Differential cytoplasmic mRNA localisation adjusts pair-rule transcription factor activity to cytoarchitecture in dipteran evolution

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Summary

Establishment of segmental pattern in the *Drosophila* syncytial blastoderm embryo depends on pair-rule transcriptional regulators. mRNA transcripts of pair-rule genes localise to the apical cytoplasm of the blastoderm via a selective dynein-based transport system and signals within their 3′-untranslated regions. However, the functional and evolutionary significance of this process remains unknown. We have analysed subcellular localisation of mRNAs from multiple dipteran species both in situ and by injection into *Drosophila* embryos. We find that although localisation of *wingless* transcripts is conserved in Diptera, localisation of *even-skipped* and *hairy* pair-rule transcripts is evolutionarily labile and correlates with taxon-specific changes in positioning of nuclei. We show in *Drosophila* that localised pair-rule transcripts target their proteins in close proximity to the nuclei and increase the reliability of the segmentation process by augmenting gene activity. Our data suggest that mRNA localisation signals in pair-rule transcripts affect nuclear protein uptake and thereby adjust gene activity to a variety of dipteran blastoderm cytoarchitectures.

Supplemental data available online

Key words: Pair-rule, Developmental evolution, mRNA localisation, Cytoarchitecture, Diptera

Introduction

Many cell types use cytoplasmic mRNA localisation to distribute protein products within cells (Kloc et al., 2002). In neurons, various transcripts are selectively sorted to different regions of the cytoplasm (Job and Eberwine, 2001), and in motile fibroblasts localisation of *β-actin* mRNA to the leading edge contributes to remodelling of the cytoskeleton (Kislauskis et al., 1997; Shestakova and Singer, 2001). Localisation can also be coupled to cell divisions in which protein determinants must be segregated asymmetrically (Long et al., 1997; Takizawa et al., 1997).

In the syncytial blastoderm embryo of the fruit fly *Drosophila melanogaster*, transcripts of the pair-rule gene class are localised asymmetrically to the apical side of a layer of peripheral nuclei (Hafen et al., 1984; Ingham et al., 1985; Kilchherr et al., 1986; Macdonald et al., 1986). Pair-rule transcripts encode transcription factors that are expressed in partially overlapping sets of seven circumferential stripes, and act in combination to establish segmental organisation (Pankratz and Jäckle, 1993). The segment polarity gene *wingless* (*wg*), which encodes an extracellular signalling molecule of the WNT family, also has its transcripts localised apically. Localisation of *wg* mRNA augments Wg signalling activity, although it is not yet clear by what mechanism this is achieved (Simmonds et al., 2001).

A shared machinery localises pair-rule and *wg* transcripts during embryogenesis (Wilkie and Davis, 2001; Bullock and Ish-Horowicz, 2001), and also translocates maternal mRNAs from their sites of synthesis in the nurse cells into the early oocyte (Bullock and Ish-Horowicz, 2001). The localisation machinery involves active transport towards the minus-ends of microtubules by the dynein/dynactin motor complex (Wilkie and Davis, 2001). Transport also depends on the proteins Egalitarian (Egl) and Bicaudal-D (BicD) (Bullock and Ish-Horowicz, 2001). The machinery may be used widely because BicD and dynein components are highly conserved in metazoans and Egl homologues are found in the nematode *C. elegans*.

All the transcript cargoes studied to date have been shown to contain localisation signals in their 3′-untranslated regions (3′-UTRs), which must direct interaction with the transport machinery. RNA recognition is dependent on secondary structure – localisation signals in different transcripts consist of double-stranded stem-loops that share no overt primary sequence similarity – although higher-order RNA folding may also be significant (Macdonald and Kerr, 1998; Bullock et al., 2003).

Although the mechanisms of pair-rule mRNA transport in the blastoderm embryo are emerging, the developmental and evolutionary significance of this process remains unclear. Pair-

rule patterning is used in many insects, but RNA signals for the dynein machinery have only been studied within the genus *Drosophila*, where signals in the pair-rule transcript *hairy* (*h*) (Bullock et al., 2003), as well as those of *wg* (Simmonds et al., 2001) and the maternal transcript *bicoid* (Macdonald, 1990), are functionally conserved. The conservation of *h* localisation in drosophilids is not surprising, however, because these flies share very similar blastoderm types. By contrast, blastoderm morphologies can differ drastically in less closely related dipteran taxa (Anderson, 1972).

In this study, we have analysed transcript localisation of a large number of newly identified homologues of the pair-rule genes *even-skipped* (*eve*) and *h*, and of *wg* in multiple families of the insect order Diptera (true flies) by in situ hybridisation to endogenous transcripts and by injection of fluorescently labelled transcripts into *Drosophila* embryos. We show that Egl-dependent localisation signals are conserved in *eve* and *h* transcripts over 145 million years of evolution in higher (cyclorrhaphan) flies, indicating that this process is functionally significant, but absent in some, but not all, branches of lower Diptera. By contrast, *wg* transcript localisation appears to be conserved throughout Diptera. The phylogenetic occurrence of pair-rule transcript localisation suggests a selective advantage of this trait in species with a thickened peripheral cytoplasm and apically residing blastoderm nuclei. Consistent with this, we find that, in *Drosophila*, localisation of pair-rule mRNAs targets their proteins apically, in close proximity to the nuclei, and that interfering with localisation lowers the activity of pair-rule genes. We provide evidence that RNA localisation augments levels of protein within the nucleus and propose that, by affecting perinuclear translation, this mechanism may be used in a wide variety of organisms to modulate the activity of nuclear factors.

Materials and methods

Fly culture

Fly cultures and egg collections of *Megaselia abdita* (Phoridae; scuttle or humpbacked flies), *Coboldia fuscipes* (Scatopsidae; scavenger flies) and *Clogmia albipunctata* (Psychodidae; moth flies) have been described previously (Rohr et al., 1999; Stauber et al., 2002). *Empis livida* (Empididae; dance flies), *Haematopota pluvialis* (Tabanidae; horse flies) and *Platypeza consobrina* (Platypezidae; flat-footed flies) were collected in the surroundings of Göttingen (Germany). Embryos of *Episyrphus balteatus* (Syrphidae; hover flies) were a gift from Peter Hondelmann (University of Hannover, Germany). *Anopheles gambiae* (Culicidae; African malaria mosquito, Suakoko strain) genomic DNA was a gift from Hans-Michael Müller (European Molecular Biology Laboratory, Germany). Throughout the text we refer to Cyclorrhapha as 'higher Diptera' (including *Drosophila*, *Episyrphus*, *Megaselia* and *Platypeza*). The term 'lower Diptera' is used for the paraphyletic assemblages of orthorrhaphous Brachycera (including *Empis* and *Haematopota*) and Nematocera (including *Coboldia*, *Clogmia* and *Anopheles*).

Wild-type *Drosophila* embryos were of the Oregon-R strain. egl^{WU50}, $eve^{1.27}$, ftz^{13}, h^{i22}, kni^{1}, Kr^{1} and wg^{CX4} are reported to be strong loss-of-function or null alleles. egl^{3e} is a partial loss-of-function allele that compromises the interaction of the protein with dynein light chain (Navarro et al., 2004). For Fig. 5B, larvae heterozygous for the pair-rule gene mutation were distinguished from wild types using balancer chromosomes marked with green fluorescent protein (GFP) driven from the *actin* promoter (Reichhart and Ferrandon, 1998). Embryos of similar zygotic genotype but different maternal origin were generated from reciprocal crosses (i.e. heterozygous pair-rule mutant males were mated to *egl* mutant females and vice versa). Larval cuticle preparations were performed as described (Wieschaus and Nüsslein-Volhard, 1986).

