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Developmental RNA processing of 3′UTRs in Hox mRNAs as a context-dependent mechanism modulating visibility to microRNAs

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SUMMARY

The *Drosophila* Hox gene *Ultrabithorax* (*Ubx*) controls the development of thoracic and abdominal segments, allocating segment-specific features to different cell lineages. Recent studies have shown that *Ubx* expression is post-transcriptionally regulated by two microRNAs (miRNAs), miR-iab4 and miR-iab8, acting on target sites located in the 3′ untranslated regions (UTRs) of *Ubx* mRNAs. Here, we show that during embryonic development *Ubx* produces mRNAs with variable 3′UTRs in different regions of the embryo. Analysis of the resulting remodelled 3′UTRs shows that each species harbours different sets of miRNA target sites, converting each class of *Ubx* mRNA into a considerably different substrate for miRNA regulation. Furthermore, we show that the distinct developmental distributions of *Ubx* 3′UTRs are established by a mechanism that is independent of miRNA regulation and therefore are not the consequence of miR-iab4/8-mediated RNA degradation acting on those sensitive mRNA species; instead, we propose that this is a hard-wired 3′UTR processing system that is able to regulate target mRNA visibility to miRNAs according to developmental context. We show that reporter constructs that include *Ubx* short and long 3′UTR sequences display differential expression within the embryonic central nervous system, and also demonstrate that mRNAs of three other Hox genes suffer similar and synchronous developmental 3′UTR processing events during embryogenesis. Our work thus reveals that developmental RNA processing of 3′UTR sequences is a general molecular strategy used by a key family of developmental regulators so that their transcripts can display different levels of visibility to miRNA regulation according to developmental cues.

KEY WORDS: 3′UTR, *Drosophila*, Hox, Ultrabithorax, miRNA

INTRODUCTION

The transformation of the fertilised egg into a complex organism largely relies on the establishment of distinct programmes of gene activity across the different regions of the developing organism. Therefore, the ultimate understanding of how development is controlled at the molecular level demands an elucidation of the full spectrum of mechanisms able to transform genomic information into local programmes of gene action. The control of gene-specific mRNA levels in time and space seems to lie at the heart of this problem; such control relies on both transcriptional and post-transcriptional mechanisms (Alonso, 2008; Alonso and Wilkins, 2005; Davidson, 2006).

Sequences located in mRNA 3′ untranslated regions (3′UTRs) contain information that determines patterns of mRNA turnover, transport, subcellular localisation and messenger translation (Moore, 2005). At the mechanistic level, such diverse mRNA outputs are thought to be dictated in trans by RNA-binding proteins and small RNAs, such as microRNAs (miRNAs), which are able to bind specific cis-regulatory elements located in transcript 3′UTRs (Bartel, 2004; Bartel and Chen, 2004). Current mechanistic models for miRNA function indicate that miRNAs can regulate gene targets by different repressive mechanisms, including the destabilisation of target mRNAs and the inhibition of protein translation (Eulalio et al., 2008). Little is known about how the information in 3′UTRs is transformed into distinct patterns of mRNA behaviour; in spite of this, gene- and developmental-specific alterations of 3′UTR sequences are predicted to be of great significance for gene regulation, as they might provide variability in the control regions seen by RNA regulators.

Bioinformatic work in the past few years has shown that a high proportion of vertebrate mRNA transcripts undergo alternative polyadenylation processes leading to transcripts with different 3′UTR sequences (Tian et al., 2005). Furthermore, recent experiments in cultured mammalian cells have expanded our understanding of the significance of 3′UTR processing processes by showing that actively proliferating cells express mRNAs with shorter 3′UTRs than those produced in stationary conditions (Sandberg et al., 2008). However, the significance of 3′UTR processing during the establishment of gene regulatory events controlling embryonic development remains largely unexplored.

Hox genes encode homeodomain-containing transcriptional regulators that operate differential genetic programmes along the anteroposterior axis of animal bodies (Alonso, 2002; Pearson et al., 2005). The *Drosophila* Hox gene *Ultrabithorax* (*Ubx*) controls the development of posterior thoracic and anterior abdominal segments, determining the segment-specific characteristics of many different cell lineages, including the epidermis, mesoderm and central nervous system (CNS) (Morata and Kerridge, 1981). The expression of *Ubx* mRNAs is very dynamic during embryogenesis. Three major phases of expression can be detected. First, an early and rather weak phase of *Ubx* mRNA expression, which resolves into a single stripe (possibly) within the anlage of parasegment 6 (PS6) (Akam et al., 1985). Second, a phase beginning at the onset of gastrulation, which demarks ectodermal expression in (1) PS6 (high expression), (2) the anterior compartments from PS7-12 giving the appearance of a

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banded pattern (see Fig. 1B), and (3) PS5 and PS13 (reduced signal) (Fig. 1B); during this phase signal is also present in the mesoderm across PS6-12. Finally, a third phase, which involves *Ubx* expression in the visceral and somatic mesoderm, ectoderm and, primarily, the CNS (Fig. 1C). Expression during this latter phase extends from PS5-12, always with uniquely high levels in PS6 (Akam et al., 1985). Given that protein expression has never been detected during the first phase, in this study we focus on the second and third phases outlined above.

The molecular control of the dynamic expression of *Ubx* relies on complex transcriptional regulation (Casares et al., 1997; Hogness et al., 1985; Peifer et al., 1987), as well as on a post-transcriptional system involving two miRNAs: miR-iab4 and miR-iab8 (also called miR-iab4AS) (Bender, 2008; Ronshaugen et al., 2005; Stark et al., 2008; Tyler et al., 2008). The ways by which these two levels of regulation are integrated to control *Ubx* expression during development are at present poorly understood. miR-iab4/8 miRNAs are produced from precursors transcribed from opposite DNA strands at the *iab4* locus within the Bithorax complex (BX-C) (Bender, 2008; Ronshaugen et al., 2005; Stark et al., 2008; Tyler et al., 2008). *Ubx* regulation by miR-iab4/8 is mediated by specific sequences located in the *Ubx* 3′UTR (Ronshaugen et al., 2005; Stark et al., 2008; Tyler et al., 2008) (Fig. 1A), and, for miR-iab4, the embryonic expression patterns of *Ubx* (mRNA and protein) seem largely, although not exactly, complementary to those of iab4 miRNAs (Ronshaugen et al., 2005), suggesting a regulatory role. However, it is difficult to reconcile the potential miRNA regulation of *Ubx* at these stages with the fact that the absence of miR-iab4/8 leads to no obvious Hox-like patterning defects (Bender, 2008) (see below).

Here, we investigate the mechanisms by which *iab4*-derived miRNAs regulate *Ubx* gene outputs. Remarkably, we report that during embryonic development *Ubx* produces mRNAs with distinct 3′UTRs that harbour different sets of miRNA targets in different tissues. Furthermore, we demonstrate that the differential distribution of *Ubx* mRNAs bearing specific 3′UTR sequences is established independently of miRNA regulation, indicating that it is not the result of miRNA-mediated transcript degradation but instead the consequence of an ‘in-built’ RNA processing system that remodels *Ubx* 3′UTRs according to developmental context. Notably, we also show that other Hox genes display similar developmental changes affecting their 3′UTR sequences, indicating that developmental 3′UTR processing is a general phenomenon that affects a key family of developmental regulators.

