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線蟲遠頂細胞遷移中 *dpy-24* 的功能探討及其異時性調控

Functional Study of *dpy-24* and its Heterochronic Regulation in Distal Tip Cell Migration in *Caenorhabditis elegans*

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本論文係學生卓峻逸在國立臺灣大學生命科學院分子與細胞生物學研究所就讀期間，所完成之碩士論文，於民國98年7月10日經考試委員審查合格。

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摘要

細胞遷移在動物發育過程中扮演很重要的角色。在線蟲(*Caenorhabditis elegans*)雌雄同體中，兩顆遠頂細胞的遷移是很好的研究模型，它們在幼蟲期會進行三個時期不同方向的遷移，牽引成蟲中兩個對稱U型的性腺的形成。先前研究發現，第二時期的背向遷移發生於L3晚期，是由Netrin受器UNC-5的轉錄啟始所啟動。此外，異時性基因也會參與決定遠頂細胞的時間身分：DAF-12/賀爾蒙受器、DRE-1/F-box蛋白質、LIN-29/鋅指轉錄調控子共同促進第二和第三時期的執行。LIN-42/Period(日光週期蛋白質)則防止第二和第三時期過早於L2執行。我們實驗室先前分離並分析了*dpy-24*突變，發現其會提早DTC第二時期的背向遷移；DPY-24蛋白質是一鋅指轉錄調控子，含有一個PR domain和五個鋅指，只會在遷移的第一時期表現在DTC中，並且會抑制*unc-5*的轉錄。在進入第二時期時，*daf-12*、*dre-1*、*lin-29*共同作用造成*dpy-24*表現量的下降。然而，DPY-24如何抑制*unc-5*的轉錄以及DPY-24的表現量如何被降低仍然未被探討清楚。在我的研究中，透過結構與功能分析，發現DPY-24的PR domain、鋅指、以及其以外的區域對於DPY-24在遠頂細胞中的功能都是重要的。我更進一步發現，DPY-24的鋅指在EMSA (Electrophoretic Mobility Shift Assay)實驗中可直接透過兩個接合位置與*unc-5*啟動子接合，表示DPY-24可能直接抑制*unc-5*的轉錄。此外，DAF-12和LIN-29的鋅指也都具有接合到*unc-5*啟動子的能力，而當*daf-12*和*lin-29*都突變後，*unc-5*的轉錄即消失了，表示DAF-12和LIN-29可能透過接合到*unc-5*啟動子來直接啟動*unc-5*的轉錄。至於*dpy-24*本身的調控，透過表現綠色螢光蛋白的基因轉殖線蟲，偵測*dpy-24*在各個基因表現階層的變化量，發現DAF-12和LIN-29共同抑制*dpy-24*的轉錄，而DRE-1則降低DPY-24蛋白質的穩定性；此外，*dpy-24*表現也會被自己的3'UTR所抑制。最後，我發現*dpy-24*在第一時期的轉錄需要LIN-42，並且*dpy-24*會透過正回饋機制維持自己的轉錄活化。這些實驗結果有助於我們了解細胞遷移中的時間調控機制。亦即在遠頂細胞遷移的第一時期，LIN-42啟動*dpy-24*的轉錄，進而抑制*lin-29*和*unc-5*的

轉錄來避免遠頂細胞過早的背向遷移。在第二時期，DAF-12和LIN-29在轉錄層次降低DPY-24的表現量，而DRE-1則在後轉譯層次降解DPY-24；這些機制疏解了DPY-24對於*unc-5*的抑制，並且容許LIN-29和DAF-12啟動*unc-5*的轉錄，最終導致遠頂細胞的遷移。

Abstract

Cell migration plays an essential role during animal development. In the hermaphrodite of *Caenorhabditis elegans*, two distal tip cells (DTCs) undergo three sequential phases of linear migration during larval stages and lead the formation of bi-lobed U-shaped gonad arm. Previous studies have shown that the initiation of the ventral-to-dorsal phase Ⅱ migration occurs in the late L3 stage and is controlled by the transcriptional up-regulation of the dorsal guidance receptor UNC-5. On the other hand, the heterochronic genes control the temporal identity of DTCs: DAF-12/nuclear hormone receptor, DRE-1/F-box protein, and LIN-29/zinc finger transcription factor function redundantly to promote phase Ⅱ and phase Ⅲ migration in the L3 stage. In contrast, LIN-42/Period prevents phase Ⅱ and phase Ⅲ to occur precociously in L2. Our laboratory has previously isolated and characterized a *dpy-24* mutation, which results in precocious DTC dorsal turn in early L3 stage. Previous studies have shown that *dpy-24* represses the transcription of *unc-5* to prevent precocious DTC dorsal turn. DPY-24 contains one PR domain and five zinc fingers and is detected in DTCs prior to, but not during or after, dorsal phase Ⅱ migration. In late L3, *lin-29*, *dre-1*, and *daf-12* function redundantly to down-regulate the *dpy-24* level to promote DTC dorsal turn. However, how DPY-24 represses *unc-5* transcription and how the DPY-24 level is maintained during phase Ⅰ and is down-regulated during the phase Ⅰ to Ⅱ transition have not been explored. My structural and functional analysis of DPY-24 reveals that the PR and zinc finger domains and the region outside of these domains are required for its complete function in regulating DTC migration. Furthermore, I demonstrated that DPY-24 zinc fingers are able to bind to *unc-5* promoter in EMSA (electrophoretic mobility shift assay), suggesting that DPY-24 may repress *unc-5* transcription directly. In addition, DAF-12 and LIN-29 each can bind to the *unc-5* promoter and when both

are mutated, no *unc-5* transcription is observed. Therefore, DAF-12 and LIN-29 may activate *unc-5* transcription by directly binding to its promoter. As for temporal regulation of *dpy-24*, I generated transgenic worms carrying *dpy-24*::GFP reporters and found that DAF-12 and LIN-29 repress *dpy-24* transcription, and DRE-1 decreases DPY-24 protein stability. Furthermore, *dpy-24* expression is also repressed through its 3'UTR. Finally, *dpy-24* transcription during phase Ⅰ is activated by LIN-42 and maintained by a positive feedback loop. These results elucidate the molecular mechanism of temporal regulation during cell migration. In phase Ⅰ, LIN-42 activates *dpy-24* transcription, which in turn blocks *lin-29* and *unc-5* transcription and hence prevents DTC from dorsalward turning. In phase Ⅱ, DPY-24 is down-regulated by LIN-29 and DAF-12 at the transcriptional level and by DRE-1 at the post-translational level, which relieves the repression of *unc-5* by DPY-24 and allows LIN-29 and DAF-12 to activate *unc-5* transcription, leading to DTC dorsal turn.

Table of Contents

Introduction

Cell migration occurs throughout our lives. During embryonic development, cells move extensively to their final destination, such as in gastrulation (Keller, 2005) or nervous system formation (Ayala et al., 2007). In adult, cell migration is required in homeostasis and immune responses (Luster et al., 2005), and increased cell motility plays a key role in metastasis (Yamaguchi et al., 2005). Although numerous pathways regulating cell polarity and motility have been identified (Ayala et al., 2007), the mechanism of temporal regulation in cell migration largely remains unknown.

The nematode *Caenorhabditis elegans* goes through four larval stages, L1 to L4, before growing into adult. In the hermaphrodite, the bi-lobed U-shape of the gonad arm is determined by the migratory paths of two distal tip cells (DTCs), which are born and positioned at the anterior and posterior tips of gonad primordium during L1 stage (Kimble and Hirsh, 1979). DTCs undergo three phases of migration and lead the gonad arm elongation during larval stages (Fig. 1A&1B). In the first phase (phase Ⅰ), the two DTCs migrate away from the mid-body along ventral body wall muscles, one toward the anterior and the other toward the posterior. In the second phase (phase Ⅱ), DTCs turn dorsally during late L3 and migrate to the dorsal side. In the third phase (phase Ⅲ), they migrate along dorsal body wall muscles to the mid-body region.

Several classes of genes have been reported to be required for proper DTC migration (Cram et al., 2006), including extracellular matrix proteins (Merz et al., 2003), metalloproteases (Blelloch et al., 1999; Nishiwaki et al., 2000), integrin receptors (Lee et al., 2001; Meighan and Schwarzbauer, 2007), src kinase (Itoh et al., 2005), and the Rac signaling pathway (Lundquist et al., 2001; Reddien and Horvitz, 2000; Wu and Horvitz, 1998); their mutations result in different types of migration defect. Specifically, the ventral-to-dorsal migration in phase Ⅱ is in part controlled by *unc-5*/*unc-6*/*unc-40*

netrin guidance system (Hedgecock et al., 1990; Wadsworth, 2002). In their loss-of-function mutants, DTCs often fail to migrate to the dorsal side, while the longitudinal migration in phase Ⅰ and Ⅲ is essentially unaffected. UNC-6 is the *C. elegans* homolog of netrin (Ishii et al., 1992), an evolutionarily conserved family of extracellular guidance cue, and is secreted from ventral neurons and hypodermis to form a dorsal-ventral gradient peaked at ventral mid-line (Wadsworth et al., 1996). UNC-5 and UNC-40 are membrane receptors for UNC-6 and function in migrating cells or axon growth cones (Chan et al., 1996; Leung-Hagesteijn et al., 1992). Previous studies support a model in which UNC-40 alone mediates attraction by UNC-6, whereas the combination of UNC-5 and UNC-40 mediates repulsion from UNC-6 (Hedgecock et al., 1990). During DTC migration, the transcriptional up-regulation of *unc-5* is coincident with the initiation of phase Ⅱ, and premature expression of *unc-5* causes precocious DTC dorsal turns (Su et al., 2000), indicating that *unc-5* is both necessary and sufficient for DTC dorsal migration.

In *C. elegans*, stage-specific temporal identities are specified by a group of genes called heterochronic genes (Ambros and Horvitz, 1984). Mutations in heterochronic genes alter the timing of stage-specific program, such as division pattern, in affected tissue relative to other tissues, resulting in precocious or retarded phenotype. The heterochronic circuit has been studied extensively in the extragonadal tissues, especially in the epidermal seams (Rougvie, 2005). Most identified heterochronic genes in the seams do not have apparent roles in DTC migration, except for *lin-42*, *daf-12*, *dre-1*, and *lin-29*. The RNAi knockdown of *lin-42* causes DTC turning occur one stage earlier, from late L3 to late L2, suggesting a role for *lin-42* during phase Ⅰ (Tennessen et al., 2006). On the other hand, *daf-12*, *dre-1*, and *lin-29* function redundantly during late L3 to promote the later programs (phase Ⅱ and Ⅲ) so that in any of their double mutants, DTCs exhibit the characteristic no-turn phenotype (Fielenbach et al., 2007). LIN-42 is

the homolog of the Period (Per) family of circadian rhythm proteins and may act by interfering with transcription activators (Jeon et al., 1999). DAF-12, DRE-1, and LIN-29 are nuclear hormone receptor (Antebi et al., 2000), F-box protein of SCF E3 ubiquitin ligase (Fielenbach et al., 2007), and zinc finger transcription factor (Rougvie and Ambros, 1995), respectively; this indicates that the temporal identity of DTCs is regulated at multiple levels in gene regulation. Genetic data show that *lin-29* is downstream of *lin-42*, and *daf-12* and *dre-1* are partially downstream or in parallel to *lin-42*. However, it is not clear how these factors interplay at the molecular level to contribute to the correct expression of downstream effectors, such as *unc-5*, at the right time in DTCs.

Our laboratory has previously identified and characterized a *dpy-24* mutation, which causes a precocious DTC dorsal turn in early L3 stage (Fig. 1C). DPY-24 is similar to mammalian transcription repressors PRDI-BF1/Blimp-1 and also contains one PR domain and five zinc fingers (Fig. 1D). Previous studies have shown that *dpy-24* represses the transcription of *unc-5* to prevent precocious dorsal turn. DPY-24 is detected in DTCs prior to, but not during or after, phase Ⅱ migration. In addition, constitutive expression of *dpy-24* prolongs phase I migrations (T. F. Huang and Y. C. Wu unpublished results). Finally, *lin-29*, *dre-1*, and *daf-12* function redundantly to down-regulate the *dpy-24* level during DTC dorsal turn, suggesting that *dpy-24* provides the link between temporal regulators (*daf-12*, *dre-1*, and *lin-29*) and the spatial regulator *unc-5*. However, how DPY-24 represses *unc-5* transcription and how DPY-24 is down-regulated during the phase Ⅰ to Ⅱ transition by *daf-12*, *dre-1*, and *lin-29* have not been explored.

In order to unravel the potential mechanism by which DPY-24 represses *unc-5* transcription, I performed structural and functional analysis of DPY-24. I found that multiple domains of DPY-24, including PR domain, the five zinc fingers, and the region outside of them, are required for its complete function in regulating both anterior and

posterior DTC migration. To reveal the role of DPY-24 zinc fingers, I conducted EMSA (electrophoretic mobility shift assay) and confirmed that DPY-24 zinc fingers are able to bind to *unc-5* promoter directly through two specific binding sites. Therefore, DPY-24 may directly and actively repress *unc-5* transcription during phase Ⅰ. Moreover, the zinc fingers of DAF-12 and LIN-29 both are able to bind to *unc-5* promoter, suggesting that they are direct activators for *unc-5*. To examine at which level *dpy-24* is down-regulated, I generated various forms of GFP reporters for *dpy-24* expression. It turns out that *dpy-24* is down-regulated at multiple levels: DAF-12 and LIN-29 act together to repress *dpy-24* transcription, and DRE-1 decreases DPY-24 protein stability. Furthermore, the *dpy-24* level is also down-regulated through its 3'UTR. Finally, *dpy-24* transcription during phase Ⅰ is activated by *lin-42* and maintained by a positive feedback loop. These results provide a molecular framework for us to understand how DTC dorsal migration is tightly controlled in wild-type.

