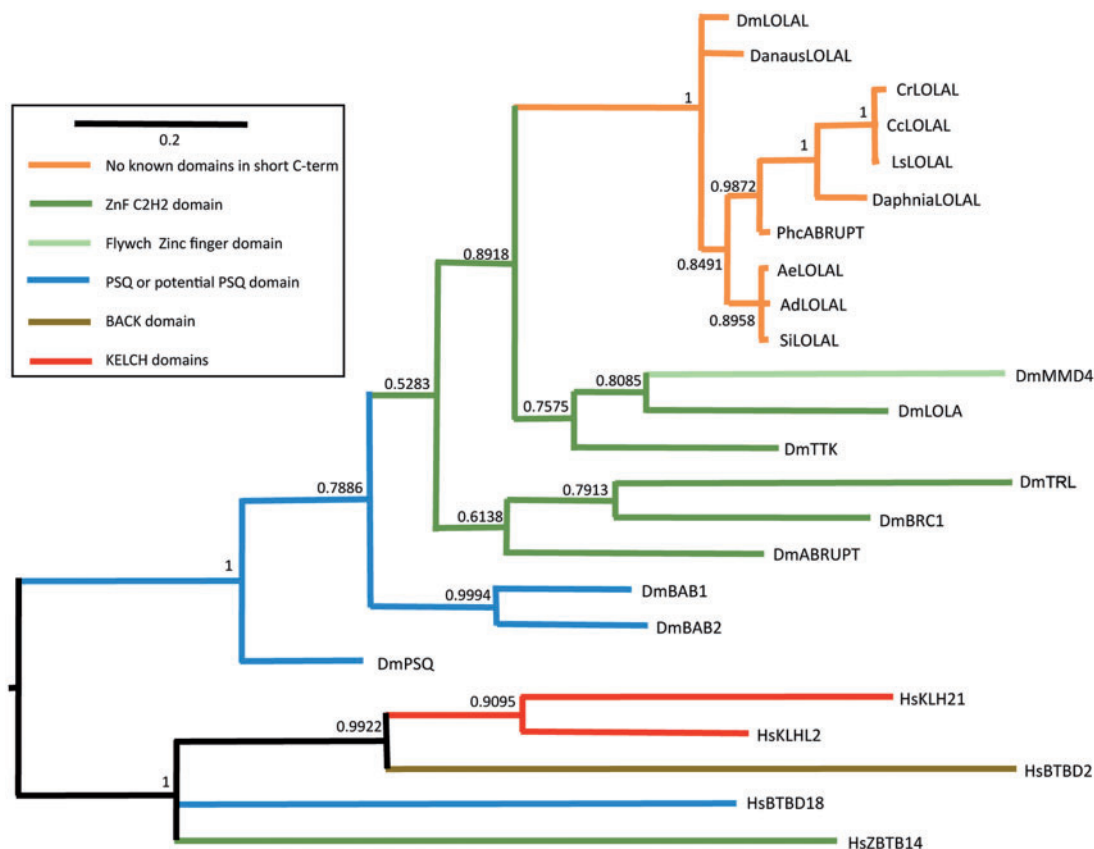


Fig. 7. Lolal is only present in insects and crustaceans. Bayesian tree of 24 proteins containing BTB domains similar to the Lolal BTB domain. These include the nine fly (Dm) proteins in the Lolal group of Fig. 6 (blue and green branches), plus nine insect, and crustacean Lolal homologs identified by BLAST (orange branches). The others are human (Hs) proteins, one from each of the five branches from Fig. 6. Pairwise alignments revealed that PhcABRUPT is misnamed as its BTB domain is 93% identical to Lolal's and it does not contain a Zinc-finger like DmABRUPT (green branch). Accession numbers are in [supplementary table S8b, Supplementary Material](#) online. The scale bar shows the number of amino acid substitutions per site. Colors of branches indicate the presence of additional functional domains as described. Posterior probabilities above 0.5 are shown. The alignment contained 89 informative positions. As described in the Materials and Methods, for alignments of between 50 and 100 informative characters posterior probabilities ≥ 0.75 should be considered statistically significant.

Stat92E GLC homozygous embryos, and 3) *sog*, early *zen*, and Twist expression are unaffected in *lotal* GLC homozygous embryos whereas they do not initiate in *Zelda* or Stat92E GLC homozygous embryos.

Mechanistically, Lolal may be needed to regulate *dpp* transcription because activation of early zygotic genes in *Drosophila* requires the combinatorial activity of transcription factors and chromatin proteins (Darbo et al. 2013; Sandler and Stathopoulos 2016). Chromatin proteins have been generally separated into the Polycomb group of repressors and the Trithorax group of activators though there are several that can perform both roles such as the Lolal group member Trl (Schuettengruber et al. 2007). Trl functions by influencing the placement of a methyl group on Histone H3 Lysine 9 (closed chromatin) or Lysine 4 (open chromatin) thereby modulating chromatin accessibility [reviewed in Kim and Kim (2012)].

The most prominent chromatin proteins active during the maternal to zygotic transition are Trl and the Lolal cluster member Ttk. Lolal has been shown to physically interact with Trl, to co-localize with Trl on larval salivary gland chromosomes and to co-localize with Trl in regions of actively

transcribing chromatin in an embryo-derived cell line (Faucheu et al. 2003; Mishra et al. 2003; Filion et al. 2010). In vitro Lolal forms heteromeric complexes with its subgroup relatives Ttk, Mmd4, and Psq (Bonchuk et al. 2011). Functionally, these four proteins form a genetic network that regulates ovariole number in *Drosophila* (Bartoletti et al. 2012).

Our hypothesis is that Lolal, with its epigenetic partner Trl, modifies chromatin near the cycle 10 *dpp* enhancers following *Zelda*/Stat92E activation of transcription. Lolal's epigenetic role is to maintain proper levels of *dpp* transcription over the next four nuclear cycles as the extracellular Dpp D/V morphogen gradient is established. Two pieces of additional evidence support this idea. First, dominant maternal enhancement assays with mutations in Lolal's closest relatives revealed a strong effect (supplementary table S1, Supplementary Material online) of a deletion allele of *pipsqueak* that removes Exon1A (*Df(2R)12*; Weber et al. 1995). *pipsqueak* has a maternal contribution and its earliest embryonic expression is dorsal-specific (Weber et al. 1995) putting it in a position to partner with Lolal in the regulation of *dpp*. Further, Pipsqueak functions are essential for sequence-

specific targeting of epigenetic complexes (Huang et al. 2002). Second, sequences in the *dpp* second intron are responsible for the cycle 10 initiation of *dpp* transcription (Huang et al. 1993; Jackson and Hoffmann 1994). An alignment of four *Drosophila* species revealed numerous conserved Zelda, Stat92E, and Trl binding sites in this intron supporting a role for epigenetic complexes in the modulation of *dpp* transcription.

New Gene Evolution in D/V Patterning

Studies of new genes in *Drosophila* showed that these could quickly become essential for viability, fertility, and behavior (Chen et al. 2013; Long et al. 2013). Indispensability for fertility and behavior results from the incorporation of new genes into existing pathways. A prediction of these studies is that the assimilation of new genes into developmental pathways underlies their requirement for viability. To gain insight into the evolutionary mechanisms behind the generation of new genes, we are currently comparing the expression patterns and upstream sequences of Lolal group proteins with the goal of correlating changes in expression with changes in transcription factor binding sites.

The architecture of D/V patterning and the Dpp/BMP pathway at its center, is among the most highly conserved processes in Bilateral development. This taxon is also called Eumetazoa and it originated roughly one billion years ago (bya; Kumar and Hedges 2011). Organisms as diverse as planarians and humans employ a common set of signaling molecules in a common format, albeit inverted in some cases (De Robertis and Sasai 1996). The Dpp/BMP pathway has other functions in development and is older than Bilateria. A complete Dpp/BMP pathway is present in *Nematostella vectensis* (a diploblast with two germ layers that originated 1.1 bya; Putnam et al. 2007). Individual members of families in the Dpp/BMP pathway including ligands, receptors, and Smads are found in all Metazoans including sponges [*Amphimedon queenslandica* originated 1.3 bya; reviewed in Konikoff et al. (2008)].

Although D/V patterning is conserved, its individual proteins are always under selective pressure. We recently showed in the vertebrate D/V program that a new gene can provide a selective advantage and become incorporated into this process. Our analysis of the ubiquitin ligases targeting vertebrate Smad4 and its fly homolog Medea revealed that a new vertebrate protein belonging to the RING class of ligases (TIF1- γ /TRIM33) replaced an older HECT class ligase (Nedd4) that performs this job in flies and presumably in the arthropod-vertebrate common ancestor. That data demonstrated that the vertebrate D/V program was not simply conserved since its divergence from flies, but has evolved by incorporating new genes (Wisotzkey et al. 2014).