MATERIALS AND METHODS

Fly strains and genetics

Flies were cultured following standard procedures at 25°C in the dark. Fly strains used in this study include Oregon Red, ‘ΔmiRNA’/TM3, *ftz-lacZ* (a gift from Welcome Bender, Harvard Medical School, Boston, USA) and *string*[AR2]/TM3, *hb-lacZ* (a gift from Jean-Paul Vincent, NIMR-MRC, London, UK). To express reporter genes in the embryonic nervous system, virgins of genotype *yw; UAS-mCherry.NLS.Ubx.3′UTR.short/CyO* and *yw; UAS-mCherry.NLS.Ubx.3′UTR.long.delta.PAS1/CyO* were crossed to males from the *Gal4* driver line *elav-Gal4/CyO* (a gift from Rob Ray, University of Sussex, Brighton, UK).

Embryo collection, RNA isolation and RT-PCR

Embryos were collected using standard procedures. For in situ hybridisations and antibody stainings, embryos were fixed following standard procedures. For RT-PCR, total RNA was extracted from staged embryo collections using Trizol reagent (Invitrogen), followed by RNA purification using the RNeasy Plus Kit (Qiagen). Total RNA (2-3 μg) was used for cDNA synthesis using random primers and Superscript II or Thermoscript First-Strand Synthesis Systems (Invitrogen). Primer sets were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm; see Tables S1-S3 in the supplementary material). Quantitative (q) PCR reactions were carried out using SYBR Green I Master Mix chemistry (Roche) on a LightCycler 480 (Roche) platform. Expression values were normalised using reference gene *RpL21*. Similar results were obtained using an alternative reference *RpL32* (*Rp49*). At least three technical replicates were performed on two independent biological samples.

RNA in situ hybridisation

RNA probes for RNA in situ hybridisation experiments were designed to target universal and distal 3′UTR sequences or the full-length open reading frame for *mCherry*; the former were designed to be of comparable length (see Table S1 in the supplementary material). RNA probes were labelled using a digoxigenin (DIG) RNA Labelling Kit (SP6/T7) (Roche) according to the manufacturer’s instructions. Probes were used in RNA in situ hybridisations according to standard protocols; RNA probes were detected using anti-DIG-AP (Roche; 1:2000) and a chromogenic reaction using NBT/BCIP substrate (Roche). Enzymatic detection reactions with NBT/BCIP (Roche) were carried out in parallel and stopped at exactly the same time for probes targeting universal and distal 3′UTR sequences to ensure comparability of results. Homozygous embryos in samples from stocks carrying *lacZ* balancer chromosomes were detected with rabbit anti-β-galactosidase (Promega; 1:100) and Rhodamine-conjugated anti-rabbit (Jackson; 1:100) antibodies after RNA probe removal and prior to enzymatic RNA detection. Fluorescent detection of RNA probes was performed using anti-DIG-POD (Roche; 1:100) followed by Cy3 tyramide (Perkin Elmer; 1:50) signal amplification. Subsequent imaging was performed on a Zeiss Axiophot confocal microscope; fluorescent signals in the CNS were quantified using ImageJ (Plot Profile function).

Antibody staining and western blot

Antibody stains were performed following standard procedures. Primary antibodies were monoclonal mouse anti-Ubx (FP3.38, a gift from Robert White, University of Cambridge, Cambridge, UK; 1:20) and anti-Engrailed 4D9 (Developmental Studies Hybridoma Bank; 1:20). Ubx protein signal was developed with biotinylated anti-mouse antibody (Jackson; 1:300) and streptavidin-alkaline phosphatase (Roche; 1:5000), followed by chromogenic detection using NBT/BCIP substrate (Roche). Engrailed protein signal was developed with streptavidin-HRP followed by signal amplification using Cy3-coupled tyramides following the manufacturer’s instructions (Perkin Elmer). Co-detection of β-galactosidase signal was performed as described for RNA in situ hybridisations. Western blots for Ubx and β-tubulin were carried out using monoclonal antibodies anti-Ubx (1:100) and anti-tubulin E7 (Developmental Studies Hybridoma Bank; 1:500), followed by HRP anti-mouse (Jackson; 1:5000) and ECL detection (GE Healthcare).

Ubx 3′UTR reporter constructs

Short and extended *Ubx* 3′UTRs were fused to an mCherry reporter, transformed into flies using site-specific integration and expressed in the CNS using the Gal4/UAS system. In brief, vector pBSIIKS_mCherry-3×NLS (gift from Markus Affolter) (Caussinus et al., 2008) was digested with *Kpn*I and *Not*I and a 837 bp fragment containing the mCherry ORF plus nuclear localisation signal (NLS) was cloned into the respective restriction sites of transformation vector pUASP.K10.attB (a gift from Beat Suter) (Koch et al., 2009). The K10 terminator sequence of resulting vector pUASP.mCherrry.3×NLS.K10.attB was then removed by *Not*I and *Nde*I double digestion and replaced with *Ubx* short (–7 to +1237 bp of annotated 3′UTR) or *Ubx* long (–7 to +2807 bp) 3′UTRs, which had been PCR amplified from genomic DNA. The first polyadenylation signal [AATAAA at +950 bp of 3′UTR (Kornfeld et al., 1989; O′Connor et al., 1988)] of the construct with the extended *Ubx* 3′UTR was deleted using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). To obtain transgenic flies, we used the ΦC31 system for site-specific integration using the ZH-attP-51C landing site (Bischof et al., 2007) (http://flyc31.frontiers-in-genetics.org/). Transformation of flies was carried out by BestGene (http://www.thebestgene.com).

Bioinformatics and data analysis

Information on alternative polyadenylation of Hox genes was compiled from FlyBase (http://flybase.org/) and the following references (Akam and Martinez-Arias, 1985; Celniker et al., 1989; Celniker et al., 1990; Kornfeld et al., 1989; Kuziora and McGinnis, 1988; Laughon et al., 1986; O'Connor et al., 1988; Rowe and Akam, 1988; Sanchez-Herrero and Crosby, 1988; Schneuwly et al., 1986; Scott et al., 1983; Stroeher et al., 1986; Tyler et al., 2008). To detect miRNA target sites, we followed methods used in previous studies (Stark et al., 2008). In brief, we screened our set of Hox 3′UTR sequences for matches with seed sequences for miR-iab4/8-5p and their -3p counterparts, as available from miRBase (http://www.mirbase.org/). We then used TargetScan (http://www.targetscan.org/fly) and the UCSC Genome Browser (http://genome.ucsc.edu/) to partition the original set of sequences into two sub-classes: (1) those showing deep evolutionary conservation, defined as being present in at least ten out of the twelve *Drosophila* species; and (2) those showing some degree of evolutionary conservation, defined as those present in more than four *Drosophila* species. Notably, the results of this analysis included the sets of miRNA target sites described previously (Ronshaugen et al., 2005; Stark et al., 2008; Tyler et al., 2008) and, in addition: (1) new sites present in longer 3′UTR forms not taken into account in prior work; and (2) sites predicted to be targeted by the star variants of the miR-iab4/8 miRNAs.

RESULTS

Ubx produces transcripts with different sets of miRNA targets in different parts of the embryo

To explore the mechanisms by which *iab4*-derived miRNAs control *Ubx* expression, we developed a series of RNA in situ hybridisation and antibody staining experiments aimed at detecting the expression of *Ubx* mRNAs and proteins during embryogenesis. During this process, we noted the existence of two *Ubx* mRNA species bearing different 3′UTR sequences and displaying clearly distinct temporal and spatial patterns during *Drosophila* embryogenesis (see below). One such *Ubx* mRNA form possesses a short 3′UTR, whereas the other bears an extended 3′UTR sequence; we termed these species *Ubx* short 3′UTR and *Ubx* long 3′UTR mRNAs, respectively (Fig. 1A).

