**lолаль 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* (*lolal*) as a modifier of *decapentaplegic* (dpp) mutations. We show that Lolal interacts genetically with the epigenetic factor *Trithorax-like* during Dpp D/V signaling. Maternally driven LolalHA is found in oocytes and translocates to zygotic nuclei prior to the point at which *dpp* transcription begins. lolal maternal and zygotic mutant embryos display significant reductions in *dpp* and, pMad, and *zerknullt* expression, but they are never absent. The data suggest that *lolal* is required to maintain *dpp* transcription during D/V patterning. Phylogenetic data revealed that *lolal* is an evolutionarily new gene present only in insects and crustaceans. We conclude that Lolal is the first maternal protein identified with a role in *dpp* D/V transcriptional maintenance, that Lolal 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 *lolal*.

**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 *zerknullt* (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--/TRIM33 (a RING class enzyme; Wisotzkey et al. 2014).

Building upon a prior genetic screen, we found that longitudinals lacking-like (lolal), 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 lolal, transcription of dpp is significantly reduced. This leads to abnormal pMad, reduced zen expression, and ventralization of the embryo. Phylogenetic studies revealed that lolal is an evolutionarily new gene present only in insects and crustaceans. Overall the data reveals three new insights: that Lolal is the first maternal enhancer of 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 lolal.

**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]ko2512 an insertion in the 5′ UTR of lolal (CG5738; also known as batman) displayed strong dominant maternal enhancement (fig. 1; supplementary table S1, Supplementary Material online). We generated two new lolal mutations via P element excision (lolal1122 and lolal1722) and confirmed that these did not affect the adjacent gene adipose via obesity tests (Wisotzkey et al. 2003). The two excision alleles and the lolal insertion allele P[EP]G0603 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 lolal 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 LolalHA rescue of lolal/hr4 mutant embryos did not reveal any Dpp-dependent reporter gene activation. These cell-based assays indicate no role for lolal in Dpp signal transduction (supplementary fig. S2, Supplementary Material online). Analysis of lolal mutant embryos did not reveal any Dpp-dependent phenotypes and stage of lethality assays showed that lolal 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 lolal in pathway. From this perspective, the rescue data suggest that lolal 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 lolal in Dpp signal transduction (supplementary fig. S2, Supplementary Material online). Analysis of lolal mutant embryos did not reveal any Dpp-dependent phenotypes and stage of lethality assays showed that lolal 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 lolal in...
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 LolalHA 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 LolalHA 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 LolalHA expression during the maternal to zygotic transition (fig. 3 and supplementary fig. S6, Supplementary Material online). LolalHA rescues lolal enhancement of dpphr4, providing confidence that LolalHA 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 coincidently 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), LolalHA, and Bonus are ubiquitous in the cytoplasm and absent from nuclei. At cycle 9, the initiation of the transition, LolalHA, 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, LolalHA 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 lolal1722 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,
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. 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 x (stack) or 100 x (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.
**Fig. 4.** Loss of maternal and zygotic lolal 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 lolal embryos. Middle column shows heterozygous lolal1122 germline clone (GLC) embryos with paternal rescue via wild-type lolal on the balancer chromosome (lolal GLC/+). Right column shows homozygous lolal mutant GLC embryos with lolal1122 on the paternal chromosome (lolal GLC/lolal). 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 lolal GLC homozygous mutants. We conclude that maternal and zygotic lolal 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 lolal 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 lolal GLC bottom group was also significantly below wild type (P = 0.004), whereas pMad expression in the top group of lolal GLC progeny was indistinguishable from wild type (P = 0.992). The data show a statistically significant reduction in pMad expression intensity in lolal 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 nonexpressing 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 lolal 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 lolal 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 lolal GLC homozygous mutants. The reduction in dpp transcription leading to decreased pMad and zen expression in lolal homozygous GLC mutant embryos provides a mechanistic explanation for lolal dominant maternal enhancement of dpp.

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

The GLC embryo was grown and stained at mid and late stage 5 for pMad expression (red). This was analyzed by quantifying pMad expression intensity in confocal microscopy for pMad expression (red). The 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, quantification 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

**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 GLC embryos with paternal rescue via wild type lolal on the balancer chromosome (lolal GLC/+). Bottom row shows homozygous lolal GLC embryos with lolal 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.