Materials and Methods

Nematode strains

Nematodes were cultured at 20℃ on NGM agar inoculated with *Escherichia coli* strain OP50 as described (Brenner, 1974). The N2 Bristol strain was used as wild type. The following alleles were used: *dpy-24(s71) I*, *dre-1(dh99) V*, and *daf-12(rh61rh411)X*. Strains other than *dpy-24(s71)* were provided by the Caenorhabditis Genetics Center CGC, which is funded by the NIH National Center for Research Resources (NCRR).

Constructs

To construct P_{lag-2}*::dpy-24△(PR or ZF)*, *dpy-24* cDNA deletion was made by inverse PCR using plasmid *d24s12* (pGEMT-easy/*dpy-24* cDNA) as templates with the following primers:

D24△PR/f: 5'-CAACAAGACCATACGTAAGAGTTCG-3'

D24△PR/r: 5'-GGTTCATAGGAAGTGTGCATTCTGC-3'

D24△Znf/f: 5'-GGATATGAAAGACTCGATGAGGGATG-3'

D24△Znf/r: 5'-GTTTTTCCATTTTCCTGTTGTTGCAC-3'

Pfu polymerase was used to avoid point mutation during PCR. The same enzyme was also used for all the following experiments except otherwise noted. The PCR products were then self-ligated again. Double deletion of PR domain and zinc fingers was achieved by deleting them sequentially. These various forms of *dpy-24* cDNA were then amplified with the following primers, cut with KpnI, and ligated into KpnI restriction site in pPD49_26/P_{lag-2}.

DPY-24-KpnI/f: 5'-GGGGTACCAATTCGCTAGCATGGGTCAAGG-3'

DPY-24-KpnI/r: 5'-GGGGTACCGAATACTCAAGCTATGCATCCAACG-3'

P_{dpy-24}*::dpy-24 (Full-length or △ZF)* were made by fusion PCR. $P_{dpy-24(5kb)}$ was first amplified from worm genomic DNA with primers:

D24-5end5kb: 5'-GATGGAAAGTTGACCTAAATGTCGG-3'

D24-n/r: CATCCCCACTTCCTTGACCCAT

dpy-24 cDNA(*full-length or △ZF*)::*unc-54* 3'UTR was amplified by PCR from plasmids *LDU* (pPD49_26/P_{lag-2}/*dpy-24* cDNA) or *LDU△Z* F with primers:

D24-5end/f: 5'-ATGGGTCAAGGAAGTGGGGATG-3'

D: 5'-AAGGGCCCGTACGGCCGACTAGTAGG-3'

The above two PCR products were then mixed and run fusion PCR using Roche Long Template PCR system with the following primers:

d24-5kbf-nest: GCCTGGAAAACGCCTTTTGAAG-3'

D': 5'-GGAAACAGTTATGTTTGGTATATTGGG-3'

The resulting PCR product was directly used for microinjection.

The plasmid pPD95_75PEST , which carries dGFP (destabilized GFP), was generated in multiple steps. The GFP-PEST fragment was first made by fusion PCR. GFP was amplified by PCR from pPD95_75 by the following primers:

GFP/f: 5'-AGCTTGCATGCCTGCAGGTCGACT-3'

GFP-PEST fusion/r:

5'-CTCCGGCGGGAAGCCATGGCTTTTGTATAGTTCATCCATGCCATGTGT-3'

PEST was amplified from pd2EGFP by the following primers:

PEST/f: 5'-AGCCATGGCTTCCCGCCGGAG-3'

PEST/r: 5'-GATCTAGAGTCGCGGCCGCGC-3'

The two PCR products were mixed and run fusion PCR with the following primers:

gfp nested/f: 5'-GCCTGCAGGTCGACTCTAGAGGATCC-3'

PEST nested/r: 5'-CCGAATTCCTACACATTGATCCTAGCAGAAGCAC-3'

The resulting fusion PCR product was then cut with XmaI and EcoRI and ligated into

the same restriction sites in pPD95_75 to replace the original GFP.

P_{dpy-24}::dGFP was generated by fusion PCR. $P_{dpy-24(5k)}$ was amplified from genomic DNA by the following primers:

D24-5end5kb: 5'-GATGGAAAGTTGACCTAAATGTCGG-3'

d24Pgfp/r:

5'-AGTCGACCTGCAGGCATGCAAGCTGTCATCCCCACTTCCTTGACCCAT-3'

dGFP::*unc-54* 3'UTR was amplified from pPD95_75PEST by the following primers:

GFP/f: 5'-AGCTTGCATGCCTGCAGGTCGACT-3'

D: 5'-AAGGGCCCGTACGGCCGACTAGTAGG-3'

The resulting two PCR products were then mixed and run fusion PCR using Roche Long Template PCR system with primers d24-5kbf-nest and D' mentioned above. The final PCR product was directly used for microinjection.

P_{lag-2}::gfp::*dpy-24* was made by fusion PCR. P_{lag-2}::gfp was amplified from pPD95_75/P_{lag-2} by the following primers:

P_{lag-2} 1k/f: 5'-GTGGAGTGAAGTCGTTTAGGG-3'

GFP-DPY-24 fusion/r:

5'-CCACTTCCTTGACCCATTTTGTATAGTTCATCCATGCCATGTG-3'

dpy-24 cDNA::*unc-54* 3'UTR was amplified from plasmid *LDU* by primers D24-5end/f and D. The two PCR products were mixed and run fusion PCR using Roche Long Template PCR system with primers:

P*lag-2* 1k nested/f: 5'-GAGATGTATTGTTTGAATTGGAGC-3' and D'. The final PCR product was directly used for microinjection.

The plasmid pPD95_75PEST*dpy-24* 3'UTR was made by subcloning the *dpy-24* 3'UTR fragment from plasmid *dgd* (P_{dpy-24}::gfp::*dpy-24* 3'UTR) into pPD95_75PEST by EcoRI and SpeI to replace *unc-54* 3'UTR.

P_{lag-2}::dGFP::*dpy-24* 3'UTR was made by PCR amplifying P_{lag-2} with the following

primers:

P*lag-2* 1k nested/f: 5'-GAGATGTATTGTTTGAATTGGAGC-3'

P*lag-2*/r txn fusion: 5'-CGATCCTTTTCTGAAAAAAGGCAAATTTG-3'

The primers were first phosphorylated by T4 polynucleotide kinase before use in PCR reaction. The PCR product was purified and blunt-end ligated into SmaI restriction site in pPD95_75PEST*dpy-24* 3'UTR.

Purification of recombinant proteins

The cDNA of *dpy-24* zinc fingers was amplified by PCR from *dpy-24* cDNA using primers:

5'-CACCGGAAAAACGAGATACGCTTGC-3' and

5'-TTACATATCCTCTTCTTTACATGTAGTTG-3'

The cDNA of *lin-29* zinc fingers was obtained by RT-PCR with primers:

5'-GGAAGATATCCACAGTTCGAACAAAAGC-3'

5'-GGAACAACATCGTTTCCAAAATTGC-3'

Then a nested PCR was run with primers:

5'-CACCGCAAAACCTTACAAGTGC-3'

5'-TTACTTCGATCGATCCGCGTGCTT-3'

The PCR products were cloned into pENTR/D-TOPO. The expression plasmid was obtained through LR recombination (Invitrogen Gateway system) with pMAL-c2x destination clone. Following induction in *E. coli* strain BL21, the cells were sonicated, and recombinant protein was purified from lysate using NEB amylase resin. The bound protein was eluted by the buffer of 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM azide, 10 mM DTT, and 10 mM maltose for use in EMSA.

EMSA (Electrophoretic mobility shift assay)

The -497 to -442 probe was obtained from annealing single stranded oligonucleotides of the following sequences:

5’-ACACAACTTTCATTTTCCTTATTGCTCCATAAACCATAAGCTTTCTCTTTCTGCCC-3’

5’-GGGCAGAAAGAGAAAGCTTATGGTTTATGGAGCAATAAGGAAAATGAAAGTTGTGT-3’.

The -540~-481 probe was obtained from annealing single stranded oligonucleotides of the following sequences:

5’-ATTGAAATGCACTTTCCACGTGGCTTCACTTAATCTCCAAAGGACACAACTTTCATTTTC-3’

5’-GAAAATGAAAGTTGTGTCCTTTGGAGATTAAGTGAAGCCACGTGGAAAGTGCATTTCAAT-3’

The mutant probes were obtained in the same way with sequence alteration as indicated.

Other larger DNA probes were amplified from genomic DNA by PCR using primers as follows:

-1000/f: 5’-CCGTTTCAGTAGATCTTCAAAGAAA-3’

-751/f: 5’-GTCTATTATAAGCGTGAGCCCAT-3’

-701/r: 5’-ATGTAGCACAAACAATCTTTTTCAAG-3’

-537/r: 5’-CAATATAGAACATTGAAAATAACTATTGGATG-3’

-499/f: 5’-GGACACAACTTTCATTTTCCTTATT-3’

-498/r: 5’-CCTTTGGAGATTAAGTGAAGCCACG-3’

-450/r: 5’-GAGAAAGCTTATGGTTTATGGAGC-3’

-442/f: 5’-CTGACTCTTCATAAAAAACAGTTTCTCC-3’

-253/f: 5’-GGGTGGACCACCGAAAAAGTG-3’

-198/r: 5’-CTGCACTATGAACTGAGGTTTCTAT-3’

-1/r: 5’-TACTGGAATAGAAATTATGATTAGTGACAA-3’

All the probes were labeled with biotin using the LightShift Chemiluminescent EMSA kit (Pierce) following manufacturer's provided protocol. EMSA was also performed as suggested by manufacturer. 20 fmol of labeled DNA and 200 ng purified protein were included in each binding reaction. Unlabeled competitors were added in 200-fold of the unlabeled DNA with the same identity of the labeled probe. They were incubated in the 10X binding buffer plus 250 μM $ZnCl_2$ at room temperature for 20 minutes and then run on 6% polyacrylamide gel in 0.5X TBE.

Transgenic worms

Transgenic worms were made by microinjection using P_{myo-2}::GFP or P_{sur-5}::GFP as co-injection marker. The injection mixtures include 1X microinjection buffer, target DNA, and co-injection marker. P_{myo-2}::GFP was injected at 1 ng/μl along with 50 ng/μl carrier DNA and 50 ng/μl 1kb ladder, and P_{sur-5}::GFP was injected at 100 ng/μl. P_{lag-2}*::dpy-24 △(PR or ZF)* and P_{lag-2}::gfp::*dpy-24* were injected at 20 ng/μl. P_{dpy-24}*::dpy-24 (Full-length or △ZF)* and P_{dpy-24}::dGFP were injected at 2 ng/μl. P_{lag-2}::dGFP::*dpy-24* 3'UTR was injected at 50 ng/μl.

RNA interference (RNAi)

To make *dre-1* RNAi construct, *dre-1* full-length cDNA was PCR amplified from yk1678a10 by the phosphorylated primers:

dre-1/f: 5'-ATGTCGTCCTCTTCGTCACCATTCTTC-3'

dre-1/r: 5'-TTAAATTTCGGTGCCAGTCTCAGTGGAG-3'

To make *lin-42* RNAi construct, *lin-42* cDNA in yk818a03 was amplified by phosphorylated primers:

lin-42 exon 4/f: 5'-CTCGATTCCATACCTTGGTCTCCTACCC-3'

lin-42 exon 9/r: 5'-TTAATTCTGAGAATCCCGTAGCATTAGG-3'

To make *lin-29* RNAi construct, *lin-29* cDNA in yk1430g04 was amplified by the phosphorylated primers:

lin-29 cDNA 359/f: 5'-GTTTTCACAATTTGGAAGATATCCACAG-3'

KpnI *lin-29*/r: 5'-GGTACCTTAATAGGAATGATTTTTCATATT-3'

The PCR products were purified and ligated into EcoRV restriction site in L4440. For *dre-1*, the resulting plasmid was further cut by AccI and purified the larger fragments which contained vector backbone and self-ligated to obtain L4440/*dre-1*(1.6kb). The RNAi for *dre-1* was performed by feeding method as described (Kamath et al., 2000).

lin-29(RNAi) and *lin-42(RNAi)* was performed by injection of dsRNA. To prepare dsRNA, the template was amplified from their RNAi constructs by primers:

L4440 T7 5'/f: 5'-GCAACCTGGCTTATCGAAATTAATAC-3'

L4440 T7 3'/r: 5'-CAGTCACGACGTTGTAAAACGACG-3'

The PCR products were purified and used as the template for *in vitro* transcription by T7 polymerase to obtain dsRNA. The reaction product was directly injected into the gonads of young adults, and their progenies were scored.

Result

DPY-24 acts cell-autonomously and requires multiple domains for its complete function in regulating DTC migration

Previous experiments have shown that *dpy-24* loss-of-function mutation causes precocious activation of *unc-5* transcription during phase Ⅰ of DTC migration, while prolonged expression of *dpy-24* prevents normal *unc-5* transcription after phase Ⅱ. Therefore, *dpy-24* functions to repress *unc-5* transcription; however, the underlying mechanism is still unknown.

To examine whether *dpy-24* acts cell-autonomously to regulate DTC migration, *dpy-24* cDNA was expressed in *dpy-24(s71)* mutant by *lag-2* promoter ($P_{lag\text{-}2}$*::dpy-24*), which is specifically expressed in DTCs throughout DTC migration, to see if it is sufficient to rescue the DTC migration defect. As a result, the percentage of DTC migration defect was reduced from about 80% to about 20% in both anterior and posterior DTCs (Table 1). This rescue of DTC migration defect is similar to the level rescued by *dpy-24* cDNA expressed by its own promoter ($P_{dpy\text{-}24}$*::dpy-24*) (Table 1). Therefore, *dpy-24* may function cell-autonomously to regulate DTC migration.

