

A microRNA that controls the emergence of embryonic movement

Jonathan A. C. Menzies, Andre M. Chagas, Tom Baden, Claudio R. Alonso 

Department of Neuroscience, Sussex Neuroscience, School of Life Sciences, University of Sussex, Brighton BN1 9QG, UK

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Version of Record

June 13, 2024

Reviewed Preprint

v2 • June 4, 2024

Revised by authors

Reviewed Preprint

v1 • March 20, 2024

Abstract

Summary

Movement is a key feature of animal systems, yet its embryonic origins are not fully understood. Here we investigate the genetic basis underlying the embryonic onset of movement in *Drosophila* focusing on the role played by small non-coding RNAs (microRNAs, miRNAs). To this end, we first develop a quantitative behavioural pipeline capable of tracking embryonic movement in large populations of fly embryos, and using this system, discover that the *Drosophila* miRNA *miR-2b-1* plays a role in the emergence of movement. Through the combination of spectral analysis of embryonic motor patterns, cell sorting and RNA *in situ*, genetic reconstitution tests, and neural optical imaging we define that *miR-2b-1* influences the emergence of embryonic movement by exerting actions in the developing nervous system. Furthermore, through the combination of bioinformatics coupled to genetic manipulation of miRNA expression and phenocopy tests we identify a previously uncharacterised (but evolutionarily conserved) chloride channel encoding gene – which we term *Movement Modulator* (*Motor*) – as a genetic target that mechanistically links *miR-2b-1* to the onset of movement. Cell-specific genetic reconstitution of *miR-2b-1* expression in a null miRNA mutant background, followed by behavioural assays and target gene analyses, suggest that *miR-2b-1* affects the emergence of movement through effects in sensory elements of the embryonic circuitry, rather than in the motor domain. Our work thus reports the first miRNA system capable of regulating embryonic movement, suggesting that other miRNAs are likely to play a role in this key developmental process in *Drosophila* as well as in other species.

eLife assessment

This **important** study presents a new quantitative imaging pipeline that describes with high temporal precision and throughput the movements of late-stage *Drosophila* embryos, a critical moment when motion first appears. A new approach is used to explore the role of miRNAs in motion onset and presents **solid** evidence that shows a role for *miR-2b-1* and its target *Motor* in embryonic motion. The data are well supported even if the mechanistic insight into the emergence of movement remains to be explored.

<https://doi.org/10.7554/eLife.95209.2.sa2>

Results and discussion

A miRNA that impacts larval movement

Movement is the main output of the nervous system allowing animals to walk, fly, crawl, swim and maintain their posture, so that they can find prey, mate partners, escape predators and relocate within habitats. Despite the key biological and adaptive roles of movement across animal systems, how developing embryos manage to organise the necessary molecular, cellular, and physiological processes to initiate patterned movement is still unknown. Although it is clear that the genetic system plays a role, how genes control the formation, maturation and function of the cellular networks underlying the emergence of motor control remains poorly understood.

Recent work in our laboratory has shown that miRNAs – which are short regulatory non-coding RNAs that repress the expression of target genes [1, 2] – have pervasive roles in the articulation of complex movement sequences such as those involved in body posture control in the young *Drosophila* larva [3–6]; these observations, as well as those from others in *Drosophila* and other systems [7–11] hinted at the possibility that these non-coding RNA molecules might also be involved in the control of more fundamental aspects of motor development and control. To explore this question, we first searched for miRNAs that might affect the simple locomotor patterns of the *Drosophila* first instar larva. The L1 larva which is a convenient model to investigate the genetics of movement given that: (i) the assembly of the machinery for movement must be fully completed by this developmental stage so as to satisfactorily propel the animal into the external world, and (ii) if analysed sufficiently early, for example during the first few minutes after hatching, the animal had no real chance of compensating or learning ways around a putative defect, increasing the probability of detection by means of a suitable motor test. To extract a signature of larval movement, we applied a whole animal imaging method based on frustrated total internal reflection (FTIR) [12, 13] which renders high resolution and high contrast movies to both normal and miRNA mutant first instar larvae. This led us to discover that a single miRNA, *miR-2b-1*, had a significant impact on larval movement (**Figure 1B–1C**). *miR-2b-1* belongs to the *miR-2* family [14] and Δ *miR-2b-1* mutant larvae show a substantial decrease in larval speed (**Figure 1C**) suggesting that absence of this specific genetic component compromises the ability of the larva to move normally. Given that these larval tests were conducted within a 30-min period post-hatching, we considered the possibility that the defects observed in the larvae stemmed from changes in earlier ontogenetic processes that occur in the embryo. More specifically, we decided to test the possibility that early embryonic movement patterns might be affected by the lack of normal expression of *miR-2b-1*.