Based on current annotated *Ubx* transcripts in FlyBase, our observations were unexpected; nonetheless, they are perfectly consistent with the original molecular work describing the cloning and expression of *Ubx* in *Drosophila*, which reported northern blot and other molecular and sequence data indicating the generation of *Ubx* mRNAs of variable 3′UTR length by alternative polyadenylation (Akam and Martinez-Arias, 1985; Kornfeld et al., 1989; O'Connor et al., 1988) (Fig. 1A). We suspect that the fact that current genomic databases fail to mention the distinct *Ubx* 3′UTRs might be the reason why previous studies investigating *Ubx* miRNA regulation did not consider this important feature of *Ubx* transcripts.

Aware of the potential significance of *Ubx* 3′UTR processing for *Ubx* regulation by miRNAs, we investigated in detail the spatial and temporal utilisation of the long and short *Ubx* transcripts during *Drosophila* embryogenesis. We generated two RNA probes that were able to specifically detect proximal and distal 3′UTR sequences in *Ubx* transcripts: the *Ubx* 3′UTR universal probe (*Ubx*-universal) and *Ubx* 3′UTR distal probe (*Ubx*-distal), respectively (Fig. 1A). The *Ubx*-universal probe is predicted to detect all *Ubx* transcripts, whereas the *Ubx*-distal probe only detects long *Ubx* 3′UTRs (Fig. 1A). Remarkably, when tested in *Drosophila* embryos the distal and universal 3′UTR probes detected signals in different tissues at different developmental times, strongly indicating that *Ubx* 3′UTRs are remodelled during embryogenesis (Fig. 1B-E,L,M). The *Ubx*-universal probe detected strong signals during *Ubx* ectodermal expression by mid-late germ band extension (stage 10, Fig. 1B), as well as in later *Ubx* expression in the CNS (stage 15, Fig. 1C). *Ubx*-distal probes revealed similar signal levels to those detected by *Ubx*-universal probes in late stages (stage 15, Fig. 1C,E), but, notably, detected no significant signal during early *Ubx* expression (stage 10, Fig. 1D).

To confirm the discrepancies in the levels of use of *Ubx* short and long 3′UTRs during embryogenesis by an independent method, we developed a quantitative real-time PCR assay (Fig. 1N), which confirmed that long *Ubx* transcripts (distal amplicon, Fig. 1A) display differential expression during embryogenesis, with higher levels reached only in later stages. In the CNS, double in situ experiments indicated that there is no obvious segregation of the spatial domains expressing long and short *Ubx* transcripts (Fig. 1F-K); however, from these experiments, we cannot rule out the possibility that short 3′UTR forms could be simultaneously expressed in cells expressing long forms. To determine whether *Ubx* 3′UTR processing is a tissue-specific feature or is determined by general developmental timing cues, we looked at *Ubx* transcripts detected in early stage 14, when *Ubx* is expressed in several tissues including the CNS and the epidermis. Whereas universal probes detected signals in both CNS and epidermis, distal probes only showed signal in the CNS (Fig. 1L-M), supporting the notion that *Ubx* 3′UTR processing is tissue specific.

Altogether, these experiments have important implications for the understanding of the post-transcriptional control of *Ubx* mRNAs by miR-iab4 and miR-iab8 (see expression patterns of these miRNAs in Fig. 2), as most of the miRNA target elements predicted to mediate miR-iab-4/8-dependent regulation are absent from *Ubx* transcripts during germ band extension (Ronshaugen et al., 2005; Stark et al., 2008; Tyler et al., 2008) (Fig. 1A,B,D).

The relationship between *Ubx* 3′UTR processing and miRNAs

We envisage two main hypotheses to explain the differential distribution of *Ubx* transcripts. One is that miR-iab4/8 are actively involved in the degradation of *Ubx* transcripts bearing long 3′UTR sequences, and, given this downregulation, no significant signals are detected with distal probes at this stage. Another possibility is that during evolution, *Ubx* transcripts have acquired a molecular mechanism (involving 3′UTR processing) that is able to modify their visibility to miRNAs during development. If the first hypothesis were to be correct, elimination of miR-iab4/8 should lead to an increase in the levels of long *Ubx* transcripts. Alternatively, if miR-iab4/8 removal leads to no significant change in the levels of long 3′UTR forms, this would support the target evolution hypothesis. To test these predictions, we used a mutant in which the locus that transcribes the precursors for miR-iab4/8 miRNAs was mutated by gene conversion (Bender, 2008). Our analysis of stage 10 wild-type and mutant embryos using universal and distal *Ubx* 3′UTR probes is shown in Fig. 3A-D. Signals detected in miRNA mutant embryos were similar to those detected in wild-type embryos of identical age, indicating that the balance between short and long *Ubx* 3′UTR forms is determined independently of miR-iab4/8 regulation. Looking at Ubx protein levels in whole-mount embryos at this stage, we could not see any significant differences between wild-type and mutant embryos (Fig. 3E-F). Furthermore, a series of qPCR reactions and western blots independently confirmed the absence of upregulation of *Ubx* mRNAs and proteins levels in the miRNA mutants (see Figs S2 and S3 in the supplementary material). In addition, systematic