Next, I want to find out the domains required for *dpy-24*'s function. The repression mechanism of DPY-24's homologs in other species, including PRDI-BF1 and Blimp-1, has been studied extensively (John and Garrett-Sinha, 2008). Their binding sites in target genes, such as IFN-beta and CIITA, have been identified and shown to be important for their repression (Agawa et al., 2007; Chen et al., 2007; Doody et al., 2007; Ghosh et al., 2001; Keller and Maniatis, 1991; Kuo and Calame, 2004; Lin et al., 1997; Magnusdottir et al., 2007; Martins et al., 2008; Shaffer et al., 2002; Tooze et al., 2006), and this binding specificity was conferred by the first two zinc fingers (Keller and Maniatis, 1992). In contrast, PR domain has been shown to have protein interaction ability

(Huang et al., 1998). Although its function in PRDI-BF1 or Blimp-1 is still unclear, several experiments suggest that PR domain also contributes to repression activity (Ghosh et al., 2001; Gyory et al., 2003; Yu et al., 2000). In addition, PRDI-BF1 and Blimp-1 have been shown to repress target genes by recruiting Groucho family co-repressors (Ren et al., 1999), histone methyltransferase (Ancelin et al., 2006; Gyory et al., 2004), and histone deacetylase (Yu et al., 2000) by regions outside of PR domain.

To assess the functional importance of different domains in DPY-24, I generated different DPY-24 mutant proteins with deletion of PR domain, all five zinc fingers, or both (DPY-24ΔPR, DPY-24ΔZF, and DPY-24ΔPRΔZF). They were then expressed in *dpy-24(s71)* mutant under the control of P_{lag-2} or P_{dpy-24} to examine their residual functions. It was found that DPY-24ΔZF can rescue anterior fully but posterior DTCs only partially, while DPY-24ΔPR and DPY-24ΔPRΔZF can only weakly rescue both anterior and posterior DTCs (Table 1). These results suggest that DPY-24 zinc fingers is important for posterior DTC, and PR domains and some other unidentified regions also plays roles in regulating both anterior and posterior DTC migration.

DPY-24 zinc fingers bind to *unc-5* promoter through D1 and D2 binding sites

Since DPY-24 zinc fingers are important for at least posterior DTCs, and since *unc-5* is one target of *dpy-24*, it is possible that DPY-24 binds to *unc-5* promoter through its zinc fingers and that this binding is important to repress *unc-5*. To examine if the *unc-5* promoter may contain DPY-24 binding site(s), the *unc-5* promoter sequence was first narrowed from 4.6 kb to 1 kb. The temporal expression pattern of the transcriptional reporter $P_{unc-5(1kb)}$:gfp in DTCs is similar to that of $P_{unc-5(4.6kb)}$:gfp (data not shown). Thus, the 1 kb region of *unc-5* promoter contains cis-elements necessary for proper expression in DTC.

The binding sites of Blimp-1 and PRDI-BF1 have already been identified previ-

ously as mentioned previously, and homology modeling of DPY-24 zinc fingers reveals the binding specificity of AAxAGxxAxAG. Further examination combined with Blimp-1 and PRDI-BF1 binding sequences shows a conserved core sequence of GAAAG (Dr. Y. S. Chen). We then searched the 1 kb *unc-5* promoter and found two potential DPY-24 binding sites: GAAAATGAAAG and GAAAGAGAAAG, which we name D1 and D2, respectively (Fig. 2A).

Next, I employed the electrophoretic mobility shift assay (EMSA) to investigate the possibility that DPY-24 binds to the *unc-5* promoter. The 1 kb region of the *unc-5* promoter was divided to 5 overlapping fragments. I found that DPY-24 zinc fingers fused with maltose binding protein (MBP) at its amino terminus (MBP::DPY-24ZF) can bind to the fragment corresponding to -497~- 442, but not the others (Fig. 2B). In addition, two forms of binding complex (D and D') were observed (Fig. 2B). The presence of D' with slower mobility suggests two DPY-24 binding sites, consistent with the prediction of homology modeling. Both forms were absent in the lane containing unlabeled competitor DNA, indicating the binding specificity of DPY-24 to the DNA sequence.

To examine whether the binding could be mediated by D1 and/or D2, I mutated either or both sites. Particularly, D1 and D2 were mutated to CAAACTCAAAC (D1m) and CAAACACAAAC (D2m) (Fig. 2A). MBP::DPY-24ZF could still bind to D1m and D2m. However, only the D, but not D', form of the binding complex was present (Fig. 2C), consistent with the binding of a single site. Double mutations (D12m) in both D1 and D2 abolished MBP::DPY-24ZF binding (Fig. 2C). These results indicate that DPY-24 binds to the *unc-5* promoter through direct interaction with the D1 and D2 sites in the EMSA assay.

DAF-12 and LIN-29 can bind to *unc-5* promoter

Previous studies have found that in *daf-12;lin-29* double mutant, DTCs usually fail

to initiate dorsal turns; furthermore, the transcription of *unc-5* after phase II is also diminished (T. F. Huang and Y. C. Wu, unpublished data), suggesting that DAF-12 and LIN-29 are activators for *unc-5* in DTCs. To investigate whether their activation is directly or indirectly through the down-regulation of DPY-24, their binding ability to *unc-5* promoter was also examined by EMSA. As a result, both the zinc fingers of DAF-12 and LIN-29 are able to bind to *unc-5* promoter (Fig. 3A&3B). LIN-29 zinc fingers have multiple binding sites in *unc-5* promoter (Fig. 3A), and DAF-12 zinc fingers can bind to -751~-450 region of *unc-5* promoter (Fig. 3B), which contains two potential DAF-12 response elements, A1 and A2 (Fig. 3C). I further split this region into two fragments and found that there is an additional DAF-12 binding site in -751~-539 (Fig. 3D). To examine whether DAF-12 directly binds to A1 and A2, I performed again mutational analysis and found that DAF-12 specifically binds A1 but not A2, because the mutation in A1 but not A2 sequence disrupts the DAF-12 binding to the probe (Fig. 3E).

To sum up, DAF-12 binds A1 and one unidentified binding site in *unc-5* promoter, and LIN-29 can also bind directly to *unc-5* promoter through multiple binding sites in EMSA. These results suggest that DAF-12 and LIN-29, besides down-regulating the *dpy-24* level, may serve as direct activators for *unc-5* to promote DTC dorsal turn.

dpy-24 is down-regulated at the transcriptional level by DAF-12 and LIN-29

It has been shown previously by immunostaining that DPY-24 can be detected in DTCs only during phase Ⅰ migration, but the double-mutation of *daf-12*, *dre-1*, or *lin-29* causes ectopic DPY-24 expression after phase Ⅰ (T. F. Huang and Y. C. Wu, unpublished data). Therefore, *daf-12*, *dre-1*, and *lin-29* function redundantly to down-regulate the *dpy-24* level, yet its molecular mechanism has not been studied.

We first investigated if *dpy-24* may be down-regulated at the transcriptional level.

In order to monitor the *dpy-24* transcription level, a GFP-PEST fusion protein (or dGFP, for destabilized GFP) was used as a reporter, which has been shown to have shorter half-life than normal GFP (Li et al., 1998). This P_{dpy-24}::dGFP was first introduced into wild-type worms to observe its expression pattern. Previous studies in the lab show that wild-type DTCs do not turn dorsalward until the vulval precursor cell P6.p divides twice, generating 4 P6.p descendant cells. Whereas in *dpy-24* mutant, DTCs precociously turn dorsalward even before P6.p divides. Therefore, for simplicity, we examine the descent number of the P6.p to define the developmental stage of the worm. The P6.p 1-cell indicates early L3, whereas P6.p 4-cell indicates late L4. In one transgenic line, 77.3% of DTCs expresses GFP during phase Ⅰ migration (Fig. 4A & Table 2). Consistent with the change in DPY-24 protein level, *dpy-24* transcription was nearly completely turned off after dorsal turn (Fig. 4D & Table 2), with only few DTCs (<4%) express GFP. P_{dpy-24}::dGFP expression after dorsal turn may be due to the residual GFP produced before dorsal turn and not completely degraded. Alternatively, the transcription of *dpy-24* is not completely repressed after dorsal turn.

Among the upstream regulators of *dpy-24* (i.e. *daf-12*, *dre-1*, and *lin-29*), *daf-12* and *lin-29* encode a nuclear hormone receptor (Antebi et al., 2000) and a zinc finger transcription factor (Rougvie and Ambros, 1995), respectively, and they have both been shown to regulate target genes at the transcriptional level (Rougvie and Ambros, 1995; Shostak et al., 2004). Therefore, it is likely that they mediate the transcriptional down-regulation of *dpy-24*. To test this possibility, the transcriptional reporter (P_{dpy-24}::dGFP) was crossed into *daf-12(rh61rh411)* null mutant or into worms treated with *lin-29(RNAi)*. The *daf-12* mutation alone does not alter the temporal expression pattern of *dpy-24* in DTCs (Fig. 4E), while *lin-29(RNAi)* prolonged the expression window of *dpy-24* in DTCs into L4 (Fig. 4F & Table 2). Although *daf-12* mutation alone has no effect on *dpy-24* expression pattern, it can enhance the ectopic *dpy-24* transcrip-

tion caused by *lin-29(RNAi)* (Fig. 4G & Table 2)). These results suggest that DAF-12 and LIN-29 function together to repress *dpy-24* transcription, while LIN-29 acts more predominantly than DAF-12

DPY-24 protein stability is decreased by DRE-1

dre-1, which encodes a F-box protein, has been shown to work in the context of SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase to promote DTC turning (Fielenbach et al., 2007), in which cullin provides the scaffold to recruit F-box substrate recognition protein through the adaptor Skp1 and transfers ubiquitin to the substrate to promote its degradation. However, the direct downstream target for DRE-1 has not been identified. Since *dre-1* is involved in the down-regulation of the *dpy-24* level in DTCs, it is possible that DPY-24 is targeted by DRE-1-containing ubiquitin E3 ligase for degradation. To test this possibility, we generated a translational fusion construct in which GFP was fused to the N-terminus of DPY-24 and was expressed by the *lag-2* promoter. The *lag-2* promoter has been shown to drive transgene experiments in the DTCs throughout larval developments. The GFP fusion protein allows us to see if DPY-24 protein stability is modulated before and after DTC dorsal turn. In the wild-type background, about 30% of DTCs express detectable GFP-DPY-24 before dorsal turn (Fig. 5A & Table 2). However, this percentage decreased to about 20% after the DTCs turned dorsalward (Fig. 5B & Table 2), indicating that DPY-24 stability decreased after DTC dorsal turn.

To see if this decrease in DPY-24 stability is caused by DRE-1, the reporter (P_{lag-2}::gfp::*dpy-24*) was crossed into the *dre-1(dh99)* weak allele background and further treated with *dre-1(RNAi)*. As a result, the percentage of DTCs expressing detectable GFP-DPY-24 before and after dorsal turns became the same (Fig. 5C&5D & Table 2). Therefore, DPY-24 protein stability is decreased by DRE-1 after dorsal turn, possibly

through E3-ubiquitin-ligase-mediated proteolysis.

dpy-24 may be down-regulated by microRNA

In extragonadal tissues, microRNAs (miRNAs) have been found to be essential components in heterochronic circuit. They are transcriptionally controlled and can repress the translation of temporal regulators in previous developmental stage, thus promoting the progression into following stages. Famous members include *lin-4* and *let-7* (Rougvie, 2005). However, no miRNA has been reported to participate in the temporal control of somatic gonad, including DTC migration. Although the *dpy-24* level is found to be down-regulated at transcriptional and post-translational level, the possibility of post-transcriptional regulation, or microRNA silencing, cannot be ruled out, especially when *dpy-24* 3'UTR is predicted to have several conserved miRNA binding sites in the miRBase database (Fig. S1)(Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008).

To reveal this potential regulation of miRNA in the *dpy-24* level, P_{lag-2}::dGFP::*dpy-24* 3'UTR was generated and sent into wild-type to see if *dpy-24* 3'UTR can interfere with the expression of reporter. About 50% of DTCs in early L2 expresses this GFP (Fig. 6A & Table 3). Surprisingly, this expression is nearly completely diminished in late L2 (Fig. 6B & Table 3), which is one stage earlier than the down-regulation of *dpy-24* transcription and protein stability. Therefore, *dpy-24* is subject to multiple but sequential down-regulation mechanisms, which may act together to facilitate switching of DTCs from phase Ⅰ into phase II and phase III.

dpy-24 transcription is activated by *lin-42* and maintained by a positive feedback

If *dpy-24* is subject to these multiple down-regulation mechanism, how do DTCs

activate and maintain the expression level of *dpy-24* during phase Ⅰ? Previous studies have shown that another heterochronic gene, *lin-42*, is expressed in DTCs before dorsal turn and suppress *lin-29* activity. Therefore, it is possible that *dpy-24* transcription is either directly or indirectly activated by LIN-42.

To confirm this possibility, transgenic worms carrying $P_{dpy\text{-}24}$::dGFP were treated with *lin-42(RNAi)*. After the treatment, the percentage of DTCs expressing GFP decreased during phase Ⅰ from about 80% to about 20% (Fig. 4B & Table 4). This result suggests that LIN-42 is required for *dpy-24* transcription during phase Ⅰ migration.