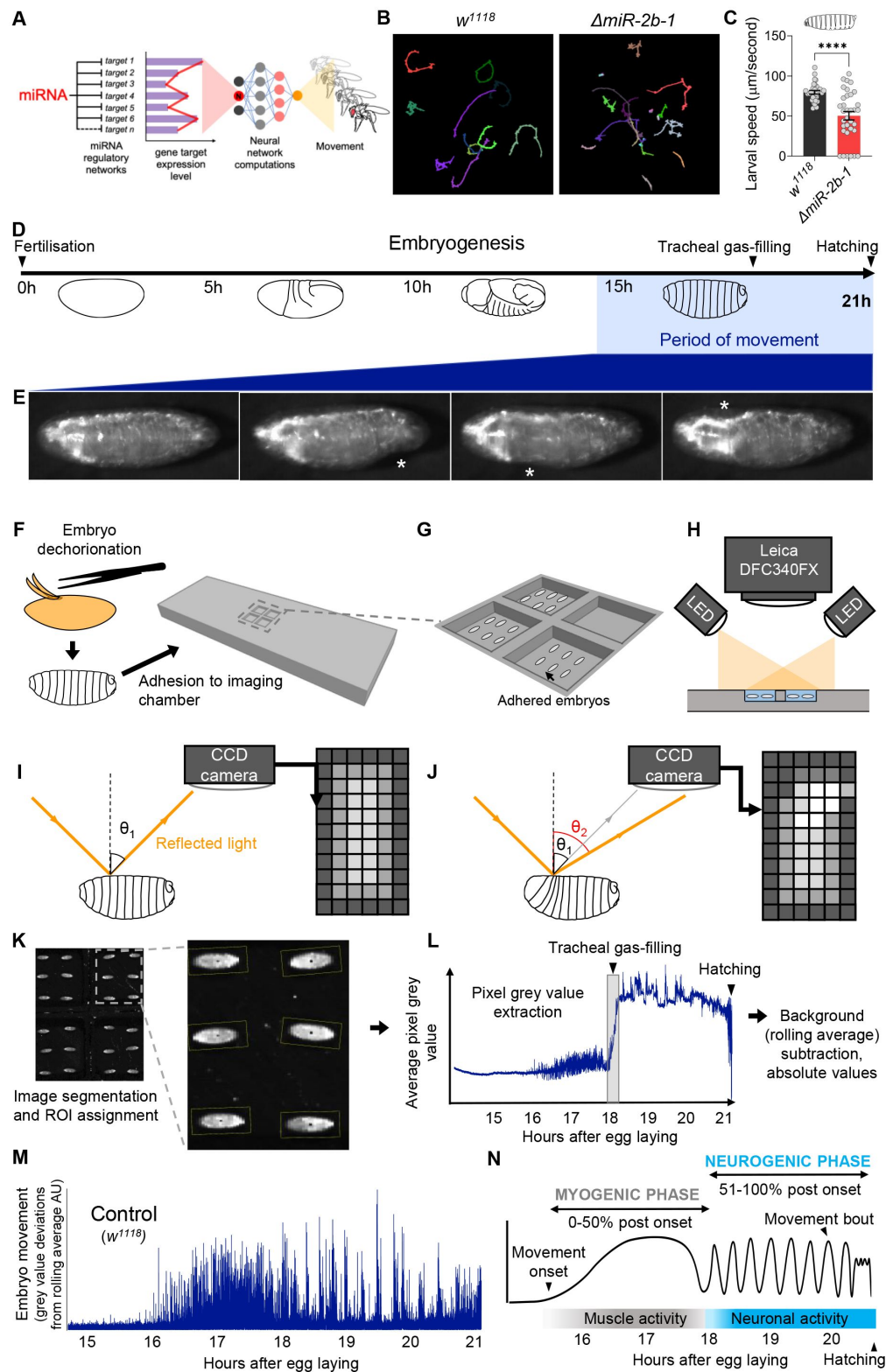


Figure 1

A novel approach for the quantification of embryonic movement (A) Diagram illustrating the point of action of miRNAs in the neural networks controlling behaviour. (B) Larval movement tracks for w^{1118} (left) and $\Delta miR-2b-1$ (right) larvae obtained using the frustrated total internal reflection based imaging system (FIM). (C) Quantification of average larval speed for w^{1118} and $\Delta miR-2b-1$ larvae using the FIM system. (D) Schematic describing the timeline of *Drosophila* embryonic development with the period of movement highlighted in blue. (E) Microscope images of a *Drosophila* embryo performing characteristic early movements, highlighted with asterisks. (F-H) Experimental pipeline for recording embryo movements. (F) eggshell removal (dechoriation) and adhesion to the imaging chamber, (G) imaging chamber design and (H) imaging set up under incident LED illumination. (I-J) Schematic describing the basis for movement detection: light is reflected from the embryo surface and internal structures and detected by a CCD sensor to generate a pixel map of the embryo (I). Embryonic movement changes the angle of reflected light, resulting in a different pixel map (J). (K-N) Pipeline for the quantification of embryonic movement. (K) representative image of the embryo movement chamber showing the assignment of regions of interest (ROIs) to individual embryos; (L) extraction of the mean grey value (MGV) for each frame (done in parallel for each ROI) allows the generation of raw movement traces for each individual embryo. Key developmental events that impact MGV (tracheal gas-filling, hatching) are indicated by arrowheads. (M) subtraction of the trace background calculated by rolling average removes slow changes in MGV that result from developmental events, allowing accurate quantification of deviations in MGV from baseline which represent movement over time (absolute values); (N) schematic of an idealised wild-type (w^{1118}) movement trace with putative phases indicated.

Previous work had provided an excellent first characterisation of the onset of embryonic movement patterns in wild type embryos [15, 16]; these early studies were based on the manual annotation of representative videotaped muscle contractions [16] or GFP-labelled muscle Z-lines analysed under spinning disc confocal microscopy [15]. Despite their attributes, these approaches were highly labour intensive and lacked the necessary throughput required to simultaneously analyse large numbers of embryos enabling a sensitive genetic screen. In consequence, we developed a new automated approach capable of quantifying movement in large populations of embryos.

A high throughput approach to quantify movement in *Drosophila* embryos

To monitor the onset of embryonic movement, which, in normal embryos, occurs during the final third of embryogenesis (i.e. 14-16h after egg laying (AEL) [15, 16] [Figure 1D, 1E] we developed a 3D printed chamber system capable of hosting multiple embryos submerged under a thin layer of halocarbon oil to ensure adequate oxygenation and hydration (Figure 1F, 1G) compatible with digital imaging by a charge coupled device (CCD) camera (Figure 1H). The camera captures the reflected light after its physical contact with the embryo; in this setting, even a subtle movement performed by the embryo results in a change in the path of reflected light, leading to variations in signal intensity detected by individual pixels in the CCD sensor, allowing for an accurate measurement of embryonic movement (Figure 1I, 1J). To extract quantitative movement information from individual embryos we applied an image segmentation protocol to define regions of interest (ROIs) corresponding to each embryo and collected pixel intensity values from all ROIs at 4 frames/sec (Figure 1K, 1L). The data allow us to plot variations in average grey pixel intensity over embryonic time, which provide a quantitative signature of the ontogeny of movement in the individual embryo (Figure 1M). From this, we were able to observe distinct phases of movement that are consistent with previous data: namely, the onset of a phase of disorganised movements ~16 hours after egg laying (hAEL) and its transition into a phase characterised by rhythmic bursts of activity and inactivity ~18 hAEL [15, 16]. These phases have been termed as ‘myogenic’ and ‘neurogenic’ based on their respective dependence on neural input (Figure 1N) [15, 17–19], and have been observed in *Drosophila*, as well as in other

systems, including vertebrates [20–24] strongly suggesting that this is a general feature of motor development. See Movie S1 for a high-resolution recording of *Drosophila* embryo movement.

To establish whether the readings of motor activity at the neurogenic phase detected by our approach were indeed dependent on neural activity, we expressed the inwardly rectifying potassium channel (*Kir*) [25] in all embryonic neurons using the pan-neuronal driver *elav-Gal4* seeking to suppress action potentials across all embryonic neuronal types. The results of this experiment show that whilst movement patterns during the early chaotic phase remain unchanged by this treatment (Figure 2C, 2E), motor activity at the rhythmic phase is almost completely eliminated (Figure 2C, 2F) strongly indicating that the emergence of this latter phase depends on normal neural activity. This is in agreement with previous observations of the effects of embryonic neural activity inhibition by other methods [15, 18]. In addition, spectral analysis demonstrates that the movement frequencies that characterise the rhythmic phase (Figure 2B, 2G) do not emerge in *Kir* embryos (Figure 2D, 2G). Altogether these observations suggest that *miR-2b-1* might exert its roles on embryonic movement, at least in part, due to action within the developing embryonic nervous system.

The miRNA *miR-2b-1* affects embryonic movement patterns

Given that the machinery for larval movement is assembled during embryogenesis [26–29] we considered the possibility that *miR-2b-1* might have an impact on the emergence of movement in the fly embryo. To explore this, we applied the approach described above to normal and Δ *miR-2b-1* mutant embryos (Figure 2H, 2J). These experiments showed that Δ *miR-2b-1* mutant embryos displayed a different pattern of embryonic movement when compared with their wild type counterparts. Although the distinct phases of embryonic movement are clearly recognisable in mutant embryos, the overall amount of movement appeared greatly reduced (Figure 2J).

Indeed, comparison of quantity of movement in wild type and Δ *miR-2b-1* mutant embryos either during the earlier myogenic phase (Figure 2L) or during the neurogenic phase (Figure 2M) shows significantly lower levels of movement in mutants. Furthermore, spectral analyses of embryonic movement traces reveal that miRNA mutant embryos shift to a higher frequency of movement bouts during the rhythmic phase when compared to normal embryos (Figure 2I, 2K) and that average bout length is also shortened (Figure S1).

miR-2b-1 expression and roles in the embryonic nervous system

The fact that removal of *miR-2b-1* impacts the neurogenic phase of embryonic movement suggests that this miRNA might be expressed in the nervous system and exert a functional role there. To further explore this model, we first conducted a spatial expression analysis in the developing embryo using fluorescence RNA *in situ* hybridisation (FISH). Taking advantage from the fact that *miR-2b-1* is located within the 3' untranslated region (3'UTR) of the *Bruton tyrosine kinase* (*Btk*) gene [30, 31] (Figure 3A) we prepared an *in situ* probe to detect *Btk* transcripts. These FISH experiments show that the *miR-2b-1* precursor miRNA transcript (pre-miRNA) [2] is expressed in multiple embryonic tissues, including the CNS (Figure 3B). To further confirm the expression of *miR-2b-1* in the nervous system, we conducted fluorescence-activated cell sorting (FACS, Figure 3C) to isolate embryonic neuronal samples (labelled by means of *elav>GFP*) followed by RT-PCR for the mature miRNA transcript and observed *miR-2b-1* specific signal (Figure 3D). Therefore, the results of these two distinct and complementary methods provide strong evidence of neural expression of the miRNA. To gain more insight on the neural roles of *miR-2b-1* in regard to embryonic movement, we conducted a genetic reconstitution experiment in which we analysed the consequences of restoring *miR-2b-1* expression in the nervous system in an otherwise Δ *miR-2b-1* null mutant (Figure 3E). Results in Figure 3F, S2A show that *elav*-driven expression of *miR-2b-1* in a Δ *miR-2b-1* mutant background leads to a phenotypic rescue, producing embryos that