Cloning of *eve*, *h* and *wg* homologues

3′ regions of *Anopheles-eve*, *-ftz*, *-h*, *-odd* and *-wg* homologues were PCR amplified with specific primers (based on sequences from the *Anopheles* genome project) on genomic DNA of *Anopheles gambiae* strain Suakoko, a gift from Hans-Michael Müller (EMBL, Heidelberg). Partial sequences of the other *eve*, *h* and *wg* homologues were amplified by PCR on genomic DNA with the degenerate primer pairs GITAYMGIACIRCITTYACNMGNGA/TANACNGCNGCRT-ANGGCCANGC (*eve*), AAYAARCCIATHATGGARAARMGNMG/GYTTIACIGTYWTYTCIARDATRTCNGC (*h*) and GARTGYAA-RTGYCAYGGNATG/RTAICCICKICCRCARCACAT (*wg*). For *Clogmia-eve1*, *Platypeza-eve* and *Platypeza-h*, phages were isolated from genomic Lambda FIX II (Stratagene) phage libraries (S. Lemke and U.S.-O., unpublished) and used as templates for PCR-amplification of 3′-regions. 3′-UTRs of all other homologues were amplified by PCR on cDNA prepared with Marathon or SMART RACE cDNA Amplification Kit (Clontech). Table S1 at http://dev.biologists.org/supplemental lists details of clones and contains information on templates, primers and products.

In situ hybridisation, immunostaining and RNA injections

In situ hybridisation was carried out essentially as described (Lehmann and Tautz, 1994; Stauber et al., 2002) using NBT/BCIP and Fast Red (Roche) for colourimetric and fluorescent detection of transcripts, respectively. Nuclei were stained by a 2 hours room temperature incubation in 5 μg/ml of Alexa 660-wheat germ agglutinin (WGA; Molecular Probes) in PBS. For immunostaining, we used mouse anti-Hairy (S. M. Pinchin, unpublished) and guinea pig anti-Run (Kosman et al., 1998) polyclonal primary antibodies and Alexa-488 or Alexa 594-conjugated secondary antibodies (Molecular Probes).

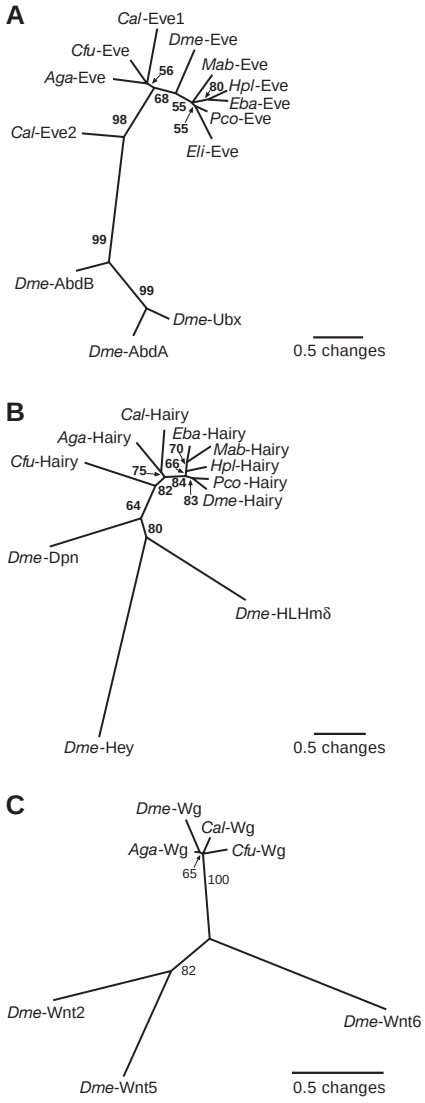
Fluorescent, capped mRNAs incorporating Alexa-488- (Molecular Probes), Cy3- or Cy5-UTP (Perkin Elmer) were synthesised as described (Bullock and Ish-Horowicz, 2001). Briefly, for each transcript, 250 ng/μl solutions were injected into nuclear cycle 14 blastoderm embryos which were fixed ~8 minutes after injection of the last embryo (~11 minutes after injection of the first). Anti-Egl antibody (Mach and Lehmann, 1997) was injected 10 minutes before the RNAs.

Expression of localising and non-localising *h* transcripts

To guard against potential dominant lethality arising from expression of non-localising transcripts, we used a conditional expression system in which an upstream FRT-stop-FRT cassette terminates transcription and prevents transgene expression unless excised using FLP recombinase (Struhl et al., 1993). We cloned the wild-type *h* cDNA, or one containing the *hΔD* deletion that removes 20 nucleotides required for localisation (Bullock et al., 2003), appended to 3′ *h* genomic sequences containing the polyadenylation signal, into a unique *Pme*I site within the *Drosophila* transformation vector $P\{w^{+mC}$ *(eve2)2 >hsp70 3′>}*, a derivative of $P\{w^{+mC}$ *(eve2)2 >hsp70 3′> eve3′}* (Wu et al., 2001) with the *eve* 3′UTR removed. *h* fragments were generated by PCR (details available upon request) and the *h*-coding regions in the final constructs were sequenced to ensure that no mutations were introduced. The resultant constructs ($P\{w^{+mC}$ *(eve2)2 >hsp70 3′>* $h^{wt}\}$ and $P\{w^{+mC}$ *(eve2)2 >hsp70 3′>* $h\Delta D^{nloc}\}$ contain two copies of a minimal *eve* stripe 2 enhancer that, following excision of the stop signal, drive expression of *h* in parasegment 3 (Kosman and Small, 1997). In the event, removal of the transcriptional terminating sequence in the male germ-line with the *β2-tubulin-FLP* transgene (Struhl and Basler, 1993) yielded viable transgenic lines which were used in all the experiments described here.

To quantitate levels of *st2-h* expression in different lines, we generated cDNA from 150-300 blastoderm embryos 2.5-3.25 hours after egg laying at 25°C from crosses of homozygous flies (*nlocA*, *B* and *C*; *wtA* and *B*) or heterozygous flies when homozygous lines were lethal (*nlocD; wtD*) or semi-lethal (*wtC*). The data shown in Fig. 6E are normalised for gene dosage. We used real-time PCR with Taqman™ probes to quantify amounts of activated *st2-h* cDNA relative to *actin 5C* cDNA using the comparative C_T method described by the manufacturers (PerkinElmer). The Taqman probes (used at 0.1 μM) and primers (0.4 μM each) were as follows: *st2-h* 5′ GTG-ACCGCCGCACAGTC; *st2-h* 3′ AACTTCAAGATCCCCATTCAA-AGT; *st2-h* Taqman™ probe CAACTAACTGCCTTCGTTAATA-TCCTCTGAATAAGCC; Actin 5′ GGTTTATTCCAGTCATTCCTT-TCAA; Actin 3′ ACTGTAAACGCAAGTGGCGA; Actin Taqman™ probe CCGTGCGGTCGCTTAGCTCAGC.

The *st2-h* oligonucleotides amplify sequences between the *eve* 5′UTR and the FRT site in the transgene. Using cDNA from embryos of the parental *yw* strain, we showed that these primers do not amplify endogenous cDNAs. Nor do they amplify products when no reverse transcription step is included. We confirmed the validity of our assay using serial dilutions of one of the transgenic cDNA samples.



Results

Apical *eve* and *h* transcript localisation in Diptera correlates with the position of blastoderm nuclei

To investigate the functional significance and phylogenetic occurrence of pair-rule mRNA localisation, we first cloned *eve* and *h* orthologues from species throughout Diptera (Fig. 1) (see Fig. S1 at http://dev.biologists.org/supplemental for full sequence alignments). We assayed transcript localisation in four of these species that can be cultured in the laboratory by whole-mount in situ hybridisations on blastoderm embryos. Two of them, *Episyrphus* (Syrphidae) and *Megaselia* (Phoridae), are cyclorrhaphan flies (i.e. higher dipterans) but, unlike *Drosophila*, belong to basal branches of this taxon; the other two, *Coboldia* (Scatopsidae) and *Clogmia* (Psychodidae), belong to different branches of lower Diptera (Fig. 2).

In each species, *eve* and *h* transcripts are expressed in circumferential stripes (Fig. 2A-E,K-O). In the higher dipterans *Drosophila*, *Episyrphus* and *Megaselia*, seven *eve* and *h* stripes are formed at blastoderm stage (Fig. 2A-C,K-M). In the lower dipterans, *Clogmia* (where *eve* has been duplicated; Fig. 1 and Fig. S1) and *Coboldia*, fewer than seven *eve* and *h* stripes are present at this stage (Fig. 2D,E,N,O) and posterior pair-rule stripes are added after the onset of gastrulation (Rohr et al., 1999). The striped expression of the *eve* and *h* transcripts in blastoderm embryos suggests that they act during segmentation in these species, although, interestingly, pair-rule expression of the putative *h* orthologue in *Clogmia* may not be conserved (Fig. 2O).