Finally, I wanted to know whether there is a positive feedback loop on *dpy-24* expression. The migratory behavior of DTCs exhibits bistability. That is, DTCs maintain high expression level of *dpy-24* and low expression level of *unc-5* before mid-L3; during late-L3, DTCs efficiently switch to the state of low *dpy-24* level and high *unc-5* level. It has been shown previously that a positive feedback loop or a double-negative feedback loop is usually necessary for such bistability (Ferrell, 2002). To identify such a loop, I crossed the transcriptional reporter ($P_{dpy\text{-}24}$::dGFP) into *dpy-24(s71)* mutant and found that the *dpy-24* mutation severely impaired its own transcription, with only 10% of DTCs before mid-L3 expressed GFP (Fig. 4F & Table 4), supporting that there is indeed a positive feedback loop to maintain *dpy-24* transcription.

Discussion

dpy-24 may repress *unc-5* transcription and regulate DTC migration by different mechanisms in the anterior and posterior DTCs

dpy-24 has been shown to be required for the repression of *unc-5* transcription in DTCs during phase Ⅰ migration, and my structural and functional analysis of DPY-24 indicates that it functions cell-autonomously to regulate DTC migration. Taken together, DPY-24 is likely a transcription repressor for *unc-5*. Surprisingly, DPY-24ΔZF can still regulate the migration of the anterior but not the posterior DTCs, giving us two implications. The first is that DPY-24 regulates the migration of the anterior and posterior DTCs by different mechanisms. Actually, it has already been recognized that the anterior and posterior DTC migration are not identical at the molecular level (Nishiwaki, 1999), because some mutations affect them to different degrees, including *dpy-24* (data not shown). Second, the lack of importance of DPY-24 zinc fingers in the anterior DTCs suggests that it may have a more global role there, while the requirement of zinc fingers in posterior DTCs suggests that it may act more specifically on a smaller subset of target genes. This is consistent with the *dpy-24* mutant phenotype, such that the anterior DTCs also display severe defect in phase Ⅲ migration, while the posterior ones are less affected. The ability of DPY-24 zinc fingers to bind to *unc-5* promoter in EMSA assay indicates that DPY-24 is potentially a direct transcription repressor for *unc-5* at least in posterior DTCs and supports the above hypothesis. Furthermore, the identification of exactly two binding sequences, D1 and D2, can help us predict other potential *dpy-24* targets (Table S1).

In contrast, DPY-24ΔPR and DPY-24ΔPRΔZF only retain partial function in regulating both anterior and posterior DTCs, suggesting that PR domain is required for both anterior and posterior DTCs and that some unidentified regions outside PR domain and

zinc fingers is also important. The PR domain has been shown to serve as protein-protein interaction interface (Huang et al., 1998). In addition, DPY-24's mammalian homologs PRDI-BF1/Blimp-1 can recruit co-repressors or histone modification enzymes to repress their target genes (Ancelin et al., 2006; Gyory et al., 2004; Ren et al., 1999; Yu et al., 2000). Therefore, it is possible that DPY-24 also forms a complex around the promoter of *unc-5* and other target genes to mediate repression. This can also explain why zinc fingers are not required in the anterior DTCs; the DNA-binding ability may be conferred by other partners in the complex.

To sum up, DPY-24 may employ different mechanisms to repress *unc-5* transcription and have different roles in the anterior and posterior DTCs, while both may involve interaction with other proteins. The identification of DPY-24 interacting protein will possibly elucidate more clearly the mechanisms of DPY-24 repression.

The heterochronic circuit in DTC migration converges on the regulation of *dpy-24* level

Previous studies have established that *lin-42* specifies the DTC phase Ⅰ temporal identity by suppressing *lin-29* (Tennessen et al., 2006) and working partly upstream or in parallel to *daf-12* and *dre-1* (Fielenbach et al., 2007). During late L3, *daf-12*, *dre-1*, and *lin-29* function redundantly to promote phase Ⅱ and phase Ⅲ programs (Fielenbach et al., 2007). However, how they coordinate together to specify temporal identity has not been studied. Here, I found that the functions of these heterochronic genes converge on the regulation of the *dpy-24* level (Fig. 7A). It has been shown previously that *dpy-24* loss-of-function causes precocious phase Ⅱ execution, and excessive DPY-24 is sufficient to prolong phase Ⅰ program, suggesting that the *dpy-24* level is a critical factor controlling the timing of DTC turning. During phase Ⅰ, LIN-42 acti-

vates *dpy-24* transcription, thereby maintaining the phase Ⅰ identity. During late L3, the *lin-42* level is declining as its innate periodic nature; DAF-12 and LIN-29 repress *dpy-24* transcription, and DRE-1 decreases DPY-24 protein stability; this multiple down-regulation mechanism, which may be weak individually, ensures the efficient switch from phase Ⅰ into phase Ⅱ and phase Ⅲ.

LIN-29, which is a zinc finger transcription factor, has been shown to bind directly to the promoter of its target genes and either activate or repress their transcription (Rougvie and Ambros, 1995). Therefore, the *lin-29* repression on *dpy-24* transcription may be a direct regulation. Further analysis of *dpy-24* promoter may help to verify this assumption. In contrast, the *daf-12* repression on *dpy-24* transcription is likely indirect. Previous experiments support a model in which the hormone for DAf-12 is produced during late L2 (Gerisch and Antebi, 2004b), and liganded DAF-12 served as a transcription activator to promote reproductive growth in L3 (Fielenbach and Antebi, 2008). Therefore, it is possible that DAF-12 activates some other intermediate regulators and cooperates with LIN-29 to repress *dpy-24* transcription. The decrease of DPY-24 stability by DRE-1 is likely through ubiquitin E3 ligase manner, and this could be confirmed by testing the interaction between DPY-24 and DRE-1 or detecting the ubiquitinated DPY-24 *in vivo*. Finally, it would also be interesting to check whether *dpy-24* and *lin-42* act in the same pathway to promote phase Ⅰ migration.

However, it should be noted that these genes possibly also have parallel functions besides regulating the *dpy-24* level to contribute to the correct expression of downstream effectors, such as *unc-5*. This is supported by the result that DAF-12 and LIN-29 can directly bind to *unc-5* promoter in EMSA. In addition, in wild-type worms carrying the transgene $P_{lag\text{-}2}$::gfp::*dpy-24*, DTCs expressing GFP-DPY-24 still managed to execute phase II and phase III programs properly, suggesting the existence of activators which outweighed the repression by DPY-24. Eventually, to turn or not to turn (dorsally)

more likely depends on the relative levels of these temporal regulators (Fig. 7A).

dpy-24 may be repressed by miRNA, which may be activated by liganded DAF-12

In addition to the down-regulation of *dpy-24* transcription and DPY-24 stability, I have found that the *dpy-24* 3'UTR also mediates gene silencing earlier at late L2 stage, likely by binding of miRNA to interfere with the translation. However, in wild type, endogenous DPY-24 can be detected in DTCs until late L3, suggesting that DPY-24 is very stable before dorsal turn so that DPY-24 produced at L2 can persist into L3 stage. This highlights the importance of the down-regulation of DPY-24 stability by DRE-1 and is consistent with the *dre-1* mutant phenotype, in which DTCs make dorsal turn slightly later than in wild-type (Fielenbach et al., 2007), perhaps because clearance of DPY-24 is defective.

It has been reported that DAF-12 directly activates miRNAs upon ligand binding but tightly represses them in the absence of ligand (Bethke et al., 2009). Mutations in DAF-12 ligand binding domain also causes severe DTC migration defect, in which DTCs fail to leave phase I migration and exhibit no-turn phenotype (Antebi et al., 2000), suggesting that hormone binding is required to release the unliganded DAF-12 repression on DTC later programs. *daf-9* encodes cytochrome P450 and is required for the production of DAF-12 ligand (Gerisch and Antebi, 2004a; Gerisch et al., 2001; Jia et al., 2002). *daf-9* expression in hypodermal cells is up-regulated in mid-L2 and act up-stream of *daf-12* to promote reproductive growth (Gerisch and Antebi, 2004a). Consid-ering all these data, it is reasonable to postulate that the miRNAs that repress *dpy-24* is also activated by liganded DAF-12. This miRNA repression on *dpy-24* translation may be the response of DTCs to global hormone signal. Further experiments will be needed to prove this hypothesis.

Multiple but sequential down-regulations of the *dpy-24* level decide the timing of DTC dorsal turn

Unlike extragonadal tissues, the temporal progression of DTC migration seems to be uncoupled from the molting cycle in *C. elegans*. For example, seam cells undergo divisions each larval stage and exhibit distinct patterns. However, the correlation between DTC temporal identity and larval stages is less clear. The phase Ⅰ of DTC migration occurs during L2 and early L3 stages, and the phase Ⅱ is finished in late L3. The phase Ⅲ occurs in late L3 and L4 stages. The hormone-dependent progression of DTC migration provides a mechanism by which DTCs integrate the signal from other tissues. However, it is still unknown how DTC turning is confined in the short window in late L3.

Here, I found that *dpy-24* expression is actually down-regulated at two steps: the translation may be blocked by miRNA at late L2, and the transcription and protein stability is down-regulated later at late-L3. This may help to resolve the puzzle of how DTCs is programmed to make turns only at late-L3. One possibility is that the low level of LIN-42 and high level of DAF-12-activated miRNA together determine the DTC turning at late L3. As a homolog of circadian rhythm protein, LIN-42 level oscillates with molting cycle in *C. elegans*, with highest level during inter-molt and lowest level at molting. At late L2, liganded DAF-12 activates the transcription of miRNAs for *dpy-24*. Until late-L3, either when DPY-24 level has dropped beyond a threshold or when miRNAs have accumulated enough, further combined with the decline in LIN-42 level before molting, *dpy-24* expression is further down-regulated at both transcriptional and post-translational level, and DTCs can now enter into phase Ⅱ and phase Ⅲ migration. In brief, two layers of temporal events, i.e. *lin-42* oscillation and *daf-12* activation by ligand, together determine the timing of DTC turning (Fig. 7B).

The switch-like behavior of DTC dorsal migration is contributed by a positive feedback loop involving *dpy-24*

During DTC phase Ⅰ migration, *dpy-24* is expressed while *unc-5* is repressed, ensuring that DTCs maintain their positions at ventral side. At late L3, there is a concomitant down-regulation of *dpy-24* and up-regulation of *unc-5*, initiating the dorsal migration. Afterward, DTCs maintain their position at dorsal side throughout the entire phase Ⅲ and migrate back to the mid-body region. Therefore, the migratory behavior of DTCs exhibits bistability, i.e. the DPY-24(+)/UNC-5(-) ventral-side migration and the DPY-24(-)/UNC-5(+) dorsal-side migration.

In my study, I found that *dpy-24* transcription is maintained by a positive feedback loop. This positive feedback loop may be important for the switch-like DTC migratory behavior. With this positive feedback loop, DPY-24 can maintain its own level during phase Ⅰ to prevent the execution of phase Ⅱ or phase Ⅲ programs, which can only occur when an outside trigger down-regulate the *dpy-24* level. Subsequently, the *dpy-24* level remains low because of the loss of activation by its own protein, so the phase Ⅱ and phase Ⅲ programs can be efficiently executed.

In conclusion, the robustness and fidelity of DTC migration is achieved in several ways (Fig. 7A). The early phase (phase Ⅰ) of DTC migration is specified by *lin-42* and *dpy-24*, while the latter is reinforced by a positive feedback to avoid precocious turn. The initiation of later phases (phase Ⅱ and phase Ⅲ) is promoted by three redundant genes (*daf-12*, *dre-1*, and *lin-29*), whose functions include down-regulation of *dpy-24*, to avoid retarded turn. Finally, the timing of this switch from phase Ⅰ to phase Ⅱ and phase Ⅲ may be precisely decided by multiple layer of regulation. These results may elucidate the principles for temporal regulation during cell migration.

References

Ambros, V. and Horvitz, H. R. (1984). Heterochronic mutants of the nematode Caenorhabditis elegans. *Science* **226**, 409-16.

Ancelin, K., Lange, U. C., Hajkova, P., Schneider, R., Bannister, A. J., Kouzarides, T. and Surani, M. A. (2006). Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat Cell Biol* **8**, 623-30.

Antebi, A., Yeh, W. H., Tait, D., Hedgecock, E. M. and Riddle, D. L. (2000). daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. *Genes Dev* **14**, 1512-27.

Ayala, R., Shu, T. and Tsai, L. H. (2007). Trekking across the brain: the journey of neuronal migration. *Cell* **128**, 29-43.

Bethke, A., Fielenbach, N., Wang, Z., Mangelsdorf, D. J. and Antebi, A. (2009). Nuclear hormone receptor regulation of microRNAs controls developmental progression. *Science* **324**, 95-8.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.

Chan, S. S. Y., Zheng, H., Su, M. W., Wilk, R., Killeen, M. T., Hedgecock, E. M. and Culotti, J. G. (1996). UNC-40, a C. elegans Homolog of DCC (Deleted in Colorectal Cancer), Is Required in Motile Cells Responding to UNC-6 Netrin Cues. **87**, 187-195.

Cram, E. J., Shang, H. and Schwarzbauer, J. E. (2006). A systematic RNA interference screen reveals a cell migration gene network in C. elegans. *J Cell Sci* **119**, 4811-4818.

Ferrell, J. E. (2002). Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Current Opinion in Cell Biology* **14**, 140-148.

Fielenbach, N. and Antebi, A. (2008). C. elegans dauer formation and the molecular basis of plasticity. *Genes & Development* **22**, 2149-2165.

Fielenbach, N., Guardavaccaro, D., Neubert, K., Chan, T., Li, D., Feng, Q., Hutter, H., Pagano, M. and Antebi, A. (2007). DRE-1: an evolutionarily conserved F box protein that regulates C. elegans developmental age. *Dev Cell* **12**, 443-55.

