Developmental Biology 366 (2012) 420–432

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Drosophila melanogaster Zelda and Single-minded collaborate to regulate an evolutionarily dynamic CNS midline cell enhancer

Joseph C. Pearson, Joseph D. Watson, Stephen T. Crews *

Department of Biochemistry and Biophysics, Program in Molecular Biology and Biotechnology, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280, USA

ARTICLE INFO

Article history:
Received 24 February 2012
Received in revised form
4 April 2012
Accepted 6 April 2012
Available online 17 April 2012

Keywords:
CG13333
CNS midline
Drosophila
link
single-minded
zelda

ABSTRACT

The *Drosophila* Zelda transcription factor plays an important role in regulating transcription at the embryonic maternal-to-zygotic transition. However, expression of *zelda* continues throughout embryogenesis in cells including the developing CNS and trachea, but little is known about its post-blastoderm functions. In this paper, it is shown that *zelda* directly controls CNS midline and tracheal expression of the *link* (*CG13333*) gene, as well as *link* blastoderm expression. The *link* gene contains a 5′ enhancer with multiple Zelda TAGteam binding sites that in vivo mutational studies show are required for *link* transcription. The *link* enhancer also has a binding site for the Single-minded:Tango and Trachealess:Tango bHLH-PAS proteins that also influences *link* midline and tracheal expression. These results provide an example of how a transcription factor (Single-minded or Trachealess) can interact with distinct co-regulatory proteins (Zelda or Sox/POU-homeodomain proteins) to control a similar pattern of expression of different target genes in a mechanistically different manner. While *zelda* and *single-minded* midline expression is well-conserved in *Drosophila*, midline expression of *link* is not well-conserved. Phylogenetic analysis of *link* expression suggests that ~60 million years ago, midline expression was nearly or completely absent, and first appeared in the *melanogaster* group (including *D. melanogaster, D. yakuba,* and *D. erecta*) > 13 million years ago. The differences in expression are due, in part, to sequence polymorphisms in the *link* enhancer and likely due to altered binding of multiple transcription factors. Less than 6 million years ago, a second change occurred that resulted in high levels of expression in *D. melanogaster*. This change may be due to alterations in a putative Zelda binding site. Within the CNS, the *zelda* gene is alternatively spliced beginning at mid-embryogenesis into transcripts that encode a Zelda isoform missing three zinc fingers from the DNA binding domain. This may result in a protein with altered, possibly non-functional, DNA-binding properties. In summary, Zelda collaborates with bHLH-PAS proteins to directly regulate midline and tracheal expression of an evolutionary dynamic enhancer in the post-blastoderm embryo.

Introduction

The *Drosophila zelda* (*zld* or *vielfältig*) gene plays an important role in regulating expression of a battery of genes in the blastoderm embryo that control the maternal-to-zygotic transition (Liang et al., 2008). *zld* encodes a zinc finger transcription factor that can act as transcriptional activator, binding to a set of sequences referred to as TAGteam sites (De Renzis et al., 2007; Liang et al., 2008; ten Bosch et al., 2006). Whole-genome analysis of Zld binding using ChIP-Seq revealed that thousands of these sites are bound by Zld in vivo (Harrison et al., 2011; Nien et al., 2011). It has also been proposed that Zld acts to increase chromatin accessibility for zygotically-expressed transcription factors to bind to its target genes and drive early developmental programs (Harrison et al., 2011). *zld* is also extensively expressed in the post-blastoderm embryo in the CNS and other cell types (Liang et al., 2008; Staudt et al., 2006). However, its role in controlling post-blastoderm gene expression and development has not been explored. In this paper, we demonstrate that *zld* activates transcription of CNS midline cell and tracheal expression.

The *Drosophila* CNS contains a specialized set of neurons and glia that reside at the midline (Wheeler et al., 2006). The *single-minded* (*sim*) gene acts as master regulator of CNS midline cell transcription and development (Nambu et al., 1991), and encodes a bHLH-PAS transcription factor that forms a heterodimer with the Tango (Tgo) bHLH-PAS protein (Sonnenfeld et al., 1997). The Sim:Tgo complex activates the transcription of midline-expressed target genes by binding the sequence ACGTG, referred to as a CNS midline element (CME) (Wharton et al., 1994). Midline primordium cells divide and differentiate into midline neurons and two populations of midline

* Corresponding author. Fax: +1 919 962 8472.
E-mail address: steve_crews@unc.edu (S.T. Crews).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.04.001

glia (MG): anterior midline glia (AMG) and posterior MG (PMG) (Wheeler et al., 2006). Not only does Sim:Tgo control midline primordium formation, but later it interacts with the Ventral veins lacking (Vvl) POU-homeobox protein and Dichaete (D) Sox proteins to control MG transcription (Ma et al., 2000; Sanchez-Soriano and Russell, 1998). Akin to the role of Sim as master regulator of midline transcription, the Trachealess (Trh) bHLH-PAS protein also forms a complex with Tgo and Vvl, binds CMEs on target genes, and acts as a master regulator of tracheal development (Isaac and Andrew, 1996; Sonnenfeld et al., 1997; Wilk et al., 1996; Zelzer and Shilo, 2000). Here, we propose that Sim and Trh collaborate with Zld to control CNS midline and tracheal expression of the *link* (*CG13333*) gene.

Increasingly, research on the mechanisms that underpin organismal and evolutionary variation is demonstrating that changes in gene expression commonly play important roles in evolution. Much of this variation is dependent on changes in enhancer sequences, although species differences in regulatory protein function can also be a factor (Gordon and Ruvinsky, 2012). While only beginning to be explored, recent data indicate that expression differences may be common in nervous system-expressed genes (Rebeiz et al., 2011). CNS midline cell gene expression has been particularly well-studied in *D. melanogaster* (Kearney et al., 2004; Wheeler et al., 2006, 2009), and represents a useful system for evolutionary study. In this paper we demonstrate how insights into midline gene regulation and evolution of cis-control regions can be mechanistically achieved.

In the studies described below, we describe a novel role for the Zld transcription factor in regulating post-blastoderm CNS midline cell and tracheal transcription. Zld protein directly activates transcription of the midline and tracheal-expressed *link* gene, interacting with Sim:Tgo to activate *link* midline expression and with Trh:Tgo to activate tracheal expression. While *zld* expression is highly conserved among *Drosophila* species, *link* midline expression is present only in species closely-related to *D. melanogaster*. We propose a two-step model in which binding sites in the *link* enhancer that promote high expression in MG arise in the lineage leading to *D. melanogaster*. Finally, we demonstrate that alternatively-spliced forms of *zld* are generated during embryogenesis, with variants expressed early in development generating a protein with 6 zinc fingers, while a CNS-specific variant encodes proteins lacking the 3 C-terminal zinc fingers, most likely generating a protein with altered or non-functional DNA-binding capabilities.

Materials and methods

Drosophila strains and genetics

The *zld* mutants, zld^{681} and vfl^{G0427}, were obtained from Christine Rushlow and Gerd Vorbrüggen, respectively (Liang et al., 2008; Staudt et al., 2006). Low levels of *zld* transcript can be detected by in situ hybridization of zld^{681} hemizygotes, indicating that this allele may be a strong hypomorph. The *Df(1)Exel6253* and *Df(1)BSC872* stocks (both deleted for *zld*) and *grainyhead* mutant null strain (grh^{lM}) were obtained from the Bloomington *Drosophila* Stock Center. These mutants were maintained over either P[*ftz-lacZ*] or P[*twi-Gal4*] P[*UAS-GFP*] balancer chromosomes. Homozygous and hemizygous mutant embryos were detected by staining for either: (1) *lacZ* or *GFP* expression from balancer chromosomes, (2) *zld* transcript, or (3) Zld protein. *D. simulans*, *D. sechellia*, *D. mauritiana*, *D. erecta*, *D. yakuba*, *D. ananassae*, *D. parabipectinata* stocks were obtained from Corbin Jones. The *D. pseudoobscura* stock was obtained from Karin Pfennig. *D. willistoni* and *D. virilis* were obtained from the *Drosophila* Species Stock Center (La Jolla, CA).

Bioinformatics

Orthologous *Drosophila* sequences corresponding to the *link*-5′ fragment were retrieved from the UCSC Genome Browser (genome.ucsc.edu), converted to FastA format using Galaxy (main.g2.bx.psu.edu), aligned using Dialign-TX (Subramanian et al., 2008), and manually adjusted using BioEdit (Hall, 1999). Motif-T sites were identified using PhyloGibbs (Siddharthan et al., 2005) and WinDotter (Sonnhammer and Durbin, 1995). Sim and Zld consensus binding sites were annotated using GenePalette (Rebeiz and Posakony, 2004).

Initial predicted gene structures of the *zld* RA, RB, RC, and RD transcripts were obtained from FlyBase. Transcription start, stop, and splice sites were determined by analysis of ModENCODE RNA-Seq data. Protein domains were predicted using InterProScan. Orthologs of *link* (*CG13333*) and *zld* were identified by reciprocal Protein BLAST searches, and were aligned with Dialign-TX using the STRAP program (Gille and Frommel, 2001).

Analysis of RNA-Seq data to obtain the fraction of *zld* splice variants was performed on ModENCODE developmental time-course Unique Mapper tracks (Graveley et al., 2011). All reads that overlap ChrX:19672268–19672269 (spanning the 5′ splice site) were downloaded in SAM format from the ModENCODE website. Each read was then categorized as "spliced" or "unspliced" based on CIGAR annotation (Li et al., 2009), and the total reads in each category were normalized to the total number of unique reads in the track.

Link enhancer cloning and germline transformation

The 285 bp region between *Roe1* and *link* (referred to as *link*-5′) and the 1197 bp region between *link* and *CG13334* (*link*-3′) were PCR-amplified from w^{1118} flies and cloned into the Gateway entry vector pENTR (Invitrogen). Binding site mutants were generated by PCR site-directed mutagenesis and cloned into pCR8 (Invitrogen). Sequences were mutated (underlined residues) as follows: T1 (CAGGTAG > CAAAAAG), T2 (TAGGTGG > TAAAAGG), T3 (CAGGTAG > CAAAAAG), T4 (GAGGTAG > GAAAAAG), and CME (AACGTG > GGATCC). All primer sequences are listed in Table S1. *link*-5′, *link*-3′, and *link*-5′ variants were cloned into pMintgate (Jiang et al., 2010) using Gateway LR Clonase II (Invitrogen). pMintgate constructs were injected into *Drosophila* embryos that contain the phiC31 destination site *attP2* (68A1-B2) (Groth et al., 2004) and possess posteriorly-localized phiC31 integrase.

