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**Article:**

Picao-Osorio, J, Johnston, J, Landgraf, M et al. (2015) MicroRNA-encoded behavior in *Drosophila*. *Science*, 350 (6262). pp. 815-820. ISSN: 0036-8075

<https://doi.org/10.1126/science.aad0217>

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Published in final edited form as:

*Science*. 2015 November 13; 350(6262): 815–820. doi:10.1126/science.aad0217.

## microRNA-encoded behaviour in *Drosophila*<sup>+</sup>

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### Abstract

The relationship between microRNA regulation and the specification of behaviour is only beginning to be explored. Here we find that mutation of a single microRNA locus (*miR-iab4/8*) in *Drosophila* larvae affects the animal's capacity to correct its orientation if turned upside-down (self-righting). One of the microRNA targets involved in this behaviour is the *Hox* gene *Ultrabithorax* whose derepression in two metameric neurons leads to self-righting defects. *In vivo* neural activity analysis reveals that these neurons, the self-righting node (SRN), have different activity patterns in wild type and miRNA mutants whilst thermogenetic manipulation of SRN activity results in changes in self-righting behaviour. Our work thus reveals a microRNA-encoded behaviour and suggests that other microRNAs might also be involved in behavioural control in *Drosophila* and other species.

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The regulation of RNA expression and function is emerging as a hub for gene expression control across a variety of cellular and physiological contexts including neural development and specification. Small RNAs such as microRNAs (miRNAs) have been shown to affect neural differentiation (1, 2) but their roles in the control of behaviour are only beginning to be explored.

Previous work in our laboratory focused on the mechanisms and impact of RNA regulation on the expression and neural function of the *Drosophila Hox* genes (3–6). These genes encode a family of evolutionarily conserved transcription factors that control specific programs of neural differentiation along the body axis (7–9) offering an opportunity to investigate how RNA regulation relates to the formation of complex tissues such as the nervous system.

Here we use the *Hox* gene system to investigate the roles played by a single miRNA locus (*miR-iab4/8*) (3, 10–14, 30) on the specification of the nervous system during early *Drosophila* development. This miRNA locus controls the embryonic expression of posterior *Hox* genes (3, 10–14). Given that we found no detectable differences in the morphological layout of the main components of the nervous system in late *Drosophila* embryos of *wild*

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*type* and *miR-iab4/8* null mutants (herein  $\Delta miR$ , (13)) (Fig S3B-F) we analysed early larval behaviour as a stratagem to probe the functional integrity of the late embryonic nervous system.

Most behaviours in early larva were unaffected by the miRNA mutation (Fig. S1, movie S1 and S2) except self-righting (SR) behaviour (Fig. 1A-C, movies S3–S4): miRNA mutant larvae were unable to return to their normal orientation at the same speed as their wild type counterparts.

By means of selective target over-expression followed by SR phenotype analyses we identified the *Drosophila Hox* gene *Ultrabithorax (Ubx)* (17, 18) as a miRNA target implicated in the genetic control of SR behaviour (Fig. 1F). Notably, overexpression of *Ubx* did not affect any larval behavior tested except self-righting in remarkable agreement with the effects observed in miRNA mutants (Fig. 1D and E). Analysis of *Ubx* 3'UTR fluorescent reporter constructs expressed in the *Drosophila* CNS (Fig. S2) indicates that the interaction between *miR-iab4/8* and *Ubx* is direct, in line with prior observations in other cellular contexts (10–12).

To identify the cellular basis for SR control we systematically over-expressed *Ubx* within sub-populations of neurons (Fig. S4). Increased levels of *Ubx* within the pattern of *Cha(7.4kb)-Gal4*, which largely targets cholinergic sensory and interneurons, phenocopied the miRNA SR anomalies (Fig. S4). Further analysis identified two metameric neurons as the minimal node required for the SR behaviour (*Self-righting node, SRN*) (Fig. 2A and B).

Several lines of evidence confirm the role of miRNA-dependent *Ubx* regulation within the SRN as a determinant of SR. First, both *Ubx* and miRNA transcripts (i.e. *miR-iab4*) derived from the *miR-iab4/8* locus are detected within the SRN (Fig. 3A-C). Second, in the context of miRNA mutation, *Ubx* protein expression is increased within SRN (Fig. 3D-F). Third, reduction of *Ubx* (i.e. *Ubx RNAi*) specifically enforced within SRN cells is able to ameliorate or even rescue the SR phenotype observed in miRNA mutants (Fig. 2C).