The results for Lolal strongly complement that for TIF1- γ /TRIM33 with this new gene incorporated into the arthropod D/V program. Although certainly possible that Lolal replaced an older gene that performs epigenetic functions in D/V patterning upstream of *dpp* transcription in the common ancestor of arthropods and vertebrates, there are no known genes upstream of BMP in vertebrate D/V axis formation to guide us. Nevertheless, employing the Nedd4 and TIF1- γ /TRIM33

replacement scenario as a reference, data reported here suggest that the function performed by Lolal (epigenetic marker) is essential to vertebrate D/V patterning and that a complex containing proteins similar to Lolal and Trl fulfill this role. Among the Trl top BLAST hits in mammals is MIZ-1, which we propose as a candidate BMP transcription regulator in D/V axis formation. MIZ-1 forms multimers with the proto-oncogene BCL6 and the complex regulates the transcription of cyclin-dependent kinase inhibitor p21 to promote cell proliferation in adult B cells (Phan et al. 2005). Taken together, the phylogenetic data for Lolal and TIF1- γ /TRIM33 shows that the D/V patterning program shared by all Bilateria contains highly conserved features such as Dpp/BMP ligands, receptors, and Smads as well as dynamic features such ubiquitin ligases and chromatin proteins that are influenced by the assimilation of new genes.

Overall, we report that Lolal is the first maternal protein identified with a role in *dpp* D/V transcriptional maintenance. Equally important corollaries are that Lolal and the epigenetic protein Trl are essential for Dpp D/V signaling and that the architecture of the Dpp D/V pathway evolved in the arthropod lineage after the separation from vertebrates via the incorporation of new genes such as *lola1*.

Materials and Methods

Flies

Mutants are *Df(2R)Pcl-11B* (Nicholls and Gelbart 1998), *Df(2R)12* (*psq*; Weber et al. 1995), *Df(3L)bab^{Ar07}* (deletion of *bab1* and *bab2*; Couderc et al. 2002), *dpp^{e87}*, *dpp^{hr4}*, *dpp^{hr27}*, *dpp^{hr56}* (St. Johnston et al. 1990), *lola^{ORE76}* (Horiuchi et al. 2003), *P{EP}lola1^{G9603}* (Bellen et al. 2004), *P{lacW}lola1^{K02512}* (Török et al. 1993), *lola1¹¹²²*, and *lola1¹⁷²²* (this work), *Mad¹²* and *Med¹⁵* (Stinchfield et al. 2012), *modifier of mdg4^{T6}* (*mmd4*; Soltani-Bejnood et al. 2007), *Trl^{R67}* (Farkas et al. 1994), and *ttk^{1e11}* (Xiong and Montell 1993). Transgenic strains are *dpp-lacZ-BS3.0* (Blackman et al. 1991), *P{lacW}brk³⁸* (*brinker-lacZ*; Minami et al. 1999), *Trl^{EY04024}* (Bellen et al. 2011), *nos.Gal4:VP16-MVD1*, *UASP.GFP- α Tub84B*, *UASP.Med^{wt}*, and *UASP.Med^{K738R}* (Stinchfield et al. 2012), *UASP.Sax** (Xie and Spradling 1998) and *UASP.Tkv** (Casaneuva and Ferguson 2004). Balancers and GLC strains are in Flybase (Marygold et al. 2013).

Genetics

Assays for adult viability, dominant maternal enhancement, stage of lethality, transgenic rescue, and zygotic lethality were conducted as described (Stinchfield et al. 2012). Germline clone (GLC) females were generated with *FRTG13 lola1¹¹²²* as described (Wisotzkey et al. 2014). GLC females were mated to *lola1¹⁷²²* heterozygous males to assay *lola1* homozygous embryos (*lola1¹¹²²/lola1¹⁷²²*) and paternally rescued heterozygous embryos. Homozygous GLC embryos were identified by quantitative comparison with wild type. Pixel intensity plots reflecting *dpp*, *sog*, *zen* (stage 5), pMad, or Twist expression were created from single channel images in ImageJ (Schneider et al. 2012). Mean pixel intensity was obtained from areas of interest drawn on the embryo. The relative mean pixel

intensity was calculated by subtracting the mean pixel intensity of background (lateral region) from that of the expression domain, thus normalizing expression for each embryo prior to statistical analysis. For *zen* at stage 6, the pixel area was obtained by calculating the number of pixels within a domain encompassing all cells expressing *zen*. Pixel intensity and area values were imported into Excel and graphed. Extensive efforts were employed to minimize variation in technique with wild-type and *lolal* GLC embryos stained side-by-side, on the same day, using a similar number of embryos, the same probe, the same antibody, the same wash solutions, and imaged on the same day with the same settings on our only confocal. In the image analysis, only wild type and *lolal* GLC embryos from the same date were compared. Wing disk clones of *FRTG13 lolal*¹¹²² were generated by standard methods, marked by the absence of *GFP* and analyzed as described (Quijano et al. 2011).

Embryos

Cuticle preparations were as described and cuticle scoring employed standard criteria (e.g., Stinchfield et al. 2012). Fluorescent RNA in situ hybridization followed by TSA-488 (Molecular Probes) was utilized to visualize *dpp* as described (Ray et al. 1991; Nagaso et al. 2001). Alkaline phosphatase RNA in situ hybridization for *sog*, *zen*, and *lolal* were conducted as described (Künnapu et al. 2014). Nuclei were visualized directly with DAPI (Sigma). Primary antibodies were: Bonus-GP37 (Beckstead et al. 2001), Digoxigenin (Zymed), Dorsal (DSHB-7A4), HA-3F10 (Roche), Hindsight (DSHB-1G9), LacZ (Organon Teknika), pSmad (Epitomics), dSRF (Marenda et al. 2004), and Twist (Roth et al. 1989). Secondary antibodies were: donkey α -sheep-HRP (for TSA; Life Technologies), or AlexaFluor 488, 546 or 633 goat α -mouse, α -rabbit, α -rat or α -guinea pig (Life Technologies). Embryos were fixed after 4–6 h, antibody labeled and imaged as described (Quijano et al. 2011).

Molecular Biology

UAST.Lolal was generated by cloning a SpeI—XhoI fragment from cDNA *pOT2-LD14505* (Berkeley Drosophila Genome Project) into the NheI—XhoI sites of pUAST2. PCR products of the coding region from this clone were inserted into two Drosophila Gateway vectors: pPW to create UASP.Lolal or pPWH to create UASP.Lolal^{HA} (Drosophila Genomics Resource Center) followed by recombination into UASP. Primers for Gateway cloning were:

UASP.Lolal: Forward 5'-CACCATGATGTCGTCGGATCAACAG-3'
Reverse 5'-TCAACCTTCCCGCTTTATCG-3'
UASP.Lolal^{HA}: Forward 5'-CACCATGATGTCGTCGGATCAACAG-3'
Reverse 5'-ACCTTCCCGCTTTATCGAAC-3'

A *lolal* RNA in situ probe was generated by PCR via a primer bearing a T7 sequence overhang. Antisense RNA was then synthesized with T7 polymerase. Primers for PCR were:

Forward 5'-TAATACGACTCACTATAGGGCGAACTGGGTGTCTCGGAGG-3'
T7promoter
Reverse 5'-GACCTCGTCCGTCACCTGCG-3'

For dsRNA, pairs of primers containing T7 sequence overhangs were designed for PCR. These products were templates for dsRNA synthesis via the MEGAscript T7 kit (Applied Biosystems). *ds-punt* and *ds-lacI* are as described (Zeng et al. 2014). Primers for two distinct *ds-lolal* RNAs:

1-F: 5'-TAATACGACTCACTATAGGGAGAAGC
GCTACTGGAGGAGAACCC-3'
1-R: 5'-TAATACGACTCACTATAGGGAGAACCTTCCCGCTTTATCGA
ACTGGG-3'
3-F: 5'-TAATACGACTCACTATAGGGAGAAAATACATAATAATAAC
AACAAACCA-3'
3-R: 5'-TAATACGACTCACTATAGGGAGATGGCCTCCACCTCTACTTT
ACAGGC-3'

One assay for Dpp signal transduction employed *Drosophila* S2 cells that were transfected with Flag-Mad and incubated with dsRNA (Ross et al. 2001). After 3 days, these cells were incubated with Dpp for 3 h. Dpp signaling in these cells was measured by western blots with the mouse α -Flag M2 (Sigma) and rabbit α -pMad. These antibodies were detected with α -mouse-680 and α -rabbit-800 (LI-COR) and analyzed with an Odyssey Infrared Imaging System as described (Künnapu et al. 2014). A second Dpp signaling assay utilized S2 cells transfected with dsRNA, Dad13-firefly plasmid, and *Renilla* luciferase plasmid. Five days after transfection, the cells were incubated with Dpp for 26 h. Cells were lysed and analyzed using a dual luciferase reporter assay system (Promega) with reporters as described (Weiss et al. 2010; Matsuda et al. 2013).

