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A microRNA that controls the emergence of embryonic movement

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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 situs*, 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 *Janus* – 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 Janus in embryonic motion. The data are well supported but do not provide mechanistic insight into the emergence of movement while the writing inflates the importance of the conclusions. The authors must change the name of Janus which is already used in Drosophila genetics.

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 *ΔmiR-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*.

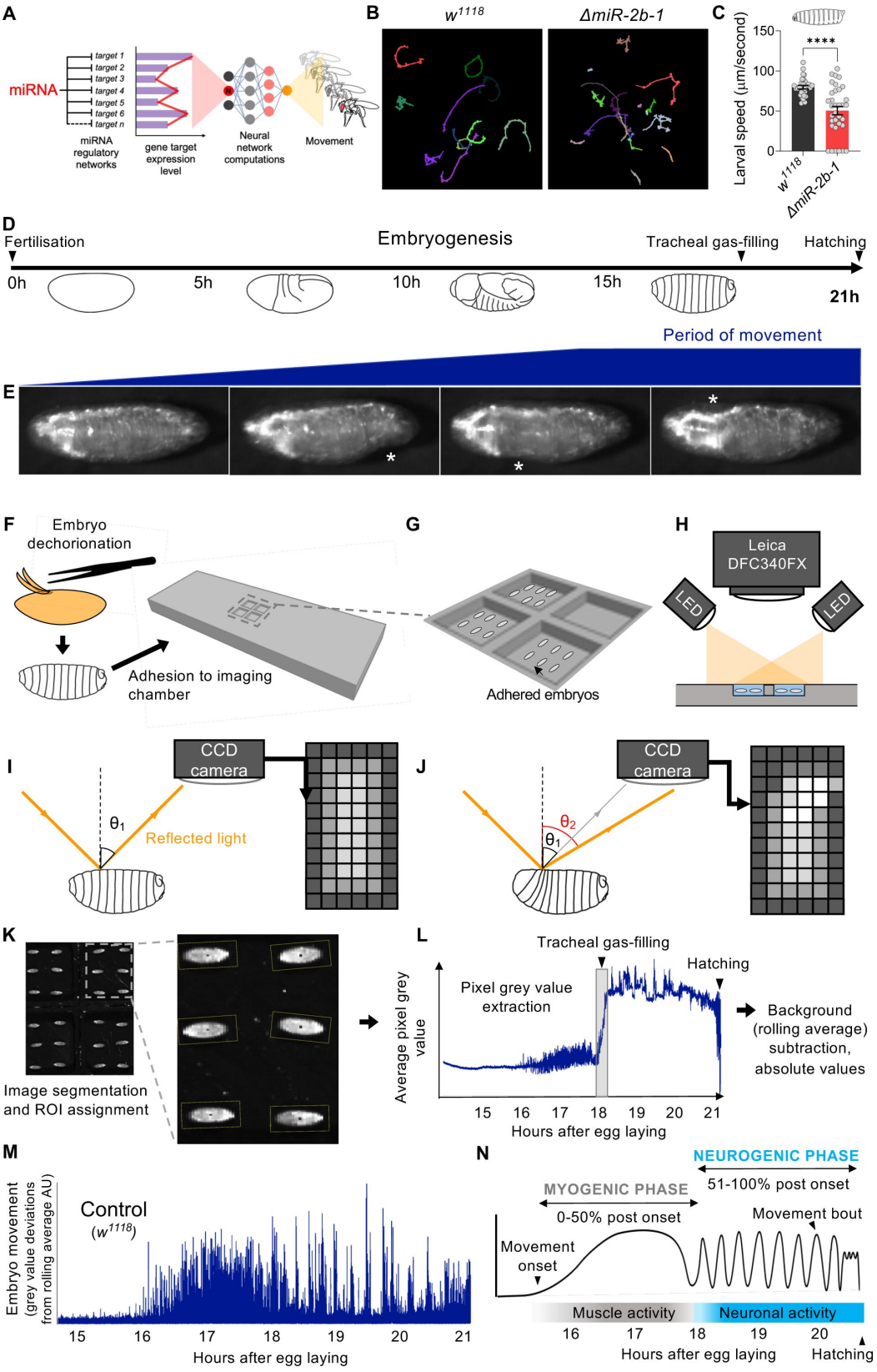
A
miRNA
target 1
target 2
target 3
target 4
target 5
target 6
target n
miRNA regulatory networks
gene target expression level
Neural network computations
Movement
B
w1118
ΔmiR-2b-1
C
Larval speed (μm/second)
150
100
50
0
****
w1118
ΔmiR-2b-1
D
Fertilisation
Embryogenesis
Tracheal gas-filling
Hatching
0h
5h
10h
15h
21h
Period of movement
E
F
Embryo dechorionation
Adhesion to imaging chamber
G
Adhered embryos
H
Leica DFC340FX
LED
LED
I
CCD camera
θ1
Reflected light
J
CCD camera
θ2
θ1
K
Image segmentation and ROI assignment
L
Tracheal gas-filling
Hatching
Pixel grey value extraction
Average pixel grey value
Background (rolling average) subtraction, absolute values
15 16 17 18 19 20 21
Hours after egg laying
M
Embryo movement (grey value deviations from rolling average AU)
Control (w1118)
15 16 17 18 19 20 21
Hours after egg laying
N
NEUROGENIC PHASE
51-100% post onset
MYOGENIC PHASE
0-50% post onset
Movement bout
Movement onset
Muscle activity
Neuronal activity
16 17 18 19 20
Hours after egg laying
Hatching