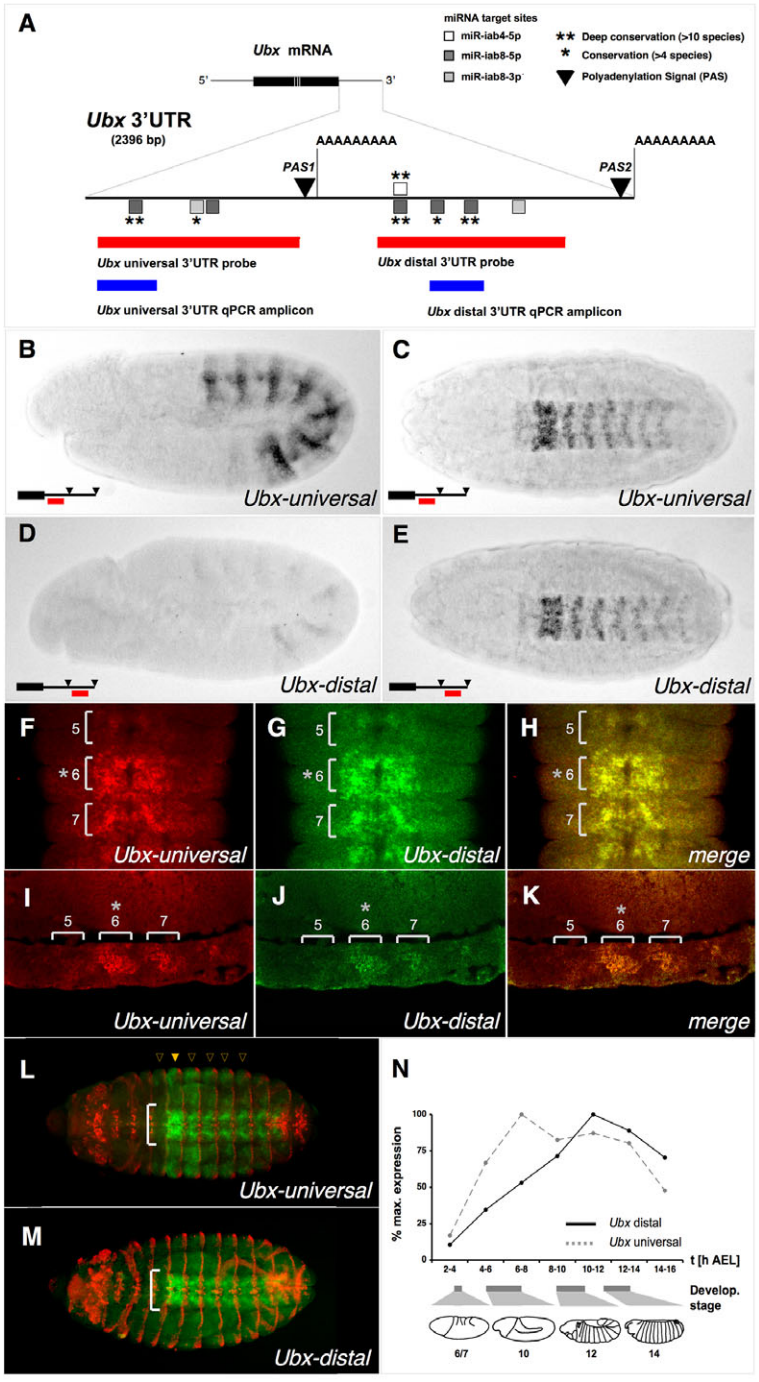

Fig. 1. The Hox gene *Ubx* produces mRNA transcripts with different 3′UTRs in different developmental contexts during *Drosophila* embryogenesis. (**A**) Structure of *Ubx* 3′UTRs. The presence of two polyadenylation signals (PAS, black triangles) within *Ubx* 3′UTR sequences determines the existence of two transcripts: the short and long 3′UTR *Ubx* mRNAs using PAS1 or PAS2, respectively. Mapping the positions of several target sites for *iab4/8*-derived miRNAs (miR-iab-4-5p, miR-iab-4-3p, miR-iab-8-5p, miR-iab-8-3p) on *Ubx* 3′UTR sequences demonstrates that *Ubx* short and long transcripts harbour very different sets of miRNA targets, many of which are evolutionarily conserved. Double asterisk indicates deep conservation, i.e. present in at least ten related species; single asterisk indicates conservation, i.e. present in four or more related species. Note that the long 3′UTR form contains the majority of deeply conserved miRNA target sites. RNA in situ probes (red rectangles) and qPCR amplicons (blue rectangles) were designed to detect both the short and long 3′UTR isoforms (universal) or just the long *Ubx* 3′UTR (distal). (**B**,**C**) *Ubx* mRNA expression using the universal probe on stage 10 and stage 15 embryos. (B) *Ubx* mRNAs detected by the universal probe are strongly expressed in mesoderm and ectodermal tissues in parasegments (PS) 6-12 (with some weaker signal in PS5). (C) The universal 3′UTR probe detects strong signals in CNS tissues along PS5-13, with highest levels in PS6. (**D**,**E**) Expression of long 3′UTR *Ubx* mRNA forms. Distal 3′UTR probes detect strong levels of *Ubx* transcripts in the CNS (E) with a pattern and intensity identical to those detected by the universal 3′UTR probes (C). Notably, distal probes show very weak signals during germ band extension (D), indicating the differential processing of *Ubx* mRNAs during embryogenesis: during germ band extension, most *Ubx* mRNAs do not include distal 3′UTR sequences, which harbour the majority of miRNA target sites. (**F-K**) Double in situ hybridisation experiments indicating that *Ubx* short and long 3′UTR forms are detected in approximately coincident expression domains within the developing CNS. Ventral (F-H) and lateral (I-K) views of embryo areas including PS5-7; note the characteristically high levels of signal in PS6 (asterisk). (**L**,**M**) *Ubx* 3′UTR isoforms show tissue-specific expression patterns. Wild-type stage 15 embryos stained for Engrailed protein (red) and simultaneously hybridised with the *Ubx* universal 3′UTR probe (L) or *Ubx* distal 3′UTR probe (M) (green). Note the comparable levels of signal detected by both *Ubx* probes in the CNS (right-facing brackets) and the presence of epidermal signal only with the universal *Ubx* 3′UTR probe (yellow triangles). (**N**) Quantification of *Ubx* transcripts by qPCR as a function of developmental time (hours after egg laying, h AEL) or developmental stage (schematic representation of selected stages). Expression values for long *Ubx* mRNAs (distal amplicon) and all *Ubx* mRNAs (universal amplicon) were obtained by normalisation of transcript concentrations to reference ribosomal transcripts; we show percentages of maximum expression during our time course for both amplicons. The experiment shows a clear increase in long 3′UTR sequences as embryogenesis proceeds.

inspection of posterior *Ubx* stripes in wild-type and miRNA mutant embryos showed, yet again, no significant expansion of *Ubx* stripes when miRNAs are removed (data not shown). However, at later stages, when *Ubx* is primarily expressed within the differentiating CNS, the situation is different. As reported previously (Bender, 2008), mutation of miR-iab4/8 leads to a visible increase in Ubx protein in PS8-13 (see Fig. S4A,B in the supplementary material). This increase could be the result of derailing miRNA-dependent *Ubx* mRNA destabilisation or protein translation. To discriminate between these two possibilities, we looked at the expression of long *Ubx* transcripts, a form highly expressed at this stage, and found a minor, yet reproducible, upregulation in *Ubx* transcript expression in posterior parasegments (see Fig. S4C,D in the supplementary material), which might account, at least to some degree, for the effects seen on protein levels.

The observations above: (1) imply the existence of a post-transcriptional system processing *Ubx* 3′UTR sequences, which produces transcripts with variable numbers of miRNA target sites at different temporal and spatial coordinates during development;

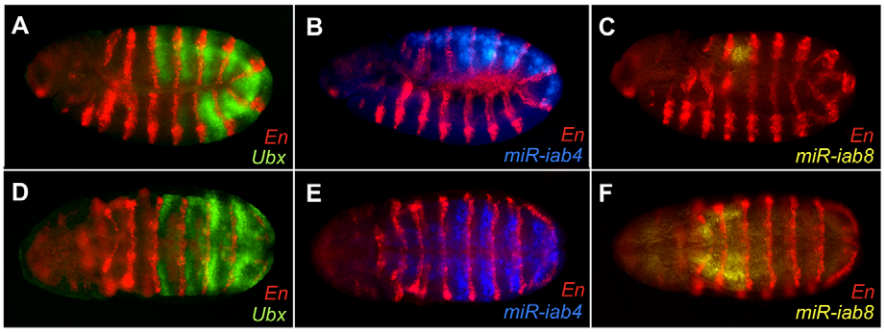

Fig. 2. *Ubx* and miR-iab4/8 expression patterns during germ band extension. (**A-F**) In situ hybridisations for *Ubx* detected with the universal 3′UTR probe (A,D; green) and precursors of miR-iab4 (B,E; blue) and miR-iab8 (C,F; yellow). Parasegmental expression of Engrailed protein (red) is also shown to provide a common spatial register. Embryos are shown in lateral (A-C) and dorsal (D-F) views. Expression patterns seem highly complementary, leading to the suggestion of a functional interaction between *Ubx* and the miRNAs. Nonetheless, genetic and gene expression analyses indicate that no major interaction takes place between miRNAs and *Ubx* mRNA molecules at this stage of development (see text for further details).

