Regulation of miR-184 gene expression in early *Drosophila* development

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By
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Abstract

In *Drosophila melanogaster*, the transcription factor Dorsal initiates dorsal-ventral (DV) patterning in the early embryo by activating or repressing genes, such as the transcription factors Twist and Snail, along the DV axis. Previous studies have shown that the microRNA gene miR-184 in the ventral portion of the embryos (the prospective mesoderm) at the early stages of *Drosophila* embryogenesis. In order to identify the tissue-specific enhancer that regulates miR-184 expression in the mesoderm, we generated transgenic fly lines and observed the expression of a reporter gene under the control of a potential *cis*-regulatory module (CRM). Our results show that this CRM is responsible for the regulation of miR-184 and furthermore, it is activated by Twist.

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List of Abbreviations

3'-UTR	3-prime untranslated region
5'-RACE	5-prime rapid amplification of cDNA ends
AJ	apple juice
Bp	base pairs
bHLH	basic Helix-Loop-Helix
CRM	*cis*-regulatory module
DV	dorsal-ventral
dsRBD	double-stranded RNA-binding domain
EGTA	ethylene glycol tetraacetic acid
hAEL	hours After Egg Laying
kb	kilobases
$MgCl_2$	Magnesium chloride
miRNA	MicroRNAs
NaCl	Sodium chloride
nt	nucleotides
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
Pol II	Polymerase II
pri-mir-184	primary transcript of miR-184
RISC	RNA-induced silencing complex
RT-PCR	reverse transcription polymerase chain reaction
t12Denh	*transfrag 12* Distal enhancer
t12Penh	*transfrag 12* Proximal enhancer
transfrags	transcribed fragments
TSS	transcription start site

Chapter 1: Introduction

Dorsal-ventral (DV) patterning of *Drosophila* is initiated by the formation of a broad nuclear gradient of the protein Dorsal. Dorsal is a transcription factor, which controls the differential expression of genes across the DV axis in a concentration-dependent manner [1]. It is the fly homolog of NFκ-B [2]. Figure 1 shows a cross-section of a cellularizing embryo; there are about 70 nuclei located at the periphery of the embryo [3]. Peak levels of Dorsal are found in the ventral-most region of the embryo (18-20 nuclei), with progressively lower levels of the protein toward the dorsal side. The 36-40 dorsal-most nuclei are devoid of nuclear Dorsal [4]. Genes that are activated by high levels of Dorsal are required for forming the mesoderm [5]. Intermediate levels of Dorsal activate genes that are important for specification of the neurogenic ectoderm [6]. There is no Dorsal or very low Dorsal expression in the ~30 dorsal-most nuclei. There, genes which are repressed by Dorsal can be expressed, and help pattering the dorsal ectoderm.

One of the earliest genes activated by the Dorsal gradient is *twist* [7]. Approximately 20 minutes after formation of the Dorsal gradient, *twist* transcripts will be detected in the prospective mesoderm [8]. Twist is absolutely required for mesoderm formation; mesodermal cells will be missing in the *twist* mutant embryos. Approximately 180 base pairs (bp) upstream of *twist*, there is an enhancer called P enhancer [9], which has two low affinity dorsal binding sites, and can direct lacZ

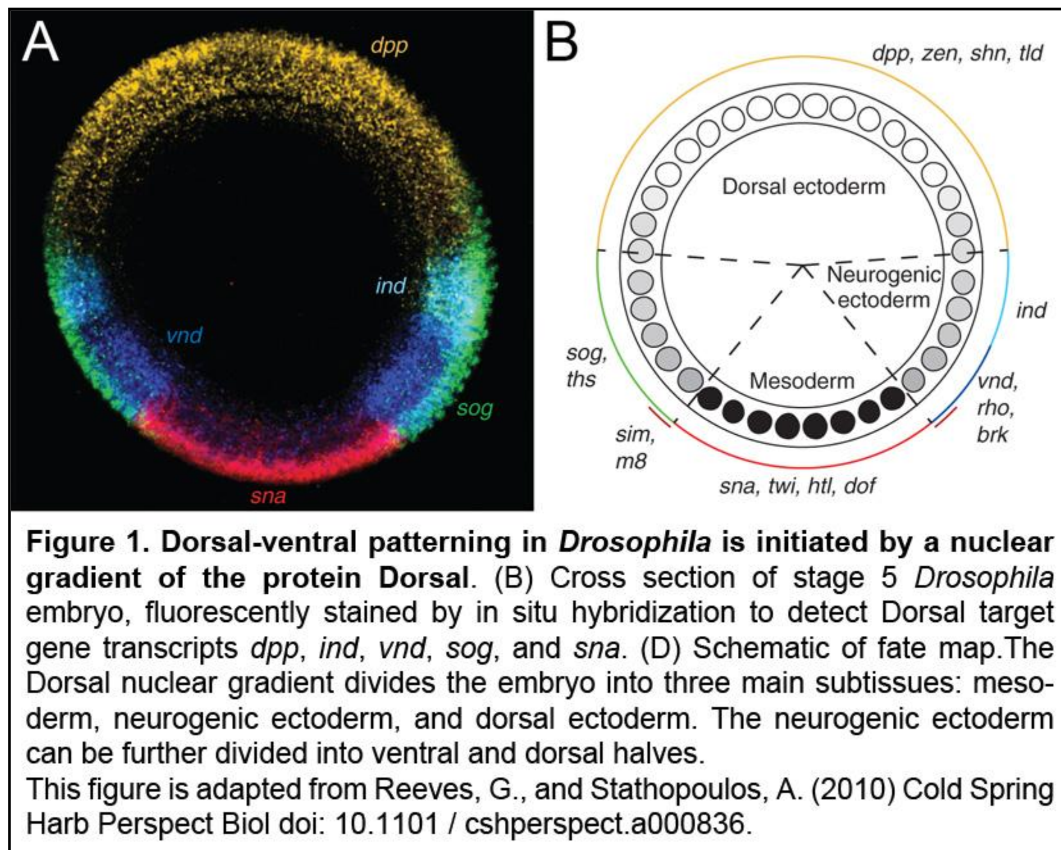

Figure 1. Dorsal-ventral patterning in *Drosophila* is initiated by a nuclear gradient of the protein Dorsal. (B) Cross section of stage 5 *Drosophila* embryo, fluorescently stained by in situ hybridization to detect Dorsal target gene transcripts *dpp*, *ind*, *vnd*, *sog*, and *sna*. (D) Schematic of fate map.The Dorsal nuclear gradient divides the embryo into three main subtissues: mesoderm, neurogenic ectoderm, and dorsal ectoderm. The neurogenic ectoderm can be further divided into ventral and dorsal halves.
This figure is adapted from Reeves, G., and Stathopoulos, A. (2010) Cold Spring Harb Perspect Biol doi: 10.1101 / cshperspect.a000836.