Phylogenetics

For figure 6, sequences most similar to Lolal were identified by BLAST from among the 83 BTB proteins in *D. melanogaster*, 181 in *Caenorhabditis elegans* and 183 in *Homo sapiens* (BTB domain database; btb.uhnres.utoronto.ca). For figure 7, a second set of sequences most similar to Lolal regardless of species was constructed. Analyses in arthropod subphyla employed these databases: *Daphnia pulex*, *Strigamia maritima*, *Tetranychus urticae* (Ensembl Genomes), *Metaseiulus occidentalis* (NCBI Refseq), and *Ixodes scapularis* (Vectorbase). Alignments were created with default settings in Clustal Omega at the EMBL-EBI website (ebi.ac.uk/Tools/msa/clustalo) and trees were created in MrBayes 3.2 as described (Wisotzkey et al. 2014). The mixed amino acid model was selected and in both cases the WAG model was employed (Whelan and Goldman 2001). Generations were 100,000 for figure 6 and 600,000 for figure 7 with a sample frequency of 100 and burn-in of 0.25. For trees with more than 150 informative positions a posterior probability of 0.95 is considered statistically significant but simulation studies (Alfaro et al. 2003) of alignments with 50–100 positions revealed that the true tree contained branches with posterior probabilities of 0.65 (50 positions) and 0.85 (100 positions). Thus, for our trees of 77 (fig. 6) and 89 (fig. 7) informative positions, we consider probabilities ≥ 0.75 to be statistically significant.

Supplementary Material

Supplementary figures S1–S10 and tables S1–S8 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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Table S1. *lola* dominant maternal enhancement of four *dpp* recessive mutations.

A. Percent of expected *dpp* adult progeny from crosses between a female heterozygous for a *lola* mutation and a male heterozygous for a *dpp* recessive mutation.^a

Maternal genotype

	wild type	<i>Mad</i> ^{l2}	<i>Med</i> ^{l5}	<i>Df(2R)Pcl-11B</i>	<i>lola</i> ^{l122}	<i>lola</i> ^{K02512}	<i>lola</i> ^{l722}	<i>lola</i> ^{G9603}	<i>Df(2R)12 - psq</i>
<i>dpp</i> ^{hr27}	62	4	0	0	0	0	36	10	4
<i>dpp</i> ^{hr4}	84	10	1	1	6	5	63	68	99
<i>dpp</i> ^{hr56}	92	85	0	30	31	13	86	100	n/d
<i>dpp</i> ^{e87}	100	100	0	39	71	62	93	100	n/d

B. Percent of expected *dpp* adult progeny from the reciprocal cross between a female heterozygous for *dpp*^{hr4} and a male heterozygous for a *lola* mutation.^a

Maternal genotype

	<i>dpp</i> ^{hr4}
<i>lola</i> ^{l122}	100
<i>lola</i> ^{K02512}	95
<i>lola</i> ^{l722}	90
<i>lola</i> ^{G9603}	99
<i>Df(2R)Pcl-11B</i>	98

C. Ventralized cuticle phenotypes in progeny from crosses between a female heterozygous for a *lola* mutation and a male heterozygous for *dpp*^{hr4}.^a

Maternal genotype

Phenotype ^b	wild type	<i>Mad</i> ^{l2}	<i>Med</i> ^{l5}	<i>Df(2R)Pcl-11B</i>	<i>lola</i> ^{l122}	<i>lola</i> ^{K02512}	<i>lola</i> ^{l722}	<i>lola</i> ^{G9603}
wild type	94%	55%	11%	66%	65%	69%	58%	78%
Herniated head	2%	21%	46%	15%	17%	19%	15%	16%
Partial U shape	3%	10%	22%	13%	15%	11%	10%	5%
Filzkörper deformed	1%	4%	10%	6%	1%	0%	11%	1%
Filzkörper missing	0%	10%	11%	0%	2%	1%	6%	0%

a. All genotypes are balanced with CyO except for wild type and *Med*^{l5}.

b. At least 200 cuticles were examined in all cases. Cuticles with multiple phenotypes are counted only once and assigned according to their most prominent feature.

Table S2. *lola* alleles and *Df(2R)Pcl-11B* fail to complement.

Percent of expected trans-heterozygous adult progeny from the indicated crosses.

Maternal genotype ^a					
Paternal genotype ^b	<i>lola</i> ¹¹²²	<i>lola</i> ^{K02512}	<i>lola</i> ¹⁷²²	<i>lola</i> ^{G9603}	<i>Df(2R)Pcl-11B</i>
<i>lola</i> ¹¹²²	-	0	0	7	2
<i>lola</i> ^{K02512}	0	-	1	14	2
<i>lola</i> ¹⁷²²	1	3	-	16	2
<i>lola</i> ^{G9603}	10 ^b	6	4	-	1

a. All genotypes are balanced with CyO.

b. In each cross at least 200 adults were scored and progeny expectations are Mendelian.

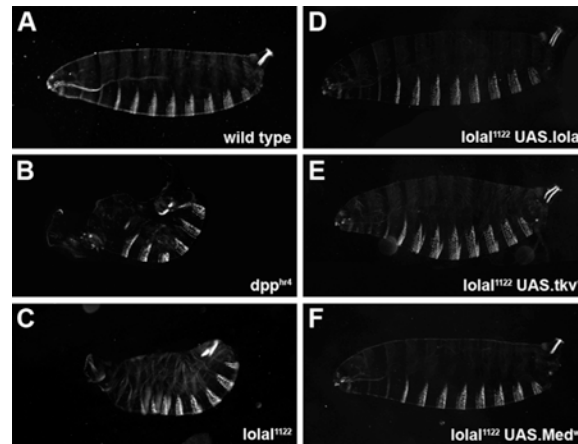


Fig. S1. Maternal expression of Lolal or Dpp signal transduction pathway components rescues *lola* dominant maternal enhancement of *dpp*^{hr4}. Cuticles with anterior to the left and dorsal up. The maternal mutant allele is shown and the indicated UASP transgene was driven by nos.Gal4. A) Wild type. B) *dpp*^{hr4} homozygous cuticle is ventralized C) *lola*¹¹²² dominant maternal enhancement yields ventralized cuticles. D) Lolal, E) Tkv* and F) Med^{wt} rescue *lola*¹¹²² enhancement as shown by progeny with wild type cuticles. Adult survival and cuticle phenotype data are in Tables S3, S4.

Table S3. Maternal expression of *Lolal* or *Dpp* signal transduction pathway components or *Trl* rescues *lolal* dominant maternal enhancement of *dpp^{hr4}* and *dpp^{hr27}*.

A. Percent of expected *dpp* adult progeny from crosses between a female heterozygous for *lolal¹¹²²* and a male heterozygous for *dpp^{hr4}*.^a

Maternal genotype includes indicated UASP transgene

	None ^b	<i>Lolal</i>	<i>Lolal^{HA}</i>	<i>Tkv*</i>	<i>Sax*</i>	<i>Med^{wt}</i>	<i>Med^{K738R}</i>	<i>Trl^{EY04024}</i>
without nos.Gal4 ^c	6	69	70	100	67	41	99	74
with nos.Gal4	-	81	86	100	79	100	100	100

B. Percent of expected *dpp* adult progeny from crosses between a female heterozygous for *lolal¹¹²²* and a male heterozygous for *dpp^{hr27}*.^a

Maternal genotype includes indicated UASP transgene

	None ^b	<i>Lolal</i>	<i>Lolal^{HA}</i>	<i>Tkv*</i>	<i>Sax*</i>	<i>Med^{wt}</i>	<i>Med^{K738R}</i>
without nos.Gal4 ^c	0	12	23	38	24	10	18
with nos.Gal4	-	39	52	58	45	62	58

C. Percent of expected *dpp* adult progeny from crosses between a female heterozygous for *Df(2R)Pcl-11B* and a male heterozygous for *dpp^{hr4}*.^a

Maternal genotype includes indicated UASP transgene

	None ^b	<i>Lolal</i>	<i>Lolal^{HA}</i>
without nos.Gal4 ^c	1	17	7
with nos.Gal4	-	20	11

- All genotypes are balanced with CyO.
- At least 200 adults were scored in all cases and progeny expectations are Mendelian.
- In the absence of nos.Gal4 functional transgene expression is generated by the P transposase minimal promoter plus first intron and the K10 3'UTR sequences in the UASP vector (e.g., Stinchfield et al. 2012).

Table S4. Maternal expression of Lolal or Dpp signal transduction pathway components rescues *lomal* dominant maternal enhancement of *dpp^{hr4}*.