(2) reveal that such an RNA processing system is set up independently of miR-iab4/8 miRNAs; and (3) show that regulatory roles of miR-iab4/8 miRNAs are only detectable in late embryogenesis, during CNS development.

Furthermore, the fact that removal of the miR-iab4/8 system does not lead to an increase in *Ubx* mRNA or protein levels during germ band elongation would be consistent with short 3′UTR *Ubx* mRNAs produced at that time not bearing a sufficient set of miR-iab4/8 target sequences (due to 3′UTR processing) to mediate functional interactions. Alternatively, the absence of any change in expression could be the result of the expression domains of *Ubx* and miR-iab4/8 miRNAs not overlapping. Evidence [see figure 1F-H in Ronshaugen et al. (Ronshaugen et al., 2005)] (Bender, 2008; Stark et al., 2008; Tyler et al., 2008) showing that some individual nuclei are indeed able to co-express miR-iab4 and *Ubx* mRNAs, supports the interpretation that short *Ubx* mRNAs are a poor substrate for miRNA regulation. To support this reasoning further, if RNA processing affecting *Ubx* 3′UTR sequences were to lead to differential regulation by miR-iab4/8, this makes two simple predictions: (1) that after miRNA removal, a change in expression should be visible only in the tissues where long 3′UTR transcripts are expressed (within the CNS); and (2) that no change should occur in tissues where short *Ubx* 3′UTR species are expressed (e.g. in the early epidermal pattern). Our results meet these two predictions in full.

Regulatory role of *Ubx* 3′UTR sequences within the embryonic CNS

To explore the potential regulatory activities mediated by *Ubx* 3′UTR sequences we tested the effects of *Ubx* 3′UTR short and long sequences on the expression of a reporter construct encoding the fluorescent protein mCherry. One of the constructs generated included the full proximal 3′UTR sequences of *Ubx*; we called this construct *mCherry.short* (Fig. 4E). The other construct encompassed the entire long 3′UTR sequence present in *Ubx* long transcripts and we called this construct *mCherry.long* (Fig. 4E). To avoid the possibility that *Ubx* 3′UTR sequences included in the *mCherry.long* construct could be processed and transformed into short 3′UTR sequences within embryonic cells, we deleted the first polyadenylation signal from *mCherry.long* constructs (Fig. 4E). In addition, to ensure that any putative expression difference among the long and short reporters was due to the differential 3′UTR sequence composition in these transgenes and not to other factors, such as variation in the integration sites of the transgenes within the host chromosomes, we made use of the recently developed ΦC31 technology (Bischof et al., 2007), which exploits the site-specific recombination features of bacteriophage ΦC31 and allows the integration of a series of transgenes into identical chromosomal positions. Both the transgenes, *mCherry.short* and *mCherry.long*, were placed downstream of a UAS promoter (using the *pUASP.attB* vector) and integrated into an attP site located in chromosome 2, 51C (Fig. 4E).

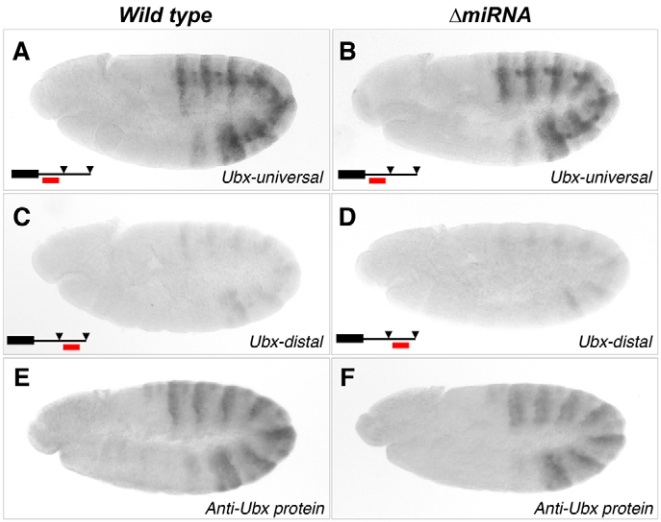

Fig. 3. Expression of *Ubx* in the absence of miR-iab4/8 miRNAs. (**A**,**B**) *Ubx* expression detected with the universal probe. Wild-type (A) and miRNA mutant (ΔmiRNA) embryos (B) during germ band extension show a similar pattern and level of expression. (**C**,**D**) Expression of *Ubx* long 3′UTR mRNAs. As in the case of the universal probe, no significant differences are detected in the expression levels of long 3′UTR transcripts between normal (C) and miRNA mutant (D) embryos. (**E**,**F**) Ubx protein levels are similar in wild-type and ΔmiRNA embryos at germ band extension. These experiments show that miRNAs derived from the *iab4* locus are not actively regulating the expression levels of *Ubx* transcripts (or protein) during germ band extension.