reporter gene to express in the ventral region of embryo [10]. The two low-affinity binding sites are sufficient to respond to the high levels of Dorsal. While the low affinity binding sites respond to high level of Dorsal, high affinity sites are necessary to respond to intermediate levels. If the two low affinity binding sites are converted into high affinity sites, the twist P enhancer will not only respond to high levels of Dorsal but also intermediate levels of Dorsal gradient. The activity of Twist is essential for the expression of multiple target genes involved in mesoderm specification as well as differentiation [11]. The second gene activated in the prospective mesoderm is *snail*. *snail* expression is controlled synergistically by both Dorsal and Twist. Snail is a transcriptional repressor that can restrict expression neuroectodermal genes, hence neural fate in the invaginating mesoderm [7, 12].

In a screen for non-protein coding genes differentially expressed along the dorsal-ventral axis in *Drosophila*, Dr. Biemar and his colleagues identified 23 uncharacterized transcribed fragments (*transfrags*) [13]. Among these, *transfrag 12* is located upstream of the microRNA gene miR-184. Further work by our lab showed that *transfrag 12* corresponds to the primary transcript of miR-184 (*pri-mir-184*; Figure 3, Faggins et al. unpublished).

MicroRNAs (miRNA) are small non-protein coding RNA approximately 22 nucleotides (nt) long. They are found in all eukaryotes and regulate mRNA expression by binding to the 3-prime untranslated region (3'-UTR) of their targets. miRNA genes are usually transcribed by the RNA Polymerase II (Pol II) into primary transcripts called the *pri-mirna*. The size of *pri-mirnas* can range from hundreds to thousands of nucleotides [14, 15]. The *pri-mirna* is cleaved into ~70-nt hairpin precursor called the pre-miRNA by a mutiprotein complex, which contains the RNase III enzyme Drosha and a double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha [16]. This hairpin precursor molecule is exported to the cytoplasm where it is cleaved into mature ~22-nt miRNA by another RNase III enzyme Dicer. The dsRBD protein Loquacious then recruits the Argonaute protein, and together with Dicer they form a ribonucleoprotein complex called RNA-induced silencing complex (RISC) [17]. The mature miRNA will be incorporated into RISC by interacting with Dicer and the dsRBD proteins, and establish imperfect base pairing with its targets [18]. Target gene expression is downregulated either by cleavage and degradation, or by translational

repression. The target mRNAs are cleaved and degraded when perfect or near perfect base pairing to the miRNA occurs. Imperfect base pairing will otherwise lead to translational repression.

miRNAs have been shown to have multiple functions, such as influences on the development of *Drosophila* to different degrees, the regulation of developmental switches in *C. elegans*, or even the regulation of cell proliferation, differentiation and apoptosis [19]. The methods of identification of miRNA targets have been improved since the first animal miRNA targets were found in *C. elegans* [20, 21]. However, due to the large number of predicted targets of miRNA and the poor understanding of miRNA-target relationship [22], the identification of biologically relevant miRNA-mRNA target interactions *in vivo* remains a challenge. The expression patterns of miRNA will help predict miRNA-target relationship *in vivo*.

In addition, as miRNAs are believed to regulate thousands of mRNAs, their misexpression may lead to down regulation of a large number of targets and can cause strong phenotypes, for instance, cancerous phenotypes [23]. For instance, work by our lab has shown that while miR-184 is strongly expressed in the mesoderm before and during gastrulation, it is absent from the mesoderm after that stage (F.Biemar, unpublished). Prolonged expression of miR-184 in the mesoderm at those later stages leads to severe defects in muscle formation. Moreover, several groups have reported potential oncogenic roles for miR-184[24].

Thus, regulation of miR-184 expression has become a very interesting subject. The temporal and spatial expression of miRNAs is regulated by tissue-specific enhancers (or *cis*-regulatory modules; CRMs), much like protein-coding genes [25]. If we can find the *cis*-regulatory module responsible for regulating miR-184, we may able to understand how miR-184 is restricted to mesoderm.

Chapter 2: Material and Methods

2.1 Plasmid constructions

To construct the plasmid *t12Penh-LacZ*, a 500 bp fragment was amplified from genomic DNA by polymerase chain reaction (PCR) using the following primers FB-p538 (5'-CGAGTCCAACAATGAGACCA-3') and FB-p539 (5'-GCCCTGAAATCCATTG AAAA-3'). The amplified fragment was cloned into the pGEM®-T easy vector (Promega) and verified by sequencing before subcloning into the -42-eveLacZ [26] *P* element vector. For subcloning, the -42-eveLacZ vector was digested by NotI and dephosphorylated using standard procedures (Sambrook 1989). The *t12Penh* NotI fragment was excised from the pGEM®-T easy vector and gel purified with the QIAquick® gel extraction kit (QIAGEN®). The *t12Penh* NotI fragment was then ligated into the NotI site of the dephosphorylated -42-eveLacZ vector using standard protocols (Sambrook 1989).

To construct the plasmid *t12PenhΔEbox-LacZ*, the QuickChange® site-directed mutagenesis kit (Stratagene) was used with the following primers: FB-p915 (5'-CCTC GTATATGTgatccaaTAAGTATACAATTGTAAGGGA-3') and FB-p937 (5'-TTTCGACCA ACCAAATTATTGA-3').