In situ hybridization and immunostaining

Embryo collection, in situ hybridization, and immunostaining were performed as previously described (Kearney et al., 2004). DGC cDNA clones LD47819 (*zld*), LD15563 (*link*), and LP11035 (*grh*) were used to generate in situ hybridization probes. The *D. melanogaster zld* α and β probes were amplified from w^{1118} genomic DNA and cloned into pCR2.1 (Invitrogen). The coding region of *EGFP* was used to make the *GFP* probe. The following primary antibodies were used for immunostaining: rabbit anti-GFP (Abcam), mouse anti-Engrailed MAb 4D9 (DSHB) (Patel et al., 1989), guinea pig anti-Sim (Ward et al., 1998), mouse anti-β-galactosidase (Promega), and rat anti-Zld (Chris Rushlow). Alexa Fluor-conjugated secondary antibodies (Invitrogen) were used except for Sim, which was detected using biotinylated goat anti-guinea pig (Vector Laboratories) with streptavidin-HRP (Jackson Laboratories) and tyramide signal amplification (TSA; Perkin Elmer). Fluorescent in situ hybridization was detected using TSA. For in situ hybridization of *Drosophila* species other than *D. melanogaster*, orthologous regions were amplified from genomic DNA using degenerate primers and cloned into pCR2.1 or pCR8.

Digoxigenin-labeled RNA antisense probes were generated to detect *zld* and *link* expression in these species. Confocal image stacks were viewed and processed using ImageJ (Abramoff et al., 2004).

Results

Post-blastoderm expression of zelda in the CNS midline and tracheal cells

It was previously demonstrated that *zld* is broadly expressed in the blastoderm (Fig. 1A) and in the developing CNS, including ventral nerve cord (VNC) and brain (Fig. 1B) (Liang et al., 2008; Staudt et al., 2006). To begin investigating potential functions of *zld* in CNS development, we stained embryos for Zld and noticed strong Zld presence in the CNS midline cells (Fig. 1C). Protein was also detected in ectodermal cells that include the trachea (Fig. 1C). Because of the diversity of midline neuronal and glial cell types, the CNS midline cells are an attractive system to study neural development (Wheeler et al., 2006), so we examined *zld* RNA expression from stages 11–16 (Fig. S1). At stages 11–13, *zld* is strongly expressed in AMG, PMG, the median neuroblast (MNB), and iVUM4 with low levels in mVUM4. By stage 14, *zld* expression is nearly absent in AMG and PMG, but persists in iVUM4 and MNB – this pattern of expression continues at least through stage 17. Midline expression of *zld* was confirmed by RNA-Seq analysis of purified midline cells, with high levels at stage 11 (122.454 FPKM) and stage 16 (194.927 FPKM) (Joe Fontana and Stephen Crews, pers. comm.). In summary, post-blastoderm expression of *zld* includes the tracheal primordium, CNS MG, and a subset of midline neurons.

Alternative splicing generates CNS-specific zelda transcripts encoding a protein lacking most of the DNA binding domain

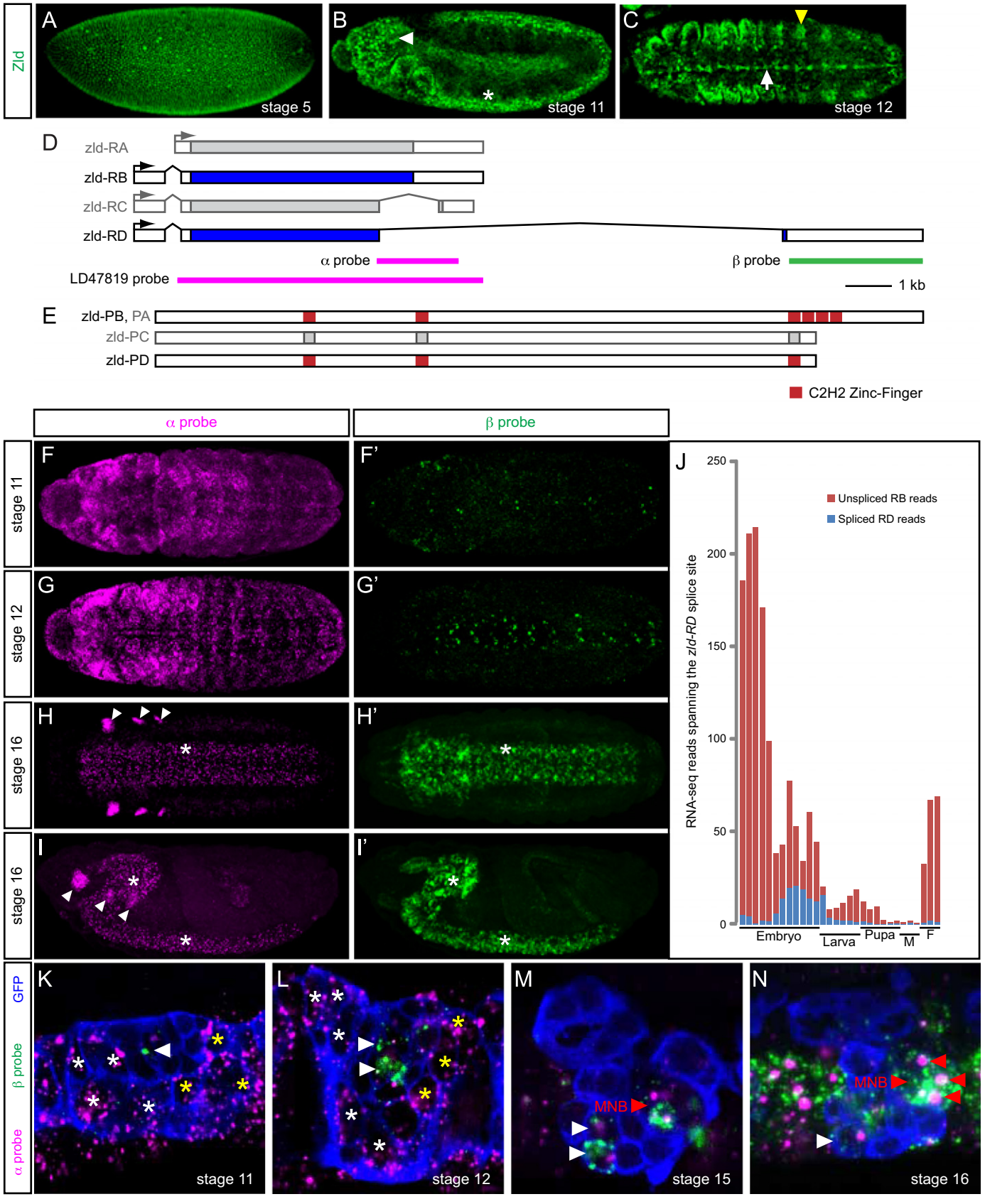
FlyBase (McQuilton et al., 2012) lists 4 different *zld* gene transcripts (RA, RB, RC, RD) (Fig. 1D) that encode 3 distinct proteins (PA, PB, PC, PD with PA and PB being identical, and PC and PD being nearly identical) (Fig. 1E). Analysis of modENCODE RNA-Seq data (Graveley et al., 2011) provided evidence for only two transcripts, RB and RD. In contrast, there are only single cDNA clones listed in FlyBase corresponding to RA and RC. Consequently, we will refer only to RB and RD and PB and PD, and assume that RA and RC are rare transcripts or cloning artifacts. Most noteworthy is that PD lacks 3 of the 4 Zld C-terminal C2H2 zinc fingers (Fig. 1E) that are sufficient to bind TAGteam sites (Liang et al., 2008). This leaves 3 other C2H2 zinc fingers that are dispersed throughout the protein. Thus, PD may carry-out a biochemical function distinct from PB with respect to target gene transcription. The probe to *zld* cDNA clone LD47819 used in Fig. S1 detects both the RB and RD transcripts. To investigate which *zld* transcripts (and proteins) were present in the post-blastoderm embryo and CNS, we generated and analyzed two probes that can recognize *zld* splice variants (Fig. 1D). The α probe detects both *zld* mRNAs: as an exonic probe for RB and an intronic probe for RD, reflecting the alternative splicing they undergo. The β probe detects only the RD mRNA transcript.

Detection of *zld* RNA with the α probe revealed strong expression at stages 11–16 (Fig. 1F–I) that resembled Zld antibody staining (Fig. 1A–C) and hybridization to the long LD47819 cDNA probe (Fig. S1). The α probe detected RNA in the developing epidermis, CNS, brain, and imaginal disc primordia. However, at stages 14–16 the CNS staining appeared punctate (*, Fig. 1H–I), resembling hybridization to unspliced primary RNA (Kosman et al., 2004), a result expected if *zld* RD is present instead of RB (Fig. 1D). In contrast, the imaginal disc staining resembled spliced mRNA transcripts, similar to the staining in all cell types at earlier stages (arrowheads, Fig. 1H and I). Confirming this interpretation, hybridization to the RD-specific β probe detected low expression in the ventral ectoderm starting at stage 11 (Fig. 1F′), but showed robust CNS expression from stages 12–16 (Fig. 1G′–I′). The RD transcript was not detected in the imaginal disc cells. Thus, *zld* transcripts at stages 11–12 and in imaginal disc cells at later stages are primarily the RB form, which encodes the PB protein isoform with 6 C_2H_2 zinc fingers. In contrast, in the CNS, the RB transcripts are reduced, but instead, the RD splice variant is present; these transcripts encode a Zld protein that lacks 3 of the zinc fingers. Analysis of modENCODE developmental time-course RNA-Seq data (Graveley et al., 2011) are consistent with these observations, in which *zld* transcripts with the 3′-end unspliced (RB) predominate early, while *zld* transcripts with 3′ splicing (RD) appear in high numbers in mid-embryogenesis and later (Fig. 1J). The 3′ unspliced transcripts present during late embryogenesis are likely due to imaginal disc expression.