In *Megaselia*, *eve* and *h* transcripts are tightly localised apically throughout blastoderm stages, much like their counterparts in *Drosophila* (Fig. 2H,R). In these species blastoderm embryos have a thickened blastoderm with nuclei that reside apically throughout the cellularisation process (Fig. 2F-H). In *Episyrphus*, transcripts are also enriched apically, but in contrast to *Drosophila* and *Megaselia*, a substantial proportion of the mRNA accumulates in the basal cytoplasm

Fig. 1. Phylogenetic relationships of dipteran *eve*, *h* and *wg* genes. Newly-identified dipteran *eve* (A), *h* (B) and *wg* (C) sequences are more closely related to one another than to paralogous *Drosophila* genes. Phylogenetic distance trees were generated from ClustalW alignments of predicted protein sequences (see legend to Fig. S1 at http://dev.biologists.org/supplemental) using the Quartet Maximum-Likelihood Method of Strimmer and von Haeseler (Strimmer and von Haeseler, 1996). Numbers refer to reliability values in percent. AbdominalA (*Dme*-AbdA), AbdominalB (*Dme*-AbdB), Deadpan (*Dme*-Dpn), E(spl)mδ (*Dme*-HLHmδ), Hairy/E(spl)-related with YRPW motif (*Dme*-Hey), Ultrabithorax (*Dme*-Ubx), *Dme*-Wnt2, *Dme*-Wnt5 and *Dme*-Wnt6 were used as outgroups. *Aga*, *Anopheles gambiae*; *Cal*, *Clogmia albipunctata*; *Cfu*, *Coboldia fuscipes*; *Dme*, *Drosophila melanogaster*; *Eba*, *Episyrphus balteatus*; *Eli*, *Empis livida*; *Hpl*, *Haematopota pluvialis*; *Mab*, *Megaselia abdita*; *Pco*, *Platypeza consobrina*. Accession numbers: *Dme*-AbdA SWP, P29555; *Dme*-AbdB SWP, P09087; *Dme*-Dpn SWP, Q26263; *Dme*-HLHmδ SWP, Q01071; *Dme*-Eve SWP, P06602; *Dme*-Hairy SPTREMBL, Q95NU9; *Dme*-Hey SPTREMBL, Q9U9U4; *Dme*-Ubx SWP, P02834; *Dme*-Wg SPTREMBL, Q8MQP9; *Dme*-Wnt2 SPTREMBL, Q9V584; *Dme*-Wnt5 SPTREMBL, Q9VWT4; *Dme*-Wnt6 SPTREMBL, Q9VM26.

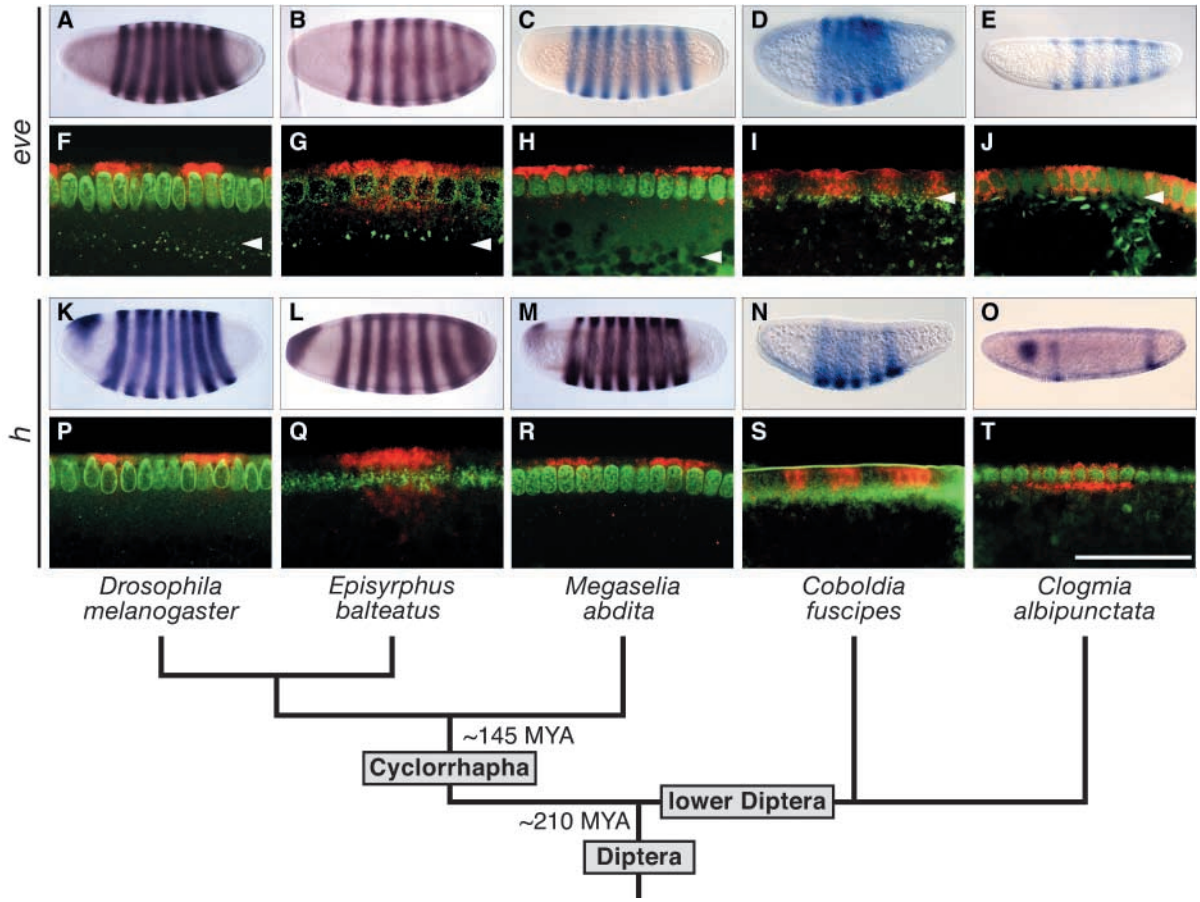


Fig. 2. Expression and localisation of *eve* and *h* transcripts in five dipteran species at blastoderm stage. (A-J) *eve* and (K-T) *h* whole-mount in situ hybridisation showing transcript expression [(A-E,K-O) blue/purple (anterior towards the left, dorsal upwards)] and subcellular localisation [(F-J,P-T) red (apical is upwards and basal downwards in this and subsequent figures)]. Nuclear envelopes are shown in green pseudocolour (Alexa 660-wheat-germ agglutinin) in this and other figures. *eve* and *h* are found at high levels in the apical cytoplasm of *Drosophila*, *Megaselia* and *Episyrphus*, although in the latter species, localisation is less efficient. In *Clogmia* and *Coboldia*, these transcripts are distributed uniformly in the apicobasal axis. We identified two *eve* homologues in *Clogmia*, both of which are expressed in stripes and do not localise asymmetrically; *Clogmia-eve2* is shown here. Arrowheads indicate the junction between the yolk and cytoplasm. In *Coboldia* and *Clogmia*, posteriormost stripes are established only after the onset of gastrulation (not shown). Unlike other dipteran *h* homologues *Episyrphus-h* is also expressed in the putative anlage of extra-embryonic tissue (not shown). *Clogmia-h* (O) is detected in a pair of anterior lateral patches and two stripes that might correspond to *h* stripes 1 and 6 in other species; expression in other stripe domains is weak or absent at blastoderm stages. Phylogenetic relationships of the species are shown below (Collins and Wiegmann, 2002; Yeates and Wiegmann, 1999). MYA, million years ago. Scale bar: 50 μm in F-J,P-T; 235 μm in A,K; 560 μm in B,J; 280 μm in C,M; 145 μm in D,N; 240 μm in E,O.