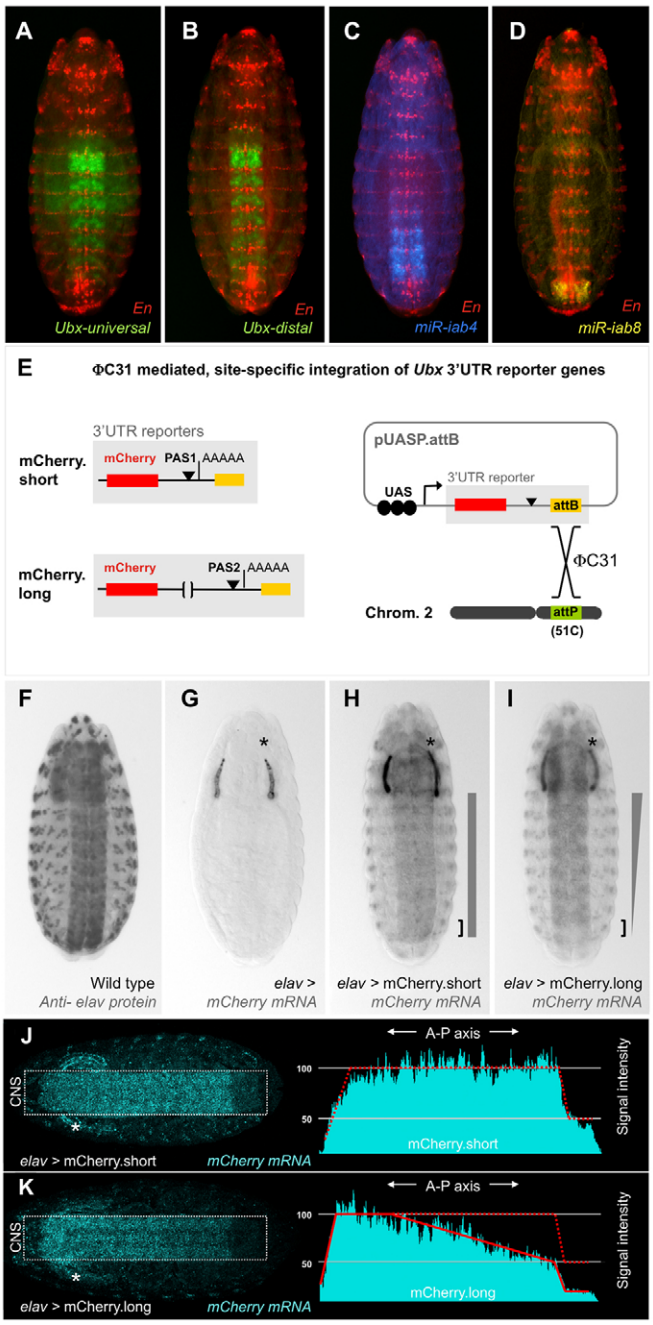

Fig. 4. *Ubx* 3′UTR reporter constructs reveal differential regulatory capacities of short and long isoforms in the CNS of *Drosophila* embryos. (**A-D**) In situ hybridisations for *Ubx* transcripts, detected with universal (A; green) or distal (B; green) 3′UTR probes, and precursors for miR-iab4 (C; blue) and miR-iab8 (D; yellow) using Engrailed protein (red) as a common spatial register across samples. Embryos are at developmental stage 15 and are shown in ventral view (also for F-I). (**E**) *Ubx* 3′UTR reporter constructs. Short and long *Ubx* 3′UTRs, the latter lacking the first polyadenylation signal (brackets), were fused to the reporter gene *mCherry*, subcloned into pUASP.attB and integrated into chromosome 2 (cytolocation 51C) of *Drosophila melanogaster* using ΦC31-mediated site-specific integration technology (Bischof et al., 2007). (**F**) Expression pattern of the pan-neural marker Elav. (**G-I**) Expression of *mCherry* mRNAs detected by in situ hybridisation. Reporter genes were expressed in the CNS using the *elav-Gal4* driver. (G) Negative control. Note the absence of specific signal within the CNS or of any other *elav* expression domains. Asterisk indicates non-specific signal detected in salivary glands. (H,I) Expression of *mCherry* reporter genes; note the even distribution of short 3′UTR reporter mRNAs within the CNS (H). By contrast, expression of long 3′UTR reporter mRNAs is progressively weaker in more posterior abdominal regions; regions showing increasing reporter downregulation are consistent with those expressing miR-iab4 (I, compare with C). Particularly weak expression in the last abdominal segment (black bracket) is noted where miR-iab8 is expressed (compare with D). (**J**,**K**). Analysis of *mCherry* reporter expression by fluorescent in situ hybridisation. (J) Expression of the *mCherry.short* construct driven by the *elav-Gal4* driver showing homogenous expression within the *elav* domain. (K) Expression of the *mCherry.long* construct driven by the *elav-Gal4* driver shows a progressive decrease in reporter expression towards the posterior areas of the embryo.

Given that our previous experiments demonstrated that *Ubx* long 3′UTR mRNAs are expressed within the embryonic CNS by stage 15 (Fig. 1C,E,N, Fig. 4A,B) and that, at this developmental stage, expression patterns of miR-iab4 and miR-iab8 are also confined to CNS cells (Fig. 4C,D), we tested the behaviour of the short and long *mCherry* constructs within the physiological environment of the embryonic CNS. We coupled our *UAS-mCherry.short* and *UAS-mCherry.long* constructs to an *elav-Gal4* driver (Luo et al., 1994). Elav is a common molecular marker of embryonic neurons and its expression at embryonic stage 15 is detected in all postmitotic neurons (Fig. 4F) (Soller and White, 2004). In the *mCherry.short* line, detection of *mCherry* RNA signal was observed in all tissues that normally express *elav*, including well-defined CNS and peripheral nervous system (PNS) domains (Fig. 4H,J); in particular, we noted that signal within the CNS was rather homogeneous and showed no detectable modulation at the parasegmental level, including the posterior regions of the CNS where miR-iab4/8 are expressed (Fig. 4C,D,H,J). Similarly, the *elav*-driven *mCherry.long* construct showed expression within the normal *elav* domain (Fig. 4I,K). However, in contrast to the behaviour of the *mCherry.short* transgene, *mCherry.long* expression within the CNS did show a progressively stronger downregulation towards the posterior abdomen (Fig. 4I,K) where miR-iab4/8 expression is increasingly prevalent (Fig. 4C,D); we also note that expression of the *mCherry.long* construct displayed a certain degree of parasegmental modulation (Fig. 4I). These results indicate that sequences present in the *Ubx* short and long 3′UTRs are able to confer differential expression control when linked to a heterologous gene. Furthermore, the differential expression observed among these constructs is consistent with the notion that *Ubx* long 3′UTR sequences are more sensitive to miR-iab4/8 regulation than *Ubx* short 3′UTR sequences within the *Drosophila* embryonic CNS.

3′UTR processing in other Hox genes

Our findings with *Ubx* prompted us to look at similar RNA processing events in other Hox genes. Based on developmental northern blots and cDNA clone analyses, earlier work had described the existence of transcripts with alternative 3′UTRs for Hox genes derived from both the Antennapedia [ANT-C; *Antennapedia* (*Antp*)] and BX-C [*abdominal-A* (*abd-A*), *Abdominal-B* (*Abd-B*)] gene complexes (Celniker et al., 1989; Celniker et al., 1990; Kuziora and McGinnis, 1988; Laughon et al.,