We also analyzed the occurrence of the two *zld* transcripts at high resolution in CNS midline cells. As described earlier, using the full-length *zld* probe (LD47819) revealed expression in MG from stages 11–13 and in VUM4 neurons and the MNB from stages 11–16 (Fig. S1). Analysis of *zld* RNA with the α probe revealed strong expression in MG at stages 11–13 (Fig. 1K and L), but not later (Fig. 1M and N). Expression in VUM4 neurons and the MNB (Wheeler et al., 2006) were present from stages 11–16, although at stages 15–16, the α probe-hybridizing RNA was present as nuclear dots in the midline cells, indicative of RD transcripts (Fig. 1M and N). Consistent with this view, β probe-hybridizing transcripts corresponding to RD were present in VUM4 neurons and MNB progeny at stages 12–16 (Fig. 1M and N). In summary, during stages 11–13, Zld protein with 6 zinc fingers

Fig. 1. *zld* expression and alternative splicing is dynamic. (A and B) Sagittal views of *zld681/FM7c* heterozygote embryos stained for Zld protein (green). Anterior left, dorsal up. (A) Zld is broadly present at stage 5. (B) Stage 11 embryo showing Zld in the brain (white arrowhead) and CNS (*). (C) Horizontal view of stage 12 embryo showing Zld in the CNS midline cells (arrow) and trachea (yellow arrowhead). (D) Schematic of *zld* transcripts. Four alternatively-spliced versions of *zld* (RA, RB, RC, RD) are shown, as annotated on FlyBase (www.flybase.org). Boxed regions indicate exons, and filled regions are coding sequences. The *zld* RA and RC transcripts are shaded gray, since they represent rare occurrences or cloning artifacts. The positions of the α, β, and LD47819 cDNA in situ hybridization probes used to detect different *zld* transcripts are shown. (E) Shown are the 4 predicted Zld proteins (PA, PB, PC, PD). Predicted InterProScan protein domains include 6 zinc finger domains. Three of the four C-terminal zinc-finger domains are absent in PD. The *zld*-PA form is identical to PB, but is shaded gray since its transcript is rare. The *zld*-PC protein isoform is shaded gray due to its rarity. (F–I) Maximum projections of *zld* RNA expression, detected by the *zld* α probe, showing widespread ectodermal expression at stages 11–12, CNS and brain expression (*) at stage 16, and imaginal disc primordia (arrowheads) at stage 16. The CNS and brain expression resemble nuclear dots, consistent with α probe detection of pre-spliced nuclear RNA corresponding to RD. In contrast, the imaginal disc expression appears to be cytoplasmic, likely corresponding to RB. (F′–I′) Same embryos as (F–I), but detecting *zld-RD* expression using the *zld* β probe. Only CNS and brain expression is detected (*), and its non-punctate appearance indicates it is detecting processed mRNA of the RD transcript. (J) RNA-Seq developmental timecourse (ModENCODE) of the abundance of unspliced (RB) and spliced (RD) reads spanning the RD-specific 5′ splice site. (K–N) Single midline segments of *sim-Gal4 UAS-tau-GFP* embryos, detecting both α probe (magenta) and β probe (green). Transcripts in AMG (white *), PMG (yellow *), and midline neurons (arrowheads) are indicated. Midline cells are outlined in blue. (K) *zld* expression at stage 11 is almost exclusively RB, and is present in AMG and PMG, except for a single midline neuron expressing low levels of RD (arrowhead). (L) During stage 12, both AMG and PMG continue to express RB, while two midline neurons express RD. (M and N) At stages 15 and 16, all *zld*-expressing cells express RD, including iVUM4, mVUM4 (white arrowheads; only iVUM4 is shown in N), and MNB progeny (red arrowheads; 3 cells are shown in N with a nuclear dot). The large nuclear transcription dots corresponding to the α probe (magenta) co-localize in the same cells with the cytoplasmic β probe (green) staining, consistent with the *zld* gene producing RD transcripts.

is present broadly in the epidermis and CNS, including MG. After stage 13, the 6 zinc finger Zld isoform is absent or greatly reduced in all cell types, except the imaginal disc primordia. In the CNS, including midline VUM4 and the MNB, Zld protein consists of a 3 zinc finger isoform with potentially altered or non-functional DNA-binding properties.



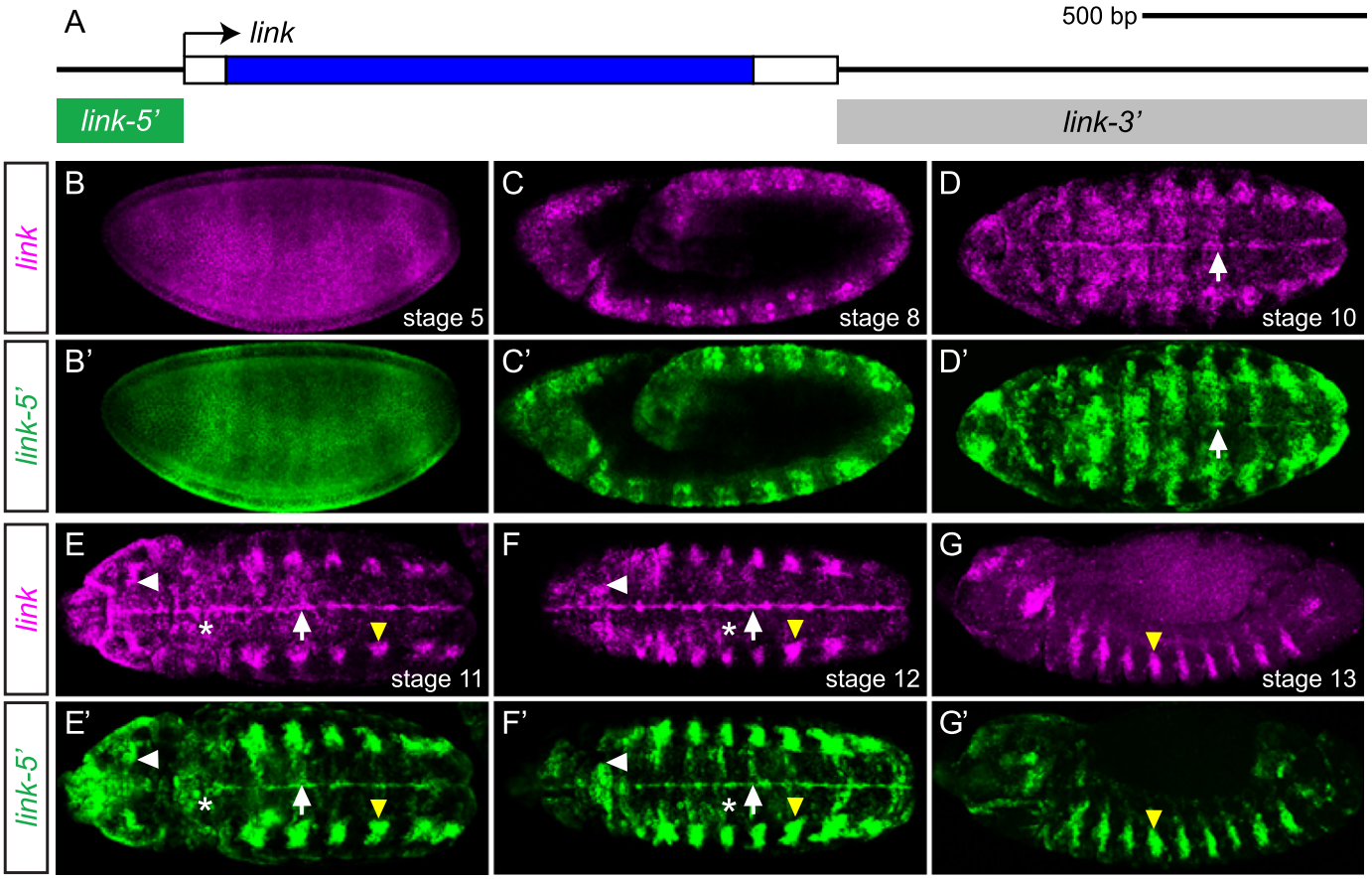


Fig. 2. *link* is expressed in CNS midline cells and a *link* 5′ 285-bp fragment controls embryonic expression. (A) Schematic of the *link* locus. Transcribed region is boxed, and coding sequence is filled blue. Arrow indicates 5′-end of the transcription unit. The intergenic regions tested for regulatory function, *link*-5′ and *link*-3′, are indicated by boxes below the locus. Scale is indicated above the schematic. (B–G′) Fluorescent in situ hybridization of *link*-5′ transgenic embryos showing (B–G) endogenous *link* expression (magenta) compared to (B′–G′) *GFP* expression from the *link*-5′ transgene in the same embryo. All views are maximum projections, except (B), which shows a single confocal slice. Shown are: (B, B′, C, C′, G, G′) sagittal views, and (D, D′, E, E′, F, F′) horizontal views. Embryonic stages are indicated in each panel. White arrows indicate midline cells, white arrowheads denote brain cells, yellow arrowheads indicate trachea, and (*) indicates ventral ectoderm.

Drosophila post-blastoderm zelda expression and alternative splicing are conserved

To study the conservation of *zld* expression in different *Drosophila* species, we utilized the α probe sequence since it contains stretches of high conservation that are sufficiently long to design primers that can amplify orthologous regions in each species tested. Near identical expression of *zld* was observed in all 5 species tested (*D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. erecta*, and *D. pseudoobscura*) that diverged up to 55 million years ago (mya) (Fig. S2A–O). In particular, strong midline expression of *zld* was observed in all species. It was also apparent that at stage 16, the CNS expression reflected the alternatively-spliced RD transcript since the transcripts were nuclear dots. Similarly, modENCODE RNA-Seq data of *Drosophila* species, including *D. mojavensis* and *D. virilis*, include reads spliced at the RD-specific junction. These data indicate that expression of the RD splice variant is conserved throughout the *Drosophila* genus.

Midline expression of link is dependent on zelda

In a separate project to identify and analyze midline enhancers, we studied the expression of the *Drosophila CG13333* midline-expressed gene, which we have renamed *link* (see *The Legend of Zelda*). The *link* gene encodes a secreted protein, and is conserved in flies and mosquitoes but is not identifiable in more distant species. The *link* gene consists of a single exon (Fig. 2A). At stage 5, *link* is initially expressed ubiquitously (Fig. 2B), but quickly develops anterior–posterior and dorsal–ventral variation, and by stage 8 expression becomes concentrated in the ectoderm in a segmentally-repeated striped pattern (Fig. 2C). At stage 10, expression in the CNS midline cells emerges (Fig. 2D), and by the end of stage 11, expression is apparent in the brain, the tracheal placodes, the lateral CNS, and in the CNS midline primordium cells (Fig. 2E). During stage 12, expression is reduced in the lateral CNS, but continues in the differentiating midline cells (primarily MG; Fig. 5C) and the trachea, as well as in the brain (Fig. 2F). CNS midline and most brain expression ceases by stage 13 (Fig. 2G), while tracheal expression is maintained until stage 15 (not shown).