A. Ventralized cuticle phenotypes in progeny from crosses between a female heterozygous for *lomal¹¹²²* (without nos.Gal4) and a male heterozygous for *dpp^{hr4}*.^a

Maternal genotype includes indicated UASP transgene

Phenotype ^{b,c}	None	Lomal	Lomal ^{HA}	Tkv*	Sax*	Med ^{wt}	Med ^{K738R}
wild type	55%	79%	71%	72%	84%	68%	67%
Herniated head	17%	13%	15%	17%	10%	23%	12%
Partial U shape	15%	6%	8%	3%	4%	6%	5%
Filzkörper deformed	11%	2%	6%	8%	2%	0%	0%
Filzkörper missing	2%	0%	0%	0%	0%	3%	16%

A'. Decreased ventralized cuticle phenotypes in progeny from crosses between a female heterozygous for *lomal¹¹²²* with nos.Gal4 and a male heterozygous for *dpp^{hr4}*.^a

Maternal genotype includes indicated UASP transgene

Phenotype ^b	None	Lomal	Lomal ^{HA}	Tkv*	Sax*	Med ^{wt}	Med ^{K738R}
wild type	-	84%	82%	94%	88%	82%	88%
Herniated head	-	6%	8%	3%	6%	13%	3%
Partial U shape	-	5%	3%	1%	6%	3%	7%
Filzkörper deformed	-	5%	4%	1%	0%	2%	2%
Filzkörper missing	-	0%	3%	1%	0%	0%	0%

B. Ventralized cuticle phenotypes in progeny from crosses between a female heterozygous for *Df(2R)Pcl-11B* (without nos.Gal4) and a male heterozygous for *dpp^{hr4}*.^a

Maternal genotype includes indicated UASP transgene

Phenotype ^{b,c}	None	Lomal	Lomal ^{HA}
wild type	51%	64%	63%
Herniated head	14%	8%	16%
Partial U shape	13%	11%	12%
Filzkörper deformed	14%	14%	4%
Filzkörper missing	8%	3%	5%

B'. Decreased ventralized cuticle phenotypes in progeny from crosses between a female heterozygous for *Df(2R)Pcl-11B* with nos.Gal4 and a male heterozygous for *dpp^{hr4}*.^a

Maternal genotype includes indicated UASP transgene

Phenotype ^b	None	Lomal	Lomal ^{HA}
wild type	-	73%	69%
Herniated head	-	17%	14%
Partial U shape	-	7%	14%
Filzkörper deformed	-	3%	2%
Filzkörper missing	-	0%	1%

a. All genotypes are balanced with CyO.

b. At least 200 cuticles were examined in all cases and scored as in Table S1C.

c. In the absence of nos.Gal4 transgene expression is generated by sequences in UASP vector.

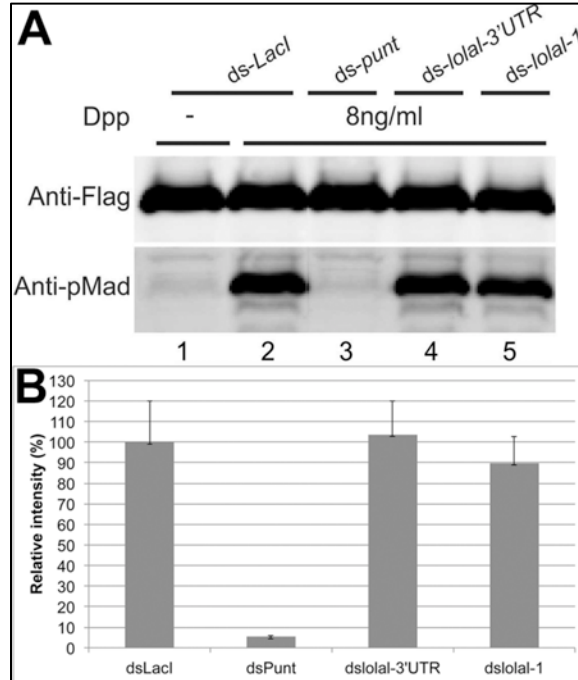


Fig. S2. *lolal* RNAi does not effect Dpp signal transduction in *Drosophila* S2 cells. A) Visualization of pMad expression. Lane 1 - S2 cells with no Dpp added resulting in no pMad. Lane 2 - Dpp added with ds-*LacI* RNA results in visible pMad. Lane 3 - Dpp added with ds-*punt* RNA eliminates pMad. Lanes 4, 5 - Dpp added with two different ds-*lolal* RNA constructs does not affect pMad. B) Visualization of a Dpp luciferase reporter. From left, Column 1 - ds-*LacI* RNA has no effect. Column 2 - ds-*punt* RNA nearly eliminates reporter expression. Columns 3, 4 - two different constructs of ds-*lolal* RNA do not effect reporter expression. Error bars indicate the standard deviation calculated from five trials. RT-PCR indicates that *lolal* is expressed in S2 cells (not shown), validating these negative results.

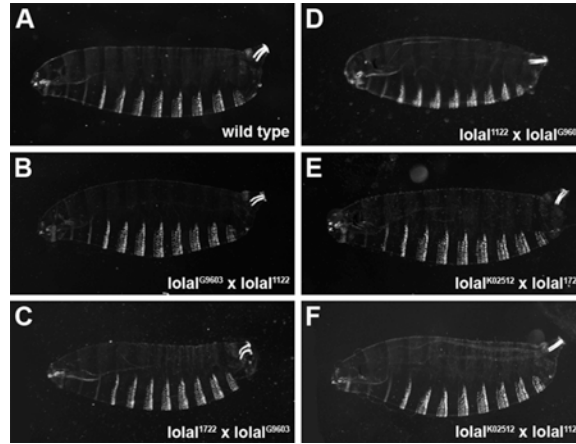


Fig. S3. *lola* trans-heterozygous mutant cuticles are rarely ventralized. Representative cuticles are shown with anterior to the left and dorsal up. The maternal allele is listed first. A) Wild type cuticle. B-F) *lola* trans-heterozygous genotypes display wild type cuticles. Numerical data are in Table S5.

Table S5. *lola* trans-heterozygous cuticles are rarely ventralized.

A. Cuticle phenotypes in progeny from crosses with a female heterozygous for *lola*^{I122}.^a

Phenotype ^b	Paternal genotype		
	<i>lola</i> ^{K02512}	<i>lola</i> ^{I122}	<i>lola</i> ^{G9603}
wild type	92%	89%	92%
Herniated head	2%	2%	2%
Partial U shape	3%	6%	4%
Filzkorper deformed	3%	3%	0%
Filzkorper missing	0%	1%	2%

B. Cuticle phenotypes in progeny from crosses with a female heterozygous for *Df(2R)Pcl-11B*.^a

Phenotype ^b	Paternal genotype			
	<i>lola</i> ^{I122}	<i>lola</i> ^{K02512}	<i>lola</i> ^{I122}	<i>lola</i> ^{G9603}
wild type	88%	93%	92%	87%
Herniated head	4%	1%	3%	1%
Partial U shape	6%	3%	2%	5%
Filzkorper deformed	0%	0%	3%	5%
Filzkorper missing	2%	3%	0%	2%

a. All genotypes are balanced with CyO.

b. At least 200 cuticles were examined in all cases and scored as in Table S1C.

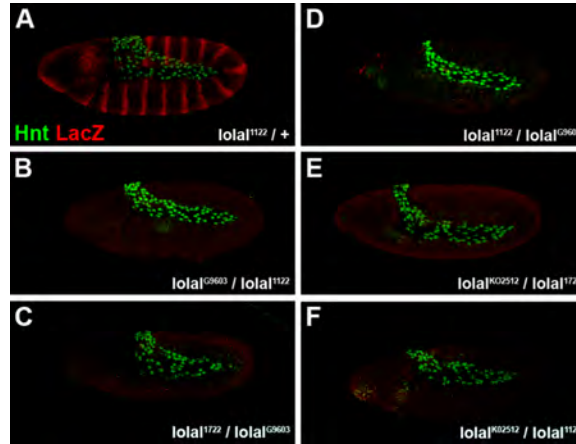


Fig. S4. *lolal* trans-heterozygous mutant embryos display normal Hindsight expression.

Embryos at stage 10 in lateral view with anterior to the left and dorsal up displaying Dpp-dependent Hindsight (green) in the amnioserosa. The maternal allele is listed first. A) Normal Hindsight in an embryo with *lolal*¹¹²² over a blue balancer chromosome (CyO wg-lacZ; red). B-F) Representative *lolal* trans-heterozygous genotypes display normal Hindsight expression.