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 euarkyotes 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 euarkyotes, 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 (Siegmund 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
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 Lolal), 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 lolal 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) lolal displays dominant maternal enhancement of dpp mutants whereas Stat92E does not, 2) dpp transcription initiates but goes awry in lolal GLC homozygous embryos whereas it does not initiate in Zelda or
Stat92E GLC homozygous embryos, and 3) sog, early zen, and Twist expression are unaffected in lolal 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 (Faucheux 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/State92E 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 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).

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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.
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 Bilaterian 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 2011). Organisms as diverse as planarians and humans employ a common set of signaling molecules in a common format, albeit (Chen et al. 2013; Long et al. 2013). Indispensability for fertility quickly become essential for viability, fertility, and behavior 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 ubiquitous 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 lolal.

Materials and Methods

Flies

Mutants are Df(2R)Pcl-11B (Nicholls and Gelbart 1998), Df(2R)12 (psq; Weber et al. 1995), Df(3L)babAv07 (deletion of bab1 and bab2; Couderc et al. 2002), dpphr27, dpphr4, dpphr27, dpphr56 (St. Johnston et al. 1990), lolalKO86 (Horiiuchi et al. 2003), P[EP]lolalG0603 (Bellen et al. 2004), P(lacW)lolalK02512 (Török et al. 1993), lolal1122, and lolal1722 (this work), Mad12 and Med15 (Stinchfield et al. 2012), modifier of mda476 (mm46; Soltani-Bejjou et al. 2007), TrlKO87 (Farkas et al. 1994), and ttk107 (Xiong and Montell 1993). Transgenic strains are dpp-lacZ-B53.0 (Blackman et al. 1991), P(lacW)brk38 (brinker-lacZ; Minami et al. 1999), TrlFG50244 (Bellen et al. 2011), nos.Gal4:VP16-MVD1, UASP.GFP, zTub84B, UASP.Medw, and UASP.MedK73BR (Stinchfield et al. 2012), UASP.Sax* (Xie and Spradling 1998) and UASP.Tkv* (Casanueva 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 lolal1122 as described (Wisotzkey et al. 2014). GLC females were mated to lolal1722 heterozygous males to assay lolal homozygous embryos (lolal1122/lolal1722) 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 Imagel (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 lolal1122 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 as described (Quijano et al. 2011; Nagaso et al. 2001). Alkaline phosphatase RNA in situ hybridization for sog, zen (Ray et al. 1991; Nagaso et al. 2001). Alkaline phosphatase RNA was visualized directly with DAPI (Sigma). Primary antibodies were: Bonus-GP37 (Beckstead et al. 2001), Digoxigenin (Zymed), pSmad (Epitomics), dSRF (Marenda et al. 2004), and Twist (Roth et al. 1989).

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-lolal are as described (Zeng et al. 2014). Primers for two distinct ds-lolal RNAs:

- 1-F: 5’-TAATACGACTCCTATAGGGAGAACCC-3’
- 1-R: 5’-TAATACGACTCCTATAGGGAGAACCTTCCGTTTTATCGA-3’
- 3-F: 5’-TAATACGACTCCTATAGGGAGAACCTTCCGTTTTATCGA-3’
- 3-R: 5’-TAATACGACTCCTATAGGGAGAACCTTCCGTTTTATCGA-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 z-Flag M2 (Sigma) and rabbit z-pMad. These antibodies were detected with z-mouse-680 and z-rabbit-800 (LI-COR) and analyzed with an Odyssey Infrared Imaging System as described (Künnapuu 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).

Molecular Biology
UAST.Lolal was generated by cloning a Spel—Xhol fragment from cDNA pOT2-1D14505 (Berkeley Drosophila Genome Project) into the Nhel—Xhol 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 pPHW to create UASP.LolalHA (Drosophila Genomics Resource Center) followed by recombination into UASP. Primers for Gateway cloning were:

- UASPLolal: Forward 5’-CACCATGATGTGCGTCCCGAATCAACAG-3’
- Reverse 5’-TCAACCTCCTGCTGCTCCAACTG-3’
- UASPLolalHA: Forward 5’-CACCATGATGTGCGTCCCGAATCAACAG-3’
- Reverse 5’-ACCTCCTGCTGCTGCTCCAACTG-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’-TAATACGACTCCTATAGGGAGAACCC-3’
- Reverse 5’-GACCTCGTCCCTCCTGCGAG-3’

Reverse 5’-CACCTCCTGCTGCTGCTCCAACTG-3’

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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