2.2 Preparation of DNA for injection

To prepare the DNA for injection, the transgenic vector and helper plasmid (∆2,3 transposase) was mixed in a 10:1 ratio (e.g., 20 ul of the transgenic vector and 2 ul of the helper plasmid in 78 ul of dH2O). The mix was precipitated by adding 1⁄2 vol. of Sodium acetate and 10 vol. of 100% Ethanol into the solution and incubation at -80ºC for 30 minutes. The mix was centrifuged for 15 minutes at 4ºC. The supernatant was discarded, and 100 ul of 70% Ethanol was added to the pellet and incubated for 5 minutes at room temperature. The pellet was then centrifuged at 4ºC for 10 minutes and dried in a speedvac. The injection mix was filtered through Millex-GV 0.22 um column (Millex-GV, Cat# SLGV004SL) into 0.5 ml microcentrifuge tube and stored at -20ºC.

2.3 Embryo injection

Young fly embryos were collected by using apple juice (AJ) plate supplemented with yeast. The first-hour collection was discarded because there might be older embryos retained by the mother. The embryos were collected every 10-15 minutes to make sure they would still be young enough when they were injected. The injected DNA can only be carried on and inherit to the next generation when they are injected into the embryo before the initiation of germline development. 100% bleach was used to remove the chorion. The embryos were then aligned on a microscope slide (Fisherbrand. Cat# 12-550-34) prepared with double-sided tape on one side. All the

embryos were kept in the same orientation, with their posterior pole facing the edge of the taped slide. Embryos were desiccated for 4-5 minutes and covered with halocarbon oil (Halocarbon Products Corporation; Series 700, cat#9002-83-9). The injection needles were prepared from borosilicate glass capillaries (WPI, Cat#1B100F-4) using a horizontal puller (Sutter instruments, PE-87) and back-loaded with ~3 ul of DNA mix. The needle was broken by moving the slide slowly toward the needle tip. If the needle is properly broken, it will have a nice sharp surface, with 0.1-0.5 nl of DNA mix per injection. The needle tip and the posterior tip of the embryo were adjusted into the same focal plane. The needle was slowly inserted into the posterior part of the embryo no further than $1/10^{th}$ of the embryo's length. After all the embryos on a slide had been injected, the embryos were incubated in a humidified chamber at room temperature in the oil for 24 hours for them to develop. Live larvae were individually picked and transferred in a vial of moist fly food. The vials were left at 25ºC for 8-10 days.

All the flies were crossed separately with wild-type flies of the opposite sex. Transformants (i.e. red-eyed flies) were isolated from the offspring and back-crossed one more time to the wild-type stock to establish stable transgenic lines.

2.4 Embryo collection and fixation

In order to collect embryos for whole-mount *in situ* hybridization, ~30-40 adult female flies were put together with ~10-20 males in mating cages, and fed regularly

with AJ plates supplemented with yeast paste for 1-2 days. To collect early stages embryos, between 2 and 4 hAEL (hours After Egg Laying), flies were allowed to lay eggs on a fresh AJ plate for 2 hours. The AJ plate was then replaced by a new one and the 0-2 hour's collection was aged for another 2 hours in a closed and clean environment. Prior to fixation, the 2-4 hAEL embryos were dechorionated in a 100% bleach solution for 3 minutes. The dechorionated embryos were rinsed abundantly with water and a solution of Triton-X/NaCl (100gr NaCl (M.W. 58.44, Fisher,cat#S271-3), 15ml TritonX-100 (Sigma, cat#T9284-500ML), up to 1L dH2O). The embryos were then placed in the fixation solution (1ml of 37% Formaldehyde, 3ml of Fixing Buffer (1.33X PBS, 66.6 uM EGTA (M.W. 380.35; Sigma, cat#E3889-100G), dH2O) and 4 ml of Heptane (Sigma, cat#H3393-250KU)). The fixation was performed on an orbital shaker for 25 minutes in order to weaken the vitelline membrane. The aqueous phase was removed and 8 ml of Methanol was added to and shaken vigorously for 2 minutes in order to remove the vitelline membrane. The embryos were further washed with methanol 3-4 times, and stored at -20ºC.

2.5 Whole-mount in situ hybridization

Fixed embryos were used, 30-50 ul per sample. The embryos were washed in ethanol 3 times for 2 minutes and rocked in 400ul ethanol and 600 ul Xylenes for 45 minutes. Then the embryos were washed with ethanol for 5 times and methanol 2 times for 5 minutes each. They were rocked 5 times in PBT (1X PBS, 0.1% Tween 20, dH2O) for 2

minutes and incubated in ProtK/PBT solution (10 mg/ml Proteinase K (Sigma; P-2308)) for 8 minutes to permeabilize the embryos. To stop the reaction, the embryos were rinsed once quickly with PBT, then fixed in PBTF (1X PBS, 5% Formaldehyde, 0.1% Tween 20 (Sigma, Ultra, 9005-64-5), dH2O) for 25 minutes. The embryos were then washed with PBT and pre-hybridized in HybMix (50% deionized Formamide, 5X SSC, 100 ug/ml heparin, 0.1% Tween 20, dH2O) at 55ºC for 2 hours. Hybridization was done at 55ºC overnight in 200 ul HybMix to which probes were added. The mixes were washed several times with HybMix and PBT and rocked the embryos overnight at 4ºC antibody solution (anti-DIG, (Roche, CAT# 11333089011)).

The embryos were washed with PBT and Staining Buffer (5 ml of 1M Tris-HCL pH 9.5, 1 ml of 5M NaCl, 2.5 ml of 1M $MgCl_2$, dH_2O) and transferred to a glass dish within 400 ul of staining solution (0.9% NBT (Sambrook 1989), 0.7% BCIP (Sambrook 1989) in staining buffer). The stained embryos were washed with ethanol and mounted on the slide with Permount (Fisher, Cat#108-88-3).