Given the similarity in expression patterns between *link* and *zld*, and previous microarray data that early blastoderm (1–2 h old) *link* expression is dependent on *zld* function (Liang et al., 2008), we examined *zld* mutant embryos for effects on *link* expression. Our genetic analysis was focused on *zld* zygotic mutant embryos at embryonic stages 11–13, stages by which maternal *zld* is likely to be largely depleted. In vfl^{G0427} mutant embryos, *link* expression was severely reduced (Fig. 3B) compared to wild-type embryos (Fig. 2) and heterozygous (staining control) embryos from the same collection (Fig. 3A). Another hypomorphic *zld* allele, zld^{681}, also showed a reduction in *link* expression (data not shown). Consistent with the single-gene mutant results, *link*

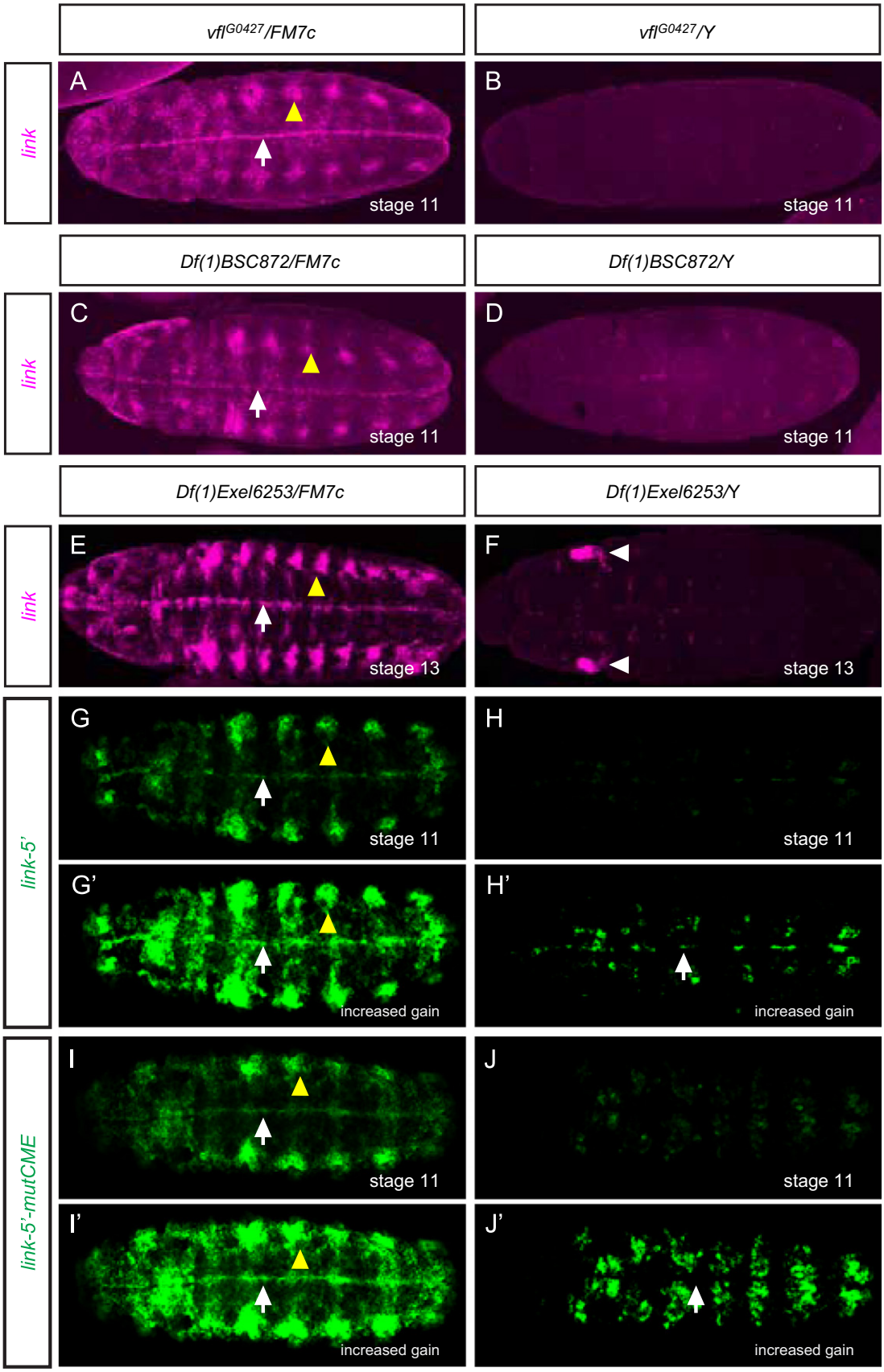


Fig. 3. *zld* regulates *link* embryonic expression. Ventral views, anterior left of maximum projections. Midline cells (white arrows) and tracheal cells (yellow arrowheads) are indicated. (A–F) *link* expression was assayed in (A) vfl^{G0427}/*FM7c* heterozygous, (B) vfl^{G0427}/Y hemizygous, (C) *Df(1)BSC872*/*FM7c* heterozygous, (D) *Df(1)BSC872*/*Y* hemizygous, (E) *Df(1)Exel6253*/*FM7c* heterozygous, and (F) *Df(1)Exel6253*/*Y* hemizygous embryos. Heterozygous embryos act as a staining control since they were stained in the same collection as the hemizygous mutant embryos. There is no significant difference in *link* expression levels between wild-type (Fig. 2) and heterozygous embryos. (B, D, F) With all 3 mutants, midline and tracheal staining was severely reduced. (F) In *Df(1)Exel6253* mutant embryos, some head staining (white arrowheads) was detected. (G–H′) *link-5′* expression was assayed in *Df(1)Exel6253* heterozygous and hemizygous mutant embryos. In G′ and H′, the gain was increased to reveal the weak presence of midline and ventral ectodermal expression and the absence of tracheal expression in H′. (I–J′) *link-5′-mutCME* was examined in *Df(1)Exel6253* heterozygous and hemizygous embryos. Note the complete absence of midline expression, although weak ventral ectoderm expression was present when the gain was increased in J′.

midline and tracheal expression was nearly eliminated in two deficiency strains that delete the *zld* gene, *Df(1)BSC872* and *Df(1)Exel6253* (Fig. 3C–F). At stage 13, *Df(1)Exel6253* (but not vfl^{G0427} or *Df(1)BSC872*) mutant embryos showed some *link* expression in the head regions (Fig. 3F) – the reason for this expression is unknown. Only 7 genes, including *zld*, are deleted in both strains, and *zld* encodes the only predicted transcription factor. We conclude that *zld* function is required for embryonic expression of *link*.

Midline expression of D. melanogaster link is evolutionarily recent

While the embryonic midline expression of *zld* is well-conserved in our analysis of *Drosophila* species, the midline expression of *link* is not well-conserved. Using species-specific *link* probes, we examined *link* expression in a number of *Drosophila* species throughout the *Drosophila* phylogeny (*D. simulans, D. mauritiana, D. sechellia, D. yakuba, D. erecta*, *D. ananassae, D. persimilis, D. pseudoobscura, D. willistoni,* and *D. virilis*). Broad expression of *link* in stage 5–10 embryos in these *Drosophila* species was similar to *D. melanogaster* (data not shown), but aspects of expression at stage 11 and later differed. Compared to *D. melanogaster* (Fig. 4A), CNS midline expression was significantly reduced in other members of the *melanogaster* subgroup consisting of *D. mauritiana* (Fig. 4B), *D. simulans* (Fig. 4C) and *D. sechellia* (Fig. 4D). These three species diverged from *D. melanogaster* ~5–6 mya (Tamura et al., 2004). Two additional species of the *melanogaster* subgroup, *D. erecta* (Fig. 4E) and *D. yakuba* (Fig. 4F) that diverged from *D. melanogaster* ~13 mya also showed reduced midline expression. More striking, in 5 more distantly related species (~44–63 mya divergence) CNS midline expression was nearly or completely absent. These species included: *D. ananassae* (Fig. 4G), *D. persimilis* (Fig. 4H), *D. pseudoobscura* (Fig. 4I), *D. willistoni* (Fig. 4J), and *D. virilis* (Fig. 4K). Thus, the high levels of *link* midline expression observed in *D. melanogaster* are likely a recently acquired trait that appeared in two steps: appearance of midline expression <44 mya and then upregulation <6 mya exclusively in *D. melanogaster* (Fig. 4L). In contrast, *link* tracheal expression was observed in all of the species and has been present for at least ~60 million years (Fig. 4A–K).

Identification of a link embryonic enhancer

To begin a molecular analysis of *link* embryonic gene expression, including addressing the questions whether Zld directly regulates *link* expression and how *link* midline expression evolved, we sought to identify a *link* embryonic enhancer. We cloned *link* flanking sequences into the *GFP* reporter vector pMintgate (Jiang et al., 2010) and analyzed reporter expression by *GFP* in situ hybridization and immunodetection. Two fragments were analyzed that encompass the entire intergenic regions: a 285 bp 5′-flanking sequence fragment, *link*-5′, and an 1197 bp 3′-flanking sequence fragment, *link*-3′ (Fig. 2A). While *GFP* expression driven by *link*-3′ did not reflect any obvious aspect of *link* endogenous expression (data not shown), *GFP* expression under the control of *link*-5′ closely matched endogenous *link* expression throughout embryogenesis (Fig. 2B′–G′). This indicated that all regulatory sequences required for the embryonic expression of *link* are contained within *link*-5′.

Identification of conserved putative transcription binding sites in the link embryonic enhancer

Initially, we took an unbiased approach to identify evolutionarily-conserved over-represented putative transcription factor binding sites in the *D. melanogaster link* 285 bp *link*-5′ fragment. Utilizing the PhyloGibbs software program, we identified a conserved sequence motif, AGGTRG (R=A/G), referred to as Motif-T, with four sites in *link*-5′ (Fig. 5A and B, S3). Two sites were identical to each other, with the sequence CAGGTAG (T1, T3) and were conserved in most sequenced *Drosophila* species (Fig. 5B). Two additional Motif-T sites in *link*-5′ were related to CAGGTAG with either a single mismatch (GAGGTAG; T4) or two mismatches (TAGGTGG; T2) (Fig. 5A and B). Motif-T sites T1 and T3 match strong sites of the TAGteam heptamers (CAGGTAG, TAGGTAG, CAGGTAA, CAGGCAG) (ten Bosch et al., 2006), which are recognized by the Zld, Grainyhead (Grh), and Bicoid Stability Factor transcription factors (De Renzis et al., 2007; Harrison et al., 2010; Liang et al., 2008). The *link* T1 and T3 sites were previously recognized as putative Zld binding sites (Liang et al., 2008), and Zelda ChIP-seq detects strong binding to the *link* 5′ region in vivo (Harrison et al., 2011).

The Sim and Trh bHLH-PAS transcription factors are known regulators of midline and tracheal expression, respectively. Both of these proteins form heterodimers with Tgo and bind the CME sequence ACGTG. We identified one CME at the promoter-proximal end of *D. melanogaster link*-5′ (C; Fig. 5A, S3), which is conserved in most *Drosophila* species from the *melanogaster* subgroup, but is not present in more distantly related *Drosophila* species (Fig. 5B). However, a putative compensatory CME is upstream of *link* in *D. willistoni* and *D. virilis* (Fig. S3). Interestingly, *D. erecta* only has a CME in the 5′-UTR, but not in the 5′ intergenic sequence. In summary, the *D. melanogaster link*-5′ fragment has two bona fide Zelda TAGteam binding sites (T1, T3) and 1 Sim:Tgo/Trh:Tgo binding site (C), in addition to two binding sites related to Zld TAGteam sites (T2, T4).

The CME binding site is not absolutely required for link midline and tracheal expression

The *sim* gene plays an important role in regulating CNS midline cell primordium and MG transcription (Nambu et al., 1990), and *trh* is required for tracheal expression (Isaac and Andrew, 1996; Wilk et al., 1996). When CMEs were mutated in the MG-expressed *slit* and *wrapper* enhancers, all MG expression was abolished (Fulkerson and Estes, 2010; Wharton et al., 1994). Similarly, when CMEs were mutated or deleted in the tracheal-expressed *breathless* and *rhomboid* enhancers, tracheal and midline expression was absent (Ohshiro and Saigo, 1997; Zelzer and Shilo, 2000). To test the requirement of the *link* CME on midline and tracheal expression, the *link*-5′ CME was mutated, tested in vivo, and compared to unmutated *link*-5′ (Figs. 5C, C′, G, 6B). Surprisingly, mutating the CME (*link*-5′-*mutCME*) caused only a slight reduction in *GFP* expression in MG (Figs. 5D, D′, 6C) and trachea (Figs. 5H, 6C) (mutational results here and below are summarized in Fig. 6A). This indicated that transcription factors besides Sim:Tgo and Trh:Tgo are necessary for *link* expression in both MG and trachea.