(Fig. 2G,Q). Early *Episyrphus* blastoderm embryos also have apical nuclei. However, in this species the nuclei adopt a more central position during the cellularisation process, at a time when pair-rule genes are active (see Fig. S2 at http://dev.biologists.org/supplemental). In *Clogmia* and *Coboldia*, however, *eve* and *h* transcripts are distributed evenly throughout the cytoplasm and these species have a thin blastoderm with little cytoplasm surrounding the nuclei (Fig. 2I,J,S,T). These results suggest that localisation of pair-rule transcripts is not associated with pair-rule patterning per se and that a requirement for the apical localisation of pair-rule genes may be influenced by the cytoarchitecture of the blastoderm. In addition, the apical localisation of *eve* and *h* mRNAs in diverse cyclorrhaphan flies that evolved independently for ~145 million years (Grimaldi and Cumming, 1999) indicates that pair-rule transcript localisation is functionally significant.

Apical localisation of *wg* transcripts throughout Diptera suggests a conserved localisation machinery

To test whether the localisation machinery is active in transporting other transcripts in *Clogmia* and *Coboldia*, we cloned homologues of *wg* (Fig. 1, Fig. 3A-C, Fig. S1 at http://dev.biologists.org/supplemental), which encodes an extracellular signalling protein, from these species. In *Drosophila*, *wg* transcripts are localised apically in late blastoderm (Fig. 3D) and cellularised postgastrular embryos (Fig. 3G). We find that *wg* transcripts are also enriched apically in *Coboldia* embryos at equivalent stages (Fig. 3E,H). This suggests that the Egl/BicD/dynein localisation machinery is active, and that the failure of *Coboldia-eve* and *-h* transcripts to localise reflects a lack of mRNA localisation signals.

In *Clogmia*, *wg* transcripts are distributed uniformly in the cytoplasm of blastoderm embryos and become apically

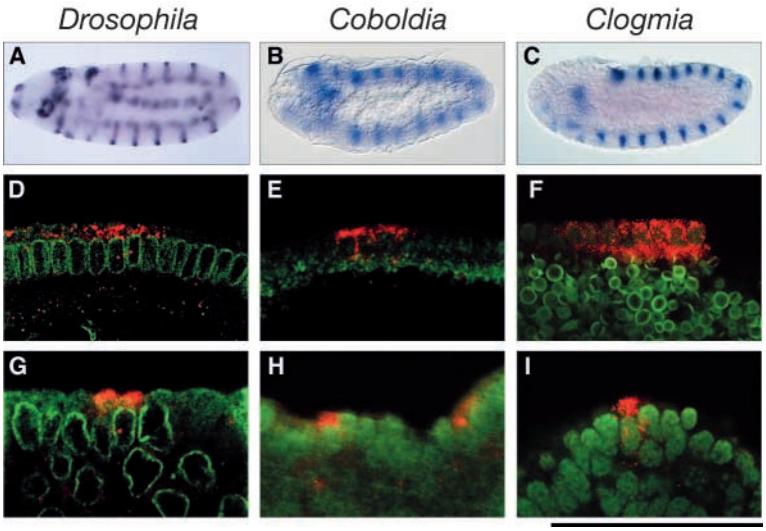


Fig. 3. Subcellular localisation of *wg* transcripts in *Drosophila*, *Coboldia* and *Clogmia* embryos. (A-C) The segmental expression pattern of *wg* in extended germband embryos is conserved in *Drosophila*, *Coboldia* and *Clogmia* (anterior towards the left, dorsal upwards). (D-F) At blastoderm stages, *wg* transcripts accumulate apically in *Drosophila* and *Coboldia*, but not in *Clogmia*. (G-I) After gastrulation, *wg* transcripts accumulate apically in all three species. Localisation of *wg* in germband extended embryos appears to be reproducibly less efficient in *Clogmia* than in *Coboldia* and *Drosophila*, with a proportion of transcripts detected basally. Scale bar: 385 μm in A; 240 μm in B; 400 μm in C; 83 μm in D,F; 50 μm in E,G,I; 27 μm in H.

enriched only in epithelial cells of postgastrular embryos (Fig. 3F,I). These observations suggest that the Egl/BicD/dynein localisation machinery is present in lower Diptera, but that it can be deployed at different stages in different species.

Localisation signals in pair-rule genes are evolutionarily labile

We wanted to survey localisation of *eve* and *h* orthologues in additional species in order to gain further insights into the phylogenetic occurrence of pair-rule mRNA localisation. However, embryos from many phylogenetically informative taxa are not available for in situ hybridisation. We therefore used injection of fluorescently labelled transcripts into *Drosophila* embryos (Bullock and Ish-Horowicz, 2001; Wilkie and Davis, 2001) to test for localisation signals in *eve*, *h* and *wg* homologues cloned from other dipteran species. Each labelled RNA included a region of coding sequence as well as the full-length 3′UTR (Table S1 at http://dev.biologists.org/supplemental). The distribution of all 11 injected *Megaselia*, *Episyrphus*, *Clogmia* and *Coboldia* transcripts mirrors closely their endogenous distributions observed by in situ hybridisation (Fig. 2, Fig. 3, Fig. 4A,B). In all cases, apical accumulation of localising transcripts is prevented by pre-injecting *Drosophila* embryos with antibodies that specifically inhibit Egl function (Bullock and Ish-Horowicz, 2001) (not shown). Based on these data, we believe that RNA injection into *Drosophila* blastoderm embryos provides a reliable tool for detecting Egl/BicD/dynein-dependent localisation signals throughout Diptera. The similarities between localisation of endogenous and injected transcripts indicates that the specificity of the RNA recognition factors has changed little during more than 210 million years of dipteran evolution, presumably because it is constrained by the need to recognise multiple cargoes in different cell types.

We therefore used this heterologous assay to probe for Egl-dependent localisation signals in transcripts of several other species where it is not possible to examine transcript localisation in situ (Fig. 4). *eve* and *h* RNAs from the cyclorrhaphan *Platypeza* (Platypezidae) localise apically in *Drosophila* blastoderm embryos, providing further evidence that localisation signals in pair-rule transcripts are common throughout Cyclorrhapha.

We detected localisation signals in *wg* and three out of four tested pair-rule transcripts of the Malaria mosquito *Anopheles* [*odd skipped* (*odd*), *fushi tarazu* (*ftz*) (not shown) and *h* (Fig. 4A); a signal was not found in the full-length *eve* transcript]. The presence of localisation signals in pair-rule genes of this lower dipteran is interesting, because similar blastoderm types appear to have evolved convergently in the cyclorrhaphan and the culicomorphan branch of Diptera to which *Anopheles* belongs (Ivanova-Kasas, 1949; Anderson, 1972; Monnerat et al., 2002). Of *Empis-eve*, *Haematopota-h* and *Haematopota-eve* – transcripts from species that are more closely related to cyclorrhaphan flies than *Anopheles*, *Clogmia* and *Coboldia* – only the last localises upon injection into *Drosophila*. Although the injection assay does not allow us to discern the developmental context in which localisation signals are used, the results corroborate our conclusion from the analysis of mRNA localisation in situ (Fig. 2, Fig. 3), that, in Diptera, localisation of *wg* transcripts is conserved, whereas localisation of pair-rule transcripts is labile.

We could not detect any significant stretches of conserved primary sequence in 3′ UTRs of localising transcripts. This is not surprising, however, because efficient signal recognition by the localisation machinery can be mediated by multiple, partially redundant interactions in which the essential features are contained within short stretches of base-paired RNA (Macdonald and Kerr, 1998; Bullock et al., 2003). Even within the genus *Drosophila*, the primary sequence of the *h* localisation signal has diverged significantly (Bullock et al., 2003).