Table S6. Stage of lethality assay for representative *lolal* trans-heterozygous progeny.^a

	<i>lolal</i> ^{G9603} x <i>lolal</i> ¹¹²²	<i>lolal</i> ¹¹²² x <i>lolal</i> ^{G9603}	<i>lolal</i> ¹⁷²² x <i>lolal</i> ^{G9603}	<i>lolal</i> ^{K02512} x <i>lolal</i> ¹⁷²²	<i>lolal</i> ^{K02512} x <i>lolal</i> ¹¹²²	<i>Df(2R)Pcl-11B</i> x <i>lolal</i> ¹¹²²
Embryo survival ^b	75%	58%	59%	66%	77%	68%
Larvae survival ^c	93%	79%	93%	93%	94%	98%
Pupae survival ^d	84%	81%	82%	76%	75%	88%

a. All genotypes are balanced with CyO.

b. Embryo survival = # hatched / # plated – # unfertilized eggs; expected 75% due to balancer chromosome homozygous embryonic lethality.

c. Larvae survival = # pupa cases / # larvae plated (new experiment - not surviving larvae from embryo survival experiment); expected 100%

d. Pupae survival = # adults / # pupa cases (continuation of larvae survival experiment); expected 100%

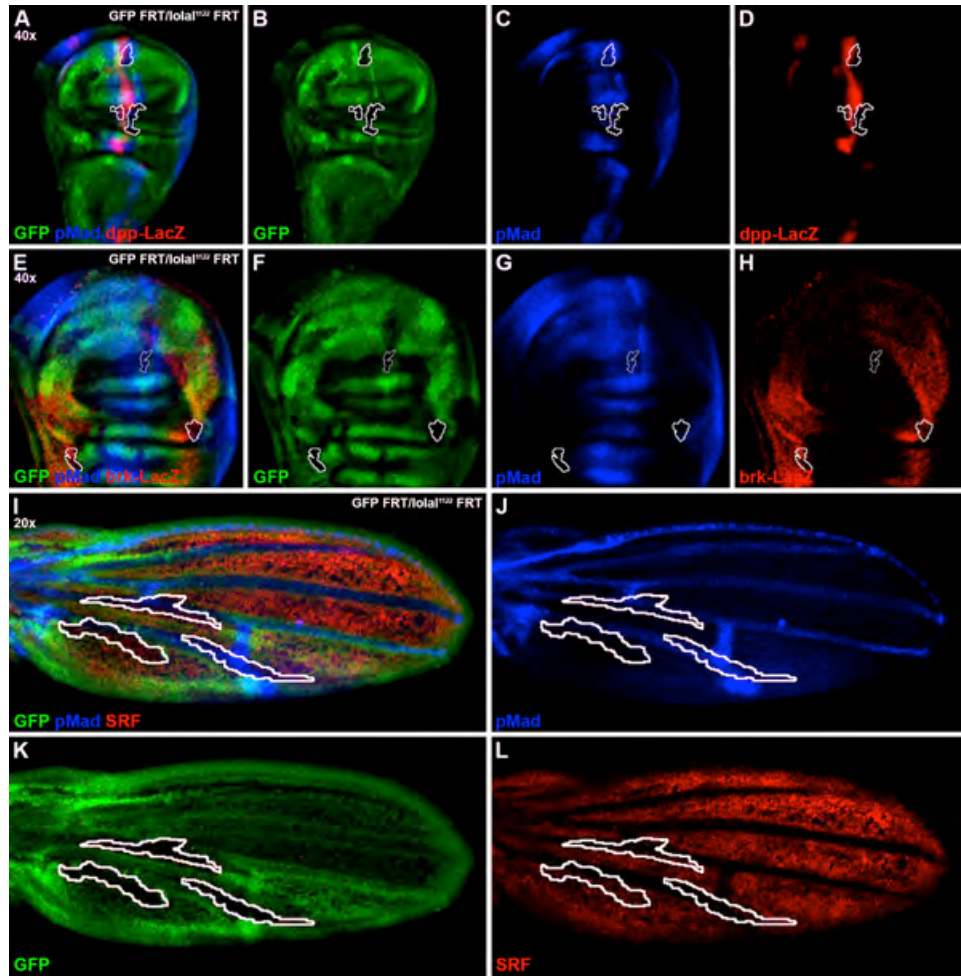


Fig. S5. *lolal* mutant clones in larval and pupal wing disks do not effect Dpp expression or signaling. A-D) Larval disk with multiple *lolal*¹¹²² clones shown in three colors and single channels. Several small clones indicated by the absence of GFP (green) are outlined in white; pMad (blue) and *dpp-lacZ* (red). All white-marked clones impinge on *dpp* with no loss of *dpp-lacZ* or pMad. E-H) Larval disk with multiple *lolal*¹¹²² clones expressing *brinker-lacZ* (red; Dpp target). The ventral-most clone (top) overlaps pMad while the other clones overlap *brinker-lacZ*. Neither pMad nor *brinker-lacZ* is affected in clones. I-L) Mid-pupal disk with multiple *lolal*¹¹²² clones marked by the absence of GFP (green); pMad (blue; marks veins) and SRF (red; marks interveins; Dpp target). The white-marked clones impinge on Dpp-dependent L4, L5 and PCV vein primordia yet neither pMad nor SRF are affected. RNA in situ hybridization (not shown) reveals that *lolal* is ubiquitously expressed in third instar and pupal wing disks, validating these negative results.

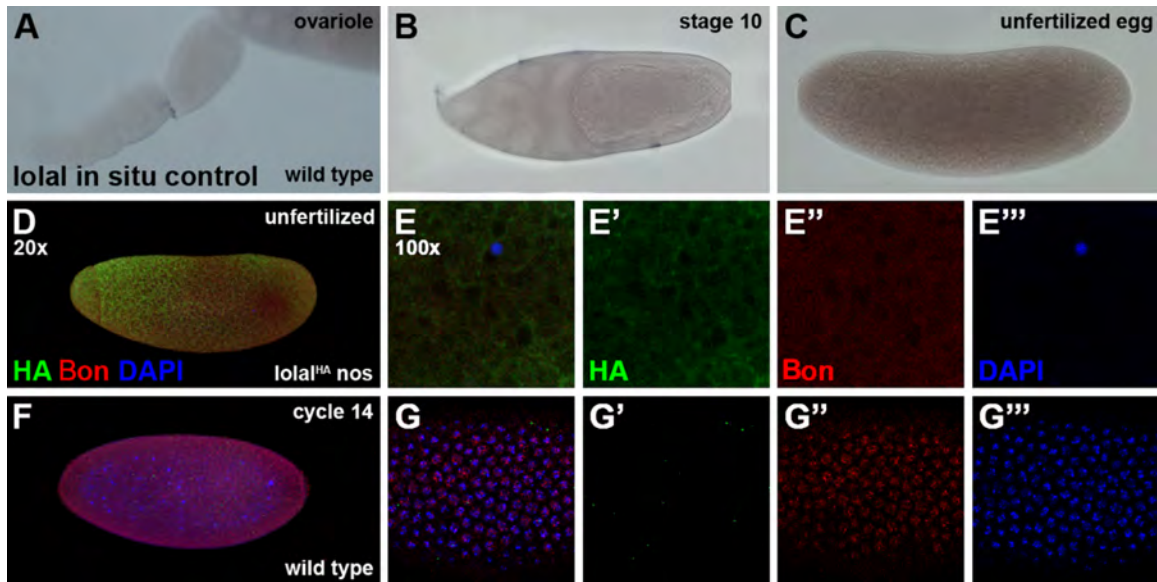


Fig. S6. Control experiments for Fig. 3 showing that *lolal* is expressed during oogenesis, maternally supplied as RNA and protein and enters nuclei prior to *dpp* transcription. Sense strand control for alkaline phosphatase in situ hybridization of *lolal* maternal RNA. A) Ovariole, B) Stage 10 egg chamber and C) Unfertilized egg display no staining. D-E) *Lolal*^{HA} maternal protein expression in an unfertilized egg with anterior to the left and dorsal up analyzed by confocal for HA (green), Bonus (red) and DAPI (nuclei; blue) at 20x (stack) or 100x (single slice as three colors and individual channels). Egg shows uniformly cytoplasmic HA and Bonus as well as a single DAPI marked nucleus. F,G) Wild type embryo at nuclear cycle 14 (Stage 5) arranged and stained similarly. Bonus and DAPI are strictly nuclear (with no HA as expected in wild type).

Table S7. Ventralized cuticle phenotypes in progeny from crosses between a *lolal*¹¹²² GLC female and a male heterozygous for *lolal*¹⁷²².

Phenotype ^{a,b}	
wild type	53%
Herniated head	13%
Partial U shape	27%
Filzkörper deformed	4%
Filzkörper missing	3%

a. At least 200 cuticles were examined and scored as in Table S1C.

b. This cross has two predicted progeny classes: heterozygous (paternal rescue) and homozygous mutant (*lolal*¹¹²²/*lolal*¹⁷²²).