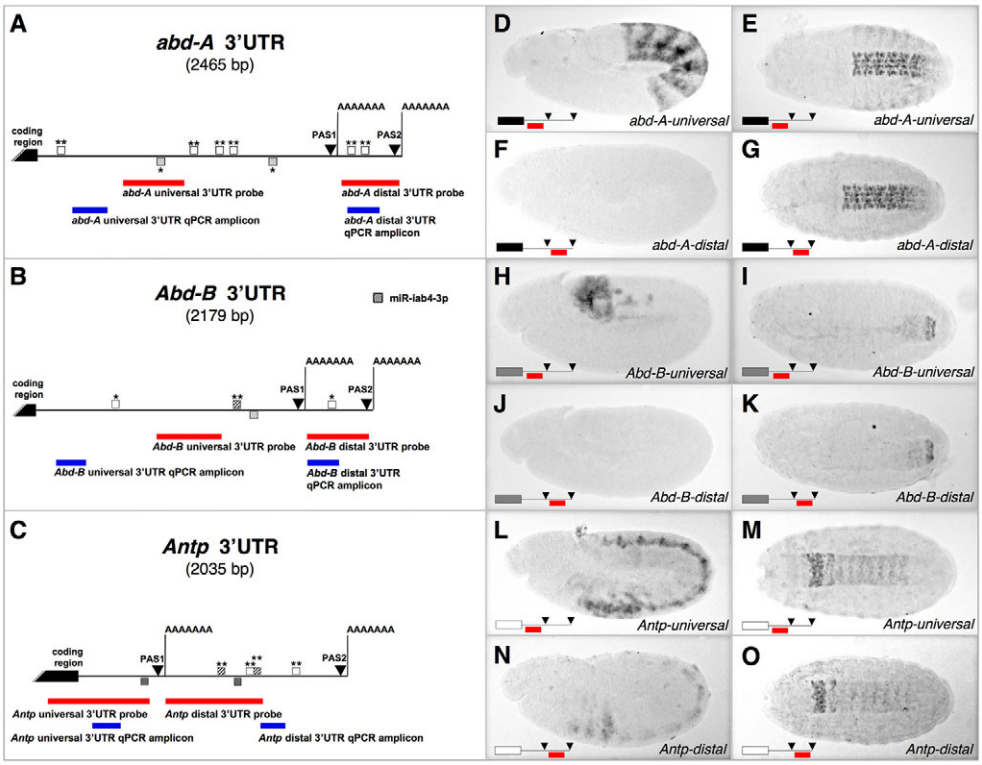

Fig. 5. Developmental processing of 3′UTRs in other Hox genes show similar dynamics to *Ubx* mRNAs. (**A-C**) Analysis of 3′UTR sequences of *abd-A* (A), *Abd-B* (B) and *Antp* (C) reveals the presence of alternative polyadenylation sites (PAS, black triangles) in the transcripts of these Hox genes. As is the case for *Ubx* (see Fig. 1A), transcripts with short and long 3′UTRs contain different sets of miRNA target sites (key for symbols as in Fig. 1A). (**D-G**) Expression patterns of *abd-A* transcripts detected by universal (D,E) and distal (F,G) *abd-A* 3′UTR probes (A). As is the case for *Ubx* transcripts, universal and distal probes show similar hybridisation patterns in late embryogenesis (see CNS expression in E and G), but no transcripts bearing the distal 3′UTR are detectable during germ band extension (compare D with F), illustrating a similar developmental usage of proximal and distal 3′UTR sequences in *Ubx* and *abd-A* transcripts. (**H-K**) Developmental detection of *Abd-B* transcripts by universal and distal 3′UTR probes (B). As for *Ubx* (see Fig. 1B-E) and *abd-A* (D-G), *Abd-B* distal 3′UTR probes do not detect significant signals during germ band extension (J), but highlight similar expression patterns to those detected by universal 3′UTR probes in the CNS of late embryos (I,K). Universal 3′UTR probes for *Abd-B* show strong signals in the posterior-most areas of the extended germ band (H), in agreement with the normal expression pattern of this gene (Celniker et al., 1989). (**L-O**) Developmental detection of *Antp* transcripts by universal and distal 3′UTR probes (C). Following the trend observed for *Ubx*, *abd-A* and *Abd-B*, *Antp* transcripts bearing long 3′UTRs are expressed at much reduced levels during germ band extension than those using the more proximal PAS (compare L with N). However, at later stages, *Antp* transcripts detected by universal and distal 3′UTR probes are visible in similar expression domains within the embryonic CNS (M,O). Altogether, our experiments confirm that four Hox genes suffer similar and synchronous rearrangements in their 3′UTR sequences during embryogenesis.

1986; O'Connor et al., 1988; Sanchez-Herrero and Crosby, 1988; Schneuwly et al., 1986; Scott et al., 1983; Stroeher et al., 1986). These studies, however, did not explore the developmental distribution of long and short isoforms in the embryo. Notably, integrating information from miRNA databases (miRBase, TargetScan Fly), the literature, and sequence analyses performed in our laboratory (see Materials and methods), we noticed that the alternative 3′UTR sequences of *abd-A*, *Abd-B* and *Antp* contain different sets of target sites for miR-iab4/8 (Fig. 5A-C). To determine the developmental distributions of short and long transcripts for these Hox genes, we prepared in situ probes that detect short and long (universal probes), as well as exclusively long (distal), 3′UTR mRNA forms and tested them in embryos (Fig. 5A-O). Strikingly, these experiments revealed that three other Hox genes undergo similar and synchronous mRNA processing events to those detected in *Ubx*: long 3′UTRs showed no (or lower) signal during germ band extension but strong signals, similar to those detected by short 3′UTR probes, in late embryonic stages within the CNS (Fig. 5D-O). A series of qPCR experiments validated the differences detected by the in situ probes (see Fig. S1 in the supplementary material). We also used the qPCR approach to independently establish the extent to which the expression of long mRNA forms is affected by the removal of miR-iab4/8 at stage 10, and found no significant differences (see Fig. S2B-D in the supplementary material).

The fact that mRNAs of four Hox genes suffer similar and coordinated 3′UTR processing suggests that a common signal might be coordinating these molecular events. In the light of recent observations in mammalian cell cultures showing that cells in proliferation produce mRNA transcripts with shorter 3′UTRs than those in stationary conditions (Sandberg et al., 2008), it seemed plausible that cell proliferation status could be coordinating Hox 3′UTR processing in vivo. To explore the role of cell proliferation in Hox 3′UTR processing, we tested the patterns of utilisation of *Ubx* long 3′UTR forms in *string* mutants, which are deficient in cell proliferation (da Silva and Vincent, 2007; Edgar et al., 1994; Edgar and O'Farrell, 1990). If cell proliferation were the primary signal behind Hox 3′UTR processing we predicted that *Ubx* long 3′UTR forms should be upregulated in *string* mutants. However, we observed no detectable upregulation of *Ubx* (or of *abd-A*) long 3′UTR mRNA forms in embryos mutant for *string* (see Fig. S5 in the supplementary material). These results suggest that, in contrast to what has been found in the mammalian cell culture system, within the physiological environment of *Drosophila* embryogenesis cell proliferation status per se is insufficient to determine transcript 3′UTR processing. Based on this and the fact that *Ubx* 3′UTR processing is tissue-specific, we propose that 3′UTR processing in vivo is primarily dictated by developmental cues in the form of specific spatial (or perhaps spatiotemporal) signals that are

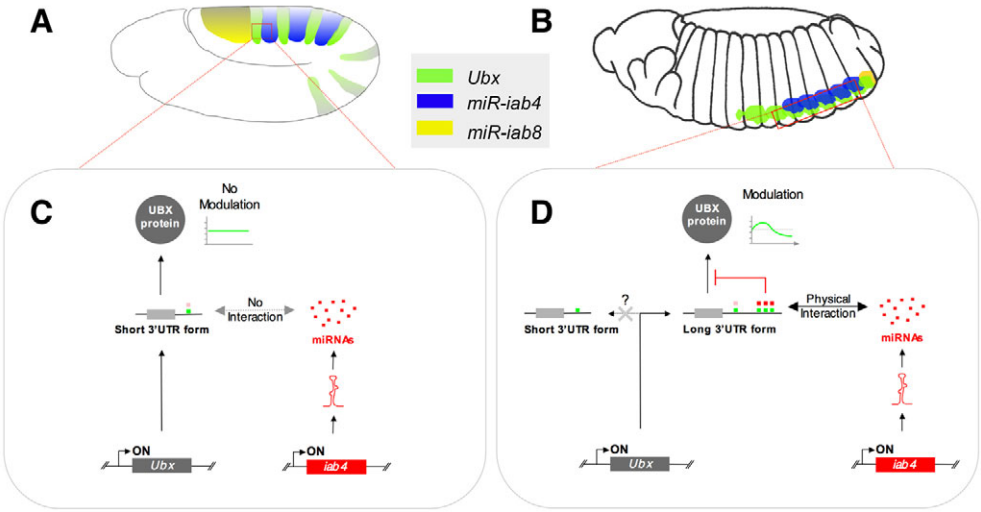

Fig. 6. An integrative model for the transcriptional and post-transcriptional regulation of *Ubx* during *Drosophila* embryogenesis. (**A**,**C**) Our expression and genetic analyses indicate that during germ band extension, the transcriptional fields of *Ubx* mRNAs and miR-iab4/8 are substantially segregated (A). Even in those cells positioned at the margin of these domains, where both *Ubx* and miRNAs may co-exist (C), functional interactions between these molecules are likely to be minimal or null due to the absence of distal 3′UTR sequences in *Ubx* mRNAs. (**B**,**D**) By contrast, genetic and expression analyses indicate that within the developing CNS, functional interactions between *Ubx* and miR-iab4/8 miRNAs do occur (B), leading to modulation of *Ubx* expression levels within this developmental context (D). The presence of an extended 3′UTR region in *Ubx* transcripts expressed at this stage is likely to mediate most interactions with miR-iab4/8. Owing to the design of our in situ hybridisation probes, we are unable to rule out the possibility that a small proportion of short *Ubx* 3′UTR mRNAs is expressed at this stage (D).

available to particular areas of the developing embryo. Alternatively, *Ubx* 3′UTR processing might be coordinated with other RNA processing events affecting *Ubx* mRNAs, such as those producing alternatively spliced products; interestingly, we do note an association between the patterns of *Ubx* 3′UTR processing and specific alternatively spliced forms of *Ubx* (see Fig. S7 in the supplementary material), suggesting that at least in some developmental contexts the two RNA processing systems are coordinated or respond to similar external factors. We are currently investigating this possibility in more detail.