Figure 1

A novel approach for the quantification of embryonic movement

(A) Schematic describing miRNA action upon target genes to control cell biology and behaviour.

(B) Larval movement tracks for w^{1118} (left) and *ΔmiR-2b-1* (right).

(C) Quantification of larval speed.

(D) Timeline of *Drosophila* embryonic movement.

(E) *Drosophila* embryo performing characteristic movements. (F-H) Experimental pipeline for recording embryo movements.

(I-J) Graphic description of how movements are detected by the experimental setup. (K-M) Analysis pipeline for the assessment of embryonic movements.

(N) Schematic of an embryo movement trace.

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.

Figure 2

miR-2b-1 controls movements during a neurogenic phase of embryonic movement

(A) Representative control (*UAS-Kir*) embryo movement trace.

(B) Control movement frequency spectrogram (FFT).

(C) Representative experimental (*elav-Gal4>UAS-Kir*) embryo movement trace.

(D) Experimental movement frequency spectrogram (FFT).

(E) Quantification of myogenic phase movement.

(F) Quantification of neurogenic phase movement.

(G) Distribution of movement across different periodicities, derived from the FFT frequency analysis shown in panels B and D. Different bars within each period are individual embryos.

(H) Representative control (w^{1118}) embryo movement trace.

(I) Control movement frequency spectrogram (FFT).

(J) Representative experimental (*ΔmiR-2b-1*) embryo movement trace.

(K) Experimental movement frequency spectrogram (FFT).

(L) Quantification of myogenic phase movement.

(M) Quantification of neurogenic phase movement.

(N) Distribution of movement across different periodicities, derived from the FFT frequency analysis shown in panels I and K. Different bars within each period are individual embryos.

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 *ΔmiR-2b-1* mutant embryos (**Figure 2H**, **2J**). These experiments showed that *ΔmiR-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 *ΔmiR-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 a 'mirtron' [30] we prepared an *in situ* probe against *miR-2b-1*'s host gene *Btk*, which encodes the Bruton tyrosine kinase [31, 32] (**Figure 3A**). 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.

Figure 3

miR-2b-1 acts within neurons to regulate embryonic and larval phenotypes

(A) Gene region of *miR-2b-1* with fluorescence *in-situ* hybridisation (FISH) probe for host *Btk*.

(B) FISH experiment.

(C) Fluorescence activated cell sorting (FACS) workflow.

(D) PCR for reference gene *RP49* (left) and mature *miR-2b-1* transcripts (right) from embryonic neurons.

(E) Graphic describing genetic reconstitution of *miR-2b-1* in neurons.

(F) Quantification of neurogenic phase movement.

(G) Quantification of larval speed.

(H) Schematic of fluorescence imaging of calcium dynamics in embryos.

(I) Representative GCaMP6s trace from control (w^{1118}) embryos.

(J) Representative tdTomato trace from the same embryo as in panel I.

(K) Representative ΔF/F trace for control (w^{1118}) embryos.

(L) Representative ΔF/F trace for (*ΔmiR-2b-1*) mutant embryos.

(M) Quantification of sum ΔF/F signal.

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 *ΔmiR-2b-1* null mutant (**Figure 3E**). Results in **Figure 3F** show that *elav*-driven expression of *miR-2b-1* in a *ΔmiR-2b-1* mutant background leads to a phenotypic rescue, producing embryos that 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 *ΔmiR-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**, S2A). 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 *ΔmiR-2b-1* mutants. 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 *ΔmiR-2b-1* mutant embryos by means of immunohistochemistry and confocal microscopy and observed no detectable differences (Figure S2B). 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 [33–35].