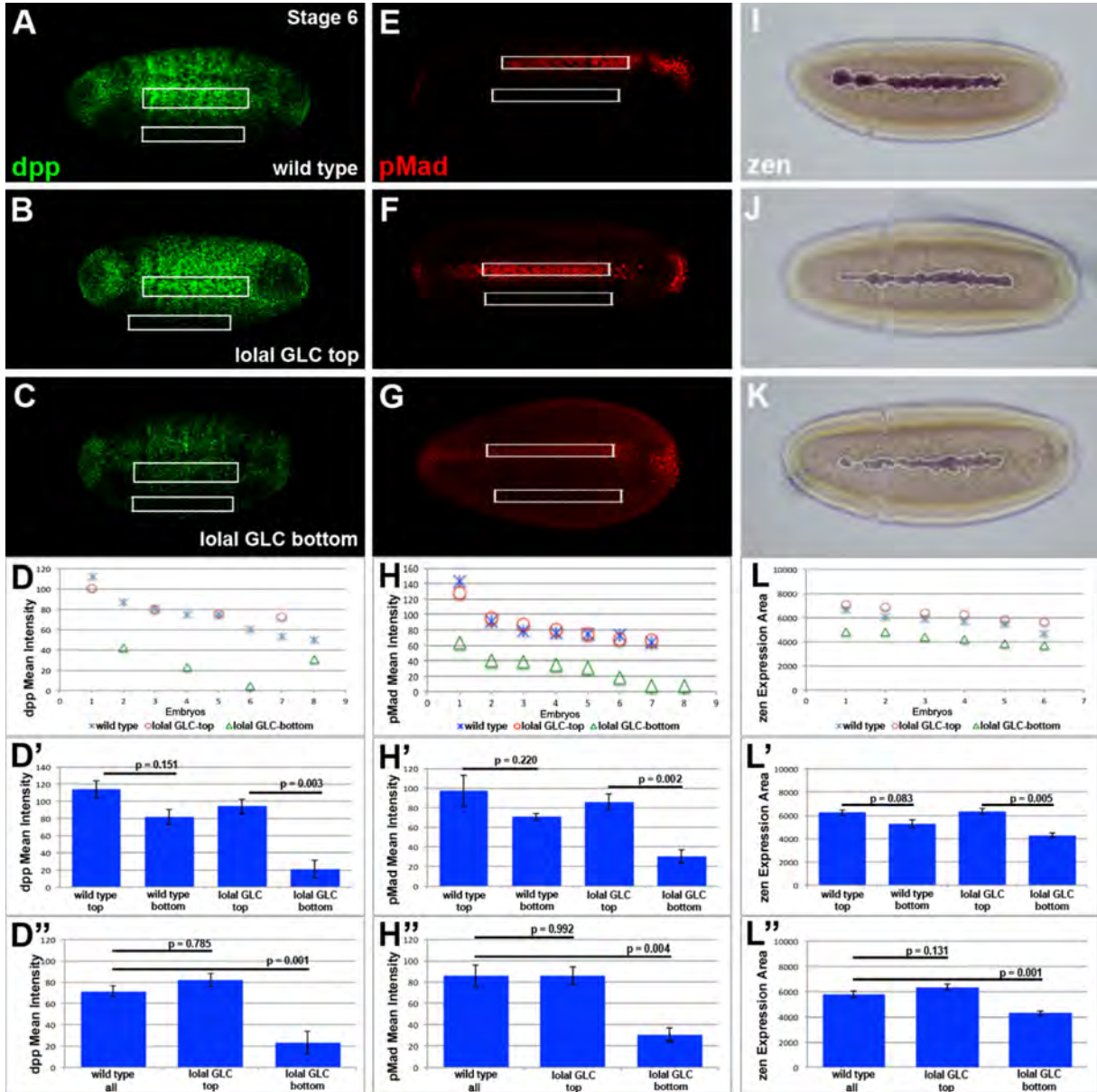


Fig. S7. Quantitation for Fig. 4 loss of maternal and zygotic *lola* significantly reduces *dpp* and *zen* transcription as well as pMad expression at stage 6. Dorsal view of stage 6 embryos displaying *dpp* transcription (A-C), pMad expression (E-G) and *zen* transcription (I-K). For *dpp* and pMad the white boxes in lateral regions (background) were employed as an internal control for dorsal regions (expression) allowing the determination of relative pixel intensity. For *zen*, the white outlined area was employed to measure the spatial extent of expression in pixels. A,E,I) Wild type. B,C; F,G; J,K) Progeny from *lola*^{l122} germline clone (GLC) females mated to *lola*^{l122} heterozygous males. A) Wild type *dpp*. B) *lola*^{l122} GLC paternally rescued embryo displaying normal *dpp* from the *lola* GLC "top" group in the graphs. C) *lola*^{l122} GLC zygotic mutant embryo displaying reduced *dpp* from the *lola* GLC

"bottom" group in the graphs. D) Plot of the relative pixel intensity for *dpp* in individual embryos that were classified as wild type (blue x's), *lolal* GLC "top" (red circles) or *lolal* GLC "bottom" (green triangles). The non-overlapping distributions of *lolal* GLC "top" and "bottom" embryos correspond to the two genotypes predicted by Mendelian principles - paternally rescued and zygotic mutant. D') Bar graph showing the mean and SEM relative pixel intensities of wild type and *lolal* GLC progeny split equally into "top" and "bottom" groups. T-tests (two-tailed, unpaired with $p < 0.050$ statistically significant) within wild type or within *lolal* GLC progeny revealed significant differences between *lolal* GLC top and bottom ($p = 0.003$) but not between wild type "top" and "bottom" ($p = 0.151$). D'') Bar graph showing the mean and SEM relative pixel intensities of pooled wild type embryos and *lolal* GLC progeny split equally into "top" and "bottom" groups. T-tests revealed significant differences between wild type and *lolal* GLC "bottom" ($p = 0.001$) but not between wild type and *lolal* GLC "top" ($p = 0.785$); this corresponds to a statistically significant decrease in *dpp* transcription in the *lolal* GLC "bottom" group. E) Wild type pMad. F) *lolal*¹¹²² GLC paternally rescued embryo displaying normal pMad from the *lolal* GLC "top" group in the graphs. G) *lolal*¹¹²² GLC zygotic mutant embryo displaying reduced pMad from the *lolal* GLC "bottom" group in the graphs. H,H',H'') Plot and bar graphs. The non-overlapping distributions of *lolal* GLC "top" and "bottom" embryos correspond to the two Mendelian genotypes. T-tests revealed a significant difference between the *lolal* GLC "top" and "bottom" ($p = 0.002$) but not between wild type "top" and "bottom" ($p = 0.220$). Significant differences are seen between wild type and *lolal* GLC "bottom" ($p = 0.004$) but not between wild type and *lolal* GLC "top" ($p = 0.992$); this corresponds to a statistically significant decrease in pMad expression in the *lolal* GLC "bottom" group. I) Wild type *zen*. J) *lolal*¹¹²² GLC paternally rescued embryo displaying normal *zen* from the *lolal* GLC "top" group in the graphs. K) *lolal*¹¹²² GLC zygotic mutant embryo displaying reduced *zen* from the *lolal* GLC "bottom" group in the graphs. L,L',L'') Plot and bar graphs. The non-overlapping distributions of *lolal* GLC "top" and "bottom" embryos correspond to the two Mendelian genotypes. T-tests revealed a significant difference between *lolal* GLC "top" and "bottom" ($p = 0.005$) but not between wild type "top" and "bottom" ($p = 0.083$). Significant differences are seen between wild type and *lolal* GLC "bottom" ($p = 0.001$) but not between wild type and *lolal* GLC "top" ($p = 0.131$); this corresponds to a statistically significant decrease in *zen* expression in the *lolal* GLC "bottom" group.