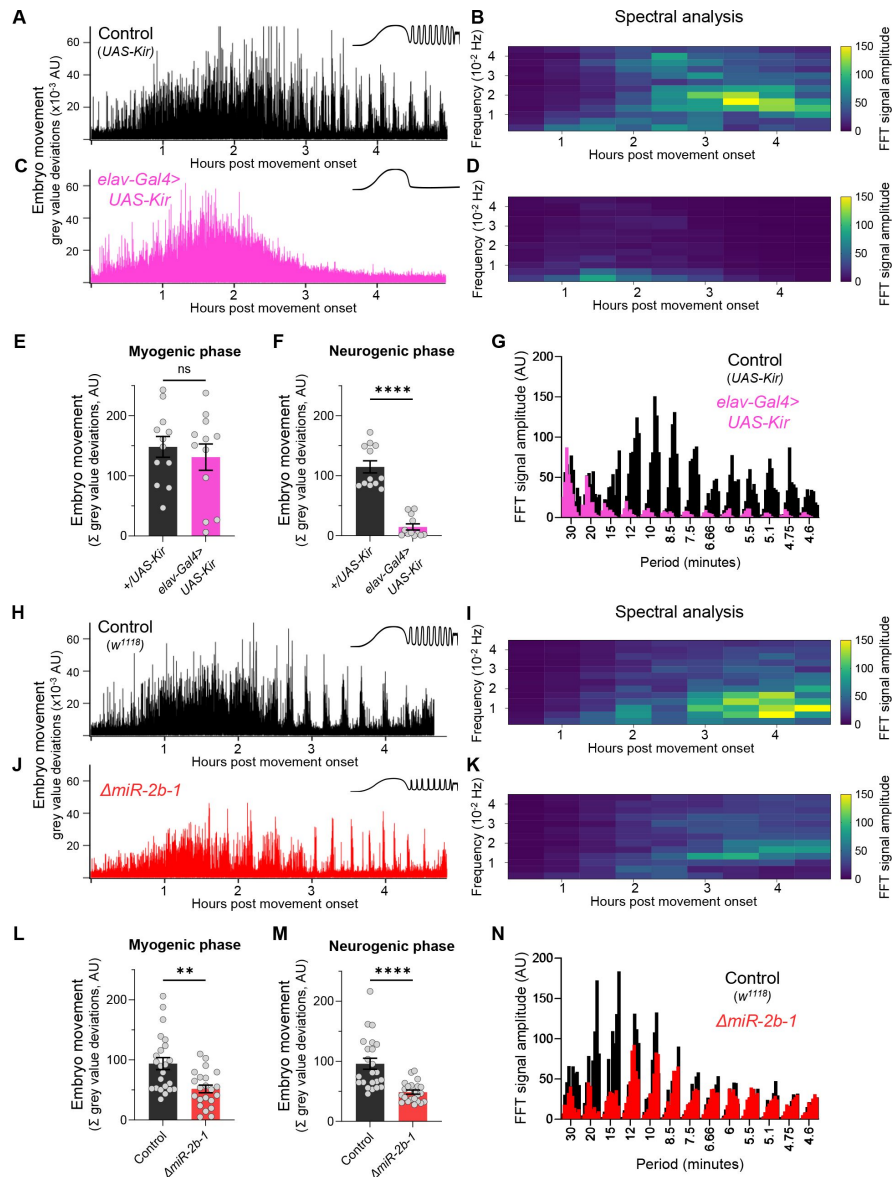


Figure 2

miR-2b-1 controls movements during the neurogenic phase of embryonic movement (A) Representative movement trace for control (*UAS-Kir*) animals. A concept diagram that summarises the pattern is shown in the top right. (B) Heat map showing the average frequency spectrogram for movements of control (*UAS-Kir*) animals, determined by fast fourier transform (FFT) analysis (1-hour sliding window with a discrete 30-min step size) from onset of embryonic movement to hatching. Brighter colours indicate a stronger amplitude of movement at a given frequency. (C) Representative movement trace for experimental (*Elav-Gal4>UAS-Kir*) animals. (D) Average frequency spectrogram for *Elav-Gal4>UAS-Kir* animals. (E) Summation of MGVD deviations during the myogenic phase in control (*UAS-Kir*, black) and experimental (*Elav-Gal4>UAS-Kir*, pink) animals. (F) Summation of MGVD deviations during the neurogenic phase in control (*UAS-Kir*, black) and experimental (*Elav-Gal4>UAS-Kir*, pink) animals. (G) Distribution of signal amplitudes across different movement periods (p) derived from the FFT frequency analysis shown in panels B and D. A higher signal amplitude is produced when more movement occurs with a particular periodicity. Bars of different height at each period sampled show data from individual embryos. (H) Representative movement trace for control (*w¹¹¹⁸*) animals. (I) Heat map showing the average frequency spectrogram for movements of *w¹¹¹⁸* control embryos. (J) Representative movement trace for experimental (*Δ miR-2b-1*) animals. (K) Heat map showing the average frequency spectrogram for movements of *Δ miR-2b-1* embryos. (L) Summation of MGVD deviations during the myogenic phase in control (*w¹¹¹⁸*, black) and experimental (*Δ miR-2b-1*, red) animals. (M) Summation of MGVD deviations during the neurogenic phase in control (*w¹¹¹⁸*, black) and experimental (*Δ miR-2b-1*, red) embryos. (N) Distribution of signal amplitudes across different movement periods derived from the FFT frequency analysis shown in panels I and K.

display statistically indistinguishable movement patterns to those recorded in control embryos, indicating that neural expression of *miR-2b-1* is sufficient to restore a normal onset of embryonic movement. To further examine the biological roles of neural *miR-2b-1* expression, we assessed the impact of restoring miRNA expression in embryonic neurons on first instar larval locomotor patterns using the FIM approach described above (**Figure 1B** [↗](#)–C) and observed that when $\Delta miR-2b-1$ mutant larvae are developmentally provided with pan-neuronal *miR-2b-1* expression, the characteristic miRNA larval mutant phenotype is rescued, with specimens moving at natural speed (**Figure 3G** [↗](#), S2B). The experiments described above strongly indicate that expression of *miR-2b-1* in the nervous system is biologically relevant and sufficient to rescue the embryonic and larval movement defects observed in $\Delta miR-2b-1$ mutants. They also suggest that the effects of *miR-2b-1* observed at earlier stages (myogenic phase) are possibly offset by normal neural expression of *miR-2b-1*.

In turn, this suggests that absence of *miR-2b-1* must impinge a morphological and/or a functional deficit in the developing nervous system of the embryo. To tease apart these potential biological effects, we examined the structure of the nervous system in normal and $\Delta miR-2b-1$ mutant embryos by means of immunohistochemistry and confocal microscopy and observed no detectable differences (Figure S2C). In contrast, analysis of neural activity patterns in the embryo by means of GCaMP6 functional imaging using a movement distortion correction approach (i.e. tdTomato, **Figure 3H** [↗](#)–**3J** [↗](#) [[17](#) [↗](#)]), shows that miRNA mutant embryos have a reduced level of calcium dynamics when compared with their control counter parts (**Figure 3K** [↗](#)–**3M** [↗](#)); notably, this occurs during a previously identified ‘critical period’ when neural activity levels are of crucial importance to the development of stable neural circuits [[32](#) [↗](#)–[34](#) [↗](#)]. Consistently with this previous work, artificial reduction of embryonic neural activity via optogenetic control leads to a significant decrease in larval speed (Figure S4A–G).