TAGteam-related sites are required for link expression

To test the role of the Motif-T sites in *link* expression, all 4 sites were mutated in the *link*-5′ fragment (*link*-5′-*mutT1234*). This resulted in the elimination of nearly all expression in the epidermis, trachea, and MG (Figs. 5E, E′, I, 6D), indicating the importance of Motif-T sites in *link* embryonic expression. In order to determine which Motif-T sites were contributing to *link* expression, we mutated either 2 or 3 sites in pairwise combinations. Motif-T sites 1 and 3 are highly conserved, identical to each other, and perfectly match canonical TAGteam sites. When mutated together, *link*-5′-*mutT13* embryonic *GFP* expression was

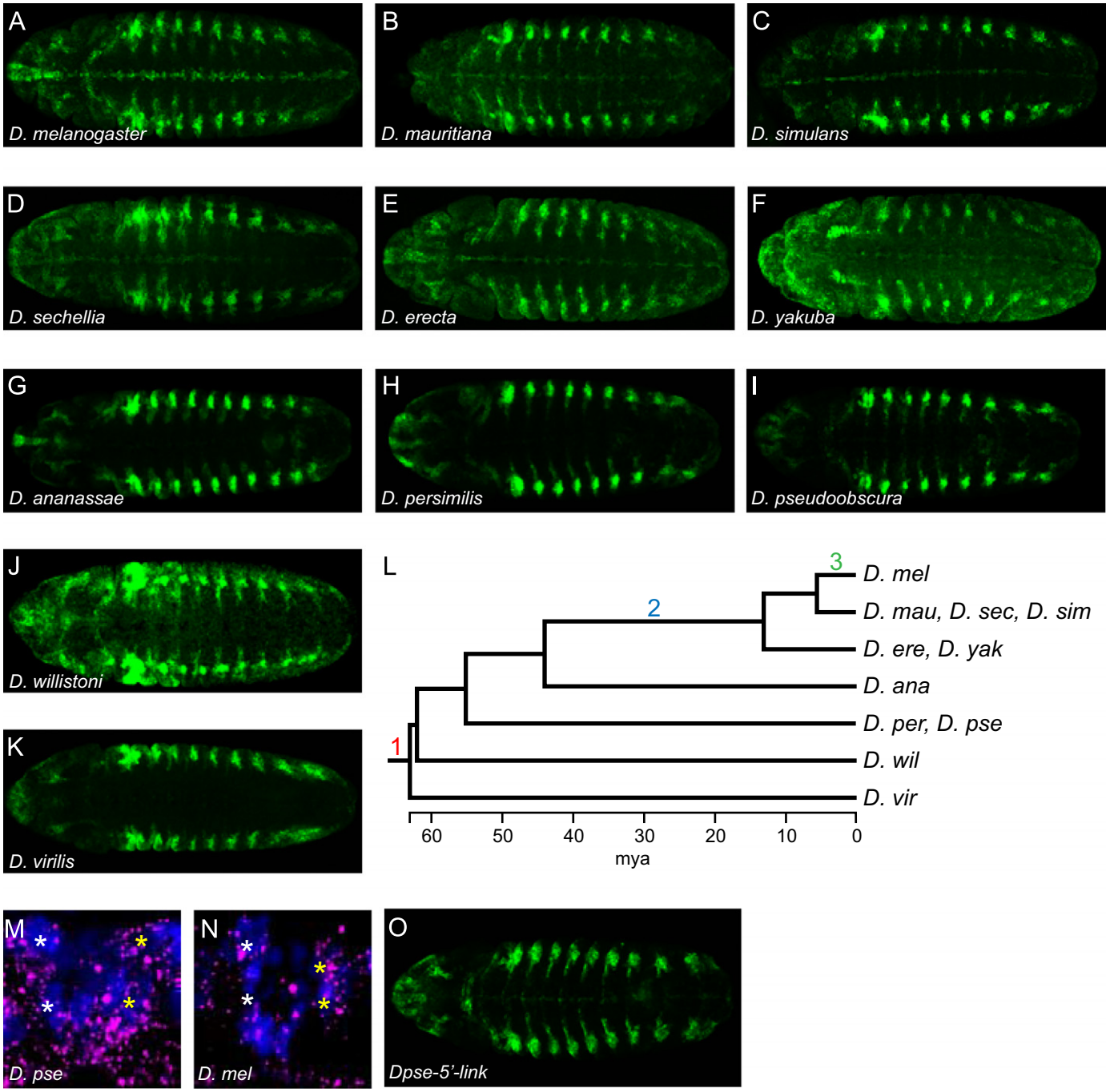


Fig. 4. *link* midline expression is evolutionarily recent. (A–K) Maximum projections of stage 12 embryos from multiple *Drosophila* species that were stained for *link* expression using species-specific probes. (A) *D. melanogaster link* is prominently expressed in CNS midline cells. (B–F) Compared to *D. melanogaster*, the closely-related species: (B) *D. mauritiana*, (C) *D. simulans*, (D) *D. sechellia*, (E) *D. erecta*, and (F) *D. yakuba*, all have midline expression, but it is considerably reduced relative to tracheal expression. (G–K) In more distantly related species, midline expression is barely detectable over background (*D. ananassae*, *D. persimilis*, *D. pseudoobscura*, *D. virilis*) or undetectable (*D. willistoni*). (L) Phylogenetic tree of the *Drosophila* species tested and a model of *link* regulatory changes. (1) ~60 mya, *link* was expressed in trachea but absent from CNS midline cells. (2) In the common ancestor of *D. melanogaster* and *D. erecta*/*D. yakuba*, a regulatory change occurred that promoted midline expression. (3) A second regulatory change occurred after the *D. melanogaster* lineage split from the *D. simulans*/*D. mauritiana*/*D. sechellia* lineage, resulting in an increase in midline expression in *D. melanogaster*. (M and N) *zld* MG (*) expression (magenta) in both (M) *D. pseudoobscura* and (N) *D. melanogaster* is robust and comparable in stage 12 segments. Midline cell nuclei are visualized by anti-Sim staining (blue). (O) The *Dpse-link-5′* transgene in *D. melanogaster* drives low levels of midline *GFP* expression, but robust tracheal expression.

present, but reduced, in MG and trachea at stage 12 (Fig. 5F, F′, J, 6E) as well as in head structures, but the broad early expression at the maternal-to-zygotic transition (data not shown) and later expression in ventral ectoderm were absent (Fig. 6E). Since sites T1 and T3 together were not absolutely required for midline and tracheal expression, but mutation of sites T1–4 were, we addressed the consequences of mutating sites T2 and T4 (*link-5′-mutT24*). While this mutant had some slight changes in head and ectodermal expression (Fig. 6F), there were no significant alterations in midline or tracheal expression.

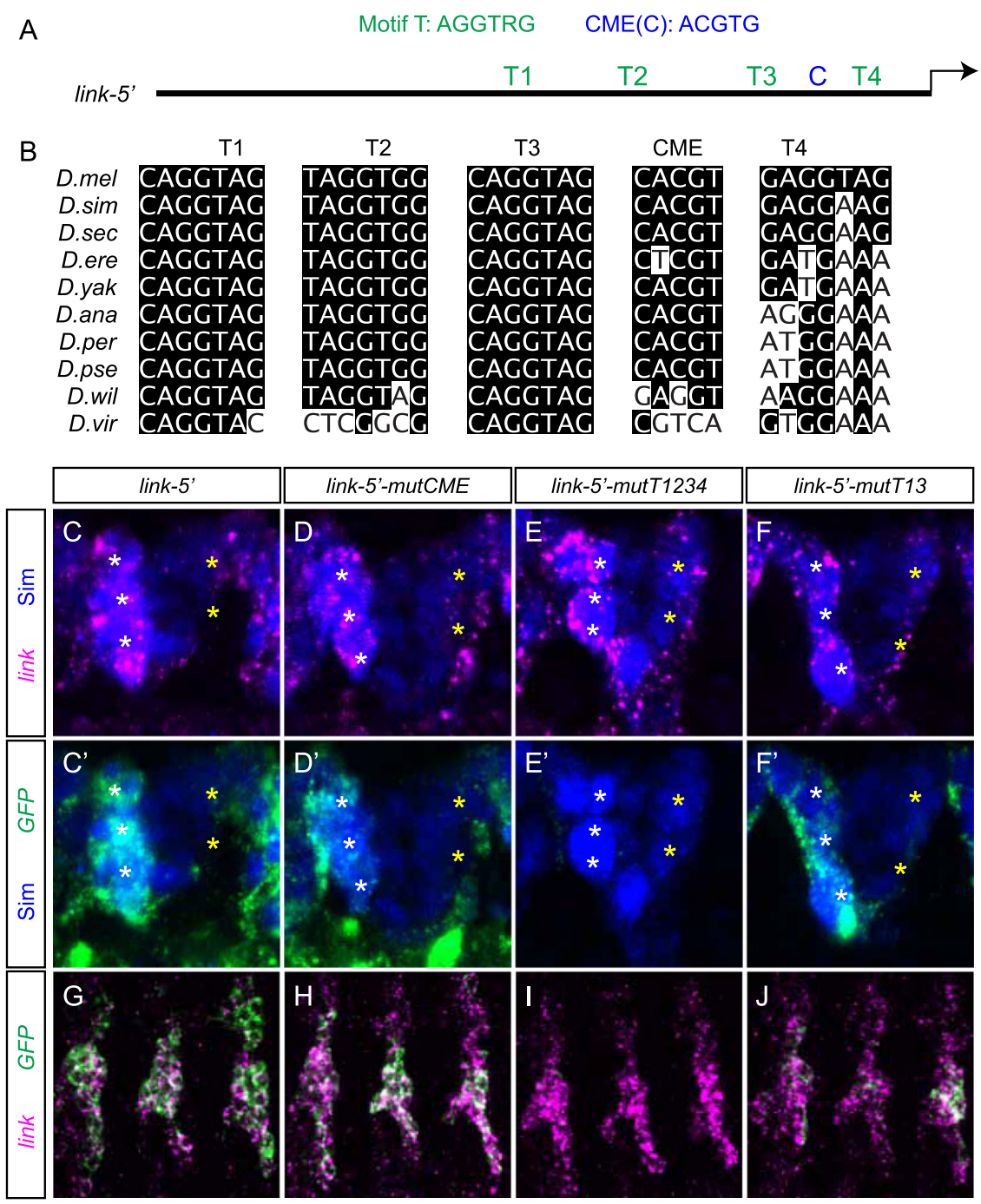


Fig. 5. The *link* Motif-T sites are required for midline and tracheal expression. (A) Schematic of the 285 bp *link*-5′ regulatory region showing the positions of Motif-T (T1–T4) and CME (C) sites. (B) Evolutionary conservation of Motif-T and CME sequences in representative *Drosophila* species. Nucleotides that match the *D. melanogaster* (*D.mel*) sequence are shaded. (C–F) Expression of *link* is visualized in stage 12 MG, and compared to (C′-F′) expression of *link*-5′ transgenes visualized by *GFP* transcript staining. Sim protein, which is present in MG and a subset of midline neurons, is blue. White * indicate AMG and yellow * indicate PMG. (C, C′, D, D′) *link*-5′-*mutCME* had a small reduction in MG *GFP* expression relative to *link*-5′. (E, E′) Mutation of all 4 Motif-T sites (*link*-5′-*mutT1234*) resulted in a loss of MG expression. (F, F′) *link*-5′-*mutT13* had a small reduction in MG expression. (G–J) *link* and *GFP* expression is visualized in stage 13 tracheal transverse connective branches (3 segments are shown). (G) Co-expression of *link* and *link*-5′ in trachea. (H) Mutation of the CME motif caused a small reduction in tracheal expression. (I) Mutation of all Motif-T sites nearly eliminated tracheal *GFP* expression. (J) Mutation of Motif-T sites 1 and 3 reduced tracheal *GFP* expression.