Chapter 3: Results

3.1 PRELIMINARY WORK IN OUR LAB

3.1.1 Identification of microRNAs expressed in the mesoderm in *Drosophila*.

Preliminary work by Dr. Biemar and colleagues identified microRNAs and other non-protein coding RNAs differentially expressed along the dorsal-ventral axis (Biemar et al. 2006). One of these non-coding RNAs, *transfrag 12*, is expressed

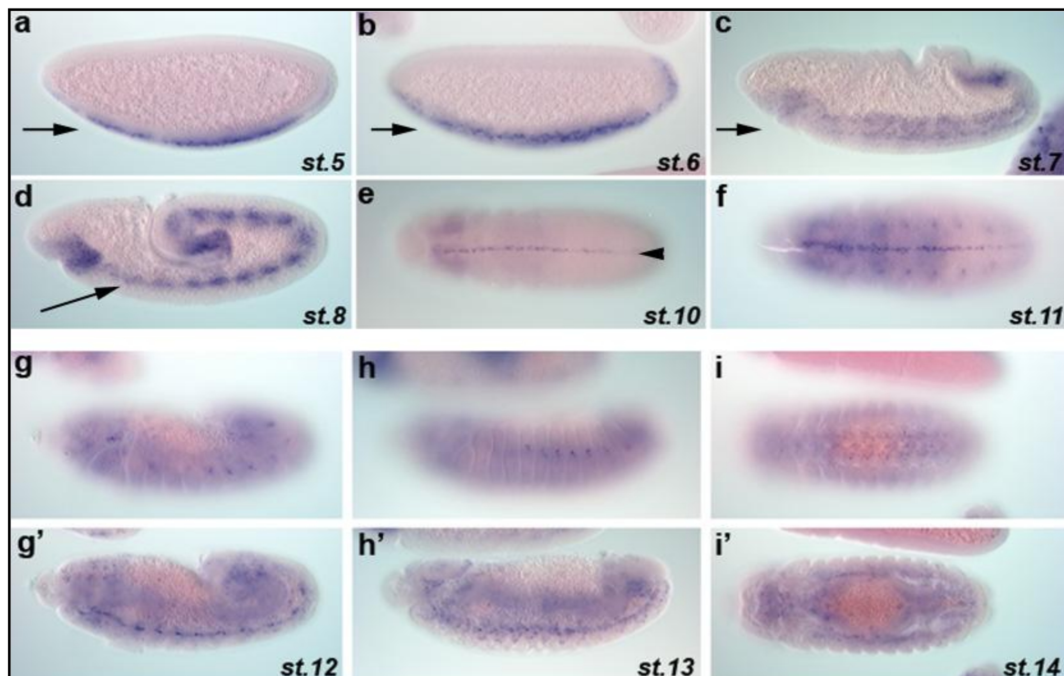

Figure 2. *transfrag 12* expression pattern throughout embryogenesis. For all embryos, anterior is to the left. Ventral views are depicted in e, f, i and i'. Lateral views are depicted for all other embryos with the dorsal surface to the top. (a-d) Mesodermal expression (arrows) is readily observed from early blastula stage (stage 5), through embryos undergoing germ-band elongation (stage 8). (e, f) ventral views of embryos after completion of germ-band elongation showing expression in the midline (black arrowhead). Additional expression is detected in the ectoderm at stage 11. (g-i') ectodermal expression of *transfrag 12* during germ-band retraction. g, h and i are "surface views", whereas g', h' and i' represent sagittal views.

specifically in the presumptive mesoderm of the early *Drosophila* embryo (stg.5; Figure 2A). Whole-mount in situ hybridization experiments revealed a highly dynamic spatial expression of *transfrag 12* during embryogenesis. *transfrag 12* remains expressed in the mesoderm until after gastrulation (Figure 2a-d). At later stages, *transfrag 12* transcripts are no longer detected in the mesoderm, but instead in the mesectoderm (stg.11; Figure 2e) and ectoderm (Figure 2f-i').

3.1.2 *transfrag 12* is the primary transcript of miR-184.

The locus encoding *transfrag 12* is located in an intergenic region that spans approximately 52 kilobases (kb) and also contains the microRNA gene miR-184. To test the hypothesis that *transfrag 12* and miR-184 are part of the same transcript,