Two plausible scenarios arise to explain the effects of *miR-iab4/8* in regards to SR behaviour. One is that miRNA input is required for the late embryonic development of the neural networks underlying SR arguing for a '*developmental*' role of the miRNA; another, is that miRNA repression affects normal physiological/behavioural functions largely without disrupting neural development in line with a '*behavioural*' role. Two independent experiments support that the primary roles of *miR-iab4/8* are behavioural. First, anatomical analysis of SRN cells in *wt*,  $\Delta miR$  and  $R54503>Ubx$  (i.e. SRN-driver line) show no significant differences in total numbers of SRN cells (Fig. S5B) nor in SRN cell body size (Fig. S5C); furthermore, analysis of *wt*,  $\Delta miR$  and  $R54503>Ubx$  show indistinguishable SRN-projection patterns (Fig. S5D and E). Second, Gal-80ts mediated conditional expression experiments show that SRN-specific *Ubx* overexpression after embryogenesis is sufficient to trigger the SR behaviour (Fig. 2D-E).

The results presented above suggest that miRNA-dependent *Hox* regulation within the SRN must somehow modify the normal physiology of SRN cells so that when the miRNA is mutated these neurons perform different functions than those in *wild type* animals. To test

this hypothesis we used genetically-encoded calcium sensors [GCaMP6, (24)] specifically expressed in SRN cells and tracked down spontaneous profiles of neural activity. SRN cells in miRNA mutants produce activity traces that are significantly different from those observed in *wild type* SRN cells (Fig. 4B-C, Fig. S6A). Quantification of maximal amplitude and proportion of active cells in each genotype also reveal significant differences (Fig. 4D, Fig. S6B) in SRN function across the genotypes, but no change in cell viability is observed (Fig S6C). Neural activity differences across genotypes are significant within regions of expression of miR-iab4 (Fig. 4E) suggesting that this miRNA (and not miR-iab8) might be the main contributor to SR control. Analysis of mutations selectively affecting miR-iab4 or miR-iab8 (13–14, 25–26) strongly suggests that miR-iab4 is the key regulator of SR (Fig. 3G-H).

To demonstrate that the changes in SRN neural activity were causal to SR behaviour we artificially activated (Fig. 4F) or inhibited (Fig. 4G) SRN cells (27, 28) and showed that this triggered the aberrant SR phenotype. This suggested that activation of SRN cells in larvae placed ‘right-side up’ might be sufficient to ‘evoke’ actions reminiscent of a self-righting response. We developed an optogenetic system where we activated SRN cells by means of R54F03-driven channelrhodopsin 2 (ChR2) in trans-retinal fed larvae. Under blue light stimulation larvae performed an atypical bending movement, frequently adopting a “lunette” position (Fig. S7 and movie S5). Neither parental line R54F03-Gal4 or UAS-Ch2R showed similar reactions to stimulation confirming the specificity of this effect (Fig. S7, movies S6 and S7).

To study the links between SRN neurons and the SR movement we labelled SRN projections with *myr-GFP* and discovered that SRN cells innervate two of the lateral transverse (LT) muscles and that they can be co-labelled with anti-Fasciclin 2 (Fas2) (Fig. 4H) demonstrating these to be motorneurons. LT muscles are innervated by Bar-H1+ motorneurons (Fig. S8A) so we used Bar-H1-Gal4 as a second driver to demonstrate that appropriate Ubx levels in these cells are required for normal self righting behavior (Fig. S8B) establishing the SRN cells as the LT-MNs.

We have therefore shown that miRNA-dependent *Hox* gene repression within a distinct group of motorneurons (SRN/LT-MNs) is required for the control of a specific locomotor behaviour in the early *Drosophila* larva. Our finding that *Hox* gene post-transcriptional regulation is involved in SR control suggests that other RNA-based regulatory processes affecting *Hox* gene expression might also impinge on specific neural outputs; we are currently investigating this possibility with especial regard to the roles of the *Hox* genes in the specification of neural lineages with axial-specific architectures and systematically testing the roles of other miRNAs on behaviour.