DISCUSSION

The work presented here shows that during embryonic development, the *Drosophila* Hox gene *Ubx* expresses mRNA forms that bear distinct 3′UTR regions in different regions of the embryo. Notably, the possession of differential 3′UTR sequences converts each *Ubx* mRNA type into a substantially different substrate for miRNA regulation.

The existence of a developmentally controlled Hox 3′UTR RNA processing system is anticipated to contribute substantially to the specificity of the regulatory interactions between miRNAs and Hox gene mRNAs: in a given cell, and according to developmental cues, individual mRNA transcripts are predicted to react differently to the presence of miRNAs depending on the processing status of their 3′UTR sequences.

Assuming that both miRNAs and mRNA targets are co-expressed in the same cell, we conceive two alternative scenarios for the evolution of alternative 3′UTRs. First, shorter 3′UTR forms might have evolved from an ancestral long 3′UTR state due to the need to escape miRNA detection at times when particularly high levels of Hox gene products are needed for normal development; according to this view, the pruning of an otherwise longer 3′UTR might have been positively selected as a system to eliminate crucial miRNA target sites from target mRNA transcripts, thus providing an ‘miRNA avoidance’ mechanism. An alternative model considers that in the ancestral state, short 3′UTR forms were produced. From such origins, the synthesis of longer, 3′UTR-extended Hox mRNAs might have evolved to provide additional regulatory surfaces that mediate interactions with miRNAs at selected spatiotemporal coordinates; we term this the miRNA ‘enhanced regulation’ hypothesis. Comparative computational analysis of Hox 3′UTRs derived from different insect groups is predicted to determine ancestral and derived modes of Hox 3′UTR use, and, ultimately, resolve this issue (P. Patraquim and C.R.A., unpublished). Nonetheless, we argue below that our observations during germ band extension and CNS development provide more support for the ‘enhanced regulation’ model.

During germ band extension, *Ubx* protein and mRNA patterns and levels in the miRNA mutant are indistinguishable from those detected in wild-type embryos. This suggests that in spite of the fact that *Ubx* mRNAs and miR-iab4/8 show largely complementary expression patterns with some degree of overlap [Fig. 2A-F; figure 1F-H in Ronshaugen et al. (Ronshaugen et al., 2005)], these miRNAs might have no obvious involvement in controlling *Ubx* expression at this point in development. It would then follow that, within this context, *Ubx* expression is primarily controlled via canonical transcriptional regulation (Fig. 6A,C) and not via post-transcriptional regulation by miRNAs. We believe that the lack of interaction between *Ubx* and the miR-iab4/8 system at this stage is therefore primarily due to the spatial segregation of the transcriptional domains of *Ubx* and the miRNAs. Even in those cells positioned at the margin of these transcriptional domains, where *Ubx* and the miRNAs seem to coexist at a certain intermediate expression level (figure 1F-H in Ronshaugen et al. (Ronshaugen et al., 2005)]), we think that interactions between these molecules is likely to be minimal or non-existent due to the fact that the only form of *Ubx* transcript available in this developmental context lacks the 3′UTR regions

that harbour most of the miRNA target sites. As they stand, these results (1) explain why removal of miR-iab4/8 does not lead to the generation of homeotic phenotypes, and (2) are consistent with earlier work showing that *lacZ* reporter 'enhancer-trap' constructs lacking *Ubx* 3′UTR sequences are able to closely recapitulate the expression pattern of *Ubx* during germ band extension (Casares et al., 1997; Maeda and Karch, 2006; McCall et al., 1994), revealing that the cis-regulatory elements that specify *Ubx* expression patterns during this phase of embryogenesis are located outside *Ubx* 3′UTRs.

Within the embryonic CNS, the situation is rather different. The observed miRNA-dependent regulation of *Ubx* protein (see Fig. S4A,B in the supplementary material) (Bender, 2008) and mRNA (see Fig. S4C,D in the supplementary material) levels within the embryonic CNS does indeed support a functional interaction between *Ubx* transcripts and the miRNA system in this developmental context, during which long *Ubx* transcripts are expressed at high level. Based on the expression patterns of *Ubx* transcripts and miR-iab4/8 and the results of our 3′UTR reporter experiments, we propose that such extended 3′UTR *Ubx* mRNA forms bearing multiple miRNA target sites allow *Ubx* mRNAs to interact with miRNA input signals (Fig. 6B,D), which can be distributed in a rather complex pattern, with clear variations at the single-cell level (see Fig. 4C,D). According to this view, the skipping of the first polyadenylation signal at stage 15 is predicted to allow active miRNA regulation during a developmental stage when Hox inputs are crucial for the normal development of the embryonic CNS (Rogulja-Ortmann et al., 2008; Rogulja-Ortmann and Technau, 2008). We thus propose that tissue-specific 3′UTR RNA processing leading to 'enhanced regulation' by miRNAs could contribute to the generation of complex and cell-specific Hox expression patterns, which cannot be explained with the current understanding of the *Ubx* transcriptional control regions (Prokop et al., 1998). Further support for this idea is provided by the existence of many unique target sites for miRNAs within long 3′UTR forms of Hox mRNAs expressed in the CNS (see Fig. S6A-C in the supplementary material). We are currently testing these ideas, dedicating significant efforts to determine the molecular mechanisms underlying Hox 3′UTR RNA processing, the effects of distinct Hox 3′UTR elements on gene expression during *Drosophila* CNS development, and how these 3′UTR regulatory events relate to the biological roles of Hox genes during CNS differentiation and embryonic and larval behaviour.

Although much remains to be learned about the subcellular and molecular mechanisms that lead to the physical contact between miRNAs and their targets, theoretically, the simple presence of a given miRNA could influence the evolution of all 3′UTRs expressed in the same cell at the same time. In certain contexts, selective pressure is anticipated to maintain miRNA target sequences unchanged, whereas in other cases, natural selection might favour the loss of miRNA sequences in mRNA 3′UTRs to ensure the lack of potentially detrimental interactions between particular miRNA species and subsets of mRNAs (Bartel and Chen, 2004); such mRNAs have been defined as 'antitargets' (Bartel and Chen, 2004; Farh et al., 2005). Earlier work has identified mRNA representatives of the miRNA antitarget classes in mammals (Farh et al., 2005) and *Drosophila* (Stark et al., 2005); in flies, however, miRNA antitargets were primarily represented by housekeeping genes with high ubiquitous expression (Stark et al., 2005). Our work here indicates that key developmental regulators, such as the Hox genes, are able to modulate their visibility to miRNA regulation by adapting their 3′UTR regions according to information derived from developmental context, becoming what might in effect be 'conditional' miRNA antitargets.

In summary, our findings in the Hox system suggest that developmental 3′UTR processing of transcript mRNAs might be a powerful regulatory system that is able to modulate 'fine-grain' developmental outputs by controlling the spatiotemporal distribution of molecular contacts between target mRNAs and mRNA regulators, such as miRNAs and RNA-binding proteins.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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