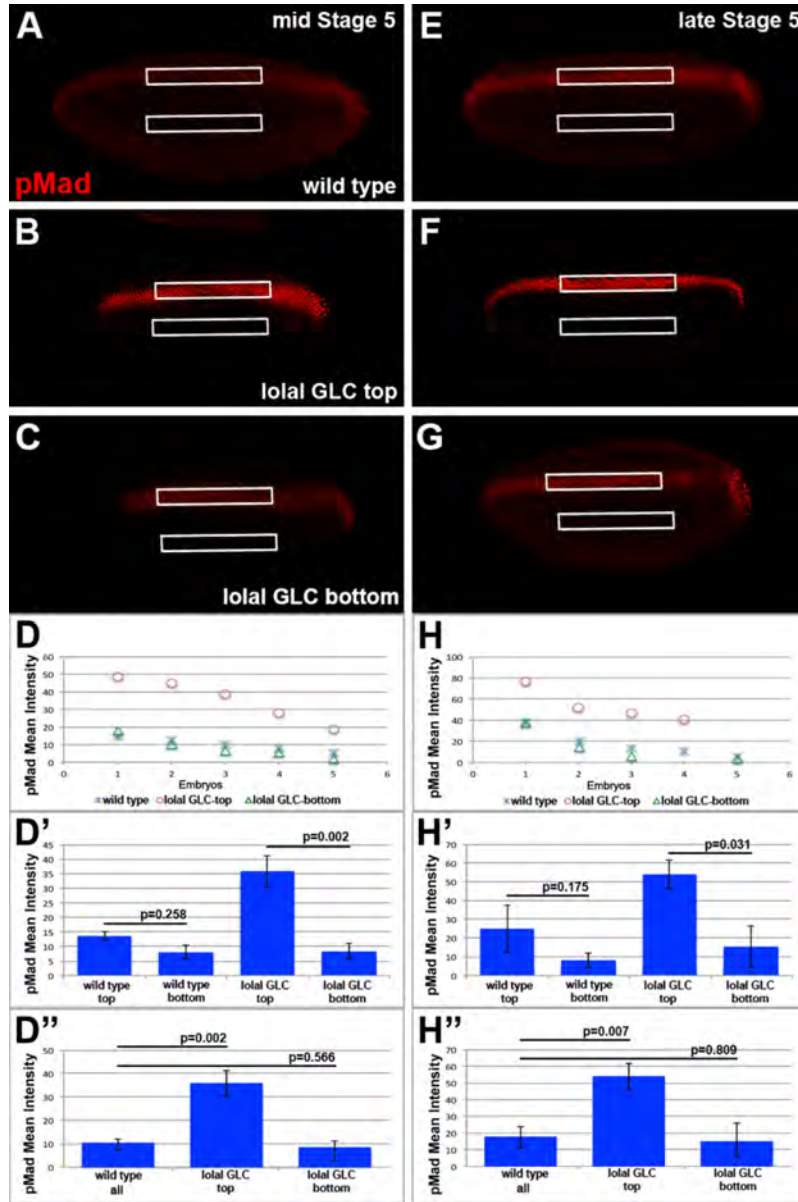


Fig. S8. Quantitation for Fig. 5 loss of maternal and zygotic *lolal* significantly increases pMad expression at stage 5. Dorsal view of mid- and late-stage 5 embryos displaying pMad expression. The white boxes were employed as in Fig. S7. A,E) Wild type. B,C; F,G) Progeny from *lolal*¹¹²² germline clone (GLC) females mated to *lolal*¹⁷²² heterozygous males. A) Wild type mid-stage 5 pMad. B) *lolal*¹¹²² GLC zygotic mutant embryo displaying increased pMad from the *lolal* GLC "top" group in the graphs. C) *lolal*¹¹²² GLC paternally rescued embryo displaying normal pMad from the *lolal* GLC "bottom" group in the graphs. D) Plot of the relative pixel intensity for *dpp* in individual embryos that were classified as wild type (blue x's), *lolal* GLC

"top" (red circles) or *lola* GLC "bottom" (green triangles). The non-overlapping distributions of *lola* GLC "top" and "bottom" embryos correspond to the two genotypes predicted by Mendelian principles - paternally rescued and zygotic mutant. D') Bar graph showing the mean and SEM relative pixel intensities of wild type and *lola* GLC progeny split equally into "top" and "bottom" groups. T-tests (two-tailed, unpaired with $p < 0.050$ statistically significant) within wild type or within *lola* GLC progeny revealed significant differences between *lola* GLC "top" and "bottom" ($p = 0.002$) but not between wild type "top" and "bottom" ($p = 0.258$). D") Bar graph showing the mean and SEM relative pixel intensities of pooled wild type embryos and *lola* GLC progeny split equally into "top" and "bottom" groups. T-tests revealed significant differences between wild type and *lola* GLC "top" ($p = 0.002$) but not between wild type and *lola* GLC "bottom" ($p = 0.566$); this corresponds to a statistically significant increase in pMad expression in the *lola* GLC "top" group. E) Wild type late stage 5. F) *lola*¹¹²² GLC zygotic mutant embryo displaying increased pMad from the *lola* GLC "top" group in the graphs. G) *lola*¹¹²² GLC paternally rescued embryo displaying normal pMad from the *lola* GLC "bottom" group in the graphs. H,H',H") Plot and graph. The non-overlapping distributions of *lola* GLC "top" and "bottom" embryos correspond to the two Mendelian genotypes. T-tests revealed a significant difference between the *lola* GLC "top" and "bottom" ($p = 0.031$) but not between wild type "top" and "bottom" ($p = 0.175$). Significant differences are seen between wild type and *lola* GLC "top" ($p = 0.007$), but not between wild type and *lola* GLC "bottom" ($p = 0.809$); this corresponds to a statistically significant increase in pMad expression in the *lola* GLC "top" group.

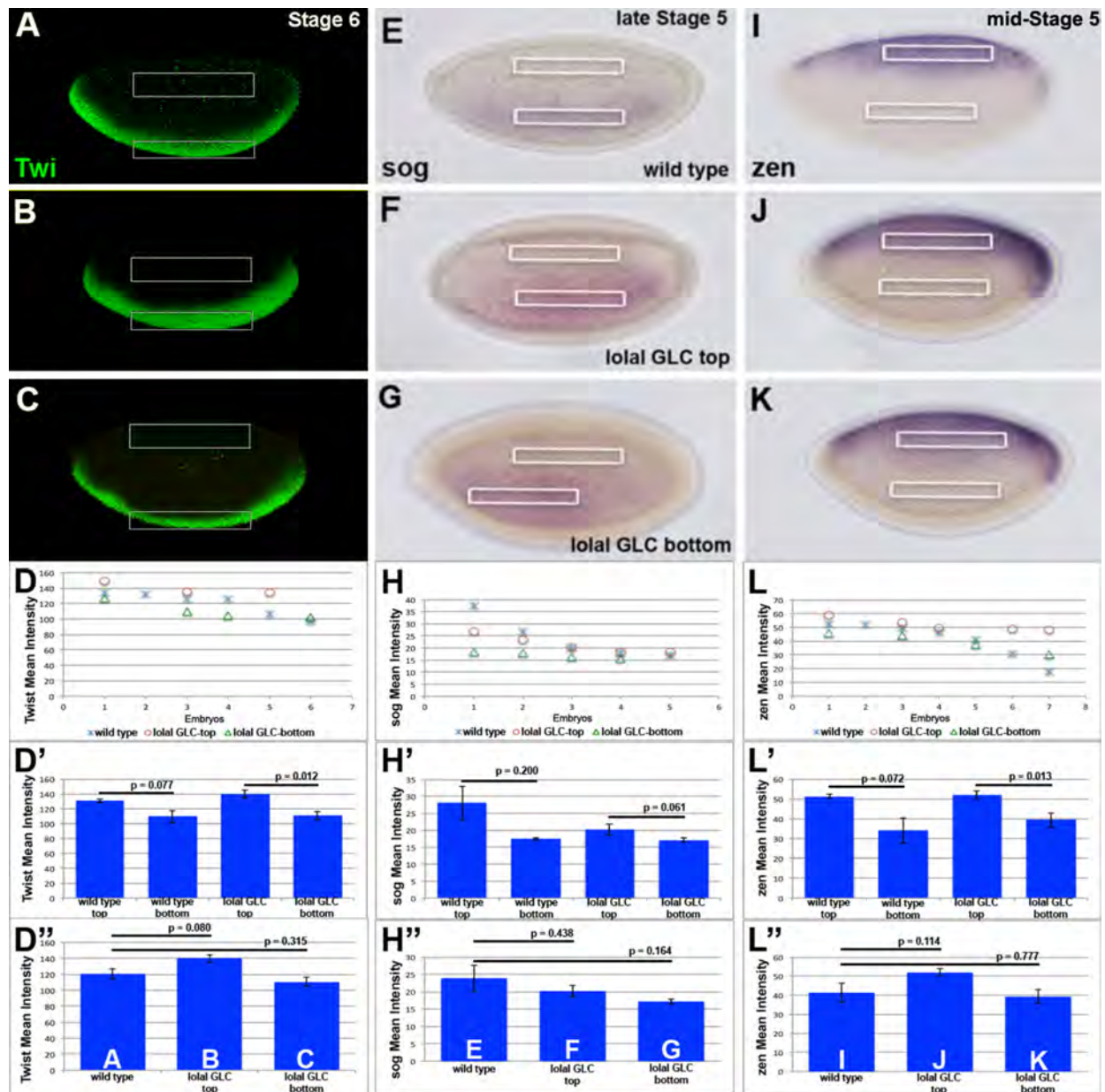


Fig. S9. Loss of maternal and zygotic *lolal* does not affect Twist, *sog* or early *zen* expression. A-C) Lateral view of stage 6 embryo displaying Twist expression. E-G) Lateral view of late stage 5 embryos displaying *sog* transcription. I-K) Lateral view of mid-stage 5 embryo displaying Dpp-independent *zen* transcription. The white boxes were employed to assay pixel intensity as in Fig. S7, including for early *zen*. A,E,I) Wild type embryos stained side-by-side with *lolal* embryos. B,C; F,G; J,K) Progeny from *lolal*^{1/22} GLC females mated to *lolal*^{1/22} heterozygous males. A) Wild type. B) *lolal*^{1/22} GLC embryo with presumed paternal rescue displaying normal Twist from the *lolal* GLC "top" group in the graphs. C) *lolal*^{1/22} GLC embryo