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**) [36, 37]. 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[38–41] – 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[36, 37]. 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[42], including humans (**Figure 4E-I**).

Figure 4

The genetic and cellular mechanisms that link miR-2b-1 to embryonic movement

(A) Experimental workflow for FACS and RT-qPCR to assess ComiR miRNA target predictions.

(B) Expression (RT-qPCR) of top 10 potential *miR-2b-1* target genes.

(C) *CG3638* transcript with *miR-2b-1* target sites in the 3' untranslated region (UTR) indicated by orange bars.

(D) Expression (RT-qPCR) of *CG3638* in whole embryos over-expressing *miR-2b-1* in neurons.

(E) Evolutionary conservation of *CG3638*.

(F, H) Transmembrane domains of *Homo sapiens* TTYH1 protein (G) and *Drosophila CG3638* proteins (H).

(G, I) Atomic protein structures for *Homo sapiens* TTYH1 protein (G) and *Drosophila CG3638* protein (I).

(K) Quantification of neurogenic phase movement under genetic inhibition of *CG3638* in all neurons.

(L) Expression (RT-qPCR) of *CG3638* under RNAi.

(M) Schematic of a feedback loop for activity dependent motor-development.

(N) Quantification of larval speed for *miR-2b-1* reconstitution in motor neurons.

(O) Quantification of larval speed for *miR-2b-1* reconstitution in chordotonal organs.

(P) Workflow for FACS isolation of chordotonal organs.

(Q) PCR for reference gene *RP49* (left) and mature *miR-2b-1* transcripts (right) from embryonic chordotonal organs.

(R) Expression (RT-qPCR) of *Janus* (*CG3638)* in chordotonal organs in the *ΔmiR-2b-1* background compared to wild-type.

(S) Quantification of larval speed under genetic inhibition of *Janus* (*CG3638)* specifically in chordotonal organs.

(T) Model for the mechanism by which *miR-2b-1* acts to control embryonic and larval 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 *ΔmiR-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[42, 43], 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 trans-membrane 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 [44] – 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 *ΔmiR-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 *ΔmiR-2b-1* mutant effectively rescued by a reduction in *CG3638* (**Figure 4J**). Based on this role of embryonic movement we termed *CG3638* as *Janus*, making reference to the Roman god of movement and the symbol of change and transitions.

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 [45, 46], 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 motorpatterns (**Figure 4L**) [17, 19, 47, 48]. 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* [49] and *iav-Gal4* [50], 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 M-O**. 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**). In contrast, re-establishing expression of the miRNA in all eight chordotonal organs leads to a full rescue of the motor phenotype (**Figure 4N**) 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-2b1* 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*, *Janus*, is also expressed in these cells in normal embryos (**Figure 4Q**). In addition, expression of *Janus* in chordotonal organs prepared from *miR-2b1* null mutants is upregulated (**Figure 4Q**) lending further support to a model in which *Janus* is de-repressed in these specific sensory elements. Furthermore, artificial reduction of *Janus* 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].

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Competing interests

The authors declare no competing interests.

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Editors

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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 Janus.

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

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.

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.

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.

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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 "Janus". Loss of the miRNA leads to movement phenotypes that can be rescued by downregulation of Janus; using specific drivers, the authors show that a larval movement phenotype (slower movement) can be rescued by knockdown of Janus in the chordotonal organs, suggesting that the increase in Janus 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. That being said, I do have a few problems with the manuscript, mostly stemming from what I feel is an inflated presentation of the importance of the findings.

Strengths:
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.

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.

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.

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Author Response

We are very pleased to hear the overall positive views and constructive criticisms of eLife Editors and Reviewers on our work. In particular, we appreciate their comments highlighting the value of our new pipeline for high-throughput quantification of fly embryonic movement and the positive views of reviewers and editors that our data on the roles of miR-2b-1 in embryonic movement are well supported.

Regarding Reviewer 1, we thank them for their positive comments that our work is experimentally sound and well-written, their kind words on the value of our new embryonic movement pipeline, and their overall appreciation of the quality, scope, and significance of our work. In a revised version of the manuscript we will consider discussing and addressing some of the interesting points raised by Rev1.

Turning to the comments by Rev2, we are grateful to them for their recognition of the novelty of our miRNA findings and appreciation of the utility of our novel quantitative pipeline for

assessing embryonic movement. Nonetheless, we politely – but strongly – disagree with their suggestion that the findings are inflated by our language. For example, 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. It is not inflation. In connection to other comments, in a revised manuscript we will propose a different name for the gene here described as Janus to avoid annotation issues at FlyBase due to other, unrelated genes that include this word as part of their names.