A genetic link between *miR-2b-1* and embryonic movement

Our gene expression, genetic reconstitution, morphological and functional imaging data support a model in which *miR-2b-1* plays a physiological role in the developing embryonic nervous system. This raises the question of how might this regulatory miRNA system interact with the physiological control of the neuron during embryogenesis. To explore the genetic elements that link *miR-2b-1* to its role in embryonic movement we searched for candidate *miR-2b-1* target genes using the ComiR bioinformatic platform (**Figure 4B** [↗](#)) [[35](#) [↗](#), [36](#) [↗](#)]. A common issue with bioinformatic predictions of miRNA targets is the generation of false positives; in this regard, ComiR integrates multiple miRNA target prediction algorithms – each one with its intrinsic strengths and weaknesses [[37](#) [↗](#)–[40](#) [↗](#)] – seeking to identify a set of consistent *bona fide* miRNA targets that satisfy the filters of multiple algorithms, thus reducing the generation of false positives [[35](#) [↗](#), [36](#) [↗](#)]. Applying ComiR to *miR-2b-1* produced a list of high probability targets organised in the form of an ascending ranking (**Figure 4B** [↗](#)). At the very top of the list was CG3638, an uncharacterised *Drosophila* gene predicted to encode a chloride channel protein; this highly ranked target was of interest to us because of its potential role in the physiological control of anionic conductances, and its broad evolutionary conservation across insects and mammals [[41](#) [↗](#)], including humans (**Figure 4E–I** [↗](#)).

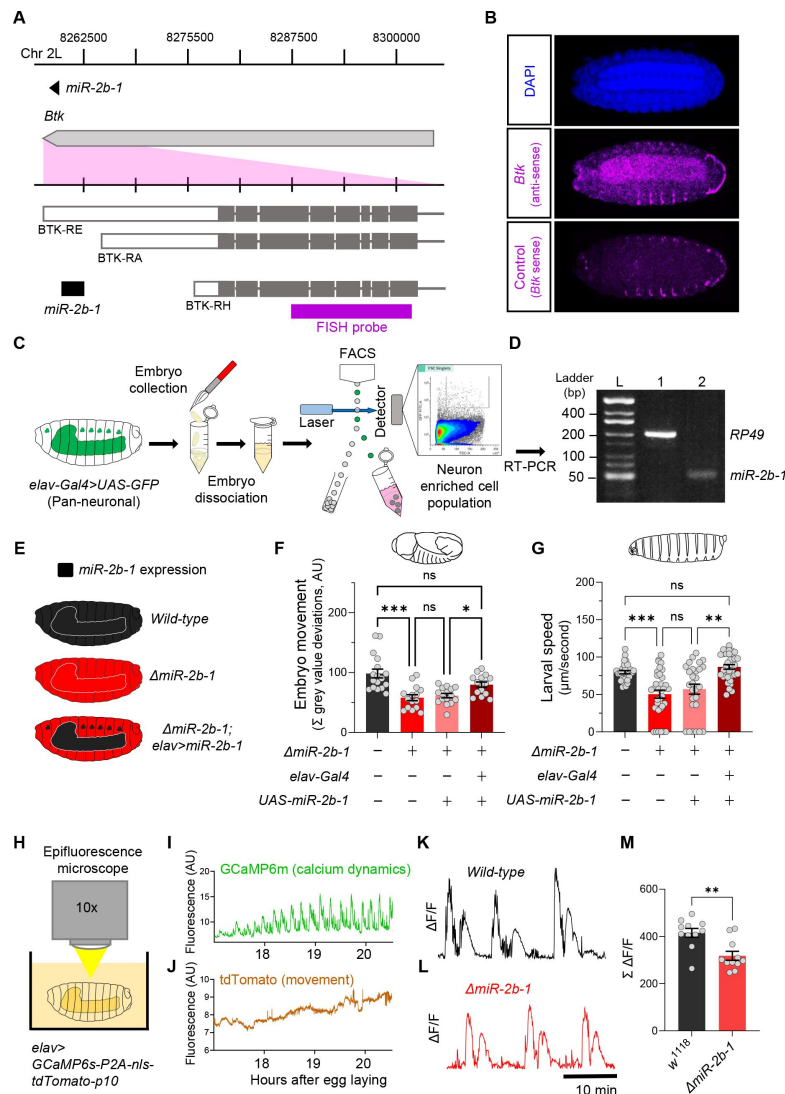


Figure 3

miR-2b-1 acts within neurons to regulate embryonic and larval movement (**A**) Gene diagram describing the *miR-2b-1* locus including the host gene *Btk* and its RNA transcripts. A fluorescence *in situ* hybridisation (FISH) probe for *Btk* is indicated by the magenta bar. (**B**) FISH experiment on control *w¹¹¹⁸* embryos showing expression of the miRNA host *Btk* transcripts (antisense probe, middle panel) [DAPI stain in blue (upper image); *Btk* sense probe in magenta (lower image)]. (**C**) Experimental workflow for a fluorescence activated cell sorting (FACS) experiment. Neurons were labelled by *elav-Gal4>UAS-GFP* (left), followed by enzymatic and mechanical separation and isolation of GFP+ neurons. (**D**) RT-PCR analysis showing expression of mature *miR-2b-1* (right) detected in neurons at the onset of the neurogenic phase (*RP49* signal (left) shown as control). (**E**) Movement patterns were assessed in embryos with normal *miR-2b-1* expression (wild-type, black), null *miR-2b-1* mutants (red) and in mutant embryos in which *miR-2b-1* expression was restored (reconstituted) specifically in neurons (red and black). (**F**) Summation of MGTV deviations during the neurogenic phase of embryonic movement in control *w¹¹¹⁸* embryos (black bar); $\Delta miR-2b-1$ mutant embryos (bright red bar); $\Delta miR-2b-1$, *UAS-miR-2b-1* parental control embryos (faded red bar); $\Delta miR-2b-1$, *Elav-Gal4>UAS-miR-2b-1* embryos (black and red lined bar). (**G**) Average L1 larval speed for the same genotypes used in the embryonic genetic reconstitution experiment. (**H**) Schematic describing the experimental setup for fluorescence imaging of *elav>GCaMP6s-P2A-nls-tdTomato-p10* embryos under an epifluorescence microscope. This design allows simultaneous detection of calcium dynamics (GCaMP6s) and movement (tdTomato). (**I**) Representative GCaMP6s trace (green) from control *w¹¹¹⁸* embryos over the neurogenic phase of embryonic movement. (**J**) Representative tdTomato trace from the same embryo as in panel I, acting as a passive fluorescence reporter used to subtract changes in GCaMP6s signal induced by embryonic movement. (**K**) Representative $\Delta F/F$ trace for *w¹¹¹⁸* embryos. (**L**) Representative $\Delta F/F$ trace for $\Delta miR-2b-1$ mutant embryos. (**M**) Summation of $\Delta F/F$ signal during embryogenesis in control *w¹¹¹⁸* embryos (black bar) and $\Delta miR-2b-1$ mutant embryos (red bar).