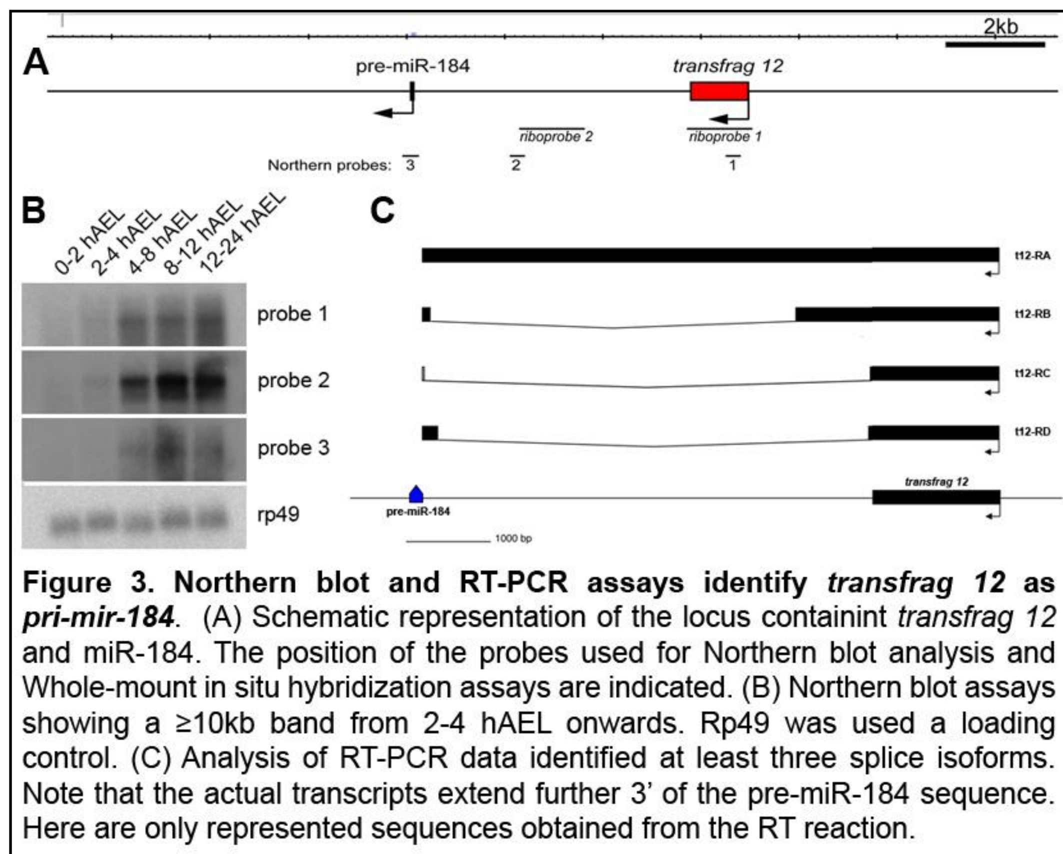

Figure 3. Northern blot and RT-PCR assays identify *transfrag 12* as *pri-mir-184*. (A) Schematic representation of the locus containint *transfrag 12* and miR-184. The position of the probes used for Northern blot analysis and Whole-mount in situ hybridization assays are indicated. (B) Northern blot assays showing a ≥10kb band from 2-4 hAEL onwards. Rp49 was used a loading control. (C) Analysis of RT-PCR data identified at least three splice isoforms. Note that the actual transcripts extend further 3' of the pre-miR-184 sequence. Here are only represented sequences obtained from the RT reaction.

Athenesia Faggins, a graduate student in the lab, performed 5'-RACE (5-prime rapid amplification of cDNA ends) and RT-PCR (reverse transcription polymerase chain reaction) assays. These experiments revealed that *transfrag 12* and miR-184 are indeed part of the same transcript. Northern blots assays detected the presence of a ~10kb transcript that encompasses both *transfrag 12* and miR-184 (Figure 3). Together, these data suggest that *transfrag 12* is the primary transcript for miR-184. We will refer to *transfrag 12* as *pri-mir-184* hereafter.

3.2 Investgation of the Regulation of miR-184 Expression

3.2.1 Computational identification of putative *cis*-regulatory modules.

The *cis*-regulatory modules (CRMs) associated with genes activated by Dorsal in the prospective mesoderm harbor common features. These include some combination of low-affinity binding sites for Dorsal itself, and one or more binding site(s) for Twist. Twist is a transcriptional activator of the bHLH (basic Helix-Loop-Helix) family. The bHLH factors bind DNA motifs called "E boxes". This stereotypical clustering of Dorsal and Twist binding sites can be identified by bioinformatics methods. Using "flyenhancer" [27], we identified two candidate CRMs (Figure 4). The first one is located approximately 1.1 kb upstream of the transfrag12 transcription start site (TSS). This putative CRM, which we named *t12Penh* (*transfrag 12* Proximal enhancer),