The fact that we could not detect any obvious neuro-anatomical changes in miRNA mutant embryos suggests these to be either very subtle or that the role of miRNA regulation may be primarily ‘behavioral’ in the sense of affecting the performance of a correctly wired neural system, rather than ‘developmental’ i.e. contributing to the development of the network (29). Given that *miR-iab4/iab8* is involved in adult ovary innervation (30) it seems that miRNAs –

much like ordinary protein-coding genes– can be involved in several distinct roles within the organism.

The results of this study contribute to the understanding of how complex innate behaviours are represented in the genetic program. Our data lead us to propose that other miRNAs might also be involved in the control of behaviour in *Drosophila* and other species.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

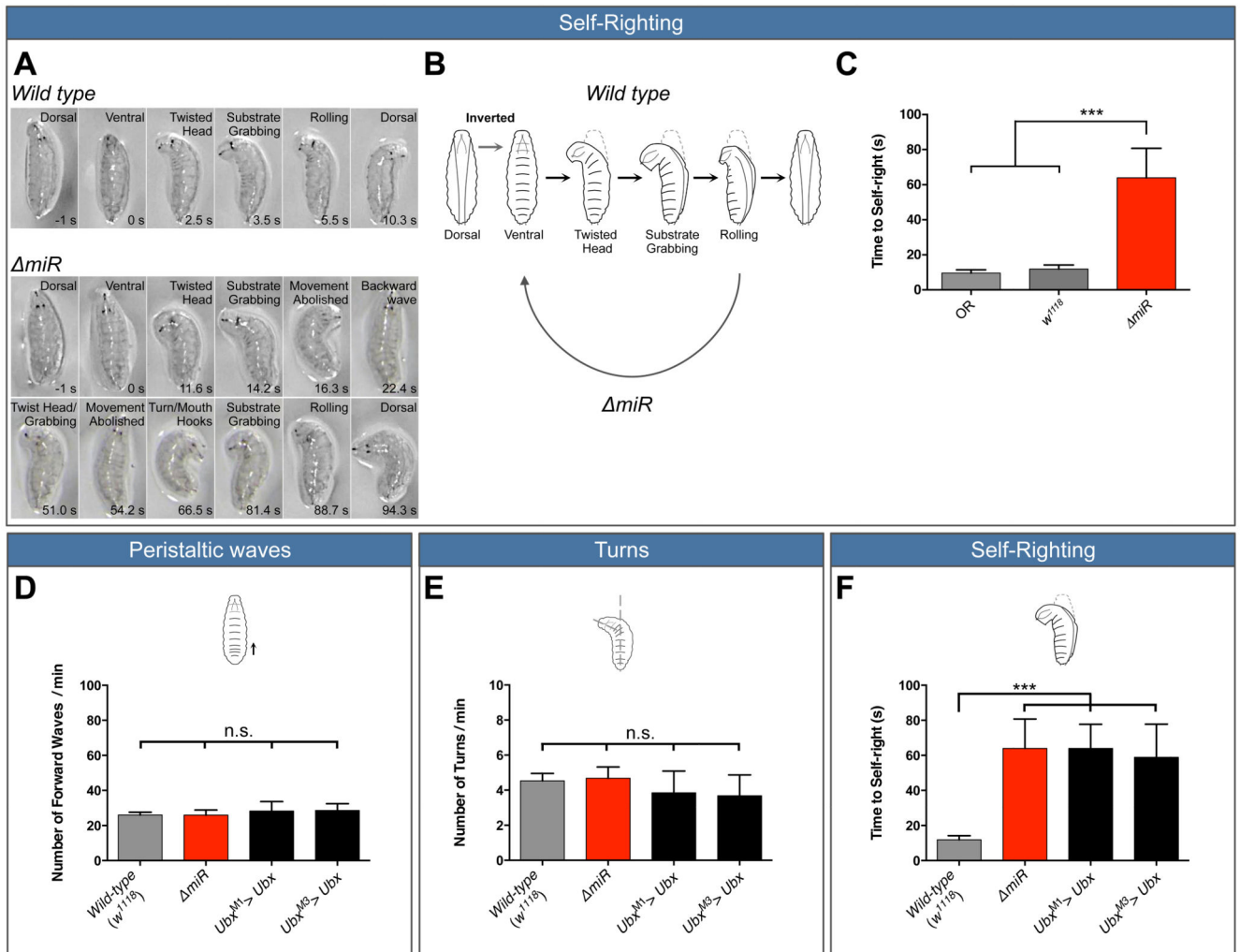
## Acknowledgements

We would like to thank Leon Lagnado for his support to this project and Sofia Pinho and Peter Reed for technical assistance. We also thank Rob White for antibodies, Welcome Bender, Ernesto Sánchez-Herrero and the Bloomington Stock Centre for *Drosophila* stocks, and Pedro Patraquim for bioinformatic support. This work was funded by a Wellcome Trust Investigator Award to C.R.A. [WT grant 098410/Z/12/Z] and a PhD studentship to J.P.O. by Fundação para a Ciência e a Tecnologia (Portugal) [FCT grant SFRH/BD/63312/2009]. J.B. is funded by a Sir Henry Dale Fellowship (Wellcome Trust and the Royal Society) Grant 105568/Z/14/Z and M.L. was supported by grants from the Biotechnology and Biological Sciences Research Council (UK) (BB/1022414/1) and the Wellcome Trust (092986/Z).