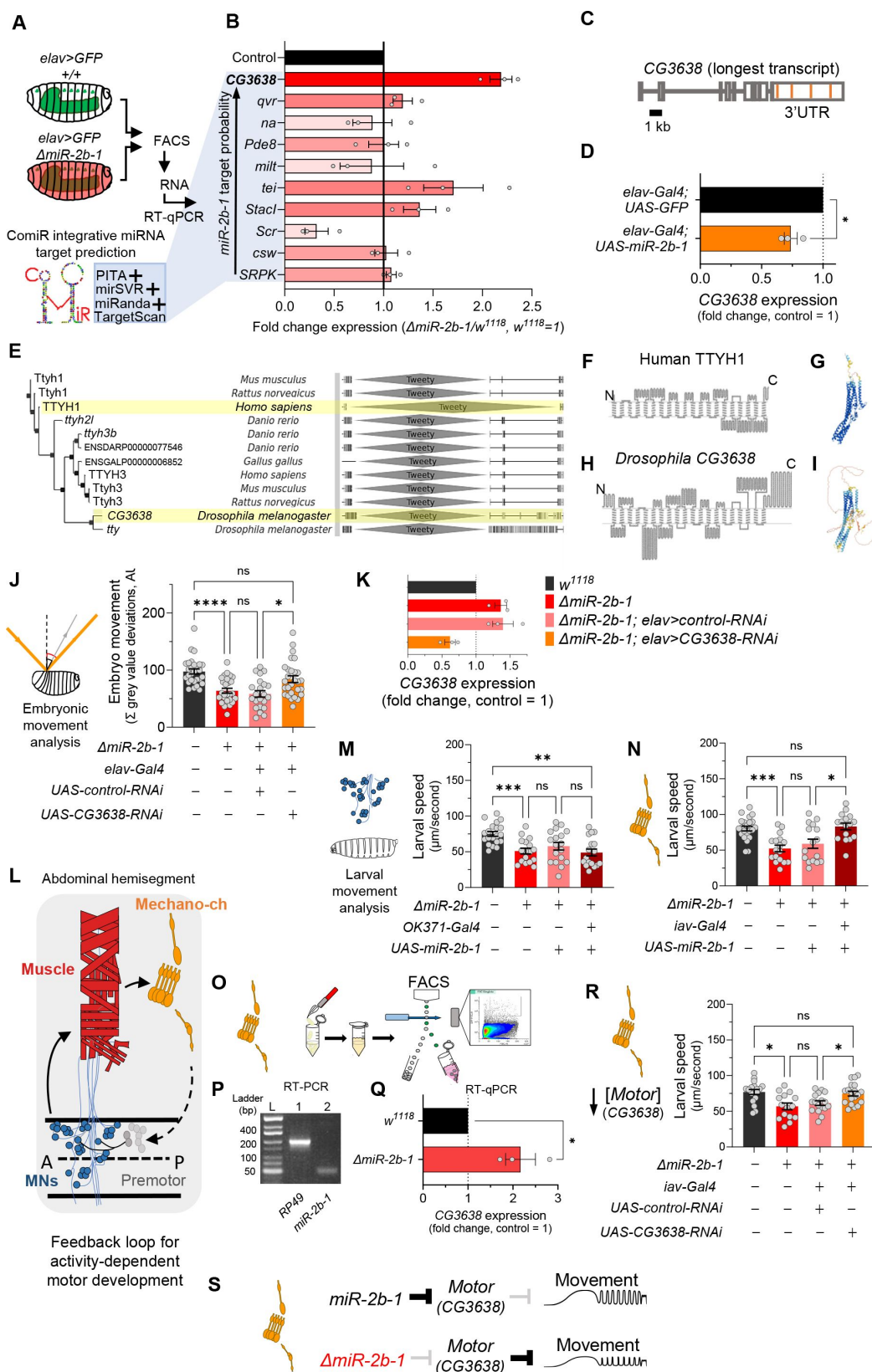


Figure 4

The genetic and cellular mechanisms that link *miR-2b-1* to embryonic movement **(A)** Workflow for the FACS and RT-qPCR experiments shown in panel B and schematic describing the ComiR miRNA target prediction tool used to generate the list of candidate *miR-2b-1* targets. **(B)** Expression analysis (qPCR) of 10 predicted *miR-2b-1* target genes shown as fold change between $\Delta miR-2b-1$ mutant and control w^{1118} embryos (three biological replicates). Targets are listed from top to bottom by descending probability score. [The black control bar, set to 1, represents expression of each gene in control w^{1118} embryos]. Note that upregulation of *CG3638* is statistically significant ($p=0.0169$). **(C)** Schematic of the *CG3638* transcript with *miR-2b-1* target sites indicated (orange lines). **(D)** Whole embryo qPCR experiment showing a reduction of *CG3638* expression in *elav-Gal4>UAS-miR-2b-1* embryos (orange bar), relative to control *elav-Gal4>UAS-GFP* embryos (black bar). **(E)** Evolutionary conservation of the *CG3638* protein across a wide range of invertebrate and vertebrate species (left), as determined with PhylomeDB 5 software (Huerta-Cepas *et al.*, 2014) [*Homo sapiens* and *Drosophila melanogaster* highlighted in yellow]. Gene schematics highlighting the conserved Tweety domain are shown on the right. **(F-I)** Transmembrane domain structure (left) and AlphaFold structural predictions (right) for Human TTYH1 (F-G) and *Drosophila CG3638* (H-I). **(J)** Embryonic movement quantification (summation of MGW deviations) of $\Delta miR-2b-1$, *elav>CG3638-RNAi* embryos (orange bar) during the neurogenic phase compared to control w^{1118} (black bar), $\Delta miR-2b-1$ mutant (bright red bar) and control $\Delta miR-2b-1$, *elav>control-RNAi* embryos (faded red bar). **(K)** qPCR expression profiling of *CG3638* in whole embryos of the genotypes tested in panel J. **(L)** Diagram describing key cell types that form a feedback loop for activity-dependent motor development. Motor neurons (MNs, blue) induce muscle (red) movements which are in turn detected by proprioceptive chordotonal organs (Mechano-ch, orange) and feed-back into the CNS to regulate activity patterns. **(M-N)** Reconstitution experiments that restore *miR-2b-1* expression in specific cellular elements related to embryonic movement circuitry. **(M)** Quantification of larval speed in control w^{1118} (black); $\Delta miR-2b-1$ mutant (red); $\Delta miR-2b-1$, *UAS-miR-2b-1* parental control (pink) and $\Delta miR-2b-1$, *OK371-Gal4>UAS-miR-2b-1* experimental embryos (brown). **(N)** Quantification of larval speed in $\Delta miR-2b-1$, *iav-Gal4>UAS-miR-2b-1* (brown) and control genotypes as in panel M. **(O)** Schematic describing FACS isolation of embryonic chordotonal organs. **(P)** Mature *miR-2b-1* (right) is expressed in chordotonal organs isolated during the neurogenic phase (RP49 expression shown on left). **(Q)** Chordotonal specific qPCR expression profiling of *CG3638* in $\Delta miR-2b-1$ mutant and control w^{1118} embryos. **(R)** Average larval speed of $\Delta miR-2b-1$, *elav>CG3638-RNAi* (orange) compared to control w^{1118} (black), $\Delta miR-2b-1$ mutant (red) and control $\Delta miR-2b-1$, *UAS-control-RNAi* (pink). **(S)** Model for the mechanism by which *miR-2b-1* acts to control embryonic movement in chordotonal organs. Under normal (control) conditions (top), *miR-2b-1* inhibits the expression of *CG3638* and thereby enables normal movement. In $\Delta miR-2b-1$ mutants (bottom), de-repression of *CG3638* expression leads to a reduction in embryonic movement.