Gerisch, B. and Antebi, A. (2004a). Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. *Development* **131**, 1765-1776.

Gerisch, B. and Antebi, A. (2004b). Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. *Development* **131**, 1765-76.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V. and Antebi, A. (2001). A Hormonal Signaling Pathway Influencing C. elegans Metabolism, Reproductive Devel-

opment, and Life Span. **1**, 841-851.

Ghosh, N., Gyory, I., Wright, G., Wood, J. and Wright, K. L. (2001). Positive regulatory domain I binding factor 1 silences class II transactivator expression in multiple myeloma cells. *J Biol Chem* **276**, 15264-8.

Griffiths-Jones, S. (2004). The microRNA Registry. *Nucl. Acids Res.* **32**, D109-111.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A. and Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucl. Acids Res.* **34**, D140-144.

Griffiths-Jones, S., Saini, H. K., van Dongen, S. and Enright, A. J. (2008). miRBase: tools for microRNA genomics. *Nucl. Acids Res.* **36**, D154-158.

Gyory, I., Fejer, G., Ghosh, N., Seto, E. and Wright, K. L. (2003). Identification of a functionally impaired positive regulatory domain I binding factor 1 transcription repressor in myeloma cell lines. *J Immunol* **170**, 3125-33.

Gyory, I., Wu, J., Fejer, G., Seto, E. and Wright, K. L. (2004). PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. *Nat Immunol* **5**, 299-308.

Hedgecock, E. M., Culotti, J. G. and Hall, D. H. (1990). The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. *Neuron* **4**, 61-85.

Huang, S., Shao, G. and Liu, L. (1998). The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. *J Biol Chem* **273**, 15933-9.

Ishii, N., Wadsworth, W. G., Stern, B. D., Culotti, J. G. and Hedgecock, E. M. (1992). UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in C. elegans. *Neuron* **9**, 873-881.

Itoh, B., Hirose, T., Takata, N., Nishiwaki, K., Koga, M., Ohshima, Y. and Okada, M. (2005). SRC-1, a non-receptor type of protein tyrosine kinase, controls the direction of cell and growth cone migration in C. elegans. *Development* **132**, 5161-5172.

Jeon, M., Gardner, H. F., Miller, E. A., Deshler, J. and Rougvie, A. E. (1999). Similarity of the C. elegans developmental timing protein LIN-42 to circadian rhythm proteins. *Science* **286**, 1141-6.

Jia, K., Albert, P. S. and Riddle, D. L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. *Development* **129**, 221-231.

John, S. A. and Garrett-Sinha, L. A. (2008). Blimp1: A conserved transcriptional repressor critical for differentiation of many tissues. *Exp Cell Res*.

Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. and Ahringer, J. (2000). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. *Genome Biology* **2**, research0002.1 - research0002.10.

Keller, A. D. and Maniatis, T. (1992). Only two of the five zinc fingers of the eukaryotic transcriptional repressor PRDI-BF1 are required for sequence-specific DNA binding. *Mol Cell Biol* **12**, 1940-9.

Keller, R. (2005). Cell migration during gastrulation. *Current Opinion in Cell Biology* **17**, 533-541.

Kimble, J. and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. *Developmental Biology* **70**, 396-417.

Lee, M., Cram, E. J., Shen, B. and Schwarzbauer, J. E. (2001). Roles for beta pat-3 Integrins in Development and Function of Caenorhabditis elegans Muscles and Gonads. *J. Biol. Chem.* **276**, 36404-36410.

Leung-Hagesteijn, C., Spence, A. M., Stern, B. D., Zhou, Y., Su, M.-W., Hedgecock, E. M. and Culotti, J. G. (1992). UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in C. elegans. *Cell* **71**, 289-299.

Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C.-C. and Kain, S. R. (1998). Generation of Destabilized Green Fluorescent Protein as a Transcription Reporter. *J. Biol. Chem.* **273**, 34970-34975.

Lundquist, E. A., Reddien, P. W., Hartwieg, E., Horvitz, H. R. and Bargmann, C. I. (2001). Three C. elegans Rac proteins and several alternative Rac regulators control axon guidance, cell migration and apoptotic cell phagocytosis. *Development* **128**, 4475-88.

Luster, A. D., Alon, R. and von Andrian, U. H. (2005). Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol* **6**, 1182-90.

Meighan, C. M. and Schwarzbauer, J. E. (2007). Control of C. elegans hermaphrodite gonad size and shape by vab-3/Pax6-mediated regulation of integrin receptors. *Genes & Development* **21**, 1615-1620.

Merz, D. C., Alves, G., Kawano, T., Zheng, H. and Culotti, J. G. (2003). UNC-52/Perlecan affects gonadal leader cell migrations in c. elegans hermaphrodites through alterations in growth factor signaling. *Developmental Biology* **256**, 174-187.

Nishiwaki, K. (1999). Mutations affecting symmetrical migration of distal tip cells in Caenorhabditis elegans. *Genetics* **152**, 985-97.

Reddien, P. W. and Horvitz, H. R. (2000). CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans. *Nat Cell Biol* **2**, 131-6.

Ren, B., Chee, K. J., Kim, T. H. and Maniatis, T. (1999). PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev* **13**, 125-37.

Rougvie, A. E. (2005). Intrinsic and extrinsic regulators of developmental timing: from miRNAs to nutritional cues. *Development* **132**, 3787-98.

Rougvie, A. E. and Ambros, V. (1995). The heterochronic gene lin-29 encodes a zinc finger protein that controls a terminal differentiation event in Caenorhabditis elegans. *Development* **121**, 2491-500.

Shostak, Y., Van Gilst, M. R., Antebi, A. and Yamamoto, K. R. (2004). Identification of C. elegans DAF-12-binding sites, response elements, and target genes. *Genes Dev* **18**, 2529-44.

Su, M., Merz, D. C., Killeen, M. T., Zhou, Y., Zheng, H., Kramer, J. M., Hedgecock, E. M. and Culotti, J. G. (2000). Regulation of the UNC-5 netrin receptor initiates the first reorientation of migrating distal tip cells in Caenorhabditis elegans. *Development* **127**, 585-94.

Tennessen, J. M., Gardner, H. F., Volk, M. L. and Rougvie, A. E. (2006). Novel heterochronic functions of the Caenorhabditis elegans period-related protein LIN-42. *Dev Biol* **289**, 30-43.

Wadsworth, W. G. (2002). Moving around in a worm: netrin UNC-6 and circumferential axon guidance in C. elegans. *Trends in Neurosciences* **25**, 423-429.

Wadsworth, W. G., Bhatt, H. and Hedgecock, E. M. (1996). Neuroglia and Pioneer Neurons Express UNC-6 to Provide Global and Local Netrin Cues for Guiding Migrations in C. elegans. **16**, 35-46.

Wu, Y. C. and Horvitz, H. R. (1998). C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. *Nature* **392**, 501-4.

Yamaguchi, H., Wyckoff, J. and Condeelis, J. (2005). Cell migration in tumors. *Current Opinion in Cell Biology* **17**, 559-564.

Yu, J., Angelin-Duclos, C., Greenwood, J., Liao, J. and Calame, K. (2000). Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Mol Cell Biol* **20**, 2592-603.

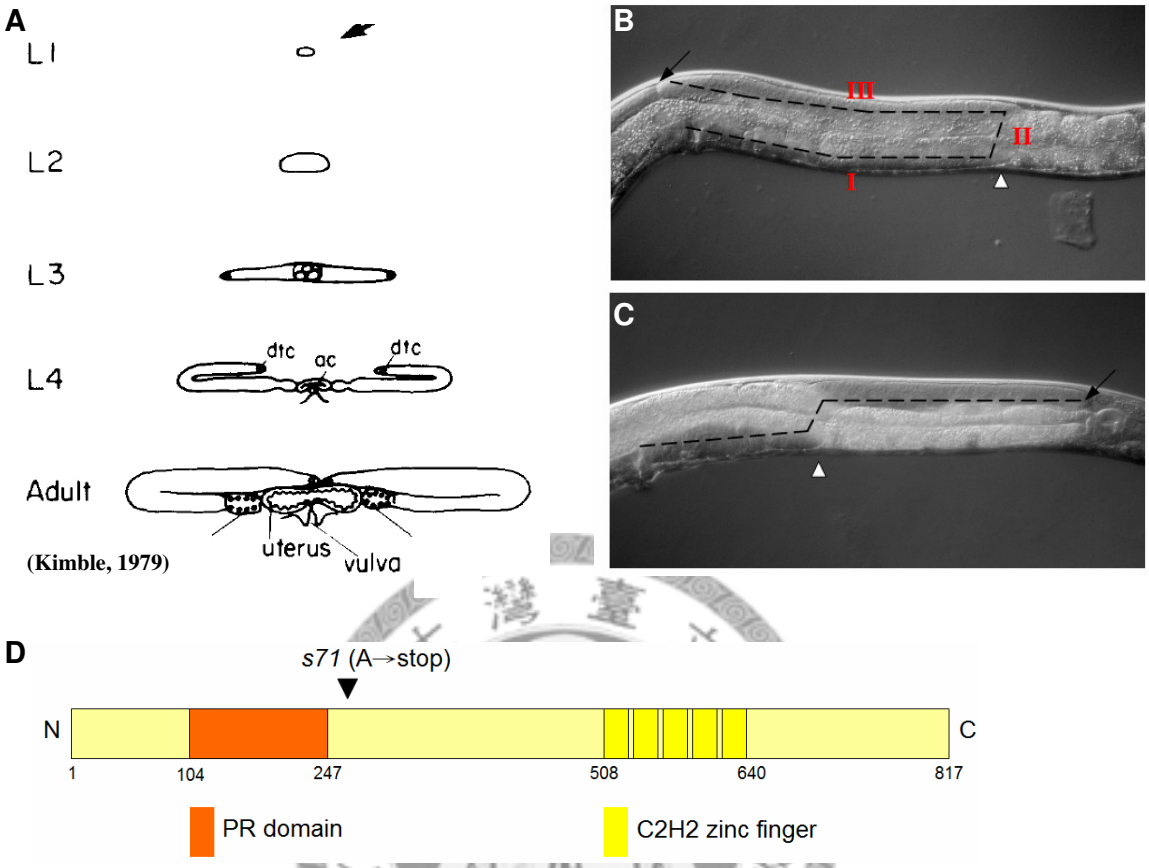


Fig. 1 *dpy-24* mutation causes precocious DTC dorsal turn

(A) Two DTCs (distal tip cells) are born in L1 stage and migrate throughout larval stages to lead the formation of bi-lobed gonad in adult (Kimble, 1979). **(B)** The gonad shape in wild-type. The corresponding phase Ⅰ, Ⅱ, and Ⅲ of DTC migration are noted. The triangle marks the position where the DTC made the dorsal turn. The DTC are pointed by the arrow. The migratory path is marked by the dotted line. **(C)** The gonad shape in *dpy-24(s71)* mutant. The DTC initiated dorsal turn precociously. **(D)** The schematic diagram of DPY-24 domain structure.