Since the Motif-T double mutants had little effect on midline and tracheal expression, mutations in 3 sites were tested. Mutating sites T1, T2, T3 (*link*-5′-*mutT123*) showed strong midline and tracheal *GFP* expression (Fig. 6G), possibly stronger than the unmutated *link*-5′, suggesting that the presence of T2 has a slight repressive effect. However, when sites T1, T3, and T4 were mutated (*link*-5′-*mutT134*) midline expression was absent and tracheal expression greatly reduced (Fig. 6H). These results indicated that site T4, along with sites T1 and T3, is functional, whereas site T2 does not positively influence *link* expression. Mutating the CME in addition to sites T2 and T4 (*link*-5′-*mutT24CME*) caused only a slight reduction in midline and tracheal expression (Fig. 6I), similar

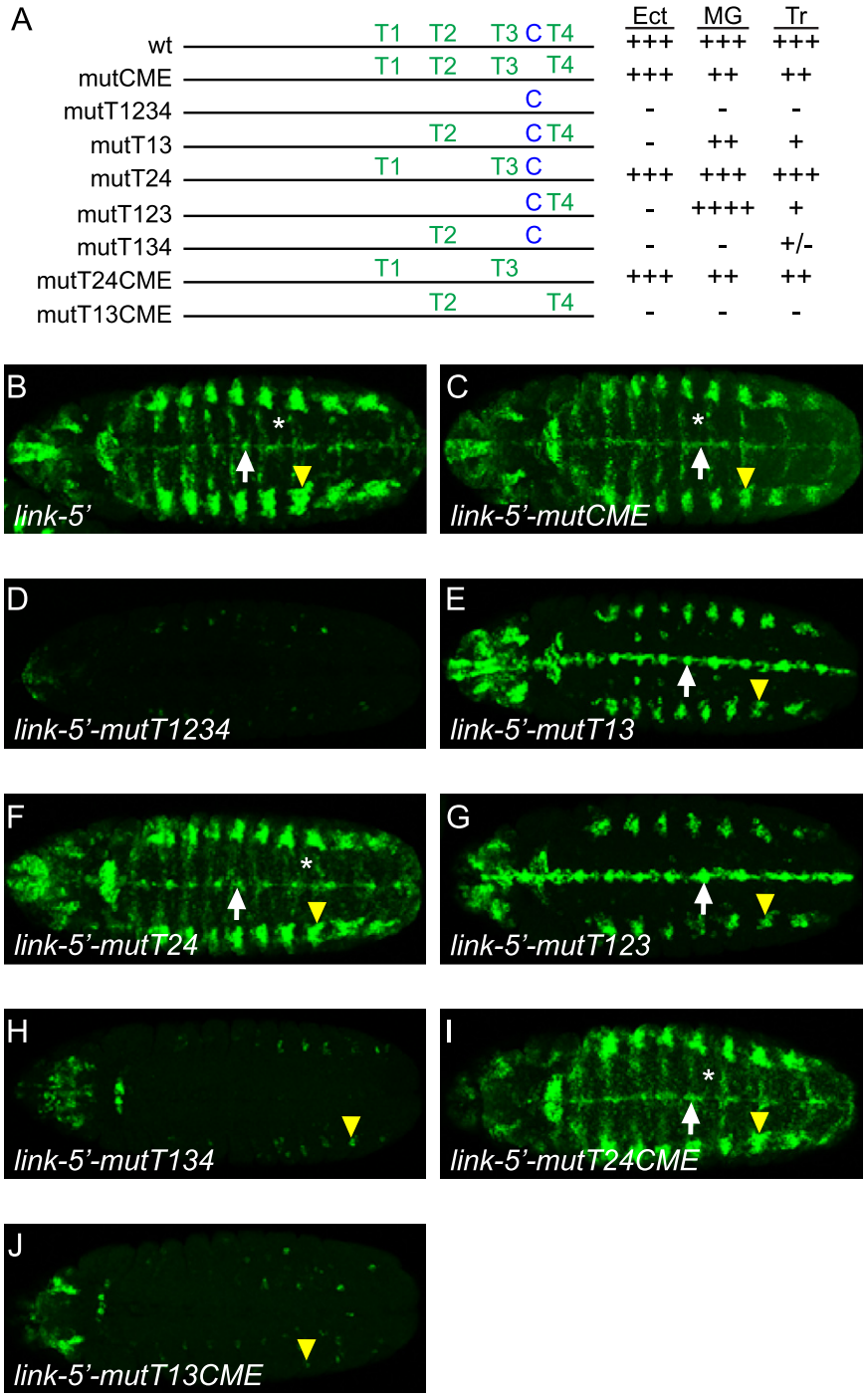


Fig. 6. Motif-T and CME sites both contribute to *link* expression. (A) Schematic of *link*-5′ transgenes tested for ventral ectodermal (Ect), midline glial (MG), and tracheal (Tr) expression. Observed levels of *GFP* expression (compared to wild-type levels) are indicated as: wild-type (+++), slightly increased (++++), slightly reduced (++), strongly reduced (+), trace levels (+/−), and absent (−). (B–J) Maximum projection horizontal views of stage 12 embryos showing *GFP* transcript levels determined by in situ hybridization. Identical settings were used in all cases for microscopy and image processing. The following were used to indicate specific cell types: CNS midline cells (white arrows), trachea (yellow arrowheads), and ventral ectoderm (*).

to mutating only the CME. However, when the CME was mutated along with T1 and T3 (*link*-5′-*mutT13CME*), midline expression was abolished and tracheal expression was strongly reduced (Fig. 6J). Thus, like site T4, the CME is required for *link* expression when mutated along with sites T1 and T3. Together, while none of these sites is absolutely required by itself, transcription factors binding Motif-T sites T1, T3, and T4 and the CME together regulate *link* midline and tracheal expression. These data argue that Zld functions together with Sim and Trh to control *link* midline and tracheal expression, respectively.

Zelda, but not Grainyhead, regulates link expression

The mutational analysis of *link*-5′ implicated TAGteam sites and the CME in regulating *link* expression. Since both Zld and Grainyhead can bind TAGteam sites (Harrison et al., 2010), we carried-out additional genetic experiments to determine which transcription factor was relevant. Similarly, we sought additional genetic data implicating *sim* in controlling *link* expression. When the *link*-5′ reporter transgene was examined in *Df(1)Exel6253 zld* hemizygous mutants, all expression was strongly reduced

(Fig. 3G and H), although some midline staining was detectable when the gain was increased (Fig. 3G′ and H′). However, when *link*-5′-*mutCME* was examined in *Df(1)Exel6253*, the midline staining was completely absent (Fig. 3I and J′). These results support a model in which both *zld* and *sim* activate CNS midline expression of *link*.

The *grh* gene is expressed during embryogenesis in CNS, epidermal, and tracheal cells (Fig. S4A–C) (Bray et al., 1989; Hemphala et al., 2003). Thus, *grh* overlaps *link* expression in these cells. In contrast, whereas *link* is expressed in MG, *grh* is only present in the MNB and is not expressed in MG (Fig. S4D–E"). Therefore, *grh* is unlikely to regulate *link* expression in MG, but could potentially regulate early embryonic *link* expression, as well as later epidermal and tracheal expression. However, in embryos homozygous for grh^{IM}, a null allele, *link* expression resembled wild-type at all developmental stages (data not shown), suggesting that *grh* does not regulate *link* expression. Together, the genetic and *link*-5′ mutational/transgenic studies provide strong evidence that *zld*, but not *grh*, directly regulates *link* expression throughout embryogenesis, via multiple TAGteam-related binding sites, and Sim:Tgo and Trh:Tgo also contribute to *link* midline and tracheal expression.

While *zld* regulates CNS midline expression of *link*, it may not be acting as a global regulator of CNS transcription, since *zld* mutant embryos did not show a reduction in expression of four additional genes (*CG7271*, *CG8965*, *escargot*, and *rhomboid*) that are expressed in the CNS, including CNS midline cells (data not shown). Two of these genes (*CG7271*, *escargot*) have conserved TAGteam sites and show reduced expression at stage 5 in *zld* mutants (Liang et al., 2008; Nien et al., 2011).

cis-regulatory alterations in the link enhancer drive evolutionary differences in midline expression

Midline expression of *link* is a relatively recent occurrence in *Drosophila* evolution, and the *Drosophila* species assayed that diverged from *D. melanogaster* >14 mya expressed little or no *link* in midline cells. In contrast, midline expression of *zld* is present in all *Drosophila* species tested, including *D. pseudoobscura* (*Dpse*) (Fig. 4M and N, S2M and N). Similarly, *sim* is expressed in the midline cells of all arthropods tested, including *D. pseudoobscura* and *D. virilis*, as well as mosquito, beetle, and honeybee (Kasai et al., 1998; Zinzen et al., 2006). Since *link* is not expressed in the midline cells of *D. pseudoobscura* (Fig. 4I), this suggests that the absence of *link* midline expression is due to alterations in the *link* regulatory region and not due to trans-acting differences. To test this, we cloned the upstream region of *D. pseudoobscura link* (*Dpse-link*-5′) into pMintgate, transformed this construct into *D. melanogaster,* and assayed expression. When normalized to tracheal expression, *Dpse-link*-5′ midline expression (Fig. 4O) was lower than stage-matched *Dmel-link*-5′ (Fig. 2F′), suggesting that *cis*-regulatory differences are at least partially responsible for the absence of *D. pseudoobscura link* midline expression. The Motif-T sites T1, T2, and T3 and the CME are identical between *D. melanogaster* and *D. pseudoobscura*, although T4 is not conserved (Fig. 5B). However, mutation of T4 (*link*-5′-*mutT24*, *link*-5′-*mutT24CME*) did not significantly affect midline expression (Fig. 6F and I), so a combination of T4 and additional diverged sequences may contribute to the alteration in expression.