A genuine genetic target for a given miRNA is predicted to: (i) be de-repressed (up-regulated) in the absence of the miRNA; and (ii) be down-regulated under miRNA ectopic expression. Analysis of *CG3638* expression shows that this target meets the predictions of a genuine *miR-2b-1* target in full: expression of *CG3638* is upregulated in $\Delta miR-2b-1$ mutants (**Figure 4B**) and reduced under neural over-expression of *miR-2b-1* (**Figure 4D**). As mentioned above, phylogenetic analysis of *CG3638* reveals that it belongs to an evolutionarily conserved family of chloride channel genes [41, 42], with representatives across distantly related lineages of insects and vertebrates including mammals, strongly indicating a functional role (**Figure 4E**). Comparison of the properties of *CG3638* and its human orthologue reveal the characteristic seven transmembrane domains with an external carboxyterminal topology (**Figure 4F**, 4H) further supporting orthology, and applying *AlphaFold* – an artificial intelligence computational method able to predict protein structures with atomic accuracy [43] – to the proteins encoded by the *Drosophila CG3638* gene and the human TTYH1 gene reveals the marked similarities between these two polypeptides (**Figure 4G**, 4I).

To explore the relationship between *CG3638* and the embryonic movement phenotype displayed by $\Delta miR-2b-1$ mutants, we tested the effects of an artificial reduction of *CG3638* in the genetic background of the miRNA mutant (**Figure 4K**). In this scenario, should the levels of expression

of *CG3638* be relevant to the triggering of the embryonic movement phenotype, we predict that a reduction in *CG3638* expression levels should compensate its cellular effects, and, accordingly, reduce or even rescue the embryonic phenotype. The results of this experiment show that this is indeed the case, with embryonic movement of the $\Delta miR-2b-1$ mutant effectively rescued by a reduction in *CG3638* (**Figure 4J**). Based on its modulatory role in embryonic movement we termed *CG3638* as *Movement modulator* (*Motor*).

Mapping the focus of action of miR-2b-1 within the known networks underlying embryonic movement

Having observed the effects of *miR-2b-1* on embryonic movement we wondered about the site of action of this miRNA in regard to circuit components previously linked to embryonic movement. In this respect, previous work has identified embryonic motor neuronal components, as well as interneurons and elements of the sensory system as playing key roles in the control of motor development. These include a motor component that includes all embryonic motor neurons which command the stereotypic array of muscles in the embryonic body wall [44, 45], as well as specific elements of the sensory system – in particular the chordotonal system – which detect early myogenic movements in the embryo and transmit the information to the pattern generators thus modulating motor patterns (**Figure 4L**) [17, 19, 46, 47]. To determine which one of these known circuitry elements might be the principal focus of action of *miR-2b-1* in connection to embryonic movement control we artificially expressed *miR-2b-1* in each motor and sensory circuit elements – using drivers *OK371-Gal4* [48] and *iav-Gal4* [49], respectively – in an otherwise null mutant background for *miR-2b-1*, asking whether these genetic restorations were sufficient to improve or perhaps even rescue normal movement patterns. To ensure that circuit-specific Gal-4 drivers were active and UAS-driven miRNA levels achieved the necessary cumulative values for biological activity we chose to measure effects on early larval movement patterns tested in 30-min old first instar larvae (L1s). The results of these experiments are shown in **Figure 4M-N**, S3A-B. Here we observe that restoring expression of *miR-2b-1* in the motor neuronal domain defined by the OK371 driver is insufficient to affect the defective movement patterns observed in *miR-2b-1* null mutants (**Figure 1B-C** and **Figure 4M**, S3A). In contrast, re-establishing expression of the miRNA in all eight chordotonal organs leads to a full rescue of the motor phenotype (**Figure 4N**, S3B) suggesting that this aspect of the sensory system might be the one where *miR-2b-1* exerts relevant actions during the normal development of movement. In line with this, we observe that the mature *miR-2b-1* transcript is indeed expressed in FACS-isolated embryonic chordotonal organs prepared from wild type embryos (**Figure 4P**) and, that the genetic target of *miR-2b-1*, *Motor*, is also expressed in these cells in normal embryos (**Figure 4Q**, top). In addition, expression of *Motor* in chordotonal organs prepared from *miR-2b-1* null mutants is up-regulated (**Figure 4Q**, bottom) lending further support to a model in which *Motor* is de-repressed in these specific sensory elements. Furthermore, artificial reduction of *Motor* implemented as a stratagem to decrease the effects of de-repression specifically within the chordotonal system is sufficient to rescue normal movement patterns (**Figure 4R**). Altogether, these findings strongly suggest that *miR-2b-1* impacts the emergence of embryonic movement, at least in part, via effects on the sensory circuit components that underlie motor development, rather than affecting the actual generation of motor patterns.

Our work identifies a miRNA system that plays a role in the emergence of embryonic movement in the fly embryo, and offers a new approach to analyse the roles of non-coding RNAs and protein coding genes at the critical period when patterned movement develops. Understanding the molecular elements controlling the onset of motor development in *Drosophila* will put us one step closer to understanding the molecular basis of embryonic movement in other species, including vertebrates, whose embryos seem to undergo remarkably similar transition phases to those reported here [20].

Acknowledgements

We wish to thank all members of the Alonso Lab for helpful discussions and feedback on this work. We are also grateful to Marta Zlatić for sharing *Drosophila* lines, and to the Bloomington Stock Center and the Vienna *Drosophila* Resource Center for providing fly stocks. This research was funded by a Wellcome Trust Investigator Award (098410/Z/12/Z) made to C.R.A., a Wellcome Trust Investigator Award (220277/Z20/Z) made to T.B. and a UK Medical Research Council Project Grant (Ref: MR/S011609/1) given to C.R.A.

Competing interests

The authors declare no competing interests.

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Editors

Reviewing Editor

Claude Desplan

New York University, New York, United States of America

Senior Editor

Claude Desplan

New York University, New York, United States of America

Reviewer #1 (Public Review):

Summary:

This is an experimentally soundly designed work and a very well-written manuscript. There is a very clear logic that drives the reader from one experiment to the next, the experimental design is clearly explained throughout and the relevance of the acquired data is well analyzed and supports the claims made by the authors. The authors made an evident effort to combine imaging, genetic, and molecular data to describe previously unknown early embryonic movement patterns and to identify regulatory mechanisms that control several aspects of it.

Strengths:

The authors develop a new method to analyze, quantitatively, the onset of movement during the latter embryonic stages of *Drosophila* development. This setup allows for a high throughput analysis of general movement dynamics based on the capture of variations of light intensity reflected by the embryo. This setup is capable of imaging several embryos simultaneously and provides a detailed measure of movement over time, which proves to be very useful for further discoveries in the manuscript. This setup already provides a thorough and quantifiable description of a process that is little known and identifies two different phases during late embryonic movements: a myogenic phase and a neurogenic phase, which they elegantly prove is dependent on neuronal activity by knocking down action potentials across the nervous system.

However, in this system, movement is detected as a whole, and no further description of the type of movement is provided beyond frequency and amplitude; it would be interesting to know from the authors if a more precise description of the movements that take place at this stage can be achieved with this method (e.g. motion patterns across the A-P body axis).

Importantly, this highly quantitative experimental setup is an excellent system for performing screenings of motion regulators during late embryonic development, and its use could be extended to search for different modulators of the process, beyond miRNAs (genetic mutants, drugs, etc.).

Using their newly established motion detection pipeline, the authors identify miR-2b-1 as required for proper larval and embryonic motion, and identify an overall reduction in the quantity of both myogenic and neurogenic movements, as well as an increased frequency in neurogenic movement "pulses".

Focusing on the neurogenic movement phenotype the authors use in situ probes and perform RT-PCR on FACS-sorted CNS cells to unambiguously detect miR-2b-1 expression in the embryonic nervous system. The neurogenic motion defects observed in miR-2b-1 mutant embryos and early larvae can be completely rescued by the expression of ectopic miR-2b-1 specifically in the nervous system, providing solid evidence of the requirement and sufficiency of miR-2b-1 expressed in the nervous system to regulate these phases of movement.