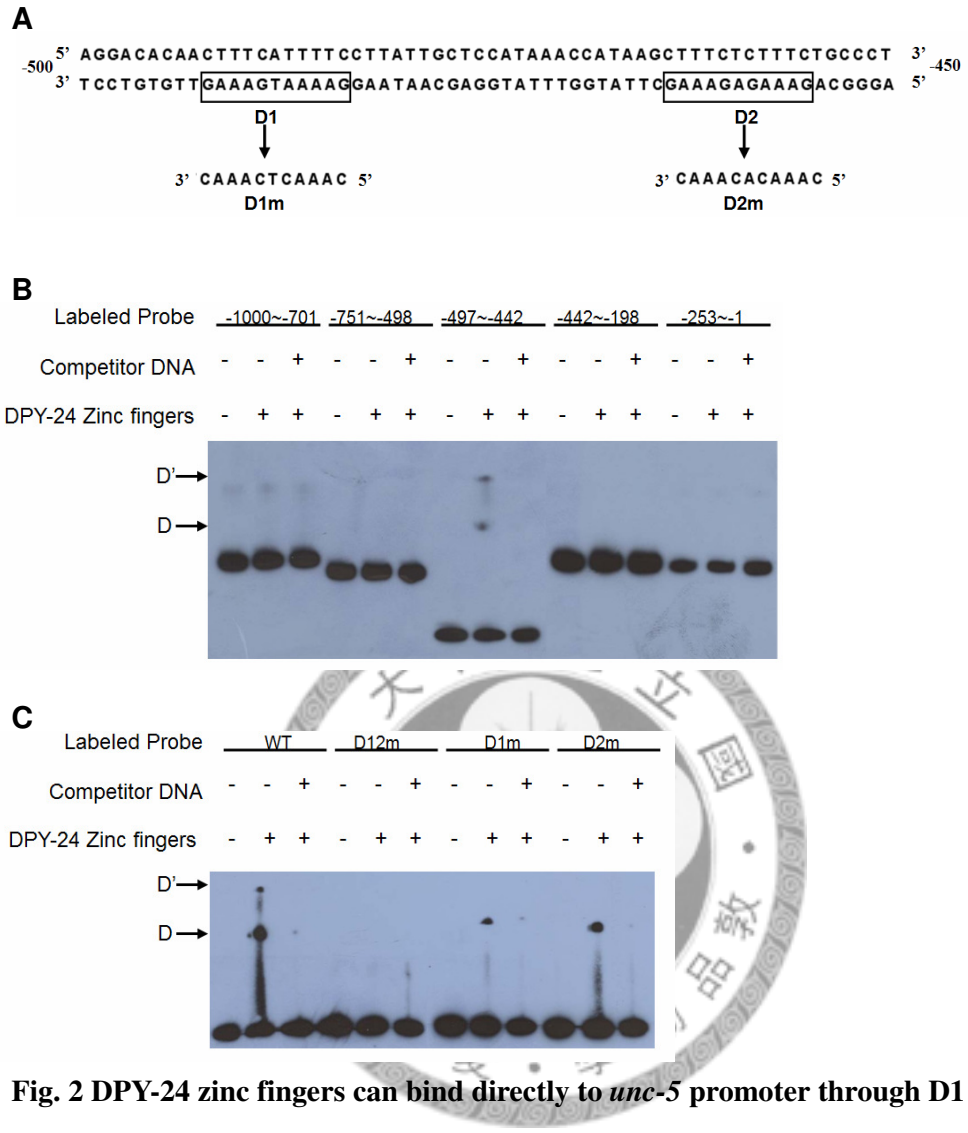
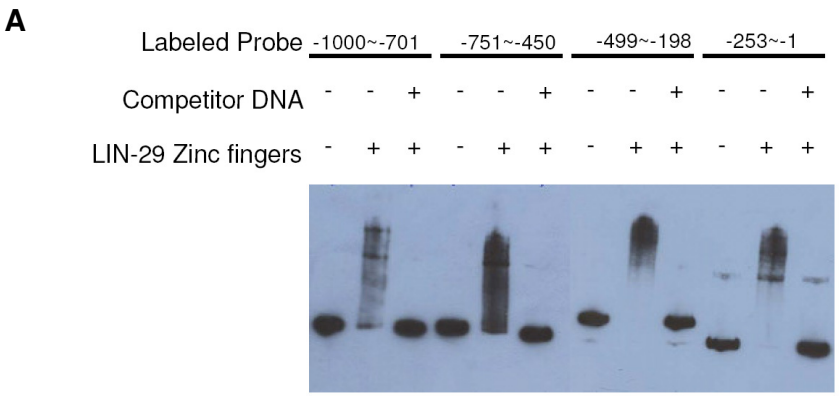
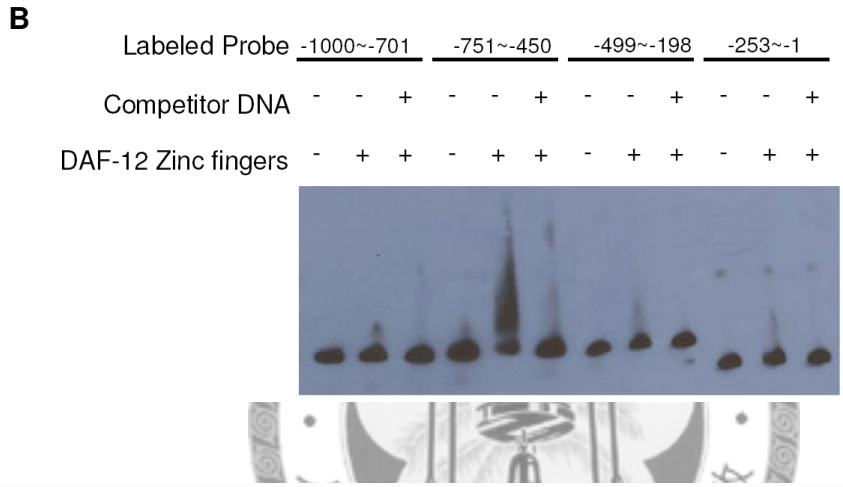
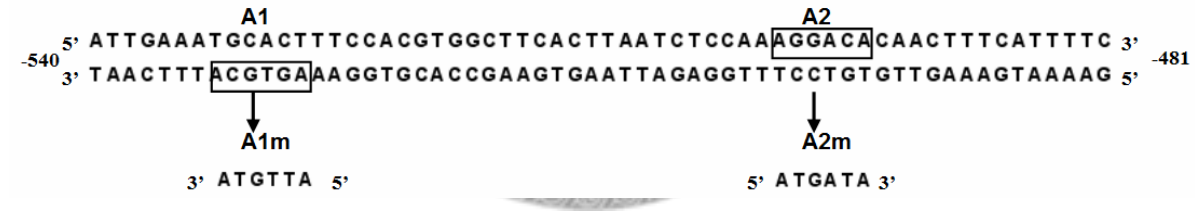


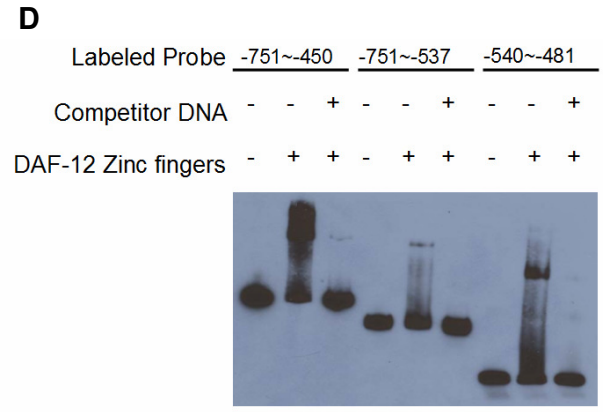
Fig. 2 DPY-24 zinc fingers can bind directly to *unc-5* promoter through D1 and D2

(A) Prediction of two DPY-24 binding sites, D1 and D2, in *unc-5* promoter by homology modeling. -500 to -450 5' regulatory region of *unc-5* was shown (+1 as the first nucleotide in coding region). This work is a favor done by Dr. Yi-Shen Chen. **(B)** DPY-24 zinc fingers have two binding sites in EMSA (Electrophoretic Mobility Shift Assay). The shifted band at position D suggests the binding of one DPY-24 zinc fingers to the probe, while the band at position D' suggests the binding of two DPY-24 zinc fingers to the same probe. **(C)** Mutation of D1 or D2 disrupts the binding of DPY-24 zinc fingers to *unc-5* promoter, suggesting that DPY-24 zinc fingers can bind specifically to D1 and D2 sites. D1m or D2m indicates the mutation of D1 and D2, respectively.


A
Labeled Probe
-1000~-701
-751~-450
-499~-198
-253~-1
Competitor DNA
LIN-29 Zinc fingers


B
Labeled Probe
-1000~-701
-751~-450
-499~-198
-253~-1
Competitor DNA
DAF-12 Zinc fingers


C
A1
A2
-540
5' ATTGAAATGCACTTTCCACGTGGCTTCACTTAATCTCCAAAGGACACAACTTTCATTTTC 3'
3' TAACTTTACGTGAAAGGTGCACCGAAGTGAATTAGAGGTTTCCTGTGTTGAAAGTAAAAG 5'
-481
A1m
A2m
3' ATGTTA 5'
5' ATGATA 3'


D
Labeled Probe
-751~-450
-751~-537
-540~-481
Competitor DNA
DAF-12 Zinc fingers

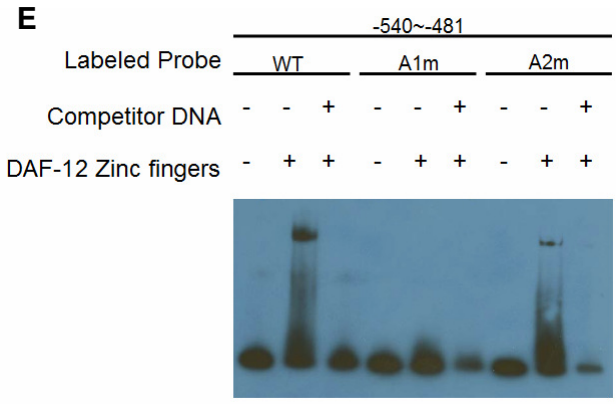

E
-540~-481
Labeled Probe
WT
A1m
A2m
Competitor DNA
DAF-12 Zinc fingers

Fig. 3 DAF-12 and LIN-29 bind directly to *unc-5* promoter in EMSA

(A) LIN-29 zinc fingers can bind to multiple regions in *unc-5* promoter. **(B)** DAF-12 zinc fingers can bind to -751~-450 of *unc-5* promoter, which contains two potential DAF-12 binding sites, namely A1 and A2 **(C)**. **(D)** Further analysis of this region reveals an unidentified binding site besides A1 and A2 in -751~-537. **(E)** Mutation in A1 disrupts the binding of DAF-12 zinc fingers, while mutation in A2 has no effect, suggesting that DAF-12 binds A1 but not A2.

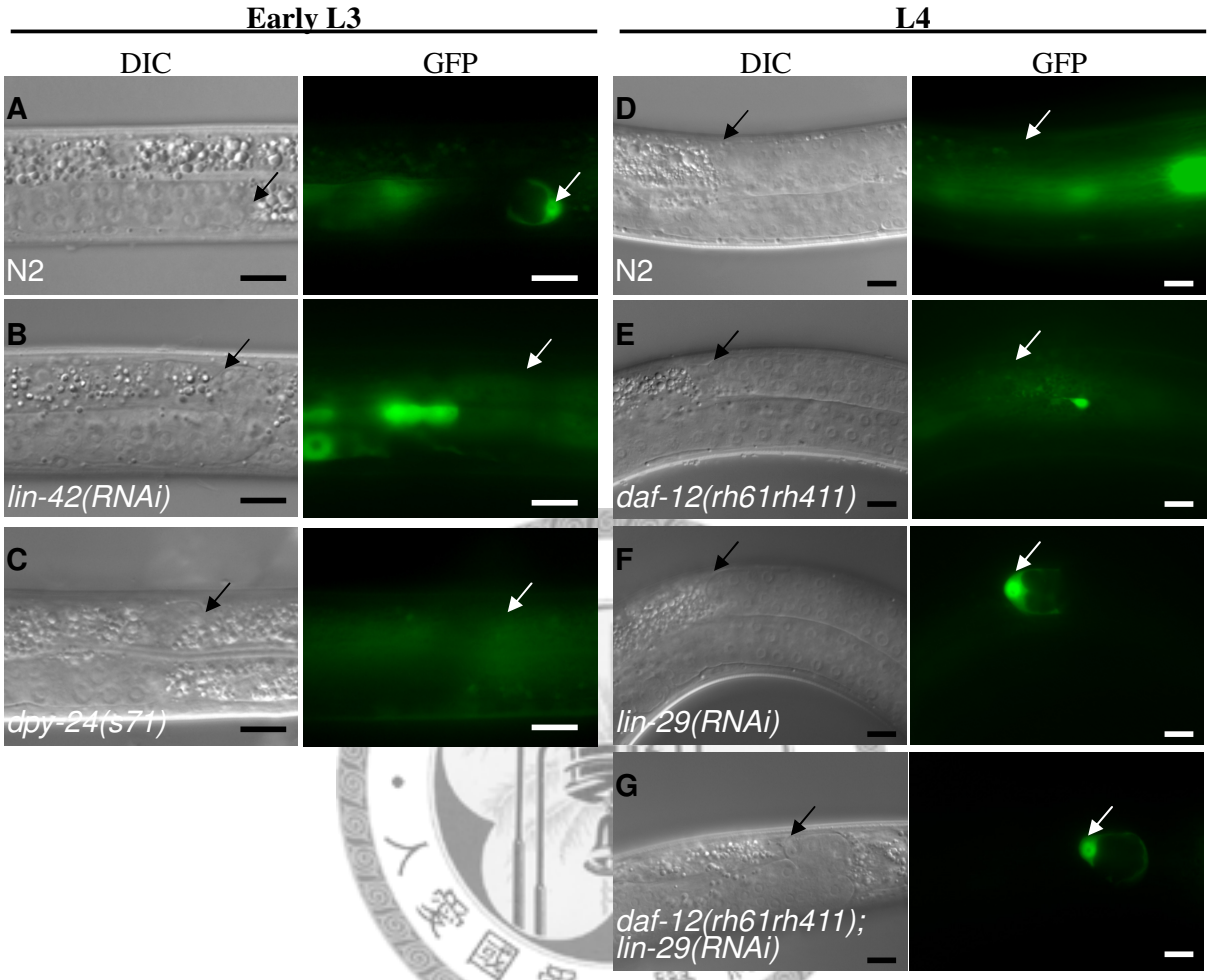


Fig. 4 Transcriptional regulation of *dpy-24*

(A) In wild-type, $P_{dpy\text{-}24}$*::*dGFP is expressed in DTCs before dorsal turn (early L3) but down-regulated after dorsal turn **(D)**. This transcriptional down-regulation is disrupted in *lin-29(RNAi)* **(F)** or *daf-12(rh61rh411);lin-29(RNAi)* **(G)** but not *daf-12(rh61rh411)* single mutant **(E)**. The expression in early L3 is diminished in *lin-42(RNAi)* **(B)** or *dpy-24(s71)* mutant **(C)**. Scale bar 10 μm.