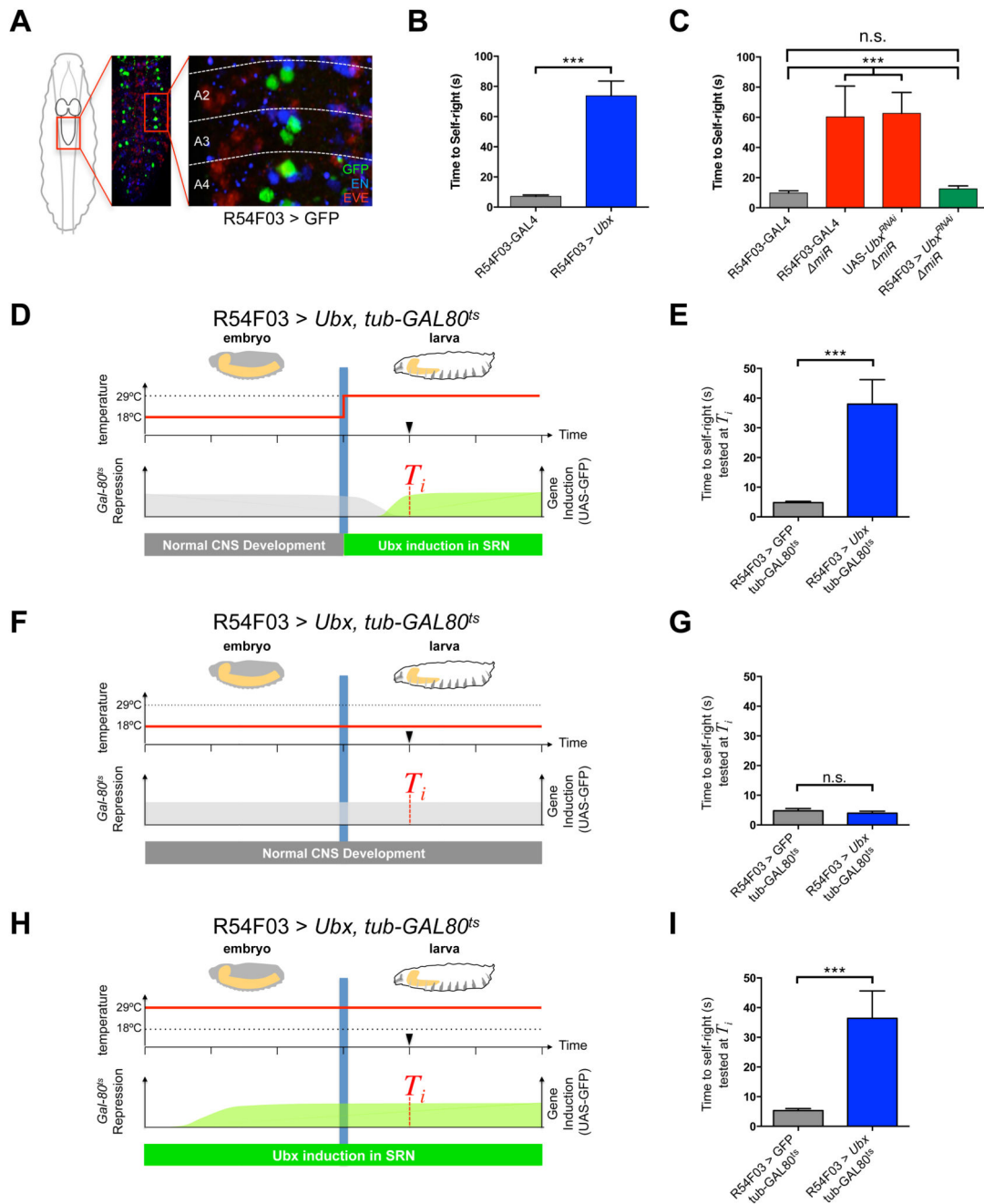
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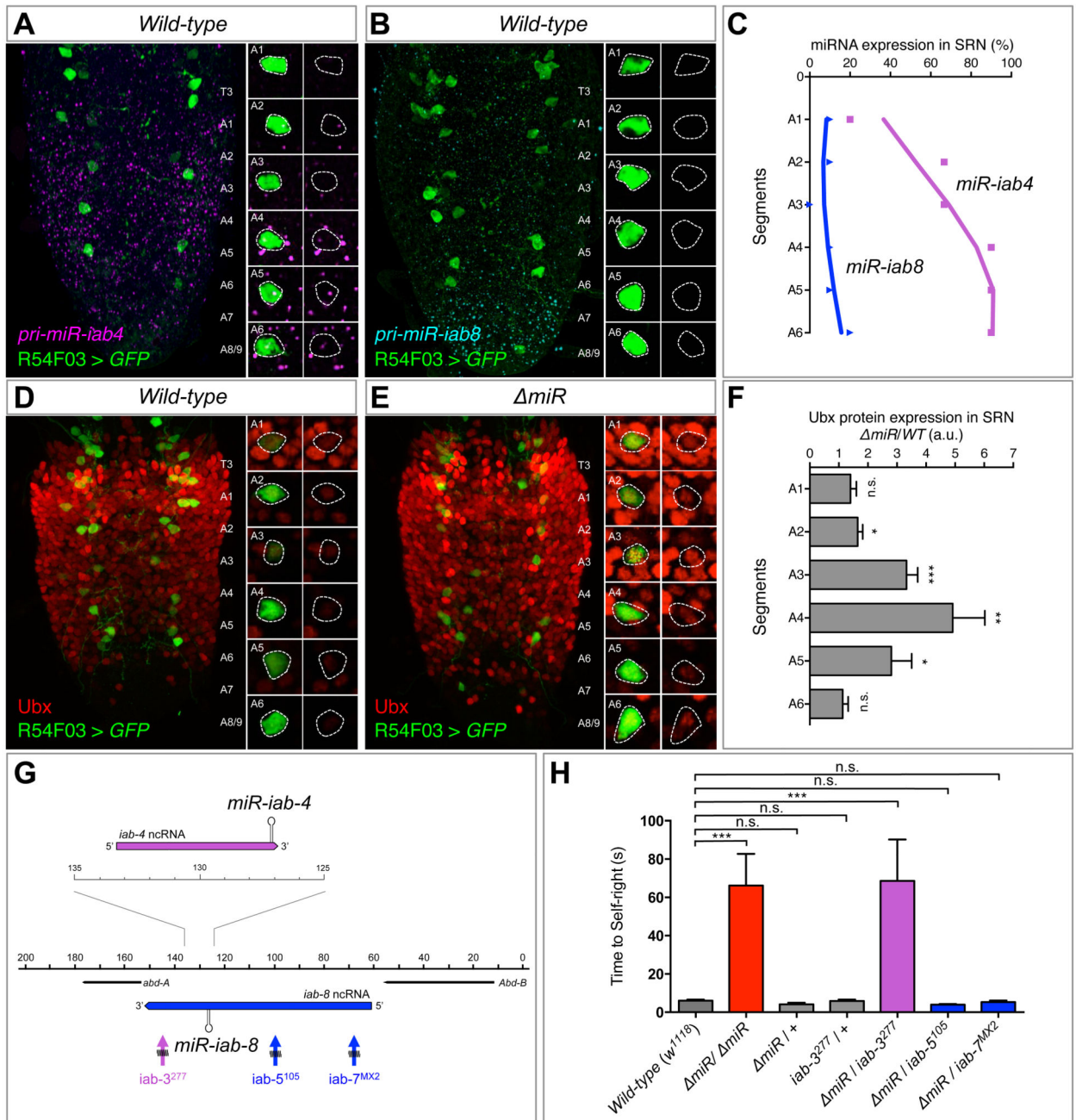
**Figure 1. Both, removal of *miR-iab4/iab8* and over-expression of *Ubx* disrupt a specific larval locomotor behaviour: self-righting**  
**(A, B)** Description of larval self-righting behaviour. **(A)** Time-lapses of larval self-righting behaviour. **(A top)** *Wild type* larvae were placed in an inverted position (ventral up), twisted their heads, grabbed the substrate with the mouth hooks and rolled their bodies onto their ventral surface (dorsal up). In contrast, **(A bottom)**  $\Delta miR$  larvae displayed problems in self-righting their bodies. **(B)** Diagram of the self-righting behavioural response. **(C)** Quantification of the time required for the successful completion of the self-righting behaviour (mean  $\pm$  SEM;  $N=27$  to 29 larvae per genotype) in the two *wild type* controls (*OR*, *w<sup>1118</sup>*, light and dark grey respectively) and  $\Delta miR$  larvae (red). **(D-F)** Quantification of larval behaviour in *Ubx* overexpression lines (*Ubx<sup>M1</sup>>Ubx* and *Ubx<sup>M3</sup>>Ubx*). Quantification of **(D)** number of forward peristaltic waves per minute, **(E)** larval turning per minute and **(F)** time to self-right in: *wild-type* (*w<sup>1118</sup>*, grey),  $\Delta miR$  (red), *Ubx<sup>M1</sup>>Ubx* and *Ubx<sup>M3</sup>>Ubx* (black) (mean  $\pm$  SEM;  $N=15$  to 29 larvae per genotype). A non-parametric Mann-Whitney *U* test was performed to compare treatments;  $p > 0.05$  (non-significant; n.s);  $p < 0.001$  (\*\*\*)