To explore the mechanism through which miR-2b-1 impacts embryonic movement, the authors use a state-of-the-art bioinformatic approach to identify potential targets of miR-2b-1, and find that the expression levels of an uncharacterized gene, CG3638, are indeed regulated by miR-2b-1. Furthermore, they prove that by knocking down the expression of CG3638 in a miR-2b-1 mutant background, the neurogenic embryonic movement defects are rescued, pointing that the repression of CG3638 by miR-2b-1 is necessary for correct motion patterns in wild-type embryos. Therefore, this paper provides the first functional characterization of CG3638, and names this gene *Motor*.

Finally, the authors aim to discriminate which elements of the embryonic motor system miR-2b-1/*Motor* are required. Using directed overexpression of miR-2b-1 and *Motor* knockdown in the motor neurons and the chordotonal (sensory) organs, they prove that the miR-2b-1/*Motor* regulatory axis is specifically required in the sensory organs to promote normal embryonic and larval movement.

Weaknesses:

The initial screening to identify miRNAs involved in motion behaviors is performed in early larval movement. The logic presented by the authors is clear - it is assumed that early larval movement cannot proceed normally in the absence of previous embryonic motion - and ultimately helped them identify a miRNA required for modulation of embryonic movement. However, it is possible that certain miRNAs play a role in the modulation of embryonic movement while being dispensable for early L1 behaviors. Such regulators might have been missed with the current screening setup.

<https://doi.org/10.7554/eLife.95209.2.sa1>

Reviewer #2 (Public Review):

Summary:

The manuscript, "A microRNA that controls the emergence of embryonic movement" by Menzies, Chagas, and Alonso provides evidence that *Drosophila* miR-2b-1 is expressed in

neurons and controls the expression of the predicted chloride channel CG3638, here named "Motor". Loss of the miRNA leads to movement phenotypes that can be rescued by downregulation of Motor; using specific drivers, the authors show that a larval movement phenotype (slower movement) can be rescued by knockdown of Motor in the chordotonal organs, suggesting that the increase in Motor found in the chordotonal organs is likely the root of the movement defects. Overall, I found the data presented in the manuscript of reasonable quality and are well enough supported by the presented data.

The genetic and phenotypic analysis seems to be correct. The nicest part of the manuscript is the connection between the loss of a miRNA and finding its likely target in generating a phenotype. The authors also develop some protocols for the analysis of the movement phenotypes which may be useful for others.

<https://doi.org/10.7554/eLife.95209.2.sa0>

Author response:

The following is the authors' response to the original reviews.

Recommendations for the Authors:

Reviewer 1:

(1) Figure legends are too sparing, and often fail to describe with enough detail and accuracy the experiments presented. Especially in a work like this one, which uses plenty of different approaches and techniques and has a concise main text, description in the figure legends can really help the reader to understand the technical aspects of the experimental design. In my opinion, this will also help highlight the effort the authors put into exploring different and often new technical approaches.

We thank Reviewer 1 for highlighting this point and agree with them that the original figure legends lacked detailed information. In this revised version of our paper we edited all figure legends providing higher detail on experiments and information displayed (see Main text p12-16, Supplementary Information p2-5). We hope this change will improve the clarity and accuracy of the description of our experiments.

Reviewer 2:

(1) Is there evidence that the early movement phenotype is actually linked to the larval movement phenotype? I noticed that the chordotonal driver experiment was only examined for larval movement. Is this driver not expressed earlier? Could the authors check the early phenotype using this driver? Are there early drivers that are expressed in chordotonal organ precursors (not panneuronal) and does the knockdown of CG3638 in these specific cells suppress the early phenotype?

(2) More broadly, I would like to understand the function of the early embryonic movements. My concern is that they may only be a sign that the nervous system is firing up. If the rescue of the late miRNA mutant phenotype with chordotonal organ expression is only through a late change in the expression of CG3638, then the larval phenotype is probably not due to a developmental change, but a change in the immediate functioning of the neurons. Would this suggest that the early pulsing is not required for anything, at least at our level of understanding? If the driver is actually expressed early and late, then perhaps the authors could test later drivers to delimit the early and late functions of the miRNA?

The comments by Reviewer 2 in the points above are important and enquire about the biological role of early embryonic movements and whether these movements are linked to later larval activity or are somewhat irrelevant to the behaviour of the animal at later stages.

To address this important question, we conducted a new experiment in which we reduced neural activity specifically in the embryo (i.e. from 10hs AEL until the end of embryogenesis) and tested whether this treatment had any impact on larval movement. If – as put by Rev2 – the ‘early pulsing is not required for anything’ and the larval phenotype emerges from an acute change in neuronal physiology, then our experiment should show no effects at the larval stage. The results shown in Figure S4 (see Supplementary Information, p5) show that this is not the case: artificial reduction of neural activity during embryogenesis leads to a statistically significant reduction in larval speed, similar to that caused by the loss of miR-2b-1. This shows that modifications of embryonic activity impact larval movement.

Furthermore, earlier work on the biological role of embryonic activity identified an activity-dependent ‘critical period’ during late embryogenesis (Giachello and Baines, 2015; Ackerman et al., 2021): manipulations at or around this critical period result in both locomotor and seizure phenotypes in larvae. We cite these papers in the main text (p7).

In addition, two recent papers (Zeng et al., 2021; Carreira-Rosario et al., 2021) – which we cite in the main text (p5) – show that inhibition of muscle activity specifically during the embryonic period prevents the generation of normal neural activity patterns in both, embryo and larva. Similar results are observed when proprioceptive sensory inputs to the central nervous system are blocked, with larval locomotion also disrupted.

Altogether, the data already in the literature plus our new addition to the paper, show that early embryonic movements play a key role in the development of the nervous system and larval locomotion.

(3) Given the role in the larval chordotonal organs, have the authors also checked the adult movements?

The question of whether miR-2b-1 action in chordotonal organs affects behaviour at later stages of the *Drosophila* life cycle is interesting and was the reason why we assessed different genetic manipulations at the larval stage. However, we believe that assessing adult locomotor phenotypes is beyond the scope of this paper.

(4) The authors state that mir-2b-1 is a mirtron. I do not believe this is correct. It is not present in an intron in Btk from what I can see. Also, in the reference that the authors use when stating that mir-2b is a mirtron, I believe mir-2b-1 is actually used as a non-mirtron control miRNA. As mirtrons are processed slightly differently from regular hairpins and often use only the 3' end of the hairpin for miRNA creation, this may not be a trivial distinction.

We are grateful to Rev2 for highlighting this point: indeed, as they say, miR-2b-1 is located in the 3'UTR of host gene Btk, rather than in an intron. Accordingly, in this revision we remove the comment on miR-2b-1 being a mirtron (p6) and deleted the citation accordingly.

(5) For miRNA detection, the authors use in situ hybridization and QPCR. Both methods show that the gene is expressed but not that the mature miRNA is made. If the authors wanted a truly independent test for the presence of the miRNA, a miRNA sensor might be a better choice and it would hint at which part of the hairpin makes the functional miRNA. This is probably not necessary but could be a nice addition.

We thank Rev2 for drawing attention to this point and allowing this clarification. The qPCR protocol we used is based on the method developed by Balcells et al., 2011 (w/303 citations) (see Materials and Methods section in Supplementary Information, p14) which allows the specific amplification of mature miRNA transcripts, and not their precursors. This method for mature miRNA PCR is so robust that it has even been patented (WO2010085966A2). To ensure that the reader is clear about our methods, we state in the main text (p6) that we perform "RT-PCR for the mature miRNA transcript". [NB: miRNA sensors provide a useful method to assess miRNA expression but can also act as competitive inhibitors of physiological miRNA functions, titrating away miRNA molecules from their real targets in tissue; therefore, results using this method are often difficult to interpret.]