Suppression of pair-rule transcript localisation in *Drosophila* alters pair-rule protein distribution and reduces pair-rule gene activity

The apparent correlation between apical pair-rule transcript localisation and apical-residing nuclei led us to the hypothesis that apical transcript localisation augments nuclear uptake of the transcription factor products of the pair-rule genes by targeting translation apically, in close proximity to the nuclei. We therefore assayed the consequences of disrupting pair-rule mRNA localisation in embryos of *Drosophila melanogaster*. Because components of the pair-rule mRNA localisation machinery are also required maternally for differentiation of the oocyte (Deng and Lin, 2001), we used a partial loss-of-function allele of *egl* (Navarro et al., 2004), which provides

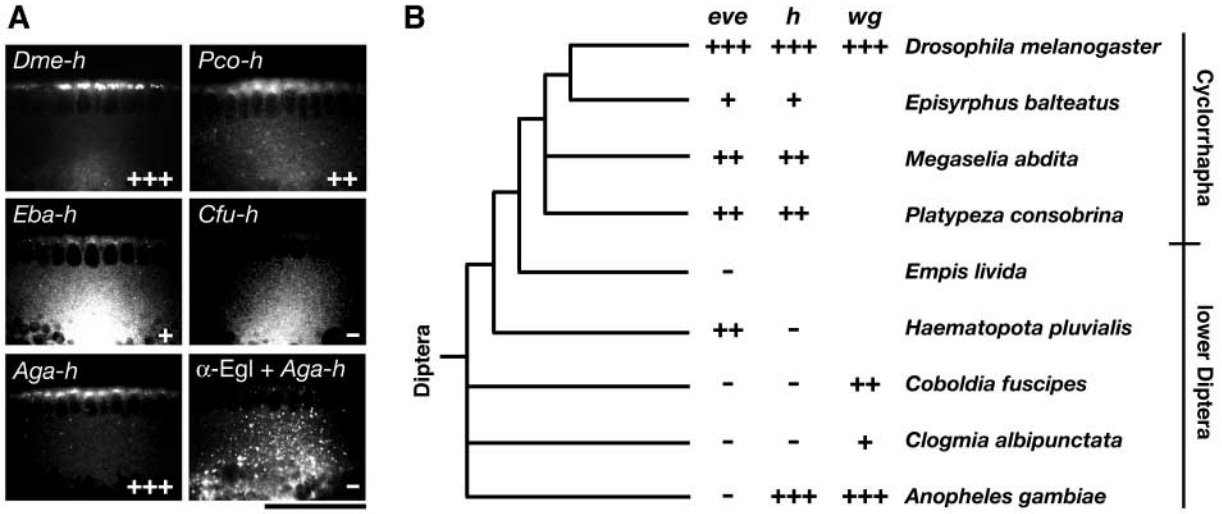


Fig. 4. Localisation of dipteran transcripts upon injection into *Drosophila* blastoderm embryos mediated by Egl-dependent mRNA localisation signals. (A) *Drosophila* embryos injected with *h* transcripts from dipteran species and fixed ~8-11 minutes later, showing representative examples of different efficiencies of mRNA localisation. Ten to 30 embryos were imaged for each transcript and used to categorise the extent of apical RNA enrichment. (A, bottom panels) *Anopheles-h* transcripts localise very efficiently upon injection (left), but localisation of this transcript is prevented by prior injection with antibodies that specifically inhibit the function of the *Drosophila* Egl protein (right). (B) Summary of the efficiency of localisation signals within dipteran *eve*, *h* and *wg* transcripts upon injection into *Drosophila* plotted onto the phylogenetic tree: +++, very efficient localisation; ++, efficient localisation; +, weak localisation; –, no apical enrichment. The efficiency of apical transport in this assay mirrors endogenous efficiencies of transcript localisation (compare with Figs 2 and 3). Both *Clogmia-eve1* and *Clogmia-eve2* fail to localise in this assay. All localising transcripts are dependent on Egl function. The occurrence of pair-rule mRNA localisation signals does not appear to be related to the absolute size of the embryo. For example, *h* transcripts contain localisation signals in *Megaselia* and *Drosophila* (length of the embryo is ~400-500 μm), but not in *Haematopota* (~1500 μm) or *Coboldia* (~260 μm). Scale bar: 50 μm.

sufficient Egl function to overcome the earlier block in oogenesis. Females that have one copy of this allele (egl^{3e}) and one copy of a null allele (egl^{WU50}) lay eggs, ~40-60% of which are fertilised and develop to blastoderm stages. Whereas embryos laid by wild-type mothers have an almost exclusively apical distribution of pair-rule mRNAs such as *eve*, *ftz*, *h* and *runt* (*run*), those laid by egl^{3e}/egl^{WU50} mothers accumulate a large proportion of these transcripts in the basal cytoplasm (Fig. 5A). A slight apical enrichment of pair-rule mRNAs is still detectable in most stripes in these mutants (Fig. 5A), consistent with their retention of some Egl activity.

The defect in transcript localisation in *egl* mutant embryos also affects the subcellular distribution of pair-rule proteins (Fig. 5A). For example, in wild-type blastoderm embryos, Run protein is first detected predominantly in the apical cytoplasm (not shown); in slightly older blastoderms, the bulk of this protein has accumulated in the nuclei but a proportion of it is still detected in the apical cytoplasm (Fig. 5A). In *egl*-deficient embryos, more diffuse cytoplasmic Run protein staining is also detected basally, similar to the distribution of its transcripts (Fig. 5A). We also observed basal accumulation of other pair-rule proteins in *egl* mutants (not shown), but the width and intensity of protein stripes as well as protein levels are not altered noticeably (not shown). Together, these observations suggest that pair-rule transcript localisation targets protein to the apical cytoplasm prior to import into the nuclei.

In embryos from *egl* mutant mothers, the apical localisation of *wg* transcripts is very strongly reduced (Fig. 5A) but, despite the inefficient localisation of these and pair-rule mRNAs, segmentation is only slightly impaired. Some (7.4%; n=136) egl^{3e}/egl^{WU50} blastoderm embryos show variable defects in the pattern of segmental *engrailed* expression (not shown), whereas only 1.3% of embryos from the reciprocal cross – i.e. wild-type mothers mated to egl^{3e}/egl^{WU50} males – exhibit such defects (n=309; P<0.01; Fisher's exact test). The frequency of mild cuticular patterning defects in first instar larvae from *egl* mutant females is also increased (3.1%, n=349) compared with wild-type controls (0.7%, n=420; P<0.05; Fig. 5B).

However, *egl* mutant embryos are acutely sensitive to a reduction in pair-rule gene dose (Fig. 5B,C). For example, 32.4% of h^{i22}/h^{+} first instar larvae from egl^{3e}/egl^{WU50} mothers have pair-rule defects, compared with 3.2% from wild-type mothers (P<0.001). These genetic interactions do not reflect a general sensitivity of early patterning processes to a reduction in Egl function, however, because phenotypes caused by heterozygosity of gap genes such as *Krüppel* (*Kr*) and *knirps* (*kni*) – which function upstream of the pair-rule genes in segmentation, and whose transcripts are not localised asymmetrically – and *wg* are not enhanced significantly by the maternal *egl* mutant genotype (Fig. 5B).

These experiments suggest that apical localisation of pair-rule mRNAs and proteins enhance their activity, although they do not rule out entirely a role for Egl independent of its function in pair-rule transcript localisation. Nor do they address the consequences of completely blocking localisation of a pair-rule mRNA, because the *egl* mutants still retain some transport activity.