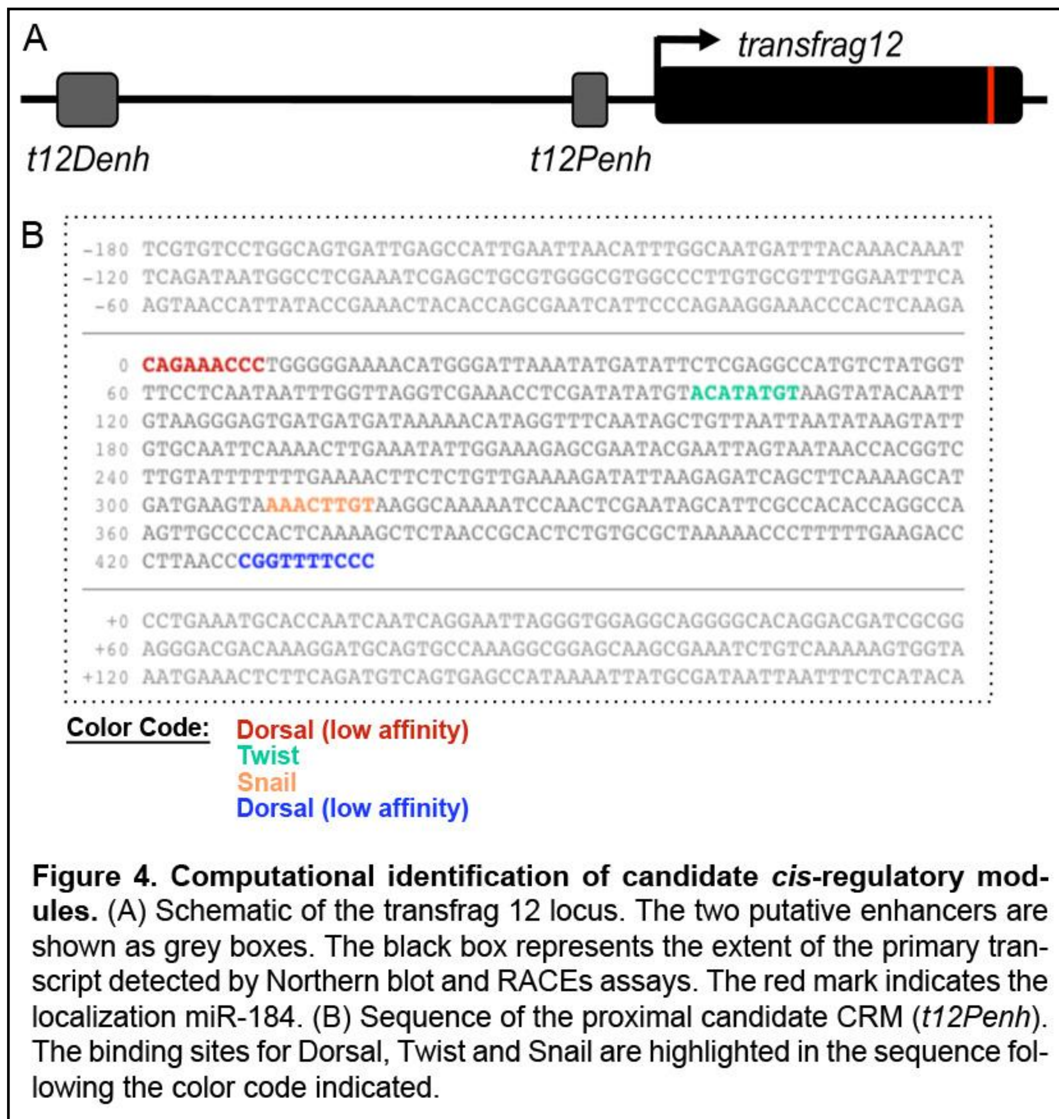

Figure 4. Computational identification of candidate *cis*-regulatory modules. (A) Schematic of the transfrag 12 locus. The two putative enhancers are shown as grey boxes. The black box represents the extent of the primary transcript detected by Northern blot and RACEs assays. The red mark indicates the localization miR-184. (B) Sequence of the proximal candidate CRM (*t12Penh*). The binding sites for Dorsal, Twist and Snail are highlighted in the sequence following the color code indicated.

contains two low affinity-binding sites for Dorsal, one Ebox (Twist binding site) and one Snail binding site. The second one is located approximately 13 kb upstream of the *transfrag 12* TSS. This putative CRM, which we named *t12Denh* (*t*ransfrag *12* Distal enhancer), contains three low affinity-binding sites for Dorsal, one Twist binding site and one Snail binding site.

3.2.2 The *t12Penh* drives mesodermal expression.

To test the hypothesis that *t12Penh* acts as a CRM to control the expression of

pri-mir-184 in the mesoderm, we prepared a reporter gene construct by placing the DNA fragment corresponding to *t12Penh* upstream of the bacterial gene encoding β-galactosidase (*t12Penh-lacZ*). This *P* element reporter construct was then injected *Drosophila* embryos in order to establish transgenic lines. *P* element-mediated transformation is frequently used in *Drosophila* to induce mutations, or simply generate transgenic lines of interest [28]. The expression pattern of *lacZ* should reflect the expression of the endogenous gene. In our case, we expect that, if the *t12Penh* drives the expression of miR-184 in the mesoderm, it will also drive the expression of *lacZ* in the same region. Flies used for the *P* element transgenesis have defect in their *white* gene; therefore, they have white eyes. When the *white* gene is successfully introduced into the embryo –as part of the *P* element– the gene will be inherited to the next generation and gave them red eyes. Thus, the progeny of these injected flies (F1 generation) will have red eyes if they are transgenic for our construct. Because the insertion of the transgene is random, four independent lines were tested to control for possible effects of the chromosomal location.
The four lines were bred separately and embryos were collected at different developmental stages. We used colorimetric whole-mount in situ hybridization to visualize the spatial distribution of RNA.

The expression pattern for LacZ in the *t12Penh 7* line is shown in Figure 5. Expression is indeed detected in the mesoderm in the cellular blastoderm embryos (stage 5) (Figure 5A). During gastrulation (stages 6-9), the LacZ expression remained in the

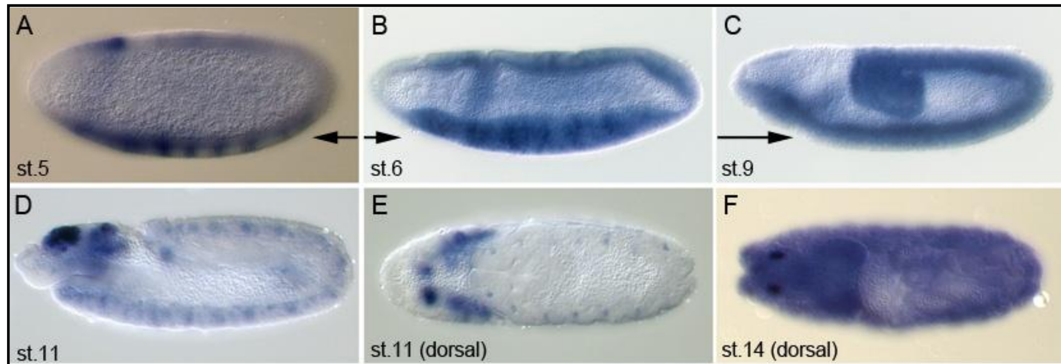

Figure 5. Distribution of LacZ under the control of t12Phenh in line 7. (A) cellular blastoderm (stage 5), (B) gastrulation stage (stage 6), (C) germ band extension (stage 9), (D, E) segmentation (stage 11), and (F) dorsal closure (stage 14). Expression is detected in the mesoderm (arrows) up until stage 11. From then on, the expression is strictly in ectoderm derivatives. Unless otherwise noted, all embryos are shown in lateral views with anterior to the left and dorsal to the top.

presumptive mesoderm, and some showed in the posterior midgut (Figure 5B, C). At later stages (stages 11-14), LacZ was no longer detected in the mesoderm (Figure 5E, F).