(6) Curious about mir-2b-1 and any overlap with the related mir2b-2 and the mir2a genes. I am just wondering about the similarity in their sequences/targets and if they might have similar phenotypes or enhance the phenotypes being scored by the authors.

This is an interesting point raised by REV2 and indeed miR-2b-1 does belong to the largest family of microRNAs in *Drosophila*, the miR-2 family, discussed in detail by Marco et al., 2012. However, we consider that performing tests of additional miRNA mutations, both individually and in combination with miR-2b-1, is beyond the scope of this paper.

(7) Related to this, the authors show that the reduction of a single miRNA target suppresses the miRNA loss of function phenotype. This indicates that this target is quite important for this miRNA. I wonder if the target site is conserved in the human gene that the authors highlight.

This is another interesting comment by Rev2. To pursue their idea, we have performed a blast for the miR-2b-1 target site in the human orthologs of CG3638 and did not find a match suggesting that the relationship between miR-2b-1 and CG3638 is not evolutionarily preserved between insects and mammals.

Public Reviews:

Reviewer #1:

Weaknesses:

The authors do not describe properly how the miRNA screening was performed and just claim that only miR-2b-1 mutants presented a defective motion phenotype in early L1. How many miRNAs were tested, and how candidates were selected is never explicitly mentioned in the text or the Methods section.

We identified miR-2b-1 as part of a genetic screen aimed at detecting miRNAs with impact on embryonic movement, but this full screen is not yet complete. Seeing the clear phenotype of miR2b-1 in the embryo prompted us to study this miRNA in detail, which is what we report in this paper.

The initial screening to identify miRNAs involved in motion behaviors is performed in early larval movement. The logic presented by the authors is clear - it is assumed that early larval movement cannot proceed normally in the absence of previous embryonic motion - and ultimately helped them identify a miRNA required for modulation of embryonic movement. However, it is possible that certain miRNAs play a role in the modulation of embryonic movement while being dispensable for early L1 behaviors. Such regulators might have been missed with the current screening setup. Although similar changes to those described for the neurogenic phase of embryonic movement are

described for the myogenic phase in miR-2b-1 mutants (reduction in motion amplitude), this phenotype goes unexplored. This is not a big issue, as the authors convincingly demonstrate later that miR-2b-1 is specifically required in the nervous system for proper embryonic and larval movement, and the effects of miR-2b-1 on myogenic movement might as well be the focus of future work. However, it will be interesting to discuss here the implications of a reduced myogenic movement phase, especially as miR-2b-1 is specifically involved in regulating the activity of the chordotonal system - which precisely detects early myogenic movements.

We thank Rev1 for their interest in that loss of miR-2b-1 results in a decrease in movement during the myogenic phase, in addition to the neurogenic phase. Indeed, two recent papers (Zeng et al., 2021; Carreira-Rosario et al., 2021) – which we cite in the main text (p5) – show that inhibition of muscle activity during a period that overlaps with the myogenic phase prevents the formation of normal neural activity patterns and larval locomotion. They also observe the same when inhibiting proprioceptive sensory inputs to the central nervous system. This could suggest that the effects of miR-2b-1 on the myogenic phase might have ‘knock-on’ effects upon the later neurogenic phase and larval movement. However, we note that genetic restoration of miR-2b-1 expression specifically to neurons completely rescues the larval speed phenotype (Fig. 3G), suggesting that the dominant effect of miR-2b-1 upon movements is through its action within neurons. To recognise Rev1’s comment we have added a short sentence to the text (p7) suggesting that ‘the effects of miR-2b-1 observed at earlier stages (myogenic phase) are possibly offset by normal neural expression of miR-2b-1’.

FACS-sorting of neuronal cells followed by RT-PCR convincingly detects the presence of miR-2b-1 in the embryonic CNS. However, control of non-neuronal cells would be required to explore whether miR-2b-1 is not only present but enriched in the nervous system compared to other tissues. This is also the case in the miR-2b-1 and Janus expression analysis in the chordotonal organs: a control sample from the motor neurons would help discriminate whether miR-2b-1/Janus regulatory axis is specifically enriched in chordotonal organs or whether both genes are expressed throughout the CNS but operate under a different regulation or requirements for the movement phenotypes.

The RNA in situ hybridisation data included in the paper (Fig. 3B) show that RNA probes for miR2b-1 precursors reveal very strong signal in neural tissue – with very low signal detected in other tissues – strongly indicating that expression of miR-2b-1 is highly enriched in the nervous system.

Reviewer #2:

Weaknesses:

As I mentioned above, I felt the presentation was a bit overstated. The authors present their data in a way that focuses on movement, the emergence of movement, and how their miRNA of interest is at the center of this topic. I only point to the title and name that they wish to give the target of their miRNA to emphasize this point. "Janus" the GOD of movement and change. The results and discussion section starts with a paragraph saying, "Movement is the main output of the nervous system... how developing embryos manage to organise the necessary molecular, cellular, and physiological processes to initiate patterned movement is still unknown. Although it is clear that the genetic system plays a role, how genes control the formation, maturation and function of the cellular networks underlying the emergence of motor control remains poorly understood." While there is nothing inherently untrue about these statements, it is a question of levels of understanding. One can always argue that something in biology is still unknown at a certain level. However, one could also argue that much is known about the molecular nature of movement. Next, I am not sure how much this work impacts the area of study

regarding the emergence of movement. The authors show that a reduction of a miRNA can affect something about certain neurons, that affects movement. The early movements, although slightly diminished, still emerge. Thus, their work only suggests that the function of some neurons, or perhaps the development of these neurons may impact the early movements. This is not new as it was known already from early work from the Bate lab. Later larval movements were also shown to be modified in the miRNA mutants and were traced to "janus" overexpression in the chordotonal organs. As neurons are quite sensitive to the levels of Cl- and Janus is thought to be a Cl- channel, this could lead to a slight dysfunction of the chordotonal neurons. So, based on this, the work suggests that dysfunction of the chordotonal organs could impact larval movement. This was, of course, already known. The novelty of this work is in the genes being studied (important or not). We now know that miR-2b-1 and Janus are expressed in the early neurons and larval chordotonal neurons and their removal is consistent with a role for these genes in the functioning of these neurons. This is not to trivialize these findings, simply to state that these results are not significantly changing our overall understanding of movement and the emergence of movement. I would call it a stretch to say that this miRNA CONTROLS the emergence of movement, as in the title.

As already mentioned in our provisional response, on this point we politely – but strongly – disagree with Rev2's suggestion that the findings are inflated by our language. We also note that they criticise our use of the verb 'control', yet this is a standard textbook term in molecular biology to describe biological processes regulated by genetic factors: given that miR-2b-1 regulates movement patterns during embryogenesis, to say that miR-2b-1 'controls' embryonic movement in the *Drosophila* embryo is reasonable and in line with the language used in the field.

Finally, the name Janus should be changed as it is already being used. A quick scan of flybase shows that there is a Janus A and B in flies (phosphatases) and I am surprised the authors did not check this. I was initially worried about the Janus kinase (JAK) when I performed the search. While I understand that none are only called Janus, studies of the jan A and B genes refer to the locus as the janus region, which could lead to confusion. The completely different molecular functions of the genes relative to CG3638 add to the confusion. Thus, I ask that the authors change the name of CG3638 to something else.

Thank you for spotting this omission. In the revised MS we propose a new name – Movement Modulator (Motor) – for the gene previously described as Janus (CG3638) to avoid annotation issues at FlyBase due to other, unrelated genes that include this word as part of their names. All instances where Janus was used are now replaced by Motor (abstract; main text pages 9–10; Figure 4).

<https://doi.org/10.7554/eLife.95209.2.sa3>