**Figure 2. miRNA-dependent *Ubx* regulation in SRN cells underlies SR behaviour**

(A) R54F03-GAL4 expression (GFP, green) in the larval abdominal CNS (Even-skipped protein in red; Engrailed protein in blue; A2 refers to abdominal segment 2). (B) Artificial increase of *Ubx* expression in two metameric neurons driven by the R54F03-GAL4 promoter (mean  $\pm$  SEM;  $N = 60$  per genotype). (C) Artificial decrease of *Ubx* expression with *Ubx*<sup>RNAi</sup> within SRN cells in  $\Delta miR$  larvae (R54F03-GAL4,  $\Delta miR$ / UAS-*Ubx*<sup>RNAi</sup>,  $\Delta miR$ , green) (mean  $\pm$  SEM;  $N = 20$  to 23 per genotype). (D-I) Conditional increase of *Ubx* expression during embryonic and early larval development with *tub-Gal80*<sup>ts</sup> (Gal80<sup>ts</sup>

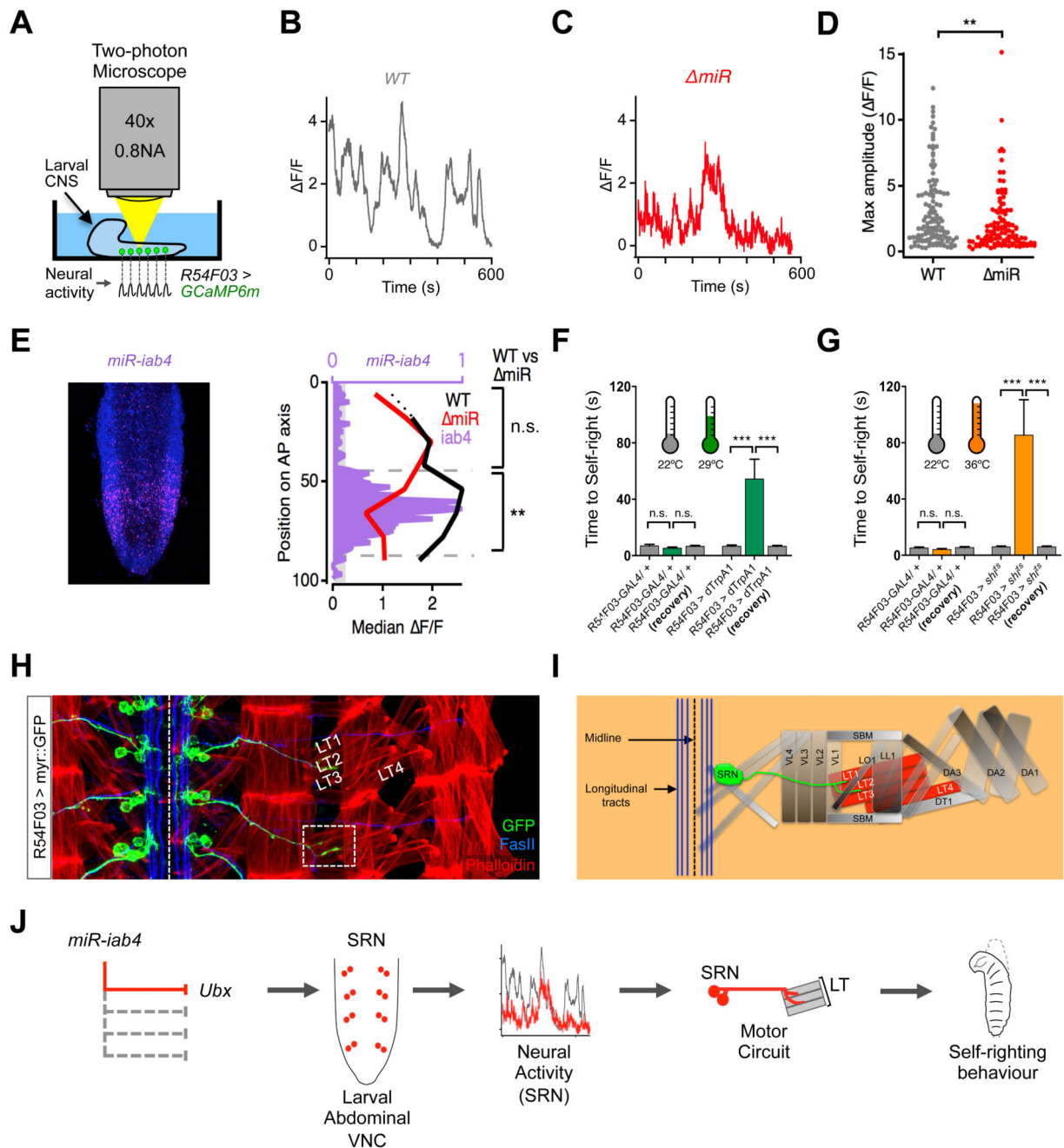
represses GAL4 activity at 18°C) within SRN cells: R54F03 > *Ubx, tub-GAL80<sup>ts</sup>* (UAS-*Ubx*/+ ; R54F03-GAL4/ *tub-Gal80<sup>ts</sup>*). Controlled increase of *Ubx* expression in SRN cells in early larvae (D and E; mean ± SEM; *N* = 20 per genotype) and from mid-embryogenesis to early larvae (H and I; mean ± SEM; *N* = 15 per genotype). (F and G) Repressed increase of *Ubx* expression in SRN cells throughout embryogenesis and early larvae (mean ± SEM; *N* = 15 per genotype). A non-parametric Mann-Whitney *U* test was performed to compare treatments; *p* > 0.05 (non-significant; n.s.); *p* < 0.001 (\*\*\*)