We therefore assayed the activity of a pair-rule protein encoded either by localising transcripts or by transcripts distributed uniformly in the cytoplasm. Wild-type *h* transcripts

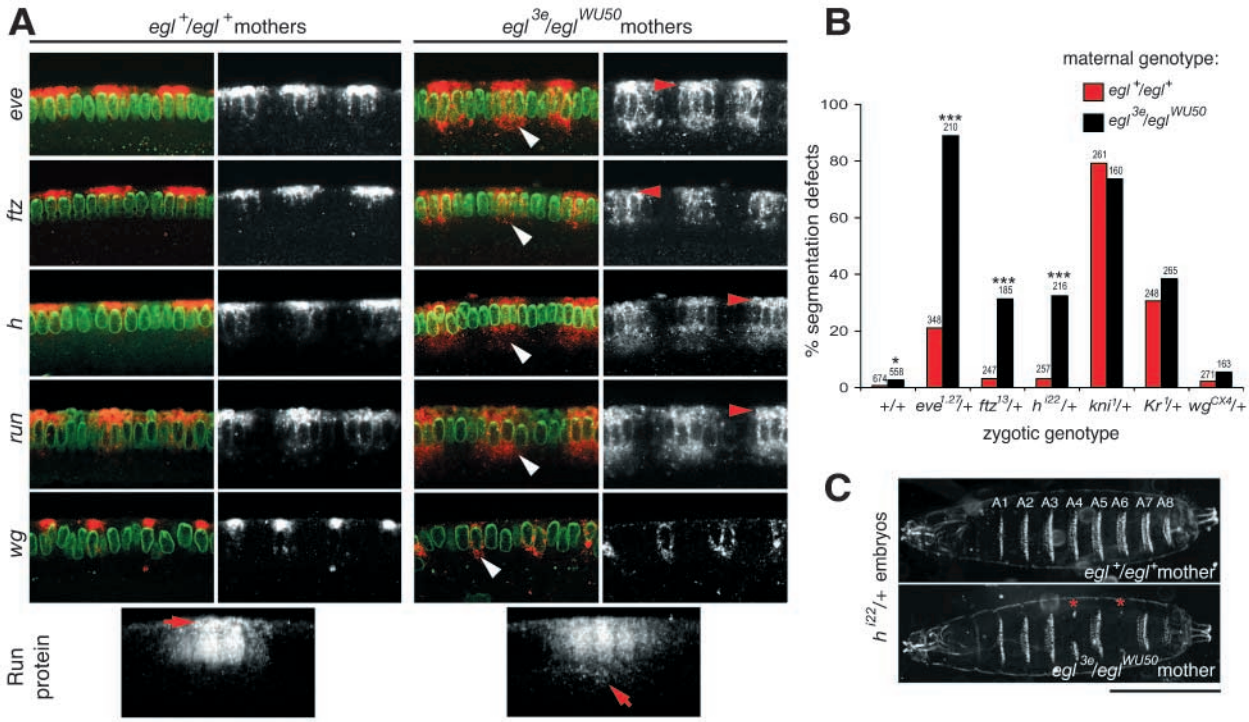


Fig. 5. Pair-rule gene activity is reduced in *egl* mutant embryos. (A) Distribution of transcripts (as indicated; red) in *Drosophila* blastoderm embryos laid by wild-type mothers (egl^{+}/egl^{+}) or partial loss-of-function *egl* mothers (egl^{3e}/egl^{WU50}). mRNA alone is shown in monochrome images. Pair-rule and *wg* transcripts accumulate apically in wild-type embryos but are detected readily in the basal cytoplasm of mutant embryos (white arrowheads), although some apical enrichment of pair-rule transcripts is observed in most stripes (red arrowheads). The bottom panels show Run protein, which is normally apical (red arrow, left) and nuclear, but can also be detected in the basal cytoplasm in *egl* mutant embryos (red arrow, right). (B) The frequency of segmentation phenotypes in first instar larvae caused by inactivation of one copy of a pair-rule gene is significantly enhanced when embryos are laid by an *egl* mutant mother. For wild-type and mutant maternal genotypes, *eve* and *h* mutations are associated with partial deletions of even-numbered segments, whereas *ftz* mutations had similar effects on odd-numbered segments. We observed similar interactions with an additional *h* mutant allele, h^{31} (data not shown). The low frequency of larvae with defects in zygotically wild-type embryos typically had small notches in either even- or odd-numbered segments or partial fusion of segments. The frequency of cuticular defects caused by heterozygosity for the gap genes *kni* and *Kr* and the segment polarity gene *wg* are not altered significantly by the *egl* mutant background although the identity of segments affected by gap gene mutations differ slightly. For example, $Kr^{1}/+$ normally gives defects in T3, A1 and A2 but in *egl* mutants defects in A3 at the expense of defects in A1 and A2 were also observed. These alterations may reflect changes in the distribution of maternal RNA determinants or reduced pair-rule activity. Numbers above the bars are total number of larvae scored. $^{*}P<0.05$; $^{***}P<0.001$ (Fisher's exact test). *egl* is provided only maternally to the blastoderm. Experiments were conducted at 25°C, except for those involving *ftz*, which were carried out at 29°C, because only a few defects were seen in either genotype at 25°C. (C) Representative examples of first instar larvae with one inactive copy of *h* (h^{i22}) laid by wild-type or *egl* mutant mothers. Red asterisks show missing regions of ventral denticle belts in abdominal segments 4 and 6 (A4 and A6). Scale bar in C: 50 μm for A (except Run protein, 30 μm); 385 μm for C.

or transcripts lacking 20 nucleotides within the 3′UTR that are essential for RNA transport [the *hΔD* mutation (Bullock et al., 2003)] were misexpressed in the *eve* stripe 2 domain, and assayed for their ability to repress stripe 2 of the *h* target gene *ftz*, which leads to deletions in the larval mesothorax (segment T2). As the effects in this assay are dose dependent (Wu et al., 2001), it is well suited to probe for subtle differences in activities. As expected, lines bearing transgenes that encode the wild-type 3′-UTR produce apically localised transcripts ($st2\text{-}h^{wt}$ lines; Fig. 6B), whereas those expressing the mutant 3′UTR give rise to transcripts distributed uniformly in the cytoplasm ($st2\text{-}h^{nloc}$ lines; Fig. 6C). We did not observe significant differences in the width of the stripe of localising or non-localising ectopic *h* transcripts using a probe that distinguishes *st2-h* from endogenous *h* (not shown).

Lines bearing localising or non-localising transcripts of *st2-h* can lead to defects in T2 (Fig. 6D), demonstrating that non-localising transcripts encode functional protein. To distinguish effects of expression level and mRNA localisation, we used real-time RT-PCR to quantitate levels of *st2-h* mRNA relative to those of endogenous *actin* mRNA levels in each transgenic line (Fig. 6E). Comparing lines expressing similar levels of *st2-h* transcript indicates that T2 defects are more severe and penetrant when transcripts localise apically (Fig. 6D). Consistent with this, localised *st2-h* RNA is more efficient at repressing *ftz* transcription than unlocalised transcripts (Fig. 6I,J; see Table S2 at http://dev.biologists.org/supplemental). Only in very strongly expressing lines ($st2\text{-}h^{wtD}$ and $st2\text{-}h^{nlocD}$) do the ectopic transcripts cause equivalent phenotypes (disruption of T2 in over 90% of embryos), indicating that high expression levels can overcome the requirement for apical mRNA localisation. We estimate that in moderately expressing lines, localising *st2-h* transcripts have similar effects to two- to threefold more non-localising transcripts (Fig. 6D,E). Consistent with our analysis