presumed to be a zygotic mutant displaying normal Twist from the *lolal* GLC "bottom" group in the graphs. D) Plot of the absolute pixel intensities for Twist in wild type (blue x's), *lolal* GLC top (red circles) and *lolal* GLC bottom embryos (green triangles). D') Bar graph showing the mean and SEM relative pixel intensities of wild type and *lolal* GLC progeny split equally into "top" and "bottom" groups. T-tests (two-tailed, unpaired with $p < 0.050$ statistically significant) within wild type or within *lolal* GLC progeny revealed no significant differences between *lolal* GLC top and bottom ($p = 0.072$) or between wild type "top" and "bottom" ($p = 0.077$). D'') Bar graph showing the mean and SEM relative pixel intensities of pooled wild type embryos and *lolal* GLC progeny split equally into "top" and "bottom" groups. T-tests revealed no significant differences between wild type and *lolal* GLC "bottom" ($p = 0.315$) or between wild type and *lolal* GLC "top" ($p = 0.080$). E) Wild type. F) *lolal*¹¹²² GLC embryo with presumed paternal rescue displaying normal *sog* from the *lolal* GLC "top" group in the graphs. G) *lolal*¹¹²² GLC embryo presumed to be a zygotic mutant displaying normal *sog* from the *lolal* GLC "bottom" group in the graphs. H,H',H'') Plot and bar graphs. T-tests revealed no significant difference between the *lolal* GLC "top" and "bottom" ($p = 0.061$) or between wild type "top" and "bottom" ($p = 0.200$). No significant differences are seen between wild type and *lolal* GLC "bottom" ($p = 0.164$) or between wild type and *lolal* GLC "top" ($p = 0.438$). I) Wild type *zen*. J) *lolal*¹¹²² GLC embryo with presumed paternal rescue displaying normal *zen* from the *lolal* GLC "top" group in the graphs. K) *lolal*¹¹²² GLC embryo presumed to be a zygotic mutant displaying normal *zen* from the *lolal* GLC "bottom" group in the graphs. L,L',L'') Plot and bar graphs. T-tests revealed a no significant difference between *lolal* GLC "top" and "bottom" ($p = 0.130$) or between wild type "top" and "bottom" ($p = 0.072$). No significant differences are seen between wild type and *lolal* GLC "bottom" ($p = 0.777$) or between wild type and *lolal* GLC "top" ($p = 0.114$).

Table S8a. Forty-five accession numbers from nematode, fly and human for Fig. 6.

Symbol	GI number	Protein number	Species
CeBAT15	97537788	P34324.2	Caenorhabditis elegans
CeBAT38	74964766	Q20681.1	Caenorhabditis elegans
CeBAT42	465837	P34371.1	Caenorhabditis elegans
CeBAT43	20981722	P34568.2	Caenorhabditis elegans
CeBAT47_domain1	353526345	O76612.3	Caenorhabditis elegans
Amino acids 16-80			
CeBAT47_domain2	353526345	O76612.3	Caenorhabditis elegans
Amino acids 576-644			
CeBATH-46	392889152	NP_494004.2	Caenorhabditis elegans
CeMEL26	75018990	Q94420.2	Caenorhabditis elegans
CeYR47	1353147	Q09563.1	Caenorhabditis elegans
DmABRUPT	17136196	NP_476562.1	Drosophila melanogaster
DmBAB1	29428068	Q9W0K7.2	Drosophila melanogaster
DmBAB2	24654988	NP_523879.2	Drosophila melanogaster
DmBRC1	13124701	Q01295.2	Drosophila melanogaster
DmPSQ	24656481	NP_728814.1	Drosophila melanogaster
DmCG15097	28573706	NP_788412.1	Drosophila melanogaster
DmCP190	93204565	Q24478.2	Drosophila melanogaster
DmDiablo	24664829	NP_524989.2	Drosophila melanogaster
DmTRL (GAGA)	83287912	Q08605.2	Drosophila melanogaster
DmGCL	417037	Q01820.1	Drosophila melanogaster
DmKelch	45549017	NP_476589.4	Drosophila melanogaster
DmKEN	41688554	O77459.1	Drosophila melanogaster
DmKLHL18	21356823	NP_650143.1	Drosophila melanogaster
DmLOLA	73920225	P42284.2	Drosophila melanogaster
DmLOLAL	24654826	NP_725756.1	Drosophila melanogaster
DmMMD4	46396378	Q86B87.1	Drosophila melanogaster
DmTTK	20455517	P17789.2	Drosophila melanogaster
HsBTBD18	223029436	NP_001138573.1	Homo sapiens
HsBTBD2	20137455	Q9BX70.1	Homo sapiens
HsENC1	12644050	O14682.2	Homo sapiens
HsKLHL2	239835722	NP_001154993.1	Homo sapiens
HsKLHL24	40018614	NP_060114.2	Homo sapiens
HsKLH21	172044863	Q9UJP4.4	Homo sapiens
HsKLH41	14285509	O60662.2	Homo sapiens
HsLZTR1	29839558	Q8N653.2	Homo sapiens
HsZBT14	115502478	O43829.2	Homo sapiens
HsZBT18	20141020	Q99592.1	Homo sapiens
HsZBT22	23396973	O15209.1	Homo sapiens
HsZBT24	66774228	O43167.2	Homo sapiens
HsZBT25	116242849	P24278.2	Homo sapiens
HsZBT39	18201965	O15060.1	Homo sapiens
HsZBT43	23396976	O43298.1	Homo sapiens
HsZBT7B	51316943	O15156.2	Homo sapiens
HsZBTB5	46577296	O15062.1	Homo sapiens
HsZBTB6	46577664	Q15916.1	Homo sapiens
HsZFBTB-9	23308693	NP_689948.1	Homo sapiens

Table S8b. Nine additional arthropod accession numbers for Fig. 7.

Symbol	GI number	Protein number	Species (common name – arthropod subphyla)
AdLOLAL	572307748	XP_006619722	<i>Apis dorsata</i> (Giant honey bee - insect)
AeLOLAL	332021216	EGI61601.1	<i>Acromyrmex echinator</i> (Panamanian Leafcutter ant - insect)
CcLOLAL	225719214	ACO15453.1	<i>Caligus clemensi</i> (Sea louse - crustacean)
CrLOLAL	225710248	ACO10970.1	<i>Caligus rogercresseyi</i> (Sea louse- crustacean)
DanausLOLAL	357628331	EHJ77703.1	<i>Danaus plexippus</i> (Monarch butterfly - insect)
DaphniaLOLAL	321464052	EFX75063.1	<i>Daphnia pulex</i> (Water flea - crustacean)
LsLOLAL	225713518	ACO12605.1	<i>Lepeophtheirus salmonis</i> (Salmon louse - crustacean)
PhcABRUPT	242010402	XP_002425957	<i>Pediculus humanus corporis</i> (Body louse - crustacean)
SiLOLAL	322802841	EFZ23037.1	<i>Solenopsis invicta</i> (Red fire ant - insect)

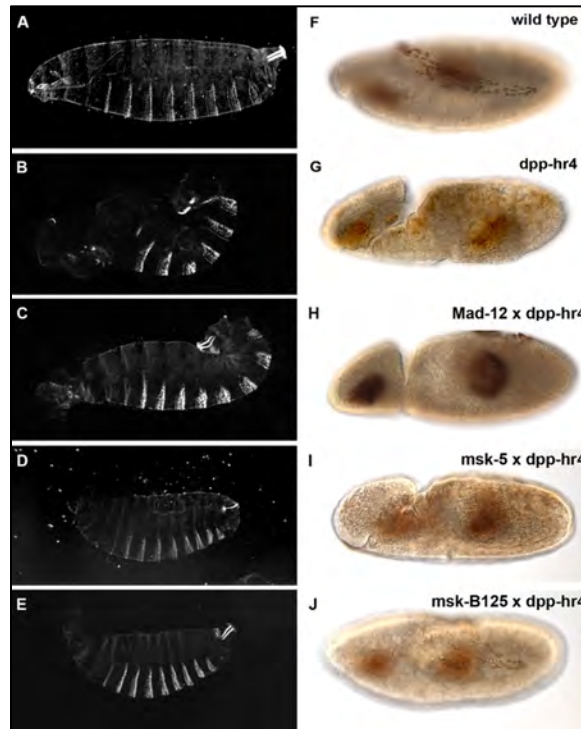


Fig. S10. *moleskin* (*msk*) dominant maternal enhancement of *dpp^{hr4}*. A-E) Cuticles as in Fig. 1. A) Wild type. B) Homozygous *dpp^{hr4}* ventralized cuticle. C,D,E) Dominant maternal enhancement of *dpp^{hr4}* by *Mad^{l2}*, *msk⁵* and *msk^{B185}* yields cuticles roughly similar to *dpp^{hr4}* homozygotes. F-J) Stage 10/11 embryos in lateral view with Dpp-dependent Hindsight in the amnioserosa visualized with DAB (orange). Dpp-independent Hindsight in the foregut and hindgut (below the plane of focus) is an internal control for staining. F) Wild type embryo. G) *dpp^{hr4}* homozygous embryo with no Hindsight in the amnioserosa. H,I,J) *Mad^{l2}*, *msk⁵* and *msk^{B185}* enhanced embryos with reduced or no Hindsight. *msk^{B185}* is not as severe as the others with a few Hindsight cells visible.