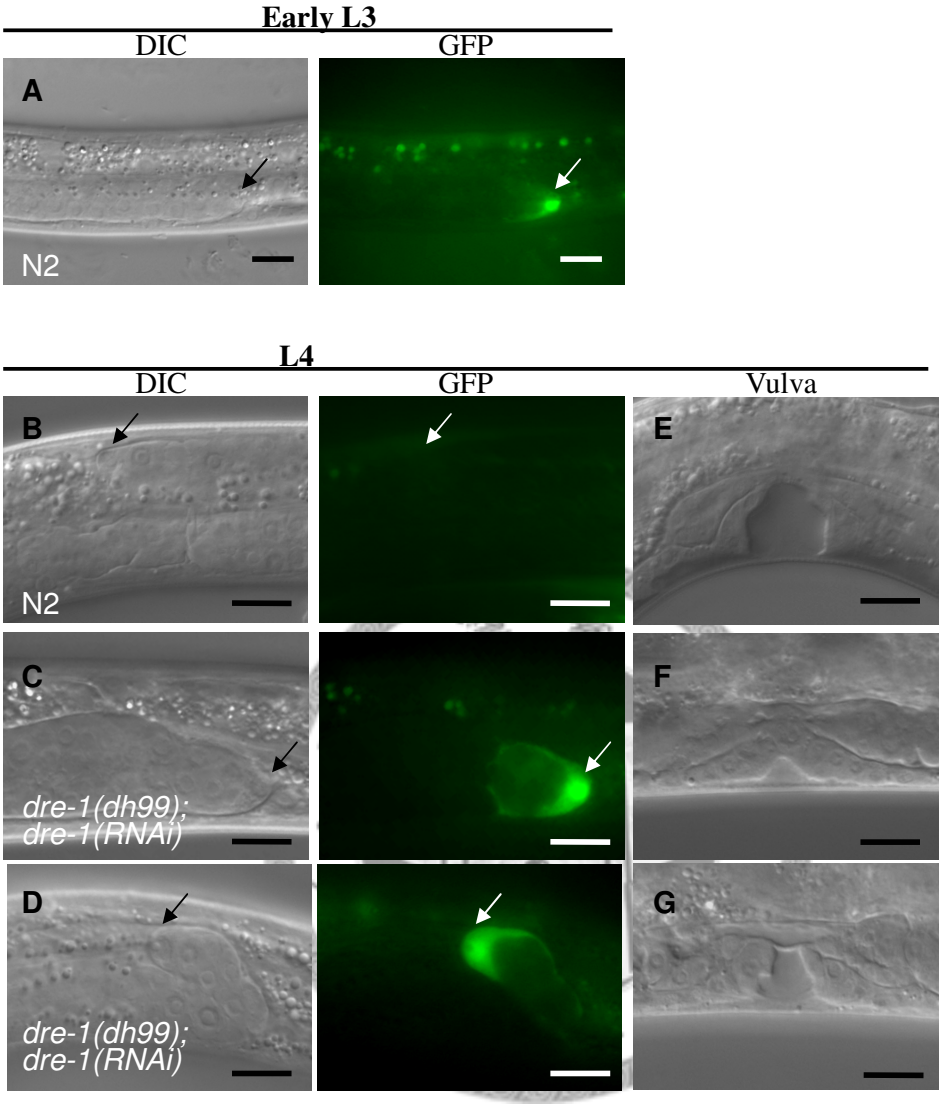


Fig. 5 DRE-1 decreases DPY-24 stability after dorsal turn.

(A) In wild-type, DTCs express $P_{lag\text{-}2}$::gfp::*dpy-24* during phase Ⅰ migration. The percentage of GFP expression decreased after dorsal turn **(B)**, suggesting that the protein stability is decreased. **(C)(D)** In *dre-1(dh99);dre-1(RNAi)*, this decrease in GFP expression disappeared. Furthermore, the timing of DTC turning is delayed. Their vulvae were shown in **(F)** and **(G)**, indicating that they are in early and late L4 stages, respectively. DTCs are pointed by arrows. Scale bar 10 μ m.

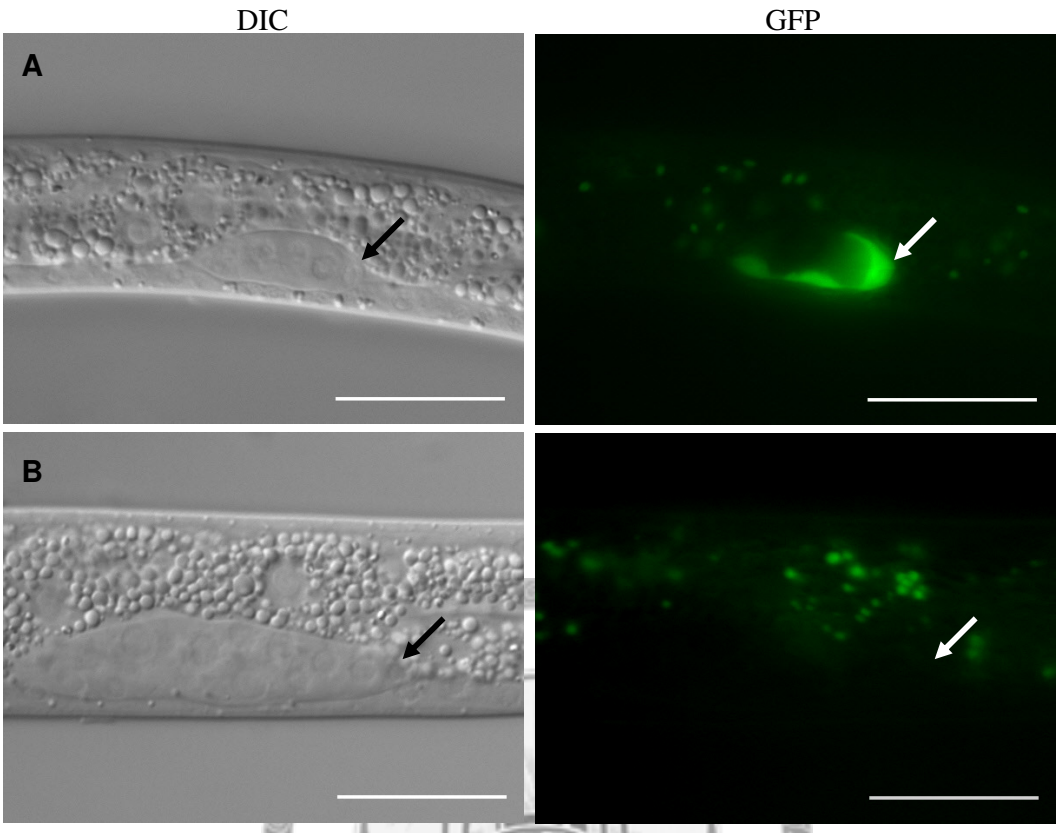


Fig. 6 *dpy-24* may be repressed by miRNA at late-L2 stage

(A) In wild-type, DTCs express P_{lag-2}::dGFP::*dpy-24* 3'UTR in early L2. **(B)** This expression is diminished at late L2 stage. The arrows indicate DTCs. Scale bar 20 μ m.

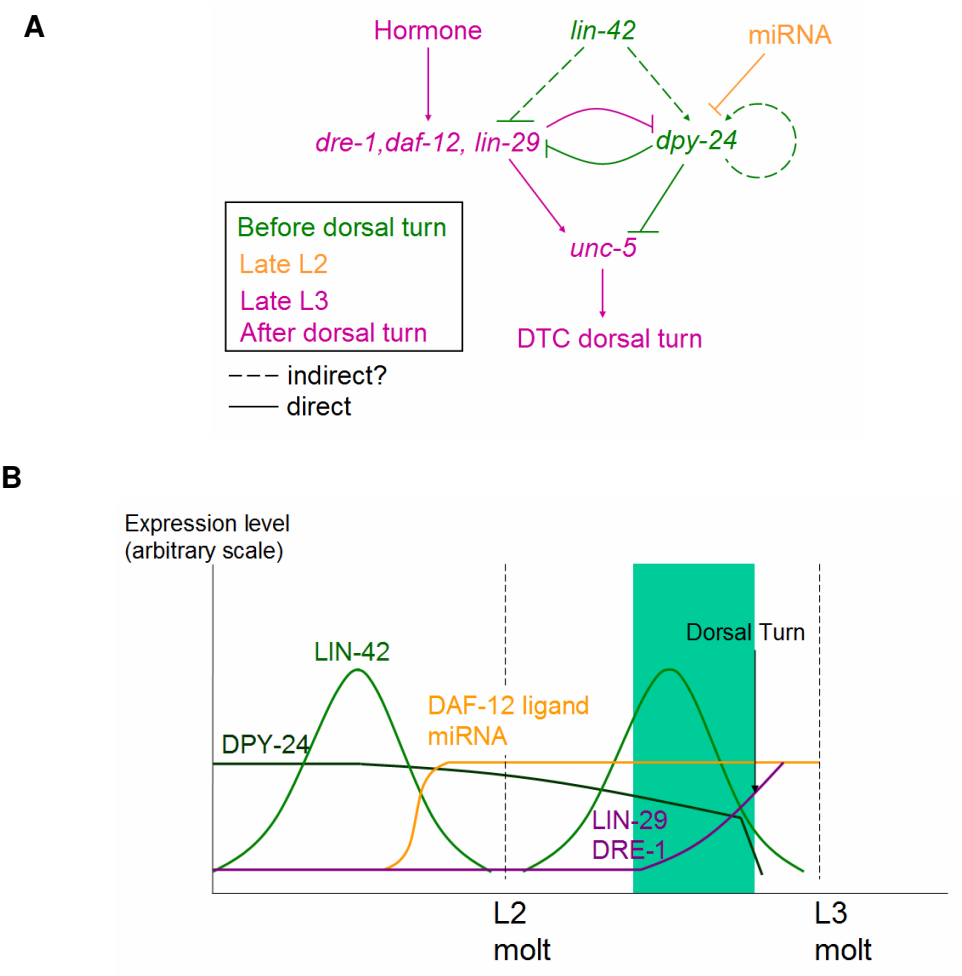


Fig. 7 DTC dorsal turn is controlled by a complex network.

(A) The structure of the molecular network controlling DTC dorsal turn. LIN-42 and DPY-24 prevents DTC dorsal turn, while DAF-12, DRE-1 and LIN-29 redundantly promote it. A positive feedback loop and a double-negative feedback loop may contribute to the switch-like behavior of DTCs. **(B)** Schematic representation of the relative levels of multiple regulators during DTC migration. The timing of DTC dorsal turn may be specified by multiple events, including production of DAF-12 ligand, low level of DPY-24 and LIN-42, and high level of LIN-29 and DRE-1.

Table 1. Functional analysis of the DPY-24 zinc fingers and PR domain

Transgene[a]	Line[b]	DTC migration defect (%)[c]	
		A	P
None		82	77
$P_{dpy\text{-}24}$::*dpy-24*	1	45	22
$P_{dpy\text{-}24}$::*dpy-24*	2	40	16
$P_{dpy\text{-}24}$::*dpy-24*Δ*ZF*	1	30	59
$P_{dpy\text{-}24}$::*dpy-24*Δ*ZF*	2	38	49
$P_{lag\text{-}2}$::*dpy-24*	1	20	19
$P_{lag\text{-}2}$::*dpy-24*	2	32	31
$P_{lag\text{-}2}$::*dpy-24*Δ*ZF*	1	24	43
$P_{lag\text{-}2}$::*dpy-24*Δ*ZF*	2	52	64
$P_{lag\text{-}2}$::*dpy-24*Δ*PR*	1	62	75
$P_{lag\text{-}2}$::*dpy-24*Δ*PR*	2	64	51
$P_{lag\text{-}2}$::*dpy-24*Δ*PR*Δ*ZF*	1	56	49
$P_{lag\text{-}2}$::*dpy-24*Δ*PR*Δ*ZF*	2	46	57

[a] *dpy-24(s71)* mutants carrying different transgene were generated by microinjection. ΔPR and ΔZF indicate deletions of PR domain and all five zinc fingers, respectively.

[b] Different numbers indicate different lines of the same transgene.

[c] Percentage of DTCs shown an abnormal migration pattern. “A” and “P” indicate anterior and posterior DTCs, respectively.

Table 2. Down-regulation of the *dpy-24* level by *daf-12*, *lin-29*, and *dre-1*

Genetic background	Line[a]	GFP percentage (%)[b]		Down-regulation (%)[c]
		Before P6.p 4-cell	After P6.p 4-cell	
I. $P_{dpy\text{-}24(5kb)}$::dGFP (Transcriptional fusion reporter)				
A. *daf-12* and *lin-29* repress *dpy-24* transcription after DTC dorsal turn				
N2	1	77.3	3.4	95.6
daf-12(rh61rh411)	1	75.0	1.2	98.4
lin-29(RNAi)	1	86.0	25.5	70.4
daf-12(rh61rh411);lin-29(RNAi)	1	80.8	58.5	27.5
II. $P_{lag\text{-}2}$::gfp::*dpy-24* (Translational fusion protein)				
B. *dre-1* down-regulates DPY-24 protein stability after DTC dorsal turn				
N2	1	27.4	16.9	38.3
N2	2	31.4	20.2	35.5
dre-1(dh99);dre-1(RNAi)	1	46.8	48.8	-4.2
dre-1(dh99);dre-1(RNAi)	2	28.4	28.2	0.7

[a] Different numbers indicate different transgenic lines.

[b] Percentage of DTCs expressing GFP reporter. The timing of vulva precursor cell P6.p division into 4-cell stage is the same with that of DTC dorsal turn in wild-type.

[c] Down-regulation=100% - (GFP percentages after P6.p 4-cell/GFP percentages before P6.p 4-cell)%

Table 3. *dpy-24* is down-regulated at post-transcriptional level at late L2 stage

P_{lag-2}::dGFP::*dpy-24* 3'UTR

Genetic background	Line[a]	GFP percentage (%)[b]	
		Early L2	Late L2
N2	1	45.2	3.3
N2	2	57.5	7.5

[a] Different numbers indicate different transgenic lines.

[b] Percentage of DTCs expressing GFP reporter.

Table 4. *dpy-24* is positively regulated by *lin-42* and *dpy-24* itself

$P_{dpy\text{-}24}$::dGFP (Transcriptional fusion reporter)

Genetic background	Line[a]	GFP percentage (%)[b]
		Before P6.p 4-cell
N2	1	77.3
lin-42(RNAi)	1	27.0
dpy-24(tp3)	1	9.3

[a] Different numbers indicate different lines.