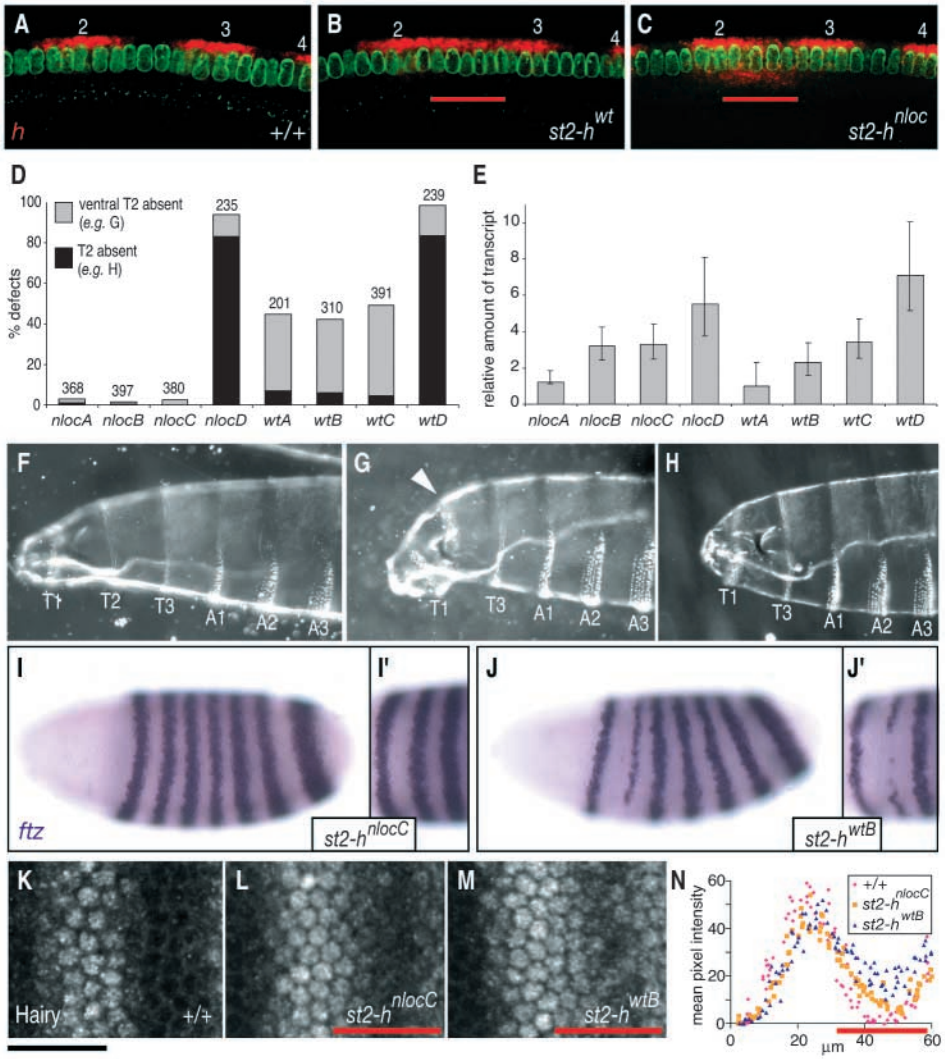


Fig. 6. Abolishing localisation of *h* transcripts reduces Hairy protein activity. (A-C) *h* transcripts (red) in wild-type (A) or transgenic (B,C) blastoderm embryos carrying one copy of a *h* transgene either with (B, *st2-h*^{wtD}) or without (C, *st2-h*^{nlocD}) a functional localisation signal under the control of the *eve* stripe 2 enhancer. Numbers of endogenous *h* stripes are indicated. Red bar indicates approximate region of enhancer activity. (D) Incidence of defects in thoracic segment 2 (T2) induced by *st2-h* activity in different transgenic lines. Numbers above bars indicate number of first instar larvae scored. (E) Levels of *st2-h* transcripts (relative to *actin 5C* transcripts) in different lines, as determined by real-time PCR, normalised to that of *wtA*. Provided RNA levels are not very high (*nlocD* and *wtD*), lines with localising *st2-h* transcripts have stronger phenotypic effects than those with non-localising transcripts, even when the localising transcript is expressed at significantly lower levels [e.g. $P<0.01$ for different transcript levels for *wtA* versus *nlocB* or *nlocC* and $P=0.05$ for *wtB* versus *nlocC* (*t*-test)]. (F-H) Lateral views of *st2-h* first instar larvae showing examples of (F) a normal T2 denticle belt, (G) a deletion of a ventral region of the T2 denticle belt leading to a characteristic curvature of the thorax (arrowhead points to remaining dorsal cuticle) or (H) a complete deletion of the T2 denticle belt. *st2-h* has been previously shown to have stronger effects ventrally (Wu et al., 2001). (I) Lateral (dorsal upwards) and (I′) ventral views of *st2-h*^{nlocC}/*st2-h*^{nlocC} blastoderm embryos stained for *ftz* mRNA. In ventral views, stripes 1, 2 and 3 are shown. Anterior is towards the left. (J,J′) Same views as in I,I′ of *st2-h*^{wtB}/*st2-h*^{wtB} embryos. Ectopic *h* expression partially deletes *ftz* stripe 2 with a stronger effect ventrally. See Table S2 for quantification of *ftz* defects in different lines. (K-M) Confocal images of Hairy protein distribution in wild-type (K, +/+), (L) *st2-h*^{nlocC}/*st2-h*^{nlocC} and (M) *st2-h*^{wtB}/*st2-h*^{wtB} blastoderm embryos. More Hairy protein accumulates in the nuclei between endogenous stripes 2 and 3 in *st2-h*^{wtB}/*st2-h*^{wtB} than it does in *st2-h*^{nlocC}/*st2-h*^{nlocC}, even though the former expresses significantly less *st2-h* mRNA. Consistent results were seen in several embryos for each genotype. Red bars in L-N show approximate regions of enhancer activity. (N) Mean fluorescent intensity/pixel within nuclei of corresponding images (data were collected with Kinetic Imaging AQM6 software). Scale bar: 50 µm in A-C; 180 µm in F-H; 130 µm in I-J′, 20 µm in K-M.

of *egl* mutant embryos, these data indicate that apical mRNA localisation augments pair-rule activity.

To investigate how localising mRNA enhances *h* activity, we compared the level of Hairy protein in the *eve* stripe 2 domain of transgenic lines encoding localising and non-localising *st2-h* transcripts. We found that levels of the protein in nuclei of the *eve* stripe 2 domain are clearly lower in *st2-h*^{nlocC} than in *st2-h*^{wtB} (Fig. 6K-N), even though the former expresses significantly more transcript. Although the anti-Hairy antibody is not sufficiently sensitive to detect cytoplasmic Hairy protein above background, we observe a diffuse distribution of several other pair-rule proteins in the basal cytoplasm when apical pair-rule RNA localisation is compromised in *egl* mutants (see above). In addition, when an excess of in vitro synthesised wild-type *h* RNA is injected, Hairy protein is detected in the apical cytoplasm, whereas when transcripts are injected that contain the same inactivating deletion in the localisation signal carried by the *st2-h*^{nloc} lines Hairy protein is detected uniformly throughout the cytoplasm (not shown). Together, these data argue that apical *h* RNA localisation targets protein apically, in close proximity to the nuclei.

Discussion

Localisation of mRNAs adjacent to the nucleus augments the nuclear concentration of pair-rule protein and improves the reliability of the segmentation process

Apical localisation of pair-rule mRNAs in *Drosophila* syncytial blastoderm embryos was first noted 20 years ago, but

the developmental and evolutionary significance of this process has remained unclear. We show that apical pair-rule mRNA localisation is conserved in cyclorrhaphan species that diverged over 145 million years ago, indicating that this process has a significant developmental role under natural conditions. Likewise, the widespread maintenance of *wg* transcript localisation in Diptera supports the importance of this process on a phylogenetic scale, even though, in *Drosophila*, *wg* appears to be less sensitive than pair-rule genes to a reduction in endogenous transcript localisation (Fig. 5B).

Unlike *wg* transcripts, pair-rule mRNAs do not localise in some branches of lower Diptera, and the phylogenetic occurrence of this process provides interesting insights into its functional significance. Enrichment of pair-rule transcripts in the apical cytoplasm correlates with the position of blastoderm nuclei: efficient apical localisation of pair-rule gene transcripts is found in species which retain an asymmetric apical position of nuclei throughout the blastoderm stage (*Drosophila*, *Megaselia*); less efficient localisation is seen when the nuclei move from an apical to a more central position during blastoderm stages (*Episyrphus*); and no apical enrichment of transcripts is seen in species where blastoderm nuclei are surrounded uniformly by a thin layer of cytoplasm (*Coboldia*, *Clogmia*). We also find localisation signals in several pair-rule transcripts of the lower dipteran *Anopheles*. Like Cyclorrhapha, but unlike many other lower Diptera and most other insects, this culicid species has evolved a thickened blastoderm with apically positioned nuclei, probably to allow rapid development as an adaptation to ephemeral larval habitats (Anderson, 1972; Ferrar, 1987): columnar cells that emerge from thickened blastoderms can enter gastrulation directly, whereas cuboidal cells that emerge from thin blastoderms still have to elongate prior to undergoing the requisite cell shape changes.