A similar expression pattern for LacZ is observed for the t12Penh 8 line shown in figure 6. Expression is seen in the mesoderm prior to and during gastrulation. As the embryos develop, the expression disappears in the mesoderm and can be found in

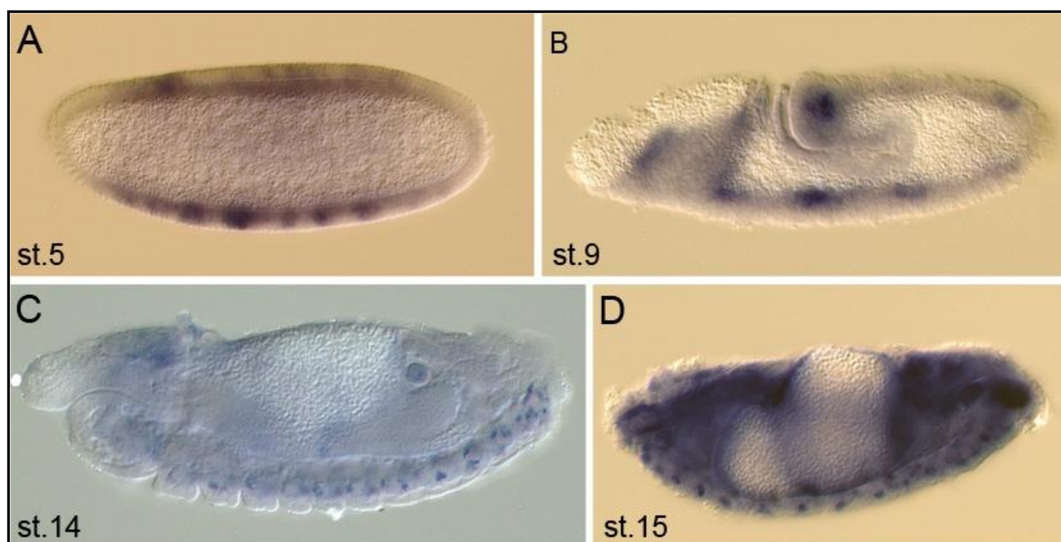

Figure 6. Distribution of LacZ under the control of *t12Phenh* in line 8. (A) stage 5 , (B) stage 9, (C) stage 14, and (D) stage 15. All embryos are shown in lateral views with anterior to the left and dorsal to the top.

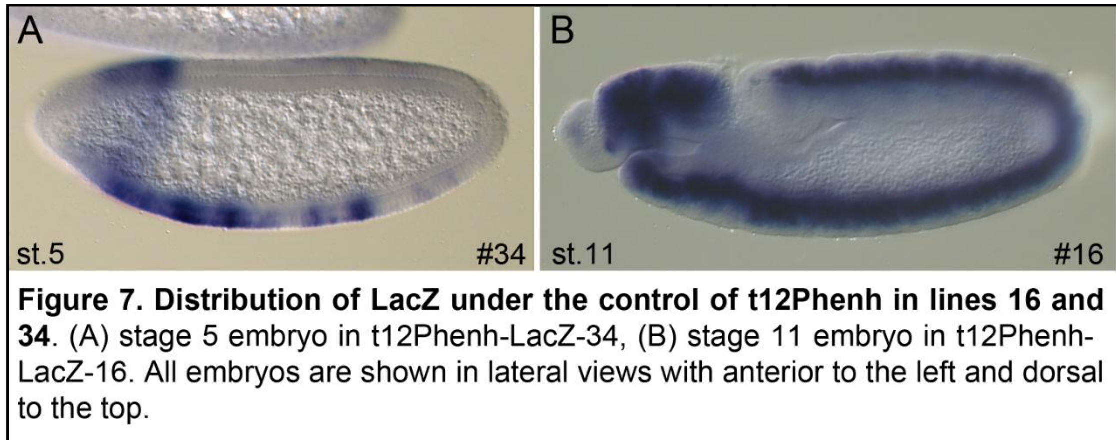

Figure 7. Distribution of LacZ under the control of t12Phenh in lines 16 and 34. (A) stage 5 embryo in t12Phenh-LacZ-34, (B) stage 11 embryo in t12Phenh-LacZ-16. All embryos are shown in lateral views with anterior to the left and dorsal to the top.

the nervous system. The same expression is found in two additionl lines. Selected pictures are showed in figure 7.

Thus, all four lines show expression of *lacZ* in the mesoderm prior to and during gastrulation, similar to the expression pattern seen for *pri-mir-184* in wild-type embryos. We conclude that the *t12Penh* represents a bone fide *cis*-regulatory module of miR-184.

3.2.3 The Ebox is required for the activity of the t12Penh.

This enhancer contains two low-affinity binding sites for Dorsal, one Twist binding site (Ebox) and one Snail binding site. To test the hypothesis that Twist is required for the expression of miR-184 in the mesoderm, we next tested the activity of an enhancer harboring a mutation in the Ebox (*t12Penh∆Ebox*). Transgenes harboring the ΔEbox construct were injected into embryos using the same procedure as t12Penhancer lines. Five independent lines were tested: t12PenhΔEbox 2, 3, 7, 8, 16. As above, we used colorimetric whole-mount *in situ* hybridization to visualize the spatial distribution of RNA in developing embryos. As shown in Figure 8, expression

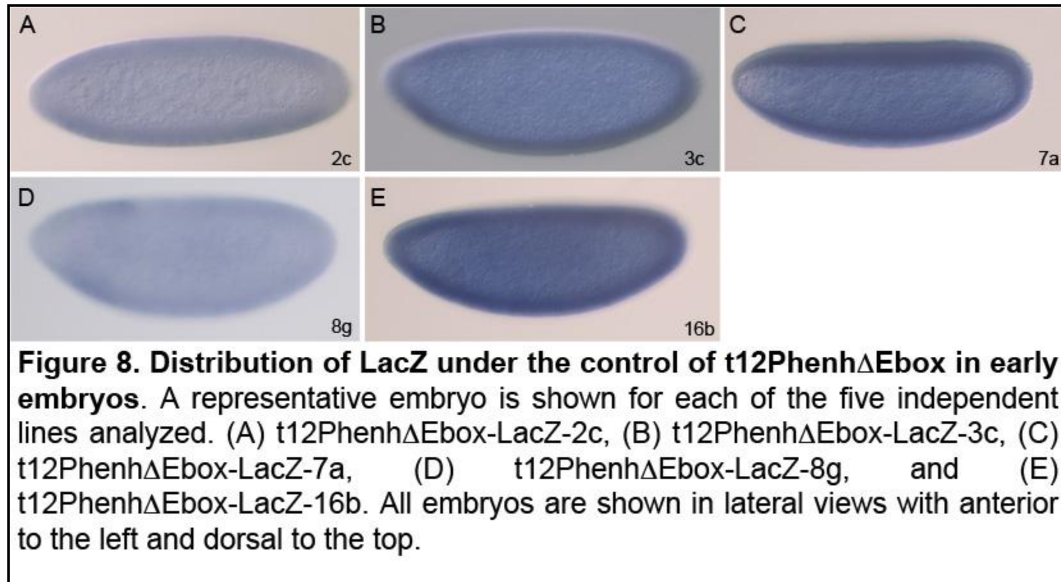

Figure 8. Distribution of LacZ under the control of t12PhenhΔEbox in early embryos. A representative embryo is shown for each of the five independent lines analyzed. (A) t12PhenhΔEbox-LacZ-2c, (B) t12PhenhΔEbox-LacZ-3c, (C) t12PhenhΔEbox-LacZ-7a, (D) t12PhenhΔEbox-LacZ-8g, and (E) t12PhenhΔEbox-LacZ-16b. All embryos are shown in lateral views with anterior to the left and dorsal to the top.