Discussion

The role of *zld* in regulating the maternal-to-zygotic transition is extensive, directly activating expression of hundreds of genes. In this paper, we demonstrate that *zld* has a post-blastoderm role in directly activating expression of *link* in the CNS midline cells, trachea, and brain. Although *zld* controls *link* MG expression, it does not control all MG expression, since *CG7271*, *CG8965*, *escargot*, and *rhomboid* MG expression was unaffected in *zld* mutant embryos. Similarly, the well-characterized MG enhancers of the *gliolectin*, *Oatp26f*, *slit*, *ventral veins lacking*, and *wrapper* genes do not reveal Zld binding in the embryo, when tested by whole-embryo ChIP (Harrison et al., 2011). Of the 120 genes that were downregulated at least twofold in embryos lacking *zld* maternal function (Liang et al., 2008), only 4 genes are listed on MidExDB (http://midline.bio.unc.edu/MDB_Home.aspx) as MG-expressed genes ($N=99$ genes), indicating no clear enrichment of MG-expressed genes as being *zld* target genes. Thus, it remains to be seen whether the function of *zld* in CNS and tracheal development is as widespread and profound as its role in the blastoderm maternal-to-zygotic transition. While *zld* may not act as a global regulator of CNS transcription, its dynamic expression pattern suggests that it can regulate transcription in a highly temporal and cell-type specific manner in combination with other transcription factors, such as Sim and Trh.

One interesting feature of *zld* expression is that transcripts are present during embryogenesis as two RNA species that encode two different proteins. In both the blastoderm embryo and during stages 11–12, when *zld* regulates midline expression, the RB transcript generates a Zld protein containing all 4 C-terminal zinc fingers required for DNA binding (Liang et al., 2008). This is consistent with the role of Zld in activating *zen* and *link* transcription by binding TAGteam sites. However, at later embryonic stages *zld* continues to be expressed in the CNS, but is alternatively spliced into the RD transcript that encodes a protein containing only one of the 4 C-terminal zinc fingers, along with two other N-terminal zinc fingers. Consequently, the PD protein is likely to have different biochemical properties compared to PB, and may be non-functional. In the latter case, termination of *zld* function in the CNS may be generated by alternative splicing rather than by termination of transcription. Consistent with this view, the *zld* RD transcript is expressed in midline iVUM4 and MNB progeny neurons and lateral CNS neurons through stage 16, yet *link* is not expressed in those neuronal cell types. In summary, midline expression of *link* is due to the midline presence of the Zld PB protein with 4 C-terminal zinc fingers. Even though the Zld PD protein with only 1 C-terminal zinc finger is present in midline and lateral CNS neurons, there is no current evidence that it can activate transcription. The alternative splicing is cell-type specific and not strictly stage-specific, since imaginal disc *zld* expression in late stage embryos consists of the RB transcript.

In this paper, we describe three aspects of *link* expression: blastoderm, midline, and trachea expression. Blastoderm expression of *link* was previously shown to be genetically dependent on *zld* function (Liang et al., 2008). We demonstrate here that this control is direct, since mutation of the two Motif-T/TAGteam sites T1 and T3 results in an absence of *link* blastoderm expression. Regulation of *link* midline and tracheal expression is different: *link* midline expression is controlled by the combined action of Zld and Sim, and tracheal expression is controlled by Zld and Trh. Sim and Trh are both bHLH-PAS transcription factors that dimerize with Tgo, and bind the same ACGTG (CME) sites (Sonnenfeld et al., 1997; Wharton et al., 1994). While there are subtle differences between *link* midline and tracheal expression, the basic mechanism of control by Zld/Trh is likely similar to Zld/Sim. Focusing on Sim, it is possible to view *link* expression as utilizing multiple Zld and Sim:Tgo binding sites in an additive manner with a threshold for expression (Fig. 7A). Mutational studies indicate that the *link* T1, T3, T4, and CME sites contribute to *link* midline/tracheal expression. Mutation of T1 and T3 together has little effect on expression, and mutation of the CME (Fig. 7B) or T4

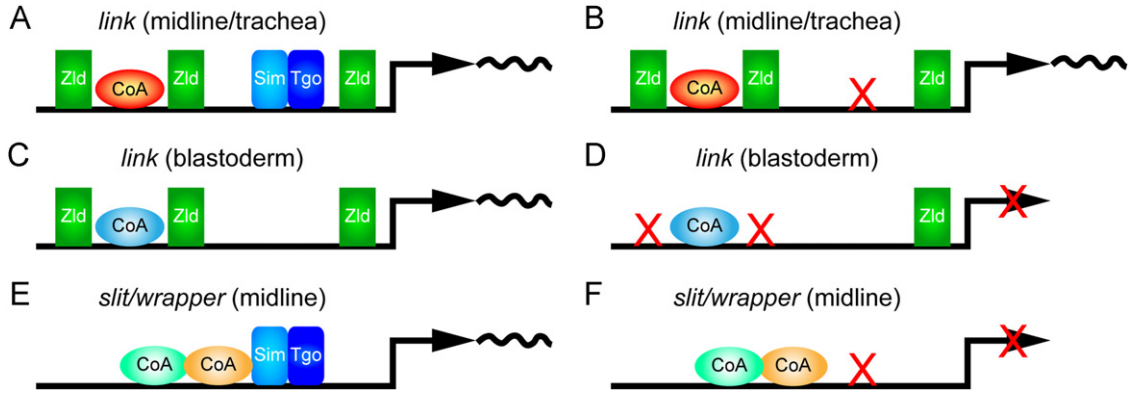


Fig. 7. Alternate modes of *Drosophila* MG gene regulation. Schematics are shown representing (A and B) *link* midline and tracheal expression, (C and D) *link* blastoderm transcription, and (E and F) *slit* and *wrapper* midline expression. (A) The *link* enhancer has multiple binding sites for Zld (sites T1, T3, and T4 are shown), Sim:Tgo/Trh:Tgo (CME), and predicted additional coactivators (red CoA). Together they result in transcription of *link* in CNS midline and tracheal cells. (B) When the CME is mutated (X), expression of *link* is still present. (C) In blastoderm cells, the *link* enhancer is occupied by Zld and additional coactivators distinct from the *link* midline/tracheal coactivators (blue CoA). (D) When the TAGteam T1 and T3 sites are mutated, *link* blastoderm expression is abolished. (E) In the *slit* and *wrapper* enhancers, it is proposed that Sim:Tgo forms an activation complex with multiple coactivators. (F) When the Sim:Tgo binding site in the *slit* and *wrapper* enhancers is mutated (X), MG expression is abolished.

and the CME together has little effect. However, mutation of 3 sites, including T1, T3 and either T4 or CME results in a dramatic loss of *link* expression.

These results also predict that additional coregulators are required for *link* expression (Fig. 7A and C). Mutation of T1 and T3 together abolishes *link* blastoderm expression (Fig. 7D), but not midline/tracheal expression, indicating that the presence of T1 and T3 is not sufficient for transcriptional activation by Zld in all cell types. This suggests that Zld interacts with a blastoderm-specific coregulator to activate *link* blastoderm expression (Fig. 7C). Similarly, the existence of additional midline/tracheal coregulators is necessary since the presence of 2 TAGteam sites is insufficient for midline/tracheal expression (e.g. *zen* has 4 TAGteam sites and is not expressed in midline cells, and *CG7271* and *escargot* have multiple TAGteam sites and are regulated in the blastoderm by *zld* but not in midline cells). Yet, the *link*-5′ fragment with intact T1 and T3 sites drives strong midline/tracheal expression even when T2, T4, and CME are mutant. This suggests that additional midline/tracheal-expressed coactivators are needed in addition to Zld and Sim/Trh (Fig. 7A). Note that there are a number of well-conserved sequences within the *link* enhancer in addition to the TAGteam and CME sites (Fig. S3).

Within the midline cells, at stages 11–12, *link* is prominently expressed in MG. Mechanistically, *link* MG expression is distinct from other MG-expressed genes, including *slit* and *wrapper*. The *slit* and *wrapper* MG enhancers have a single CME (similar in number to *link*) (Fig. 7E), yet mutation of the *slit* and *wrapper* CME results in loss of MG expression (Fig. 7F) (Estes et al., 2008; Wharton et al., 1994). This contrasts with *link* in which mutation of the CME by itself has little effect (Fig. 7B). This result also indicates that the presence of a single CME is insufficient for midline transcriptional activation. Also unlike *link*, there is no evidence that *zld* regulates *slit* and *wrapper* MG expression, since neither enhancer possess TAGteam sites nor detectably binds Zld in vivo (Harrison et al., 2011), and *wrapper* expression is not reduced in *zld* mutant embryos (not shown). However, genetic, biochemical, and mutational studies have provided evidence that Sox proteins (e.g. Dichaete), POU-HD proteins (e.g. Ventral veins lacking), ETS proteins (e.g. Pointed), and poly(T) sequences may act as MG co-activators along with Sim:Tgo (Estes et al., 2008; Ma et al., 2000; Sanchez-Soriano and Russell, 1998). We propose that Sim:Tgo forms a strong association with the *slit* and *wrapper* co-activators (Fig. 7E and F), such that when the CME is mutated, the co-activators are either poorly bound or unable to activate transcription on their own. In contrast, Zld and co-activators are able to still activate *link* MG transcription, even when the CME is mutated. Thus, there are at least two distinct modes of MG enhancers. Each uses Sim:Tgo, but one class employs multiple Zld TAGteam sites to activate *link* expression along with Sim:Tgo in an additive/threshold manner, whereas the other class (*slit* and *wrapper*) is more dependent on an intertwined Sim activation complex. These data further reinforce the view that there exist multiple ways to regulate genes in a similar manner.

The *link* gene has recently gained midline expression in the *melanogaster* subgroup, although blastoderm and tracheal expression are stable. Another example of recent evolutionary change in midline expression is the *Drosophila α methyl dopa-resistant* gene (Wang et al., 1996). Since *zld* and *sim* midline expression is well-conserved, the differences are likely due to *cis*-regulatory changes in the *link* midline enhancer. This view is supported by the inability of the *D. pseudoobscura link* regulatory region to drive significant midline expression in *D. melanogaster*. We propose a two-step model in which $\sim$60 mya, *link* was weakly or not expressed in midline cells. It acquired midline expression $>$13 mya, and then $<$6 mya a second change occurring in the *D. melanogaster* lineage resulted in increased levels of *link* midline expression.

The exact alterations that led to the changes in midline expression are unclear. It is unlikely to be due to changes in the T1, T3, and CME sequences since these are identical between *D. melanogaster* and several species that either lack or have trace levels of midline expression, including *D. ananassae*, *D. persimilis*, and *D. pseudoobscura*. Changes in site T4 are also unlikely to be causative in acquiring midline expression, since it differs significantly in sequence even among species in the *melanogaster* subgroup that have equivalent *link* midline expression. Most likely the acquisition of midline expression in the *melanogaster* subgroup was due to additional uncharacterized sequences in *link*-5′ sequences. However, the high levels of *link* expression present in *D. melanogaster* may be due, in part, to an alteration in T4, since only *D. melanogaster* T4 contains the TAG nucleotide sequence (Fig. 5B) common among high-affinity TAGteam sites (Liang et al., 2008).