In *Drosophila*, we find that pair-rule proteins are enriched in the apical cytoplasm prior to import into the nuclei in wild-type blastoderms, whereas they are detected basally in *egl* mutant embryos, in which transcript localisation is inefficient. The apical accumulation of pair-rule proteins under normal circumstances is consistent with the observation that apical RNA targeting restricts diffusion of cytoplasmic β-galactosidase (Davis and Ish-Horowicz, 1991). Apically targeted protein is most likely confined by the cellularisation process, in which the plasma membrane invaginates between the nuclei and encloses the apical compartment first (Fig. 7).

Davis and Ish-Horowicz (Davis and Ish-Horowicz, 1991) speculated that mRNA localisation prevents pair-rule proteins from moving into inter-stripe regions, where they would cause dominant patterning defects. However, when pair-rule mRNA localisation is compromised, either by interfering with the localisation machinery or the RNA signals, we do not observe expansion of RNA or protein stripes or ectopic phenotypic effects. Rather, we see a reduction of pair-rule activity in their domains of expression in these experiments, indicating that transcript localisation augments gene function. Pair-rule mRNA localisation does not appear to be obligatory for protein activity in *Drosophila* but makes the segmentation process more reliable: *egl* mutants, in which transcripts localise very inefficiently, have a mild increase in segmentation defects and are acutely sensitive to the reduction of pair-rule gene dose.

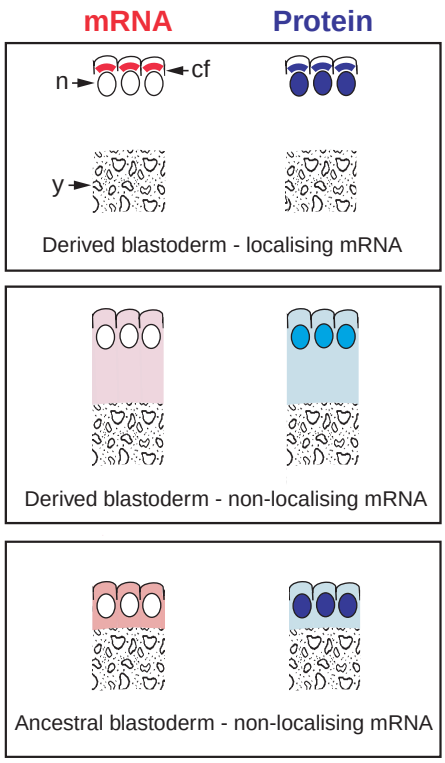


Fig. 7. A model for the role of apical pair-rule mRNA localisation in Diptera. Cartoon illustrating mRNA and protein distributions in syncytial blastoderm embryos. Darker shading of nuclei represents a higher concentration of pair-rule protein. See Discussion for details. cf, cellularisation front; n, nucleus; y, yolk.

By what mechanism does pair-rule mRNA localisation augment the activity of their transcription factor products? We demonstrate for *h* that suppression of transcript localisation reduces nuclear levels of its protein. Pair-rule proteins could be specifically modified in the apical cytoplasm, or localising transcripts could be translated more efficiently. However, given the diffuse distribution of pair-rule proteins in the basal cytoplasm when RNA localisation is disrupted in *egl* mutants and the correlation between cytoarchitecture and pair-rule transcript localisation in Diptera, we favour a third possibility, namely that apical mRNA localisation increases nuclear uptake of their proteins by targeting translation in close proximity to the nuclei (Fig. 7). Proteins from non-localising mRNAs would not be available at high levels in the immediate vicinity of the nuclei, which would result in a decreased nuclear uptake. Such a role for apical pair-rule mRNA localisation would be redundant in lower Diptera with only a thin layer of cytoplasm surrounding the nuclei, which provides little room for diffusion of pair-rule proteins prior to nuclear import. A mechanism for perinuclear protein targeting might be particularly significant for nuclear proteins with short half-lives, such as those encoded by pair-rule genes (Edgar et al., 1987). Interestingly, localisation of mRNA in the vicinity of the nucleus to aid import of nuclear proteins has also been reported in cultured mammalian cells (Levadoux et al., 1999) and may be a widespread mechanism to efficiently exploit a limited pool of transcripts in cells that are polarised or have a high cytoplasmic:nuclear ratio.

The relationship between cytoarchitecture and apical pair-rule transcript localisation does not appear to be absolute because we detected a signal in *eve*, but not *h*, from *Haematopota*, which has retained the ancestral, cuboidal blastoderm morphology (U.S.-O., unpublished) and because we did not detect a localisation signal in *Anopheles-eve*. Although we cannot yet discern the developmental context in which these signals are used (in situ hybridisation is currently not possible in these species because of egg shells that are difficult to remove and because of difficulties in obtaining embryos) these data raise the possibility that, within a single species, the differential ability of transcripts to be recognised by the localisation machinery is used to fine-tune transcriptional control of target genes in the blastoderm by modulating the nuclear concentration of pair-rule proteins.

The efficiency of transcript localisation is modified gradually in evolution

The ability of *eve* and *h* pair-rule transcripts to use the localisation machinery varies in Diptera. We observe a range of localisation efficiencies in situ that are mirrored in all 11 cases upon injection into *Drosophila* embryos. Thus, differences in localisation efficiency appear to reflect changes in the respective localisation signals, rather than alterations in the specificity of the protein machinery. These findings are consistent with previous studies with artificial variants of the *Drosophila-h* localisation signal, which suggest that the character of localisation signals modulates the efficiency of localisation by determining the kinetics of both the initiation of transport and the transport process itself (Bullock et al., 2003). Localisation efficiency appears to be determined by multiple RNA:protein interactions, the sum of which affects the stability and/or activity of the RNA:motor complex (Macdonald and Kerr, 1998; Chartrand et al., 2002; Bullock et al., 2003). Therefore, the efficiency of the localisation process can be modified gradually during evolution by the addition, loss or modification of individual recognition sites within mRNAs.

It seems that localisation signals in pair-rule genes have emerged multiple times within Diptera. For example, although we cannot rule out the possibility that localisation signals in *h* have been lost in multiple different lineages of lower Diptera, the most parsimonious explanation for the phylogenetic distribution of signals in this transcript is that they evolved independently in response to changes in cytoarchitecture in the lineages leading to Cyclorrhapha and Culicomorpha. Injection of transcripts from additional species into *Drosophila* will determine whether *eve* localisation signals emerged independently in the lineages leading to *Haematopota* and Cyclorrhapha, or were lost in the lineage leading to *Empis*.

Work in mammalian cells has provided insights into how localisation signals might initially appear (Fusco et al., 2003). These studies suggest that non-localising mRNAs can also interact with a motor complex, albeit with a comparatively small probability, and undergo short movements on microtubules. Localisation signals appear to augment these interactions and lead to the net translocation of an RNA population along a polarised cytoskeleton by increasing the frequency and duration of directed transport (Fusco et al., 2003). The localisation machinery in Diptera may also have a general, weak affinity for mRNAs because a small proportion of particles of injected non-localising transcripts are transported over short distances in *Drosophila* embryos (M. Wainwright and S.B., unpublished). Asymmetric accumulation of a population of transcripts may therefore evolve gradually as a result of selection for increased interaction between a specific transcript and the localisation machinery.

Concluding remarks

Using a combination of functional and phylogenetic analyses, we have provided evidence that the alteration of mRNA localisation signals is an important mechanism by which the activity of pair-rule transcription factors is regulated in flies. Apical localisation of these transcripts appears to augment the nuclear concentration of their protein products and makes the segmentation process less sensitive to perturbation of gene activity. It seems that different species have made use of the localisation machinery to adapt the deployment of specific pools of transcripts to evolutionary changes in blastoderm cytoarchitecture. Thus, the mRNA localisation mechanism may permit networks of patterning genes to tolerate changes in cell morphology, such as those imposed by reproductive adaptations.

In *Drosophila*, transport of mRNAs by the Egl/BicD/dynein machinery determines the distributions of several different kinds of proteins in diverse cell types such as oocytes, epithelial cells and neuroblasts (Bullock and Ish-Horowicz, 2001) (J.H., unpublished). Our studies of pair-rule mRNAs imply that the repertoire of other RNA cargoes for the machinery, and their efficiency of transport, may also be modulated readily in evolution through changes in localisation signals. Therefore, differential mRNA localisation is potentially an important factor in facilitating morphological evolution.

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