of LacZ is not detected in the early embryo in any of the five independent lines analyzed.

We next analyzed the t12PenhΔEbox2c line in greater detail (Figure 9). Compared to the corresponding stages in t12Penh lines (Figures 5-7), which show strong and clear expression patterns, the t12PenhΔEbox2c embryos show no obvious expression which can be distinguished from the background.

We conclude that the Twist binding site is absolutely required for the activity of the *pri-mir-184* proximal enhancer.

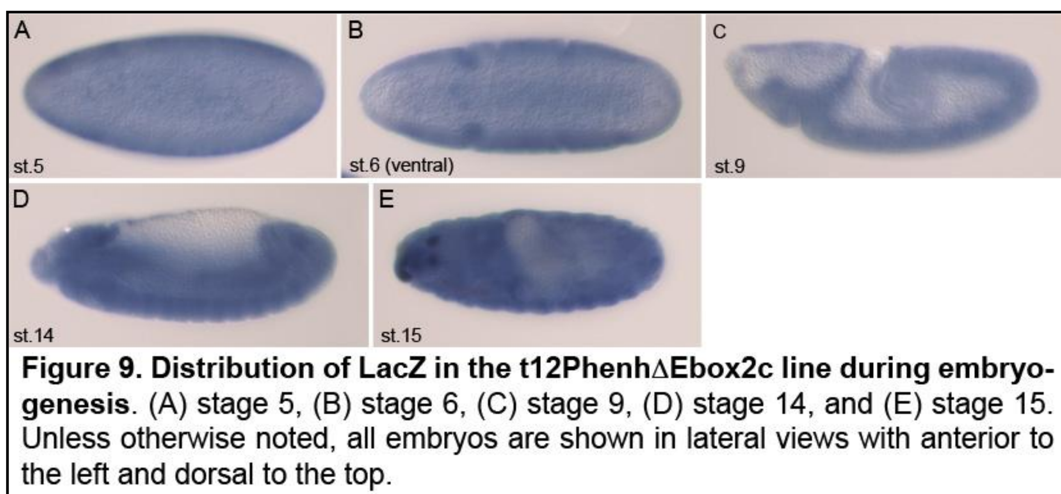

Figure 9. Distribution of LacZ in the t12PhenhΔEbox2c line during embryogenesis. (A) stage 5, (B) stage 6, (C) stage 9, (D) stage 14, and (E) stage 15. Unless otherwise noted, all embryos are shown in lateral views with anterior to the left and dorsal to the top.

Chapter 4: Discussion

In this study, we focused on *t12Penh*, one of the two putative *cis*-regulatory modules for *pri-mir-184* identified by computational methods. We used *P* element-mediated transgenesis to show that *t12Penh* drives mesodermal expression of a reporter gene. In the early embryo, the expression pattern of *t12Penh-lacZ* is essentially identical to the expression pattern of *pri-miR-184*. Future work will involve the study of the other module, *t12Denh*, and its probable long-range interactions between the enhancer and the mRNA. As the *t12Denh* is located approximately 13 kb upstream of the TSS of *transfrag12*, the *t12Denh* may loop to the promoter region of miR-184 in order to regulate expression [29]. Those looping enhancers require a distance from the gene. The regulation cannot process when the enhancer is placed just next to the gene [30]. It has been proposed that this might be because after looping takes place, other transcription factors such as the Mediator complex, might be activated [31]. It will also be interesting to see if the *t12Denh* acts as a “shadow enhancer” [32]. If this is the case, both *t12Denh* and *t12Penh* would drive the same expression pattern and either of them might be sufficient [33], but they are both required for the accurate and reliable expression pattern for miR-184 and in response to environmental and genetic variation [34, 35].

Furthermore, by mutating the Ebox within the *t12Penh-lacZ*, we also showed that the binding site for Twist is required for the activity of t12Penh. This suggests that Twist is required for the activation of miR-184 expression in the mesoderm. These results

should be confirmed by analyzing the expression of both endogenous miR-184 and *t12Penh-lacZ* in *twist* mutant embryos. If Twist is required, we predict that expression will be abrogated in both situations. There are two low affinity dorsal binding sites in the t12Penh. What is the role of the Dorsal binding site? Will there be differences of expression if we mutate the Dorsal binding site? If there are differences, will they be shown as the level of expression pattern or by some other ways?

By combining data of all the stages, we are able to track miR-184 throughout the entire embryo development. The expression of miR-184 is restricted to the mesoderm at early stages, but disappears at later stages. Similarly, the *t12Penh* is not longer active at post-gastrulation stages. This suggests that the protein binding site for the transcription factor responsible for the repression of miR-184 in the mesoderm is present in the sequence of *t12Penh*.

These data show us spatial and temporal expression of *transfrag12*. From those data, we can conclude that *t12Penh* drives mesoderm expression, and the Ebox is required for the activity of *t12Penh.* However, in the absence of additional evidence, we cannot draw conclusions about the function of miR-184. These data do not imply that miR-184 is actually needed in all the cells in which it is expressed, neither necessarily suggest the presence of miR-184 plays a role during all the stages it is expressed. Those are questions that need to be addresses in future experiments.

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