[b] Percentage of DTCs expressing GFP reporter. The timing of vulva precursor cell P6.p division into 4-cell stage is the same with that of DTC dorsal turn in wild-type.

```
     GUAUUGUUGUUGGUGGGCGG  -mir-264
     :||:| ||| ||:||||||:|
19-  TATGAAAAC-ACTACCCGTC  -dpy-24 3' UTR


     GGGUUCUUAUGGUCUGUAUAGU  -mir-50
     :|| | ||| |||| |||||||
38-  TCCCACAAT-CCAGTCATATCA  -dpy-24 3' UTR


     CGUAAGUGGCGCACGGA  -mir-124
     || | |||:||||||||
88-  GCCTCCACTGCGTGCCT  -dpy-24 3' UTR


     GUCGAUUGGUGUGACGGA  -mir-34
     :: || || :||||||||
98-  TGCCTCACAGCACTGCCT  -dpy-24 3' UTR


     UCACAGUGUCUUAUU  -mir-1020
     | | | ::|||||||
115- ATTTTGGTAGAATAA  -dpy-24 3' UTR
```

Fig. S1 Potential miRNA binding sites in *dpy-24* 3'UTR

The alignments between miRNAs and their binding sites in *dpy-24* 3'UTR are shown. Starts of binding sites are indicated on the left, with number 1 as the first nucleotide after the stop codon. These binding sites are also conserved in *C. briggsae* as predicted by miRBase (http://microrna.sanger.ac.uk).

Table S1. Conserved *dpy-24* binding sites in *C. elegans* genome

Rank	Mismatch	*C. elegans* Binding Sites	*C. Briggsae* Binding Sites	ORF 1	Gene Name	ORF 2	Gene Name
0	0	GAAAGAGAAAG	GAAAGAGAAAG	F57G12.1	-	C23H4.1	cab-1
1	0	GAAAGTGAAAG	GAAAGTGAAAG	C24B5.3	ptr-1	C24B5.1	-
2	0	GAAAGTGAAAG	GAAAGTGAAAG	C24F3.4	-	C24F3.6	col-124
3	0	GAAAGGGAAAG	GAAAGGGAAAG	C52A11.4	-		-
4	0	GAAAGAGAAAG	GAAAGAGAAAG	D2021.2a	-		-
5	0	GAAAGAGAAAG	GAAAGAGAAAG	T28B8.4	-	F08A10.1a	-
6	0	GAAAGTGAAAG	GAAAGTGAAAG	F11H8.3	col-8	F11H8.1	rfl-1
7	0	GAAAGTGAAAG	GAAAGTGAAAG	F38B7.1b	-		-
8	0	GAAAGTGAAAG	GAAAGTGAAAG	T10B10.5	-	H03A11.1	-
9	0	GAAAGTGAAAG	GAAAGTGAAAG	B0205.4	-	R13H8.1b	daf-16
10	0	GAAAGAGAAAG	GAAAGAGAAAG	T27A10.6	-	T27A10.7	-
11	0	GAAAGCGAAAG	GAAAGCGAAAG	Y15E3A.1	nhr-91		-
12	0	GAAAGAGAAAG	GAAAGAGAAAG	Y40H7A.5	srd-23	Y40H7A.6	-
13	0	GAAAGAGAAAG	GAAAGAGAAAG	ZK180.5a	-	ZK180.6	-
14	1	GAAAGGGAAAG	GAAAGAGAAAG	B0035.5	-	B0035.6	-
15	1	GAAAGTGAAAG	GAAAGCGAAAG	F46F2.2a	kin-20	C02D4.1	-
16	1	GAAAGGGAAAG	GAAAGCGAAAG	C16D9.5	-	C16D9.6	-
17	1	GAAAGGGAAAG	GAAAGAGAAAG	C32D5.6	-	C32D5.7	-
18	1	GAAAGTGAAAG	GAAAGGGAAAG	C53A5.4	-	C53A5.5	-
19	1	GAAAGAGAAAG	GAAAGTGAAAG	C56G2.1a	-		-
20	1	GAAAGCGAAAG	GAAAGGGAAAG	F38B6.6	-	F38B6.3	-
21	1	GAAAGCGAAAG	GAAAGTGAAAG	F43C9.1	-		-
22	1	GAAAGCGAAAG	GAAAGTGAAAG	F57B7.3	col-156	T11F9.1	-
23	1	GAAAGCGAAAG	GAAAGAGAAAG	K08F8.1a	-	K08F8.2	-
24	1	GAAAGGGAAAG	GAAAGAGAAAG	R08D7.5	-	R08D7.6	-
25	1	GAAAGAGAAAG	GAAAGTGAAAG	T10A3.1a	unc-10	K03A1.2	-
26	1	GAAAGGGAAAG	GAAAGTGAAAG	ZK520.5	-	W06F12.1a	liv-5
27	1	GAAAGAGAAAG	GAAAGTGAAAG	ZC449.4	-	ZC449.5	-
28	1	GAAAGAGAAAG	GAAAGGGAAAG	ZK669.1a	-		-
29	1	GAAAGTGAAAG	GAAAGAGAAAG	ZK682.2	-	ZK682.4	hlh-10
30	1	GAAAGAGAAAG	GAAAAAGAAAG	C02F4.3	-	C02F4.4	-
31	1	GAAAAGGAAAG	GAAAGGGAAAG	C05E7.2	-	C05E7.1b	-
32	1	GAAAAAGAAAG	GAAAGAGAAAG	C07G2.3a	cct-5	F25F2.1a	-
33	1	GAAAGAGAAAG	GAAAAAGAAAG	C36F7.1	-	C36F7.2	-
34	1	GAAAGAGAAAG	GAAAAAGAAAG	T12B3.4	-	C48A7.2	-
35	1	GAAAAAGAAAG	GAAAGAGAAAG	C49F8.1	-	C49F8.2	-

Table S1. (Continued)

Rank	Mismatch	*C. elegans* Binding Sites	*C. Briggsae* Binding Sites	ORF 1	Gene Name	ORF 2	Common2
36	1	GAAAGTGAAAG	GAAAATGAAAG	T28C12.6	-	F13H6.1	-
37	1	GAAAAAGAAAG	GAAAGAGAAAG	F14F11.1e	-		-
38	1	GAAAGGGAAAG	GAAAAGGAAAG	F35D2.4	-	F35D2.5a	syd-1
39	1	GAAAAAGAAAG	GAAAGAGAAAG	F38H12.5	-	F38H12.3	-
40	1	GAAAAAGAAAG	GAAAGAGAAAG	D1022.8	cah-2	R10H1.1	-
41	1	GAAAGAGAAAG	GAAAAAGAAAG	F52E10.4	-	T01B4.1	twk-21
42	1	GAAAAAGAAAG	GAAAGAGAAAG	T06D10.1	-	T06D10.2	-
43	1	GAAAGGGAAAG	GAAAAGGAAAG	T21B6.5	-	T21B6.1	dgn-1
44	1	GAAAATGAAAG	GAAAGTGAAAG	ZK524.4	-	T28F4.1	-
45	1	GAAAGAGAAAG	GAAAAAGAAAG	W03A5.4	-	W03A5.5	-
46	1	GAAAGTGAAAG	GAAAATGAAAG	Y65B4A.3	-	Y65B4A.2	-
47	1	GAAAAAGAAAG	GAAAGAGAAAG	Y75B8A.1	php-3	Y75B8A.2b	nob-1
48	1	GAAAGAGAAAG	GAAAAAGAAAG	ZK177.8a	-		-
49	2	GAAAACGAAAG	GAAAGTGAAAG	B0495.10a	-	B0228.4b	-
50	2	GAAAGAGAAAG	GAAAATGAAAG	B0353.1	-		-
51	2	GAAAGCGAAAG	GAAAATGAAAG	C09G1.4	-	B0395.1	nhx-1
52	2	GAAAATGAAAG	GAAAGAGAAAG	C01H6.8	-	C01H6.9	-
53	2	GAAAAGGAAAG	GAAAGTGAAAG	C12C8.1	HSP16B	F26H9.8	-
54	2	GAAAAAGAAAG	GAAAGGGAAAG	C12D12.5	-	C12D12.6	-
55	2	GAAAAAGAAAG	GAAAGGGAAAG	C13B4.2	uts-4	K10H10.1	-
56	2	GAAAGTGAAAG	GAAAAAGAAAG	C18B12.6	-		-
57	2	GAAAGGGAAAG	GAAAAAGAAAG	C18D1.1	die-1	ZK945.1	-
58	2	GAAAAGGAAAG	GAAAGAGAAAG	C32D5.7	-	C32D5.8	-
59	2	GAAAATGAAAG	GAAAGAGAAAG	F13D2.4	-	C34F6.1	-
60	2	GAAAGAGAAAG	GAAAAGGAAAG	C36F7.1	-	C36F7.2	-
61	2	GAAAATGAAAG	GAAAGAGAAAG	C37F5.1	lin-1		-
62	2	GAAAGAGAAAG	GAAAATGAAAG	C48G7.1	-		-
63	2	GAAAATGAAAG	GAAAGAGAAAG	C54D2.1	-	C54D2.5a	cca-1
64	2	GAAAAGGAAAG	GAAAGCGAAAG	D1065.3	-	D1065.4	srh-210
65	2	GAAAAGGAAAG	GAAAGCGAAAG	D1069.3	-		-
66	2	GAAAAAGAAAG	GAAAGGGAAAG	D2024.6	cap-1	D2024.4	-
67	2	GAAAGAGAAAG	GAAAACGAAAG	C53C11.1	-	F10D7.1	-
68	2	GAAAATGAAAG	GAAAGAGAAAG	F10G8.5	ncs-2	F10G8.7	-
69	2	GAAAGTGAAAG	GAAAAAGAAAG	R153.1b	-	F18A1.6a	-
70	2	GAAAGAGAAAG	GAAAATGAAAG	F28D1.9	-	F28D1.10	gex-3
71	2	GAAAGCGAAAG	GAAAAGGAAAG	F28F5.6	-	F28F5.1	-

Table S1. (Continued)

Rank	Mismatch	*C. elegans* Binding Sites	*C. Briggsae* Binding Sites	ORF 1	Gene Name	ORF 2	Common2
72	2	GAAAAAGAAAG	GAAAGCGAAAG	F34D10.6	-	C44F1.1	-
73	2	GAAAAGGAAAG	GAAAGCGAAAG	F40A3.7	-	Y97E10B.6	-
74	2	GAAAGAGAAAG	GAAAATGAAAG	F55E10.7	-	F40B5.2a	-
75	2	GAAAGTGAAAG	GAAAAAGAAAG	F42G10.1	-	F42G10.2	mkk-4
76	2	GAAAATGAAAG	GAAAGAGAAAG	F42H10.5	-		-
77	2	GAAAAAGAAAG	GAAAGTGAAAG	F46C8.5	ceh-14	F46C8.6	dpy-7
78	2	GAAAACGAAAG	GAAAGAGAAAG	F47D12.9a	-	F47D12.1a	gar-2
79	2	GAAAGTGAAAG	GAAAAAGAAAG	F47F6.2	lin-43	F47F6.5	-
80	2	GAAAACGAAAG	GAAAGTGAAAG	F48B9.5	-	F48B9.4	-
81	2	GAAAGAGAAAG	GAAAATGAAAG	F52F12.2	col-64	F52F12.3	mom-4
82	2	GAAAGAGAAAG	GAAAAGGAAAG	F57F5.5	pkc-1	F10C2.2	kup-1
83	2	GAAAAAGAAAG	GAAAGCGAAAG	H22D07.1	-	T08H10.3	-
84	2	GAAAAGGAAAG	GAAAGAGAAAG	K01A12.3	-	F12D9.1	-
85	2	GAAAAAGAAAG	GAAAGGGAAAG	M01A8.2	-	K01B6.1	-
86	2	GAAAAAGAAAG	GAAAGCGAAAG	K10D2.6	-		-
87	2	GAAAAAGAAAG	GAAAGGGAAAG	K11E8.1g	unc-43	Y43C5B.2	-
88	2	GAAAACGAAAG	GAAAGAGAAAG	M04C9.6a	-	F16A11.3	-
89	2	GAAAATGAAAG	GAAAGAGAAAG	R05H10.3	-		-
90	2	GAAAGGGAAAG	GAAAAAGAAAG	R06A10.4	-	ZK993.2	-
91	2	GAAAGGGAAAG	GAAAAAGAAAG	T15H9.1	-	T15H9.7	dnj-20
92	2	GAAAGGGAAAG	GAAAATGAAAG	T22C1.6	-	T22C1.7	jsP301
93	2	GAAAGTGAAAG	GAAAAGGAAAG	F48C5.1	-	T25C12.1a	lin-14
94	2	GAAAGTGAAAG	GAAAAAGAAAG	W02G9.4	-	W02G9.3	-
95	2	GAAAGCGAAAG	GAAAATGAAAG	Y111B2A.12	-		-
96	2	GAAAGAGAAAG	GAAAATGAAAG	Y38E10A.22	-	Y38E10A.23	-
97	2	GAAAGTGAAAG	GAAAAAGAAAG	Y40B10A.9	-	F36F12.3	-
98	2	GAAAATGAAAG	GAAAGAGAAAG	Y47D3B.7	hlh-20		-
99	2	GAAAATGAAAG	GAAAGAGAAAG	Y53C10A.3	-	Y53C10A.4	-
100	2	GAAAATGAAAG	GAAAGAGAAAG	Y61A9LA.3b	-	Y61A9LA.1	-

* Cisortho program (http://dev.wormbase.org/cisortho/) was used for the prediction. GAAA$^{G}/_{A}$NGAAAG was presumed to be the DPY-24 binding motif. Adjacent ORFs and their gene names near each binding site were shown. Only binding sites with the top 100 highest scores were listed.