Acknowledgments

The authors would like to thank Christine Rushlow for kindly providing anti-Zld antibody, *zld* mutant stocks, and advice, and Gerd Vorbrüggen for *vfl* stocks. We thank Corbin Jones, Hung-Jui

Shih, Sumit Dhole, and Karin Pfennig for *Drosophila* strains and rearing advice, Nasser Rusan, David Roberts, Mira Pronobis, Derek Applewhite, Kimberly Peters, and Joshua Currie for reagents and advice, and Daniel McKay and Joseph Fontana for help analyzing high-throughput sequencing data. We are grateful to the Bloomington *Drosophila* Stock Center for providing *Drosophila* stocks. The project was supported by NRSA postdoctoral awards to JCP (NICHD) and JDW (NINDS), fellowships from the UNC/NIH Developmental Biology Training Program to JCP and JDW (HD046369), and NIH grants R01 NS64264 (NINDS) and R37 RD25251 (NICHD) to STC.

Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.04.001.

References

Abramoff, M.D., Magalhaes, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophoton. Int. 11, 36–42.

Bray, S.J., Burke, B., Brown, N.H., Hirsh, J., 1989. Embryonic expression pattern of a family of *Drosophila* proteins that interact with a central nervous system regulatory element. Genes Dev. 3, 1130–1145.

De Renzis, S., Elemento, O., Tavazoie, S., Wieschaus, E.F., 2007. Unmasking activation of the zygotic genome using chromosomal deletions in the *Drosophila* embryo. PLoS Biol. 5, 1036.

Estes, P., Fulkerson, E., Zhang, Y., 2008. Identification of motifs that are conserved in 12 Drosophila species and regulate midline glia vs. neuron expression. Genetics 178, 787–799.

Fulkerson, E., Estes, P.A., 2010. Common motifs shared by conserved enhancers of *Drosophila* midline glial genes. J. Exp. Zool. B. Mol. Dev. Evol. 316, 61–75.

Gille, C., Frommel, C., 2001. STRAP: editor for STRuctural Alignments of Proteins. Bioinformatics 17, 377–378.

Gordon, K.L., Ruvinsky, I., 2012. Tempo and mode in evolution of transcriptional regulation. PLoS Genet. 8, e1002432.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., Brown, J.B., Cherbas, L., Davis, C.A., Dobin, A., Li, R., Lin, W., Malone, J.H., Mattiuzzo, N.R., Miller, D., Sturgill, D., Tuch, B.B., Zaleski, C., Zhang, D., Blanchette, M., Dudoit, S., Eads, B., Green, R.E., Hammonds, A., Jiang, L., Kapranov, P., Langton, L., Perrimon, N., Sandler, J.E., Wan, K.H., Willingham, A., Zhang, Y., Zou, Y., Andrews, J., Bickel, P.J., Brenner, S.E., Brent, M.R., Cherbas, P., Gingeras, T.R., Hoskins, R.A., Kaufman, T.C., Oliver, B., Celniker, S.E., 2011. The developmental transcriptome of *Drosophila melanogaster*. Nature 471, 473–479.

Groth, A.C., Fish, M., Nusse, R., Calos, M.P., 2004. Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. Genetics 166, 1775–1782.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95–98.

Harrison, M.M., Botchan, M.R., Cline, T.W., 2010. Grainyhead and Zelda compete for binding to the promoters of the earliest-expressed *Drosophila* genes. Dev. Biol. 345, 248–255.

Harrison, M.M., Li, X.Y., Kaplan, T., Botchan, M.R., Eisen, M.B., 2011. Zelda binding in the early *Drosophila melanogaster* embryo marks regions subsequently activated at the maternal-to-zygotic transition. PLoS Genet. 7, e1002266.

Hemphala, J., Uv, A., Cantera, R., Bray, S., Samakovlis, C., 2003. Grainy head controls apical membrane growth and tube elongation in response to Branchless/FGF signalling. Development 130, 249–258.

Isaac, D.D., Andrew, D.J., 1996. Tubulogenesis in *Drosophila*: a requirement for the t*rachealess* gene product. Genes Dev. 10, 103–117.

Jiang, L., Pearson, J.C., Crews, S.T., 2010. Diverse modes of *Drosophila* tracheal fusion cell transcriptional regulation. Mech. Dev. 127, 265–280.

Kasai, Y., Stahl, S., Crews, S., 1998. Specification of the *Drosophila* CNS midline cell lineage: direct control of *single-minded* transcription by dorsal/ventral patterning genes. Gene Expr. 7, 171–189.

Kearney, J.B., Wheeler, S.R., Estes, P., Parente, B., Crews, S.T., 2004. Gene expression profiling of the developing *Drosophila* CNS midline cells. Dev. Biol. 275, 473–492.

Kosman, D., Mizutani, C.M., Lemons, D., Cox, W.G., McGinnis, W., Bier, E., 2004. Multiplex detection of RNA expression in *Drosophila* embryos. Science 305, 846.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R. 1000 Genome Project Data Processing Subgroup, 2009. The Sequence Alignment/Map format and SAM tools. Bioinformatics 25, 2078–2079.

Liang, H.L., Nien, C.Y., Liu, H.Y., Metzstein, M.M., Kirov, N., Rushlow, C., 2008. The zinc-finger protein Zelda is a key activator of the early zygotic genome in *Drosophila*. Nature 456, 400–403.

Ma, Y., Certel, K., Gao, Y., Niemitz, E., Mosher, J., Mukherjee, A., Mutsuddi, M., Huseinovic, N., Crews, S.T., Johnson, W.A., Nambu, J.R., 2000. Functional interactions between *Drosophila* bHLH/PAS, Sox, and POU transcription factors regulate CNS midline expression of the *slit* gene. J. Neurosci. 20, 4596–4605.

McQuilton St, P., Pierre, S.E., Thurmond, J., 2012. FlyBase 101–the basics of navigating FlyBase. Nucleic Acids Res. 40, D706–D714.

Nambu, J.R., Franks, R.G., Hu, S., Crews, S.T., 1990. The *single-minded* gene of Drosophila is required for the expression of genes important for the development of CNS midline cells. Cell 63, 63–75.

Nambu, J.R., Lewis, J.L., Wharton, K.A., Crews, S.T., 1991. The Drosophila *single-minded* gene encodes a helix-loop-helix protein which acts as a master regulator of CNS midline development. Cell 67, 1157–1167.

Nien, C.Y., Liang, H.L., Butcher, S., Sun, Y., Fu, S., Gocha, T., Kirov, N., Manak, J.R., Rushlow, C., 2011. Temporal coordination of gene networks by Zelda in the early *Drosophila* embryo. PLoS Genet. 7, e1002339.

Ohshiro, T., Saigo, K., 1997. Transcriptional regulation of *breathless* FGF receptor gene by binding of TRACHEALESS/dARNT heterodimers to three central midline elements in *Drosophila* developing trachea. Development 124, 3975–3986.

Patel, N.H., Martin-Blanco, E., Coleman, K.G., Poole, S.J., Ellis, M.C., Kornberg, T.B., Goodman, C.S., 1989. Expression of *engrailed* proteins in arthropods, annelids, and chordates. Cell 58, 955–968.

Rebeiz, M., Posakony, J.W., 2004. GenePalette: a universal software tool for genome sequence visualization and analysis. Dev. Biol. 271, 431–438.

Rebeiz, M., Jikomes, N., Kassner, V.A., Carroll, S.B., 2011. Evolutionary origin of a novel gene expression pattern through co-option of the latent activities of existing regulatory sequences. Proc. Natl. Acad. Sci. USA 108, 10036–10043.

Sanchez-Soriano, N., Russell, S., 1998. The *Drosophila* SOX-domain protein Dichaete is required for the development of the central nervous system midline. Development 125, 3989–3996.

Siddharthan, R., Siggia, E.D., van Nimwegen, E., 2005. PhyloGibbs: a Gibbs sampling motif finder that incorporates phylogeny. PLoS Comput. Biol. 1, e67.0534–e67.0556.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S., Crews, S., 1997. The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian Arnt and controls CNS midline and tracheal development. Development 124, 4571–4582.

Sonnhammer, E.L., Durbin, R., 1995. A dot-matrix program with dynamic threshold control suited for genomic DNA and protein sequence analysis. Gene 167, GC1–10.

Staudt, N., Fellert, S., Chung, H.R., Jackle, H., Vorbruggen, G., 2006. Mutations of the *Drosophila* zinc finger-encoding gene *vielfältig* impair mitotic cell divisions and cause improper chromosome segregation. Mol. Biol. Cell 17, 2356–2365.

Subramanian, A.R., Kaufmann, M., Morgenstern, B., 2008. DIALIGN-TX: greedy and progressive approaches for segment-based multiple sequence alignment. Algorithms Mol. Biol. 3, 6.

Tamura, K., Subramanian, S., Kumar, S., 2004. Temporal patterns of fruit fly (*Drosophila*) evolution revealed by mutation clocks. Mol. Biol. Evol. 21, 36–44.

ten Bosch, J.R., Benavides, J.A., Cline, T.W., 2006. The TAGteam DNA motif controls the timing of *Drosophila* pre-blastoderm transcription. Development 133, 1967–1977.

Wang, D., Marsh, J.L., Ayala, F.J., 1996. Evolutionary changes in the expression pattern of a developmentally essential gene in three *Drosophila* species. Proc. Natl. Acad. Sci. USA 93, 7103–7107.

Ward, M.P., Mosher, J.T., Crews, S.T., 1998. Regulation of bHLH-PAS protein subcellular localization during *Drosophila* embryogenesis. Development 125, 1599–1608.

Wharton, J.,.K.A., Franks, R.G., Kasai, Y., Crews, S.T., 1994. Control of CNS midline transcription by asymmetric E-box elements: similarity to xenobiotic responsive regulation. Development 120, 3563–3569.

Wheeler, S.R., Kearney, J.B., Guardiola, A.R., Crews, S.T., 2006. Single-cell mapping of neural and glial gene expression in the developing *Drosophila* CNS midline cells. Dev. Biol. 294, 509–524.

Wheeler, S.R., Stagg, S.B., Crews, S.T., 2009. MidExDB: a database of *Drosophila* CNS midline cell gene expression. BMC. Dev. Biol. 9, 56.

Wilk, R., Weizman, I., Glazer, L., Shilo, B., 1996. *trachealess* encodes a bHLH-PAS protein and is a master regulator gene in the *Drosophila* tracheal system. Genes Dev. 10, 93–102.

Zelzer, E., Shilo, B., 2000. Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein Drifter, specifies tracheal cell fates. Mech. Dev. 19, 163–173.

Zinzen, R.P., Cande, J., Ronshaugen, M., Papatsenko, D., Levine, M., 2006. Evolution of the ventral midline in insect embryos. Dev. Cell. 11, 895–902.