**Figure 3. Regulation of Ubx protein expression in SRN cells by *miR-iab4/iab8***

(A) *Wild type* expression of precursor *miR-iab4* transcripts (RNA-FISH, magenta) in SRN cells (R54F03>GFP, green) of the ventral nerve cord (VNC) of first-instar *Drosophila* larvae. (B) *Wild type* expression of precursor *miR-iab8* transcripts (RNA-FISH, cyan) in SRN cells (R54F03>GFP, green) of the VNC of first-instar *Drosophila* larvae. (C) Percentage of SRN cells expressing *miR-iab4* (purple, square) and *miR-iab8* (blue, triangle) precursors across A1 to A6 (N = 10). (D and E) Ubx protein expression (red) in SRN cells of *wild type* (D) and  $\Delta miR$  (E) first-instar larvae VNCs. (F) Quantification of Ubx protein expression ratio of

*ΔmiR* over *wild type* within the SRN cells (red) by fluorescent intensity (N = 8 per genotype; arbitrary units, a.u.). (G) Diagram of a sub-region of the bithorax complex based on (13) showing *iab-4* (purple) and *iab-8* (blue) non-coding RNAs (ncRNA), and rearrangement breakpoints affecting *miR-iab-4* (*iab-3<sup>277</sup>*, purple) and *miR-iab-8* (*iab-5<sup>105</sup>* and *iab-7<sup>MX2</sup>*, blue). (H) Genetic complementation tests to determine the involvement of *miR-iab4* or *miR-iab8* in SR behaviour using trans-heterozygote larvae for *ΔmiR* and different chromosomal rearrangement breakpoints that disrupt the bithorax complex (mean ± SEM; N = 17 to 20 per genotype). A non-parametric Mann-Whitney *U* test was performed to compare treatments;  $p > 0.05$  (non-significant; n.s.);  $p < 0.05$  (\*);  $p < 0.01$  (\*\*);  $p < 0.001$  (\*\*\*)).



**Figure 4. *ΔmiR* mutants have abnormal patterns of neural activity in SRN cells**

(A) Schematic of the lateral larval CNS expressing *GCaMP6m* in SRN cells (*R54F03 > GCaMP6m*, green) imaged in a two-photon microscope. (B) Examples of spontaneous activity recorded over 10 mins from *wild type* (*WT*: *UAS-GCaMP6m/+*; *R54F03-GAL4/+*) and (C) *ΔmiR* mutants (*UAS-GCaMP6m/+*; *R54F03-GAL4*, *ΔmiR/ΔmiR*) in SRN cells. (D) Maximum amplitude of spontaneous activity in SRN cells: *WT* (median ΔF/F=1.91; N = 120) and in *ΔmiR* mutants (median ΔF/F=1.27; N = 115) (\*\* *p*<0.01, Mann-Whitney *U* test). (E) Expression pattern of *miR-iab4* (purple) and DAPI (blue) in the VNC of a freshly

hatched larva (left panel). Median  $\Delta F/F$  in SRN cells of WT (black line) and  $\Delta miR$  (red line) larval VNCs, and relative expression of *miR-iab4* (purple) along the anterior-posterior (A-P) axis. Median  $\Delta F/F$  of WT (median of 2.132, n=73) and  $\Delta miR$  (median of 1.122, n=68) in regions of high *miR-iab4* expression (\*\*  $p < 0.01$ , Mann-Whitney *U* test). Regions of low *miR-iab4* expression have a median  $\Delta F/F$  of 1.763 in WT (N = 47) and 1.749 (N = 47) in  $\Delta miR$  specimens (n.s.,  $p > 0.05$ ; Mann-Whitney *U* test). (F and G) Thermogenetic manipulation of neural activity in SRN cells. Activation (F, R54F03>*dTrpA1*) and inhibition (G, R54F03>*sh<sup>1A5</sup>*) of SRN neural activity (\*\*\*) [ $p < 0.0001$ ] [29°C (green) for activation (H) and 36°C (orange) for inhibition (I)]. (H) *Wild-type* motor axonal projections of SRN cells (UAS-*myr::GFP*/UAS-*myr::GFP*; R54F03-GAL4/ R54F03-GAL4, green) into muscles (phalloidin, red) lateral transverse 1 and 2 (LT1 and LT2) in late embryos (stage 17) (Fasciclin II, FASII, blue). Scale bars (white bars) represent 10µm (I) Diagram of SRN cells projecting to the LT1 and LT2 muscles. (J) A model that summarises the data reported in this study. Mutation of *miR-iab4* (left) leads to *Ubx* de-repression in the SRN node affecting SRN neural activity patterns and triggering an anomalous self-righting behaviour (right).